TISSUE-SPECIFIC DIFFERENTIAL INDUCTION OF DUPLICATED FATTY ACID-BINDING PROTEIN GENES BY THE PEROXISOME PROLIFERATOR, CLOFIBRATE, IN ZEBRAFISH (*Danio rerio*)

by

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MY SON, ISHAAN, AND MY WIFE, VALAR

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ABSTRACT

Duplicated genes are present in the teleost fish lineage owing to a whole-genome duplication (WGD) event that occured ~ 230-400 million years ago. In the duplication-degeneration-complementation (DDC) model, partitioning of ancestral functions (subfunctionalization) and acquisition of novel functions (neofunctionalization) have been proposed as principal processes for the retention of duplicated genes in the genome. The DDC model was tested by analyzing the differential tissue-specific distribution of transcripts for the duplicated fatty acid-binding protein 10 (*fabp10*) genes in embryos, larvae and adult zebrafish (*Danio rerio*). The distribution of zebrafish *fabp10a* and *fabp10b* transcripts show a strikingly different tissue-specific pattern leading us to suggest that the zebrafish *fabp10* duplicates had been retained in the genome owing to neofunctionalization. In another experiment to test the DDC model, transcriptional regulation of duplicated *fabp* genes was analyzed in zebrafish fed clofibrate, a peroxisome proliferator-activated receptor (PPAR) agonist. Clofibrate increased the steady-state level of both the duplicated copies of *fabp1a/fabp1b.1*, and *fabp7a/fabp7b* mRNA and heteronuclear RNA (hnRNA), but in different tissues of zebrafish. The steady-state level of *fabp10a* and *fabp11a* mRNA and hnRNA was elevated in liver of zebrafish, but not for *fabp10b* and *fabp11b*. We also investigated the effect of dietary fatty acids (FAs) and clofibrate on the transcriptional regulation of single copy *fabp* genes, *fabp2*, *fabp3* and *fabp6* in zebrafish. The steady-state level of *fabp2* transcripts increased in intestine, while *fabp3* mRNA increased in liver of zebrafish fed diets differing in FA content. In zebrafish fed clofibrate, *fabp3* mRNA in intestine, and *fabp6* mRNA in intestine and heart, was elevated. Whether the regulation of *fabp* gene transcription by clofibrate is controlled either directly or indirectly, the regulatory elements in the zebrafish *fabp* genes have diverged markedly since the WGD event, thereby supporting the DDC model.

LIST OF ABBREVIATIONS USED

acox1	Acyl-CoA oxidase 1 gene
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
DDC	Duplication-degeneration-complementation
dpf	Days post-fertilization
ef1α	Elongation factor 1 alpha gene
FA	Fatty acid
Fabp	Fish fatty acid-binding protein
fabp	Fish fatty acid-binding protein gene
FABP	Mammalian fatty acid-binding protein
FABP	Mammalian fatty acid-binding protein gene
Fabp10b	Fatty acid-binding protein 10b
fabp10b	fatty acid-binding protein 10b gene
h	Hour
HD	Highly unsaturated fatty acid-rich diet
hnRNA	Heterogenous nuclear ribonucleic acid
hpf	Hours post-fertilization
iLBP	Intracellular lipid-binding protein
kb	Kilobase
kDa	Kilodalton

LD	Linoleic acid-rich diet
LFD	Low fat diet
LG	Linkage group
LND	Linolenic acid-rich diet
min	Minute
mRNA	Messenger ribonucleic acid
mya	Million years ago
NCBI	National centre for biotechnology information
PCR	Polymerase chain reaction
p*I*	Isoelectric point
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
RAR	Retinoic acid receptor
rpl13α	Ribosomal protein large subunit 13 alpha gene
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RXR	Retinoid X receptor
s	Second
SEM	Standard error of means
WGD	Whole-genome duplication
TF	Transcription factor

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CHAPTER 1 : INTRODUCTION

1.1 Nomenclature, classification and function of *FABP* genes

The fatty acid-binding proteins (FABPs) are low-molecular-mass (14-16 kDa) polypeptides belonging to the members of the multigene family of intracellular lipid-binding proteins (iLBPs) that bind fatty acids (FAs) and other hydrophobic ligands (Bernlohr et al., 1997). FABPs were first identified from the cytosol of rat liver, intestinal mucosa and myocardium by Ockner and co-workers in 1972. To date, twelve different FABPs have been identified in vertebrates (Table 1.1). An interesting fact about the multigene family of iLBPs is that, they have only been found in animals and not in plants or fungi leading Schaap et al. (2002) to suggest that a single ancestral iLBP gene emerged in animals after their divergence from plants and fungi some 930 million years ago (mya). Presumably, a series of gene or genome duplications or both followed by their sequence divergence led to the diversity of the iLBP multigene family (Schleicher et al., 1995; Postlethwait et al., 2000; Woods et al., 2000; Furlong and Holland, 2002; Jaillon et al., 2004).

FABP/fabp genes and their proteins were named according to the initial tissue of isolation, *e.g*., liver-type (L-FABP), intestinal-type (I-FABP), *etc*. However, this system of nomenclature is confusing as no FABP is specific for a given tissue. Hertzel and Bernlohr (2000) proposed a new nomenclature for FABPs, in which the Hindu-Arabic numerals distinguish the different FABP proteins and their genes based on the chronological order of their discovery, *i.e*., FABP1 (liver-type), FABP2 (intestinal-type), *etc*.

Table 1.1 Fatty acid-binding protein (*FABP*/*fabp*) genes in different vertebrate species

Gene	Common name	Chromosome and amino acid length							
		Human	**Mouse**	**Rat**	**Chicken**	**Zebrafish**	**Medaka**	**Three-spined stickleback**	**Green-spotted pufferfish**
FABP1/fabp1	Liver FABP	2 (127aa)	6 (127aa)	4 (127aa)	23 (126aa)	*fabp1a* - 5 (127aa) *fabp1b.1* - 8 (129aa) *fabp1b.2* - 8 (128aa)	*fabp1* - 9 (127aa)	*fabp1* - XIII (127aa)	*fabp1* - 12 (128aa)
FABP2/fabp2	Intestinal FABP	4 (132aa)	3 (132aa)	2 (132aa)	4 (132aa)	*fabp2* - 1 (132aa)	*fabp2a* - Sf 461 (132aa) *fabp2b* - 18 (132aa)	*fabp2a* - IX (132aa) *fabp2b* - VII (132aa)	*fabp2* - UR (132aa)
FABP3/fabp3	Heart FABP	1 (133aa)	4 (133aa)	5 (133aa)	23 (133aa)	*fabp3* - 19 (133aa)	*fabp3* - 11 (133aa)	*fabp3* - X (134aa)	*fabp3* - 21 (133aa)
FABP4	Adipocyte FABP	8 (132aa)	3 (132aa)	2 (132aa)	2 (132aa)	-	-	-	-
FABP5	Epidermal FABP	8 (135aa)	3 (135aa)	2 (135aa)	2 (134aa)	-	-	-	-
FABP6/fabp6	Ileal FABP	5 (128aa)	11 (128aa)	10 (128aa)	13 (129aa)	*fabp6* - 21 (131aa)	*fabp6a* - 17 (126aa) *fabp6b* - 10 (125aa)	*fabp6a* - III (127aa) *fabp6b* - IV (125aa)	*fabp6a* - UR (127aa) *fabp6b* - 1 (125aa)
FABP7/fabp7	Brain FABP	6 (132aa)	10 (132aa)	20 (132aa)	3 (132aa)	*fabp7a* - 17 (132aa) *fabp7b* - 20 (132aa)	*fabp7a* - 22 (132aa) *fabp7b* - 24 (132aa)	*fabp7a* - XV (132aa) *fabp7b* - XVIII (132aa)	*fabp7a* - 10 (132aa) *fabp7b* - 14 (132aa)
FABP8	Myelin FABP	8 (132aa)	3	2	2	-	-	-	-
FABP9	Testis FABP	8 (132aa)	3 (132aa)	2 (132aa)	-	-	-	-	-
FABP10/fabp10	Liver-basic FABP	-	-	-	23 (127aa)	*fabp10a* - 16 (126aa) *fabp10b* - 19 (128aa)	*fabp10a* - 16 (126aa) *fabp10b* - 11 (126aa)	*fabp10a* - XX (126aa) *fabp10b* - X (126aa)	*fabp10* - UR (127aa)
fabp11	-	-	-	-	-	*fabp11a* - 19 (134aa) *fabp11b* - 16 (134aa)	*fabp11a* - 22 (134aa) *fabp11b* - 16 (134aa)	*fabp11a* - X (134aa) *fabp11b* - XX (134aa)	*fabp11* - 21 (134aa)
FABP12	-	8 (140aa)	3 (132aa)	2 (132aa)	-	-	-	-	-

Note: Sf, Scaffold; UR, un_random; aa, Amino acid.

The iLBP multigene family has been divided into four subfamilies based on phylogenetic analysis of the amino acid sequences of the proteins (Haunerland and Spener, 2004). The members of subfamily 1 consist of cellular retinoic acid-binding proteins (CRABP1 and 2) and retinol-binding proteins (RBP1, 2, 5, and 7). Subfamily 2 contains FABP1, FABP6, and FABP10. Subfamily 3 has only one member, *i.e*., FABP2 and subfamily 4 is comprised of FABP3, FABP4, FABP5, FABP7, FABP8, FABP9, FABP11 and FABP12.

Lipids and their constituent FAs play a vital role in the physiology of fishes (Tocher, 2003). FAs have more energy per gram than carbohydrates and they are an important source of energy in animals. A major constituent of biological membrane is FAs and they also function as signalling molecules (Hotamisligil et al., 1996). The transport of FAs across the plasma membrane and to other organelles inside the cell is carried out by FABPs, which play an important role in FA metabolism. Several gene-knockout studies in mice have tried to provide direct evidence for the biological functions of FABPs (Binas et al., 1999; Coe et al., 1999; Schaap et al., 1999), but the precise physiological role(s) played by each FABP has not been defined. The proposed functions of FABPs include: (1) uptake and utilization of FAs; (2) intracellular targeting of FAs to specific organelles and metabolic pathways; (3) protection of other proteins and cellular structures from the detergent effects of high concentration of FAs and acyl-CoA; (4) regulation of gene expression, influence mitosis, cell growth and differentiation; and (5) a role in insulin signal transduction (Ockner et al., 1972; Veerkamp and van Moerkerk, 1993; Ho et al., 2002; Storch et al., 2002; Corsico et al., 2004; Murota and Storch, 2005). Deficiencies in or malfunctioning of the FABP's in humans may be involved in the

etiology of several diseases such as diabetes, hyperlipidaemia, obesity, atherosclerosis and cardiac hypertrophy, suggesting that the FABPs may be a suitable target for intervention in these cases.

1.2 The zebrafish: an experimental system

At over 23,000 living species, teleost fishes are the most evolutionary diverse of all vertebrate groups. They inhabit both marine and freshwater habitats and are worldwide in their distribution. Although iLBPs have been identified in fishes, our understanding of the characteristics of iLBPs and their genes are mainly from studies in mammals. Fishes differ from mammals in lipid metabolism and homeostasis. Studying these non-mammalian systems may, therefore, provide us with greater knowledge of iLBP functions, and offer insight into the mechanism governing their functions. Zebrafish (a teleost fish) has become an increasingly popular vertebrate model organism for developmental biologists and geneticists (Detrich et al., 1999; Westerfield, 2000). Zebrafish made its start in developmental biology and its popularity is increasing in numerous research areas spanning from neuroscience to oncological studies. More established model systems like mice and rats are an expensive choice for large-scale analysis, so, zebrafish researchers find the economy required for high throughput studies, in a vertebrate model. Zebrafish has several advantages over mammalian systems, which includes rapidly developing clear embryos, allowing visualization of organ systems. The relatively small size of zebrafish allows large numbers to be maintained in a small space. In addition, high reproductivity, low cost and ease of maintenance makes them an ideal model for large-scale analysis. The sequencing of the zebrafish genome is almost complete. Techniques like whole-mount *in situ* hybridization, green fluorescent protein

reporter systems are commonly used in zebrafish studies (Amsterdam et al., 1996; Moss et al., 1996; Jowett, 1999; Westerfield, 2000). Certainly, studies using zebrafish will broaden our understanding of vertebrate gene function and development.

1.3 Molecular evolution of zebrafish *fabp* genes and their genomic organization

Genes are duplicated in different ways. Small scale gene duplication like the duplication of a single gene or many genes on a segment of chromosome occurs by tandem duplication or retrotransposition (Hurles, 2004). Tandem duplication occurs by unequal crossing over between homologous chromosomes, during meiosis (Hurles, 2004). The integration of reverse transcribed mRNAs at random sites in a genome are termed as retrotransposition (Hurles, 2004) and the mechanism of transposition can be described as '*copy and paste*'. The resultant duplicated genes are called as retrogenes which lack introns but have a poly-A tail (Hurles, 2004). Duplicates resulting from retrotransposition often do not produce a viable mRNA transcript as they lack regulatory elements which are required to activate transcription (Hurles, 2004). Large scale gene duplications like the whole-genome duplication (WGD) occur due to polyploidy (Prince and Pickett, 2002). Polyploid organisms possess more than two sets of haploid chromosomes. Allopolyploidy results from the hybridization of two closely related species followed by chromosome doubling and is very common in plants (Klug et al., 2012), whereas autopolyploids can be the result of accidents during meiosis or mitosis e.g., nondisjunction of all chromosomes in mitosis, which doubles the chromosomes, all derived from a single species.

Duplication of individual genes, chromosomal segments or whole-genomes plays an important role in the increasing complexity of life (Ohno, 1970; Holland et al., 1994;

Sidow, 1996). In 1936, Bridges discussed the role of gene duplicates in the evolution of morphological and functional diversity. But, it was Ohno who suggested two possible fates for the duplicated genes by combining the findings on gene and genome duplications (Hahn, 2009). The most common evolutionary fate of a duplicated gene proposed by Ohno is nonfunctionalization, in which mutations accumulate in the protein coding region, leading to gene silencing and subsequent loss from the genome. The other fate of a duplicated gene is neofunctionalization, wherein mutation in the protein coding region of a gene results in a novel function for that protein of benefit to the organism. As such, the process of neofunctionalization leads to the retention of both copies of the duplicated sister genes in the genome.

The ancestor of present day teleost fishes is believed to have undergone a whole-genome duplication (WGD) approximately 230–400 mya, which explains why teleost fishes have two copies of some genes which exist as single copy in most other vertebrates (Amores et al., 1998; Postlethwait et al., 2000; Jaillon et al., 2004; Woods et al., 2005; Kasahara et al., 2007). As predicted, iLBP genes have duplicates. To date, 18 paralogous iLBP genes have been identified in the animal kingdom, which includes 12 *FABPs*, 4 cellular retinol-binding proteins (*RBPs*) and 2 cellular retinoic acid-binding proteins (*CRABPs*) (Fig. 1.1). In zebrafish, as of today, we have identified 22 zebrafish iLBP genes and 18 (nine pair of duplicate genes) of them are sister duplicates of each other (Fig. 1.2). Of the 22 zebrafish iLBP genes so far identified, 12 *fabp* genes have been characterized (Denovan-Wright et al., 2000a; Denovan-Wright et al., 2000b; Pierce et al., 2000; Liu et al., 2003a; Liu et al., 2003b; Liu et al., 2004; Sharma et al., 2004; Sharma et al., 2006; Liu et al., 2007; Alves-Costa et al., 2008; Karanth et al., 2008; Venkatachalam

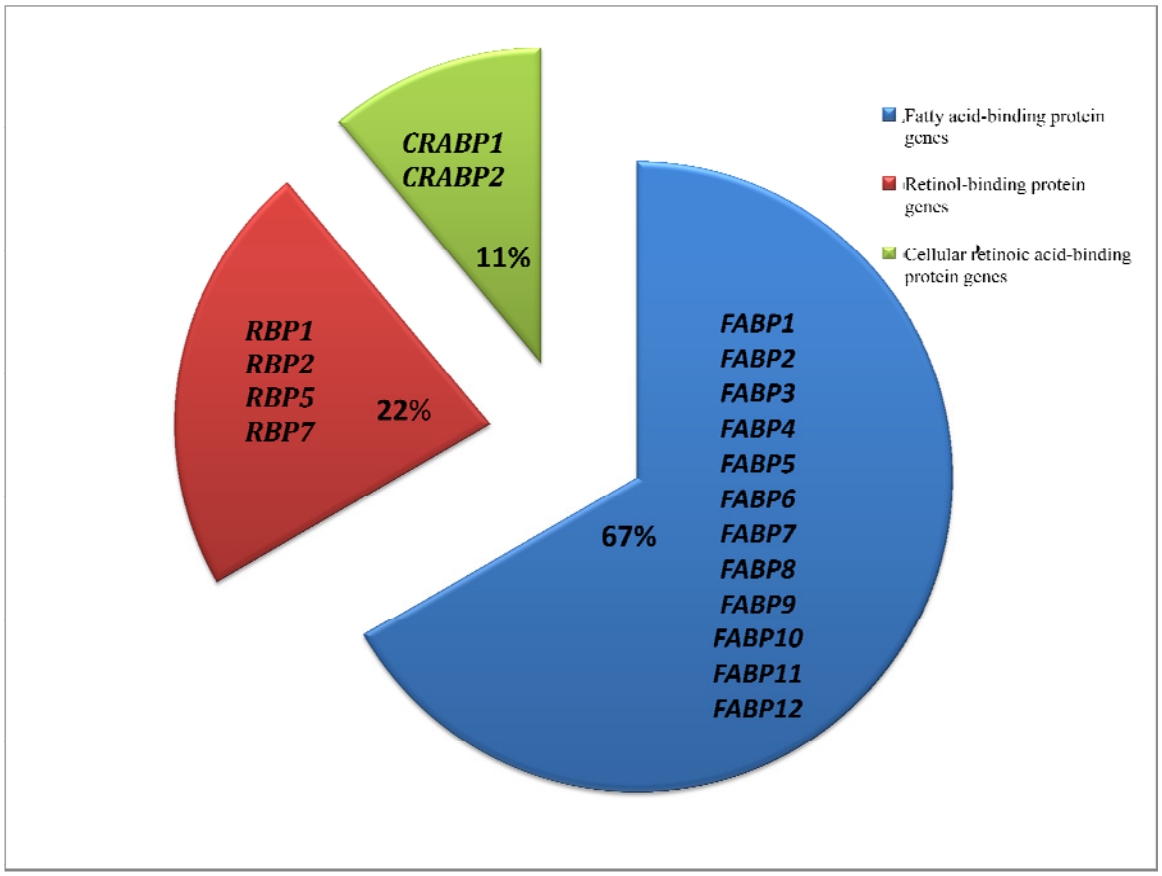

Figure 1.1 Intracellular lipid-binding protein (iLBP) genes of the animal kingdom.

This pie chart represents the members of the multigene family of iLBP genes identified in the animal kingdom, which includes 12 *fabp* genes (in blue), 4 *rbp* genes (in red) and 2 *crabp* genes (in green).

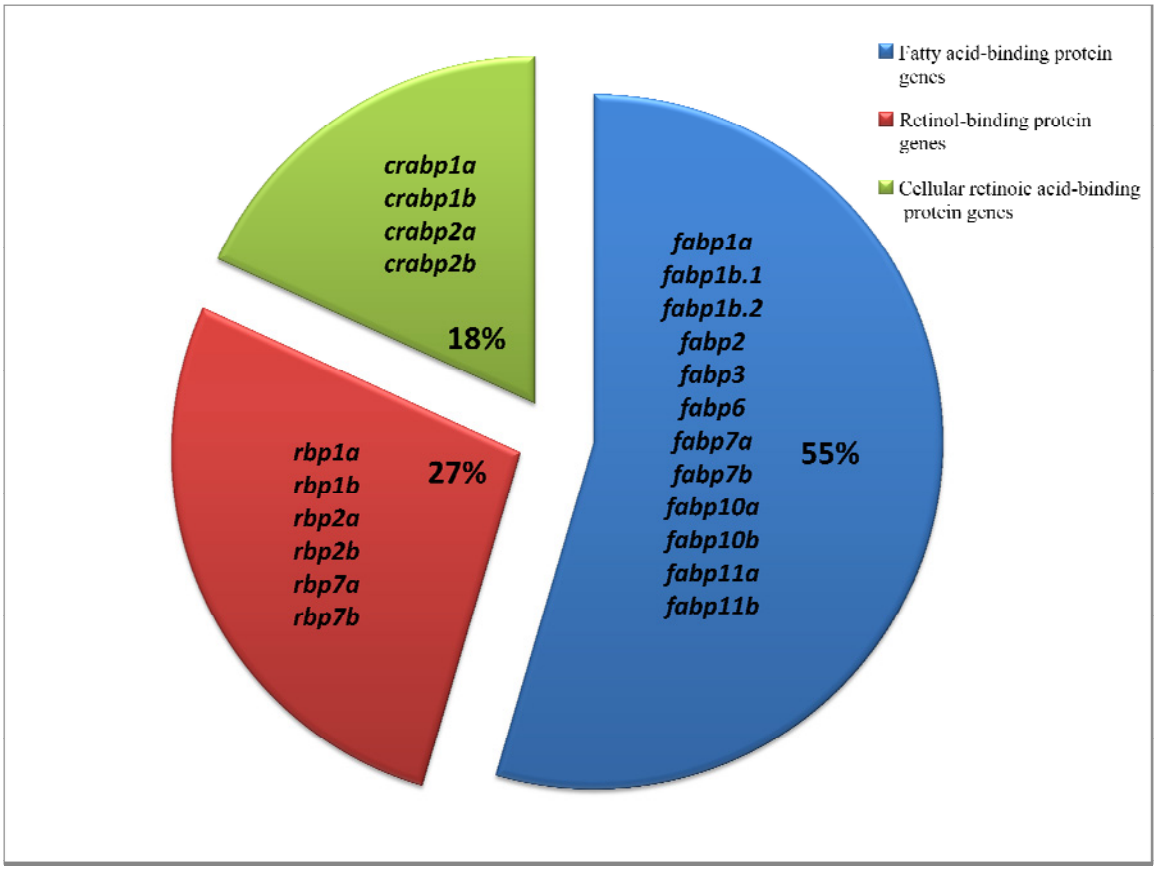

Figure 1.2 Intracellular lipid-binding protein (iLBP) genes of zebrafish. This pie chart represents the members of the multigene family of iLBP genes of zebrafish, which includes 12 *fabp* genes (in blue), 6 *rbp* genes (in red) and 4 *crabp* genes (in green).

et al., 2009; Karanth et al., 2009a). Eight genes (four pair of duplicate genes *fabp1a/fabp1b, fabp7a/fabp7b*, *fabp10a/fabp10b* and *fabp11a/fabp11b*), out of the 12 zebrafish *fabp* genes were formed as a result of teleost-specific WGD (Fig. 1.3) (Liu et al., 2004; Sharma et al., 2006; Karanth et al., 2008; Venkatachalam et al., 2009). In addition, Karanth et al. (2009a) identified *fabp1b.2* gene which is a tandem duplicate of *fabp1b* gene. Estimates show 14-30% of the duplicated genes are retained in the zebrafish genome following the WGD event (Postlethwait et al., 2000; Woods et al., 2005). Interestingly, the percentage of duplicated *fabp* genes retained in the zebrafish genome was found to be 73% (Fig. 1.3). Sufficient evidence has been provided to support that most of these duplicated *fabp* genes arose as a result of the teleost-specific WGD.

In zebrafish, some duplicate genes (including *fabp* genes) are clustered on duplicated chromosomal segments (LG 2/ LG 24, LG 3/ LG 12, LG 5/ LG 10, LG 7/ LG 25, LG 16/ LG 19, and LG 17/ LG 20) containing a copy of the duplicate gene in each chromosome pair (Woods et al., 2005). Our lab has identified the precise location of the four zebrafish *fabp* duplicate pairs on chromosomes *fabp1a* (LG 5)/ *fabp1b.1 and fabp1b.2* (LG 8), *fabp7a* (LG 17)/ *fabp7b* (LG 20), *fabp10a* (LG 16)/ *fabp10b* (LG 19), *fabp11a* (LG 19)/ *fabp11b* (LG 16), with each chromosome containing a copy of its sister duplicate, using radiation hybrids of the LN54 panel (Liu et al., 2004, 2007; Sharma et al., 2006; Karanth et al., 2008, 2009a; Venkatachalam et al., 2009).

Certain regions of the ancestral chromosomes have not changed over the course of evolution, so, to identify those regions, conserved gene synteny has been used (Woods et al., 2005). Duplicated *fabp* gene pairs, *fabp1a/fabp1b, fabp7a/fabp7b, fabp10a/fabp10b, fabp11a/fabp11b,* found on the different zebrafish linkage groups, together share the

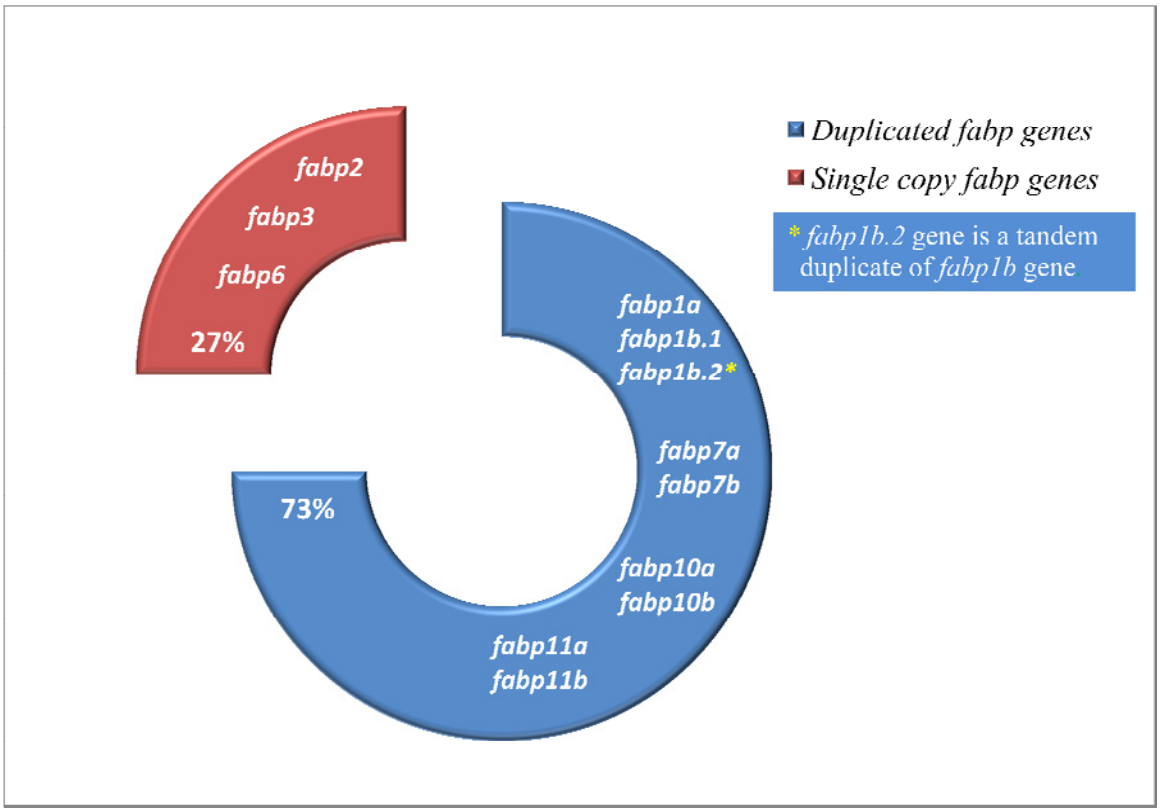

Figure 1.3 Zebrafish fatty acid-binding protein (*fabp*) genes. This chart represents all the identified *fabp* genes in zebrafish. Duplicated *fabp* genes comprise 73% of all the identified *fabp* genes in zebrafish (in blue) and 27% comprises of the single copy *fabp* genes in zebrafish (in red).

same gene order found in mammalian/chicken chromosomes (Liu et al., 2004, 2007; Sharma et al., 2006; Karanth et al., 2008, 2009a; Venkatachalam et al., 2009). The single copy zebrafish *fabp* genes namely the *fabp2*, *fabp3* and *fabp6* also show conserved gene synteny with their mammalian orthologs (Liu et al., 2003b; Sharma et al., 2004; Alves-Costa et al., 2008) inferring that the ancestral vertebrate karyotype is conserved in both zebrafish and mammals after the divergence from each other 400 mya (Kumar and Hedges, 1998).

All *fabp* genes, except *fabp2*, *fabp3* and *fabp6*, exist as duplicates in the zebrafish genome, whereas three-spined stickleback, medaka and fugu has two copies of *fabp2* and *fabp6* (Table 1.1) (Lai et al., 2009; Parmar et al., 2012b; Parmar and Wright, 2013) and in some Antarctic teleost fishes two *fabp3* isoforms has been detected (Vayda et al., 1998). Probably, the duplicates of zebrafish *fabp2, fabp3* and *fabp6* have been lost due to the accumulation of mutations leading to nonfunction. Mammalian orthologs have been described for all the zebrafish *fabp* genes excepting *fabp10* and *fabp11* genes (see Table 1.1). *Fabp10/fabp10* gene is present in the genomes of birds, reptiles and fishes, and is thought to have been lost from the mammalian genomes (Sharma et al., 2006). iLBPs play an important role in the intracellular transport of nutrients and also caters to various other physiological functions in the teleosts, which would have been one of the many factors leading to the retention of the duplicated iLBP genes in the teleost's genome.

Although *FABP* genes exhibit distinct, but overlapping, tissue-specific patterns of expression, the *FABP* gene structure and their proteins are highly conserved among all paralogous members from all vertebrate species (Ong et al., 1994; Glatz and van der

Vusse, 1996). Each *FABP*/*fabp* gene, except *FABP3* gene in desert locust (Wu et al., 2001), the *fabp1a* gene from zebrafish (Sharma et al., 2006) and *fabp11a* gene from medaka (Parmar et al., 2012a), consists of four exons separated by three introns (Ong, 1994; Bernlohr et al., 1997; Schaap et al., 2002) (Fig. 1.4). The mammalian genome has only a single functional copy of *FABP* gene excepting mice *FABP3* (*H-FABP*), where some non-transcribed intronless pseudogene have been identified (Treuner et al., 1994). Based on some evolutionary analyses, it has been estimated that the iLBP multigene family has undergone a minimum of 14 gene duplications (Schleicher et al., 1995). FABP1, 2, 6 and 10 clade diverged from the FABP3, 4, 5, 7, 8 and 9 lineage some 700 mya at or prior to the vertebrate/invertebrate divergence around 600–700 mya (Schleicher et al., 1995).

1.4 Structural and functional analysis of FABPs

The first FABP was isolated forty years ago (Ockner et al., 1972) and since then there has been an intensive study of this group of proteins. FABPs have been isolated from various species and their structure and physical properties have been studied. FABPs are single chain polypeptides consisting of 10 anti-parallel β-strands and two short α-helices. Each β-strand is hydrogen-bonded to the adjacent strand to form two 5-stranded β-sheets, which forms the β-barrel (Ong et al., 1994; Glatz and van der Vusse, 1996; Bernlohr et al., 1997). The proteins encoded by the *FABP* genes are small, ranging in size from 125 to 140 amino acid residues (Table 1.1). Although the amino acid sequence varies from 20% to 70% among the different paralogous members, the "β-barrel" structure is strikingly similar for all the members of the FABP/Fabp family. The "β-barrel" tertiary structure of FABPs forms a central cavity, which accommodates the

Figure 1.4 Genomic organization of *FABP*/*fabp* genes in mammals and fishes. The *FABP*/*fabp* gene structure is highly conserved among all paralogous members from all vertebrate species. This figure shows the genomic organization of *fabp* genes in fishes (zebrafish and medaka) and *FABP* genes in mammal (human). Each *FABP*/*fabp* gene consists of four exons separated by three introns with exceptions like *fabp1a* gene of zebrafish which has an additional exon (exon 0) and *fabp11a* gene of medaka which is intronless.

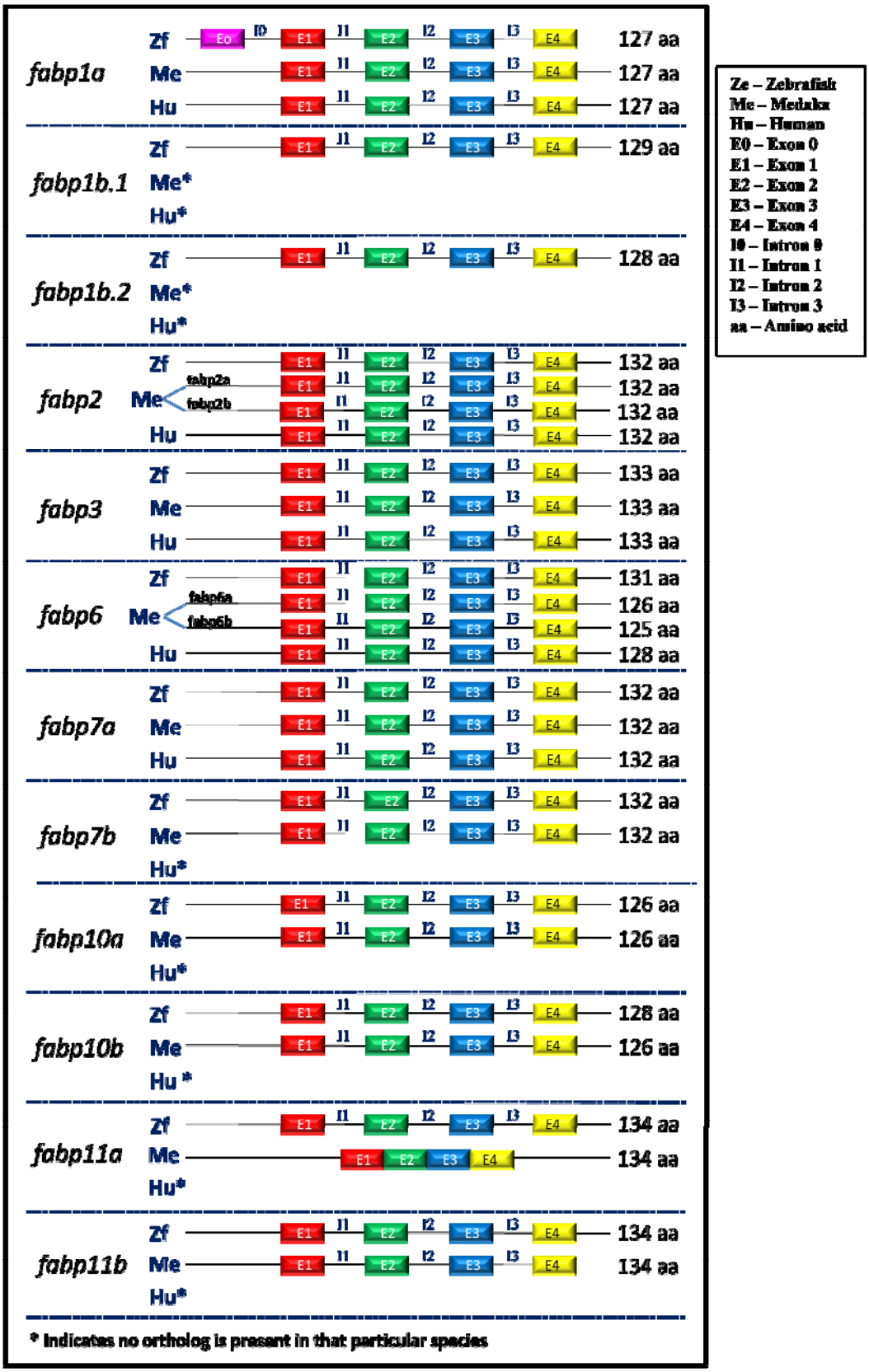

Figure 1.4 Genomic organization of *FABP*/*fabp* genes in mammals and fishes.

FA ligands and increases their solubility in the cytoplasm, thereby facilitating their movement to the target sites within the cell where they exert their biological effects (Glatz and van der Vusse, 1996; Bernlohr et al., 1997).

Regarding the functional analysis of FABPs, researchers working on catfish FABP10 observed and reported that a single fatty acid molecule binds the FABP10 protein (Di Pietro et al., 1997), whereas Nichesola et al. (2004) reported that the chicken FABP10 binds two ligand molecules as does FABP1. Recently, researchers have reported the crystallization and three-dimensional structure of zebrafish Fabp10a (Lb-fabp) (Capaldi et al., 2007). Capaldi et al. (2007) also suggested that fish Fabp10s could accommodate a single cholate molecule, whereas amphibians and other vertebrate Fabp10s can accommodate two cholate molecules. Recently, the three-dimensional structure of zebrafish Fabp6 (I-Babp) has been determined, which also accommodates two cholate ligands leading to structural changes (Capaldi et al., 2009).

The ligand binding preferences is varied for different FABPs. For example, brain FABP (B-FABP) binds docosahexaenoic acid (DHA) with higher affinity than other FAs (Xu et al., 1996). A broad range of ligands including heme, steroids, acyl CoA, leukotrienes, peroxisome proliferators, prostaglandins, and long-chain FAs binds FABP1 (L-FABP) (Raza et al., 1989; Miller and Cistola, 1993; Richieri et al., 1994; Sorof, 1994) and can bind to two ligands at the same time (Haunerland et al., 1984; Thompson et al., 1997). Schaap et al. (2002) have suggested that the ancestral iLBP gene encoded a universal hydrophobic ligand-binding protein that acquired specialized binding affinity during evolution. Different iLBPs were believed to display selective affinity for different ligands. However, recent reports suggest that FABPs demonstrate more fluidity for a

range of FAs (Richieri et al., 2000). So, the question is why the vertebrate genomes have so many iLBP genes? The answer for the iLBPs unique functional properties may lie in the structural characteristics of the individual rather than their ligand binding specificity (Storch and McDermott, 2009). More evidence from the zebrafish studies (Haunerland et al., 1984; Di Pietro et al., 1997; Thompson et al., 1997; Capaldi et al., 2007; Capaldi et al., 2009) also support Storch and McDermott (2009)'s hypothesis that change in structural conformation of iLBPs upon ligand binding gives them unique functional properties, which are different from each other, and enables iLBPs to carry out their distinctive functions inside the cell.

1.5 Spatiotemporal distribution of zebrafish *fabp* mRNA transcripts in developing embryos and larvae

The distribution of iLBP gene transcripts is highly conserved in mammals but, with a few exceptions (Ong et al., 1994; Bernlohr et al., 1997). During the evolution of vertebrates, the expression pattern of iLBP genes might have diverged to fulfill the specialized physiological requirements of each species. In zebrafish, the *fabp* gene transcripts are detected at different stages of embryos and larvae (Table 1.2). The spatio-temporal distribution of zebrafish *fabp* mRNA transcripts during embryonic and larval development was analyzed by whole-mount *in situ* hybridization using probes generated from their specific cDNA sequences. Zebrafish has seven broad periods of embryogenesis, the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching periods (Westerfield, 2000). In zebrafish embryo, during the oblong stage ($3^{2/3}$ hours post fertilization (hpf)) of the blastula period, the marginal blastomere tiers collapse, releasing their cytoplasm and nuclei into the adjoining cytoplasm of the yolk

Table 1.2 Tissue-specific distribution of *fabp* transcripts in embryos and larvae of zebrafish.

Gene	Zebrafish tissues													References
	Liver	**Intestine**	**Muscle**	**Brain**	**Heart**	**Retina**	**Ofv**	**Pharynx**	**CNS**	**YSL**	**Yolk sac**	**Pancreas**	**Swim bladder**	
fabp1a	-	-	-	-	-	-	-	-	-	-	-	-	-	Sharma et al., 2006
fabp1b.1	-	+	-	-	-	-	-	-	-	+	-	-	-	Sharma et al., 2006
fabp1b.2	-	-	-	-	-	-	-	-	-	-	+	-	-	Karanth et al., 2009a
fabp2	-	+	-	-	-	-	-	-	-	+	-	-	-	Sharma et al., 2004
fabp3	+	+	-	+	-	+	-	-	-	-	-	+	-	Liu et al., 2003b; Liu et al., 2007
fabp6	-	+	-	-	-	-	-	-	-	-	-	-	-	Alves-Costa et al., 2008
fabp7a	-	-	-	+	-	+	-	-	+	-	-	-	-	Liu et al., 2004
fabp7b	-	-	-	+	-	+	-	+	-	-	-	-	+	Liu et al., 2004
fabp10a	+	-	-	-	-	-	-	-	-	-	-	-	-	Sharma et al., 2006
fabp10b	-	-	-	-	-	-	+	-	-	-	-	-	-	Venkatachalam et al., 2009
fabp11a	-	-	-	+	-	+	-	-	-	-	-	-	-	Liu et al., 2007
fabp11b	-	-	-	-	-	+	-	-	-	-	-	-	-	Karanth et al., 2008

Note: +, transcripts detected in the tissue; -, transcripts not detected in the tissue; Ofv, Olfactory vesicles; CNS, Central Nervous System; YSL, Yolk Syncytial Layer.

cell, thus forming the yolk syncytial layer (YSL) (Kimmel and Law, 1985). The YSL is an organ unique to teleosts. Whole-mount *in situ* hybridization of zebrafish embryos detected *fabp1b.1*, *fabp1b.2* and *fabp2* mRNA transcripts in the YSL suggesting that these genes could be involved in the transfer of nutrients from the yolk to the blastoderm (Sharma et al., 2004; Sharma et al., 2006; Karanth et al., 2009a).

In zebrafish embryos, neurulation starts during the segmentation period (10-24 hpf). The brain rudiment forms along the anterior neural keel with ten distinctive swellings, termed neuromeres. The first three neuromeres are large and correspond to the two forebrain subdivisions, the diencephalon and telencephalon, and the midbrain or mesencephalon. Whole-mount *in situ* hybridization of zebrafish embryos detected *fabp1b.2*, *fabp3*, *fabp7a*, *fabp7b* and *fabp11a* mRNA transcripts in the developing forebrain (Liu et al., 2004; Liu et al., 2007; Karanth et al., 2009a). The remaining seven neuromeres are called rhombomeres, subdivide the hindbrain in the zebrafish embryo. Transcripts of the zebrafish *fabp7a* are detected in rhombomere 4 (Liu et al., 2004). Transcripts of zebrafish *fabp3* and *fabp7a* are detected in the hindbrain (Liu et al., 2004; Liu et al., 2007). The *fabp3* and *fabp7a* mRNA transcripts are detected at the early segmentation stage, along the posterior keel, which develops into the spinal cord (Liu et al., 2004; Liu et al., 2007). In zebrafish embryos, the optic primordial develops from the lateral walls of the diencephalon. Zebrafish embryos detected *fabp3*, *fabp7a*, *fabp7b*, *fabp11a* and *fabp11b* mRNA transcripts in retina (Liu et al., 2004; Liu et al., 2007; Karanth et al., 2008).

In the pharyngula period (24-48 hpf), simple blood vessel branches can be seen in the head of the zebrafish embryos and a complex vascular system is formed at the early

hatching period (48 hpf). The zebrafish heart starts to beat at the beginning of this period. Transcripts of zebrafish *fabp11a* are detected in the developing head vasculature and are also detected in low levels in the intersegmental blood vessels and in the aorta wall (Liu et al., 2007; Flynn et al., 2009). The development of the zebrafish intestine also starts around the pharyngula period (24 hpf), and continues until the larval stage (120 hpf) (Ng et al., 2005). Zebrafish *fabp1b.1*, *fabp2* and *fabp3* mRNA transcripts are detected in the pharyngula stage (24-36 hpf) (Sharma et al., 2004; Sharma et al., 2006; Liu et al., 2007). The zebrafish *fabp2* mRNA transcripts are detected at the posterior intestine by late hatching period (72 hpf) (Mudumana et al., 2004). Mudumana et al. (2004) suggest that *fabp2* can be used as a differential marker for intestinal epithelium because of its unique pattern of expression in the developing zebrafish intestine. At 24–48 hpf, zebrafish *fabp3* mRNA transcripts are detected in liver, muscle and pancreas (Liu et al., 2007). In addition, transcripts of *fabp10a* and *fabp10b* are detected in zebrafish liver and olfactory vesicles respectively (Sharma et al., 2006; Venkatachalam et al., 2009). In zebrafish, until the end of 3rd day, the creatures are called "embryos", and afterwards, "larvae" whether they have hatched or not. The zebrafish swimbladder gets inflated at the larval stage (120 hpf). Transcripts of zebrafish *fabp7b* are detected in a region associated with swimbladder (Liu et al., 2004).

1.6 Tissue-specific distribution of *fabp* mRNA transcripts in adult zebrafish

Each zebrafish *fabp* gene transcript exhibit a clearly defined, but occasionally overlapping tissue-specific pattern of distribution with that of other *fabp* gene transcripts (Table 1.3 and the references therein). The tissue-specific distribution pattern of zebrafish *fabp* mRNA transcripts in adult zebrafish tissues was determined by reverse

Table 1.3 RT-PCR analysis of the tissue-specific distribution of *fabp* transcripts in adult zebrafish.

Gene	Zebrafish tissue												References
	Liver	**Intestine**	**Muscle**	**Brain**	**Heart**	**Eye**	**Gills**	**Ovary**	**Testis**	**Skin**	**Kidney**	**Swim bladder**	
fabp1a	-	+	-	-	-	-	-	-	-	-	-	-	Sharma et al., 2006
fabp1b.1	+	+	-	-	+	-	+	+	+	-	-	-	Sharma et al., 2006
fabp1b.2	-	+	-	+	+	+	-	+	-	+	-	-	Karanth et al., 2009a
fabp2	+	+	+	+	-	NA	-	-	+	-	NA	NA	Sharma et al., 2004
fabp3	+	+	+	+	+	NA	-	+	+	+	NA	NA	Liu et al., 2003b; Liu et al., 2007
fabp6	+	+	-	-	+	-	-	+	NA	-	+	-	Alves-Costa et al., 2008
fabp7a	+	+	-	+	-	NA	-	-	+	-	NA	-	Liu et al., 2004
fabp7b	+	+	-	+	-	NA	-	-	+	+	NA	-	Liu et al., 2004
fabp10a	+	+	-	-	-	NA	-	-	+	-	NA	NA	Sharma et al., 2006
fabp10b	+	+	+	+	+	+	+	+	+	+	+	+	Venkatachalam et al., 2009
fabp11a	+	+	+	+	+	NA	-	-	-	-	NA	NA	Liu et al., 2007
fabp11b	-	-	+	+	+	+	-	+	-	-	-	NA	Karanth et al., 2008

Note: +, transcripts detected in the tissue; -, transcripts not detected in the tissue; NA, not assayed.

transcription-polymerase chain reaction (RT-PCR). Transcripts of most zebrafish *fabp* genes are detected in liver and intestine except *fabp11b* (Table 1.3). A probable reason for this pattern of expression might be because of the ancestral gene from which all these *fabp* genes have descended may have expressed in the midgut of the common ancestor of the present day vertebrates and invertebrates (Schaap et al., 2002). Zebrafish *fabp1a* and *fabp1b.2* mRNA transcripts are detected in intestine but not in liver (Sharma et al., 2006; Karanth et al., 2009a). Arrese et al. (2001) pointed out that digestion and absorbtion of lipids occurs in the midgut of insects. Smith et al. in 1992 detected two *FABPs* namely *MFB1* and *MFB2* in the midgut of Tobacco horn worm *Manduca sexta*. The promoters of *fabp* genes must have gained the *cis*-acting regulatory promoter or enhancer elements in the subsequent duplications giving rise to the present day complexity of the spatial distribution of transcripts observed in zebrafish, which is responsible for the intestine and liver specific distribution. In mammals, transcripts of *FABP1, FABP2, FABP4,* and *FABP6* are detected in the intestine and transcripts of *FABP1* and *FABP7* are detected in the liver (Yamamoto et al., 2009). Mammals, which evolved much later than fishes in the evolutionary time scale, might have lost the regulatory elements in some *FABP* genes, which drive only the intestine and liver specific distribution. In addition to liver and intestine, transcripts of zebrafish *fabp* genes are predominantly detected in ovaries, testis, muscle and heart, the sites of intense lipid metabolism in fish. Transcripts of zebrafish *fabp10b* are detected in all the tissues assayed, whereas *fabp10a* mRNA transcripts are detected only liver, intestine and testis (Sharma et al., 2006; Venkatachalam et al., 2009). Zebrafish *fabp11a* and *fabp11b* mRNA transcripts are detected in muscle, brain and heart (Liu et al., 2007; Karanth et al., 2008). Zebrafish *fabp11b* mRNA transcripts are also

detected in eye and ovary (Karanth et al., 2008). The *fabp1b.1* mRNA transcripts of zebrafish are detected in heart, gills, ovary and testis (Sharma et al., 2006). The *fabp1b.2* mRNA transcripts of zebrafish are detected in brain, heart, eye, ovary and skin (Karanth et al., 2009a). Zebrafish *fabp2* mRNA transcripts are detected in muscle, brain and testis (Sharma et al., 2004). The *fabp3* mRNA transcripts of zebrafish are detected in muscle, brain, heart, ovary, testis and skin (Liu et al., 2003b; Liu et al., 2007). The *fabp6* mRNA transcripts of zebrafish are detected in heart, ovary and kidney (Alves-Costa et al., 2008). Zebrafish *fabp7a* and *fabp7b* mRNA transcripts are detected in brain and testis (Liu et al., 2004). Zebrafish *fabp7b* mRNA transcripts are also detected in skin (Liu et al., 2004).

1.7 Subfunction partitioning in the tissue-specific distribution of *fabp* mRNA transcripts in embryos and adult zebrafish

To explain the retention or fate of duplicated genes in the genome, Force et al. (1999), subsequently elaborated by Lynch and Conery (2000), proposed the duplication-degeneration-complementation (DDC) model. In the DDC model, they proposed three fates for the duplicated genes. The first and the most common fate of a duplicated gene is a process termed as 'nonfunctionalization', where one of the sister duplicates accumulate deleterious mutation in the protein coding region, leading to gene silencing and subsequent loss of one of the duplicate gene from the genome. The second fate of a duplicated gene is by a process called 'neofunctionalization', where one of the sister duplicate gene gains a new function, a function which was not present in the ancestor of the duplicates. Duplicated genes may also be retained in the genome by 'subfunctionalization', where the regulatory functions of the ancestral gene are sub-divided between sister duplicate genes. In the DDC model, subfunctionalization was

proposed as an alternate mechanism to Ohno's neofunctionalization (1970), to explain the higher retention of duplicated genes in the genome. DDC model also elaborates that subfunctionalization and neofunctionalization occurs by either loss or gain of *cis*-regulatory elements in the promoters of the duplicated genes.

In 2004, Postlethwait and co-workers suggested a process called subfunction partitioning where the duplicated genes may diverge in their pattern of transcript distribution in adults and embryos and together they may complement the pattern of transcript distribution of the ancestral gene. Since mammals have not undergone WGD after they diverged from teleost fishes, a single mammalian ortholog can be considered as an ancestral gene of zebrafish duplicates for comparison purposes. Subfunctionalization is thought to have played a role in the retention of *fabp1a* and *fabp1b.1*, *fabp7a* and *fabp7b* because of a shared transcript distribution pattern in embryos and adults (Liu et al., 2004; Sharma et al., 2006). However recent reports suggest that the transcript distribution patterns differ among the duplicates and subfunctionalization alone may not explain their retention in the zebrafish genome (Karanth et al., 2009a). *fabp10a* and *fabp10b* transcripts in embryos, larvae and adult show strikingly different tissue-specific patterns of distribution (Venkatachalam et al., 2009) leading to conclude that the zebrafish *fabp10a* and *fabp10b* genes may have retained owing to neofunctionalization. Similarly, *fabp11a* and *fabp11b* transcripts also exhibit different patterns of tissue distribution in adult zebrafish (Karanth et al., 2008). Since there is no apparent ortholog of the fish *fabp11* gene in other vertebrate species (Agulleiro et al., 2007), it is hard to decide whether the duplicated *fabp11* genes are retained in the zebrafish genome by any processes mentioned in the DDC model (Force et al., 1999).

1.8 Transcriptional regulation of the *fabp* genes by ligands

To date, only few reports have been published describing the effect of feeding and starvation, and the effect of different ligands, on the regulation of *fabp* genes in zebrafish (Jury et al., 2008; Flynn et al., 2009; Karanth et al., 2009b; Venkatachalam et al., 2012) compared to numerous reports in mammals. Jury et al. (2008) fed zebrafish either high or low calorie diets for five weeks and detected an increase in the protein levels of Fabp7a in high calorie diet compared to low calorie diet. In 2009, Flynn et al. investigated the regulation of *fabp11a* and *fabp11b* genes under physiological conditions of feeding and starvation in zebrafish larvae. The authors (Flynn et al., 2009) observed that feeding stimulates the formation of adipocytes expressing the *fabp11a* transcripts in zebrafish larvae. To summarize, *fabp* genes are regulated not only by the composition of lipids, but also by the quantity of lipid influx. Our lab has published two extensive reports investigating whether the duplicated *fabp* genes in zebrafish are differentially regulated by dietary FAs and by clofibrate, which is a PPAR agonist (Karanth et al., 2009b; Venkatachalam et al., 2012). The FA study has shown that the transcriptional initiation of only one copy in each of the three sets of duplicated *fabp* genes of zebrafish, *fabp1/fabp1b.1/fabp1b.2, fabp7a/fabp7b,* and *fabp11a/fabp11b*, is modulated by dietary FAs in a given tissue (Karanth et al., 2009b). The differential induction of only one of the sister pair of duplicated *fabp* genes by FAs provides evidence for retention of duplicated genes in the zebrafish genome by either subfunctionalization or neofunctionalization. However, the FA study did not answer the questions on the regulatory cascades involved in the FA mediated induction of *fabp* genes. The differential transcriptional activation by the nuclear receptors like peroxisome proliferator-activated receptors (PPARs) involved

in the transcriptional regulation of *fabp* genes may explain their differential induction by dietary FA (Desvergne and Wahli, 1999). The transcriptional modulation of the duplicated *fabp* genes in various tissues of zebrafish fed different FAs might have been mediated by PPARs. So, our lab undertook another extensive study to investigate whether the duplicated *fabp* genes in zebrafish are differentially regulated by PPAR, by using clofibrate, a PPAR agonist. Transcription of several mammalian *FABP* genes are induced by FAs and peroxisome proliferators such as clofibrate (Meunier-Durmort et al., 1996; Poirier et al., 2001). Molecular mechanisms involved in the induction of mammalian *FABP* gene by FAs and peroxisome proliferators have been proposed (Ockner and Manning, 1974; Bass et al., 1985; Reddy, 2004; Schachtrup et al., 2004, Mochizuki et al., 2007; Schroeder et al., 2008). Long-chain FAs are carried from the cytoplasm to the nucleus by FABPs (Huang et al., 2004; Schroeder et al., 2008). Inside the nucleus, the nuclear receptors, PPARα and PPARγ, accept the long-chain FAs and peroxisome proliferators (Delva et al., 1999; Budhu and Noy, 2002; Tan et al., 2002). Upon receiving the FAs and peroxisome proliferators, PPARs form heterodimers with retinoic-acid receptors (RAR) or retinoid X receptors (RXR) (*e.g.,* PPAR-RAR and PPAR-RXR), which in turn bind to reponse elements in the promoters of *FABP* genes to stimulate initiation of transcription (Gottlicher et al., 1992; Keller et al., 1993; Lemberger et al., 1996; Desvergne and Wahli, 1999; Escher and Wahli, 2000; Wolfrum et al., 2001; Wilk et al., 2005). The molecular mechanism has been explained in a figure format (Fig. 1.5). Our study showed that clofibrate has induced the transcriptional initiation of both pairs of some duplicated *fabp* genes in zebrafish (Venkatachalam et al., 2012).

Figure 1.5 Molecular mechanism for the transcriptional initiation of *FABP/fabp* genes by fatty acids and peroxisome proliferators. Long-chain FAs are carried from the cytoplasm to the nucleus by FABPs. Inside the nucleus, the nuclear receptors, PPARα and PPARγ accept the long-chain FAs and peroxisome proliferators. Upon receiving the FAs and peroxisome proliferators, they form heterodimers with retinoic-acid receptors (RAR) or retinoid X receptors (RXR), which in turn binds to response elements in the promoters of *FABP* genes, thereby, stimulating the initiation of transcription.

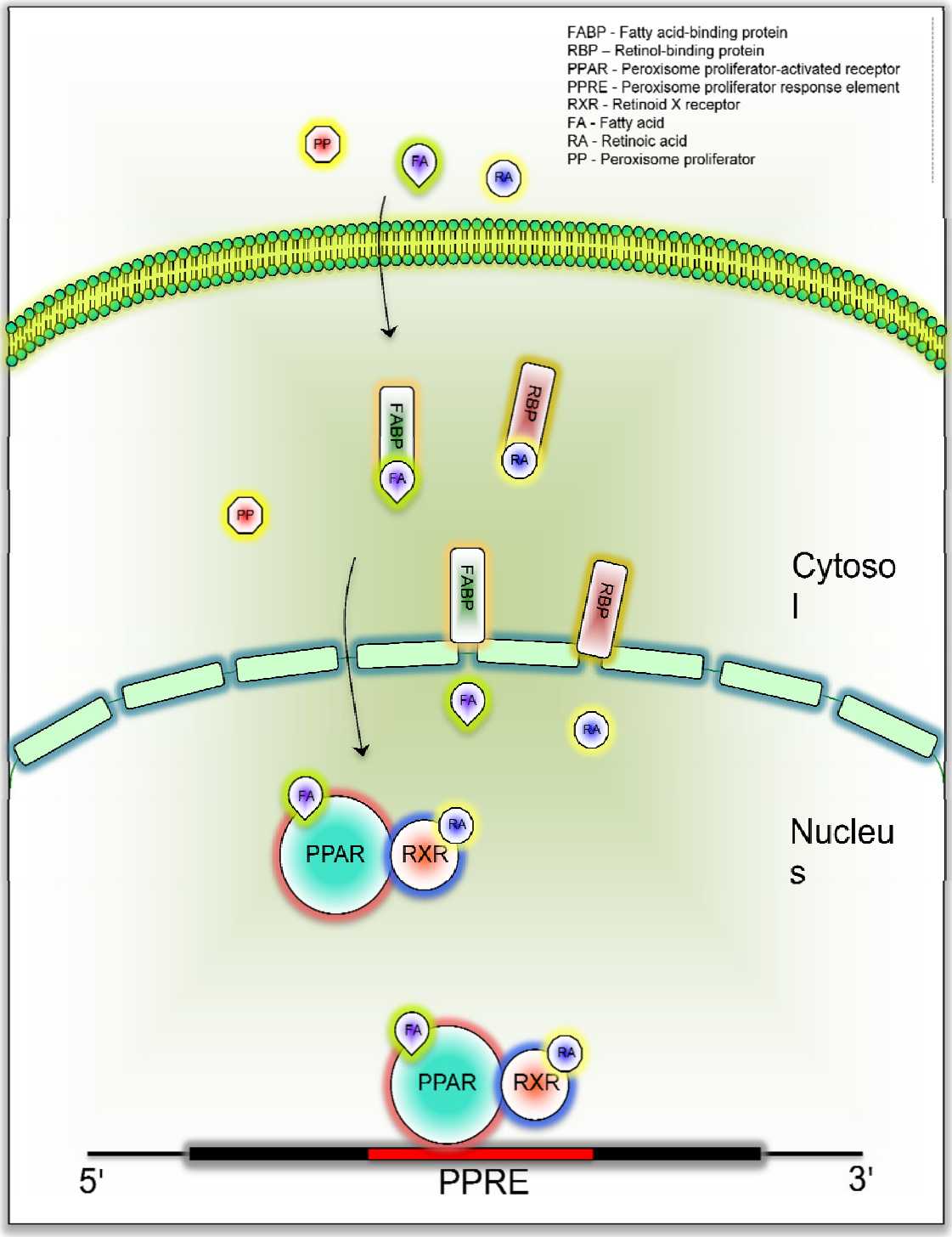

Figure 1.5 Molecular mechanism for the transcriptional initiation of *FABP/fabp* genes by fatty acids and peroxisome proliferators.

Currently, our knowledge about the *cis*-regulatory elements involved in the regulation of zebrafish *fabp* genes is limited (Meunier-Durmort et al., 1996; Poirier et al., 2001; Wu et al., 2001; Her et al., 2003a; Her et al., 2003b; Her et al., 2004a; Her et al., 2004b; Schachtrup et al., 2004; Qu et al., 2007). Her et al. (2003b) identified two distinct regulatory elements, A and B, in a 435-bp distal region of the *L-FABP* (*fabp10a*) promoter which regulates the expression of the gene in the liver of larvae and juveniles of transgenic zebrafish. The authors have characterized two consensus hepatocyte fork head (HFH) and a hepatocyte nuclear factor (HNF-1 alpha) site in element A and a HNF-3 beta site in element B, mutations which will lead to loss or decrease of *fabp10a* expression in liver of zebrafish (Her et al., 2003b). However, *fabp10a* transcripts are also detected in intestine and testis in addition to liver of adults (Venkatachalam et. al., 2009) and Her et al. (2003b) fail to mention whether mutations in any of these elements have an effect on the expression of *fabp10a* in intestine and testis of adult zebrafish. Her et al. (2004b) have also analyzed the promoter of zebrafish *fabp2* gene, and reported that the proximal 192-bp region of the zebrafish *fabp2* promoter is sufficient to direct intestine-specific expression during larval development. The reason behind the retention of both duplicated *fabp10* genes (*fabp10a/fabp10b*) in the zebrafish genome or why a copy of the zebrafish *fabp2* gene was lost from the genome still remains elusive and neither of the above two studies provided insight to this.

1.9 Objectives of this study

FABPs are involved in lipid metabolism by the intracellular transport of long chain FAs. A diet-mediated change in the metabolism of FAs would influence the requirement of FABPs for cellular processes and thus affect the transcription of their

genes. Previous studies in zebrafish have shown that transcriptional initiation of only one copy in each of the three sets of duplicated *fabp* genes of zebrafish, *fabp1a*/*fabp1b.1*/*fabp1b.2*, *fabp7a*/*fabp7b* and *fabp11a*/*fabp11b*, is modulated by dietary FAs in a given tissue (Karanth et al., 2009b). Since FAs are known to be ligands of PPARs that leads to transcriptional up-regulation of target genes, we anticipated that the transcriptional modulation of *fabp* genes in various tissues of zebrafish fed different FAs might be mediated by PPARs. The objective of the present research was, therefore, to investigate whether the duplicated *fabp* genes, *fabp1a*/*fabp1b.1*/*fabp1b.2*, *fabp7a*/*fabp7b*, *fabp10a*/*fabp10b* and *fabp11a*/*fabp11b* in zebrafish are differentially regulated by PPAR, by using clofibrate, a PPAR agonist. Clofibrate has been used extensively to investigate the regulation of gene transcription in vertebrates, owing to its specific binding with PPARα, and to a lesser extent to PPARγ, and its effect on the transcription of specific genes involved in lipid metabolism (Yamoto et al., 1996; Haasch et al., 1998; Akbiyik et al., 2004; Nunes et al., 2004; Konig et al., 2007; Luci et al., 2007; Ringseis et al., 2007; Rorvik et al., 2007). The current study focuses on newly available data and insights from various literatures, to better understand the evolution and transcript distribution of the *fabp* genes of zebrafish.

CHAPTER 2 : DIFFERENTIAL TISSUE-SPECIFIC DISTRIBUTION OF TRANSCRIPTS FOR THE DUPLICATED FATTY ACID-BINDING PROTEIN 10 (*fabp10*) GENES IN EMBRYOS, LARVAE AND ADULT ZEBRAFISH (*Danio rerio*)

The manuscript based on this study is presented below.

Co-authors for this manuscript are Christine Thisse, Bernard Thisse and Jonathan M. Wright.

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Author's contributions:

ABV and JMW conceived and designed the research. ABV conducted the experimental work except for whole-mount *in situ* hybridization of zebrafish embryos and larvae (CT and BT). ABV and JMW drafted the manuscript. All authors read and approved the final version of the manuscript.

2.1 Abstract

Genomic and complementary DNA (cDNA) sequences coding for a fatty acid-binding protein (FABP) in zebrafish were retrieved from DNA sequence databases. The cDNA codes for a protein of 14.7 kDa (pI = 5.94), and the gene consists of four exons, properties characteristic of most vertebrate *FABP* genes. Phylogenetic analyses using vertebrate FABPs indicated that this protein is most similar to zebrafish Fabp10. Currently, only one *fabp10* gene is annotated in the zebrafish genome. In this article, the notations '*fabp10a*' and '*fabp10b*' are used to refer to the duplicate copies of *fabp10*. The zebrafish *fabp10a* and *fabp10b* genes were assigned by radiation hybrid mapping to chromosomes 16 and 19, respectively. On the basis of conserved gene synteny with chicken FABP10 on chromosome 23, zebrafish *fabp10a* and *fabp10b* are duplicates resulting from a whole-genome duplication event early in the ray-finned fish lineage some 230-400 million years ago (mya). Whole-mount *in situ* hybridization detected *fabp10b* transcripts only in the olfactory vesicles of embryos and larvae, whereas *fabp10a* transcripts have been shown previously to be present only in the liver of embryos and larvae. In adults, reverse transcription, polymerase chain reaction (RT-PCR) detected *fabp10b* transcripts in all tissues assayed. By contrast, *fabp10a* transcripts were detected only in adult liver, intestine and testis. This differential tissue distribution of transcripts for the duplicated *fabp10* genes suggests considerable divergence of their *cis*-acting regulatory elements since their duplication.

2.2 Introduction

The fatty acid-binding proteins (FABPs), members of the multigene family of intracellular lipid-binding proteins (iLBPs), are low-molecular-mass (~ 14 kDa) polypeptides that bind fatty acids, eicosanoids and other hydrophobic ligands (Bernlohr et al., 1997). To date, 18 paralogous iLBP genes, including 12 FABPs and six cellular retinol and retinoic acid-binding proteins, have been identified, but only in vertebrates and not in plants or fungi. This led Schaap et al. (2002), to suggest that a single ancestral iLBP gene emerged in animals after their divergence from plants and fungi approximately 930 million years ago (mya). Presumably, a series of gene duplications, followed by their sequence divergence, led to the diversity of the iLBP multigene family (Schleicher et al., 1995).

Previously, FABP/Fabps and their genes were named on the basis of the tissue from which they were initially isolated, *e.g*. liver-type FABP (L-FABP), intestinal-type FABP (I-FABP), *etc*., and subsequently on the basis of sequence similarity to the prototypic FABP of that tissue. This nomenclature is confusing because different types of FABP have been isolated from the same tissue, and even some orthologous FABPs from different species show distinctly different tissue-specific expression patterns (Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002). Moreover, Sharma et al. (2006) reported that the transcripts for two liver-type *FABP* genes were not detected in the liver of zebrafish. In this paper, we have chosen to follow the nomenclature proposed by Hertzel and Bernlohr (2000), where each FABP and its gene are given an Arabic numeral presumably reflecting the chronological order of its discovery, *i.e*. FABP1 (liver-type), FABP2 (intestinal-type), *etc*. The recommendations of the Zebrafish Model Organism

Database (http://www.zfin.org) for the gene and protein designations are also followed here.

Several *FABP* gene knockout experiments in mice have provided evidence for the biological function(s) of FABPs (Binas et al., 1999; Coe et al., 1999; Schaap et al., 1999), but our understanding of the precise physiological role(s) of FABPs remains elusive. Proposed physiological roles for FABPs include the uptake and utilization of fatty acids, intracellular targeting of fatty acids to specific organelles and metabolic pathways, and the protection of cellular structures from the detergent effects of fatty acids (Veerkamp and van Moerkerk, 1993; Ho et al., 2002; Storch et al., 2002; Corsico et al., 2004; Murota and Storch, 2005). Although different *FABP* genes exhibit distinct, but sometimes overlapping, tissue-specific patterns of expression (Ong et al., 1994; Glatz and van der Vusse, 1996), the structure of *FABP* genes and their encoded proteins are highly conserved. Each *FABP* gene, with the exception of the *FABP3* gene from desert locust (Wu et al., 2001) and the *fabp1a* gene from zebrafish (Sharma et al., 2006), consist of four exons of comparable coding capacity between paralogous and orthologous *FABP* genes in different species. All FABPs are approximately 130 amino acids in length and have a common tertiary structure consisting of a fold in which 10 strands of antiparallel β-sheet surround the ligand-binding site (Zimmerman and Veerkamp, 2002).

Most FABPs have an isoelectric point (p*I*) that is either acidic or neutral, with the exception of an FABP first isolated from chicken liver (Ceciliani et al., 1994), which has a p*I* of 9.0. This FABP was termed liver basic-type FABP owing to its basic isoelectric point. Primarily on the basis of sequence similarity, phylogenetic analysis and tissue-specific patterns of expression, so-called 'liver basic-type' FABPs have subsequently

been identified in fishes (Di Pietro et al., 1997; Denovan-Wright et al., 2000b; Di Pietro and Santome, 2001; Jordal et al., 2006), salamander (Di Pietro et al., 1999), toad (Schleicher and Santome, 1996), iguana (GenBank accession number U28756) and nurse shark (Cordoba et al., 1999). Several of these FABPs (Di Pietro and Santome, 2001; Schleicher and Santome, 1996), although showing phylogenetic relatedness to the chicken liver basic-type FABP, have acidic p*I* values. As such, the term 'liver basic-type' FABP seems inappropriate, and FABP10 is therefore used here throughout.

The tissue-specific pattern of expression of FABP10 appears to be restricted to the liver of nonmammalian vertebrate species. No FABP10 has been detected thus far in mammalian species. In an initial study based on *in vitro* binding assays, catfish FABP10 binds a single fatty acid molecule (Di Pietro et al., 1997), whereas Nichesola et al. (2004) have shown that chicken FABP10 binds two ligand molecules, a property uniquely shared with FABP1 among FABPs.

Previously, we have described a FABP10 from zebrafish with a calculated p*I* value of 8.8, and a tissue-specific pattern of expression restricted to liver, intestine and testis of adult zebrafish (Denovan-Wright et al., 2000b; Sharma et al., 2006). Here, we report another *fabp10* (hereafter referred to as *fabp10b*) gene in zebrafish which, based on sequence similarity, phylogenetic analysis and conserved gene synteny with the chicken *FABP10* gene, is a duplicated copy of the previously described zebrafish *fabp10* (hereafter referred to as *fabp10a*) gene. These duplicated copies of the *fabp10* gene most probably arose as a result of a whole-genome duplication (WGD) event that occurred early in the radiation of the ray-finned fishes approximately 230–400 mya (Furlong and Holland, 2002; Mulley and Holland, 2004; Vandepoele et al., 2004).

Furthermore, we show differential tissue-specific distribution of *fabp10a* and *fabp10b* transcripts in developing and adult zebrafish, evidence of the divergence of regulatory elements in the promoters of the *fabp10a* and *fabp10b* genes compared with the ancestral gene illustrated by the single-copy *FABP10* gene in chicken.

2.3 Materials and methods

2.3.1 Zebrafish husbandry

Zebrafish (AB strain) were raised according to established protocols (Westerfield, 2000). Experimental protocols were reviewed by the Animal Care Committee of Dalhousie University in accordance with the recommendations of the Canadian Council on Animal Care.

2.3.2 Identification of the zebrafish *fabp10b* gene

Using a *fabp10a* cDNA sequence (Denovan-Wright et al., 2000b), as a query, a gene sequence (ENSDARG00000069449) and a transcript (ENSDART00000101095) were retrieved from a BLASTN search of the zebrafish genome sequence database at the Wellcome Trust Sanger Institute, Cambridge, UK (version Zv7, http://www.ensembl.org /Danio_rerio/Info/Index). The transcript sequence, ENSDART00000101095, was then used to identify expressed sequence tags and genomic DNA sequences from GenBank of the National Center for Biotechnology Information (NCBI). Based on the expressed sequence tag sequence, EH467748, the primers clf and clr (Fig. 2.1) were synthesized and used to amplify by PCR a 503-bp fragment from a cDNA template prepared from total RNA isolated from a whole zebrafish. The single product of the expected size was generated and cloned into the pGEM-T vector (Promega, Madison, WI, USA) and five clones were sequenced. The sequences of all five clones were identical to the coding

Figure 2.1 Nucleotide sequence of zebrafish *fabp10* and its proximal 5' upstream promoter region. Exons are shown in capital letters, with the coding sequences of each exon underlined and the deduced amino acid sequence indicated below. The nucleotide positions in the gene sequence are indicated by the numbers on the right. +1 indicates the transcription initiation site. The 5′ upstream sequence of *fabp10b* is shown in lower case letters, with a putative TATA box highlighted and underlined. The square symbol indicates the stop codon. A putative polyadenylation signal sequence AATAAA is highlighted in bold and underlined. The PCR primers used for RT-PCR detection of *fabp10b* transcripts in RNA extracted from adult zebrafish tissues (rtf, rtr), for radiation hybrid mapping (rhf, rhr) and for cloning (clf, clr) are overlined.

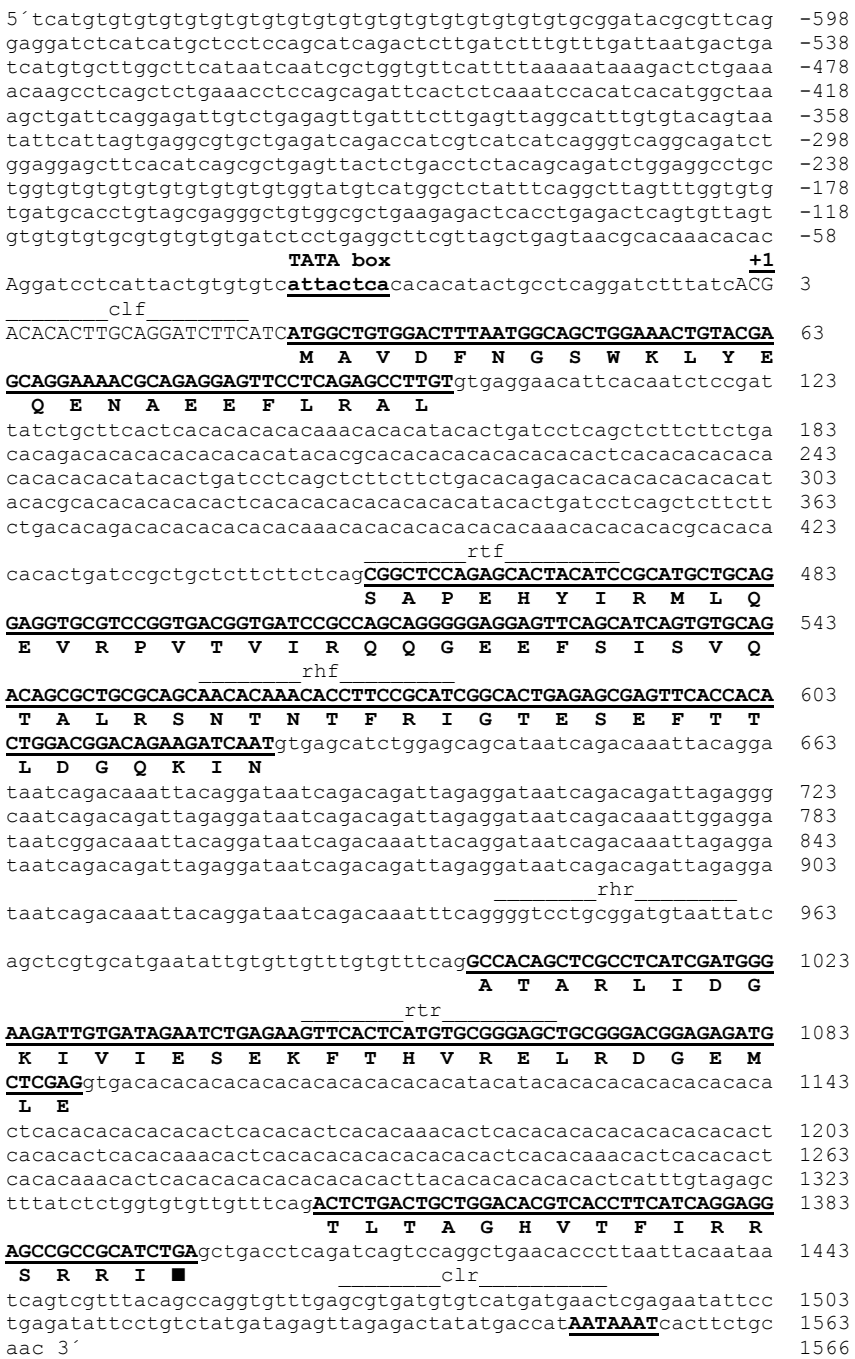

Figure 2.1 Nucleotide sequence of zebrafish *fabp10* and its proximal 5' upstream promoter region.

sequence of the *fabp10b* gene found in the genomic DNA scaffold CU644170 (NCBI), and also found at Zv7_NA3232 (NCBI). The molecular mass and p*I* of the zebrafish Fabp10b polypeptide and other vertebrate FABPs/Fabps were determined using the program at http://ca.expasy.org/tools/pi_tool.html.

2.3.3 Phylogenetic analysis

The sequence alignment and percentage amino acid sequence identity and similarity of FABP/Fabp sequences from zebrafish and other vertebrates were performed using CLUSTALW (Thompson et al., 1994). To reveal the evolutionary relationship of the zebrafish *fabp10b* gene with other vertebrate *FABP/Fabp* genes, a bootstrap neighbour-joining phylogenetic tree was constructed from various FABPs using MEGA4 software (Tamura et al., 2007). Human Von Ebner's gland protein, lipocalin 1 (LCN1, NP_002288), served as an outgroup sequence.

2.3.4 Chromosome assignment of the zebrafish *fabp10b* gene by radiation hybrid mapping

To assign the *fabp10b* gene to a specific zebrafish chromosome, we used the LN54 radiation hybrid panel (Hukriede et al., 1999). The sequences of the primers rhf and rhr used to PCR amplify a portion of the *fabp10b* gene from genomic DNA of LN54 radiation hybrids are shown in Fig. 2.1. PCR conditions included initial denaturation at 94.0 °C for 4 min, followed by 35 cycles at 94.0 °C for 30 s (denaturation), 54.1 °C for 30 s (primer annealing) and 72.0 °C for 1 min (elongation), with a final elongation step at 72.0 °C for 5 min. The radiation hybrid panel was scored and analysed according to the directions at http://zfin.org/action/ln54mapper.

2.3.5 Spatiotemporal distribution of *fabp10b* transcripts in embryos and larvae

To determine the spatiotemporal distribution of *fabp10b* transcripts, whole-mount *in situ* hybridization of zebrafish embryos and larvae was performed using riboprobes synthesized from the cloned *fabp10b* cDNA, according to the method of Thisse and Thisse (2008).

2.3.6 Tissue-specific distribution of *fabp10b* transcripts in adult zebrafish

To determine the tissue-specific distribution of *fabp10b* transcripts, total RNA was extracted from adult zebrafish tissues by the Trizol reagent according to the protocol recommended by the supplier (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from each RNA sample using an Omniscript RT kit (Qiagen, Mississauga, Canada). For PCR amplification, primers were synthesized based on the cDNA sequence shown in Fig. 2.1 (rtf, rtr). PCR conditions for the amplification of *fabp10b* transcripts included initial denaturation of DNA at 94.0 °C for 2 min, followed by 30 cycles at 94.0 °C for 30 s (denaturation), 56.8 °C for 30 s (primer annealing) and 72.0 °C for 1 min (elongation), with a final elongation step at 72.0 °C for 5 min. The constitutively expressed mRNA for elongation factor 1 aplha (*ef1α*) was used as a positive control. PCR primers for amplification of *ef1α* transcripts have been described previously by Pattyn et al. (2006). The PCR conditions employed an initial denaturation step at 94.0 °C for 2 min, followed by 30 cycles at 94.0 °C for 30 s (denaturation) 57.9 °C for 30 s (primer annealing) and 72.0 °C for 1 min (elongation), with a final elongation step of 72.0 °C for 5 min.

For quantitative analysis, RT-qPCR was performed for zebrafish *fabp10b* and *ef1α* cDNA using the Rotor-Gene (RG-3000) thermal cycler system according to the

manufacturer's instructions (Corbett Research, Sydney, NSW, Australia). Primers and conditions for RT-qPCR amplification of *fabp10b* and *ef1α* transcripts were the same as those employed for RT-PCR (see above). Serial dilutions of gel-purified *fabp10b* and *ef1α* RT-PCR products were allowed to bind SYBR® Green I dye (Qiagen, Mississauga, Canada), and the amount of bound SYBR® Green I was determined by fluorimetry. The concentrations of *fabp10b* and *ef1α* RT-PCR products were determined by extrapolation from the standard curve. The PCR conditions used included an initial denaturation step for 15 min at 95.0 °C, followed by 40 cycles of 20 s at 94.0°C (denaturation), 30 s at 56.8 °C and 57.9 °C for *fabp10b* and *ef1α*, respectively (annealing of primers) and 30 s at 72.0 °C (elongation). The fluorescent signal was measured at the end of each elongation phase. Melting curve analysis was performed to assess the purity of the PCR products after the 40 cycles by continuous measurement of the total fluorescent signal in each PCR whilst slowly heating the samples from 65 to 95 °C. The copy number for *fabp10b* transcripts in different tissues was determined using the standard curves as outlined by Bustin et al. (2005). As a negative control, cDNA was omitted from the reactions: no product was detected in these samples. The copy number of *fabp10b* transcripts was divided by the copy number of *ef1α* transcripts determined in each tissue sample, and represented as the relative abundance of mRNA for the *fabp10b* gene in each tissue.

2.4 Results and discussion

2.4.1 Identification of a duplicated *fabp10* gene in the zebrafish genome

Using the GenBank sequence AF254642, coding for *fabp10a* (Denovan-Wright et al., 2000b), as a query in a search of the zebrafish genome database (http://www.ensembl.org/index.html), we identified a paralogous gene to the previously described zebrafish

fabp10a. We predicted that this newly found *fabp10b* gene (GenBank accession no. BC122459) might be a duplicate copy of the previously described zebrafish *fabp10a*. This duplicate zebrafish *fabp10b* had a relatively small gene size that spanned 1.5 kb of genomic DNA and consisted of four exons separated by three introns (Fig. 2.1), a gene organization common to all members of the iLBP multigene family in vertebrates (Bernlohr et al., 1997). The sizes of each of the three introns of this duplicated *fabp10b* were 353, 375 and 258 bp, respectively. Each of the intron/exon splice junctions in this duplicated zebrafish *fabp10b* conformed to the GT/AG rule proposed by Breathnach and Chambon (1981). A 387-bp cDNA sequence for duplicated *fabp10b* was identified, which codes for a peptide of 128 amino acids (Fig. 2.1). The molecular mass of the duplicate Fabp10b was 14.7 kDa with a p*I* of 5.94, which is in contrast with Fabp10a, which has a p*I* of 8.87 (Denovan-Wright et al., 2000b). FABP10 was first isolated from chicken and named liver basic-type FABP owing to its p*I* of 9.0 (Ceciliani et al., 1994), whereas the p*I* values of all other FABPs identified at that time were acidic. Subsequently, FABP10s were identified primarily on the basis of amino acid sequence identity with the chicken FABP10 in several other nonmammalian vertebrates. Most, but not all, of these FABP10s have basic *pI*s (Table 2.1) (Ceciliani et al., 1994; Schleicher and Santome, 1996; Di Pietro et al., 1997; Cordoba et al., 1999; Di Pietro et al., 1999; Denovan-Wright et al., 2000b; Di Pietro and Santome, 2001; Jordal et al., 2006). As such, the more appropriate name for this protein and its gene should be FABP10, as proposed by Hertzel and Bernlohr (2000), not liver basic-type FABP.

Multiple sequence alignment of the duplicated zebrafish Fabp10b sequence with amphibian, reptile, fish, bird and mammal FABP/Fabp sequences was performed using

Table 2.1 Isoelectric point (*pI*) of FABP10s in different species.

Species	**Protein**	***pI***
Zebrafish	Fabp10b	5.94
Zebrafish	Fabp10a	8.87
Shark	FABP10	8.69
Catfish	Fabp10	9.1
Lungfish	Fabp10	6.97
Salamander	FABP10	7.1
Toad	FABP10	6.8
Iguana	FABP10	8.64
Chicken	FABP10	9.0
Salmon	Fabp10	8.52
Stickleback	Fabp10a	8.83
Stickleback	Fabp10b	6.61
Medaka	Fabp10a	8.40
Medaka	Fabp10b	7.77
Tetraodon	Fabp10	8.69

CLUSTALW (Thompson et al., 1994). Zebrafish Fabp10b showed the highest sequence identity and similarity (48% and 74%, respectively) with shark FABP10, and the next highest sequence identity and similarity with zebrafish Fabp10a (46% and 75%, respectively) (Fig. 2.2). The sequence alignment strongly suggests that the expressed sequence tags and the genomic sequence found in our database search code for the duplicated copy of Fabp10 in zebrafish. The evolutionary relationship of the zebrafish *fabp10b* gene with other identified vertebrate iLBP genes was revealed by phylogenetic analysis (Fig. 2.3). A bootstrap neighbour-joining phylogenetic tree was constructed using MEGA4 software (Tamura et al., 2007), with the human lipocalin 1 protein sequence as an outgroup to root the tree. The zebrafish Fabp10a and Fabp10b sequences clustered with the amphibian, reptile, fish and bird FABP10/Fabp10 sequences in the same clade (bootstrap value of 56/100). The single copy of the *fabp10* gene found in the Tetraodon genome sequence database may indicate that the genome of this fish has lost one of the duplicated copies of this gene following the fish-specific WGD event, or that the genome sequence database is incomplete.

2.4.2 The zebrafish *fabp10a* and *fabp10b* genes arose from a fish-specific WGD event

Chromosome [linkage group (LG)] assignment of zebrafish *fabp10b* was determined by radiation hybrid mapping using the LN54 panel (Hukriede et al., 1999). The *fabp10b* gene was mapped to chromosome (LG) 19 at a distance of 0.30 CentiRays from marker Z160 with a logarithm of odds to the base10 score of 16.6. Zebrafish *fabp10a* has been assigned previously to chromosome (LG) 16 by the same LN54

Figure 2.2 Sequence alignment of zebrafish Fabp10 with FABPs from various vertebrates. The deduced amino acid sequences of zebrafish Fabp10b (Zf-Fabp10b; XP_001335329), Fabp10a (Zf-Fabp10a; NP_694492), Fabp1b (Zf-Fabp1b; NP_001019822), Fabp3 (Zf-Fabp3; NP_694493), Fabp7a (Zf-Fabp7a; NP_571680), Fabp1a (Zf-Fabp1a; NP_001038177), chicken FABP10 (Ch-FABP10; P80226), FABP1 (Ch-FABP1; NP_989523), shark FABP10 (Sh-FABP10; P81653), catfish Fabp10 (Cf-Fabp10; P80856), iguana FABP10 (Ig-FABP10; AAA68960), salamander FABP10 (Sa-FABP10; P81400), toad FABP10 (To-FABP10; P83409), fugu Fabp1 (Fu-Fabp1; O42494), stickleback Fabp10a (St Fabp10a, BT027383), stickleback Fabp10b (St Fabp10b, Ensembl no. ENSBACG00000002234), medaka Fabp10a (Me Fabp10a, Ensembl no. ENSORLG00000014794) and medaka Fabp10b (Me Fabp10b, Ensembl no. ENSORLG00000007702) were aligned using CLUSTALW. Dots specify amino acid identity and dashes represent gaps. The percentage sequence identity and similarity of zebrafish, shark, chicken, iguana, salamander, toad, fugu, stickleback and medaka FABP sequences with zebrafish Fabp10b are shown at the end of each sequence.

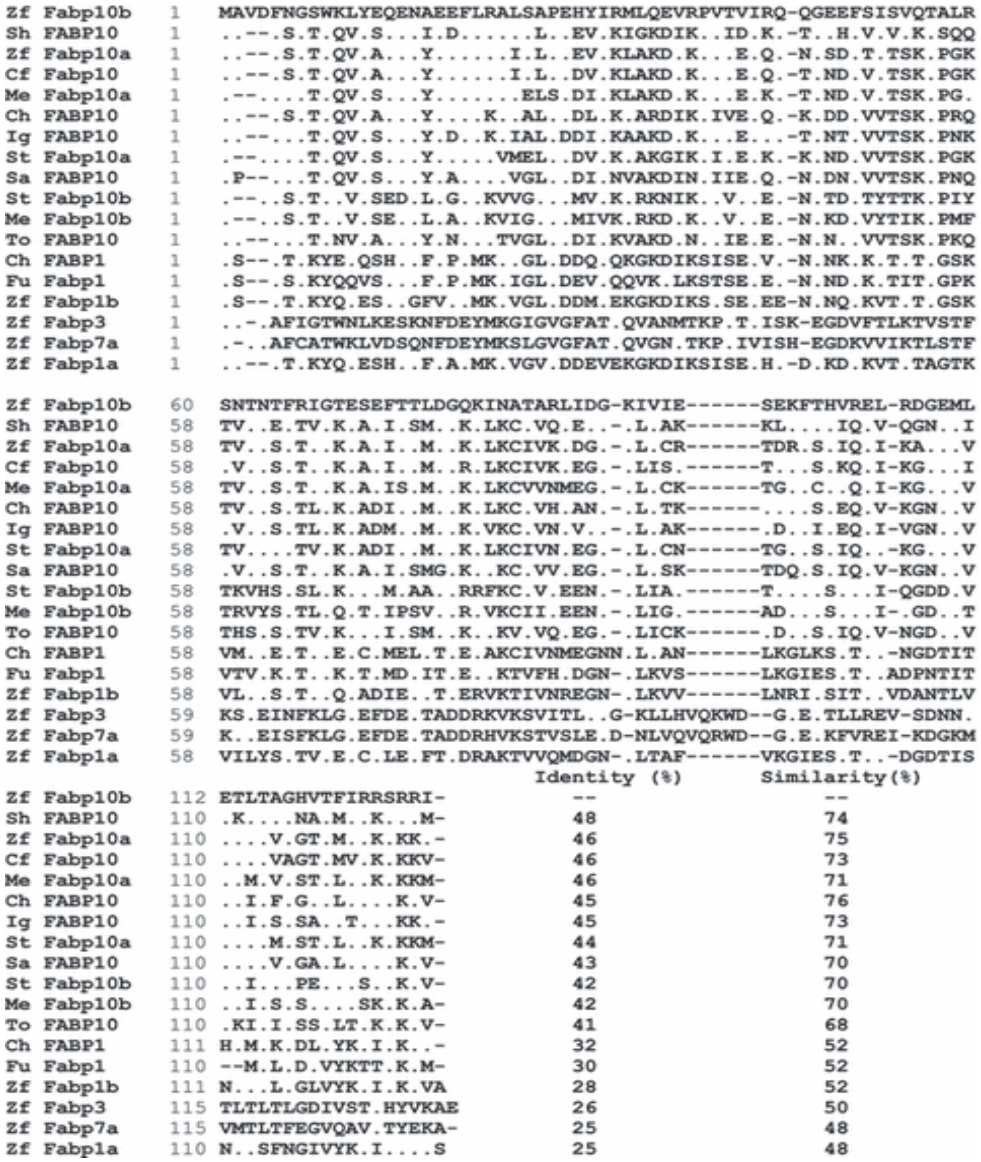

Figure 2.2 Sequence alignment of zebrafish Fabp10 with FABPs from various vertebrates.

Figure 2.3 A neighbour-joining tree showing the phylogenetic relationship of zebrafish Fabp10b with selected paralogous and orthologous FABPs/Fabps from zebrafish and mammals. The human lipocalin 1 protein sequence (Hu LCN1, GenBank accession no. NP_002288) was used as an outgroup. The bootstrap values, as a percentage (based on 100 replicates), are indicated above or under each node. The distinct clade of FABP10/Fabp10s is shaded in grey. The amino acid sequences used in this analysis were zebrafish Fabp10a (Zf Fabp10a, NP_694492), zebrafish Fabp10b (Zf Fabp10b, XP_001335329), zebrafish Fabp1a (Zf Fabp1a, NP_001038177), zebrafish Fabp1b (Zf Fabp1b, NP_001019822), zebrafish Fabp2 (Zf Fabp2, NP_571506), zebrafish Fabp3 (Zf Fabp3, NP_694493), zebrafish Fabp6 (Zf Fabp6, NP_001002076), zebrafish Fabp7a (Zf Fabp7a, NP_571680), zebrafish Fabp7b (Zf Fabp7b, NP_999972), zebrafish Fabp11a (Zf Fabp11a, NP_001004682), zebrafish Fabp11b (Zf Fabp11b, NP_001018394), human FABP1 (Hu FABP1, NP_001434), human FABP2 (Hu FABP2, NP_000125), human FABP3 (Hu FABP3, NP_004093), human FABP4 (Hu FABP4, NP_001433), human FABP5 (Hu FABP5, NP_001435), human FABP6 (Hu FABP6, NP_001436), human FABP7 (Hu FABP7, NP_001437), chicken FABP1 (Ch FABP1, NP_989523), chicken FABP2 (Ch FABP2, NP_001007924), chicken FABP10 (Ch FABP10, P80226), rat FABP1 (Ra FABP1, NP_036688), rat FABP2 (Ra FABP2, NP_037200), mouse FABP1 (Mo FABP1, NP_059095), mouse FABP2 (Mo FABP2, NP_032006), pig FABP1 (Pi FABP1, NP_001004046), pig FABP6 (Pi FABP6, NP_999380), shark FABP10 (Sh FABP10, P81653), catfish Fabp10 (Cf Fabp10, P80856), lungfish Fabp10 (Lf Fabp10, P82289), salamander FABP10 (Sa FABP10, P81400), toad Fabp10 (To Fabp10, P83409), iguana Fabp10 (Ig FABP10, AAA68960), fugu Fabp1 (Fu Fabp1, O42494), Tetraodon Fabp10 (Te Fabp10, CAF89192), stickleback Fabp10a (St Fabp10a, BT027383), stickleback Fabp10b (St Fabp10b, Ensembl no. ENSBACG00000002234), medaka Fabp10a (Me Fabp10a, Ensembl no. ENSORLG00000014794) and medaka Fabp10b (Me Fabp10b, Ensembl no. ENSORLG00000007702). Scale bar, 0.2 substitutions per site.

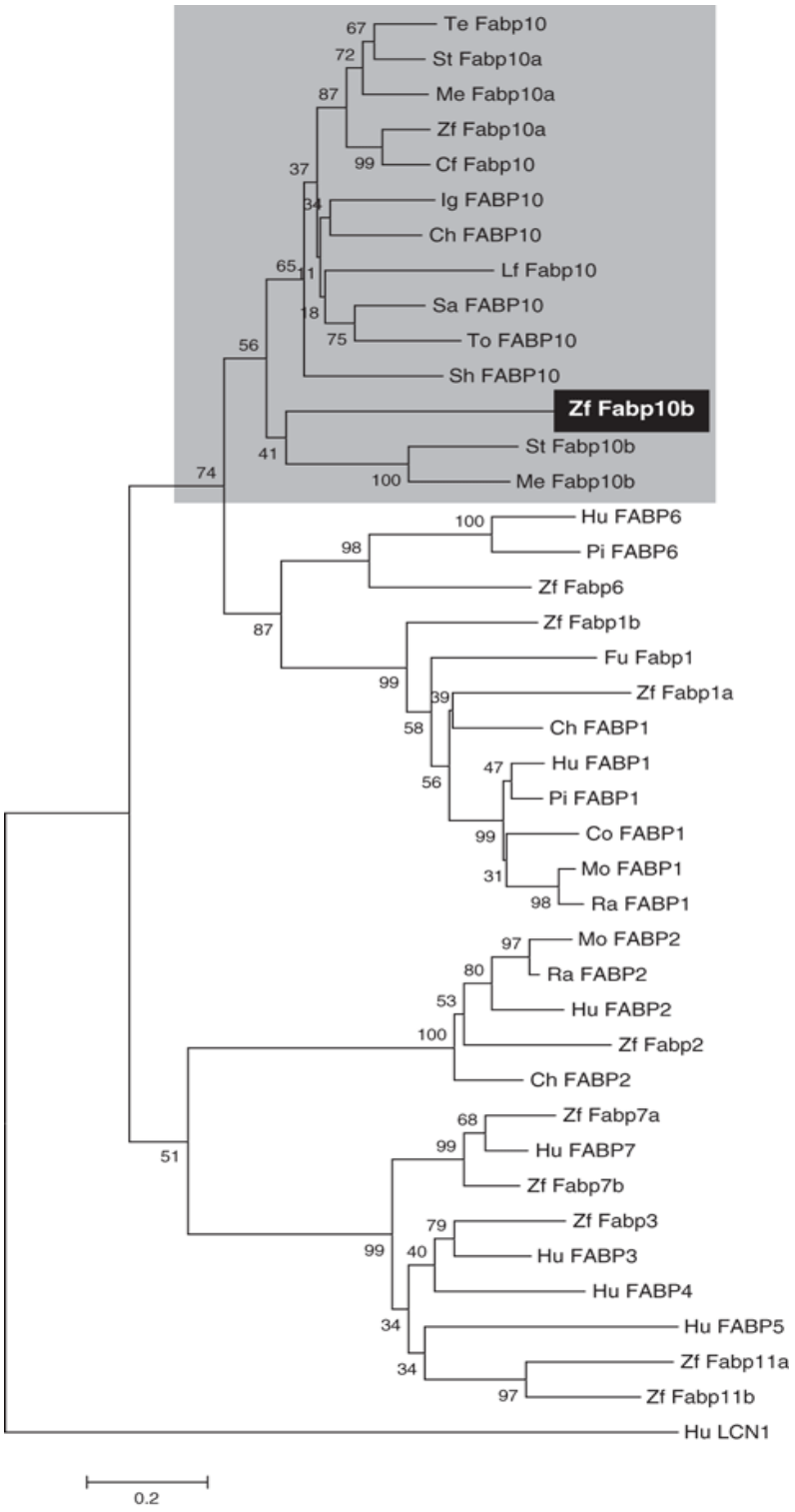

Figure 2.3 A neighbour-joining tree showing the phylogenetic relationship of zebrafish Fabp10b with selected paralogous and orthologous FABPs/Fabps from zebrafish and mammals.

radiation hybrid panel (Sharma et al., 2006). Based on data obtained from LocusLink (http://www.ncbi.nlm.nih.gov/), we found that the zebrafish *fabp10a* and *fabp10b* genes exhibit conserved gene synteny with the chicken *FABP10* gene on chromosome 23 (Fig. 2.4), indicating that they are orthologous genes arising from the same ancestral gene, most probably as a result of a WGD event early in the radiation of the ray-finned fishes (Furlong and Holland, 2002; Mulley and Holland, 2004; Vandepoele et al., 2004).

2.4.3 Distribution of *fabp10b* transcripts in zebrafish embryos and larvae

The spatiotemporal distribution of zebrafish *fabp10b* transcripts during embryonic and larval development was determined by whole-mount *in situ* hybridization (Fig. 2.5). Transcripts of zebrafish *fabp10b* were not detected in embryos at 24 h post-fertilization (hpf), but a distinct hybridization signal was detected in the olfactory vesicles of the developing embryos at 36 hpf (Fig. 2.5A, B). The hybridization signal remained in the olfactory vesicles throughout development and was more prominent by 5 days post-fertilization (dpf) (Fig. 2.5C, D), the last stage of development assayed. Initiation of *fabp10b* gene transcription therefore occurred between 24 and 36 hpf. In contrast with *fabp10b*, transcripts of *fabp10a* were only detected in liver in 48 hpf embryos, and continued to be detected only in the liver up to the last developmental stage assayed: 5 dpf larvae (Sharma et al., 2006). Kurtz et al. (1994), reported high levels of *fabp7* transcripts in the olfactory bulb of mice and also characterized this protein as a potential brain morphogen during development. The presence of zebrafish *fabp10b* transcripts in olfactory vesicles indicates a potential role for this protein in the early development of the zebrafish brain.

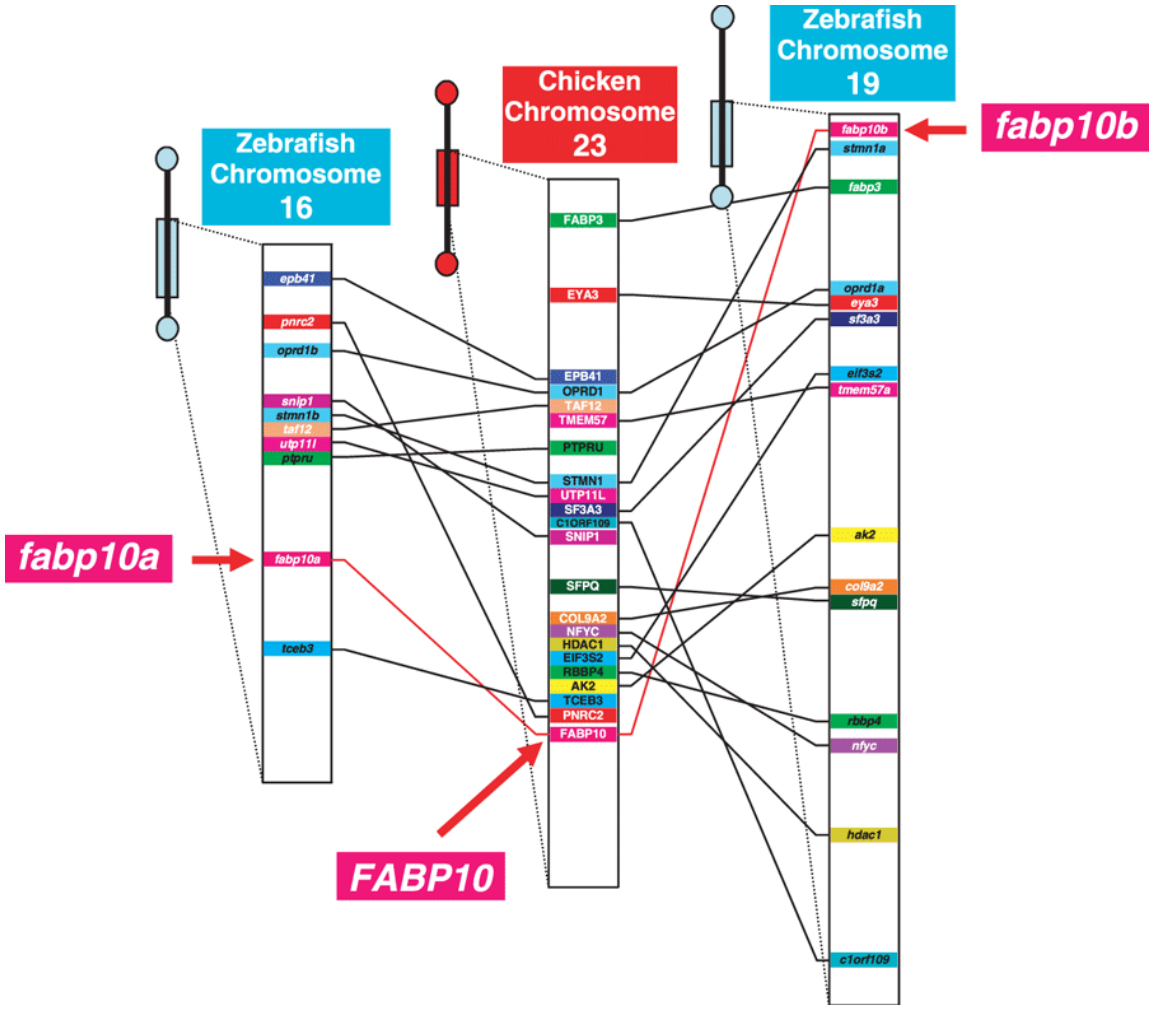

Figure 2.4 Conserved gene synteny of the duplicated copies of zebrafish *fabp10* with chicken *FABP10*. Both the zebrafish *fabp10a* gene on chromosome 16 and *fabp10b* gene on chromosome 19 show conserved gene synteny with the chicken *FABP10* gene on chromosome 23, which suggests that the zebrafish chromosomes 16 and 19 arose from duplication of a chromosome homologous with the chicken chromosome 23.

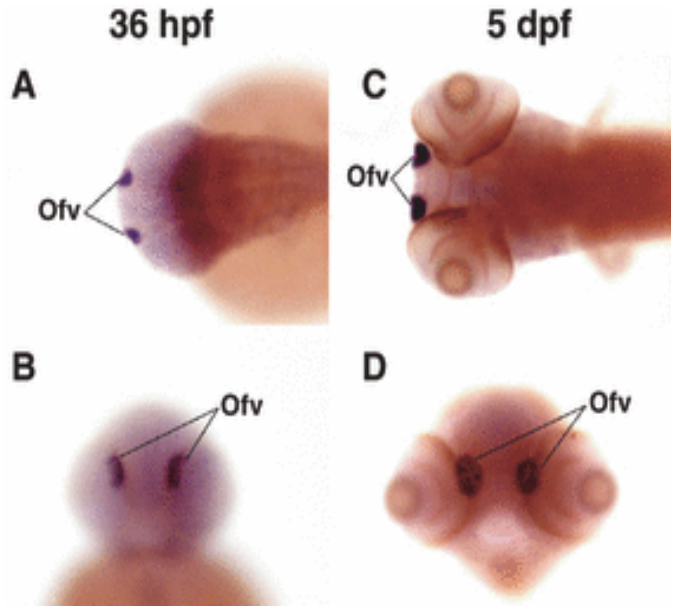

Figure 2.5 Spatiotemporal distribution of *fabp10b* gene transcripts in zebrafish embryos and larvae determined by whole-mount *in situ* hybridization. *fabp10b* transcripts were first detected in olfactory vesicles (Ofv) at 36 h post-fertilization (hpf) (A, B). The hybridization signal from*fabp10b* transcripts in the olfactory vesicles became more intense during development up to 5 days post-fertilization (dpf), the last developmental stage assayed (C, D). Dorsal view of head (A, C). Frontal view of head (B, D).

2.4.4 Tissue-specific distribution of *fabp10b* gene transcripts in adult zebrafish

The tissue-specific distribution of *fabp10b* transcripts in adult zebrafish was determined by RT-PCR amplification from total RNA extracted from various tissues. A *fabp10b*-specific RT-PCR product of the expected size was amplified from total RNA extracted from liver, intestine, muscle, brain, heart, eye, gills, ovary, testis, skin, kidney and swimbladder (Fig. 2.6A, top panel). To determine the integrity of the RNA samples used in these assays, transcripts for the constitutively expressed *ef1α* gene were amplified by RT-PCR, and an RT-PCR product of the expected size was generated from total RNA extracted from all the tissues assayed (Fig. 2.6A, bottom panel). We quantified the *fabp10b* transcripts in the same tissues of adult zebrafish by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using the *ef1α* transcripts as a positive control. The steady-state level of *fabp10b* transcripts in the tissue samples ranged between 1.9×10^2 and 5.1×10^4 copies per microlitre of cDNA. RT-PCR products of *ef1α* were amplified from each cDNA sample, and the levels ranged from 3.2×10^3 to 2.6×10^6 copies per microlitre. The ratio of the steady-state levels of transcripts for *fabp10b*/*ef1α* for each experimental sample was calculated (Fig. 2.6B). This analysis showed that the levels of *fabp10b* mRNA in muscle and heart were 6–24 times higher than in brain, eye, gills, testis, skin, kidney and swimbladder, and 320–650 times higher than in liver, intestine and ovary. Both RT-PCR and RT-qPCR showed similar tissue-specific patterns of distribution for *fabp10b* transcripts in which *fabp10b* mRNA was most abundant. The abundance of *fabp10b* transcripts in muscle and heart suggests that *fabp10b* may play an important role in lipid homeostasis in these tissues. In contrast

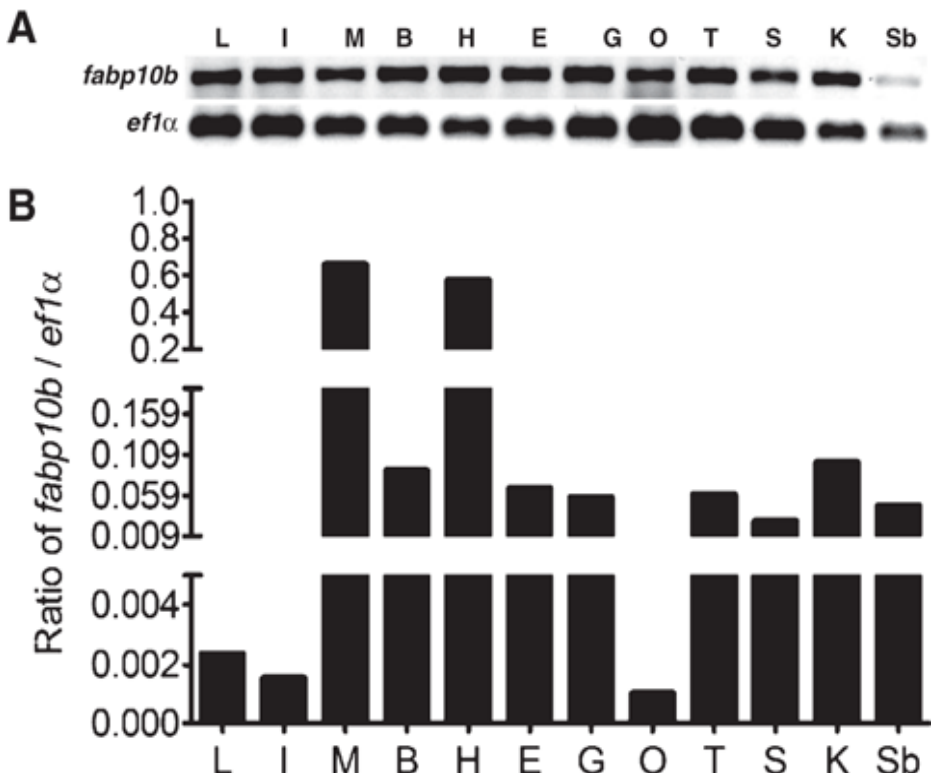

Figure 2.6 Tissue-specific distribution of *fabp10b* transcripts in adult zebrafish. (A) Zebrafish *fabp10b* cDNA-specific primers amplified an RT-PCR product from total RNA extracted from the liver (L), intestine (I), muscle (M), brain (B), heart (H), eye (E), gills (G), ovary (O), testis (T), skin (S), kidney (K) and swimbladder (Sb) (top panel). As a positive control, constitutively expressed *ef1α* transcripts were detected by RT-PCR in RNA extracted from the same tissues (bottom panel). (B) RT-qPCR showed that zebrafish*fabp10b* transcripts were most abundant in RNA extracted from adult zebrafish muscle (M) and heart (H). *fabp10b* mRNA was also detected in liver (L), intestine (I), brain (B), eye (E), gills (G), ovary (O), testis (T), skin (S), kidney (K) and swimbladder (Sb).

with zebrafish *fabp10b*, *fabp10a* transcripts were detected only in the liver, intestine and testis of adult zebrafish (Sharma et al., 2006).

2.4.5 Are the duplicated *fabp10* genes retained in the zebrafish genome owing to the neofunctionalization of *fabp10b*?

Based on sequence identity, phylogenetic analysis and conserved gene synteny with the chicken *FABP10* gene, zebrafish *fabp10a* and *fabp10b* arose by either a chromosomal duplication or, more likely, by the WGD event in the ray-finned fishes. In previous studies, we have identified pairs of genes for several paralogous members of the iLBP multigene family in the zebrafish genome, *fabp7a*/*fabp7b* (Liu et al., 2004), *rbp1a*/*rbp1b*, *rbp2a*/*rbp2b* (Liu et al., 2005), *crabp2a*/*crabp2b* (Sharma et al., 2005) and *fabp11a*/*fabp11b* (Karanth et al., 2008), that were duplicated by the same WGD event. Retention of duplicated copies of iLBP genes in the zebrafish genome appears to be a common feature for this multigene family. Moreover, this observation is consistent with the hypothesis of 'large-scale gene duplication in fishes' (Gates et al., 1999; Postlethwait et al., 2000; Woods et al., 2000; Robinson-Rechavi et al., 2001; Taylor et al., 2001; Taylor et al., 2003; Van de Peer, 2004).

In 1970, Ohno suggested two possible fates for duplicated genes: nonfunctionalization, in which mutations accumulate in the protein coding region, leading to gene silencing and subsequent loss from the genome, the most common fate of a duplicated gene; and neofunctionalization, in which mutation in the protein coding region of a gene results in a novel function for that protein of benefit to the organism. As such, the process of neofunctionalization leads to the retention of both copies of the duplicated sister genes in the genome. Data derived from genome sequencing projects

suggest that a much higher proportion of gene duplicates is preserved in the genome than predicted by Ohno's neofunctionalization model. Force et al. (1999), subsequently elaborated by Lynch and Conery (2000), however, proposed the degeneration–duplication–complementation model in which subfunctionalization, an alternative mechanism to neofunctionalization, is responsible for the retention of duplicated genes in the genome. In subfunctionalization, the functions of the ancestral gene are subdivided between the duplicated genes. Moreover, this new conceptual framework for understanding the fate of duplicated genes focused on the regulatory complexity of eukaryotic genes, *i.e.* the evolution of DNA elements that regulate the spatiotemporal transcription of duplicated genes. Although subfunctionalization was proposed as an alternative mechanism to neofunctionalization in the degeneration–duplication–complementation model to explain the high retention rate of duplicate genes in the genome, Force et al. (1999), did not exclude neofunctionalization in their degeneration–duplication–complementation model, where one of the duplicated genes acquires new regulatory elements in its promoter, as a possible process for the retention of duplicated genes in the genome.

Transcripts of *fabp10a* were detected in liver, intestine and testis of adult zebrafish and only in the liver of zebrafish embryos and larvae (Sharma et al., 2006). By contrast, zebrafish *fabp10b* transcripts were only detected in the olfactory vesicles of embryos and larvae (Fig. 2.5), and in the liver, intestine, muscle, brain, heart, eye, gills, ovary, testis, skin, kidney and swimbladder in adult zebrafish (Fig. 2.6A, B). Clearly, *fabp10a* and *fabp10b* transcripts in embryos, larvae and adult zebrafish show strikingly different tissue-specific patterns of distribution. On the basis of the distribution of

fabp10b transcripts in many tissues of adult zebrafish, compared with the limited distribution of zebrafish *fabp10a transcripts*, and chicken *FABP10* (the presumed ancestral state of the *fabp10* gene prior to duplication) transcripts, which are restricted to the liver in adults (Murai et al., 2009), we propose that both the zebrafish *fabp10a* and *fabp10b* genes were retained in the genome owing to neofunctionalization of *fabp10b*.

CHAPTER 3 : TISSUE-SPECIFIC DIFFERENTIAL INDUCTION OF DUPLICATED FATTY ACID-BINDING PROTEIN GENES BY THE PEROXISOME PROLIFERATOR, CLOFIBRATE, IN ZEBRAFISH (*Danio rerio*)

The manuscript based on this study is presented below.

Co-authors for this manuscript are Santosh P. Lall, Eileen M. Denovan-Wright and Jonathan M. Wright.

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Author's contributions:

ABV and JMW conceived and designed the studies. ABV carried out the experimental work and statistical analysis. SPL assisted in the formulation of zebrafish diet. EMD-W assisted in the design and interpretation of RT-qPCR analysis. ABV and JMW drafted the manuscript with subsequent editorial comments from SPL and EMD-W. All authors read and approved the final version of the manuscript.

3.1 Abstract

Force, Lynch and Conery proposed the duplication-degeneration-complementation (DDC) model in which partitioning of ancestral functions (subfunctionalization) and acquisition of novel functions (neofunctionalization) were the two primary mechanisms for the retention of duplicated genes. The DDC model was tested by analyzing the transcriptional induction of the duplicated fatty acid-binding protein (*fabp*) genes by clofibrate in zebrafish. Clofibrate is a specific ligand of the peroxisome proliferator-activated receptor (PPAR); it activates PPAR which then binds to a peroxisome proliferator response element (PPRE) to induce the transcriptional initiation of genes primarily involved in lipid homeostasis. Zebrafish was chosen as our model organism as it has many duplicated genes owing to a whole genome duplication (WGD) event that occurred ~230-400 million years ago in the teleost fish lineage. We assayed the steady-state levels of *fabp* mRNA and heterogeneous nuclear RNA (hnRNA) transcripts in liver, intestine, muscle, brain and heart for four sets of duplicated *fabp* genes, *fabp1a/fabp1b.1/fabp1b.2*, *fabp7a/fabp7b*, *fabp10a/fabp10b* and *fabp11a/fabp11b* in zebrafish fed different concentrations of clofibrate. Electron microscopy showed an increase in the number of peroxisomes and mitochondria in liver and heart, respectively, in zebrafish fed clofibrate. Clofibrate also increased the steady-state level of *acox1* mRNA and hnRNA transcripts in different tissues, a gene with a functional PPRE. These results demonstrate that zebrafish is responsive to clofibrate, unlike some other fishes. The levels of *fabp* mRNA and hnRNA transcripts for the four sets of duplicated *fabp* genes was determined by reverse transcription, quantitative polymerase chain reaction (RT-qPCR). The level of hnRNA coded by a gene is an indirect estimate of the rate of

transcriptional initiation of that gene. Clofibrate increased the steady-state level of *fabp* mRNAs and hnRNAs for both the duplicated copies of *fabp1a/ fabp1b.1*, and *fabp7a/fabp7b*, but in different tissues. Clofibrate also increased the steady-state level of *fabp10a* and *fabp11a* mRNAs and hnRNAs in liver, but not for *fabp10b* and *fabp11b*. Some duplicated *fabp* genes have, most likely, retained PPREs, but induction by clofibrate is over-ridden by an, as yet, unknown tissue-specific mechanism(s). Regardless of the tissue-specific mechanism(s), transcriptional control of duplicated zebrafish *fabp* genes by clofibrate has markedly diverged since the WGD event.

3.2 Introduction

In 1970, Ohno proposed that duplication of individual genes, chromosomal segments or whole genomes plays an important role in genome evolution, provides for increasing organismal complexity and contributes to morphological diversification among vertebrates (Holland et al., 1994; Sidow, 1996; Robinson-Rechavi et al., 2001). The role of gene duplicates in generating morphological and functional diversity has been discussed by various researchers (see (Taylor and Raes, 2004) and references therein). To our knowledge, Ohno was the first to suggest possible fates for duplicated genes by the process of either nonfunctionalization or neofunctionalization. Nonfunctionalization of a duplicated gene occurs due to deleterious mutations accumulating in the protein coding region, leading to gene silencing and subsequent loss of one of the duplicate genes from the genome. Ohno further argued that nonfunctionalization is the common fate of a duplicated gene. Neofunctionalization results from mutations in the protein coding region that gives rise to a novel function for a gene product. If this novel function benefits the organism, the gene will be retained in the genome. With complete genomic DNA sequences becoming increasingly available, it is apparent that a greater proportion of gene duplicates are preserved in genomes than that predicted by Ohno's model (Force et al., 1999). In light of these observations, Force et al. (1999), subsequently elaborated by Lynch and Conery (2000), proposed the duplication-degeneration-complementation (DDC) model. In the DDC model, subfunctionalization is the process by which the functions of the ancestral gene are subdivided between the duplicated genes. Subfunctionalization in the DDC model was proposed as an alternative mechanism to Ohno's neofunctionalization (1970) to explain the high retention rate of duplicated genes

in the genome. Force et al. (1999), however, did not exclude neofunctionalization, in which one of the duplicated genes acquires a novel function. In the DDC model, subfunctionalization and neofunctionalization occur by either loss or gain of *cis*-regulatory elements in the promoters of the duplicated genes.

Fatty acid-binding protein (*FABP*) genes belong to the multigene family of intracellular lipidbinding protein (iLBP) genes that also includes the cellular retinol-binding protein (*CRBP*) and the cellular retinoic acid-binding protein (*CRABP*) genes (Bernlohr et al., 1997; Schaap et al., 2002; Haunerland and Spener, 2004; Wolfrum, 2007; Storch and Corsico, 2008). To date, eighteen paralogous iLBP genes, including 12 *FABPs*, 4 *CRBPs* and 2 *CRABPs* have been identified in the animal kingdom. No *FABP* genes have been found in plants or fungi, leading Schaap et al. (2002) to suggest that the first FABP gene emerged after the divergence of animals from plants, some 930–1000 million years ago (mya). About 230–400 mya, the iLBP multigene family was further augmented in teleost fishes by a whole genome duplication (WGD) event early in this fish lineage (Woods et al., 2000; Robinson-Rechavi et al., 2001; Furlong and Holland, 2002; Christoffels et al., 2004; Jaillon et al., 2004; Vandepoele et al., 2004). Based on complementary DNA (cDNA) sequence, gene structure, conserved gene synteny with their mammalian, avian and fish orthologs, and spatio-temporal patterns of expression, we have characterized 12 zebrafish *fabp* genes (Denovan-Wright et al., 2000a; Denovan-Wright et al., 2000b; Pierce et al., 2000; Liu et al., 2003a; Liu et al., 2003b; Liu et al., 2004; Sharma et al., 2004; Sharma et al., 2006; Liu et al., 2007; Alves-Costa et al., 2008; Karanth et al., 2008; Karanth et al., 2009a; Venkatachalam et al., 2009). Of these 12 zebrafish *fabp* genes, eight (four pairs) *fabp1a/fabp1b*, *fabp7a/fabp7b*, *fabp10a/fabp10b*

and *fabp11a/fabp11b* arose as a result of the teleost fish-specific WGD (Liu et al., 2004; Sharma et al., 2006; Karanth et al., 2008; Venkatachalam et al., 2009). One pair of duplicated genes, *fabp1b.1* and *fabp1b.2*, is tandemly arrayed on chromosome 8 separated by 3.8 kb of DNA (Karanth et al., 2009a). This duplication, subsequent to the WGD early in the fish lineage, is presumably the result of unequal crossing-over between homologous chromosomes during meiosis. The total number of duplicated genes retained in the zebrafish genome following the WGD event is estimated to be 14-30% (Postlethwait et al., 2000; Woods et al., 2005). Surprisingly, 73% of the duplicated *fabp* genes have been retained in the zebrafish genome. Only three zebrafish *fabp* genes exist as single copies, *fabp2*, *fabp3* and *fabp6*. Originally, FABPs were named according to their initial tissue of isolation. This nomenclature has become increasingly confusing as some tissues contain more than one FABP, and some FABPs are found in many tissues. As such, we have chosen to use the nomenclature proposed by Hertzel and Bernlohr (2000) *e.g*. FABP1, FABP2, *etc*. Although different *FABP* genes exhibit distinct, but sometimes overlapping, tissue-specific patterns of expression, the tertiary structure of *FABP* genes and their genomic organization are highly conserved (Ong et al., 1994; Glatz and van der Vusse, 1996; Storch and Corsico, 2008). Almost all *FABP/fabp* genes, with the exception of the *FABP3* gene in desert locust (Wu et al., 2001), the *fabp1a* gene from zebrafish (Sharma et al., 2006) and *fabp11a* gene from medaka (Parmar et al., 2012a), consist of four exons of comparable coding capacity separated by three introns of varying sizes between paralogous and orthologous *FABP/fabp* genes in different species (Ong, 1994; Veerkamp and Maatman, 1995; Bernlohr et al., 1997; Schaap et al., 2002; Zimmerman and Veerkamp, 2002). Despite extensive studies on the structure of FABPs,

binding properties and *in vitro* lipid transfer mechanisms, their precise physiological role remains elusive. However, several studies [reviewed in Storch and Corsico, 2008] have implicated FABPs in myriad cellular processes that include: (1) binding and sequestering of long-chain fatty acids, bile salts and other hydrophobic ligands; (2) transport of these ligands to intracellular compartments for metabolism and energy production; (3) interaction with other enzyme systems and transport proteins; and (4) transport of fatty acids (FAs) to the nucleus to regulate gene transcription *via* activation of the nuclear receptors, the peroxisome proliferator-activated receptors (PPARs) (see (Leaver et al., 2005) and references therein). Currently, our knowledge of the regulatory elements controlling the expression of the *FABP* genes is limited and based mainly on studies of mammalian *FABP* genes and one *FABP* gene in desert locust (Meunier-Durmort et al., 1996; Poirier et al., 2001; Wu and Haunerland, 2001; Schachtrup et al., 2004; Qu et al., 2007). Her et al. (2003b, 2004b) cloned the 5' upstream regions, including the basal promoters, of the zebrafish *fabp10a* and *fabp2* genes. They identified a 435 base pair (bp) region with two distinct liver regulatory elements in the liver basic fatty acid-binding protein (*fabp10a*) gene, which is sufficient to modulate liver regional expression in transgenic zebrafish (Her et al., 2003b). A 192 bp region was identified in the 5' upstream region of the intestinal-type fatty acid-binding protein (*fabp2*) gene sufficient to direct intestine-specific expression in zebrafish larval development (Her et al., 2004b). Neither of these studies provided insight into why both duplicated *fabp10* genes, *fabp10a* and *fabp10b* were retained in the zebrafish genome, or why a copy of the duplicated *fabp2* gene was lost from the zebrafish genome following the WGD in the teleost fishes.

In previous studies, we have shown that transcriptional initiation of only one copy in each of three sets of duplicated *fabp* genes of zebrafish, *fabp1a/fabp1b.1/fabp1b.2*, *fabp7a/fabp7b* and *fabp11a/fabp11b*, is modulated by dietary FAs in a given tissue (Karanth et al., 2009b). Since FAs are known to be ligands of PPARs that leads to transcriptional up-regulation of target genes, we anticipated that the transcriptional modulation of *fabp* genes in various tissues of zebrafish fed different FAs might be mediated by PPARs. The goal of the present research was, therefore, to investigate whether the duplicated *fabp* genes in zebrafish are differentially regulated by PPAR, by using clofibrate, a PPAR agonist. Clofibrate has been used extensively to investigate the regulation of gene transcription in vertebrates, owing to its specific binding with PPARα, and to a lesser extent to PPARγ, and its effect on the transcription of specific genes involved in lipid metabolism (Yamoto et al., 1996; Haasch et al., 1998; Akbiyik et al., 2004; Nunes et al., 2004; Konig et al., 2007; Luci et al., 2007; Ringseis et al., 2007; Rorvik et al., 2007). We assayed the steady state levels of *fabp* mRNA and heterogeneous nuclear RNA (hnRNA) transcripts for four sets of duplicated *fabp* genes, *fabp1a/fabp1b.1/fabp1b.2*, *fabp7a/fabp7b*, *fabp10a/fabp10b* and *fabp11a/fabp11b* in zebrafish fed different concentrations of clofibrate to determine if clofibrate induced transcriptional initiation of only one of a pair of duplicated *fabp* genes. We show here, however, that clofibrate induced the transcriptional initiation of both pairs of some duplicated *fabp* genes in zebrafish, but the induction is differentially regulated by an, as yet, unknown tissue-specific mechanism(s).

3.3 Materials and methods

3.3.1 Experimental diet and zebrafish husbandry

Experimental diets containing five different concentrations (0, 0.25, 0.50, 0.75 and 1.00% w/w) of clofibrate (Sigma-Aldrich, Oakville, Ontario, Canada) were formulated (Table 3.1). Clofibrate concentrations and basic feed formulation were based on previous dietary studies (National Research Council, 1993; Goolish et al., 1999; Meinelt et al., 1999; Rorvik et al., 2007) and the United States National Research Council's nutrient requirement recommendations for warm-water fishes (National Research Council, 1993). The dry ingredients were mixed using a Hobart mixer for 20 min. Choline chloride was dissolved in distilled water and clofibrate mixed incorn oil prior to addition to dry ingredients. Boiling water was added to the dry ingredients tomake wet dough (40% v/v). The dough was spread on tray and freeze-dried for 36–48 h. The freeze-dried diet was then passed through a 0.8 mm mesh to yield particles of less than 800 μm, which were then stored at −20°C.

To reduce genetic variance, four female and two male adult zebrafish of the AB strain (Bradford et al., 2011), obtained from the Aquatron at Dalhousie University, were bred in a single tank to produce embryos. Embryos, larvae and adult fish were maintained in aerated water at 28.5 °C on a 14 h light and 10 h dark cycle (Westerfield, 2000). One hundred and fifty day-old zebrafish were acclimatized in 25 L aquaria for four weeks prior to feeding fish diets containing clofibrate. Three replicates of five different dietary groups of fish were distributed in 15 tanks in a randomized complete block design. Each tank contained 15 zebrafish. Fish in each tank were maintained under the same light intensity and photoperiod. After acclimatization for a week, fish were fed the

Table 3.1 Composition of diets (% by weight)

Ingredients	0% clofibrate diet	0.25% clofibrate diet	0.50% clofibrate diet	0.75% clofibrate diet	1.00% clofibrate diet
Vitamin free casein[a]	33	33	33	33	33
Wheat gluten[b]	10	10	10	10	10
Gelatin[a]	4	4	4	4	4
Corn oil[c]	4	4	4	4	4
Fish oil[d]	4	4	4	4	4
Corn starch[e]	33	33	33	33	33
Celufil[l]	8.00	7.75	7.50	7.25	7.00
Vitamin mix[f]	1.30	1.30	1.30	1.30	1.30
Mineral mix[g]	1	1	1	1	1
Betaine[h]	1.50	1.50	1.50	1.50	1.50
DL-Methionine[a]	0.20	0.20	0.20	0.20	0.20
Clofibrate[i]	0	0.25	0.50	0.75	1.00
Total	100	100	100	100	100

[a] US Biochemical. (Cleveland, OH, USA).
[b] Dover Mills Ltd. (Halifax, NS, Canada).
[c] Sobeys Inc. (Halifax, NS, Canada).
[d] Corey Feeds Ltd. (Fredericton, NB, Canada).
[e] National Starch and Chemical Co. (Bridgewater, NJ, USA).
[f] Vitamin added to supply the following (per kg diet): vitamin A, 8000 IU; vitamin D3, 4000 IU; vitamin E, 300 IU; menadione sodium bisulfite, 40 mg; Thiamine HCl, 50 mg; riboflavin, 70 mg; d-Ca pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin B12, 0.15 mg; niacin, 300 mg; pyridoxine HCl, 20 mg; ascorbic acid, 300 mg; inositol, 400 mg; choline chloride, 2000 mg; butylated hydroxy toluene, 15 mg; butylated hydroxy anisole, 15 mg.
[g] Mineral added to supply the following (per kg diet): manganous sulphate (32.5% Mn), 40 mg; ferrous sulphate (20.1% Fe), 30 mg; copper sulphate (25.4% Cu), 5 mg; zinc sulphate (22.7% Zn), 75 mg; sodium selenite (45.6% Se), 1 mg; cobalt chloride (24.8% Co), 2.5 mg; sodium fluoride (42.5% F), 4 mg.
[h] Betaine anhydrous (96% feed grade). (Finnfeeds, Finland).
[i] Sigma-Aldrich Inc. (St. Louis, MO, USA).

experimental diets twice a day to satiation. At the end of four weeks, fish were anaesthetized by immersion in a solution of 0.20% (v/v) MS-222 prior to tissue dissection. Dissection of fish was done on ice. From each fish, liver, intestine, muscle, brain and heart were removed. All experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals in accordance with the recommendations of the Canadian Council on Animal Care.

3.3.2 Electron microscopy to visualize peroxisomes and mitochondria

Tissue samples (liver, intestine, muscle, brain and heart) from 0, 0.25, 0.50, 0.75 and 1.00% (w/w) clofibrate-fed fish were dissected and transferred to centrifuge tubes containing 2% glutraldehyde fixative (osmolarity ~300 mOsm) for 30–40 min on ice (Saito and Tanaka, 1980). The tissue samples were subjected to three 10-minute washes in 0.1 M cacodylate buffer. The samples were transferred to 0.1 M Tris–HCl buffer and washed twice for 10 min. The samples were pre-incubated in 1% diaminobenzidine (DAB) solution for 30 min at 37 °C with shaking. Ten µl of 30% hydrogen peroxide solution was added and the samples were incubated for 20–30 min at 37 °C with shaking. Tissues were washed in 0.1 M TBS for 10 min and transferred to centrifuge tubes and subjected to three 10-minute washes with 0.1 M cacodylate buffer at room temperature (Bozzola and Russell, 1999). Finally, the tissues were post-fixed in 1% osmium in 0.1 Mcacodylate buffer for 1 h at 4 °C and washed in filtered, deionized H2O for 15 min. Tissues were dehydrated for transmission electron microscopy, infiltrated overnight and late rembedded in epon resin (Luft, 1961). Ten electron microscopy images of tissues for peroxisomes and mitochondria were counted in tissues of adult zebrafish fed different concentrations of clofibrate.

3.3.3 RNA isolation, cDNA synthesis and RT-qPCR

Total RNA was extracted from adult zebrafish tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocol recommended by the supplier. The quality and quantity of extracted RNA was assessed by agarose gel-electrophoresis and spectrophotometry at 260 nm, respectively. cDNA was synthesized from mRNA using an oligo (dT) primer according to the manufacturer's protocol for the omniscript RT kit (Qiagen, Mississauga, Canada). cDNA was synthesized from hnRNA using random hexamers. Primer sequences for the quantification of mRNA and hnRNA encoded by different *fabp* genes and their annealing temperature (AT) for primer pairs of each *fabp* gene during PCR are shown in Table 3.2. To assay specific hnRNAs, one primer was complementary to an intronic sequence, while the other was complementary to an exonic sequence. *Acox1*, a gene known to be induced by clofibrate in many organisms (Reddy and Hashimoto, 2001; Akbiyik et al., 2004) was used as a positive control.

Amplification of cDNA samples and DNA standards was carried out using the SYBR Green Quantitect PCR Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's instructions. For thermal cycling and fluorescence detection, a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia) was used. PCR conditions were: initial hold for 15 min at 95 °C followed by 40 cycles of 15 s denaturation at 94 °C, 20 s annealing of primers at different temperatures depending on the primer pairs (see Table 3.2), and 30 s of elongation at 72 °C. Following completion of the PCR cycles, the melting temperature of the PCR product was determined as an indication that total fluorescence was derived from a single gene specific product. Fluorescence was

Table 3.2 Primer sequences used for RT-qPCR

Gene symbol	Entrez Gene ID	Forward primer 5' -› 3'	Reverse primer 5' -› 3'	AT[a]
		mRNA quantification		
fabp1a	791610	TAAGCTGACAGCGTTTGTGAAGGG	AGATGCGTCTGCTGATCCTCTTGT	60.0
fabp1b.1	554095	AAGCTGAAGGTGGTGCTGAACA	CACGTTTGCTGATGCGCTTGTA	59.0
fabp1b.2	EB880179	TGCCGTTCTCTGGGAAGTTTGAGT	TGACTTTGTCTCCGCTCAGCATCT	61.0
fabp7a	58128	TGTGCCACTTGGAAACTGGTTGAC	AACATTGCCTACTTGCCTGGTAGG	60.0
fabp7b	407736	AAACCACTGCTGATGACCGACACT	AGTGGTCTCTTTCCCATCCCACTT	61.0
fabp10a	171481	TTACGCTCAGGAGAACTACG	CTTCCTGATCATGGTGGTTC	55.0
fabp10b	795210	CGGCTCCAGAGCACTACATC	GTTCACTCATGTGCGGGAGC	60.0
fabp11a	447944	TGTGCAGAAACAGACCTGGGA	ACAGCCACCACATCACCCATCTT	60.0
fabp11b	553579	GCTGTCACTACATTCAAGACCTG	AGTTTACCATCCGCAAGGCTCA	60.0
acox1	449662	AGTCAGCACGAGCTCTCTCC	GCCCTACAAAGTGAAAGGCA	58.0
rpl13α	560828	AGCAAGTGCTGTTGGGCCAC	GTGTGGCGGTGATGGCCTGG	61.0
		hnRNA quantification		
fabp1a	791610	ATCAATGGAGGTCAACGGCGAC	CAGCATGCGTGAAGCCGCCC	62.5
fabp1b.1	554095	GAACTAACGTGTGCTGCTTGTG	CACGTTTGCTGATGCGCTTGTA	57.0
fabp7a	58128	CCATCCATCAGATTTCTATGTGGG	CATTATGCCTTCTCGTATGTGCG	56.5
fabp7b	407736	TTGGAAATGTGACCAAACCGACGC	TCGTCTCGAAAGGGAATGCAGTGT	61.5
fabp10a	171481	TCCAGCAGAACGGCAGCGAC	CGCCTGTAAAGTGAAGCCATTTCCA	61.0
fabp11a	447944	CCAAGCCGTTTTTGATGATGTGAG	GCTATTAATTTCCCATCCGACACC	57.0
acox1	449662	GGCTACTCCCGCTGCAGCAG	GGCCTGAGGGTTGTTGGGCC	63.0
rpl13α	560828	ACCAACCCTTCCCGTGGACCA	AGCCAATGCTTGCTTCTACAACAGA	61.5

[a]AT, annealing temperature (°C)

measured following each cycle. The copy number of mRNA and hnRNA for each *fabp* gene was determined using the standard curves as explained by Bustin et al. (2005). As negative controls, reverse transcriptase was omitted from cDNA synthesis reactions for each sample and these controls were subjected to quantitative PCR. To determine the relative steady-state level of *fabp* mRNA and hnRNA transcripts in each tissue, the absolute copy number of *fabp* mRNA and hnRNA transcripts was divided by the copy number of ribosomal protein large subunit 13 α (*rpl13α*) (Tang et al., 2007) mRNA and hnRNA transcripts in each sample.

3.3.4 Statistical analysis

Statistical analyses were performed using the GraphPad PRISM® software version-5 (SanDiego, California, USA). Data were analyzed using one-way analysis of variance (ANOVA). Post hoc comparisons were conducted using the Tukey's Multiple Comparison Test. The level of significance was chosen at $p < 0.05$ and the results were presented as means ± S.E.M.

3.4 Results and discussion

3.4.1 Zebrafish is responsive to the peroxisome proliferator, clofibrate

Peroxisome proliferators, such as clofibrate, are known to cause a marked proliferation of peroxisomes in the hepatocytes of animals (Hess et al., 1965; Svoboda and Azarnoff, 1966; Lazarow and de Duve, 1976; Alvares et al., 1990; Tanaka et al., 1992; Donohue et al., 1993; Paul et al., 1994; Ibabe et al., 2005). Proliferation of peroxisomes is also associated with a predictable pleiotropic response, characterized by hepatomegaly, and the increased steady-state level of mRNAs coding for peroxisomal enzymes (Alvares et al., 1990). In this study, we first wished to determine if clofibrate

acts as a peroxisome proliferator in zebrafish as vertebrate species show different responses to clofibrate as assayed by peroxisome proliferation or induction of steady-state transcript levels for several clofibrate-responsive genes. Rats and mice are more responsive to clofibrate than hamsters and humans (Mizumoto et al., 1988; Holden and Tugwood, 1999), while some fish, such as medaka and rainbow trout, show little response (Haasch et al., 1998), and sea bass is essentially refractory to clofibrate treatment (Pretti et al., 1999). The number of peroxisomes was higher in hepatocytes of zebrafish fed ≥ 0.75% clofibrate (Figure 3.1B) compared to livers of zebrafish not fed clofibrate (Figure 3.1A). The number of peroxisomes in liver increased 4-fold in zebrafish fed ≥ 0.75% clofibrate compared to the control (Figure 3.1C). The peroxisomal numbers in intestine did not change with clofibrate treatment, whereas, in other tissues like muscle, brain and heart, we could not observe any peroxisomes (data not shown). Previous studies in rats and mice fed clofibrate showed an increase in the number of mitochondria in the liver (Lundgren et al., 1990; Meijer et al., 1991; Eagles and Chapman, 2007). In this study, zebrafish fed ≥ 0.75% clofibrate showed an increase in the number of mitochondria only in heart cells (Figure 3.2B) compared to the control (Figure 3.2A). The number of mitochondria in heart cells increased 2-fold in zebrafish fed ≥ 0.75% clofibrate compared to the control (Figure 3.2C). The mitochondrial number in other tissues (liver, intestine, muscle and brain) examined did not change in zebrafish fed clofibrate.

Clofibrate has been widely used in vertebrates to activate PPARα, and in some instances PPARγ, to induce transcriptional initiation of genes involved in lipid homeostasis, such as the acyl-CoA oxidase 1 (*Acox1*) gene, a gene that contains a

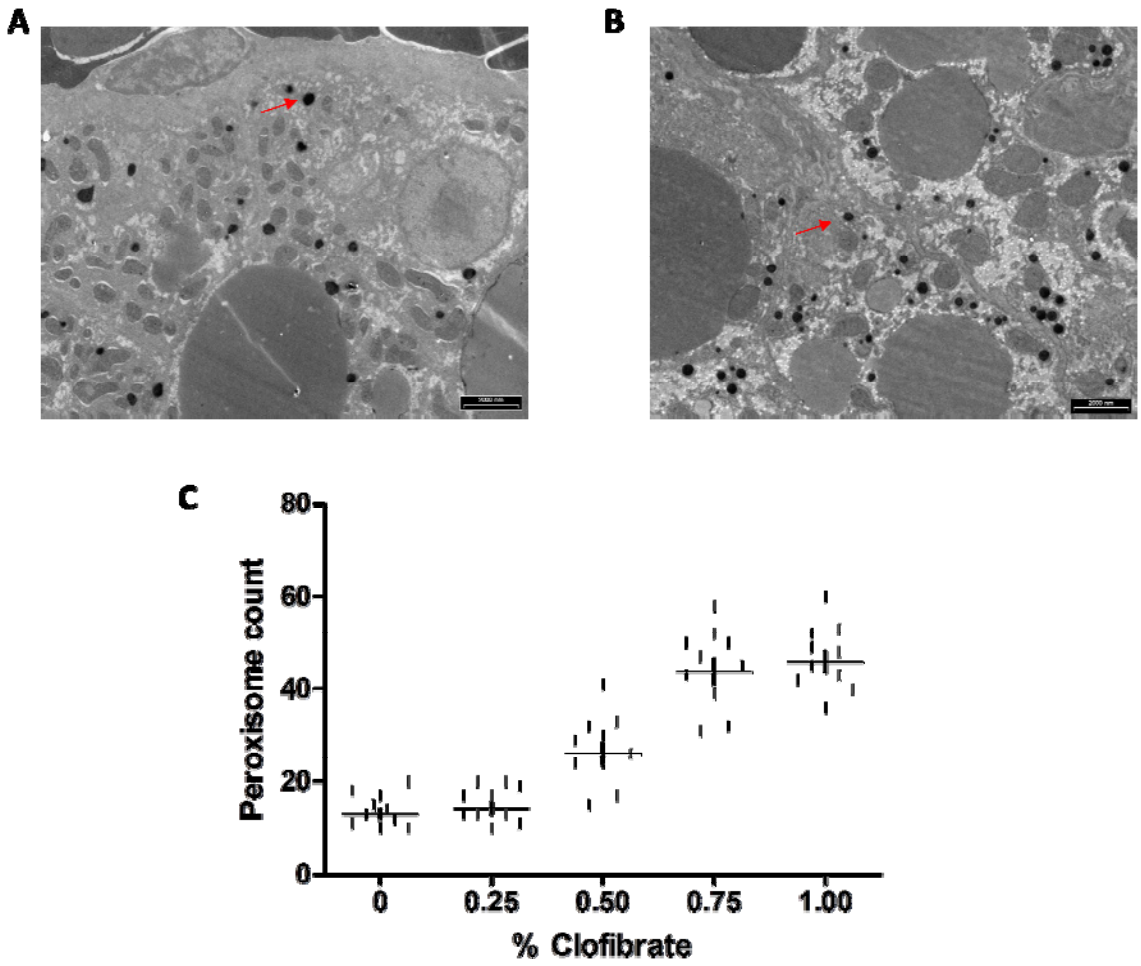

Figure 3.1 Electron micrographs of hepatocytes of zebrafish after clofibrate treatment. Staining of peroxisomes in the hepatocytes of zebrafish fed 0% clofibrate (A) and 1.00% clofibrate (B). Number of peroxisome per field of view in liver increased with increasing concentration of clofibrate fed zebrafish (C). Arrows point to peroxisomes. Bar = 2 μm

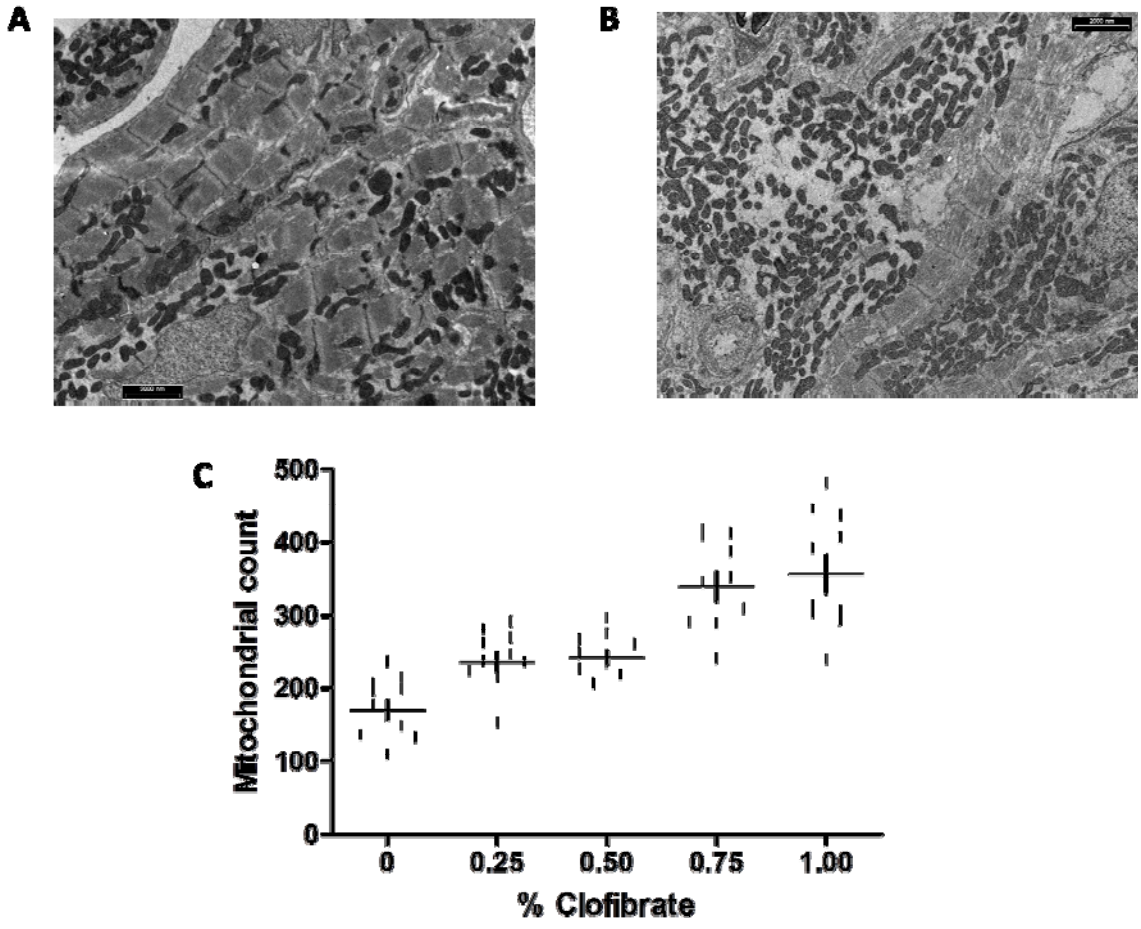

Figure 3.2 Electron micrographs of heart cells of zebrafish after clofibrate treatment. Mitochondria in the heart cells of zebrafish fed 0% clofibrate (A) and 1.00% clofibrate (B). Number of mitochondria per field of view in heart increased with increasing concentration of clofibrate fed zebrafish (C). Bar = 2 μm

peroxisome proliferator response element (PPRE) (Reddy and Hashimoto, 2001; Helledie et al., 2002; Akbiyik et al., 2004; Ibabe et al., 2005). In rats, transcriptional initiation of PPARα-responsive genes are up-regulated in the liver, moderately up-regulated in the small intestine and to a lesser extent up-regulated in other tissues, such as skeletal muscle, heart and kidney by clofibrate (Ringseis et al., 2007). The level of *Acox1* mRNA in liver, heart, kidney, duodenum and jejunum is increased in rats fed clofibrate compared to controls, but not in ileum and brain (Ringseis et al., 2007). Clofibrate was also shown to increase the level of *Acox1* mRNA in liver of chicken (Konig et al., 2007), liver and adipose tissue of pigs (Luci et al., 2007), hepatocytes of rainbow trout (Donohue et al., 1993) and liver of rats (Paul et al., 1994). In this study, the steady-state level of *acox1* mRNA increased 3-fold in liver (Figure 3.3A), 3-fold in intestine (Figure 3.3B), 2-fold in muscle (Figure 3.3C) and 2.5-fold in heart (Figure 3.3D) of zebrafish fed ≥ 0.50% clofibrate compared to zebrafish fed < 0.50% clofibrate.

To determine if the increased levels of *acox1* mRNA transcripts by clofibrate in various tissues was due to an increased rate of transcriptional initiation, we assayed the steady-state level of hnRNA coded by the *acox1* gene. The level of hnRNA for a given gene is an indirect estimate of the rate of transcriptional initiation for that gene as the processing of hnRNA to mRNA occurs rapidly (Watson et al., 2008). Zebrafish *acox1* hnRNA increased 2.5-fold in liver (Figure 3.3F), 2-fold in intestine (Figure 3.3G), 8-fold in muscle (Figure 3.3H) and 4-fold in heart (Figure 3.3I) of fish fed ≥ 0.50% clofibrate compared to zebrafish fed < 0.50% clofibrate indicating an increase of transcriptional initiation of the *acox1* gene in these tissues. In brain, *acox1* mRNAand hnRNA levels did not change in zebrafish fed clofibrate (Figure 3.3E, 3.3J). The lack of affect on the level

Figure 3.3 The steady-state level of *acox1* mRNA and hnRNA in various tissues of zebrafish fed clofibrate. The level of mRNA and hnRNA of the *acox1* gene in liver (A, F), intestine (B, G), muscle (C, H), heart (D, I) and brain (E, J) was determined by RT-qPCR using gene-specific primers. The steady-state level of *acox1* transcripts was normalized to the steady-state level of *rpl13α* transcripts in the same sample. Data are presented as the mean ratio ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *acox1* mRNA and hnRNA between zebrafish [n = 12, (male = 6, female = 6)] fed different concentrations of clofibrate compared to zebrafish not fed clofibrate are indicated by an asterisk.

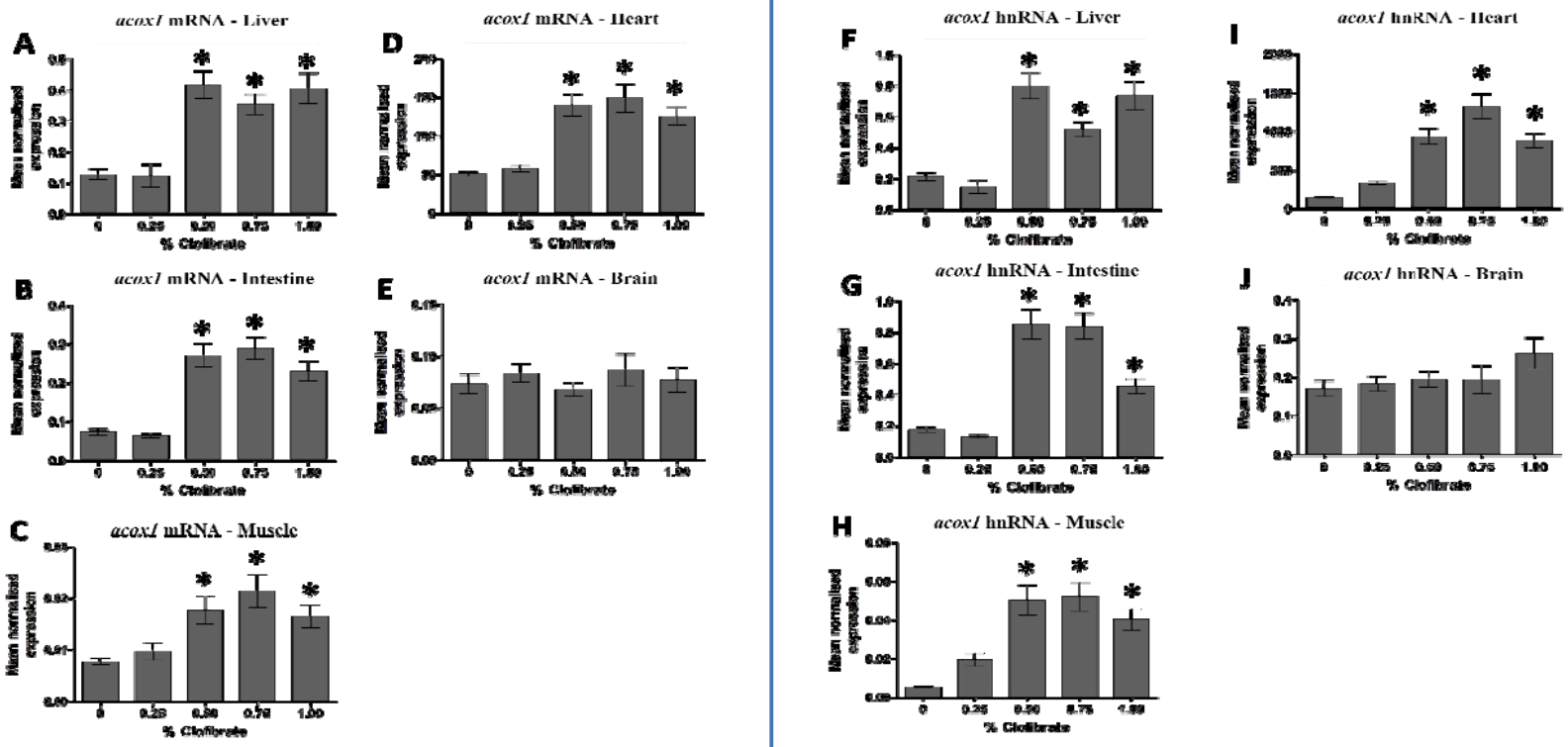

Figure 3.3 The steady-state level of *acox1* mRNA and hnRNA in various tissues of zebrafish fed clofibrate.

of *acox1* transcripts in the brain of zebrafish fed clofibrate may be due to: (i) clofibrate does not cross the blood-brain barrier, or (ii) if clofibrate does cross the blood-brain barrier, the *acox1* gene is not induced by clofibrate in the brain of zebrafish. The increased number of peroxisomes and mitochondria in the liver and heart, respectively, and the induction of the transcriptional initiation of the *acox1* gene by clofibrate in liver, intestine, muscle and heart is compelling evidence that zebrafish is responsive to this peroxisome proliferator, like many other vertebrates (Hess et al., 1965; Svoboda and Azarnoff, 1966; Lazarow and de Duve, 1976; Mizumoto et al., 1988; Alvares et al., 1990; Lundgren et al., 1990; Meijer et al., 1991; Tanaka et al., 1992; Donohue et al., 1993; Paul et al., 1994; Holden and Tugwood, 1999; Pretti et al., 1999; Reddy and Hashimoto, 2001; Colton et al., 2004; ; Ibabe et al., 2005; Eagles and Chapman, 2007).

3.4.2 Tissue-specific up-regulation of zebrafish *fabp* transcription by clofibrate

Some mammalian *FABP* genes are induced by various FAs and peroxisome proliferators, and molecular mechanisms for their induction have been proposed (Ockner and Manning, 1974; Bass et al., 1985; Reddy, 2004; Schachtrup et al., 2004; Mochizuki et al., 2007; Schroeder et al., 2008). FABPs transport long-chain FAs from the cytoplasm to the nucleus (Huang et al., 2004; Schroeder et al., 2008). Inside the nucleus, FABPs transfer their long-chain FAs to nuclear receptors, such as PPARα and PPARγ (Delva et al., 1999; Budhu and Noy, 2002; Tan et al., 2002). Dietary long chain FAs and peroxisome proliferators activate these nuclear receptors, and once activated, these nuclear receptors form heterodimers with retinoic-acid receptors (RAR) or retinoid X receptors (RXR) (e.g., PPAR-RAR and PPAR-RXR), which in turn bind to response elements in *FABP* genes, and thereby, stimulate initiation of transcription (Gottlicher et

al., 1992; Keller et al., 1993; Lemberger et al., 1996; Desvergne and Wahli, 1999; Escher and Wahli, 2000; Wolfrum et al., 2001; Wilk et al., 2005). Previous reports have shown that FAs and peroxisome proliferators increase the steady-state level of *L-FABP* (*FABP1*) and *I-FABP* (*FABP2*) gene transcripts in the mammalian liver and small intestine (Ockner and Manning, 1974; Bass et al., 1985; Kaikaus et al., 1993; Wolfrum et al., 1999; Poirier et al., 2001; Mochizuki et al., 2007). Peroxisome proliferators also increase the transcriptional activity of *A-FABP* (*FABP4*) in adipocytes of mice (Tan et al., 2002; Schachtrup et al., 2004).

3.4.3 Up-regulation of transcription of duplicated zebrafish *fabp1* genes by clofibrate

In this study, the steady-state level of *fabp1a* mRNA increased 1.5-fold in the intestine of zebrafish fed 0.50% and 0.75% clofibrate (Figure 3.4A) and increased 4-fold in muscle of zebrafish fed 0.50% clofibrate compared to zebrafish not fed clofibrate (Figure 3.4E). In heart cells of zebrafish fed ≥ 0.75% clofibrate, *fabp1b.1* mRNA increased 2-fold compared to zebrafish not fed clofibrate (Figure 3.4J). To determine if the increased levels of *fabp1a* mRNAs was the result of transcriptional initiation, we assayed the levels of hnRNA for these *fabp* genes in various tissues of zebrafish. The steady-state level of *fabp1a* hnRNA increased 6-fold in intestine of zebrafish fed ≥ 0.50% clofibrate (Figure 3.4D) and > 5-fold in muscle of zebrafish fed 0.50% clofibrate (Figure 3.4H). In zebrafish fed ≥ 0.50% clofibrate, the level of *fabp1b.1* hnRNA in heart increased 3-fold compared to zebrafish fed the control diet. (Figure 3.4L). The levels of *fabp1a* mRNA in heart (Figure 3.4I), *fabp1b.1* mRNA in intestine (Figure 3.4B), *fabp1b.1* mRNA in muscle (Figure 3.4F), *fabp1b.2* mRNA in intestine (Figure 3.4C),

Figure 3.4 The steady-state level of *fabp1a*/*fabp1b.1*/*fabp1b.2* mRNA and hnRNA in intestine (A, B, C, D), muscle (E, F, G, H) and heart (I, J, K, L) of zebrafish fed clofibrate. The level of mRNA and hnRNA was determined by RT-qPCR using gene-specific primers. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α* transcripts in the same sample. Data are presented as the mean ratio ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNAs between zebrafish [n = 12, (male = 6, female = 6)] fed different concentrations of clofibrate compared to zebrafish not fed clofibrate are indicated by an asterisk.

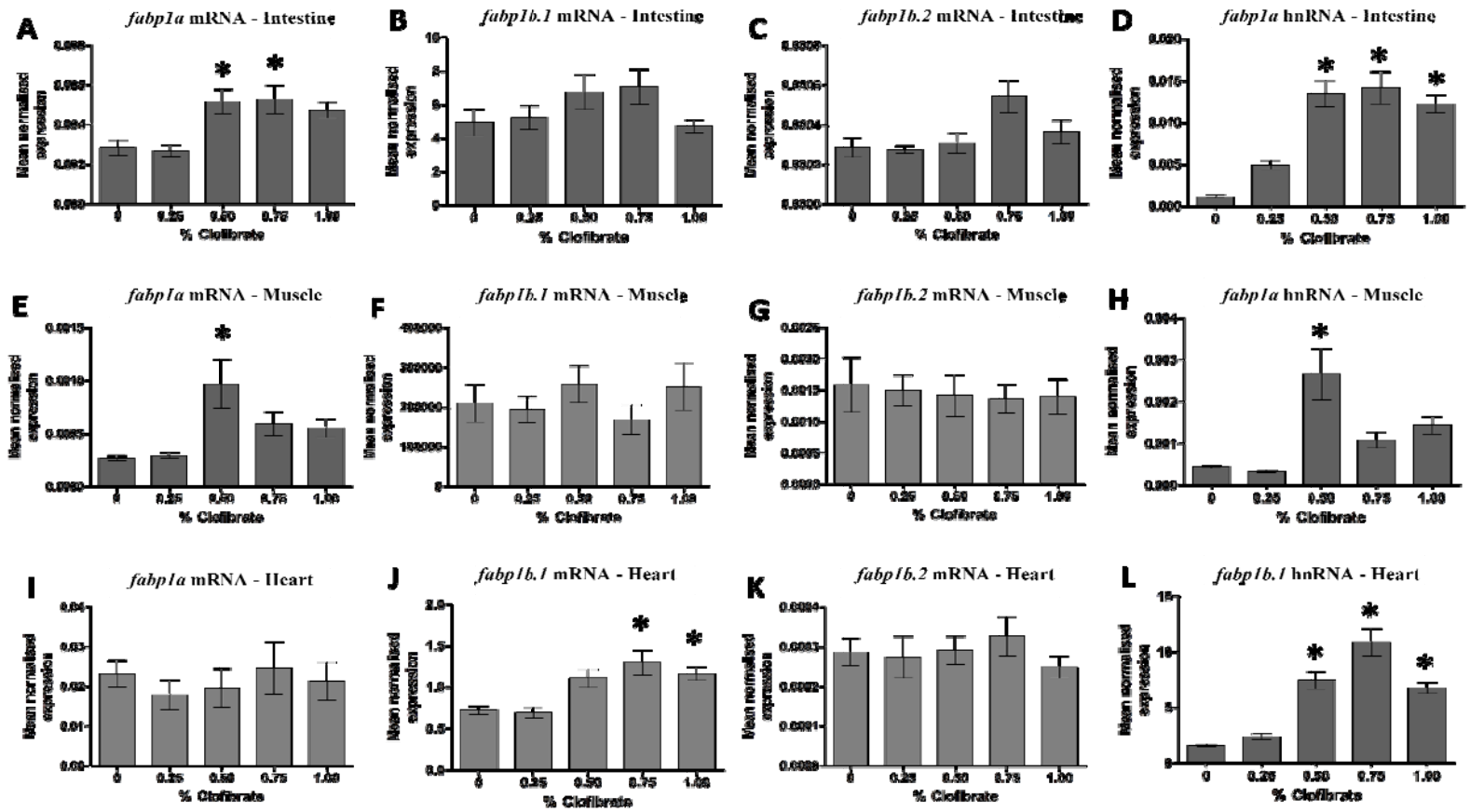

Figure 3.4 The steady-state level of *fabp1a*/*fabp1b.1*/*fabp1b.2* mRNA and hnRNA in intestine (A, B, C, D), muscle (E, F, G, H) and heart (I, J, K, L) of zebrafish fed clofibrate.

fabp1b.2 mRNA in muscle (Figure 3.4G) and *fabp1b.2* mRNA in heart (Figure 3.4K) remained unchanged in zebrafish fed clofibrate.

3.4.4 Up-regulation of transcription of zebrafish *fabp7* genes by clofibrate

Duplicated copies of zebrafish *fabp7* (*fabp7a* and *fabp7b*) exhibited distinct tissue-specific patterns of up-regulation by clofibrate of levels of both mRNA and hnRNA (Figure 3.5). The *fabp7a* mRNA increased > 7-fold in liver of zebrafish fed 0.50% clofibrate (Figure 3.5A) and > 2-fold in intestine of zebrafish fed 1.00% clofibrate (Figure 3.5D), while *fabp7b* mRNA levels increased 6-fold only in muscle of zebrafish fed ≥ 0.50% clofibrate compared to zebrafish not fed clofibrate (Figure 3.5H).

The increase in the mRNA levels of zebrafish *fabp7* genes correlated with the increase in the levels of their hnRNA. The *fabp7a* hnRNA increased > 3-fold in liver of zebrafish fed 0.50% clofibrate (Figure 3.5C) and 7-fold in intestine of zebrafish fed ≥ 0.75% clofibrate (Figure 3.5F), while *fabp7b* hnRNA increased 6-fold in muscle of zebrafish fed ≥ 0.50% clofibrate (Figure 3.5I) compared to control zebrafish. No change was observed in the levels of *fabp7b* mRNA transcripts in liver (Figure 3.5B) and intestine (Figure 3.5E), and *fabp7a* mRNA in muscle (Figure 3.5G) in zebrafish fed clofibrate.

3.4.5 Up-regulation of zebrafish *fabp10* gene transcription by clofibrate

The steady-state level of *fabp10a* mRNA increased > 2-fold in liver of zebrafish fed 0.50% clofibrate compared to control (Figure 3.6A), whereas the level of *fabp10b* mRNA (Figure 3.6B) did not change in the liver of zebrafish fed clofibrate. A 3-fold increase of *fabp10a* hnRNA mirrored the increase of mRNA coded by this gene in liver of zebrafish fed 1.00% clofibrate compared to control zebrafish (Figure 3.6C).

Figure 3.5 The steady-state level of *fabp7a*/*fabp7b* mRNA and hnRNA in liver (A, B, C), intestine (D, E, F) and muscle (G, H, I) of zebrafish fed clofibrate. The level of mRNA and hnRNA was determined by RT-qPCR using gene-specific primers. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α* transcripts in the same sample. Data are presented as the mean ratio ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNAs between zebrafish [n = 12, (male = 6, female = 6)] fed different concentrations of clofibrate compared to zebrafish not fed clofibrate are indicated by an asterisk.

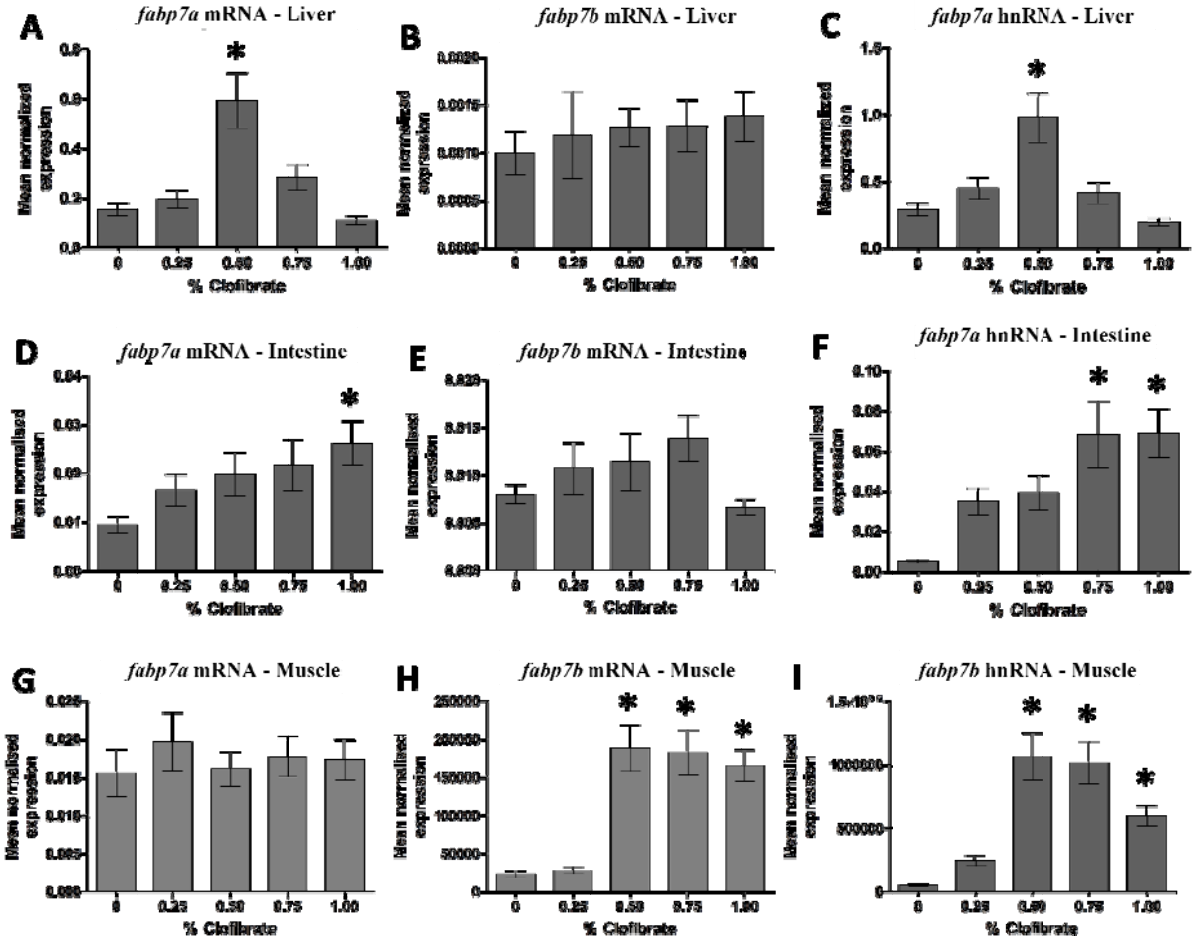

Figure 3.5 The steady-state level of *fabp7a*/*fabp7b* mRNA and hnRNA in liver (A, B, C), intestine (D, E, F) and muscle (G, H, I) of zebrafish fed clofibrate.

Figure 3.6 The steady-state level of *fabp10a*/*fabp10b* mRNA and hnRNA in liver (A, B, C) and *fabp11a*/*fabp11b* mRNA and hnRNA in liver (D, E, F) of zebrafish fed clofibrate. The level of mRNA and hnRNA was determined by RT-qPCR using gene-specific primers. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α* transcripts in the same sample. Data are presented as the mean ratio ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNAs between zebrafish [n = 12, (male = 6, female = 6)] fed different concentrations of clofibrate compared to zebrafish not fed clofibrate are indicated by an asterisk.

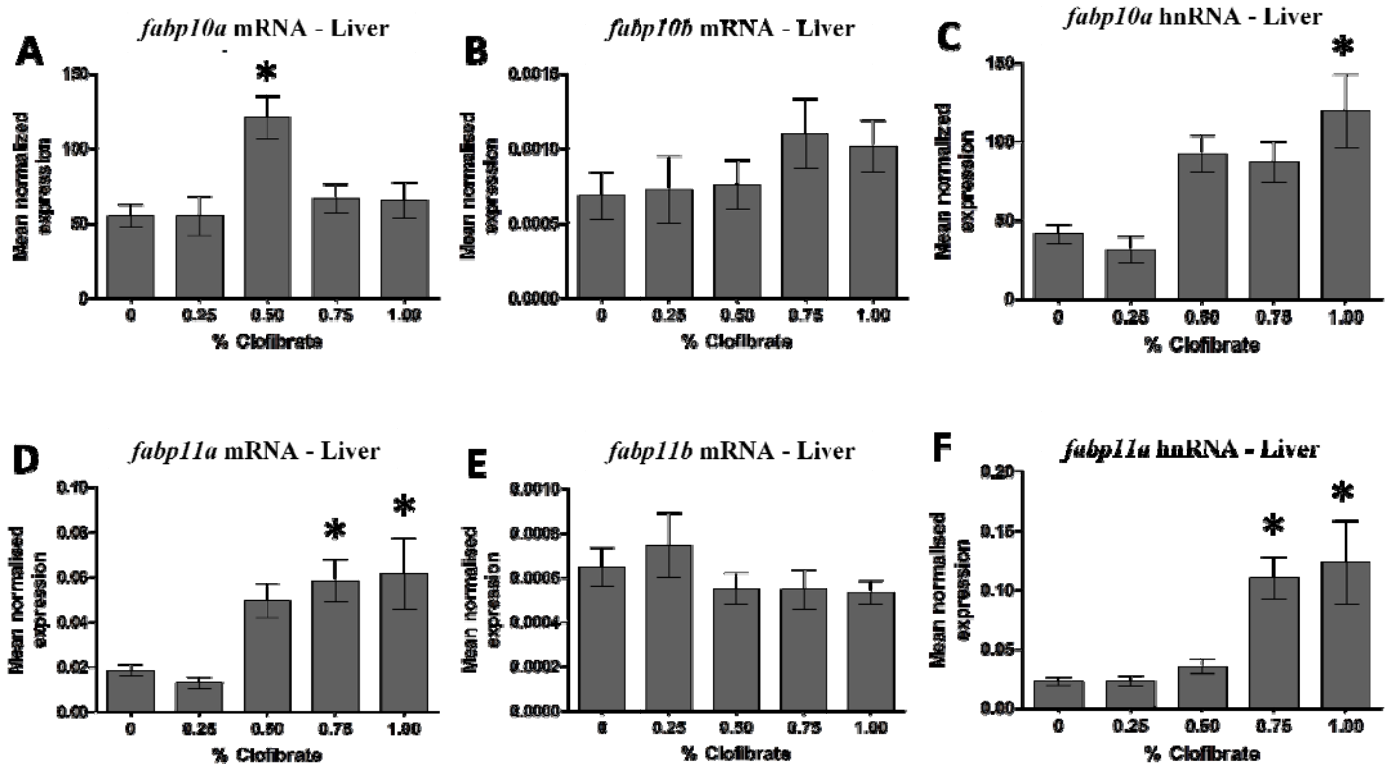

Figure 3.6 The steady-state level of *fabp10a*/*fabp10b* mRNA and hnRNA in liver (A, B, C) and *fabp11a*/*fabp11b* mRNA and hnRNA in liver (D, E, F) of zebrafish fed clofibrate.

3.4.6 Up-regulation of zebrafish *fabp11* gene transcription by clofibrate

The steady-state level of *fabp11a* mRNA (Figure 3.6D) increased 3-fold in liver of zebrafish fed ≥ 0.75% clofibrate compared to control, but the steady-state level of *fabp11b* transcripts (Figure 3.6E) did not change in the liver of zebrafish fed clofibrate. Similarly, *fabp11a* hnRNA increased > 4-fold in liver of zebrafish fed ≥ 0.75% clofibrate compared to control (Figure 3.6F). No difference in the steady-state level of any fabp mRNA and hnRNA assayed was observed between male and female zebrafish (data not shown).

3.5 Conclusion

We report here that zebrafish fed clofibrate exhibited distinct patterns of up-regulation of the steady-state level of mRNAs of duplicated *fabp* genes (Table 3.3). None of the levels of *fabp* mRNA transcripts assayed changed in the brain of zebrafish fed clofibrate (data not shown). Furthermore, changes in the levels of mRNA for a specific *fabp* gene were directly correlated with changes in the steady-state level of hnRNA for that particular *fabp* gene indicating that clofibrate induced transcriptional initiation of zebrafish *fabp* genes (Table 3.3). Clofibrate induction of some zebrafish *fabp* genes appears, however, to be controlled by a tissue specific mechanism(s), as induction of the steady-state level of *fabp* mRNAs and hnRNAs by clofibrate was seen for both duplicated copies of *fabp1a/fabp1b.1*, and *fabp7a/fabp7b*, but in different tissues. Clofibrate also increased the steady-state level of *fabp10a* and *fabp11a* mRNAs and hnRNAs in liver, but not for *fabp10b* and *fabp11b*.

Based on *in silico* analyses, we found that most zebrafish *fabp* genes contain putative PPREs within 7 kilobase pairs (kb) of DNA upstream of the transcriptional

Table 3.3 Steady-state levels of specific mRNA and hnRNA of *fabp* genes increased in tissues of zebrafish after clofibrate treatment.

Gene	Liver	Intestine	Muscle	Heart	Brain
acox1	+	+	+	+	-
fabp1a	-	+	+	-	-
fabp1b.1	-	-	-	+	-
fabp1b.2	-	-	-	-	-
fabp7a	+	+	-	-	-
fabp7b	-	-	+	-	-
fabp10a	+	-	-	-	-
fabp10b	-	-	-	-	-
fabp11a	+	-	-	-	-
fabp11b	-	-	-	-	-

+, increase relative to control
-, no change relative to control

initiation site of each *fabp* gene (data not shown). Since functional PPREs that activate gene transcription *via* peroxisome proliferators have also been found in introns (Helledie et al., 2002; Hansmannel et al., 2003), we screened the intronic sequences of each of the zebrafish *fabp* genes described in this paper for PPREs. Many of these *fabp* genes contained putative PPREs in their introns (data not shown). While *in silico* analyses can be very useful in determining the direction of future experimental work, the results of *in silico* analyses must be interpreted cautiously. To illustrate this cautionary point, *in silico* analysis of the rat peroxisomal thiolase B gene identified a putative PPRE in the promoter region of this gene that did not bind PPARα *in vitro*, but subsequent studies showed that a functional PPRE in intron 3 of this gene did bind an activated PPARα *in vitro* (Hansmannel et al., 2003). To demonstrate that the putative PPREs we have found by *in silico* analysis in the various zebrafish *fabp* genes are indeed functional will require studies involving deletion of PPREs and/or site directed mutation of putative PPRE sequences in the *fabp* promoters and introns to demonstrate loss of function in various cell culture lines and transgenic zebrafish. If functional PPREs are identified in the zebrafish *fabp* genes, the most parsimonious explanation of the tissue-specific differential induction of transcriptional initiation of the duplicated zebrafish *fabp1a*/*fabp1b.1* and *fabp7a/fabp7b* genes by clofibrate is that both duplicated copies of these gene have retained a functional PPRE, but that induction by clofibrate is over-ridden by an, as yet, unknown tissue-specific mechanism(s). An alternative explanation is that induction of *fabp* transcriptional initiation by clofibrate is mediated *via* an indirect mechanism wherein the induction of *fabp* genes occurs by an intermediate or "upstream" gene activated by PPAR coding for a transcription factor, which in turn activates zebrafish

fabp genes. Again, however, this indirect induction of *fabp* gene transcription by clofibrate-activated PPAR must be mediated by an over-riding tissue-specific mechanism(s). Whether clofibrate-induced transcription of zebrafish *fabp* genes is the result of clofibrate activated PPAR directly at a *fabp* PPRE or indirectly *via* an "upstream" gene coding for a regulatory protein, the regulatory DNA elements in the duplicated *fabp* genes have certainly diverged markedly since the WGD event ~230-400 mya (Robinson-Rechavi et al., 2001; Furlong and Holland, 2002; Jaillon et al., 2004; Woods et al., 2000; Christoffels et al., 2004; Vandepoele et al., 2004), thereby supporting the DDC model (Force et al., 1999; Lynch and Conery, 2000) for the retention of these duplicated *fabp* genes in the zebrafish genome.

CHAPTER 4 : TISSUE-SPECIFIC TRANSCRIPTIONAL MODULATION OF FATTY ACID-BINDING PROTEIN GENES, *fabp2*, *fabp3* AND *fabp6*, BY FATTY ACIDS AND THE PEROXISOME PROLIFERATOR, CLOFIBRATE, IN ZEBRAFISH (*Danio rerio*)

The manuscript based on this study is presented below.

Co-authors for this manuscript are Daniel L. Sawler and Jonathan M. Wright.

Originally published online as:

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Author's contributions:

We thank Manoj Parmar for assistance during qPCR analysis and Santhosh Karanth for providing tissue samples of fish fed diets differing in fatty acid content. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (to JMW). ABV is a recipient of a Faculty of Graduate Studies Scholarship from Dalhousie University.

4.1 Abstract

All *fabp* genes, except *fabp2*, *fabp3* and *fabp6*, exist as duplicates in the zebrafish genome owing to a whole genome duplication event ~230–400 million years ago. Transcription of some duplicated *fabp* genes is modulated by fatty acids (FAs) and/or clofibrate, a peroxisome proliferator-activated receptor (PPAR) agonist. We had also shown previously that the steady-state level of acyl-CoA oxidase 1 (*acox1*) mRNA, a marker of PPARα activation, was elevated in liver, intestine, heart and muscle of fish fed clofibrate demonstrating that zebrafish, unlike some fishes, is responsive to this drug. *acox1* transcripts were not induced in the brain of fish fed clofibrate, which suggests this drug may not cross the blood brain barrier. Here, we investigated the effect of dietary FAs and clofibrate on the transcription of single copy *fabp* genes, *fabp2*, *fabp3* and *fabp6*, in five tissues of inbred zebrafish. The steady-state level of *fabp2* transcripts increased in intestine, while *fabp3* mRNA increased in liver of fish fed diets differing in FA content. In fish fed clofibrate, *fabp3* mRNA in intestine, and *fabp6* mRNA in intestine and heart, were elevated. Based on these findings, modulation of *fabp2*, *fabp3* and *fabp6* transcription by FAs and/or clofibrate in zebrafish implicates control of these genes by PPAR interaction with peroxisome proliferator response elements (PPRE) most likely in *fabp* promoters. Moreover, transcriptional induction of these *fabp* genes by dietary FAs and/or clofibrate is over-ridden by a tissue-specific mechanism(s), e.g., transcriptional activator or repressor proteins.

4.2 Introduction

Fatty acid-binding proteins (FABPs) are 14–16 kDa cytosolic proteins, which bind fatty acids (FAs) and other hydrophobic ligands, such as eicosanoids and retinoids (Bernlohr et al., 1997). FABPs belong to the multigene family of intracellular lipid-binding proteins (iLBPs) that includes the retinol-binding (RBPs) and the cellular retinoic acid-binding proteins (CRABPs) (Bernlohr et al., 1997; Haunerland and Spener, 2004; Schaap et al., 2002; Storch and Corsico, 2008; Wolfrum, 2007). The tertiary structure of iLBPs is highly conserved and consists of two short α-helices and ten antiparallel β-strands. The β-strands are arranged into two orthogonal β-sheets giving the iLBPs a clamshell-like structure (Bernlohr et al., 1997). The iLBP multigene family includes 12 FABPs, 4 RBPs and 2 CRABPs. Schaap et al. (2002) reported that the first *FABP* gene emerged after the divergence of animals from plants, some 930–1000 million years ago (mya). A whole-genome duplication (WGD) event around 230–400 mya early in the teleost fish lineage has further augmented the iLBP multigene family (Furlong and Holland, 2002; Jaillon et al., 2004; Mulley and Holland, 2004; Postlethwait et al., 2000; Woods et al., 2000).

So far, we have characterized 12 *fabp* genes in zebrafish (Alves-Costa et al., 2008; Denovan-Wright et al., 2000a, 2000b; Karanth et al., 2008; Liu et al., 2003a, 2003b, 2004, 2007; Pierce et al., 2000; Sharma et al., 2004; Sharma et al., 2006; Venkatachalam et al., 2009). Eight zebrafish *fabp* genes (four pair of duplicate genes) *fabp1a/fabp1b*, *fabp7a/fabp7b*, *fabp10a/fabp10b* and *fabp11a/fabp11b* arose as a result of the teleost fish-specific WGD (Karanth et al., 2008; Liu et al., 2004; Sharma et al., 2006; Venkatachalam et al., 2009). A tandem gene duplication of the *fabp1b* gene

resulted in the formation of *fabp1b.1* and *fabp1b.2* (Karanth et al., 2009a). The retention rate of duplicated genes in zebrafish is estimated to be 14–30% (Postlethwait et al., 2000; Woods et al., 2005). Interestingly, the zebrafish genome has retained 73% of the duplicated *fabp* genes. *fabp2*, *fabp3* and *fabp6* are the only *fabp* genes identified, thus far, extant as single copies in the zebrafish genome. The sister duplicates of these genes have presumably been lost due to accumulation of mutations leading to functional decay.

FABPs were first isolated from the cytosol of rat liver, intestinal mucosa and myocardium (Ockner et al., 1972). Since the discovery of the first FABP, subsequent FABPs have been named according to the initial tissue where each FABP was first isolated: e.g., liver (L-FABP), intestinal (I-FABP), heart (H-FABP), etc. However, this nomenclature has become increasingly confusing as different FABPs have been isolated from the same tissue and some FABPs show overlapping patterns of tissue-specific distribution. We prefer to use the nomenclature proposed by Hertzel and Bernlohr (2000) where each FABP is given a number according to the chronological order of its discovery, e.g., FABP1 corresponds to L-FABP, FABP2 to I-FABP, FABP3 to H-FABP.

The biological functions of FABPs are myriad (Binas et al., 1999; Coe et al., 1999; Kaikaus et al., 1990; Schaap et al., 1999), and include: (1) a role in binding, sequestering and metabolism of long-chain FAs, eicosanoids, bile salts, and other hydrophobic ligands; (2) transport of lipids to lipid droplets for storage, to mitochondria for β-oxidation; (3) modulation of cell growth and proliferation; (4) lipid-mediated regulation of gene transcription via activation of nuclear receptors (see Leaver et al., 2005 and references therein). Regulatory elements in some mammalian FABP promoters have been defined (Meunier-Durmort et al., 1996; Poirier et al., 2001; Qu et al., 2007;

Schachtrup et al., 2004). Wu and Haunerland (2001) identified a fatty acid response element in the flight muscle FABP of desert locust (*Schistocerca gregaria*), which is a 19 base pair (bp) inverted repeat (− 162/− 180), an element responsible for the fatty acid-mediated induction of gene expression. Her et al. (2003b, 2004b) defined a 435 bp region in the promoter of the liver-basic fatty acid-binding protein (*fabp10a*) gene that modulates liver specific expression and a 192 bp region in the promoter of the intestinal-type fatty acid-binding protein (*fabp2*) gene which directs intestine-specific expression in transgenic zebrafish.

Transcription of several mammalian *FABP* genes is induced by FAs and peroxisome proliferators such as clofibrate (Meunier-Durmort et al., 1996; Poirier et al., 2001). Molecular mechanisms involved in the induction of mammalian *FABP* gene by FAs and peroxisome proliferators have been proposed (Bass et al., 1985; Mochizuki et al., 2007; Ockner and Manning, 1974; Reddy, 2004; Schachtrup et al., 2004; Schroeder et al., 2008). Long-chain FAs are carried from the cytoplasm to the nucleus by FABPs (Huang et al., 2004; Schroeder et al., 2008). Inside the nucleus, the nuclear receptors, PPARα and PPARγ, accept the long-chain FAs or peroxisome proliferators (Budhu and Noy, 2002; Delva et al., 1999; Tan et al., 2002). Upon receiving the FAs or peroxisome proliferators, the activated PPARs form heterodimers with retinoic-acid receptors (RAR) or retinoid X receptors (RXR) (e.g., PPAR–RAR and PPAR–RXR), which in turn bind to response elements in the promoters of *FABP* genes to stimulate initiation of transcription (Desvergne and Wahli, 1999; Escher and Wahli, 2000; Gottlicher et al., 1992; Keller et al., 1993; Lemberger et al., 1996; Wilk et al., 2005; Wolfrum et al., 2001).

Transcriptional initiation of only one copy in each of the three sets of duplicated *fabp* genes of zebrafish, *fabp1a/fabp1b.1/fabp1b.2*, *fabp7a/fabp7b* and *fabp11a/fabp11b*, is modulated by dietary FAs in a given tissue (Karanth et al., 2009b). Since FAs are ligands of PPAR that lead to transcriptional upregulation of target genes, we surmised that the transcriptional modulation of *fabp* genes in various tissues of zebrafish fed different FAs might be mediated by PPARs. We, therefore, investigated the potential regulation of duplicated *fabp* genes in zebrafish by PPAR using the peroxisome proliferator, clofibrate, a PPAR agonist (Venkatachalam et al., 2012). Clofibrate specifically binds with PPARα, and to a lesser extent with PPARγ, and upregulates the transcription of specific genes involved in lipid homeostasis (Luci et al., 2007; Ringseis et al., 2007). Clofibrate increased the steady-state level of *fabp10a* and *fabp11a* mRNAs in liver, but not the steady-state level of *fabp10b* and *fabp11b* transcripts in this tissue (Venkatachalam et al., 2012). Some duplicated *fabp* genes of zebrafish show a different pattern of induction by clofibrate, where both the duplicated copies of *fabp1a/fabp1b.1*, and *fabp7a/fabp7b* were induced by clofibrate, but in different tissues (Venkatachalam et al., 2012). Here, we report the transcriptional regulation of the single copy *fabp* genes, *fabp2* (Sharma et al., 2004), *fabp3* (Liu et al., 2003b) and *fabp6* (Alves-Costa et al., 2008), in zebrafish by FAs and clofibrate.

4.3 Materials and methods

4.3.1 Diets and zebrafish husbandry

In a preliminary study of the transcriptional regulation of the single copy *fabp* genes of zebrafish, *fabp2*, *fabp3*and *fabp6*, zebrafish were fed four isoproteic diets (highly unsaturated FA-rich diet (HD), linoleic acid-rich diet (LD), linolenic acid-rich

diet (LND), low fat diet (LFD)) differing in FA composition and lipid content. The experimental design for this study was the same as that reported in Karanth et al. (2009b). In a second study, zebrafish were fed diets containing different concentrations of clofibrate, (0, 0.25, 0.50, 0.75 and 1.00% w/w) (Venkatachalam et al., 2012). Genetic variance was controlled by breeding four female and two male adult zebrafish obtained from a local aquarium supply store. Fish were maintained in aerated water at 28.5 °C on a 14 h light and 10 h dark cycle (Westerfield, 2000). Five month-old zebrafish were acclimatized in 25 L aquaria for four weeks prior to feeding fish FA and clofibrate diets. In the FA diet study, 12 aquaria (25 L) were arranged with 15 fish per tank. In the clofibrate diet study, 15 aquaria (25 L) were arranged with 15 fish per tank. To reduce potential environmental variance in both studies, fish were distributed in tanks in a randomized, complete block design and each dietary treatment had three tank replicates maintained under the same light intensity and photoperiod. After acclimatization for a week, fish were fed the FA diet for 10 weeks or the clofibrate diet for 4 weeks, twice a day to satiation. After the feeding period, the fish were deprived of food for 24 h before dissection. Fish were anesthetized with 0.2% (v/v) MS-222. The fish were placed on ice and liver and intestine were extracted from each fish for the FA study. For the clofibrate study, liver, intestine, muscle, brain and heart were extracted from the anesthetized fish. Experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals in accordance with the recommendations of the Canadian Council on Animal Care.

4.3.2 Reverse transcription, quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissue using TRIzol® (Invitrogen, Carlsbad, CA, USA).

Omniscript RT kit (Qiagen, Valencia, CA, USA) and an oligo (dT) primer was used to prime synthesis of cDNA from mRNA. The rationale for design of primer sequences for RT-qPCR of *fabp* gene transcripts and their annealing temperature (AT) are outlined in Karanth et al. (2009b) and Venkatachalam et al. (2012) (Table 4.1). The acyl-CoA oxidase 1 (*acox1*) gene, a gene known to be induced by clofibrate in many organisms (Akbiyik et al., 2004; Reddy and Hashimoto, 2001) including zebrafish (Venkatachalam et al., 2012) was chosen as a positive control in the clofibrate study.

QuantiTect® SYBR® Green PCR Kit (Qiagen) was used for the amplification of cDNA samples. Thermal cycling and fluorescence detection were carried out using a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). The thermal cycling conditions were as follows: initial hold for 15 min at 95 °C followed by 40 cycles of 15 s denaturation at 94 °C, 20 s annealing of primers at different temperatures depending on the primer pairs (see Table 4.1), and 30 s of elongation at 72 °C. Following each qPCR cycle, fluorescence was measured and transcript copy number for each *fabp* gene was determined using standard curves as outlined by Bustin et al. (2005). To determine the relative steady-state level of *fabp* mRNA transcripts in each tissue, the copy number of *fabp* mRNA transcripts was divided by the copy number of elongation factor 1α (*ef1α*) mRNA transcripts for the FA study and ribosomal protein large subunit 13α (*rpl13α*) mRNA transcripts for the clofibrate study (Tang et al., 2007). As *ef1α* mRNA transcripts showed variation following clofibrate treatment in different tissues, *rpl13α*, which exhibited highly stable steady-state levels of its transcripts in all tissues assayed, was chosen as the reference gene for the clofibrate study (data not shown).

Table 4.1 RT-qPCR primer sequences for *ef1α, rpl13α, fabp2, fabp3* and *fabp6* genes of zebrafish.

Gene	Entrez Gene ID	Sense primer 5' -› 3'	Antisense primer 5' -› 3'	AT[a]
		Fatty acid study primers[b]		
fabp2	30708	TCATCATGACCTTCAACGGGACCT	ATTTCCAGTGTGCGGAAAGTGCTG	59.0
fabp3	171478	TTGACACTTGGCGACATCGTCT	TTGGGAAACGGTGCAAAGGAGA	59.0
fabp6	415166	CGTCCAGAACGGAGATGACT	TTGGGGAAGCTTATGGTCAG	61.0
ef1α	30516	TTGAGAAGAAAATCGGTGGTGCTG	GGAACGGTGTGATTGAGGGAAATTC	59.6
		Clofibrate study primers[c]		
fabp2	30708	TCACCCTGGAGCAGACCGGAG	AGGATCCTGTGAGCTCAGTGCC	63.4
fabp3	171478	TCGGCACGTGGAACTTGAAGGA	GCGTGGCAAAGCCAACACCAAT	61.0
fabp6	415166	CATGGAAGGAGGCAAGCTGACCA	GCCACTGGCTGTGGAGGTCTCC	63.2
rpl13α	560828	AGCAAGTGCTGTTGGGCCAC	GTGTGGCGGTGATGGCCTGG	59.1

[a] AT, annealing temperature (°C).
[b] Primers designed across introns.
[c] Primers designed spanning an exon-exon junction.

4.3.3 Statistical analysis

Statistical analysis employed GraphPad PRISM® software version-5 (San Diego, California, USA) with a significance level of $p < 0.05$. The relative abundance of mRNA encoded by each *fabp* gene is presented as mean ± S.E.M. The effects of diet on the relative abundance of mRNA encoded by each *fabp* gene were analyzed using one-way analysis of variance (ANOVA). Post hoc comparisons were conducted using the Tukey's multiple comparison test.

4.4 Results and discussion

4.4.1 No difference in the steady-state level of *fabp* gene transcripts between male and female zebrafish fed diets differing in FAs or clofibrate

Several reports indicate that sex of mammals influences lipid metabolism (Bass et al., 1985; Luxon and Weisiger, 1993). Luxon and Weisiger (1993) reported that long-chain FAs in the plasma are cleared faster in female rats than male rats. Bass et al. (1985) showed that cellular fatty acid metabolism and *FABP3* mRNA levels in rats were influenced by sex, i.e., female rats had twice as much *FABP3* mRNA levels than male rats. Karanth et al. (2009b) and Venkatachalam et al. (2012) demonstrated that transcriptional initiation of zebrafish duplicated *fabp* genes is modulated by dietary FAs and clofibrate, but observed no difference in the steady-state level of any *fabp* mRNA between male and female zebrafish in experimental treatments with dietary FAs and clofibrate. In the present study, no difference was observed in the steady-state level of *fabp2*, *fabp3* and *fabp6* mRNAs between male and female zebrafish fed diets differing in FA content or clofibrate concentration (data not shown).

4.4.2 Effect of FA diet on the steady-state levels of *fabp2, fabp3 and fabp6* mRNAs in tissues of zebrafish

In a preliminary study, we assessed the effect of dietary FAs on the steady-state level of *fabp2*, *fabp3* and *fabp6* gene transcripts in liver and intestine of zebrafish. The steady-state level of *fabp2* mRNA did not change in the liver (Fig. 4.1A) of zebrafish fed any of the FA diets. The steady-state level of *fabp2* mRNA, however, was 2.5-fold higher in the intestine of fish fed LND compared to the *fabp2* mRNA levels in the intestine of zebrafish fed LFD, but was not different in the intestine of zebrafish fed HD or LD (Fig. 4.1B). The steady-state level of *fabp3* mRNA did not change in the intestine (Fig. 4.1D) of zebrafish fed any of the FA diets. The steady-state level of *fabp3* mRNA was 2.5-fold higher in the liver of zebrafish fed LND compared to the mRNA levels in the liver of zebrafish fed LFD, but was not different in the liver of zebrafish fed HD or LD (Fig. 4.1C). No difference was observed in the steady-state level of *fabp6* mRNA in the liver (Fig. 4.1E) or intestine (Fig. 4.1F) of zebrafish fed any of the FA diets. Based on the conclusions of the effect of dietary FAs on the transcriptional regulation of mammalian *FABP* genes (Drozdowski et al., 2004; Liu et al., 2008; Mochizuki et al., 2007; Puskas et al., 2004), the effect of FA diet on the steady-state level of *fabp2* and *fabp3*, but not *fabp6*, in the liver and intestine of zebrafish suggests that the transcriptional control of *fabp2* and *fabp3* may be mediated, in part, by PPARs in these tissues.

Figure 4.1 The steady-state levels of *fabp2, fabp3 and fabp6* mRNAs in tissues of zebrafish fed diets differing in FA content. The steady-state levels of *fabp2* mRNAs in liver (A) and intestine (B), *fabp3* mRNA in liver (C) and intestine (D) and *fabp6* mRNA in liver (E) and intestine (F), of fish fed either highly unsaturated FA-rich diet (HD), linoleic acid-rich diet (LD), linolenic acid-rich diet (LND) and low fat diet (LFD). The steady-state level of *fabp* transcripts was normalized to the steady-state level of *ef1α*. Data are presented as mean ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNA between zebrafish [n = 6, (♂ = 3; ♀ = 3)] fed different FA diets are indicated by an asterisk.

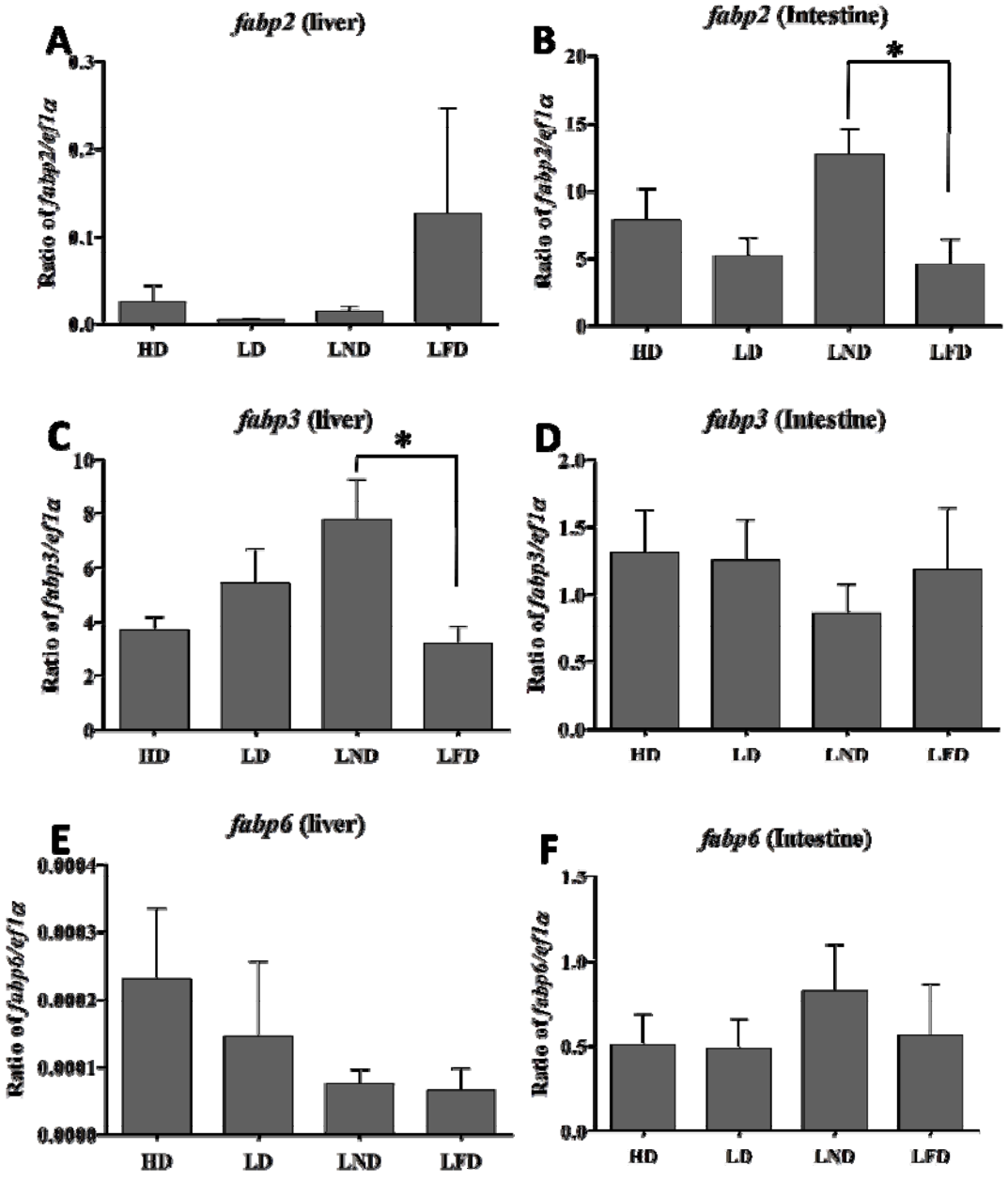

Figure 4.1 The steady-state levels of *fabp2, fabp3 and fabp6* mRNAs in tissues of zebrafish fed diets differing in FA content.

4.4.3 Effect of clofibrate on the steady-state levels of *fabp2, fabp3 and fabp6* mRNAs in tissues of zebrafish

To determine if the modulation of zebrafish *fabp* transcripts by dietary FAs was mediated via PPARs, we assayed the steady-state levels of *fabp2*, *fabp3* and *fabp6* mRNA transcripts in zebrafish fed clofibrate, a well studied PPARα agonist (Akbiyik et al., 2004; Haasch et al., 1998; Konig et al., 2007; Luci et al., 2007; Nunes et al., 2004; Ringseis et al., 2007; Yamoto et al., 1996). Clofibrate has been used to induce genes involved in FA metabolism, such as the acyl-CoA oxidase 1 (*Acox1*) gene, which is a marker of PPARα activation (Akbiyik et al., 2004; Reddy and Hashimoto, 2001). The levels of *Acox1/acox1* mRNA are induced by clofibrate in the liver, heart, kidney, duodenum and jejunum of rats (Ringseis et al., 2007), hepatocytes of rainbow trout (Donohue et al., 1993), liver of chicken (Konig et al., 2007), and liver and adipose tissue of pigs (Luci et al., 2007). In a previous study, we had shown that the steady-state level of zebrafish *acox1* mRNA was elevated in the liver, intestine, heart and muscle of fish fed clofibrate demonstrating that zebrafish, unlike some fishes, is responsive to this drug (Venkatachalam et al., 2012). The steady-state level of *fabp2*, *fabp3* and *fabp6* gene transcripts in the liver, intestine, muscle, brain and heart were determined in zebrafish fed diets differing in clofibrate content. The steady-state level of *fabp2* mRNA in liver (Fig. 4.2A), intestine (Fig. 4.2B), muscle (Fig. 4.2C), brain (Fig. 4.2D) and heart (Fig. 4.2E) did not change in zebrafish fed clofibrate. The steady-state level of *fabp3* mRNA, however, increased 4-fold in the intestine of zebrafish fed 0.75% clofibrate diet (Fig. 4.3B) compared to zebrafish not fed clofibrate. No change was observed in the steady-state level of *fabp3* mRNA in liver (Fig. 4.3A), muscle (Fig. 4.3C), brain

(Fig. 4.3D) or heart (Fig. 4.3E) of zebrafish fed clofibrate. The steady-state level of *fabp6* mRNA was 8-fold higher in the intestine of zebrafish fed 0.50% clofibrate compared to *fabp6* mRNA levels in intestine of zebrafish fed 0.25% clofibrate and zebrafish not fed clofibrate (Fig. 4.4B). Also, the steady-state level of *fabp6* mRNA was 5-fold higher in the heart of zebrafish fed 1.00% clofibrate compared to zebrafish not fed clofibrate (Fig. 4.4E). No change was observed in the steady-state level of *fabp6* mRNA in liver (Fig. 4.4A), muscle (Fig. 4.4C) or brain (Fig. 4.4D) of zebrafish fed clofibrate. The steady-state level of *fabp2*, *fabp3* and *fabp6* transcripts did not change in the brain of fish fed clofibrate, which might indicate that clofibrate does not cross the blood brain barrier.

4.4.4 Mechanisms for modulation of the steady-state levels of *fabp* mRNAs by dietary FAs and clofibrate

Studies in mammals have shown that gene transcripts of *FABP1* (*L-FABP*) and *FABP2* (*I-FABP*) in the liver and small intestine, respectively, are up-regulated by dietary FAs and peroxisome proliferators (Bass et al., 1985; Besnard et al., 1993; Kaikaus et al., 1993; Wolfrum et al., 1999; Poirier et al., 2001). In cultured human monocytes, *FABP4* (*A-FABP*) transcripts are increased by peroxisome proliferators (Pelton et al., 1999). More recently, Karanth et al. (2009b) showed that the tissue-specific changes in FA composition modulated the steady-state levels of mRNA and heterogeneous nuclear RNA for only one sister duplicate of three pairs of duplicated *fabp* genes in a given tissue, suggesting that transcriptional initiation of these FA-responsive *fabp* genes is regulated by FAs via PPAR and its response element, peroxisome proliferator response element (PPRE). Further implicating PPAR in the transcriptional regulation of *fabp* genes,

Figure 4.2 The steady-state level of *fabp2* mRNA in tissues of zebrafish fed clofibrate. The steady-state level of *fabp2* mRNA in liver (A), intestine (B), muscle (C) brain (D) and heart (E) of fish fed 0, 0.25, 0.50, 0.75 or 1.00% (w/w) clofibrate. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α*. Data are presented as mean ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNA between zebrafish [n = 12, (♂ = 6; ♀ = 6)] fed different concentrations of clofibrate are indicated by an asterisk.

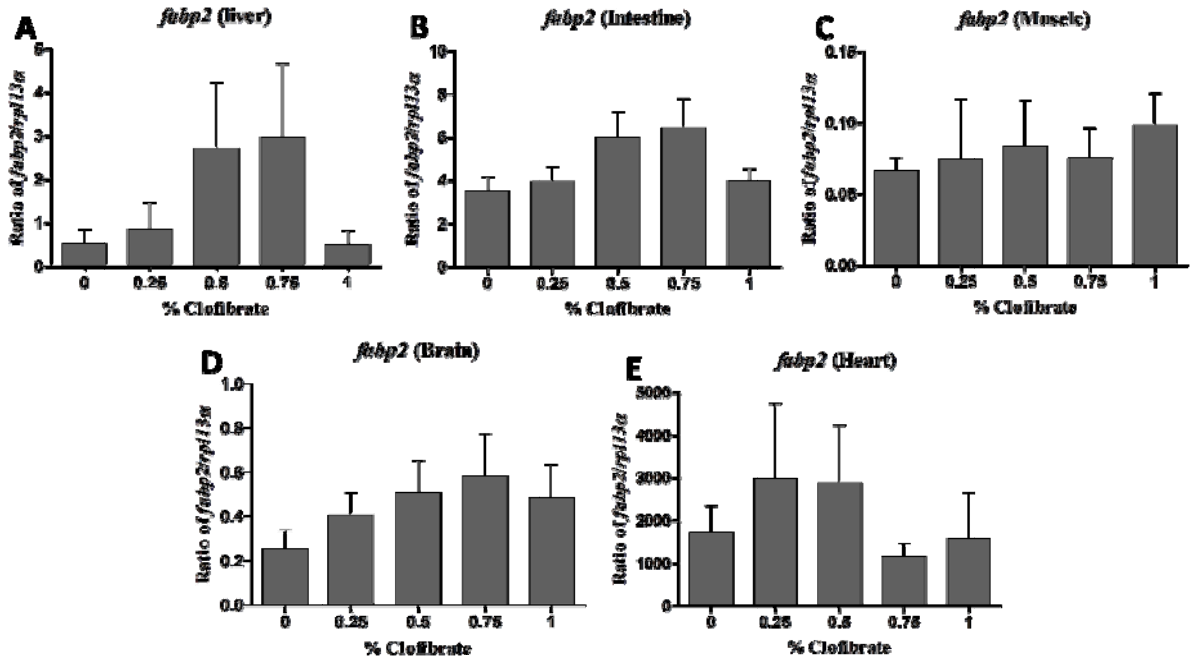

Figure 4.2 The steady-state level of *fabp2* mRNA in tissues of zebrafish fed clofibrate.

Figure 4.3 The steady-state level of *fabp3* mRNA in tissues of zebrafish fed clofibrate. The steady-state level of *fabp3* mRNA in liver (A), intestine (B), muscle (C) brain (D) and heart (E) of fish fed 0, 0.25, 0.50, 0.75 or 1.00% (w/w) clofibrate. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α*. Data are presented as mean ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNA between zebrafish [n = 12, (♂ = 6; ♀ = 6)] fed different concentrations of clofibrate are indicated by an asterisk.

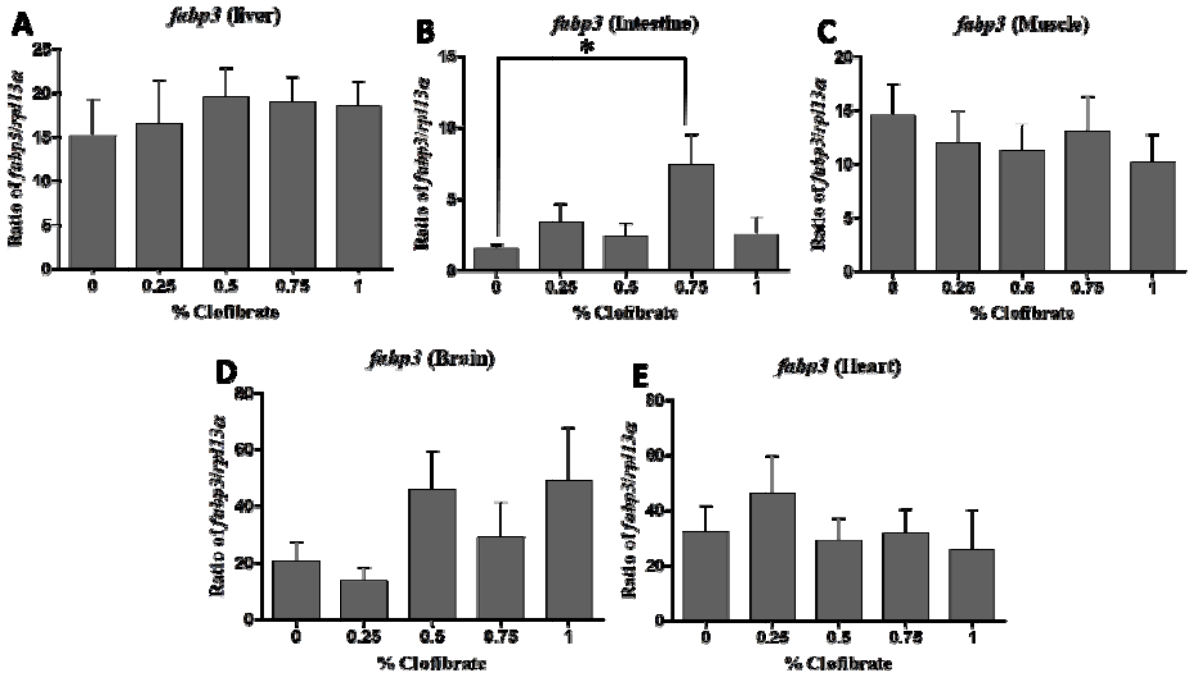

Figure 4.3 The steady-state level of *fabp3* mRNA in tissues of zebrafish fed clofibrate.

Figure 4.4 The steady-state level of *fabp6* mRNA in tissues of zebrafish fed clofibrate. The steady-state level of *fabp6* mRNA in liver (A), intestine (B), muscle (C) brain (D) and heart (E) of fish fed 0, 0.25, 0.50, 0.75 or 1.00% (w/w) clofibrate. The steady-state level of *fabp* transcripts was normalized to the steady-state level of *rpl13α*. Data are presented as mean ± S.E.M. Significant differences ($p < 0.05$) in the relative steady-state level of *fabp* mRNA between zebrafish [n = 12, (♂ = 6; ♀ = 6)] fed different concentrations of clofibrate are indicated by an asterisk.

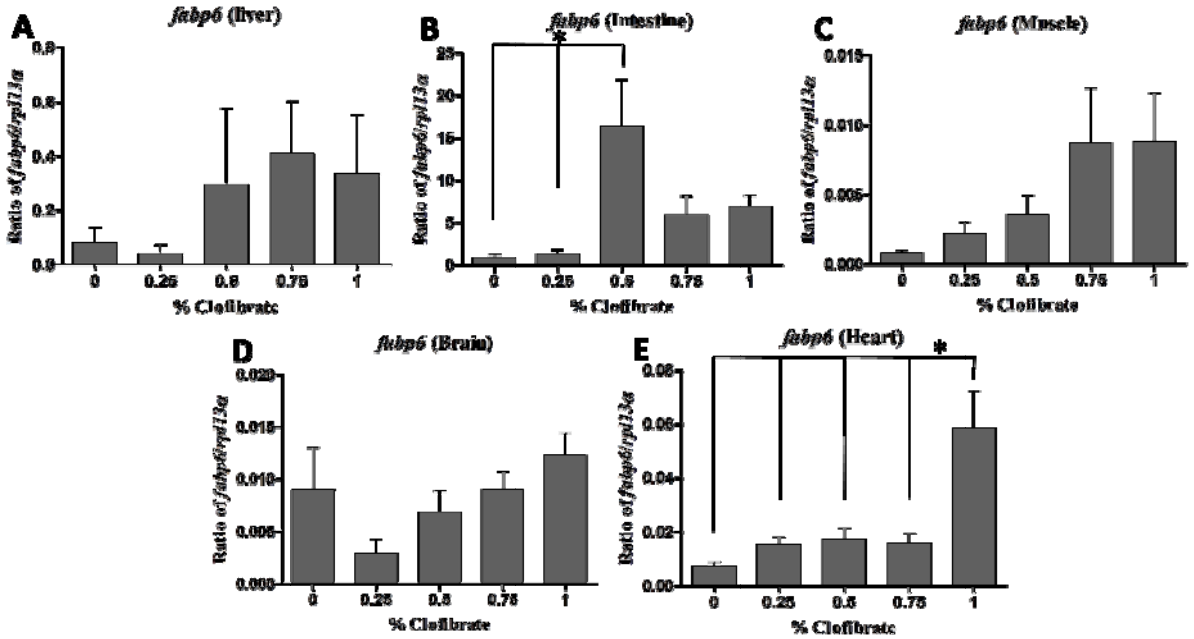

Figure 4.4 The steady-state level of *fabp6* mRNA in tissues of zebrafish fed clofibrate.

Venkatachalam et al. (2012) demonstrated that zebrafish fed clofibrate, a PPAR agonist, induced the steady-state level of several duplicated *fabp* genes. In the present study, we observed the transcriptional up-regulation of the steady-state level of *fabp2* transcripts in the intestine and *fabp3* transcripts in the liver of fish fed diets differing in FA content. In zebrafish fed clofibrate, *fabp3* mRNA in the intestine and *fabp6*mRNA in the intestine and heart were elevated (Table 4.2).

Based on the studies described here and in Venkatachalam et al. (2012), it is evident that PPAR is involved in the regulation of several *fabp* genes in zebrafish. Induction of *fabp* transcription by clofibrate via PPAR may be mediated directly, in which activated PPAR interacts with a PPRE in the promoter or an intronic region of the clofibrate-inducible *fabp* gene (Fig. 4.5A). Many putative PPREs were identified in the 5′ upstream region and introns of the zebrafish *fabp2*, *fabp3* and *fabp6* genes by in silico analysis (data not shown). We searched intronic regions for PPREs as several genes are induced by an interaction of PPAR with intronic PPREs (Hansmannel et al., 2003; Helledie et al., 2002). Alternatively, induction of *fabp* transcription by clofibrate may be indirect, wherein the *fabp* gene is controlled by an "upstream" transcription factor (TF) gene activated by PPAR, which produces a TF-activator to stimulate *fabp* gene transcription (Fig. 4.5B). Mutational studies of PPREs identified by *in silico* analysis, however, will be required to confirm their functionality, and to distinguish between direct or indirect control of *fabp* transcription by clofibrate-activated PPAR. Whether clofibrate induction of *fabp* transcription is controlled directly or indirectly by PPAR, clofibrate induction must be overridden by a tissue-specific mechanism(s), as *fabp3* transcription was induced by clofibrate in the intestine, but not the liver, muscle or heart, while *fabp6*

Table 4.2 Induction of *fabp2, fabp3* and *fabp6* mRNAs by clofibrate in various zebrafish tissues.

Gene	Liver	Intestine	Muscle	Heart	Brain
fabp2	-	-	-	-	-
fabp3	-	+	-	-	-
fabp6	-	+	-	+	-

+, increase relative to control
-, no increase relative to control

Figure 4.5 Transcriptional regulation of *fabp* genes in zebrafish by FAs and clofibrate. (A) *Direct regulation* of *fabp genes* – A peroxisome proliferator-activated receptor (PPAR), activated by either FAs or clofibrate, heterodimerizes with the retinoid X receptor (RXR), which in turn binds to the peroxisome proliferator response element (PPRE) in the *fabp* promoters, to induce transcriptional initiation of *fabp* genes by the preinitiation complex (PIC). **(B) *Indirect regulation* of *fabp genes* –** A PPAR, activated by either FAs or clofibrate, heterodimerizes with the RXR, which in turn binds to the PPRE in the promoter of an upstream gene coding for a transcription factor (TF), to induce transcriptional initiation of the TF gene. The TF then binds to a specific TF-binding site in the *fabp* gene to induce transcriptional initiation by the PIC. **(C) *Direct regulation* of *fabp* genes *by a tissue-specific activator or repressor* –** The heterodimer, PPAR-RXR, binds to PPRE in the *fabp* promoters, but induction of *fabp* transcription is modulated by tissue-specific transcription factors. The transcription of *fabp* genes is enhanced by PPAR-RXR only in the presence of bound activator in the *fabp* promoter. Alternatively, PPAR-RXR induction of *fabp* transcription is inhibited by a tissue-specific repressor, thereby maintaining the basal level of transcription. The recruitment of the tissue-specific activator or repressor is regulated by an as yet unknown ligand.

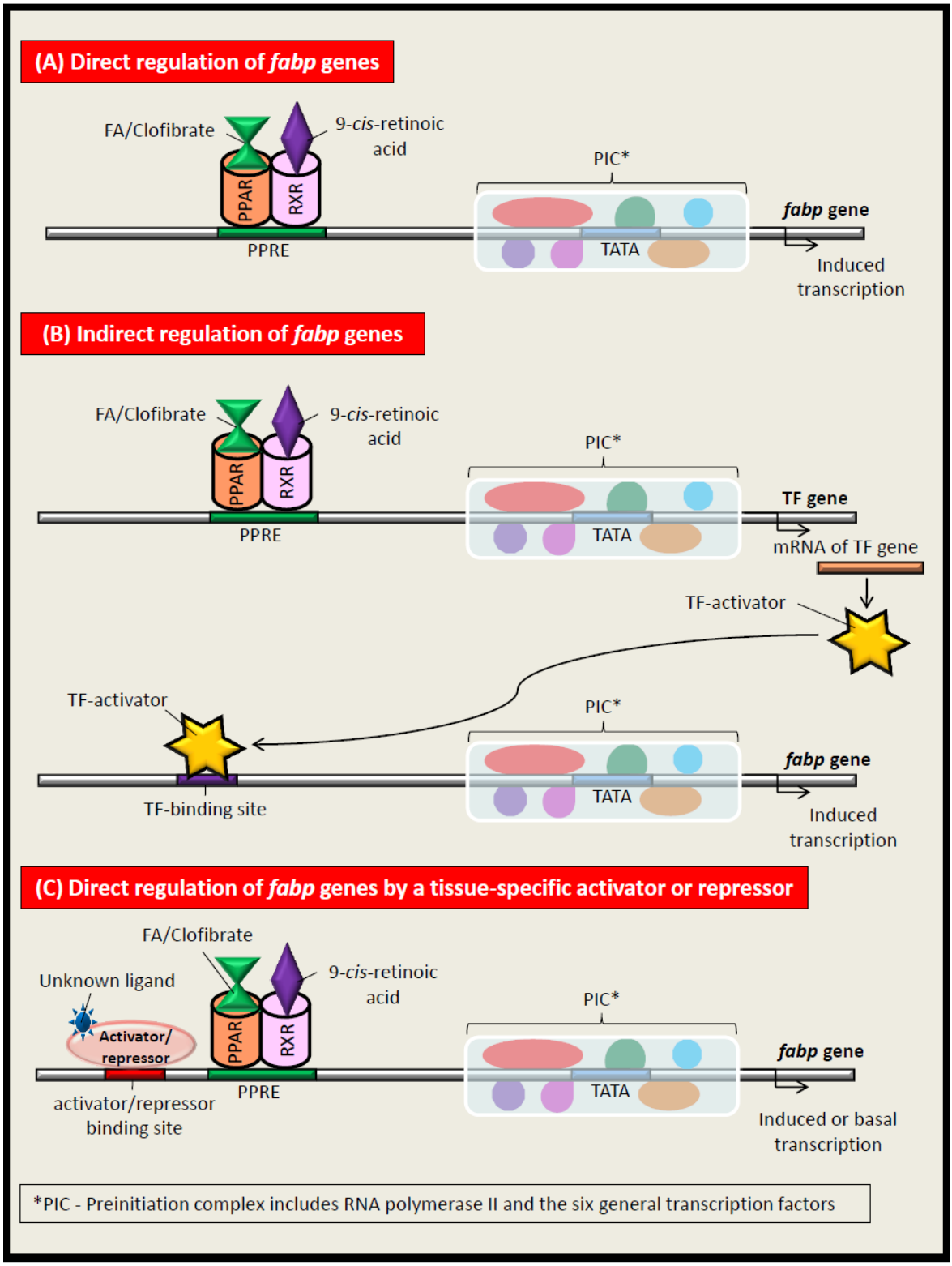

Figure 4.5 Transcriptional regulation of *fabp* genes in zebrafish by FAs and clofibrate.

transcription was induced by clofibrate in the intestine and heart, but not the liver and muscle (Fig. 4.3 and Fig. 4.4; Table 4.2). Venkatachalam et al. (2012) came to the same conclusion for the tissue-specific induction by clofibrate of duplicated copies of the zebrafish *fabp1*, *fabp7*, *fabp10* and *fabp11* genes. We propose, therefore, that clofibrate-induction may be modulated by either: (1) a tissue-specific *fabp* gene activator or repressor, which binds an as yet unidentified ligand (Fig. 4.5C); or (2) by tissue-specific remodeling of chromatin (Clapier and Cairns, 2009).

CHAPTER 5 : CONCLUSIONS

5.1 Evolution of the zebrafish *fabp* genes

The ancestor of present day teleost fishes underwent a WGD ~ 230 – 400 mya (Amores et al., 1998; Postlethwait et al., 2000; Jaillon et al., 2004; Woods et al., 2005; Kasahara et al., 2007). This teleost-specific WGD might explain the presence of numerous duplicate genes found in fish genomes (Amores et al., 1998; Woods et al., 2000; Taylor et al., 2003; Robinson-Rechavi et al., 2001). Phylogenetic analysis and conserved gene synteny of the zebrafish duplicated *fabp* genes suggests that these duplicated genes resulted from a duplication of the whole genome. Twelve *fabp* genes have been identified, thus far, in zebrafish, out of which eight genes (four pairs) *fabp1a/fabp1b*, *fabp7a/fabp7b*, *fabp10a/fabp10b*, *fabp11a/fabp11b* are sister duplicates of each other formed as a result of teleost-specific WGD (Liu et al., 2004; Sharma et al., 2006; Karanth et al., 2008; Venkatachalam et al., 2009). Karanth et al. (2009a) identified the *fabp1b.2* gene in zebrafish, which is a tandem duplicate of the *fabp1b* gene. Only three zebrafish *fabp* genes exist as single copies, namely *fabp2*, *fabp3* and *fabp6*. The duplicates of the zebrafish *fabp2*, *fabp3* and *fabp6* have been lost, most likely, owing to accumulation of deleterious mutations leading to nonfunction.

5.2 Why have so many *fabp* genes been retained in the zebrafish genome?

Estimates predict that 14 – 30% of the duplicated genes in the zebrafish genome are retained following the WGD event (Postlethwait et al., 2000; Woods et al., 2005). Surprisingly, the retention of the duplicated *fabp* genes in the zebrafish genome was found to be 73% (Figure 1.3). What might be the cause for the higher retention of duplicated *fabp* genes compared to other genes in the zebrafish genome? After the WGD

event, the resultant genome can revert back to its original state by elimination of genes, but certain selective constraints can lead to the retention of some genes. Lynch and Conery (2000) estimated that in eukaryotic genomes, duplicate genes arise at a rate of 0.01 per gene per generation on average, suggesting that gene duplication is a frequent event. In theory, the majority of the duplicated genes will be silenced and eventually lost due to accumulation of deleterious mutations in a relatively short time (Bailey et al., 1978; Li, 1980; Lynch and Conery, 2000). The fate of the duplicated genes and the mechanism for preservation of abundant ancient duplicate genes remains unclear and controversial. Aury et al. (2006) suggested that the high level of retention of duplicated copies of genes in the ciliate *Paramecium tetraurelia*, a unicellular eukaryote, is due to constraints arising from gene dosage. The retention of duplicated copies of gene occurs only if it is 'fixed' in the population (Hughes et al., 2007). The probability of fixation depends on the fate of initial duplication event. If the initial duplication event is evolutionarily neutral, then the duplicated copies of genes gets fixed in the population, whereas, if the initial duplication event proves deleterious for the gene duplicates, then it reduces the probability of fixation of the duplicated copies of genes (Hughes et al., 2007). The genes involved in the control of metabolism are found to have higher retention than other genes (Aury et al., 2006). To explain the retention of duplicated genes in *Arabidopsis*, Bekaert et al. (2011) proposed an hypothesis called the "dosage balance hypothesis", which explains that certain metabolic genes having key interactions with other metabolic components should be retained because, the loss of such genes would lead to an imbalance of metabolic homeostasis. Comparison of the duplicated genes involved in metabolism, signal transduction and transcription, have found no difference

between the retention of duplicated genes involved in these various cellular processes (Sato et al., 2009). As such, the higher retention of the duplicated *fabp* genes compared to other genes in the zebrafish genome is not solely dependent on the metabolic function of Fabps. Transcriptional regulation of the duplicated *fabp* genes in the zebrafish genome may explain their high retention. As proposed in the DDC model, the increasing independent mutable subfunctions in the regulatory region of the duplicate genes increase the probability of subfunctionalization in those duplicate genes (Force et al., 1999). With the passage of time, these subfunctionalized duplicates are fixed in the population by attaining new functions (neofunctionalization).

5.3 Tissue-specific distribution of *fabp* mRNA transcripts in zebrafish

To determine the tissue-specific distribution of *fabp* mRNA transcripts in zebrafish adults and embryos, RT-PCR and whole-mount *in situ* hybridization was performed. The pattern of transcript distribution of the duplicated genes in adults and embryos may deviate, but collectively they complement the pattern of transcript distribution of the ancestral gene (Postlethwait et al., 2004). A suitable ancestral gene for the zebrafish duplicate genes may be a mammalian/avian ortholog, because mammals or birds have not undergone a WGD after the divergence from teleost fishes. In this study, by analyzing the tissue-specific distribution of gene transcripts of the duplicated pair of zebrafish *fabp10* using RT-PCR and whole-mount *in situ* hybridization, a distinct pattern of transcript distribution was observed (Table 5.1) (Venkatachalam et al., 2009). Clearly, *fabp10a* and *fabp10b* transcripts in embryos, larvae and adult zebrafish show strikingly different tissue-specific patterns of distribution. On the basis of the distribution of *fabp10b* transcripts in several tissues of adult zebrafish, compared with the limited

Table 5.1 Tissue-specific distribution of *fabp10* (*fabp10a* and *fabp10b*) transcripts in embryos and adults of zebrafish.

Gene	Zebrafish tissue												
	Liver	**Intestine**	**Muscle**	**Brain**	**Heart**	**Eye**	**Gills**	**Ovary**	**Testis**	**Skin**	**Kidney**	**Swim bladder**	**Ofv**
fabp10a	+	+	-	-	-	NA	-	-	+	-	NA	NA	NA
fabp10b	+	+	+	+	+	+	+	+	+	+	+	+	+

Note: +, transcripts detected in the tissue; -, transcripts not detected in the tissue; NA, not assayed;
Ofv, olfactory vesicle

distribution of zebrafish *fabp10a* transcripts, and chicken FABP10 (the presumed ancestral state of the *fabp10* gene prior to duplication) transcripts, which are restricted to the liver in adults (Murai et al., 2009), we propose that both the zebrafish *fabp10a* and *fabp10b* genes were retained in the genome owing to neofunctionalization of *fabp10b*.

5.4 Tissue-specific up-regulation of *fabp* gene transcription by FAs and clofibrate in zebrafish

Transcriptional initiation of mammalian *FABP1* (*L-FABP*) and *FABP2* (*I-FABP*) in liver and small intestine, respectively, and *FABP4* (*A-FABP*) in monocytes are up-regulated by dietary FAs and peroxisome proliferators (Bass et al., 1985; Besnard et al., 1993; Kaikaus et al., 1993; Pelton et al., 1999; Wolfrum et al., 1999; Poirier et al., 2001). Recently, Karanth et al. (2009b) showed that transcriptional initiation of only one sister duplicate of three sets of duplicated *fabp* genes, *fabp1a/fabp1b.1/fabp1b.2, fabp7a/fabp7b* and *fabp11a/fabp11b* is regulated by dietary FAs in a specific tissue. As transcriptional regulation of these *fabp* genes by dietary FAs might be mediated via PPARs (Pelton et al., 1999), we fed fish clofibrate, a PPAR agonist, and assayed the steady-state levels of *fabp* transcripts. The steady-state level of duplicated *fabp* mRNA transcripts (Venkatachalam et al., 2012) and the single copy *fabp* mRNA transcripts (Table 4.2) of zebrafish fed clofibrate exhibited a distinct pattern of up-regulation. These studies have demonstrated that the regulation of *fabp* genes in zebrafish is indeed mediated via PPARs interaction with a PPRE present in the promoter region/introns of *fabp* genes. *In silico* analyses of the zebrafish *fabp* genes identified many putative PPREs in the 5' upstream region and introns of the zebrafish *fabp* genes (data not shown), however, mutational studies of these potential PPREs are necessary to confirm their

functionality. To explain the mechanisms involved in the induction of zebrafish *fabp* genes by clofibrate, we propose two exclusive testable models: (1) the regulation of *fabp* gene transcription may be either direct, in which PPAR interacts with a PPRE in the promoter or an intronic region of the *fabp* gene and initiates an activated level of transcription (Fig. 4.5A) or indirect, where the induction of the *fabp* genes is mediated by an intermediate or 'upstream' transcription factor (TF) gene activated by PPAR, which in turn activates transcriptional initiation of *fabp* genes (Fig. 4.5B); and (2) to explain the tissue-specific pattern of *fabp* gene regulation, either a direct mechanism is involved together with a tissue-specific activator or repressor which is activated by an unknown ligand (Fig. 4.5C) or an indirect mechanism is involved together with a TF acting as an activator on the TF gene itself (Fig. 5.1). The tissue-specific remodeling of chromatin should also be considered for the clofibrate-induction of *fabp* genes. Whether the regulation of *fabp* gene transcription by clofibrate is controlled either directly or indirectly, the regulatory elements in the zebrafish *fabp* genes have diverged markedly since the WGD event ~ 230 – 400 million years ago (Robinson-Rechavi et al., 2001; Furlong and Holland, 2002; Jaillon et al., 2004; Woods et al., 2000; Christoffels et al., 2004; Vandepoele et al., 2004).

Figure 5.1 Transcriptional regulation of *fabp* genes in zebrafish by FAs and clofibrate. Indirect regulation of *fabp* genes with a transcription factor (TF) activator binding to the TF gene. A PPAR, activated by either FAs or clofibrate, heterodimerizes with the RXR, which binds to the PPRE in the promoter of an upstream gene coding for a transcription factor (TF), to induce transcriptional initiation of the TF gene. The TF then binds to a specific TF-binding site in the TF gene, which in turn regulates the transcription of *fabp* genes.

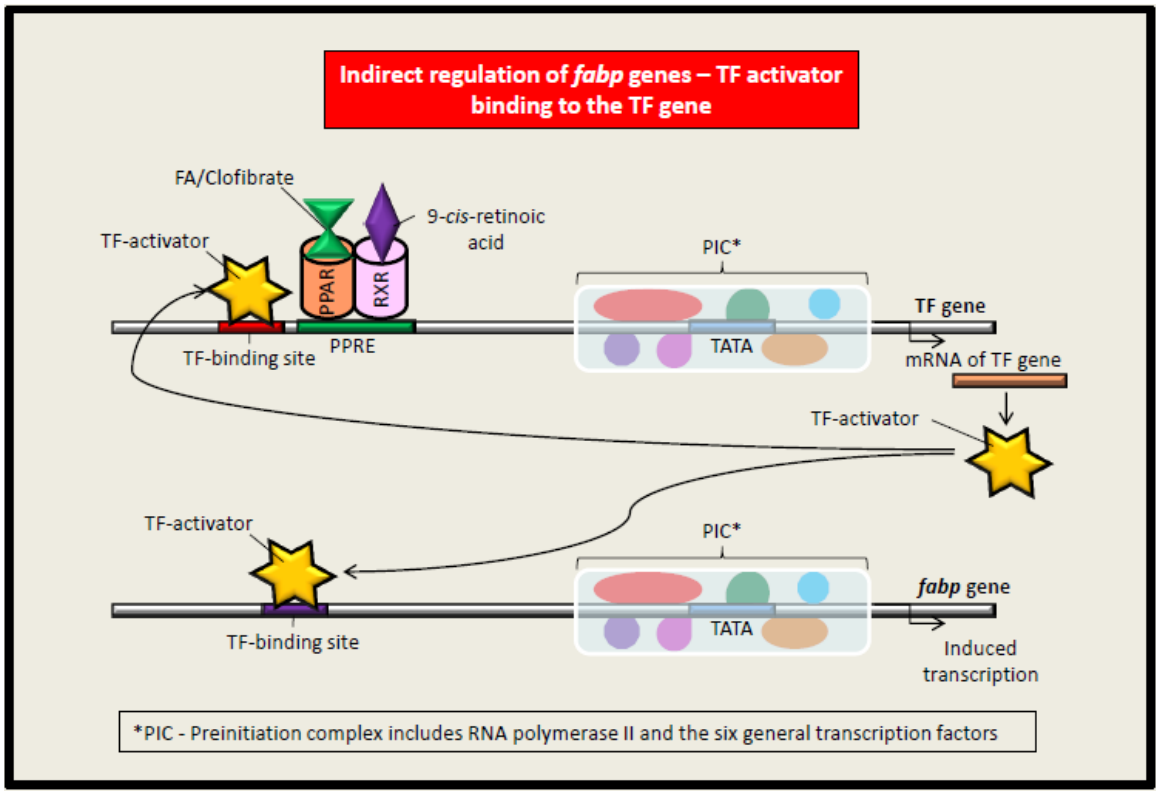

Figure 5.1 Transcriptional regulation of *fabp* genes in zebrafish by FAs and clofibrate. Indirect regulation of *fabp* genes with a transcription factor (TF) activator binding to the TF gene.

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Appendix A

Copyright Permission Letter from FEBS Journal

Appendix B

Copyright Permission Letter from BMC Evolutionary Biology Journal

Date: Mon, 16 Jul 2012 16:52:10 +0100 [07/16/12 12:52:10 ADT]
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Tim

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Copyright Permission Letter from GENE Journal

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