博士論文（要約）

Physiological functions of lipoprotein lipase for controlling triacylglycerol levels in medaka

（メダカのトリアシルグリセロールレベル調節におけるリポプロテインリパーゼの機能）

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Abbreviations

The abbreviations were used in the thesis as follows:

ANOVA: analysis of variance

Arg: arginine

Asn: asparagine

Asp: aspartic acid

bp: base pairs

cDNA: complementary deoxyribonucleic acid

Ct: threshold cycle

Cys: cysteine

ddH2O: double distilled water

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleic acid

DTT: dithiothreitol

EF-1α: elongation factor-1 alpha

EL: endothelial lipase

GAPDH/G3PDH: glyceraldehyde-3-phosphate dehydrogenase

His: Histidine

HL: hepatic lipase

HMMPS: N-(3-sulfopropyl)-3-methoxy-5-methylaniline

HUFA: highly unsaturated fatty acids

LPL: lipoprotein lipase

Lys: lysine

mRNA: messenger ribonucleic acid

NJ: Neighbor-Joining

nt: nucleotide

ORF: open reading frame

PCR: polymerase chain reaction

PI: isoelectric point

PL: pancreatic lipase

Pro: proline

QPCR: real-time quantitative polymerase chain reaction

RACE: Rapid Amplification of cDNA Ends

RNA: ribonucleic acid

RNase: ribonulease

RPL-7: ribosomal protein L7

RT-PCR: reverse transcription polymerase chain reaction

Ser: serine

TAG: triacylglycerol

Trp: tryptophan

UNG: uracil-N glycosylase

UTR: untranslated region

Val: valine

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ABSTRACT

Triacylglycerol (TAG) is the major lipid providing energy required for metabolic process in organisms. It is composed of one glycerol molecule esterified with three fatty acids (FA). In general, glycerol backbone is derived from glucose metabolism, while the three fatty acid components are derived either from dietary lipids or from acetyl CoA via *de novo* FA biosynthesis. Acetyl CoA is well known as one important intermediary metabolite from glucose and amino acid metabolisms. In contrast to mammals, fish utilize TAG as a main energy source, in preference to carbohydrate. They store TAGs in several sites, such as adipose, liver and muscle tissues.

Fasting is known to be a natural occurrence in fish life. During fasting, FA portion of TAG is used for the synthesis of acetyl CoA, a critical precursor for the syntheses of ketone bodies. These derivatives are considered as available energy fuels in muscle and brain. Glycerol portion is generally used as a gluconeogenic substrate, and thus TAG reserves in fish are considered to play an important role in the metabolic adaptation to fasting. The molecular mechanism in the regulation of TAG metabolism under the condition of food deprivation, however, remains unclear. The aim of this study is to clarify the correlation of changes in TAG reserves and the regulation of related genes in response to fasting and re-feeding.

Four groups of medaka were acclimated to laboratory environment for 3 weeks. One group of medaka was used as the control, and two groups were fasted for

4 and 8 days, while the remaining one group was re-fed for 4 days after the 8-day fasting. Measurement of TAG levels in muscle and liver of medaka under each period was performed using colorimetric method. Fasting for 8 days reduced hepatic TAG levels, followed by their recovery after 4-day re-feeding. In contrast, muscle TAG levels were increased after fasting for 4 days, and the further 4-day fasting did not affect the levels. Re-feeding reduced muscle TAG levels again. These results suggest the tissue-specific effects of fasting and re-feeding on TAG stores in medaka tissues.

Comprehensive assay of gene expression profile was performed using a next generation sequencing (NGS) technique. Overall, eight clusters of gene expressions were identified in both tissues using hierarchical clustering method. Fasting for 4 days resulted in the up-regulations of the genes in four clusters, which were involved mainly in glycolysis and proteolysis. However, most genes related to TAG metabolism and FA oxidation were expressed at low levels. Notably, several up-regulated genes were found to participate in the pathway of TAG synthesis. These findings suggest that medaka reduces TAG metabolism and conserves TAG stores in muscle, while consumes glycogen, glucose and protein as energy fuels for the maintenance of basic cellular functions at the early stage of fasting, in order to adapt to the nutritional restriction. In addition, the induction of TAG synthesis would be associated with the elevation of TAG levels in muscle. During the post period of fasting, the six clusters contained approximately 50 genes showing high expressions in muscle. These genes were involved in TAG metabolism, FA oxidation, gluconeogenesis and protein/amino acids catabolism. These observations suggest that medaka would acquire the acclimation stage against nutrient restriction with the

dynamic metabolic regulations. In contrast, re-feeding reduced most of the genes related to proteolysis and amino acid catabolism, whereas the genes with high expressions in four clusters were related to TAG metabolism and FA oxidation. These findings suggest that re-feeding should promote the metabolic levels of TAG and FA in medaka muscle, accompanied by the conservation of protein stores.

On the other hand, during the early period of fasting, the genes with elevated expressions in liver were involved in lipolysis, FA oxidation, gluconeogenesis, ketone bodies synthesis and proteolysis, while the expressions of the genes in the other clusters were decreased. These observations imply that liver would rapidly respond to fasting and consume its stores for the provision of energy fuels required by peripheral tissues as the center of metabolic control. The post period of fasting induced the down-regulations of most genes, whereas only two clusters containing about 18 genes were expressed at high levels. This complete inversion of gene expression patterns suggests that medaka should acclimate to the low energy status, and thus reduce the metabolic regulation in liver. Re-feeding induced the up-regulations of four groups of genes that were mainly related to TAG synthesis and de novo FA biosynthesis. These observations suggest that re-feeding should reset the state of acclimation to restricted nutritional condition and recover TAG stores in liver.

During the adaptation periods of fasting and re-feeding, the patterns of metabolic regulations in both tissues suggest that TAG transport has a large effect on the maintenance of TAG stores. Especially, the expression patterns of LPL gene that has a critical role in the regulation of TAG transport were found to have close correlations with TAG levels in both tissues. Therefore, the molecular characteristic

and tissue-distribution pattern of medaka LPL gene were further investigated in this study. The regulation of LPL transcripts in response to fasting and re-feeding was also confirmed by quantitative real-time RT-PCR (qRT-PCR) technique.

The cDNA sequence of medaka LPL gene was cloned from liver of adult medaka using rapid amplification of cDNA ends technique. The deduced amino acid sequence of LPL was found to share a high identity (78.3–84.7 %) with LPL1 sequences from several fish species, and therefore was termed LPL1. The 2,259–bp cDNA of medaka LPL1 contained an open reading frame of 1,551 bp encoding 516 amino acids, a 415–bp 5' untranslation region (UTR) and a 563–bp 3' UTR. The deduced amino acid sequence of medaka LPL1 was found to share the common conserved sites with that of LPL1 genes from several fish species and LPL genes from mammals, i.e., one catalytic triad (Ser179, Asp203 and His291), one heparin-binding site (329–332 residues), a putative polypeptide 'lid' (263–289 residues), eight cysteine residues, one lipid-binding site (Trp440, Trp443 and Trp444) and one putative *N*-linked glycosylation site (Asn409).

The tissue-distribution pattern of LPL1 gene indicated that LPL1 transcripts were ubiquitously expressed in various tissues. The highest levels of LPL1 transcripts were found in liver, followed by visceral adipose tissue, whereas the lowest levels were found in the intestine. The observation in the relatively high expression of LPL1 gene in the brain suggests the potential function of LPL1 in this organ.

The expressions of LPL1 gene in muscle and liver modulated by fasting and re-feeding were further analyzed by qRT-PCR assay using three internal reference genes. In general, the relative mRNA levels of LPL1 normalized to each internal

reference gene showed a similar tendency in both tissues. The results in muscle confirmed the NGS assay that a higher rate of the increase in the expression of LPL1 gene was found during the early period of fasting. In contrast, a slight decrease was found during the post period of fasting by qRT-PCR assay. Overall, the continuously high expression of LPL1 gene suggests that this gene has a large effect on the maintenance of TAG levels in muscle during fasting.

In conclusion, the gene expression profile observed in this study suggests that medaka modulates the metabolic patterns of TAG stores in tissue-specific manners for the adaptation to the different stages of fasting. A rapid response of liver to fasting was reflected by the transient lipolysis of its TAG stores for the provision of various energy substrates required by peripheral tissues. However, medaka was likely to prefer to conserve muscle TAG stores for the adaptation to the low energy status. The observations during the post period of fasting indicate that medaka enters the acclimation stage to the poor nutritional condition through the induction of the dynamic metabolic regulations and the reduction of metabolic reactions in muscle and liver, respectively. The potential transport of energy sources among the tissues regulated by LPL gene implied its critical function on the maintenance of muscle TAG stores during fasting.

INTRODUCTION

Basic background

Lipids play a number of major roles in organisms such as metabolism, signal transduction and supply of lipid-soluble vitamins. Fish utilize lipids and proteins as their major macronutrients, in preference to carbohydrates (Cowey and Cho, 1993; Sargent et al., 2002). Dietary lipid has three primary functions in fish. They can be incorporated into cell membranes and hence the flesh of fish, they can be oxidized to provide energy, or lipid can be deposited in adipose or other tissues for storage. Depending on the species, fish have a varying requirement for lipid. Marine fish require strictly for n-3 highly unsaturated fatty acids (HUFA), because they have limited capability to synthesize n-3 HUFA from shorter chain fatty acids (Zheng et al., 2004; Hastings et al., 2004; Seiliez et al., 2003). Consequently, fish oil, rich in n-3 HUFA and derived from industrial fisheries, is used as the only commercially available source of the essential fatty acids in marine fish diets. However, it is clear that increasing demand from aquaculture for fish oil will soon exceed supply and threaten the viability of fish farming activities as well as wild fish stocks (Tacon, et al., 2010). For these reasons, identifying suitable alternatives to fish oil in cultured fish diets or improving efficiency of fish oil use are becoming urgent issues.

In addition, a number of studies focused on the evaluation of lipid levels of muscle in cultured fish, because the lipid content in muscle is an evaluating factor of the fish quality (Shimizu et al., 1973; Konosu and Watanabe, 1976; Saeki and

Kumagai, 1984; Nakagawa et al., 1991; Umino et al., 1991; Ando et al., 1993). With the development of fin-fish aquaculture, artificial diets or feeds are formulated to satisfy essential requirements, and provide macronutrients and energy to the cultured fish. However, in order to satisfy commercial pressure to increase growth rates and reduce production times, dietary formulations have maximized lipid content. This phenomenon is driven by the views that the more energy can be supplied by dietary lipid, the less protein will be used for energy and so more dietary protein can be spared for synthesis of new flesh (Hemre and Sandnes, 1999), but the fact that there is a strong relationship between dietary lipid levels and undesirable lipid levels in fish tissues is ignored (Cowey and Cho, 1993). Therefore, from sustainable fisheries and aquaculture, it is necessary to research fish nutrition including lipid deposition, metabolism and their regulation to ensure fish health and maintain the nutritional benefits of fish for human consumer (Tocher, 2003).

Fish accumulate lipids in several storage sites such as mesenteric adipose tissue, subcutaneous adipose tissue, muscle and liver (Babin and Vernier, 1989). It has been reported that the distribution of lipids among these tissues largely depends on the species of fish. Pufferfish and flounder contain lipids mainly in liver, whereas other commercially valuable fish such as red seabream and amberjack accumulate lipid both in liver and muscle (Ando et al., 1993). Some studies also found that the lipid contents in different tissues of fish showed the variances (Johnstone and Goldspink, 1973; Umino et al., 1991). The studies in rainbow trout by Black and Skinner (1986) found that the weight of heart muscle was little affected by fasting whereas the weight of liver was markedly decreased during fasting. It appears that

tissues essential for the animal's existence, such as nervous tissue and heart muscle, are little affected by fasting (Black and Skinner, 1986). The tissue-specific distribution and transport of lipid may relate to the regulation of lipoprotein lipase (LPL) expression and activity.

LPL is a member of the lipase gene family, which consist of other lipases such as hepatic lipase (HL), endothelial lipase (EL) and pancreatic lipase (PL) (Kurtoic et al., 2009). All these enzymes would have evolved from a common ancestor and they play a critical role in the deposition and metabolism of lipids (Wong and Schotz, 2002; Kurtoic et al., 2009). LPL has its physiological site of action at the luminal surface of capillary endothelial cells through the interaction with heparan sulfate proteoglycans, where the enzyme hydrolyses the triacylglycerol, a component of circulating lipoprotein particles including chylomicrons and very low density lipoproteins, to provide free fatty acids and glycerol for tissue utilization (Mead et al., 2002). Mammalian LPL is primarily synthesized in heart, skeletal muscle and adipose tissue, but not in the adult liver (Kurtoic et al., 2009). In contrast to the mammals, LPL is also synthesized in the liver of fish.

There are a number of evidences for a regulated LPL expression and activity in response to a number of physiological conditions affected by endogenic factors such as hormone including insulin, feeding conditions such as dietary lipid levels and feeding/fasting, and environmental conditions such as season (Auwerx et al., 1992; Enerbäck and Gimble, 1993; Ruge et al., 2005; Doolittle et al., 1990) that help to direct fatty acid utilization according to specific metabolic demands. In mammals such as human and rodents, adipose tissue LPL activity is decreased during fasting

(Bergö et al., 1996; Doolittle et al., 1990) whereas a concomitant increasing or remaining unchanged in heart and skeletal muscle is found (Tan et al., 1977; Ruge et al., 2005).

In contrast to mammals, LPL expression has been also detected in the liver of adult fish (Liang et al., 2002; Saera-Vila et al., 2005). Black et al. (1986) demonstrated that rainbow trout LPL activity decreased in adipose tissue and liver during fasting, whereas there were no changes in heart and red muscle. The studies reported by Saera-Vila (2005) showed that the LPL gene expression of gilthead seabream was regulated in a tissue-specific manner by dietary and seasonal condition.

Small fish species such as zebrafish (Danio rerio) and medaka (Oryzias latipes) are widely accepted as model vertebrate animals in developmental and molecular biological fields. Both teleost fish have been used as models in various fields of biological research for the past several decades, such as mutation, carcinogenesis and toxicology (Powers, 1989; Kubota et al., 1992; Brown-Peterson et al., 1999; Toussaint et al., 2001). Medaka and zebrafish have several species-specific features for different genetic studies, such as sex determination and adult pigment patterning, which are best studied in medaka and zebrafish, respectively (Schartl, 2004; Haffter et al., 1996).

In comparison with the spawning of zebrafish for twice a week, medaka spawns eggs every day and the time of spawning can be controlled by regulating light conditions (Furutani-Seiki and Vittbrodt, 2004). Until now, little is known about sex determination in zebrafish, and there is none of the many markers on the zebrafish genetic map found to be sex linked (Woods et al., 2000). Conversely, medaka has an

XX, XY sex-determination system like mammals, with the male determining locus on the Y chromosomes, and its chromosome number is 48, which is similar with the chromosome number of human for a total of 46 (Wittbrodt et al., 2002). It is the only fish species with more than ten inbred strains and its small genome size, about 800 Mb, which is one-half of zebrafish and one-fourth of mammalian sizes, enables sequencing of the whole genome (Wittbrodt et al., 2002). In addition, medaka is easy to work with because it tolerates a wide range of temperatures (10-40 °C) and highly resists to common fish diseases.

Objectives of this study

TAG is considered as one important energy source required for metabolic process in fish during fasting. The distribution pattern of TAG in tissues is intimately related to the whole of intermediary metabolism, and thus TAG reserves in fish are considered to play an important role in the metabolic adaptation to fasting. The molecular mechanism in the regulation of TAG metabolism under the condition of food deprivation, however, remains unclear. The objectives of this study are as follows:

1) Investigation of the profile of TAG storage in medaka tissue.
2) Clarification of the effect of food restriction and re-feeding on the changes in TAG reserves and their correlations with the regulation of related genes in response to fasting and re-feeding.
3) Investigation of the correlations of the expressions of LPL gene with TAG

storage in medaka tissues.

Outline of chapters

This thesis is composed of four chapters.

In Chapter I, the distribution profile of TAG stores in medaka tissues were measured. In addition, the TAG levels in liver and muscle of fed and fasted medaka were examined, which is the major lipid for energy provision and storage, in order to understand the effects of feeding conditions on the characteristics of lipid deposition.

In Chapter II, comprehensive analyses of gene expression patterns were performed to clarify the molecular mechanism in the regulation of TAG storage during fasting and re-feeding.

In Chapter III, identification of the LPL gene was performed using rapid amplification of 3’ and 5’ end (RACE) approaches. Tissue-distribution pattern of LPL gene were investigated by quantitative real-time RT-PCR (qRT-PCR). In addition, the regulations of LPL1 gene on TAG storage in liver and muscle of medaka in response to fasting and re-feeding were further confirmed by qRT-PCR using three internal reference genes.

Chapter IV is the general discussion of all the results and perspective on future work.

CHAPTER I

Distribution profile and the regulation of TAG stores in response to fasting and re-feeding in medaka

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CHAPTER II

Expression pattern of the genes related to TAG metabolism under the condition of fasting and re-feeding

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CHAPTER III

Molecular characterization and regulation of medaka lipoprotein lipase gene

Lipoprotein lipase (LPL) is one of the members in lipase superfamily that contributes to vascular lipoprotein degradation and plays major rules in hydrolyzing TAG that is a component of circulating chylomicrons and very-low density lipoproteins (Wong and Schotz, 2002; Mead et al., 2002). LPL thus plays a primary role in TAG metabolism and is widely distributed in tissues such as adipose tissues, heart, muscle, liver (neonatal period in mammals), small intestine, kidney and lung (Kurtoic et al., 2009). In contrast to mammals, LPL is also synthesized in the liver of adult fish (Kurtoic et al., 2009).

The effects of feeding conditions, such as dietary compositions and feeding/fasting, on LPL gene expression and activity have been investigated in liver, adipose tissue, muscle and heart (Tan et al., 1977; Doolittle et al., 1990; Ruge et al., 2005). It has been found that, in mammals, the LPL mRNA levels in adipose tissues are decreased during fasting, whereas the levels are increased or remain no changes in heart or muscle (Doolittle et al., 1990; Ladu et al., 1991). Black et al. (1986) demonstrated that fasting decreased LPL activity in adipose tissue and liver of rainbow trout, but not in heart and red muscle. Liang et al. (2002) founded that the tissue-specific regulation of LPL gene expression in red seabream was affected by dietary compositions.

Real-time PCR has been widely used in the field of gene expression analysis in living organisms. In comparison to classical reverse transcription-polymerase chain reaction (RT-PCR), the main advantages of real-time PCR are its higher sensitivity and specificity (Bustin, 2002; Walker, 2002). In the course of measurement of gene expression, some experimental conditions such as the inconsistencies in the procedures of tissue collection, RNA isolation and complementary cDNA synthesis may lessen the reliability of qRT-PCR (Thellin et al., 1999). To correct the sample-to-sample variation during determining the gene expression, housekeeping genes, such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH/G3PDH), 18S rRNA, elongation factor 1α (EF1α) and so on, are used as internal references in order to adjust for intrinsic experimental variation and normalize the target gene expression levels (Stürzenbaum et al., 2001; de Kok et al., 2005). However, numerous studies showed that even these genes could vary in given situations (Nicot et al., 2005; Radonić et al., 2004; Olsivk et al., 2005; Filby et al., 2007 and Vandesompele et al., 2002). Some references demonstrated that because the use of a single gene for normalization could lead to relatively large errors. At least two or three housekeeping genes should be used as an internal control, which may be partly explained by the fact that housekeeping proteins are not only implicated in the basal cell metabolism but also participate in other functions (Singh and Green, 1993; Ishitani et al., 1996).

Actin is a major component of the protein scaffold that supports the cell and determines its shape, and is the most abundant intracellular protein in eukaryotic cells (Olsivk et al., 2005). Beta-actin has been widely used in the measurement of gene expression, highlighting that it can exhibit good invariable characteristics within a

defined context, and can be enhanced its specificity by designing probes (Raff et al., 1997). The EF-1α is a ubiquitous protein binding aminoacyl-transfer RNA to ribosomes in the course of protein synthesis (Stürzenbaum et al., 2001). It has been used to be a good invariant control to adjust for differences in tube-to-tube loading and/or degradation (Dostal et al., 1994). RPL-7 was found in human by Von Mikecz et al. in 1999 and then was used as an internal control to normalize gene expression in mammals and teleost fish such as medaka (Muroya et al., 2005; Zhang and Hu, 2007).

The aim of this charpter was to understand the effects of regulated LPL expression on the lipid deposition in medaka tissues during fasting. Clarification of the structure, function and regulation of LPL gene expression in tissues of teleost fish may enable us to better understand the lipid deposition in fish. As the first step, the full-length cDNA of LPL from liver was cloned and its characteristic was estimated. Then, the relative mRNA levels of LPL in liver and muscle of fed and fasted medaka were compared, using real-time PCR technology with three housekeeping genes, β-actin, EF-1α and RPL-7 as internal controls.

Section 1

Molecular cloning of lipoprotein lipase full-length cDNA in medaka

In this section, the total RNA was isolated from liver and first-strand cDNA was synthesized for the cloning of the full-length cDNA of LPL. Characteristic of the nucleotide sequence and amino acid sequence of LPL was also estimated.

Materials and methods

Materials

Medaka were purchased from fish center (Ichigaya, Tokyo, Japan) and cultured in the indoor tank in the laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan. The fish were fed with a commercial diet under condition of temperature ranged from 27 to 28 °C. The body masses of fish used for the cloning were ranged from 400 mg to 500 mg.

Isolation of total RNA

Liver tissues of medaka were taken under microscopic observation. Total RNA was isolated from 10 mg of liver using the RNeasy Lipid Tissue Mini Kit (Qiagen) and eluted from the RNeasy Mini column with 30 µl of RNase-free water, then stored at -80 °C. The quantity and purity of total RNA was determined by absorbance measures at 260 nm and 280 nm using Nano photometer (Implen GmbH,

Munich, Germany), and its integrity was assessed by 1% agarose-formaldehyde denaturing gels electrophoresis stained with SYBR Safe DNA Stain (Invitrogen, Carlsbad, CA, USA).

First-strand cDNA synthesis for partial sequence

Synthesis of first-strand cDNA was performed with 1 μg of total RNA from liver of medaka as template and oligo(dT)18 as primer using SuperScriptTM III Reverse Transcriptase from Invitrogen, according to the manufacturer's instructions. The reaction mixture was composed of 1 μg total RNA, 500 ng oligo(dT)18, 4 μl dNTP Mix (2.5 mM each at neutral pH) and RNase-free water to 13 μl. The mixes were denatured at 70 °C for 10 min and then quickly cooled on ice for at least 1 min. Reverse transcription was carried out in 4μl of 5X First-Strand Buffer, 1μl of 0.1 M DTT, 1 μl of RNaseOUTTM Recombinant RNase Inhibitor (40 U/μl), 1μl of SuperScriptTM Ⅲ RT (200 U/μl). The reaction was incubated at 50 °C. for 60 min followed by 70 °C for 15 min to inactivate the reverse transcriptase and then add 1μl of E. coli RNase H (2 U) followed by incubation at 37 °C for 20 min, to remove RNA complementary from the cDNA. The cDNA was cleaned up using MinElute Reaction Cleanup Kit (Qiagen) and eluted from the MinElute column with 10 μl of RNase-free water.

Partial cloning of LPL gene

First-strand cDNA was diluted to 1:10 with ddH2O using as a template for PCR. To amplify a partial fragment of LPL gene, a pair of specific primers, LPL-F and LPL-R (Table 3-1) was designed based on alignment of mRNA sequence of red

seabream (Pagrus major) LPL1 obtained from GenBank (AB243791) and EST of medaka obtained from NBRP Medaka (http://www.shigen.nig.ac.jp/medaka/top/top.jsp), using oligo version 7.37 software (MBI, Cascade, USA).

PCR reaction was carried out in a 20 µl volume reaction mixture composed of 1 µl diluted first-strand cDNA, 2 µl 10X Ex Taq Buffer with 20 mM Mg2+/ml, 1.6 µl dNTP Mix (2.5 mM each), 1µl each primer (10 µM), 0.2 µl Ex Taq DNA polymerase (5 U/µl), and ddH2O to 20 µl. Amplifications were performed starting with a 5 min initial denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min 40 s and a final extension at 72 °C for 10 min.

Amplified DNA fragments were gel purified using QIAquick Get Extraction Kit (Qiagen), and subcloned into pCR4-TOPO vector from Invitrogen, according to the manufacturer's instructions, followed by transformed into JM109 competent cells. Purified plasmids were sequenced by ABI PRISM 3100 genetic analyzer (Applied Biosystems) after labeled using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The sequences were analyzed by BioEdit and assembled using SeqMan II software in DNAStar package version 5.0. The primers for 5' RACE and 3' RACE were designed based on the partial sequence of LPL.

Rapid amplification of 3' UTR (3' RACE)

To obtain the complete 3' end of LPL, amplification of 3' UTR was performed using GeneRacerTM Kit (Invitrogen, Carlsbad, CA, USA). GeneRacer Oligo $(dT)_{24}$ was used for the synthesis of first-strand cDNA by SuperScriptTM III Reverse Transcriptase from Invitrogen. The primers for PCR and nested-PCR were shown in Table 3-1. First amplification of DNA with LPL3-R1 and GeneRacer 3' was performed by touchdown PCR program as follows: the initial denaturation at 94 °C for 2 min, followed by 5 cycles of denaturation at 94 °C for 30 s and annealing/extension at 72 °C for 35 s and another 5 cycles of denaturation at 94 °C for 30 s and annealing/extension at 70 °C for 35 s, then followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 50 s and a final extension at 72 °C for 10 min. After first PCR, the reaction mixture was diluted by 50 times for nested PCR with LPL3-R2 and GeneRacer 3' nested under the same conditions as the first PCR. The amplified fragments were subcloned and sequenced as described previously.

Rapid amplification of 5' UTR (5' RACE)

To obtain the complete 5' end, cDNA was synthesized using a modified lock-docking oligo(dT) primer, termed the 5'-RACE CDS primer (Table 3-1), and the SMART II A oligo from Clontech (Palo Alto, CA, USA). After RNase H treatment, cDNA was cleaned up as described above. The primers used for first and nested PCR were shown in Table 3-1. Amplification was performed with a nested universal primer and LPL5-R1. The reaction mixture was diluted 50 times for nested PCR with the same nested universal primer as first PCR and LPL5-R2. The first and nested PCR reactions were carried out under the same conditions as follows: the initial

denaturation at 95 °C for 2 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min. The amplified fragments were subcloned and sequenced as described previously.

Sequence analysis

Three partial sequences, containing first partial sequence of LPL, 5' UTR and 3' UTR sequence were assembled using SeqMan II software in DNAStar package version 5.0 to obtain the full-length cDNA of LPL. The full-length nucleotide sequence was edited and analyzed by BioEdit v7.0.9 (Ibis Biosciences, Carlsbad, CA, USA) to search ORF and translate it into amino acid sequence. The deduced amino acid sequence of medaka LPL was aligned with other species sequences (Table 3-2) obtained from GenBank and the protein sequence similarities were also calculated, using EMBL-EBI ClustalW2 at http://www.ebi.ac.uk/Tools/msa/clustalw2/. The predicted medaka LPL amino acid sequence was analyzed for the presence of signal peptide using SignalP v3.0 at http://www.cbs.dtu.dk/services/SignalP/. The potential N-linked glycosylation sites were predicted using NetNGlyc v1.0 at http://www.cbs.dtu.dk/services/NetNGlyc/. The conserved domains of LPL were blasted against conserved domain database (CDD) in NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The Phylogenetic tree based on LPL amino acid sequences was constructed by maxium likelihood (ML) method of MEGA software version 5.10 (Tamura et al., 2011) with JTT + G model which was determined by the evaluation of best-fit substitution. Bootstrap analysis (10000 replicates) was used to estimate the branches of the tree.

Table 3-1 Primers used for LPL gene cDNA RT-PCR and RACE

Primers name	Sequence 5'→3'	Use
LPL-F	ATGGGAAAGGAACTCCTCTGC	Used with LPL-R for PCR of core fraction
LPL-R	TCAGGCGTCGCTCTTTC	Used with LPL-F
GeneRacer Oligo dT	GCTGTCAACGATACGCTACGTAACG GCATGACAGTG$(T)_{24}$	Synthesis of the first-strand cDNA for 3'RACE
GeneRacer3'	GCTGTCAACGATACGCTACGTAACG	Used with LPL3-R1
GeneRacer3' Nested	CGCTACGTAACGGCATGACAGTG	Used with LPL3-R2
LPL3-R1	GGGCAGCAGCAAGTTCCACATCCGA	Used with GeneRacer3' for first PCR of 3'RACE
LPL3-R2	CTCGTCAGGGGAGGAGAAGATGCC GT	Used with GeneRacer3' Nested for nested PCR of 3'RACE
SMART II A oligo	AAGCAGTGGTATCAACGCAGAGTA CGCGGG	Used with 5'-RACE CDS
5'-RACE CDS A	$(T)_{25}$V N (N=A, C, G or T; V=A, G, or C)	Synthesis of first-strand cDNA for 5'RACE, used with SMART II A oligo
Nested Universal	AAGCAGTGGTATCAACGCAGAGT	Used with LPL5-R1 or LPL5-R2
LPL5-R1	GCTCTCAAACATCCCCGTTACCGTC CAG	Used with nested universal primer for first PCR of 5'RACE
LPL5-R2	AGCCGTGTATGACGATGAAGGTCTG AG	Used with nested universal primer for nested PCR of 5'RACE
M13 Forward	GTAAAACGACGGCCAG	Used with M13 Reverse primer for sequencing
M13 Reverse	CAGGAAACAGCTATGAC	Used with M13 Forward

Table 3-2. Amino acid sequences of LPL genes used in alignment and their identities (%) with that of medaka

Source	Organism	Accession number	Identity (%)
Medaka	*Oryzias latipes*	-	-
Red seabream 1	*Pagrus major*	BAE95413	83
Red seabream 2	*Pagrus major*	BAB20997	47
Mandarin fish	*Siniperca chuatsi*	ACZ55136	84
Zebrafish	*Danio rerio*	NP_571202	66
Rainbow trout	*Oncorhynchus mykiss*	NP_001118076	70
Gilthead seabream	*Sparus aurata*	AAS75120	83
European seabass	*Dicentrarchus labrax*	CAL69901	84
Human	*Homo sapiens*	CAG33335	58
House mouse	*Mus musculus*	CAJ18552	58

Results

Molecular characteristic of LPL sequence

The cDNA coding LPL was obtained from medaka liver by assembling the partial fragment, 5'- and 3'-UTR sequences. The cDNA of medaka LPL was 2279 bp in length with an open reading frame (ORF) of 1551 bp encoding 516-amino-acid protein. The 5' UTR was 145 bp as well as the 3'-terminal UTR was 583 bp with 3'-poly A tail. One AAAATA motifs (nt 2240-2245), which represent putative polyadenylation signals was found in the downstream of 3' region.

The theoretical molecular weight of 57.98-kDa and isoelectric point (PI) of 7.26 for medaka LPL protein were obtained using Expasy web tools at http://web.expasy.org/protparam/. Sequence analysis of the predicted medaka LPL amino acid sequence using SignalP 3.0 Server and NetNGlyc 1.0 Server program predicts the presence of putative signal peptide region (1-23 aa) and four potential N-linked glycosylation sites as Asn29, Asn36, Asn409 and Asn493 which is given as Asn-X-Ser/Thr, X being any amino acid except Pro (Mead et al. 2002). Some functional sites were also found, including one catalytic triad (Ser179, Asp203 and His291), nine cysteine residues, one lipid-binding site (Trp440, Trp443 and Trp444) and one polypeptide 'lid' (residues 263-289) which covers the active site and contributes to the preference for lipid substrate specificities of neutral vascular lipases (Dugi et al., 1995; Santamarina-Fojo and Brewer, 1994). Cys74 and Cys87, Cys263 and Cys289, Cys314 and Cys333, Cys325 and Cys328 are involved in the formation

of four disulfide bridges. Cys408 is found free in medaka LPL protein sequence because of N-linked glycosylation site beside it. Important sites for heparin-binding were also identified at residues 194-198, 312-335 and 340-356 in the N-terminal region, and one site (residues 452-464) at the C-terminal region. The site (residues 312-335), particularly Arg329, Lys330 and Arg332 fulfill the criteria for the first consensus heparin-binding sequence BBXB (Beg et al. 1998) and two sites (residues 194-198 and 340-356) partially fulfill the type 2 consensus sequence BXBBXXB (Table 3-3), where B represents a positively charged basic amino acid and X represents a neutral amino acid without Pro, while the site (residues 452-464) does not meet the criterion of consensus heparin-binding sequence.

Alignment of medaka LPL amino acid sequence with 9 vertebrates ones

The deduced amino acid sequence of medaka LPL was compared with the corresponding sequences from *Pagrus major* LPL1 (BAE95413), *P. major* LPL2 (BAB20997), *Oncorhynchus mykiss* (NP_001118076), *Danio rerio* (NP_571202), *Sparus aurata* (AAS75120), *Dicentrarchus labrax* (CAL69901), *Siniperca chuatsi* (ACZ55136), *Homo sapiens* (CAG33335) and *Mus musculus* (CAJ18552), shown in Fig. 3-1. With the exception of the putative signal peptide regions, most of function sites in these sequences were strictly conserved, which may reflect that these residues were essential for the contribution to LPL structure and function, such as Ser-Asp-His catalytic triad and one polypeptide 'lid'.

Medaka LPL sequence shared eight of the ten cysteine residues observed for human LPL sequence, which involved in the formation of four disulfide bridges (Cys74 and Cys87, Cys263 and Cys289, Cys314 and Cys333, Cys325 and Cys328,

Cys numbers refer to medaka LPL sequence) located in the N-terminal domain. The disulfide bridge (Cys445 and Cys 465) located in the C-terminal domain of human LPL sequence (Raisonnier et al., 1995) was also found in red seabream LPL2 sequence.

A comparative analysis of potential *N*-linked glycosylation sites for 10 vertebrates LPL was shown in Table 3-4. Although there are 6 sites overall, the numbers and positions of potential N-linked glycosylation sites are different among species. Only two of these sites designated as sites 2 and 5 are conversed in 10 vertebrate sequences, except that site 2 is conversed in teleost fish which is not found in human and house mouse.

Heparin-binding sites have been previously shown to play key roles in binding LPL and related neutral vascular lipases, HL and EL, to heparin sulfate proteoglycans on the luminal side of endothelial cells (Hill et al., 1996; Sendak and Bensadoun, 1998). Table 3-3 summarizes the comparison of medaka putative heparin-binding sites with several sites of other vertebrate species previously investigated by Beg et al. 1998. Cluster 1 (residues 312-335) retained Arg313, Lys317, Lys322, Arg329, Lys330 and Arg332 (medaka LPL numbers used) for all of the vertebrate LPL sequences examined with the exception of Lys317 substituted by Arg for zebrafish or by Ser for red seabream LPL2, and Lys322 substituted by Arg for red seabream LPL2. Especially, Arg329, Lys330 and Arg332 fulfill the criteria for the first consensus heparin-binding sequence BBXB in all of vertebrate LPL sequences examined. Cluster 2 retained Arg321, Lys323, Arg324 and Lys327 (human LPL numbers used) only for human, mouse and zebrafish LPL sequences, which strictly

correspond to the second consensus heparin-binding sequence BXBBXXB, not for medaka and other fish LPL sequences, of which residues 340-356 (medaka LPL numbers used) partially correspond to the second consensus heparin-binding sequence. Residues 174-178 (human LPL numbers used) retained Lys174, Lys175 and Arg178 for human, house mouse and gilthead sea bream LPL with the exception of Lys174 substituted by Arg for gilthead sea bream, while Lys195 and Arg198 (medaka LPL numbers used) were retained for medaka and other fish LPL sequences. Cluster 4 (residues 452-464) used medaka LPL numbers does not meet the criterion of consensus heparin-binding sequence in all of vertebrate LPLs examined. These results are consistent with synthetic peptide heparin-binding properties for corresponding clusters in human LPL (Beg et al. 1998), showing that the strong binding was observed for cluster 1, 2 and 3 peptides, whereas cluster 4 peptide showed little or no heparin affinity, under the conditions used in their study.

The conserved tryptophan clusters at Trp440, Trp443 and Trp444 used medaka LPL numbers, which play an important role in lipid substrate binding (Krapp et al., 1995) were found in all of vertebrate LPL sequences, except that Trp440 was substituted by Val for red seabream LPL2 and Trp443 was substituted by Ile for rainbow trout LPL.

The amino acid sequence of medaka LPL showed the identities between 58 % and 84 % with other vertebrate LPL amino acid sequences, suggesting that these belong to the same family of genes, whereas the comparison of sequence identity of medaka LPL protein with red seabream (*P. major*) LPL2 showed the lower levels of

identity (47 %), suggesting that red seabream LPL2 is a member of distinct lipase family from medaka LPL (Table 3-2).

Phylogenetic analysis of LPL

A phylogenetic tree calculated by the progressive alignment of 18 vertebrate LPLs was shown in Fig. 3-2. The phylogram showed clustering of the most fish into the same group that was consistent with their evolutionary relatedness, distinguished from red seabream LPL2 and torafugu LPL2, which formed another clade with a bootstrap of 100 %. The group for mammals LPL formed a distinct cluster of sequences from all fish. Red seabream (*P. major*) LPL1 and gilthead sea bream (*S. aurata*) formed a first group which belong to the same family *Sparidae* which was searched from FishBase (http://www.fishbase.org/search.php) with a high bootstrap value of 91 %, and then formed two clades with mandarin fish (*S. chuatsi*) and European seabass (*D. labrax*) which belong to the same order *Perciformes* perch-likes with bootstrap values of 84 % and 100 %, respectively. Medaka (*O. latipes*) belongs to the same class *Actinopterygii* Ray-finned fishes with four fish as mentioned above with a bootstrap value of 58 %, and shared a high bootstrap value of 99 % with torafugu LPL1.

Table 3-3. Amino acid sequence alignments for predicted heparin binding sites in 10 vertebrate lipoprotein lipase. K-lysine residue shown in shaded yellow; R-arginine residue shown in shaded grey. B refers to positively charged basic amino acid; X refers to neutral amino acid. Consensus heparin-binding sequences: type 1, BBXB; type 2, BXBBXXB.

Cluster Consensus	3 BXXB BBXXB	Position	1 BBXB	Position	2 or 3 BXBBXXB BXBXXBXXB BXBBXBXXB	Position	4 BBXBXBXXXXXXB BXBXBXXXXXBB	Position
Medaka	NKISR	194-198	YRCNSKEAFNKGVCLSCRKNRCNK	312-335	INKVRTARSTKMYLKTR	340-356	RKLRIKSGETQSK	452-464
Red seabream 1	HKISR	201-205	YRCNSKEAFNKGLCLSCRKNRCNK	319-342	INKVRRTRSTKMYLKTR	347-363	RKLRIKSGETQSK	459-471
Red seabream 2	NKVGR	190-194	YRCGSSDMFNRGMCLSCRKGRCNT	305-328	ISKVRKARNVQMYTKTR	333-349	HKIRIRAGETQQK	457-469
Mandarin fish	HKISR	193-197	YRCNSKEAFNKGLCLSCRKNRCNK	311-334	INKVRRARSTKMYLKTR	339-355	RKLRIKSGETQSK	451-463
Zebrafish	HKVNR	193-197	FRCSSRDSFNKGMCLSCRKNRCNK	308-331	VNKIRTRRSSKMYMKTR	336-352	RKLRIKSGETQSK	447-459
Gilthead seabream	RKISR	203-207	YRCNSKEAFNKGLCLSCRKNRCNK	321-344	INKVRRTRSTKMYLKTR	349-365	RKLRIKSGETQSK	461-473
Rainbow trout	HKVSR	183-187	YRCSSKEAFMKGMCLNCRKNRCNK	299-322	VNKVRLPRNTKMYLKTR	327-343	RKMRVKAGETQSR	439-451
European seabass	HKISR	196-200	YRCNSKDAFNKGLCLSCRKNRCNT	314-337	INKVRMARSTKMYLKTR	342-358	RKLRIKSGETQSK	454-466
House mouse	KKVNR	174-178	YRCNSKEAFEKGLCLSCRKNRCNN	289-312	INKVRAKRSSKMYLKTR	317-333	ERIRVKAGETQKK	429-441
Human	KKVNR	174-178	YRCSSKEAFEKGLCLSCRKNRCNN	289-312	INKVRAKRSSKMYLKTR	317-333	QKIRVKAGETQKK	429-441

Table 3-4. Predicted N-glycosylation sites for 10 vertebrate lipoprotein lipase. Numbers refer to the positions of Asp in amino acid sequences. N-glycosylation sites were identified using the NetNGlyc 1.0 web server (http://www.cbs.dtu.dk/services/NetNGlyc/). The sites of human are referred to Kobayashi et al., 1996. N-asparagine; K-lysine; H-histidine; S-serine; T-threonine; Q-glutamine; I-isoleucine; L-leucine; A-alanine; R-arginine.

Vertebrate	Species	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	No. of Sites
Medaka	*Oryzias latipes*	29 NTT	36 NST			409 NNT	493 NIS	4
Red seabream 1	*Pagrus major*		43 NTT			416 NTT	500 NLS	3
Red seabream 2	*Pagrus major*		51 NQT		370 NRS	403 NKT		3
Mandarin fish	*Siniperca chuatsi*		35 NTT			408 NTT		2
Zebrafish	*Danio rerio*		45 NAT			405 NST		2
Gilthead seabream	*Sparus aurata*	29 NTT	45 NTT			418 NTT	502 NLS	4
Rainbow trout	*Oncorhynchus mykiss*		35 NST			396 NST		2
European seabass	*Dicentrarchus labrax*	32 NST	38 NTT			411 NTT	495 NRS	4
House mouse	*Homo sapiens*			70 NHS		386 NKT		2
Human	*Mus musculus*			70 NHS		386 NKT		2

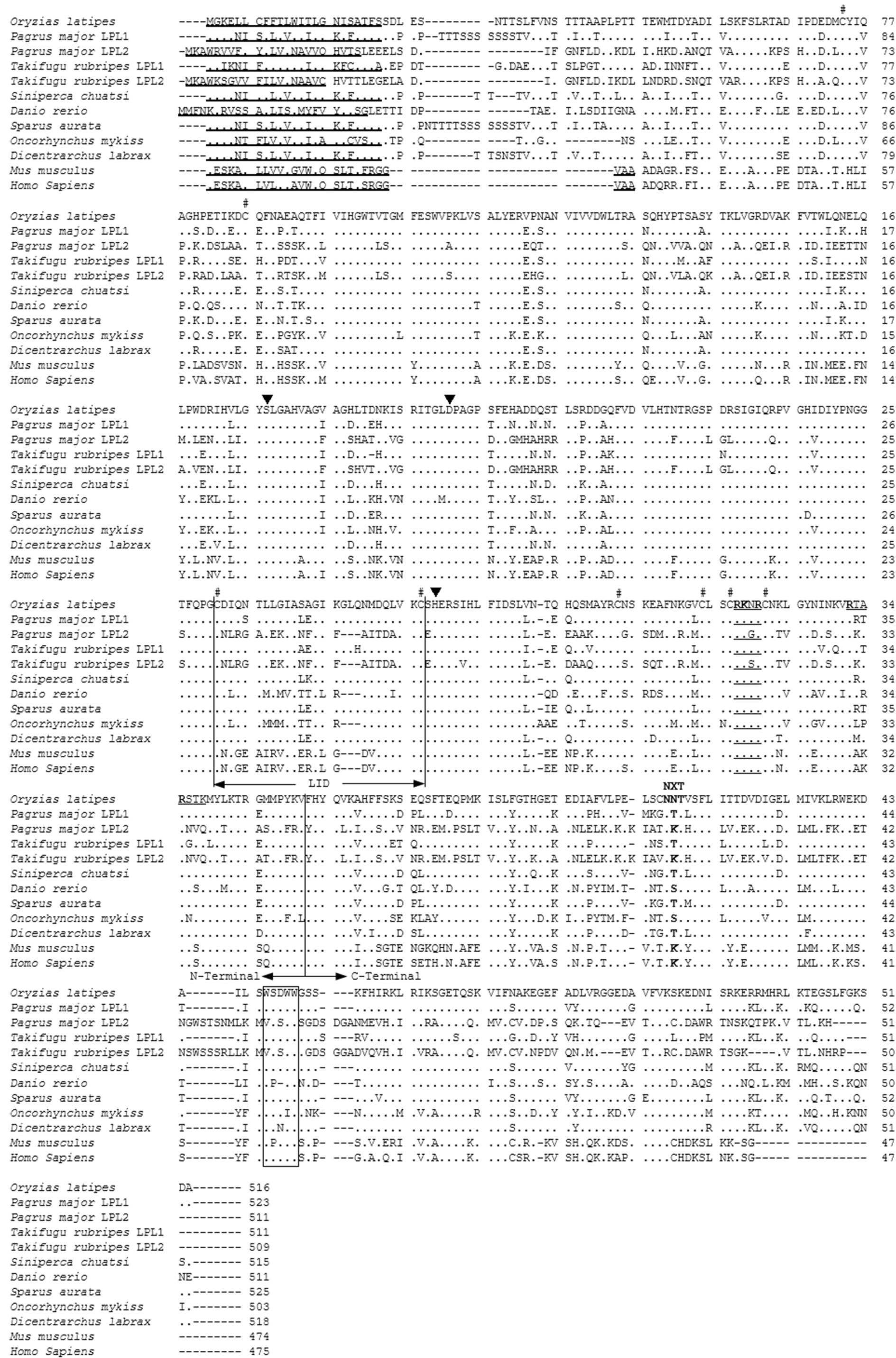

Fig. 3-1 Multiple alignment of the deduced amino acid sequence of medaka lipoprotein lipase 1 (LPL1) with LPL isoforms (LPL1, LPL2) from other animals. Dots Amino acids identical to those of medaka (Oryzias latipes, top line), dashes

alignment gaps, bold underlining the signal peptide, hatch marks conserved cysteine residues, inverted filled triangle active site residues, single underline putative heparin binding domains, bold letters in putative heparin binding domains conserved residues in all species, *NXT* a potential conserved N-linked glycosylation site, box a putative lipid binding site

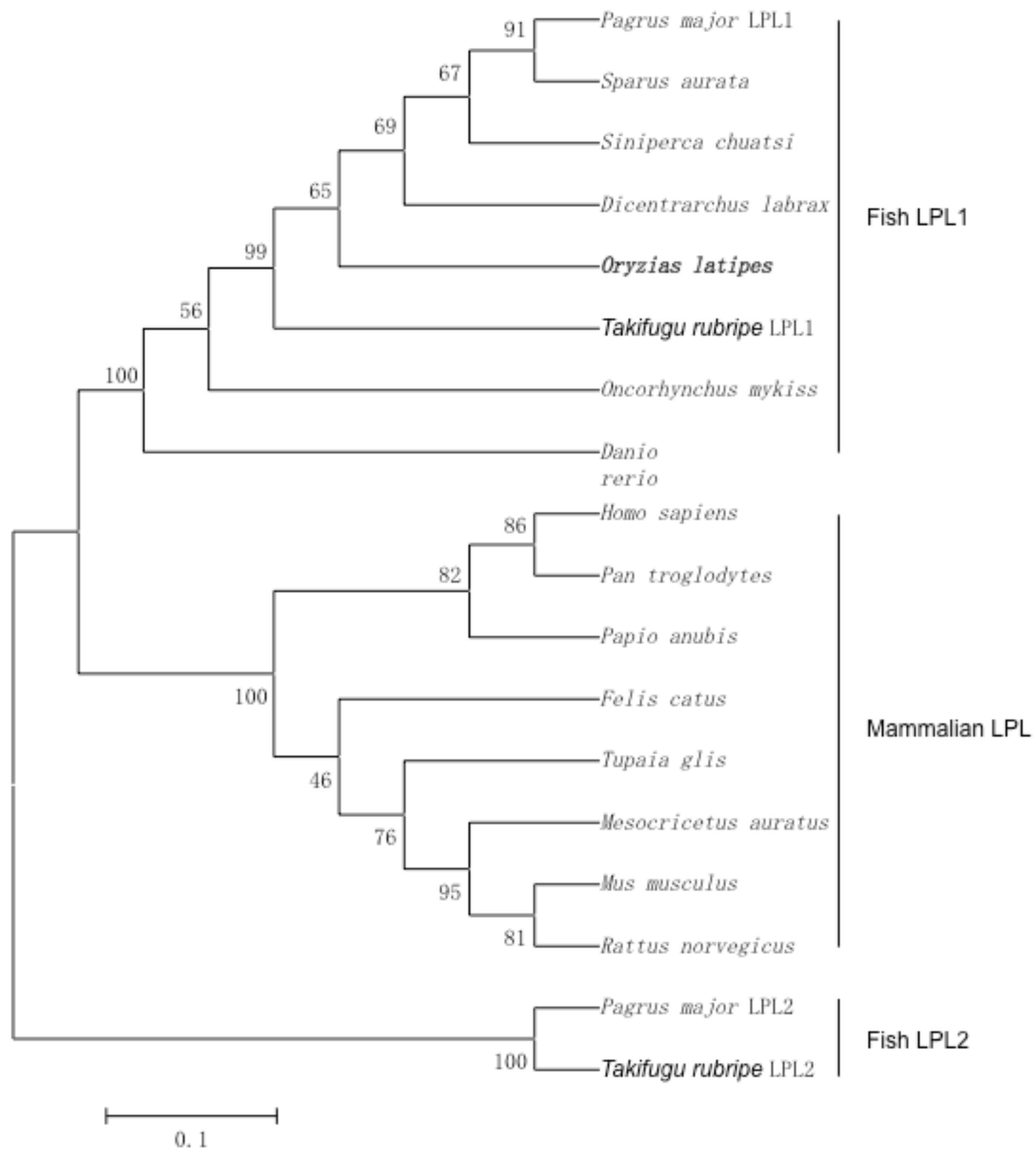

Fig. 3-2 Phylogenetic tree based on LPL amino acid sequences, constructed with MEGA v5.10 software using the maximum likelihood method. The topology was tested using bootstrap analyses (10,000 replicates). Numbers at internal branches bootstrap percentages.

Section 2

Distribution profile of LPL1 gene in medaka tissues

In this section, the relative mRNA levels of LPL in seven tissues of medaka were compared, using real-time PCR technology with three housekeeping genes, β-actin, EF-1α and RPL-7 as internal controls.

Materials and methods

Materials

Medaka specimens (orange red strain) were obtained from a local fish market (Ichigaya Fish Center, Tokyo, Japan) and reared in the indoor tanks at a constant water temperature of 28 ± 0.5 °C. Fish were fed with commercial diets (Kamihata Fish Industry Group; Kyorin Co. Ltd., Himeji, Japan) twice a day. 315-410 mg of fish (n=10) were used for the measurement of the distribution of LPL1 gene.

RNA isolation

Total RNAs were isolated from approximately 10 mg of liver using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and stored at -80 °C until further analysis. The quantity and purity of total RNAs was determined by absorbance measurements at 260 nm and 280 nm using a Nano photometer (Implen

GmbH, Munich, Germany). The integrity of RNA was assessed by 1 % agarose-formaldehyde denaturing gel electrophoresis.

Real-time PCR assay for LPL transcripts

The expression of LPL at mRNA level was determined in various tissues of medaka by quantitative real-time RT-PCR using three internal reference genes, ribosomal protein L-7 (RPL-7), elongation factor 1α (EF1α) and β-actin genes. Gene-specific primers and TaqMan probes were designed based on the LPL nucleotide sequence identified in the present study and the sequences of RPL-7, EF1α and β-actin genes obtained from the GenBank database (DQ118296, NM_001104662 and S74868, respectively). Total RNAs were extracted from visceral adipose tissue, brain, gill, heart, intestine, liver and muscle of medaka. First-strand cDNA was synthesized from each tissue sample (n=10) as described above except for GeneRacer Oligo $(dT)_{24}$ primer substituted by a blend of oligo (dT)18 and random hexamer. The reaction system for real-time PCR was prepared according to the manufacturer's protocol of TaqMan Fast Advanced Master Mix Kit (Applied Biosystems), followed by running on the ABI 7300 Real Time PCR System (Applied Biosystems). The relative LPL mRNA levels were determined by the comparative C_T method.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer HSD test using the statistical program JMP version 9 (SAS Institute Inc., Cary, NC, USA).

Results

Quantitative real-time PCR was performed to determine the expression levels of LPL1 transcripts in various tissues of medaka. Overall, LPL1 transcripts were ubiquitously expressed in all tissues examined (Fig. 3-4). The highest expression was found in liver, followed by visceral adipose tissue. The relatively high mRNA levels of LPL1 were detected in the brain and muscle, while LPL1 transcripts were expressed in the intestine at the lowest level.

In general, the gene expression patterns of medaka LPL1 normalized to the three internal control genes showed similar to each other. LPL1 transcripts were detected at the highest level in liver followed by visceral adipose tissue, and at low levels in heart, gill and intestine. In muscle and brain, meanwhile, there were minor inconsistencies in LPL transcript levels normalized to those of RPL-7, EF1α and β-actin. Comparison of cycle threshold (C_T) values of these housekeeping genes revealed that these inconsistencies are caused by relatively low expressions of EF1α and RPL-7 transcripts in muscle and brain, respectively (Fig. 3-5). However, compared with those of EF1α and β-actin, the levels of the RPL-7 gene showed less variation among tissues tested as reported in a previous report by Zhang et al. Thus, as an internal control, the RPL-7 gene is more suitable for the current analysis than two other housekeeping genes.

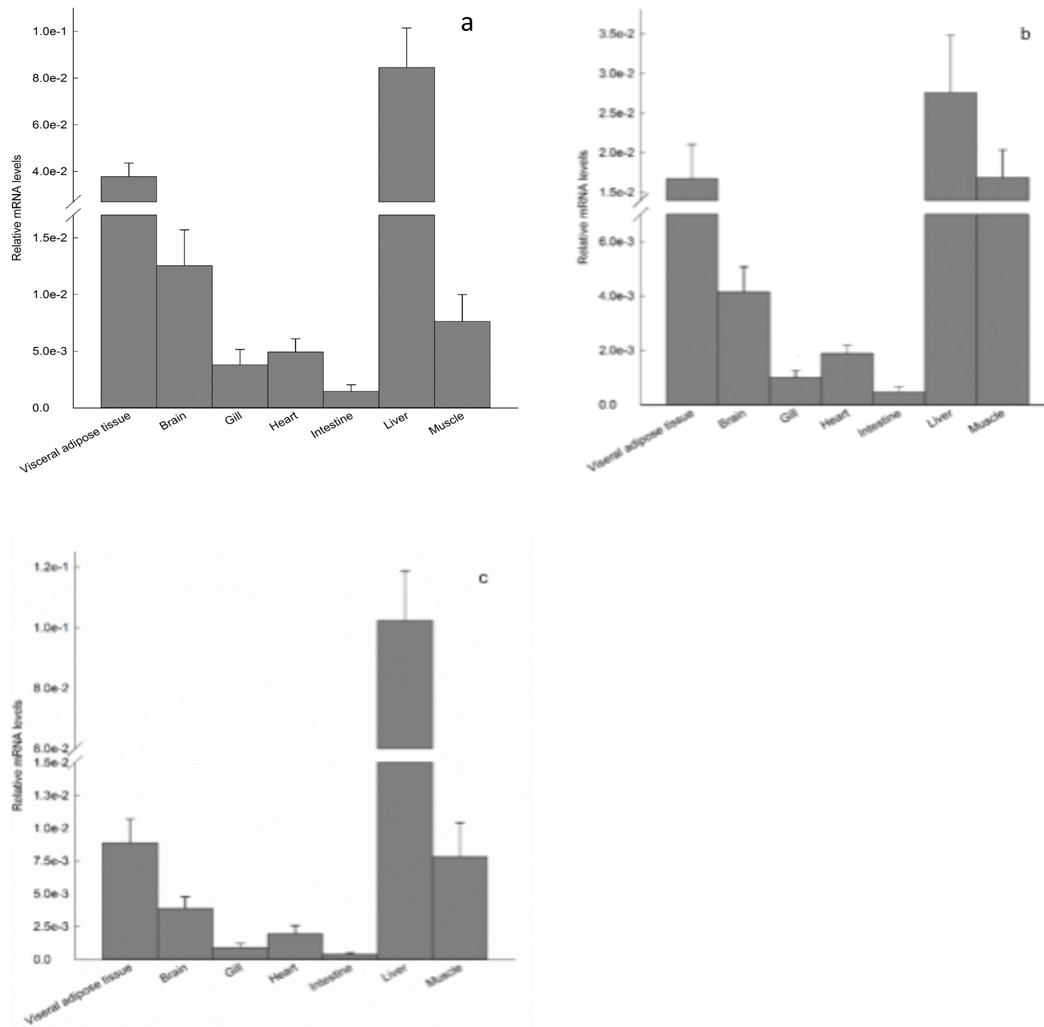

Fig. 3-3 Relative mRNA levels of medaka LPL in various tissues determined by quantitative real-time PCR. RPL-7 (a), EF1α (b) and β-actin (c) were used as internal controls. Each bar represents the mean + standard deviation (n = 10).

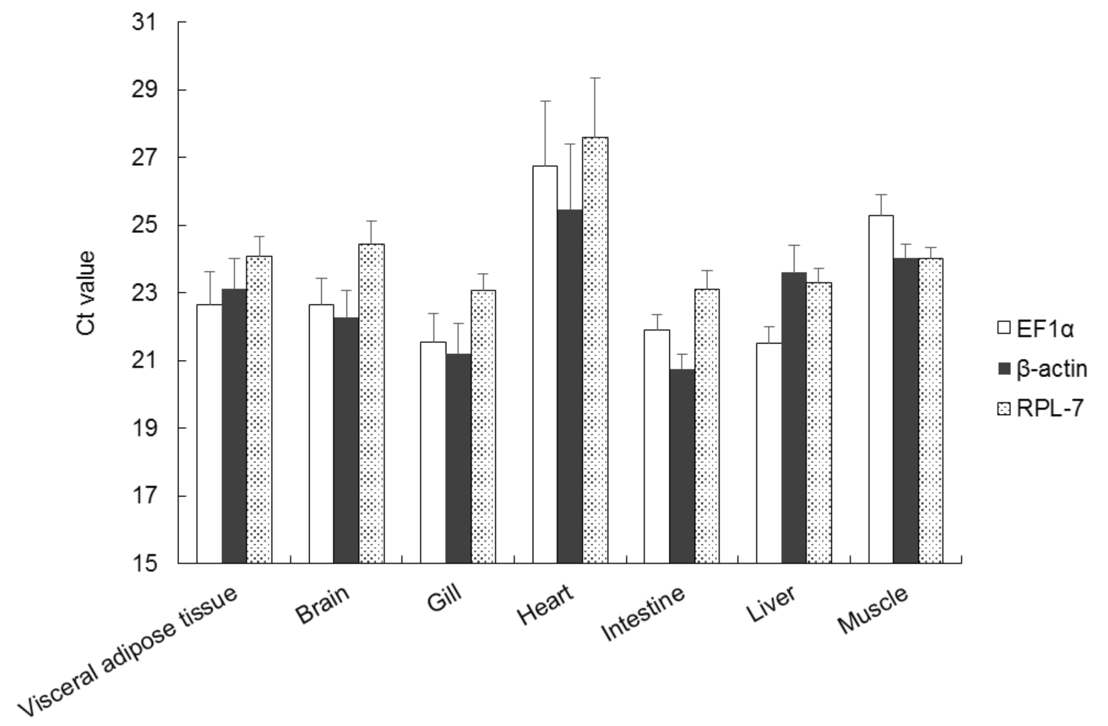

Fig. 3-4 Comparison of cycle threshold values of three housekeeping genes in the different tissues of medaka. Each bar represents the mean + standard deviation (n = 10).

Section 3

Regulation of LPL1 gene in medaka tissues during fasting and re-feeding

本節の内容は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定。

Section 4

Discussion

In this chapter, the full-length cDNA of LPL from medaka liver was cloned and analyzed, using RT-PCR and rapid amplification of 3' and 5' end (RACE) approaches. The resulting cDNA sequence of medaka LPL was 2279 bp in length with 5' UTR of 145 bp, 3' UTR of 583 bp with 3'-poly A tail and an ORF of 1551 bp encoding 516-amino-acid protein which showed the high identities of 84 % with mandarin fish and European seabass, whereas medaka LPL showed the lower identity of 47 % with red seabream LPL2.

Like other vertebrate LPL protein sequences, medaka LPL is also organized into two distinct structure regions, composed by N-terminal region (1-363) and C-terminal region (364-516). N-terminal region is the region responsible for catalysis whereas C-terminal region is required for the binding to lipoprotein substrate (Mead et al. 2002). N-terminal region contains one putative signal peptide region (1-23 aa), one highly conserved catalytic triad (Ser179, Asp203 and His291), eight cysteine residues (Cys74 and Cys87, Cys263 and Cys289, Cys314 and Cys333, Cys325 and Cys328), one polypeptide 'lid' (residues 263-289), three putative heparin-binding sites (residues 194-198, 312-335 and 340-356) and two potential N-linked glycosylation sites (Asn29 and Asn36). At the C-terminal region, Cys408 is the ninth cysteine residue which is found free in medaka LPL protein sequence because of N-linked glycosylation site (Asn409) beside it and Asn493 is the fourth N-linked

glycosylation site. One conserved lipid-binding site (Trp440, Trp443 and Trp444) and the fourth putative heparin-binding site (residues 452-464) were also found.

Alignment of medaka LPL protein sequence with other vertebrate corresponding sequences showed that some strictly conserved active sites were found at the N-terminal region of all vertebrate LPLs examined, containing the Ser-Asp-His catalytic triad where hydrolysis of TAG is carried out (Santamarina-Fojo and Brewer, 1994), eight cysteine residues, a polypeptide 'lid', and one conserved heparin-binding site (Arg329, Lys330 and Arg332). One N-linked glycosylation site (Asn409) and one conserved lipid-binding site (Trp440, Trp443 and Trp444) were also found in the C-terminal region.

Phylogenetic tree analyzed using eight teleost fish LPL and ten mammals LPL indicated that the group for mammals LPL formed a distinct cluster of sequences from all teleost fish and medaka (O. latipes) LPL belongs to the same class Actinopterygii Ray-finned fishes with Red seabream (P. major) LPL1, gilthead sea bream (S. aurata), mandarin fish (S. chuatsi) and European seabass (D. labrax) LPL.

To investigate the nutritional regulation of LPL, the effects of fasting and re-feeding on the gene expression levels of LPL in muscle and liver tissues of medaka were measured by two-step real-time RT-PCR method. First-strand cDNAs were synthesized from muscles and livers of medaka using a combination of oligo$(dT)_{18}$ primers and random hexamers and were subjected to the subsequent Q-PCR reactions using EF-1α, β-actin and RPL-7 as internal controls.

Integrated with the changes in lipid depositions of medaka tissues, re-feeding increased LPL mRNA and TAG levels in liver, whereas the opposite trend was found in fasted one. These results imply that re-feeding induced the lipid deposition in liver through the increased LPL expression, whereas fasting decreased the TAG levels in liver because of the decreased LPL expression. These observations are similar to those found in rainbow trout (Black and Skinner, 1986) in which fasting results in decreased LPL activity and TAG levels of liver. In contrast to the changes in liver, the TAG levels in muscle increased with increased LPL mRNA levels during fasting, whereas re-feeding decreased the TAG levels through the decreased LPL mRNA levels.

The observations represent an interesting example that LPL expression levels change rapidly under certain physiological conditions in association with the change of tissue-specific lipid accumulation. Upon no exogenous lipids for storage, the LPL expression levels in liver are decreased. To maintain the essential body functions, liver increases the secretion of lipid. Following these changes in liver, muscle increases LPL expression levels to take advantage of circulating lipid for energy during fasting.

CHAPTER IV

General discussion

As a major industry in many countries, aquacultural production will continue to grow because of the increased demand for fisheries products. This tendency will, on the other hand, lead to the decreased supply from natural sources. Not only to avoid the excessive consumption of wild fish sources but also to ensure the fish health and provide energy, artificial feeds containing essential nutrition, such as amino acids, lipids, vitamins and minerals, are widely used for cultured fish. Fish oil, which is rich in HUFA, is also used in feed, because of the strict requirements of marine fish (Hastings et al., 2004; Seiliez et al., 2003). However, the requirement for diet compositions, especially for lipid, is different among fish species (Oku and Ogata, 2000; Shimeno et al., 1980). Oku and Ogata (2000) found that lipid intakes of the three fish were increased with increased dietary lipid levels, weight gain of Japanese flounder tended to decrease with increased lipid intake, but not red seabream and yellowtail. In addition, the lipid deposition ratios in three fish showed significantly variances with increased lipid intakes of the whole bodies. Especially, the lipid deposition ratio of Japanese flounder was markedly decreased with increasing lipid intake, whereas that of yellowtail was not affected markedly. It is likely that fish differ in the ability to use lipid for energy provision and storage. Furthermore, appropriate supplement of lipid to dietary diet is efficient in improving the fish growth (Shimeno et al., 1980). Therefore, in order to improve the efficient utilization

of diets and ensure the fish health and growth, it is necessary to understand the lipid deposition and metabolism in fish.

The aim of this study was to investigate the effects of feeding conditions on the characteristics of lipid deposition using medaka, one of the model vertebrate animals.

In Chapter 2, the characteristics of lipid deposition in muscle and liver affected by fasting and re-feeding were found. The liver weight was markedly decreased with the increasing period of fasting, followed by a recovery during re-feeding. Although body weight was also significantly decreased during fasting, the recovery tendency was not found in body weight during re-feeding. It is likely that medaka was re-fed only for a short period so that the significant recovery of weight was not found in medaka body. In addition, the increase and decrease of the TAG levels were found in muscle and liver during fasting, respectively, whereas the opposite trend was observed in each tissue during re-feeding. The ratio of muscle to liver TAG levels further explained the characteristics of lipid deposition during fasting and re-feeding. During feeding and re-feeding, the ratios were lower than 1, whereas fasting increased the ratio higher than 1. These results suggest that while fasting promotes lipid accumulation in muscle, lipids are accumulated in liver during feeding and re-feeding.

Black and Skinner (1986) also found that 8-week fasting decreased liver weight remarkably. Johnston and Goldspink (1973) compared the total lipid levels of white and red muscle in plaice that belongs to ‘non-fatty’ fish, and found that fasting significantly decreased the lipid levels of red muscle, but not white muscle. In

contrast to the results reported by Johnston and Goldspink (1973), Umino et al. (1991) found that the TAG levels in muscle of red seabream were markedly decreased during long-term fasting. Especially, not only TAG and free fatty acid but also phospholipid levels in muscle of red seabream in small size were significantly decreased. These results implied that the response of lipid levels in muscle to fasting is likely to depend on tissue-specific deposition of lipids in fish species. 'Fatty fish', such as herring and mackerel, which accumulate high levels of lipids in muscle, is likely to deplete muscle lipids susceptibly for biological activities, whereas lipids stored mainly in liver of 'non-fatty' fish, such as pufferfish and flounder, are easy to be depleted for biological energy during fasting. Furthermore, a large number of lipid droplets were observed in the liver of pufferfish, but less in the thin muscle fiber, whereas high levels of lipid droplets were found in both muscle and liver of red seabream (Ando et al., 1993). These observations indicate that fish differ in the abilities of the generation of lipid-storing cells in tissues. Thus, the histological analyses of medaka, especially muscle and liver, are required for the elucidation of lipid deposition in fish tissues.

In chapter 3, to investigate the effects of feeding conditions on LPL expression, the full-length cDNA of LPL was cloned from liver and its characteristic was firstly estimated. The full- length cDNA sequence of medaka LPL was 2279 bp in length with 5' UTR of 145 bp, 3' UTR of 583 bp with 3'-poly A tail and an open reading frame of 1551 bp encoding 516-amino-acid protein which showed the high identities of 84 % with mandarin fish and European seabass, whereas showed the lower identity of 47 % with red sea bream LPL2. Like other vertebrate LPL protein sequences, medaka LPL protein sequence also contains some strictly conserved active

sites, including the catalytic triad (Ser179, Asp203 and His291), eight cysteine residues (Cys74 and Cys87, Cys263 and Cys289, Cys314 and Cys333, Cys325 and Cys328), a polypeptide 'lid' (residues 263-289), one conserved heparin-binding site (Arg329, Lys330 and Arg332), one N-linked glycosylation site (Asn409), and one conserved lipid-binding site (Trp440, Trp443 and Trp444).

After the analysis of LPL sequence, the relative mRNA levels of LPL in liver and muscle of fed and fasted medaka were examined to understand the roles of LPL gene expression in lipid deposition.

This study found that fasting decreased LPL mRNA and TAG levels in liver, whereas the opposite trend was found in re-fed one. These results imply that fasting decreased the TAG levels in liver because of the decreased LPL expression, whereas re-feeding induced the lipid deposition in liver through the increased LPL expression. In contrast to the changes in liver, the TAG levels in muscle increased with increased LPL mRNA levels during fasting, whereas re-feeding decreased the TAG levels through the decreased LPL mRNA levels.

The results of LPL expression in medaka muscle coincide with the previous studies by Ladu et al. (1991) that LPL mRNA levels in cardiac, soleus, red vastus and white vastus muscles of rat were significantly increased during fasting. Black and Skinner (1986) found that eight week fasting results in decreased LPL activity and TAG levels in liver of rainbow trout. However, the different results were found in the liver of red seabream that both LPL1 and LPL2 mRNA levels were increased during 48 h fasting (Oku et al., 2006). It is difficult to compare the results on LPL mRNA levels in medaka during 8 day fasting with those in red seabream under short-term

fasting (48 h). Furthermore, Liang et al. (2002) found that LPL mRNA levels in liver of red seabream fed with low lipid diet were increased drastically during fasting, whereas liver LPL expression in the fish fed with high lipid diet was not affected by fasting. These observations indicate that dietary lipid levels also affected the LPL expressions in response to the fasting in fish tissues. Therefore, it is necessary to research on the effects of dietary lipid levels on the LPL expression and lipid deposition in their responses to fasting, in order to improve the elucidation of the regulation of LPL expression on lipid deposition in fish.

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