Post-feeding molecular responses of cobia (Rachycentron canadum): RNA-sequencing as a tool to evaluate postprandial effects in hepatic lipid metabolism

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Abstract

We used transcriptome sequencing to investigate the hepatic postprandial responses of *Rachycentron canadum* (cobia), an important commercial fish species. In total, 150 cobia juveniles (50 per tank, triplicate) were fed *ad libitum* with a commercial diet for 7 days, fasted for 24 h and fed for 10 min. Liver samples were sampled 10 min before and 30 min, 1, 2, 4, 8, 12, and 24 h after the feed event. Posteriorly, it was evaluated liver fatty acid profile, transcriptome sequencing, and differential gene expression focusing on fatty acid synthesis and oxidation pathways. In general, liver fatty acid profile reflected the diet composition. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) levels increased at 8 to 12 h and decreased at 24 h after the feed event. It was observed a high number of differentially expressed genes (DEGs) comparing fish fasted by 8 h with those fasted by 30 min and 24 h, while a reduced number of DEGs was observed comparing individuals fasted by 30 min compared with those fasted by 24 h. Similarly, the main differences in the expression of genes related to the fatty acid biosynthesis and oxidation pathways were noticed in individuals fasted by 8 h compared with those fasted by 30 min and 24 h. The results suggested that the adequate time to sample the individuals ranged between 8 and 12 h after meal, since apparently, after 24 h, differential gene expression was not necessarily influenced by feed intake.

Introduction

The main aim to perform nutritional trials with commercial finfish species is to define adequate levels of nutrients (primarily lipids and proteins) in the diets aiming to promote optimum performance and health (Glencross 2009; Wade et al. 2014). Lipids and proteins are molecules fundamentally important to animals, including fish since are related to several essential functions such as energy production, cellular structuring, fat-soluble vitamin transport, and eicosanoid synthesis (Higgs and Dong 2000; Turchini et al. 2009). Thus, the knowledge of the metabolic pathways that modulate processes related to the lipid synthesis and oxidation is essential in the definition of specific nutritional strategies based on the physiological characteristics of the target species (Glencross 2009; Tocher et al. 2013; Wade et al. 2014; Araújo et al. 2016). For most fish species, the liver is the main lipogenic and lipolytic tissue, and it is related to the modulation of lipid metabolism, including fatty acid synthesis and oxidation processes (Tocher et al. 2013). Thus, to understand the dynamics of the lipid metabolism pathways influenced by diet composition in fish, firstly it is essential to investigate the hepatic molecular responses after a single meal (Leaver et al. 2008; Wade et al. 2014; Araújo et al. 2016).

Molecular tools like transcriptome sequencing have been effective in different applications such as molecular markers identification, discovery and description of new genes and pathways, and differential analyses of gene expression, and it has been applied to several aquaculture commercial fish species such as rainbow trout (*Oncorhynchus mykiss*, Rivas-Aravera et al. 2019), Atlantic salmon (*Salmo salar*, Glencross et al. 2015) barramundi (*Lates calcarifer*, Wade et al. 2014), dusky grouper (*Epinephelus marginatus*, Araújo et al. 2018), sea bass (*Dicentrarchus labrax*, Magnanou et al. 2014), sea bream (*Sparus aurata*, Mininni et al. 2014), and tilapia (*Oreochromis niloticus*, Zhao et al. 2018). Additionally, the

development of transcriptome sequence technology and the advance of bioinformatics tools helps in providing a broad overview of the post-feeding effect on the expression of genes related to the growth, immunological system, and energetic metabolism (*e.g.*, synthesis, oxidation, and deposition of specific fatty acids) in finfish species (Leaver et al. 2008; Calduch-Giner et al. 2013; Qian et al. 2014; Glencross et al. 2015). In a study performed with barramundi, Wade et al. (2014) using information generated by liver transcriptome sequencing mapped out, identified, and quantified the expression of several metabolic-relevant genes (mainly those related to carbohydrates, lipids, and proteins metabolism) improving the knowledge about the physiology of this species, and consequently contributing to the development of future studies with the target species.

The knowledge regarding the postprandial effects on the expression of metabolic-relevant genes is essential to precisely investigate the physiological effects of the nutrients in diets and which, is only possible if the adequate moment to sample the animals is known. Aiming to avoid stress by handling, and poor water quality, several previous nutritional studies investigating the influence of diet composition on gene transcription, fasted the animals for 24 h before sampling. However, previous studies performed with finfish species such as Atlantic salmon (Valente et al. 2012), barramundi (Wade et al. 2014; Araújo et al. 2016; Poppi et al. 2019), and rainbow trout (Mente et al. 2017), showed that metabolic-relevant genes, especially those related to the modulation of fatty acid synthesis and oxidation pathways, such as fatty acid synthase (*fas*), stearoyl CoA desaturase (*scd*), ATP citrate lyase (*acyl*), carnitine palmitoyltransferase (*cpt*), and 3-hydroxybutyrate dehydrogenase (*bdh2*), were drastically down-regulated after 8 to 12 h after the last meal, presenting consistent expression compared to the moment when these animals were fed. Therefore, based on that it is assumed that in 24 h of fasting the expression of protein and lipid-relevant genes reached basal levels, probably not integrally reflecting the influence of the diet on their expression. However, it is worth mentioning that these responses will be modulated not only by fasting but also by interacting several parameters, such as health condition, life stage, diet composition, and temperature (Leaver et al. 2008; Wade et al. 2014). Therefore, the characteristics of every single nutritional trial are unique and the time to sample the individuals/tissues after the last meal needs to be always considered to properly understand the animal physiological response.

Rachycentron canadum, popularly known as cobia, is an important worldwide commercial species and a targeted species for research to inform the aquaculture industry. However, no studies related to the modulation of energetic metabolism during the postprandial period have been performed. Therefore, this study aimed to evaluate the molecular responses on the hepatic tissue of cobia juveniles after a single feed event and the results provide insights regarding the physiological mechanisms modulated by the influence of the postprandial period, generating essential knowledge about the physiology of this important commercial fish species.

Material And Methods

Experimental Design

All procedures performed in this study were conducted following the Mogi das Cruzes University Institutional Animal Care and Use Ethics Committee (approval number: #008/2017). In total, 150 cobia juveniles (90.85 ± 11.75 g, mean ± SD) were acquired by Redemar Alevinos (Ilha Bela, SP, Brazil), acclimated during 15 days in a 10,000 L tank (temperature average of 28 ± 0.4 ºC; dissolved oxygen 6.57 ± 0.7 mg L^{-1}; and total ammonia nitrogen – TAN 0.05 mg L^{-1}, mean ± SD), and fed twice a day until apparent satiation with a fish commercial diet (Guabipirá, Guabi Nutrição e Saúde Animal S.A., SP, Brazil). After the acclimatization period, the animals were transferred to three 1,000 L tanks (50 animals per tank, triplicate) and were fed twice a day for seven days with the same commercial diet. At the end of the seventh day, the animals were fasted for 24 h. After the fasting period, it was carried out an uninterrupted single feed event (*ad libitum*) for 10 min. Ten min before and after 30 min, 1, 2, 4, 8, 12, and 24 h of the feed event, three fish from each tank (a total of 9 individuals) were anesthetized, euthanized, and aliquots of liver were immediately frozen in liquid nitrogen, and then transferred to the ultra-freezer (-80 ºC) until the analysis.

Diet and liver fatty acid profile

The total lipids of the diet and liver were extracted using a chloroform/methanol/water mix (2:1:0.5) (modified from Folch et al. 1957). The lipid extracts were methylated using 5% HCl methanol prepared by dissolving 10% vol acetyl chloride in methanol according to Christie (2003). The fatty acid analysis was performed using a gas chromatograph (GC, model 3900, CA, USA) coupled with a flame ionization detector (FID) and a CP-8410 autosampler. The FAME analysis was carried out using a capillary column (CP-Wax 52 CB, 0.25-µm thick, 0.25-mm inner diameter, and 30-m length), and hydrogen was used as a carrier gas at a linear velocity of 22 cm/s. The column was programmed to start at 170 °C for 1 min followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and FID temperature were kept at 250 and 260 °C, respectively. The FAMEs were identified by comparing their retention times to those obtained with commercial standards (Supelco, 37 components; Sigma-Aldrich; mixture Me93, Larodan, and Qualimix; PUFA fish M, menhaden Oil, Larodan). The fatty acid profile of the diet used in the trial is presented in Table 1.

Table 1 Fatty acid composition (% of total fatty acids) of the experimental diet

Fatty acids	
14:0	2.23 ± 0.08
16:0	22.46 ± 0.24
18:0	7.30 ± 0.13
ΣSFA	31.99 ± 0.40
16:1n-7	6.16 ± 0.21
18:1n-9	26.95 ± 0.52
18:1n-7	3.46 ± 0.48
ΣMUFA	37.88 ± 0.61
18:3n-3	1.40 ± 0.05
20:5n-3	3.45 ± 0.32
22:5n-3	1.22 ± 0.11
22:6n-3	6.66 ± 0.29
Σn-3 PUFA	12.74 ± 0.34
18:2n-6	14.45 ± 0.41
20:4n-6	1.49 ± 0.15
22:4n-6	0.58 ± 0.03
22:5n-6	0.43 ± 0.16
Σn-6 PUFA	16.96 ± 0.45
ΣPUFA	30.13 ± 0.33
ΣLC-PUFA	13.84 ± 0.50
Σn-3/Σ n-6	0.75 ± 0.04
Others	0.43 ± 0.03

ΣSFA, ΣMUFA, ΣPUFA, n-3 ΣPUFA, n-6 ΣPUFA are the sum of saturated, monounsaturated, n-3 polyunsaturated, polyunsaturated and n-6 polyunsaturated fatty acids respectively

Rna Isolation, Library Preparation, And Sequencing

Total RNA from the liver was extracted using an RNeasy Lipid Tissue kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. The quantity of extracted RNA was assessed by Nanodrop™

Spectrophotometer (Thermo Fisher Scientific, USA), and the quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). The RIN values of the RNA used were all above 7.5.

RNA from the liver of three animals of each tank was used in the library construction using the TruSeq RNA Sample Preparation kit (Illumina Inc., USA) according to the manufacturer's specifications. Transcriptome sequencing was performed with Illumina Nextseq® platform (Illumina Inc., San Diego, CA, USA) using a paired-end sequencing strategy (2×75 bp).

De novo assembly, functional annotation, and differential expression

The detailed methodology, statistics, and summary of the *de novo* transcriptome assembled of cobia liver tissue were previously described by Barbosa et al. (2021). Due to the massive dataset generated by the transcriptome sequencing, the differential expression and quantitative real time PCR (qRT-PCR) validation were restricted to three comparisons (30 min *vs.* 8 h, 30 min *vs.* 24 h, and 8 h *vs.* 24 h) based on the main differences observed in the liver fatty acid profile. Raw libraries were submitted to quality control checking, using FastQC (Andrews 2010) and Fastp was used to remove low-quality ($Q < 30$) reads, adapters, and other contaminant sequences. To remove rRNA reads, the high-quality reads were aligned to sequences in the SILVA ribosomal RNA (rRNA) gene database (http://www.arbsilva.de/) using SortMeRNA (Kopylova et al., 2012). The National Center for Biotechnology Information (NCBI) Univec database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/) was used to remove contaminants from libraries. Quality-filtered reads were then mapped to *R. canadum* transcriptome available and described by Barbosa et al. (2021), using HISAT2 (Kim et al., 2019) with default parameters. StringTie (Pertea et al. 2015) was then used to assemble the mapped reads into transcripts, using the *de novo* transcriptome reconstruction method, allowing the identification of all transcripts present in each sample (including currently annotated genes, as well as newly identified elements and isomorphs). StringTie Merge was next used to combine redundant transcription structures, providing a non-redundant reference transcriptome, with unique identifiers. Cufflinks (Trapnell et al. 2012) were next used to estimate expression values (FPKM) for each element in the StringTie-generated reference transcriptome. Transcriptome completeness was assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO) software, version 3.0 (Simão et al. 2015). Finally, the reads and the merged assembly were also used to calculate expression levels and the effect size and statistical significance of observed differences using Cuffdiff (Trapnell et al. 2010). Heatmaps based on expression patterns were built with Complex Heatmap package (Gu et al. 2016) in a custom R script.

We applied Eukaryotic Non-Model Transcriptome Annotation Pipeline (EnTAP) (Hart et al. 2020) on the query transcripts (blastx, e-value $\leq e^{-5}$) for homology against the National Center for Biotechnology Information non-redundant protein database (NCBI nr), NCBI proteins reference database (RefSeq), the curated Swiss-Prot database from UniProt Knowledgebase (UniProtKB), and the EggNOG proteins database. The EggNOG hits also helped to assign the biological function to the genes, identifying GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) terms. EnTAP functional

annotation process was carried out using a Dugong container environment (Menegidio et al. 2018) in BioPortainer Workbench (Menegidio et al. 2019).

Kegg terms were annotated and organized in Pathway Level 2 classes, from KEGG Pathway Maps page (https://www.genome.jp/kegg-bin/show_brite?&htext=br08901.keg&option=-a&panel=collapse). Kegg Automatic Annotation Server (KAAS, https://www.genome.jp/kegg/kaas/) was used to annotate the transcript sequences in KEGG Orthologs (KO) terms, with the bi-directional best hit (BBH) method. KOs were utilized as input in the KEGG Mapper-Search&Color Pathway (https://www.genome.jp/kegg/tool/map_pathway2.html) to map the differentially expressed transcripts based on their modulation; Up-regulated elements were assigned as green, while Down-regulated ones received the color red. Barramundi was set as the background species because it is closely related to the cobia contained in KEGG.

Validation Of Degs

Total RNA from liver samples was purified using Kit Pure Link RNA (12183018A Invitrogen™) according to the manufacturer's instructions. To eliminate the potential contamination by genomic DNA, samples were treated with DNase using Turbo DNAfree (AM1907 Invitrogen ™), for this, previously the RNA samples were diluted to 100 ng/uL and after treatment were stored at -80 ºC. 500 ng of total RNA were used as a template to synthesize cDNA using SuperScript III Reverse Transcriptase (18080093 Invitrogen™) and random primers were used according to the manufacturer's instructions. Negative controls were also performed, without reverse transcriptase and the cDNA was stored at -20 ºC.

It was selected a total of 10 differentially expressed unigenes (DEGs) from the liver to verify the reliability of the RNA-Seq data. The primers were previously designed using the primer design software tools: IDT Oligo Analyzer (www.idtdna.com/calc/analyzer). The elongation factor 1 alpha (*ef1a*) and *β-actn* were used as endogenous reference genes. The oligonucleotides used as primers and the parameters of efficiency are described in Table 2. All primers were synthesized by Thermofisher (Invitrogen™).

Table 2
Nucleotide sequence of primers used for quantitative real-time PCR (qRT-PCR)

Gene	Primer Sequence 5’ – 3’	Amplicon Size (bp)
acads_fw	GATGAGGTGATGGCGACTTATC	94
acads_ rv	GTACCAGTCATAGGCACACTTT	
acsl_fw	CTCAATCTTCTCTGGTGCTATGT	146
acsl_rv	CTGCAGAGGCCTTGGATAAA	
cpt1_fw	GTCTCACCGCACGAGTATTT	101
cpt1_rv	TTCGGTCCTGTTGCTGATG	
fas_fw	TGTGGAGCCAACCATCTTTATC	97
fas _rv	GGAGGTCTGTATCAAGGGTAGA	
pdk_fw	GGCTGTTCACCTACACATACTC	123
pdk_rv	CTTGAAAGTAGCGGGCATAGA	
scd_fw	CAGTCAGTGTAGTCAGTGGTTAG	99
scd_rv	TAGGGAACAGGAAGCAGAGA	
elovl6-like_fw	GGTCTGACTCCACTTTGAGAAG	96
elovl6-like_rv	AAGACACAGAAGGAGCCAATAG	
elovl6_fw	TGAGTGACCACAGCACTAAC	105
elovl6_rv	TCATGTTCTGTGGCCTGTATG	
mcm3_fw	GACGATGAGTCGGGCTTTATT	102
mcm3_rv	CAGAGGGACTACCTGGACTT	
hspa1S_fw	TCTTGCTAACCCACTCCAATTA	75
hspa1S_rv	GAGGGTGGGAGGTAAGAATAAG	
β-act_fw	AGCCATGGAAGATGAAATCG	190
β-act_rv	TCTCTTGCTCTGGGCTTCAT	
ef1α_fw	AGTGCGGAGGAATCGACAAG	188
ef1α_rv	TGCTGGTCTCGAACTTCCAC	

fw: forward and *rv*: reverse

Statistical analysis

Data related to the liver fatty acid profile is presented as mean ± SD (Table 3). The comparisons between hepatic fatty acid composition of different sample times were performed by one-way analysis of variance (ANOVA), followed by Tukey's HSD test using the R package, with a significance level of 5% ($P < 0.05$).

Results

Liver fatty acid profile

Several significant differences were observed in the hepatic fatty acid profile of the experimental animals sampled at different postprandial periods, with the most relevant observed in the levels of n-3 and n-6 PUFAs (Table 3). Lower levels of DHA, EPA, 22:5n-3, 20:4n-6, 22:4n-6, and 22:5n-6, were observed in animals before the feed event and 30 min after feeding, especially compared with animals sampled at 8 and 12 h, and the percentage of these same fatty acids was significantly reduced in 24 h after the meal. These changes resulted in the same profile of total n-3 polyunsaturated fatty acids (n-3 PUFA) and consequently total long chain polyunsaturated fatty acids (LC-PUFA).

Table 3
Liver fatty acid composition (% of total fatty acids) of cobia juveniles sampled before the feeding event and at different postprandial periods

Fatty Acids	Pre-feeding	30 minutes	1 hour	2 hours	4 hours	8 hours	12 hours	24 hours	P-value
14:0	1.33 ± 0.15^{a}	1.29 ± 0.14^{ab}	1.21 ± 0.14^{abc}	1.22 ± 0.08^{abc}	1.11 ± 0.06^{bc}	1.08 ± 0.13^{c}	1.12 ± 0.09^{bc}	1.19 ± 0.11^{ab}	*< 0.001*
16:0	22.02 ± 1.20	21.50 ± 0.63	22.14 ± 1.25	21.43 ± 0.67	20.90 ± 1.00	21.12 ± 0.81	21.13 ± 1.13	22.30 ± 1.67	*0.099*
18:0	6.42 ± 0.49	6.23 ± .047	6.56 ± 0.50	6.42 ± 0.37	6.53 ± 0.41	6.75 ± 0.53	6.70 ± 0.51	6.21 ± 0.67	*0.259*
ΣSFA	29.77 ± 1.32	29.02 ± 1.53	29.91 ± 1.23	29.06 ± 1.11	28.54 ± 1.27	28.95 ± 1.05	28.96 ± 1.50	29.70 ± 1.39	*0.285*
16:1n-7	7.02 ± 0.38^{ab}	6.97 ± 0.25^{ab}	6.66 ± 0.55^{abc}	6.71 ± 0.29^{abc}	6.38 ± 0.41^{bc}	6.17 ± 0.46^{c}	6.25 ± 0.28^{c}	6.84 ± 0.59^{b}	*< 0.001*
18:1n-9	33.09 ± 1.10	33.26 ± 0.84	33.27 ± 0.67	32.71 ± 0.70	33.36 ± 0.72	33.04 ± 0.59	32.87 ± 0.75	33.21 ± 0.59	*0.614*
18:1n-7	4.38 ± 0.15	4.38 ± 0.11	4.36 ± 0.10	4.30 ± 0.10	4.41 ± 0.16	4.32 ± 0.11	4.27 ± 0.17	4.33 ± 0.11	*0.388*
ΣMUFA	44.49 ± 1.08	44.62 ± 0.91	44.29 ± 0.96	43.72 ± 0.71	44.16 ± 1.11	43.52 ± 0.72	43.39 ± 0.96	44.37 ± 1.04	*0.056*
18:3n-3	1.00 ± 0.08	1.00 ± 0.06	0.95 ± 0.06	1.00 ± 0.07	0.98 ± 0.09	0.95 ± 0.04	0.96 ± 0.08	0.94 ± 0.09	*0.628*
20:5n-3 (EPA)	1.86 ± 0.27^{a}	1.89 ± 0.14^{a}	1.96 ± 0.27^{ab}	2.11 ± 0.09^{ab}	1.99 ± 0.26^{ab}	2.12 ± 0.07^{b}	2.18 ± 0.17^{b}	1.81 ± 0.57^{a}	*0.048*
22:5n-3	0.88 ± 0.20^{a}	0.96 ± 0.13^{ab}	0.98 ± 0.24^{ab}	1.07 ± 0.06^{ab}	1.08 ± 0.21^{ab}	1.19 ± 0.10^{b}	1.20 ± 0.10^{b}	0.96 ± 0.35^{ab}	*0.005*
22:6n-3 (DHA)	3.92 ± 0.89^{a}	4.22 ± 0.59^{a}	4.48 ± 1.13^{ab}	4.85 ± 0.43^{ab}	4.99 ± 1.10^{ab}	5.55 ± 0.35^{b}	5.56 ± 0.65^{b}	4.33 ± 1.30^{a}	*< 0.001*
Σn-3 PUFA	7.66 ± 1.30^{a}	8.07 ± 0.80^{a}	8.36 ± 1.65ab	9.03 ± 0.46^{ab}	9.05 ± 1.43^{ab}	9.81 ± 0.54^{b}	9.90 ± 0.86^{b}	8.04 ± 2.23^{a}	*0.002*
18:2n-6	13.69 ± 0.85	13.69 ± 0.78	12.89 ± 0.99	13.51 ± 0.74	13.43 ± 1.33	12.81 ± 0.63	12.86 ± 1.05	13.45 ± 0.81	*0.344*

Pre-feeding is related with individuals sampled 10 min before the feeding event, and 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h are related with the time of sampled post feed event. ΣSFA, ΣMUFA, ΣPUFA, n-3 ΣPUFA, n-6 ΣPUFA are the sum of saturated, monounsaturated, polyunsaturated, n-3 polyunsaturated and n-6 polyunsaturated fatty acids, respectively. “Others” are the sum of no representative fatty acids (lower than 0.3% of total fatty acids). $^{a.b}$Different letters represent significant differences between fish sampled at different times, by Tukey’s test, ANOVA ($P > 0.05$, $n = 3$)

Fatty Acids	Pre-feeding	30 minutes	1 hour	2 hours	4 hours	8 hours	12 hours	24 hours	P-value
20:4n-6	1.64 ± 0.12[a]	1.65 ± 0.08[a]	1.66 ± 0.11[ab]	1.69 ± 0.05[ab]	1.73 ± 0.12[b]	1.76 ± 0.04[b]	1.73 ± 0.11[b]	1.57 ± 0.24[a]	*0.041*
22:4n-6	0.44 ± 0.08[a]	0.44 ± 0.06[a]	0.47 ± 0.09[ab]	0.52 ± 0.02[ab]	0.55 ± 0.09[ab]	0.59 ± 0.04[b]	0.58 ± 0.04[b]	0.48 ± 0.11[a]	*< 0.001*
22:5n-6	0.38 ± 0.07[a]	0.40 ± 0.05[a]	0.43 ± 0.09[ab]	0.46 ± 0.03[ab]	0.46 ± 0.09[ab]	0.51 ± 0.03[b]	0.51 ± 0.05[b]	0.41 ± 0.13[a]	*0.001*
Σn-6 PUFA	16.15 ± 1.00	16.25 ± 1.36	15.45 ± 1.00	16.19 ± 0.96	16.18 ± 1.36	15.67 ± 0.69	15.69 ± 1.05	15.91 ± 0.65	*0.630*
ΣPUFA	23.81 ± 1.93	24.32 ± 1.78	23.81 ± 1.50	25.21 ± 0.84	25.23 ± 1.50	25.48 ± 0.59	25.59 ± 1.22	23.95 ± 1.99	*0.422*
ΣLC-PUFA	9.12 ± 1.48[a]	9.63 ± 0.91[ab]	9.97 ± 1.91[ab]	10.70 ± 0.55[ab]	10.81 ± 1.66[ab]	11.72 ± 0.62[b]	11.77 ± 1.00[b]	9.56 ± 2.61[a]	*0.001*
Σn-3/Σ n-6	0.47 ± 0.08[a]	0.50 ± 0.06[ab]	0.55 ± 0.12[ab]	0.56 ± 0.05[ab]	0.57 ± 0.11[ab]	0.63 ± 0.06[b]	0.63 ± 0.08[b]	0.51 ± 0.15[a]	*0.028*
Others	1.94 ± 0.12	2.04 ± 0.06	1.98 ± 0.10	2.00 ± 0.05	2.08 ± 0.07	1.99 ± 0.18	2.06 ± 0.10	1.97 ± 0.12	*0.120*

Pre-feeding is related with individuals sampled 10 min before the feeding event, and 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h are related with the time of sampled post feed event. ΣSFA, ΣMUFA, ΣPUFA, n-3 ΣPUFA, n-6 ΣPUFA are the sum of saturated, monounsaturated, polyunsaturated, n-3 polyunsaturated and n-6 polyunsaturated fatty acids, respectively. “Others” are the sum of no representative fatty acids (lower than 0.3% of total fatty acids). [a.b]Different letters represent significant differences between fish sampled at different times, by Tukey’s test, ANOVA ($P > 0.05$, $n = 3$)

Deg Analysis

The Fig. 1A presents the DEGs in three comparison pairs according to the different postprandial periods. The results showed 2,507 DEGs between animals sampled at 30 min *vs.* 8 h, with 1,591 transcripts up-regulated and 916 down-regulated in fish sampled at 30 min, compared with those sampled at 8 h after meal. Comparing individuals sampled at 30 min *vs.* 24 h it was observed a reduced number of 448 DEGs, with 220 up-regulated and 228 down-regulated in fish sampled at 30 min compared to those sampled at 24 h after meal. Finally, a total of 2,401 DEGs were observed comparing fish sampled at 8 h *vs.* 24 h, with 1,071 up-regulated and 1,330 down-regulated in individuals sampled at 8 h compared to those sampled at 24 h after meal. The heatmap based on the DEGs generated by the transcriptome sequence corroborated the same expression profile observed in Fig. 1A, with a higher number of DEGs and an opposite profile between 30 min *vs.* 8 h and 8 h *vs.* 24 h, and a reduced number of DEGs at 30 min *vs.* 24 h (Fig. 1B).

Enrichment Analysis Of Gene Ontology (Go) And Kegg Pathways Of The Degs

The GO annotation was used to classify the DEGs of each comparison by three categories, (1) biological process, (2) cellular component, and (3) molecular function (Fig. 2). In general, the top 10 GO annotations were similar between different comparisons (with a few exceptions mainly in 30 min *vs.* 24 h). A higher number of transcripts were observed in all three categories for 30 min *vs.* 24 h and 8 h *vs.* 24 h, while a reduced number of transcripts were observed at 30 min *vs.* 24h.

The top 30 KEGG pathways of the Tier 2 classification were analyzed to detect the main pathways for the DEGs (Fig. 3). The profile of KEGG pathway classification, was consistent between different comparisons. The “signal transduction”, “cell growth, and death”, and “carbohydrate metabolism” were the most prominent pathways observed in 30 min *vs.* 8 h, 30 min *vs.* 24 h, and 8 h *vs.* 24 h, respectively. The “lipid metabolism”, which is the main metabolic pathway explored in this study, was also represented by a high number of transcripts in the three comparisons, with a higher number of DEGs noticed in 30 min *vs.* 8 h, followed by 8h *vs.* 24 h, and 30 min *vs.* 24 h (Fig. 3).

To further explore the physiological response profiles induced by fasting at specific pathways (fatty acid synthesis and oxidation), KEGG mapper analysis was performed to identify the DEGs (Fig. 4). Several differences were observed in the expression of genes related to the fatty acid biosynthesis and degradation pathways (Fig. 4A and B, respectively). The expression of fatty acid synthase (*fasn* – 2.3.1.85), acetyl CoA-carboxylase (*acc* – 6.4.1.2.), and medium-chain acyl dehydrolase (*mch* – 3.1.2.21) were up-regulated in animals sampled at 30 min compared to those sampled at 8 h after the meal, while animals fasted by 8 h showed lower expression of these genes compared with those fasted by 24 h. The long-chain acyl-CoA synthetase (*acsl* – 6.2.1.3) expression was up-regulated in animals fasted by 30 min compared to those fasted by 8 h, while 3-oxoacyl-synthase II (*fabf* – 2.3.1.179) was down-regulated in fish fasted by 8 h compared with those fasted by 24 h (Fig. 4A). Regarding the fatty acid catabolism pathway, fish fasted by 30 min showed higher expression of long-chain acyl-CoA synthetase (*acsl* – 6.2.1.3), and butyryl-CoA dehydrogenase (*acads* – 1.3.8.1) compared to individuals sampled at 8 h. The acyl-CoA oxidase (*acox* – 1.3.3.6) expression was down-regulated, and carnitine palmitoyltransferase 1 (*cpt1–2*.3.1.21) was up-regulated, in fish sampled at 30 min and 24 h compared to those sampled at 8 h after last meal, while acyl-CoA dehydrogenase (*acadm* – 1.3.8.7) was up-regulated in fish sampled at 30 min compared to fish sampled at 8 and 24 h. Finally, the expression of alcohol dehydrogenase 1/7 (*adh1_7–1*.1.1.1) and aldehyde dehydrogenase (*aldh* – 1.2.1.3) were upregulated in animals fasted by 30 min and 24 h compared to those fasted by 8 h (Fig. 4B).

Verification And Validation Of Degs By Qrt-pcr

To verify the accuracy of the RNA-seq data, we screened ten DEGs, analyzed them with qRT-PCR, and compared the differences. The expression profile of the ten genes (comparing different sample periods)

was consistent with those observed in the RNA-seq analysis (Fig. 5). Therefore, the qRT-PCR results confirmed the reliability and accuracy of the RNAseq data.

Discussion

In order to understand the nutritional requirements of finfish species, it is crucial to investigate the molecular responses that occur each time that the animals receive food, since several metabolic pathways are differentially modulated during the postprandial period (Valente et al. 2012; Wade et al. 2014). However, studies focusing on the lipid metabolism of finfish species after feeding are limited/scarce, and despite the commercial relevance, no studies on this space have been performed using cobia as a model. Thus, the data generated in this study are essential in providing bases for future physiological and nutritional studies using this important marine commercial species.

Such as commonly observed in previous studies performed with finfish species (Rombenso et al. 2016; Araújo et al. 2018; Gou et al. 2020; Fisher et al. 2022), and specifically with cobia (Araújo et al. 2021; Marques et al. 2021), with a few exceptions, the liver fatty acid profile reflected the composition of the diet. However, it was observed a lower percentage of important LC-PUFA, such as DHA and EPA, in the liver compared to the diet reflecting also in the same profile as the total n-3 PUFA. The LC-PUFAs, mainly DHA and EPA, play several essential physiological functions in marine fish, such as cell membrane structuring and eicosanoid synthesis, and it is commonly retained by the animals in the same proportion as they are included in the diet (Bell 2003; Tocher 2003; Glencross 2009). Therefore, this paradigm can be changed by the influence of several other variables, especially tissue specificity, diet composition, and temperature (Tocher 2003). Based on the profile observed in the liver fatty acid composition, with several statistical differences in the n-3 and n-6 PUFA levels, especially between 8 and 12 h compared to 30 min and 24 h after the feed event, aligned with consistent results from previous studies that also investigated the molecular responses of finfish species during the postprandial period (Valente et al. 2012; Wade et al. 2014; Araújo et al. 2016; Mente et al. 2017), it was defined the assessments to be explored in the molecular analysis. Thus, as previously mentioned, the molecular results were focused on three main comparisons, (1) 30 min *vs.* 8 h; (2) 30 min *vs.* 24 h; and (3) 8 h *vs.* 24 h after feeding.

A reduced number of DEGs comparing individuals sampled at 30 min with those sampled at 24 h (448 transcripts in total, 220 up-regulated and 228 down-regulated at 30 min) after feeding was observed, while animals sampled at 8 h showed many DEGs compared to those sampled at 30 min (2,507 transcripts in total, 1,591 up-regulated and 916 down-regulated at 8 h) and 24 h (2,401 transcripts in total, 1,071 up-regulated and 1,330 down-regulated at 8 h) after feeding, such as presented in Fig. 1A. These results (DEGs and qRT-PCR), clearly showed an opposite profile in the expression of metabolic-relevant genes at 8 h compared with 30 min and 24 h (which showed consistent expression) after the feed event, as demonstrated in Fig. 1B. In previous studies performed with barramundi juveniles (Wade et al. 2014; Araújo et al. 2016; Poppi et al. 2019), it was observed a differential expression of several metabolic-relevant genes after 8 h and 12 h compared with 1 h and 24 h after feeding. Additionally, Mente et al. (2017) observed significant changes (up or down-regulation) in the expression of several protein-relevant

genes in the liver of rainbow trout 6 to 12 h after the last meal, while at 24 h the expression levels of these same genes were consistent to the moment after the feed event. Similarly, in Atlantic salmon juveniles, growth-relevant genes such as insulin-like growth factor (*igf*) and ubiquitin ligase MAFbx/atrogin-1 were initially down-regulated but restored to the basal expression levels after 12 h of fasting (Valente et al. 2012). Aiming to avoid stress by handling and poor water quality (due to the possibility of fish regurgitating during handling), several previous studies performed with finfish species standardized a period of 24 h of fasting to sample the experimental animals (Zheng et al. 2004; Manor et al. 2015; Jin et al. 2017; Zhang et al. 2019; Gou et al. 2020). However, based on our results with cobia and previous results with other finfish species (Valente et al. 2012; Wade et al. 2014; Araújo et al. 2016; Diez et al. 2017; Mente et al. 2017; Poppi et al. 2019), it is suggested that after 24 h of the last meal the expression of metabolic-relevant genes did not necessarily reflect the influence of the feed intake/composition, with this large interval (24 h) between the last meal and sample resulting potentially in a misinterpretation of the gene expression results.

Lipids are the major source of energy for finfish, and the liver is considered the main lipolytic tissue (especially under fasting) and notably the most important fat deposit for energy in several species (Tocher 2003; Turchini et al. 2009). Thus, previous knowledge regarding the hepatic fatty acid synthesis and catabolism pathways is essential for designing physiological and nutritional trials aiming to define the nutrient requirements of finfish species. Specifically related to the lipid-relevant pathways (biosynthesis and *β*-oxidation) it was observed a similar profile in the expression of the global transcripts, with only one DEG (*acadm)* comparing fish fasted by 30 min *vs.* 24 h, while all the other DEGs were observed comparing fish fasted by 8 h *vs.* 30 min and 24 h. These results suggest that after 24 h of a single meal the expression of lipid-relevant genes (such as the expression of other metabolic genes) reached basal levels (30 min after meal). Most of the lipogenic genes (mainly *fas*, *acc*, and *acsl*) were significantly down-regulated in fish sampled at 8h after meal compared with those sampled at 30 m or 24 h (or both periods). In general, the expression of fatty acid synthesis-relevant genes is strictly related to the tissue, and consequently to the diet fatty acid composition (Leaver et al. 2008; Torstensen et al. 2009). Previous studies *in vivo* (Alvarez et al. 2000) and *in vitro* (Menoyo et al. 2003) showed that higher levels of hepatic EPA and DHA inhibited lipogenesis in salmonids, corroborating the results observed in this study, since it was observed higher levels of both fatty acids, and total n-3 PUFAs, in fish sampled at 8 h compared to those sampled at 30 min and 24 h after meal. Acetyl CoA is activated to malonyl-CoA by ACC and iteratively assembled in the cytosol by FAS to form 16- and 18-carbon saturated fatty acids, thus, consistent expression levels between both genes are expected. Consistent expression of *fas* and *acc* was also observed in zebrafish (*Danio rerio*) under fasting (Seiliez et al. 2013). However, differently than observed in cobia, zebrafish juveniles showed an up-regulation of these genes (and other lipid-relevant genes) after 6 h of the last meal, while at 24 h the expression levels of these genes reached basal levels. Similarly, Wade et al. (2014) observed that fatty acids synthesis-relevant genes (e.g., *fas* and *scd*) were up-regulated in barramundi sampled at 12 h compared with those fish sampled at 1 h and 24 h after feeding. These contradictory results in the expression of lipogenic genes during a postprandial period between different finfish species corroborate the hypothesis that lipogenesis is highly modulated by the

interaction of several variables, primarily species specificity (physiological characteristics), diet composition, and culture conditions (Tocher 2003; Leaver et al. 2008).

A different profile in the expression of genes related to fatty acid catabolism was observed in cobia sampled at different postprandial periods. While *acox* expression was significantly up-regulated, *cpt1* was significantly down-regulated in fish sampled at 8 h compared with those sampled at 30 min and 24 h after the meal. In general, fatty acid catabolism can occur in two different organelles, mitochondria, and peroxisomes (Leaver et al. 2008). ACOX catalyzes the rate-limiting step in the peroxisomal *β*-oxidation, while CPT1 is related to the fatty acid transport over the inner mitochondrial membrane to be *β*-oxidized (mitochondrial oxidation) (Leaver et al. 2008). This opposite profile in the *acox* and *cpt1* expression suggests that fish sampled at 8 h catabolized hepatic fatty acids preferentially by peroxisomal *β*-oxidation, while fish sampled at 30 min and 24 h after feeding preferentially by mitochondrial *β*-oxidation. According to Leaver et al. (2008), typically in fish liver, peroxisomal *β*-oxidation contributes more significantly to the total *β*-oxidation. However, the capacity to catabolize fatty acids depends on the interaction of several biotic and abiotic factors mainly temperature, fish size, life stage, and diet composition, that will differently modulate fatty acid catabolism processes in fish. Additionally, malonyl-CoA produced from ACC activity inhibits CPT1, justifying an opposite profile in the expression of both genes. Similar to those found in cobia, Diez et al. (2007) observed higher peroxisomal *β*-oxidation (ACOX activity) in sea bream juveniles after 6 h of the last meal, accompanied by a decrease in a mitochondrial *β*-oxidation (L-3-hydroxyacyl-CoA dehydrogenase - L3HOAD activity), but interestingly, these effects were not observed at 24 h after feeding. Unfortunately, studies investigating fatty acid catabolism of finfish species during the postprandial period are scarce in the literature. However, the results obtained in this study and those found in sea bream by Diez et al. (2007) and Wade et al. (2014) reinforce the importance of evaluating the expression of fatty acid catabolism-relevant genes during the postprandial period in finfish species.

Conclusion

This study performed a transcriptional profiling analysis of fasting in cobia juveniles to identify metabolism-related genes and pathways, with a focus on lipid metabolism. The results showed that the main differences in liver fatty acid profile and molecular responses were observed between 8 and 12 h after the last meal, while after 24 h the n-3 LC-PUFAs, global transcription, and differential expression of lipid-relevant genes reached basal levels (30 min after the last meal). Our results, differently than previous studies performed with other teleost species, suggest an adequate time to sample the animals/tissues ranging between 8 and 12 h after the last meal, since apparently, after 24 h gene expression is not more influenced by feed intake/composition. However, these molecular responses should be evaluated for every single species/trial since the interaction of several biotic and abiotic variables such as species specificity, life stage, diet composition, and temperature, can directly influence the hepatic molecular responses in finfish species, especially post-feeding.

Declarations

Author's Contributions Bruno C. Araújo, David A. Barbosa, Fabiano B. Menegidio, Giovana S. Branco, Renato M. Honji: Conceptualization, data curation, formal analysis, investigation, writing, editing; Victor H. Marques, Paulo H. de Mello, Marcelo V. Kitahara, Artur N. Rombenso: Formal analysis, writing, review, editing; Renata G. Moreira, Alexandre W.S. Hilsdorf: Conceptualization, methodology, resources, review.

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Availability of Data and Material Sequencing raw data were deposited in the Sequence Read Archive (SRA) repository of the National Center for Biotechnology Information (NCBI), under accession numbers SRR13009897, SRR13009896, SRR13009895, SRR13009894, SRR13009893, SRR13009892, SRR13009891, SRR13009890, SRR13009889, SRR13009888, SRR13009887, and SRR13009886, associated to the BioProject numbers PRJNA675281 and BioSamples numbers SAMN16708758, SAMN16708759, SAMN16708760, SAMN16708761, SAMN16708762, SAMN16708763, SAMN16708764, SAMN16708765, SAMN16708766, SAMN16708767, SAMN16708768, and SAMN16708769. The Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under accession number GIWT00000000.

Ethics Approval This study was conducted according to the guidelines and approval of the Mogi das Cruzes University Institutional Animal Care and Use Ethics Committee (#008/2017).

Disclaimer The authors alone are responsible for the content and the writing of the paper.

Conflict of Interest The authors declare no competing interests.

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Figures

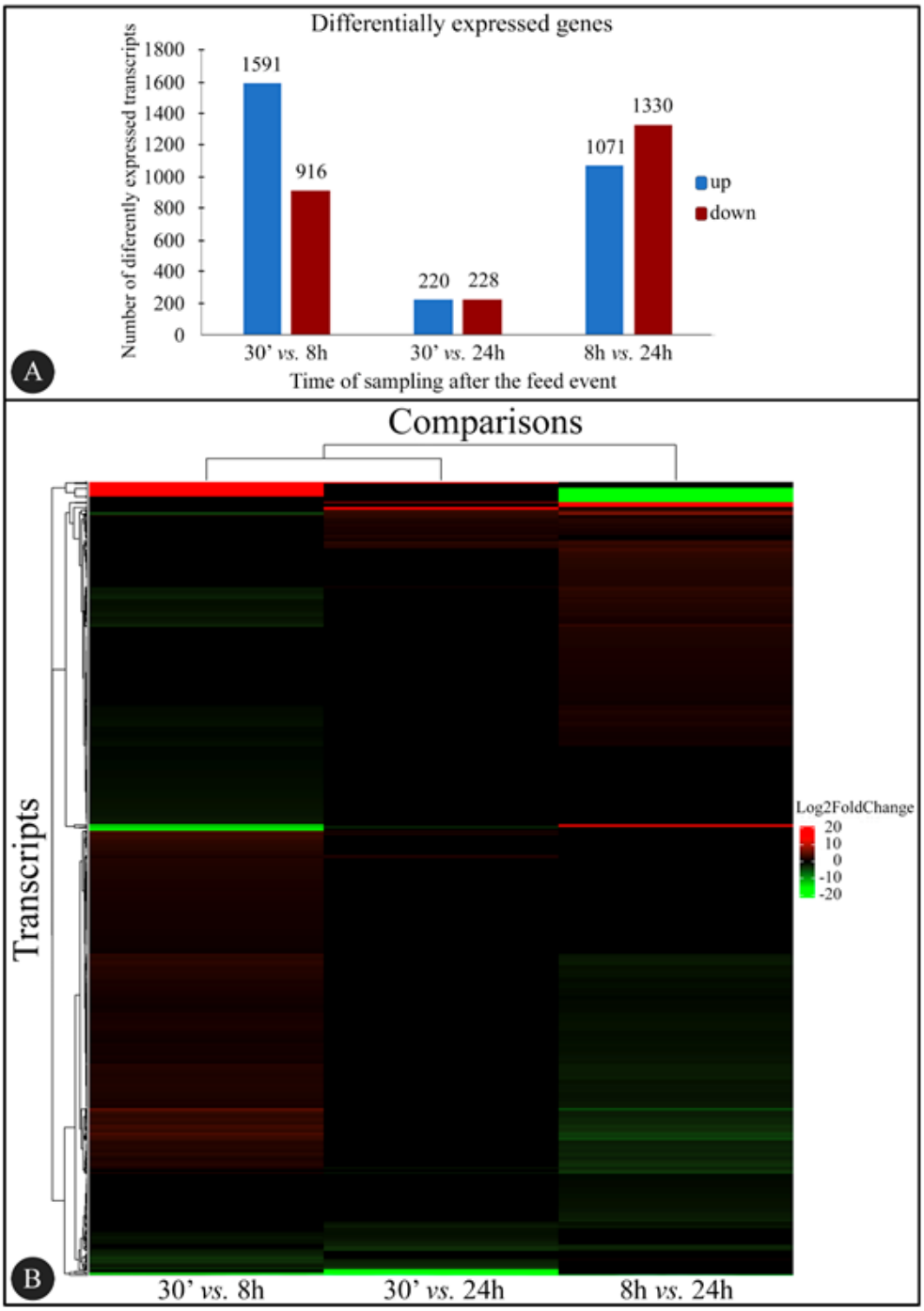

Figure 1

(A) Histogram for differentially expressed genes (DEGs). The blue bar indicated up-regulated genes, and the red bar indicates down-regulated genes. (B) Heatmap of transcripts with expression change higher than 2 log2 fold change

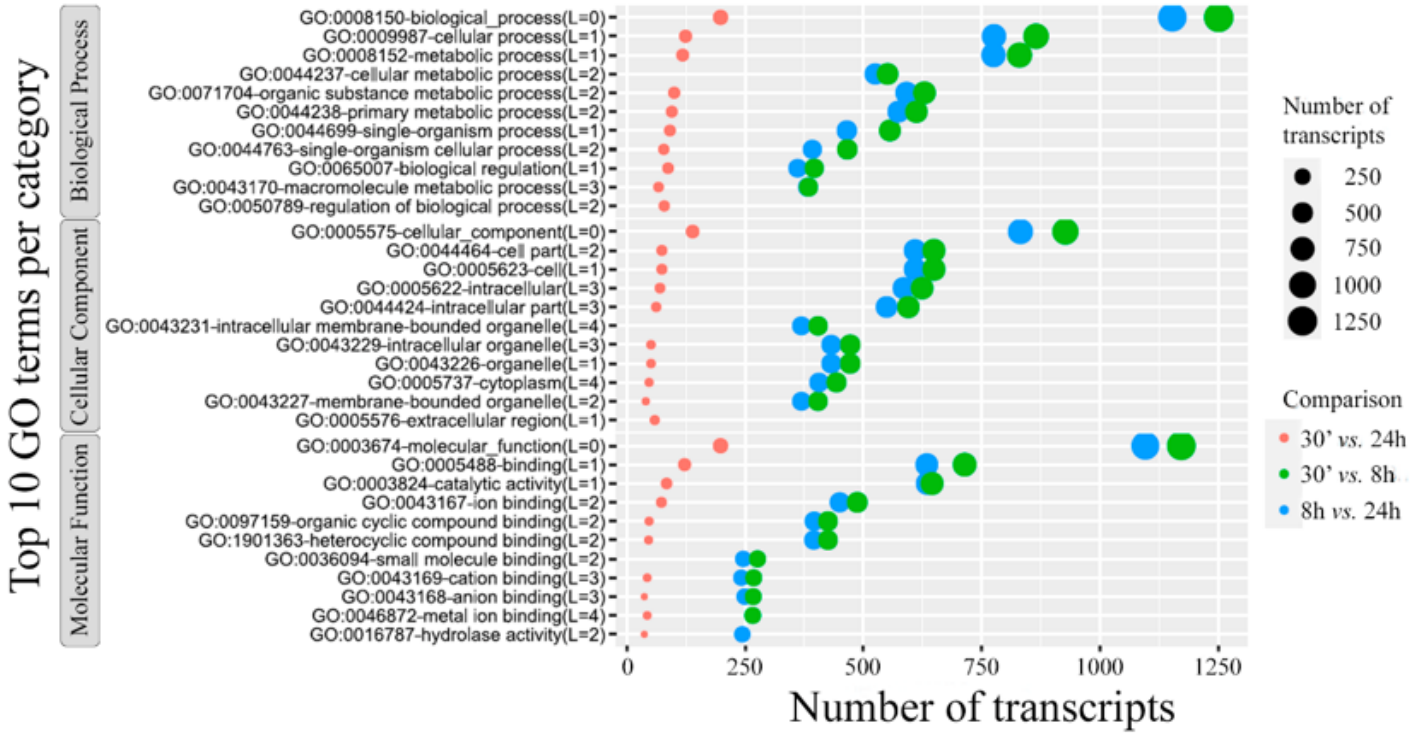

Figure 2

Gene ontology (GO) enrichment distribution for the 10 most assigned biological processes, cellular component, and molecular function of assembled isoforms according to different comparisons (30 min *vs.* 24 h, 30 min *vs.*8 h, and 8 h *vs.* 24 h)

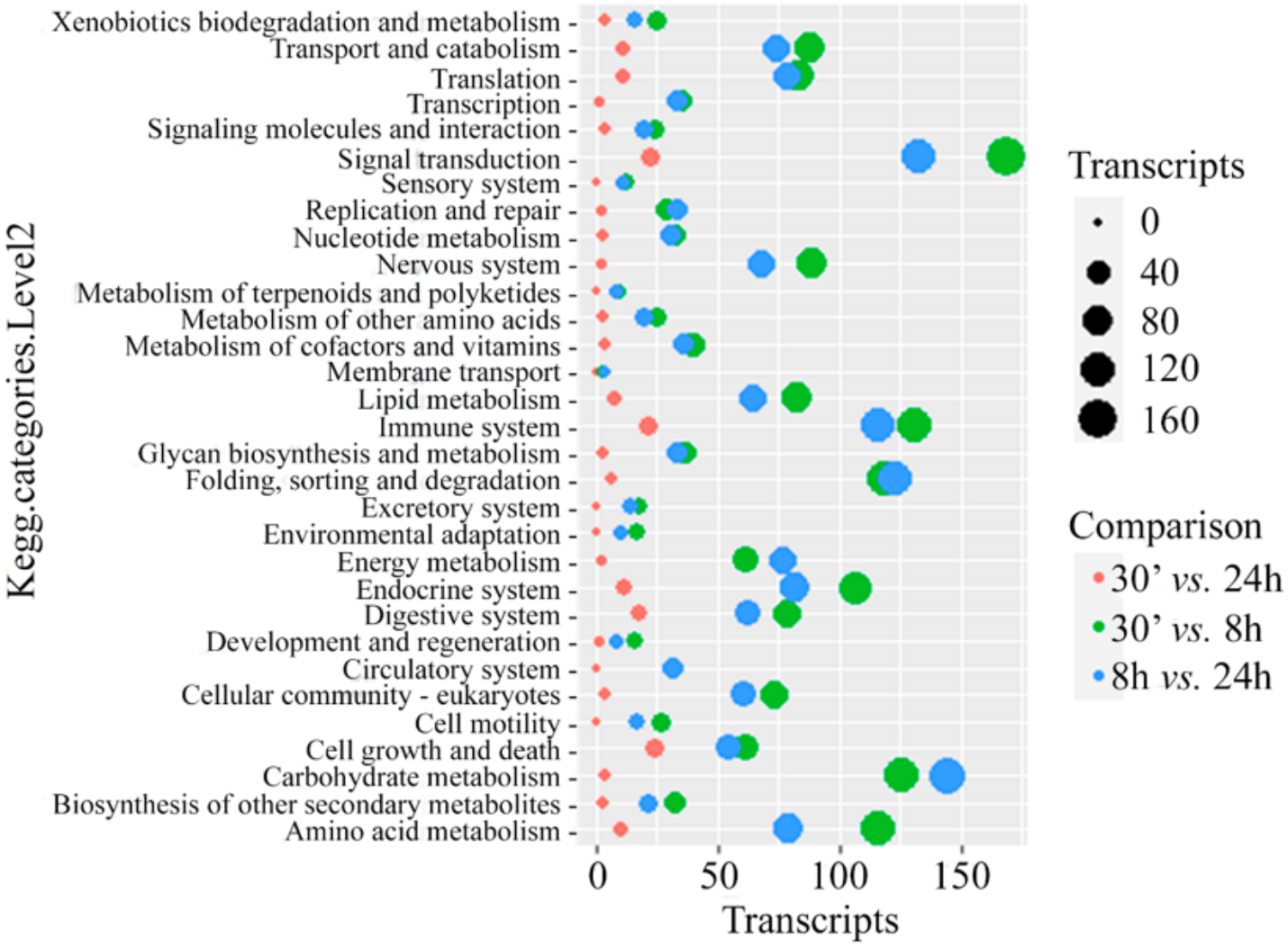

Figure 3

Number of DEGs in the Top 30 KEGG pathway classifications according to different comparisons (30 min *vs.* 24 h, 30 min *vs.* 8 h, and 8 h *vs.* 24 h)

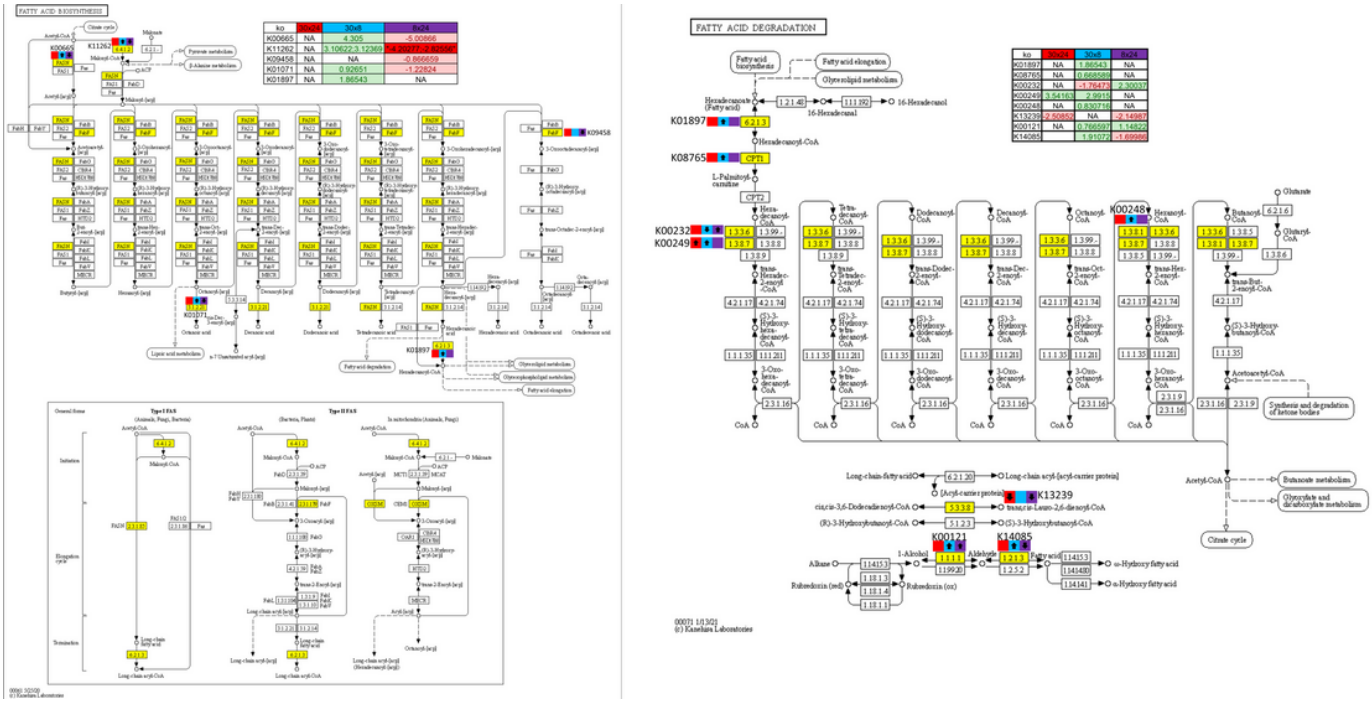

Figure 4

Lipid-relevant DEGs identified by KEGG in the (A) fatty acid biosynthesis, and (B) degradation pathways. The genes highlighted in yellow were significantly expressed in at least one of the three comparisons. Red squares represent a comparison between 30 min *vs.*24 h, blue squares between 30 min *vs.*8 h, and purple squares between 8 h *vs.*24 h. The arrows in the colorful squares represent: (↑) up-regulation of the specific transcript in the lower fasting time compared to the higher fasting time in each of the three comparisons, and (↓) down-regulation of the specific transcript in the lower fasting time compared to the higher fasting time in each of the three comparisons. Arrow absence indicates no significant difference in the specific comparison

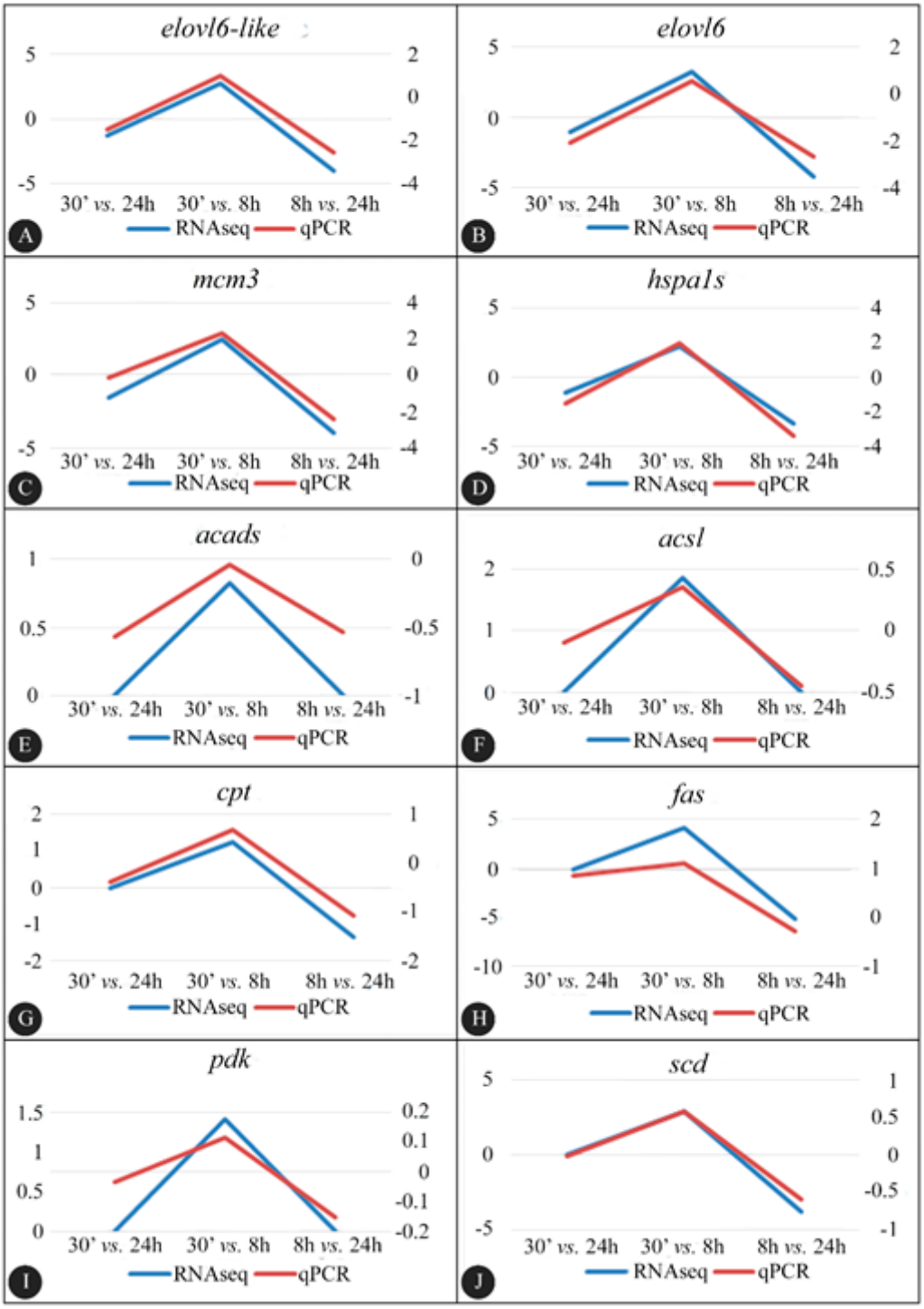

Figure 5

Comparison of gene expression patterns obtained using comparative transcriptome analysis and qRT-PCR. (A) *elovl6-like* (elongase 6-like), (B) *elovl6* (elongase 6), (C) *mcm3* (minichromosome maintenance complex component 3), (D) *hspa1s* (heat shock 70 kDa protein 1), (E) *acads*(acyl-CoA dehydrogenase short chain), (F) *acsl* (long-chain acyl-CoA synthetases), (G) *cpt* (carnitine palmitoyltransferase), (H) *fas* (fatty acid synthase), (I) *pdk* (pyruvate dehydrogenase kinase), and *scd* (stearoyl-CoA desaturase). The transcript expression levels of the target genes were normalized by the *β*-actin expression