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Molecular aspects of lipid metabolism in the midgut gland of the brown shrimp *Crangon crangon*

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ABSTRACT

The brown shrimp, *Crangon crangon*, is well adapted to the variable environmental conditions in the southern North Sea. It is very abundant, has high reproduction rates, and holds a key position in coastal ecosystems. This species has very low lipid deposits in the midgut gland, suggesting that the main function of the midgut gland is metabolic turnover rather than energy storage. Based on seasonal gene expression studies and established transcriptome data, we investigated key components of lipid metabolic pathways. Gene expression of triacylglycerol lipase, phospholipase, and fatty acid desaturase were analyzed and compared with that of other digestive enzymes involved in lipid, carbohydrate, and protein catabolism. Our results suggest that gene expression of digestive enzymes involved in lipid metabolism is modulated by the lipid content in the midgut gland and is related to food availability. Brown shrimp seem to be capable of using cellular phospholipids during periods of food paucity but high energetic (lipid) requirements. Two of three isoforms of fatty acid binding proteins (FABPs) from the midgut gland involved in fatty acid transport showed specific mutations of the binding site. We hypothesize that the mutations in FABPs and deficiencies in anabolic pathways limit lipid storage capacities in the midgut gland of *C. crangon*. In turn, food utilization, including lipid catabolism, has to be efficient to fulfill the energetic requirements of brown shrimp.

1. Introduction

The brown shrimp, *Crangon crangon*, is a key species of the southern North Sea ecosystem with major commercial importance. It has high reproduction rates and is well adapted to the variable environmental conditions. Lipid stores are important energy reserves to survive periods of food scarcity, especially in species confronted with highly variable conditions. However, *C. crangon* shows rather low lipid levels of about 14–17% of dry mass (%DM) in the midgut gland (also referred to as hepatopancreas), the typical storage organ in decapods. In contrast, the midgut gland of the pink shrimp *Pandalus montagui*, which inhabits southern but also central parts of the North Sea, has lipid levels of 47–70%DM (Martínez-Alarcón et al., 2019b). Therefore, it has been suggested that the main function of the midgut gland of *C. crangon* is metabolic turnover rather than energy storage (Clarke, 1982; Martínez-Alarcón et al., 2019b).

In invertebrates, lipid metabolism is highly coordinated and involves multiple catabolic and anabolic processes. The lipid metabolism of crustaceans has been poorly investigated. Studies of their catabolic capacities showed dynamic gene expression of two lipases after starvation of the shrimp *Penaeus vannamei* (Rivera-Pérez and García-Carreño, 2011). In the crayfish *Cherax quadricarinatus* a lipase was differentially expressed during moulting and fasting periods (Yudkovski et al., 2007). At the transcriptional level, dietary lipids had effects on fatty acid biosynthesis and β-oxidation in the hepatopancreas of the Chinese mitten crab *Eriocheir sinensis* (Wei et al., 2017). The transcriptome of *C. crangon* exhibits a high level of polymorphism in triacylglycerol lipase and phospholipase A_2 (Martínez-Alarcón et al., 2019a) indicating a high flexibility of the lipid catabolism.

In addition to the catabolic lipases, other proteins without catalytic power also have key functions in the lipid metabolism, for example those involved in lipid transport. The transfer of lipids between organs and organelles is an essential biological process. Inside and outside the cell, specialized proteins redistribute hydrophobic lipid molecules (Van der Horst and Ryan, 2012) and many lipid transfer proteins have been described so far. The metabolic functions of these molecules are targeted by several investigations. However, the majority of these studies focus on the lipid metabolism of insects (Van der Horst and Ryan, 2012) or humans (Chen and Davidson, 2012). There are still large gaps in our knowledge of this topic, especially concerning crustacean lipid

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metabolism. Furthermore, the majority of research done on lipid transport of crustaceans is focused on the transport between tissues via the hemolymph (O'Connor and Gilbert, 1968) rather than the transport of lipid molecules within cells. There are vesicular and non-vesicular routes for intracellular traffic. In non-vesicular routes, the so-called lipid-transfer proteins (LTPs) mediate lipid transfer across the cytoplasm. LTPs stimulate the extraction of lipids from a membrane, their mobilization in the cytoplasm, and their re-insertion into different membranes (Wong et al., 2017). LTP specificity varies, but many of them shield the hydrophobic portion of lipids in internal cavities. In this way, lipids are transported in a mobile protein segment into the cytoplasm. There are several families of proteins that are capable of mobilizing lipids (Wong et al., 2017). Also, in the cytoplasm the flux of fatty acids between organelles seems to be controlled by fatty acid binding proteins (FABPs) (Maatman et al., 1994). These proteins bind non-covalently to fatty acids and were identified for instance in the Chinese mitten crab (Gong et al., 2010). A thorough review of invertebrate intracellular FABPs is given by Esteves and Ehrlich (2006).

Metabolic pathways for the synthesis of triacylglycerols (TAG) and phospholipids (PL) were already proposed by Kennedy and Weiss (1956). The Kennedy pathway is the principal route to TAG synthesis. This pathway involves the addition of fatty-acyl groups to a glycerol-3-phosphate to form phosphatidic acid (PA), which is converted to diacylglycerol (DAG) by phosphatidic acid phosphatase. DAG is further acylated by diacylglycerol acyltransferase and forms TAG (Kennedy and Weiss, 1956).

The aim of this study is to investigate principal components of the lipid metabolism in the midgut gland of *Crangon crangon* to better understand its function and, particularly, its limited lipid storage capacities. Therefore, we addressed the following objectives: 1) to study seasonal expression patterns of key lipase digestive enzymes, 2) to compare the gene expression of lipases with that of other important digestive enzymes, 3) to analyze the occurrence and structure of FABPs, and 4) to identify enzymes of principal anabolic pathways.

2. Material and methods

2.1. Origin of samples

Brown shrimp, *Crangon crangon* (Linnaeus, 1758), were collected by bottom trawling with R/V *Uthörn* during four sampling campaigns in February, April, July, and October 2016 in the southern North Sea near the island of Helgoland (Table 1). Total body length (rostrum to tip of telson) and sex of adult animals were determined onboard ship. Thereafter, animals were dissected and midgut glands (30–50 mg) were transferred to individual 1.5-ml reaction tubes containing 0.5 ml of RNAlater (AM7020, Life Technologies, Ontario, Canada). The samples were shock-frozen in liquid nitrogen, shipped frozen to the laboratories of the Alfred Wegener Institute in Bremerhaven, and stored at −80 °C until further processing and analysis. All applicable European and German guidelines for the use of marine invertebrates were followed in this study.

2.2. Reference genes and primer design

Forward and reverse primer sequences used for the qPCR are shown in Table 2. The cell skeleton protein β actin, the chromatin protein histone H3, and the hypoxia-inducible factor (HIF) were selected as possible reference genes for the normalization of target gene expression. The choice of these reference genes was based on a similar analysis by Koenigstein et al. (2013). We focused our gene expression study on selected enzymes involved in lipid metabolism (fatty acid desaturase, phospholipase, triacylglycerol lipase), carbohydrate digestion (alpha-amylase, chitinase), and protein utilization (trypsin and cathepsin L). All primers in the study were designed based on the sequence information obtained from the transcriptome data (Martínez-Alarcón et al., 2019a) using Primer-Blast from NCBI (National Center for Biotechnology Information) considering CG or GC ration > 55% and a fragment length between 100 and 250 bp. Candidate primer pairs were double-checked for primer dimers using Oligo Analyzer 3.1. Each primer pair was evaluated on the grounds of the absence of artifacts in their melting curve and their efficiency was assessed by serial template dilution. The specificity of the RT-qPCR amplification was confirmed by melt analysis of the reactions and by sequencing of the PCR products.

2.3. Extraction of mRNA from midgut glands

Midgut gland samples of *C. crangon*, previously stored in RNAlater at −80 °C, were thawed and about 30 mg of the tissue was used for RNA extraction. The Precellys Keramik-Kit (PEQLAB 91-PCS-CKM, Erlangen, Germany) was used for cell lysis, performing three cycles of 15 s shaking and 30 s pauses in between shakes. Thereafter, samples were centrifuged at 13,000*g* for 3 min at room temperature. Total RNA was isolated using RNeasy Mini Kit spin columns (Qiagen 74104, Hilden, Germany) following the manufacturer's instructions. RNA quantities and purities were determined with a NanoDrop ND-1000 device at 260 nm and 260/280 nm, respectively. A high capacity cDNA RT Kit (Applied Biosystems, USA) was used to generate cDNA.

2.4. Real-time qPCR

Gene expression levels were studied by real-time PCR (q-PCR, Rotor-Gene Q, Qiagen). The tested samples were 100-fold diluted in molecular water and the primers were used in a final concentration of 700 nM in a Master Mix (Type-it HRM PCR kit (400), Qiagen 206544) in 0.1 ml Qiagen tubes for the Rotor-Gene. Every sample was tested in duplicate. The PCR amplification program was 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 30 s at 55 °C. PCR products were sequenced and the results were analyzed in CodonCodeAligner software and compared for matching with the sequences from the NCBI database.

2.5. Data processing and statistics

Relative levels of gene expression were calculated by the Comparative Quantification feature in the Rotor-Gene Q Series software V. 1.7.94 (Qiagen) with the deltadeltaC_T method (Joehanes and Nelson, 2008). Data were transferred to Excel (Microsoft) and the stability of the candidate reference genes was tested with the macros GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). Shapiro-Wilk normality test was used to determine normal distribution. To test for differences in gene expression the data were analyzed by

Table 1
Origin and biological data of *Crangon crangon* samples.

Date (2016)		Location	Ind. analyzed (n)	Males (n)	Females (n)	Total length (mm)
February	19	54°08′N 007°52′E	10	2	8	55–70
April	18	53°44′N 008°15′E	10	2	8	53–70
July	19–21	53°44′N 008°15′E	10	5	5	55–68
October	24–26	54°08′N 007°52′E	10	2	8	62–74

Table 2
List of primers used for the gene expression analysis of the midgut gland of *Crangon crangon*.

Category	Gene	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Amplicon length (bp)
Housekeeper	Actin	CCGATAGTGATGACCTGACCG	TACCACTGCCGAGAGGGAAA	150
	HIF-1 alfa	CGCCGCTGACGATGTAATTG	TCAGGCCACTCTCATCAACG	77
	Histone H3	AACAGACCGACAAGGTAGGC	TGGTACTGTTGCCCTTCGTG	183
Lipid metabolism	Triacylglycerol lipase	CGTGGTACCCATCTTGCAGT	GGCATGGAATGGGAGACACA	219
	Phospholipase	TGGGTCATGTATCCCTCGTC	TTGGGGAGTTTGTGGCATTC	233
	Fatty acid desaturase	GCGTCTGTCTCGCTGTACTT	CTGGGTATCACCATGGGAGC	167
Protein metabolism	Trypsin	AATGTCGTTGGAGCGTCTGT	GTGCTGCCCAGTGTTTTCAG	167
	Cathepsin L	TTCATGCCATTCTCTTCGCC	CACCCAAGGGAAAACTCCAC	571
Carbohydrate metabolism	Alpha amylase	AGAGTCGTCAGTCGGGTACA	TTGCTGGAACTCTGCGACAT	185
	Chitinase	TGCTTTGCCGGCAGATACAG	TGGGAATACCCTACTCAGCGA	132

one-way ANOVA. If there was no homogeneity of variances, Krustal-Wallis test was used. Differences among seasons were subsequently identified by pairwise comparison with the Tukey's post-hoc test. The level for statistical significance was set at $P < 0.05$. The correlation matrix was obtained using the Pearson correlation coefficient which measures the linear dependence between two variables. RStudio software (version 0.99.491) was used to carry out the statistical test and graphical representations.

2.6. Fatty acid binding protein (FABP) sequences and 3D structure

DNA sequences from the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al., 2019a) identified as FABPs were translated into amino acid sequences based on the standard code. Amino acid sequences were aligned using ClustalW at EMBL-EBI (Larkin et al., 2007) against ten previously reported FABPs in NCBI database and two sequences reported by Söderhäll et al., (2006) (Table 3). A cladogram based on amino acid sequences was constructed using CLC Genomics Workbench version 12.0.1 (QIAGEN). Evolutionary history was interfered by the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The initial tree was obtained by applying the Neighbor Joining method. A bootstrap analysis with 10,000 replicates was performed for assessing confidence in the analysis of the clades. 3D structures of the different isoforms of *C. crangon* FABPs were predicted with the online software SWISS-MODEL (https://swissmodel.expasy.org) (Waterhouse et al., 2018).

2.7. Transcriptome screening for enzymes and proteins involved in lipid metabolism

Transcriptome data of *C. crangon* midgut glands, previously reported by Martínez-Alarcón et al. (2019a), was screened for the presence of enzymes involved in the catabolism and anabolism of alimentary lipids. Particular attention was paid to the enzymes involved in the Kennedy pathway facilitating the synthesis of triacylglycerols (TAG) and diacylglycerols (DAG).

3. Results

3.1. Identification of reference genes

Stability of candidate reference gene expression was calculated by two algorithms (GeNorm and NormFinder). Both algorithms identified actin as the most stable gene, hypoxia-inducible factor (HIF) ranked second, and histone 3 (H3) was the least stable gene (Table 4). GeNorm calculates an average expression stability value (M) based on the standard deviation between all genes and samples, while NormFinder provides a model-based stability value between groups (Koenigstein et al., 2013).

3.2. Seasonal expression of digestive enzyme genes

The digestive enzyme triacylglycerol lipase involved in the catabolism of TAG showed deviating expressions between seasons. From autumn to winter, this enzyme was down-regulated ($p = 0.008$), and up-regulated from winter to summer ($p = 0.004$) (Fig. 1a). The gene expression of phospholipase, which is involved in the catabolism of phospholipids (Fig. 1b) and fatty acid desaturase (Fig. 1c) important for the anabolism of lipids, did not show statistical differences between seasons. The ratio of gene expression between phospholipase and triacylglycerol lipase showed significant seasonal changes (Fig. 2). In spring, expression of phospholipase was twice as high as the expression of triacylglycerol lipase. In summer, the ratio changed and the expression of triacylglycerol lipase became higher. This tendency continued in autumn, showing twice as high expression of triacylglycerol lipase than phospholipase. Towards winter, the ratio changed again, showing a five times higher expression of phospholipase compared to triacylglycerol lipase.

Table 3
List of fatty acid binding protein (FABP) sequences used for the alignment with FABPs of *Crangon crangon* (NCBI ID: Reference sequence ID in the database of the National Center for Biotechnology Information) (ns: no specified).

Species	Tissue	Taxon	NCBI ID	Reference
Homo sapiens	Adipocyte	Mammalia	NP_001433.1	Baxa et al., 1989
Homo sapiens	Muscle	Mammalia	AAB02555.1	Peeters et al., 1991
Homo sapiens	Muscle	Mammalia	CAA39889.1	Peeters et al., 1991
Homo sapiens	Heart	Mammalia	NP_001307925.1	Börchers et al., 1990
Locusta migratoria	Muscle	Insecta	AAB30739.1	Maatman et al., 1994
Drosophila melanogaster	ns	Insecta	NP_001027181.1	Matthews et al., 2015
Anopheles gambiae	ns	Insecta	Q17017	Holt et al., 2002
Schistocerca gregaria	Muscle	Insecta	P41496	Price et al., 1992
Pacifastacus leniusculus	Hemocyte	Malacostraca	NA	Söderhäll et al., 2006
Penaeus monodon	Hemocyte	Malacostraca	NA	Söderhäll et al., 2006
Schistosoma japonicum	ns	Trematoda	AAG50052.1	Liu et al., 2004
Echinococcus granulosus	ns	Cestoda	AAK51437	Unpubl.

Table 4
Stability ranking of candidate reference gene expression in the midgut gland of *Crangon crangon* by two different algorithms.

GeNorm			NormFinder		
Rank	Gene	M	Rank	Gene	Stability
1st + 2nd	Actin + HIF	0.691	1st	Actin	0.352
1st + 3rd	Actin + H3	0.768	2nd	HIF	0.434
			3rd	H3	0.467

3.3. Correlation analysis of digestive enzyme gene expression

Seasonal gene expression of phospholipase was always negatively correlated with the other digestive enzymes studied here. The correlation coefficient with triacylglycerol lipase was −0.17 ($p = 0.31$) and with fatty acid desaturase −0.29 ($p = 0.09$). Also, with cathepsin L involved in protein catabolism, phospholipase showed a negative correlation coefficient of −0.13 ($p = 0.47$). Comparison with carbohydrases produced correlation coefficients of −0.31 ($p = 0.07$) for chitinase and −0.20 ($p = 0.24$) for alpha amylase. Strongly positive relationships were determined for triacylglycerol lipase and cathepsin L (0.52, $p < 0.001$), for triacylglycerol lipase and chitinase (0.53, $p < 0.001$), and for trypsin and cathepsin L (0.64, $p < 0.001$) (Table 5).

3.4. Alignment and cladogram of fatty acid binding protein (FABP) sequences

BLAST analysis confirmed the coding sequences obtained from the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al., 2019a) as FABPs. The three isoforms were aligned with other previously reported FABPs in the NCBI database (Table 3). Secondary structure characteristics of FABPs were identified in the three isoforms of *C. crangon*. One of the three amino-acid residues of the P2 motif, which is characteristic of FABPs (Söderhäll et al., 2006) (involved in the interaction with ligand carboxylate) was found mutated in one isoform of *C. crangon* FABPs. This mutation substituted valine (V) in the position of arginine (R) 110. This substitution was found in the isoform 3 of *C. crangon* but not in the isoforms 1 and 2. The isoform 2 contained another mutation in the P2 motif, where tyrosine (Y) was substituted by a phenylalanine (F) 132 (numbers according to *Penaeus monodon* sequence). Isoform 1 did not show any mutation in P2 motif (Fig. 3).

A cladogram was created with the FABP amino acid sequences from the midgut gland of *C. crangon* and several other FABPs reported in the NCBI data base (Table 3). We obtained two main branches, one with *Homo sapiens* sequences and the other with crustacean sequences. Separate branches represent the insects *Drosophila melanogaster* and *Anopheles gambiae* and the same branch contains the insects *Schistocerca gregaria* and *Locusta migratoria*. Within the crustacean branch, the cladogram shows that the isoforms 2 and 3 of *C. crangon* are closely related. Isoform 1 of *C. crangon* is more closely related to *Pacifastacus leniusculus* than to the other two isoforms of *C. crangon*. Interestingly the shrimp *P. monodon* branch is closer to the insects than to the crustaceans (Fig. 4).

3.5. 3D structure of C. crangon FABPs

3D structures of the three isoforms of FABPs found in the midgut gland of *C. crangon* are shown in Fig. 5a. Isoform 1 possesses the three binding residues characteristic of the FABP family (Arg110, Arg130, Tyr132) (number based on *P. monodon* sequence). In the isoform 2 the tyrosine 132 (Y) was substituted by a phenylalanine (F). Isoform 3 has a valine (V) residue instead of arginine 110 (R). The three isoforms present the ten anti-parallel β strands forming a β-barrel and helix-turn-helix motifs characteristic of FABPs (Esteves and Ehrlich, 2006).

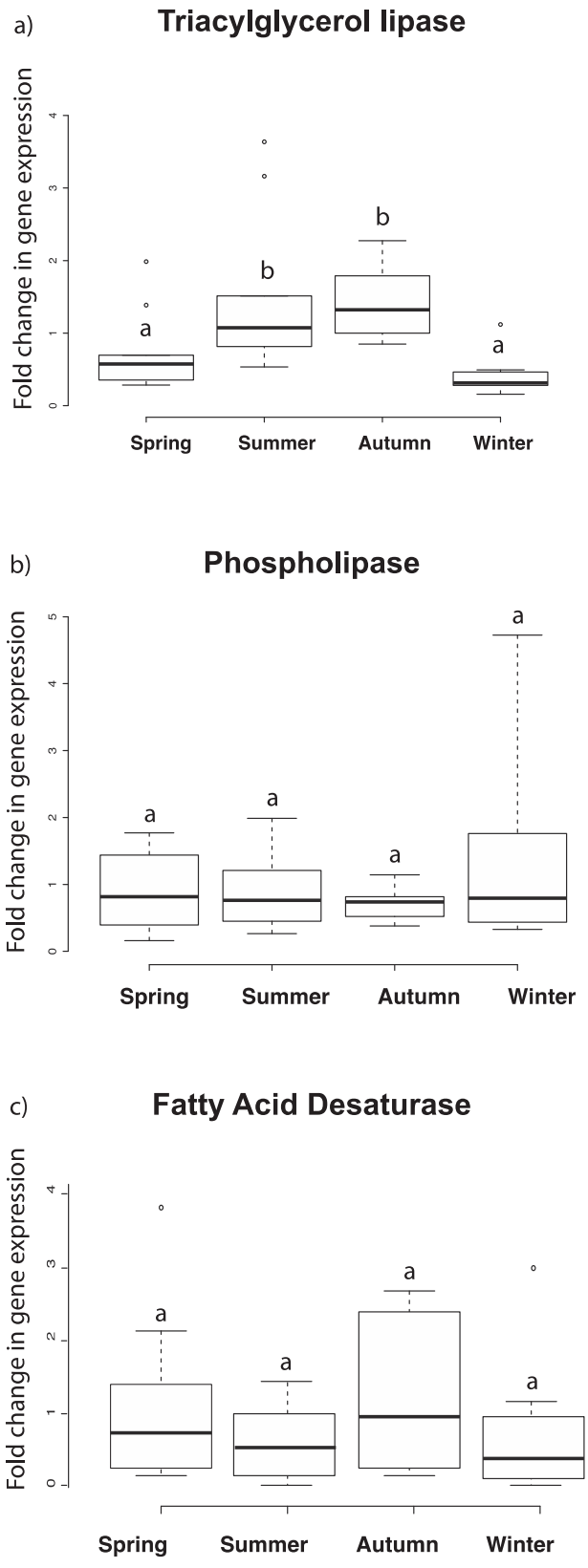

Fig. 1. *Crangon crangon* midgut glands: Seasonal expression of digestive enzymes involved in lipid metabolism (mean ± SD, n = 10). Different letters above bars indicate significantly different values.

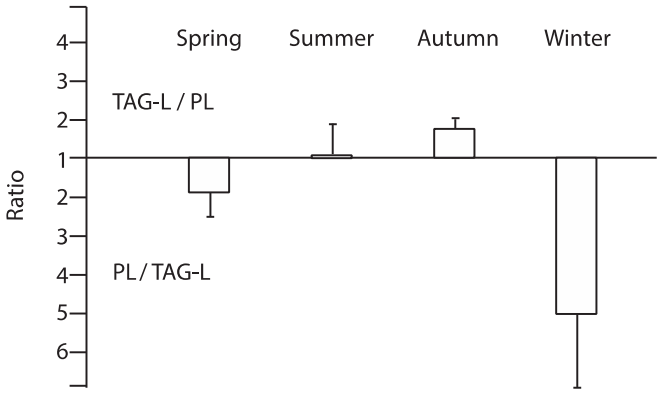

Fig. 2. *Crangon crangon* midgut glands: Seasonal ratio of gene expression of phospholipase (PL) and triacylglycerol lipase (TAG-L).

Table 5
Correlation matrix of gene expressions of digestive enzymes in midgut glands of *Crangon crangon:* triacylglycerol lipase (TAG lipase), alpha amylase (α-amy), fatty acid desaturase (FAD), phospholipase (PL), trypsin (Try), cathepsin L (CatL), and chitinase (Chit). *P* values in parentheses.

	α-amy	FAD	PL	Try	CatL	Chit
TAG lipase	0.21 (0.22)	0.05 (0.80)	−0.17 (0.31)	0.38 (0.02)	0.52 (0.00)	0.53 (0.00)
α-amy		0.23 (0.17)	−0.20 (0.24)	0.32 (0.05)	0.38 (0.02)	0.44 (0.01)
FAD			−0.29 (0.09)	0.05 (0.78)	0.04 (0.81)	0.15 (0.39)
PL				0.01 (0.97)	−0.13 (0.47)	−0.31 (0.07)
Try					0.64 (0.00)	0.14 (0.43)
CatL						0.26 (0.12)
Chit						

3.6. Enzymes and proteins involved in lipid metabolism

Proteins involved in lipid metabolism found in the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al., 2019a) are listed in Table 6. We focused on key proteins involved in lipid catabolism, anabolism, and transport (see Fig. 6).

Lipophorin (LPP), which transports lipids in the intracellular space to other organs, showed the highest number of transcripts, followed by glycerol-3-phosphate acyltransferase (GPAT), which participates in the synthesis of triacylglycerols, and phospholipase A_2 (PLA_2) that is involved in the catabolism of phospholipids. Low numbers of transcripts of other enzymes that are involved in the last steps of triacylglycerol synthesis were found: two transcripts of phosphatidic acid phosphohydrolase (PAP) and only one transcript of the acylglycerophosphate acyltransferase (AGPAT). Finally, we found nine transcripts of diacylglycerol acyltransferase (DGAT), a key enzyme involved in the last step of TAG synthesis (Table 6).

4. Discussion

4.1. Seasonal gene expression of digestive lipases

Triacylglycerol (TAG) lipases hydrolyze dietary triacylglycerol (Watt and Steinberg, 2008). The expression of TAG lipase in the midgut gland of *C. crangon* followed a distinct seasonal cycle. The up-regulation of this enzyme from spring over summer to autumn parallels the seasonal cycle of primary and secondary production. Phytoplankton production in the southern North Sea starts with a spring bloom between March and June and continues at a lower level until autumn (Wiltshire et al., 2015). Hence, the expression of TAG lipases apparently reflects the utilization of dietary TAG during the productive period. Concomitantly, total lipid levels in the midgut glands increased towards autumn (Martínez-Alarcón et al., 2019b), supporting this assumption. Moreover, expression of TAG lipase was positively correlated with the expression of chitinase, especially during spring ($r^2 = 0.66$) and summer ($r^2 = 0.81$), and with the proteinase cathepsin L ($r^2 = 0.52$), indicating that *C. crangon* is capable of utilizing chitin and protein from prey organisms such as smaller crustaceans and polychaetes (Plagmann, 1939; Pihl and Rosenberg, 1984). The high correlation of the gene expression between the two analyzed proteases (trypsin and cathepsin L) suggests that both enzymes are co-expressed.

Gene expression of phospholipase A_2, in contrast, did not show a clear seasonal pattern and it did not correlate with productivity, since expression was higher in winter than in other seasons. Phospholipases A_2 form a superfamily of enzymes that release free fatty acids by hydrolysis of phospholipids at the sn-2 position (reviewed by Dennis et al., 2011). Phospholipids are the main components of biomembranes and therefore, important in maintaining cell integrity. Phospholipases contribute to the intracellular and extracellular hydrolysis of phospholipids (Leslie, 2015; Yamamoto et al., 2017). Our results showed a slight up-regulation of phospholipase gene expression during winter. This increase is, however, unlikely to be causally linked to more intense feeding activities, since neither lipid content nor trophic marker indices increased during winter (Martínez-Alarcón et al., 2019b). Likewise, no up-regulation of gene expression of other digestive enzymes was observed.

The midgut gland is considered as the major metabolic and storage organ in crustaceans (Vogt, 2019). It accounts on average for about 5% of body mass, but may even reach 8–10% in well-fed and lipid-rich species, and decrease to about 2% in starving species. Accordingly, this parameter, usually expressed as midgut gland index or hepatosomatic index ($HSI = m_{MGG} \cdot 100 / m_{total}$) (m = mass), is often used as an indicator of the nutritional status of an animal (e.g. Chu, 1999; Sánchez-Paz et al., 2007). Previous studies on the feeding ecology and physiology of *C. crangon* showed that the HSI of freshly caught specimens from summer and autumn ranged between 4 and 5. Acute starvation for ten days caused a rapid decrease of the HSI to half of the initial values. Simultaneously, the total lipid content decreased from 15% to 5%DM (Pöhlmann, pers. comm.). Accordingly, upon starvation these shrimp utilize a significant fraction of their midgut gland, which contains only minor lipid reserves in the form of TAG, but plenty of polar lipids as components of biomembranes (Martínez-Alarcón et al., 2019b).

Phospholipase A_2 enzymes may in fact contribute to the metabolic utilization of phospholipids in the midgut gland during periods of food scarcity. The transcriptome of *C. crangon* shows at least twelve putative isoforms of phospholipase A_2 (Martínez-Alarcón et al., 2019a). The most frequent isoform is the calcium-independent phospholipase A_2, which may play a major role in membrane phospholipid remodeling (Murakami and Kudo, 2002). This suggestion is supported by the seasonal course of the gene expression ratios between triacylglycerol lipases and phospholipases (Fig. 2). During the productive seasons, the relative expression of TAG lipases is higher than that of phospholipases, indicating the primary utilization of dietary triacylglycerols. In early spring and particularly in winter, when food is scarce, expression of phospholipases seems to favor utilization of intracellular phospholipids. Between November and April, up to 75% of brown shrimp showed signs of starvation (Hufnagl et al., 2010). The degradation of midgut gland biomembranes is unlikely as rapid and drastic in nature as reported above from the acute starvation experiment. The shrimp will not entirely run out of food and the low winter temperatures will strongly reduce metabolic rates and, thus, the steady energy demand. A minimum level of polar lipids (ca. 2–3%DM) is essential to maintain the physiological function of the biomembranes, cells, and the organ. Besides the midgut gland, other tissues like the large abdominal muscle assemblage may provide phospholipids for energetic purposes.

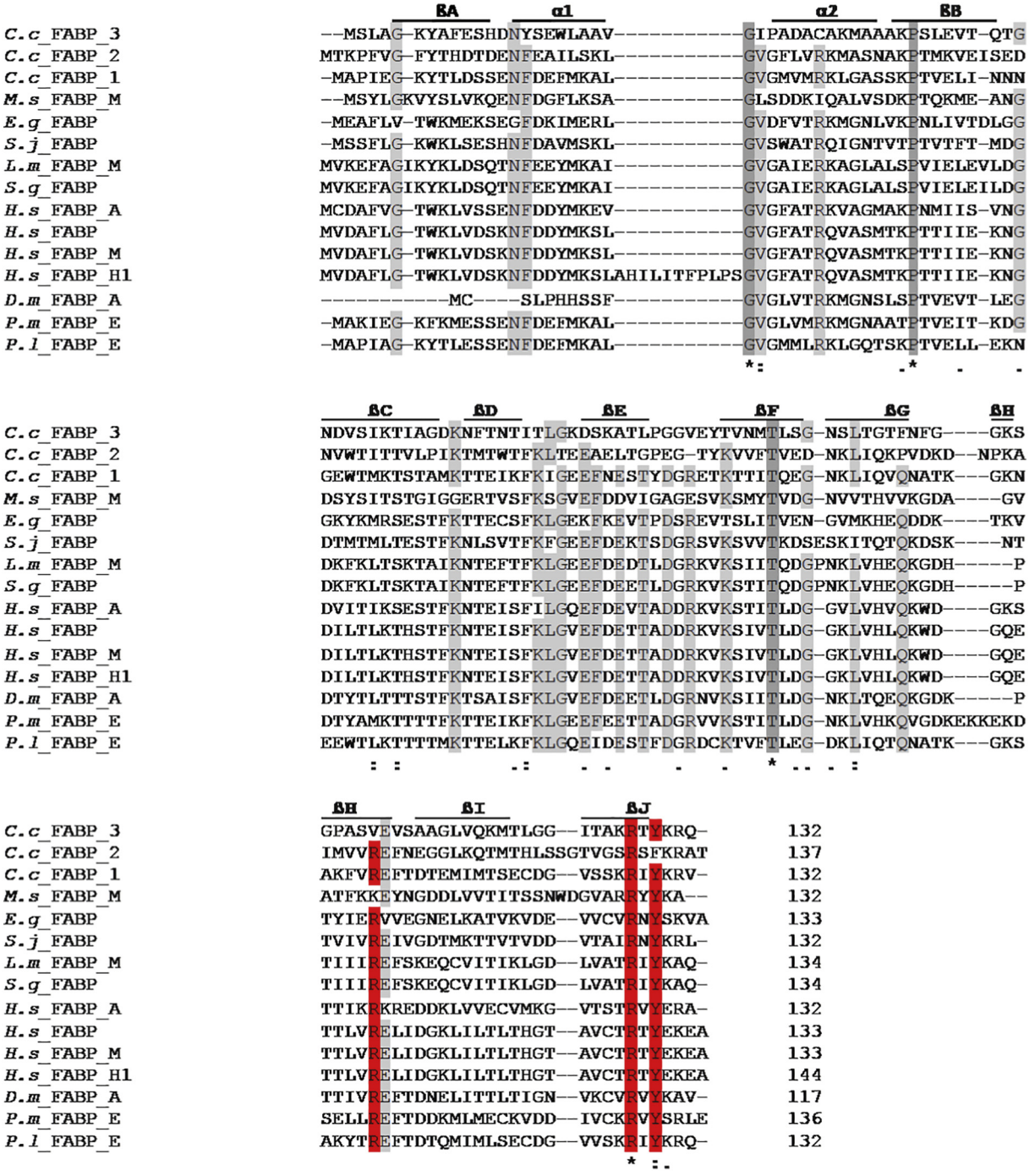

Fig. 3. Alignment of FABP sequences. Residues that are conserved 100% are highlighted in dark grey and residues conserved at least 75% in clear grey. The residues highlighted in red represent the three amino-acid residues (Arg110, Arg130, Tyr132) (numbers according to *Penaeus monodon* sequences) of the P2 motif characteristic of FABPs. The following sequences were aligned: *Crangon crangon* FABP isoforms 1–3 (*C.c*_FABP_1, *C.c*_FABP_2, *C.c*_FABP_3), *Schistosoma japonicum* FABP (*S.j*_FABP), *Locusta migratoria* FABP (*L.m*_FABP), *Drosophila melanogaster* FABP isoform A (*D.m*_FABP_A), *Homo sapiens* FABP from adipocyte (*H.s*_FABP_A), *Homo sapiens* FABP from muscle (*H.s*_FABP), *Homo sapiens* FABP from muscle (*H.s*_FABP_M), *Homo sapiens* FABP from heart isoform 1 (*H.s*_FABP_H1), *Echinococcus granulosus* FABP (*E.g*_FABP), *Anopheles gambiae* FABP (*A.g*_FABP), *Schistocerca gregaria* FABP (*S.g*_FABP), *Penaeus monodon* FABP from hemocytes (*P.m*_FABP_E), and *Pacifastacus leniusculus* FABP from hemocytes (*P.l*_FABP_E). Secondary structures are indicated: α, α helix and β, β strands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, the utilization of phospholipids appears to be an emergency strategy after most TAG has been depleted. This view agrees with a recently proposed concept considering plasma membranes as capacitors for energy and metabolism (Ray et al., 2016). Membrane compounds may act as storage molecules that can be utilized under stress conditions, providing energy, signaling molecules, and various metabolites. So far, only polar euphausiids, e.g. Antarctic krill *Euphausia superba*, are known to accumulate significant amounts of phospholipids (in the form of phosphatidylcholine) as energy reserves to buffer the pronounced seasonality in food supply in polar oceans (Hagen et al., 1996, 2001).

Fatty acid desaturases (FAD) are involved in the anabolism of lipids. They introduce double bonds into acyl chains, thus generating unsaturated fatty acids (Los and Murata, 1998). We found a positive correlation between the expression of FAD and TAG lipases, especially

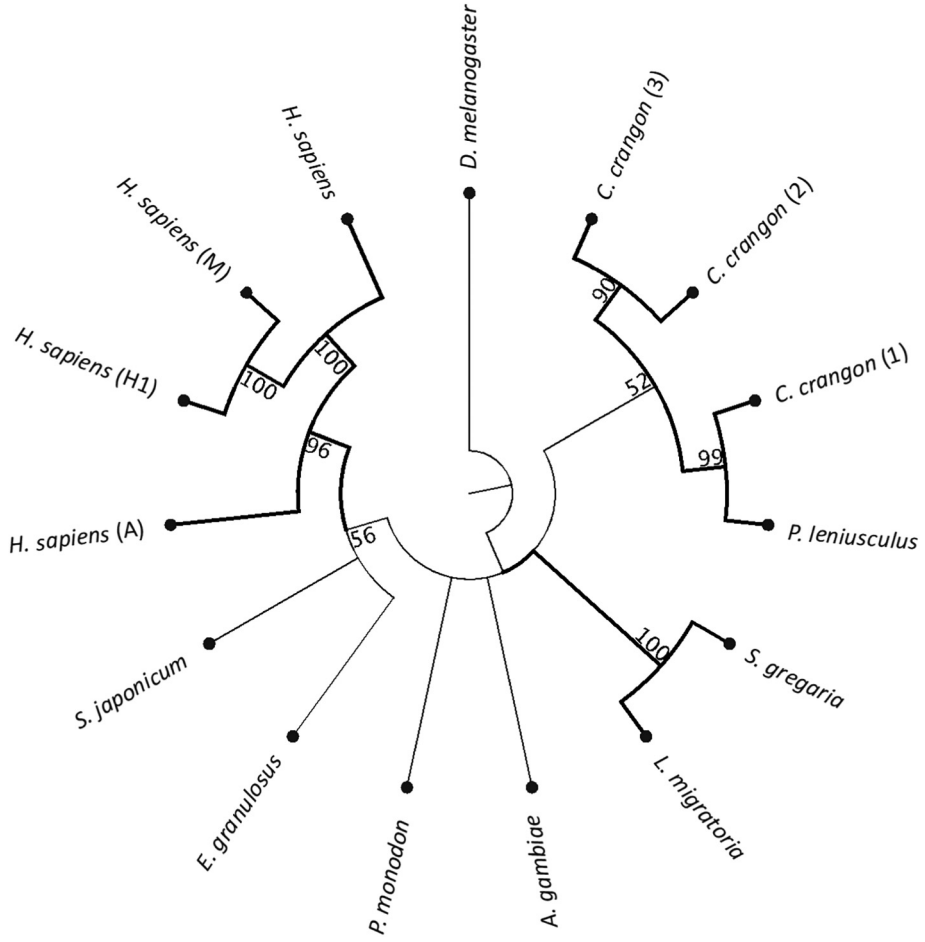

Fig. 4. Maximum likelihood cladogram of amino-acid sequences of fatty acid binding proteins of *Crangon crangon* (isoforms 1 to 3) and FABPs of other taxa from NCBI data. Bootstrap threshold was set at > 50%, values are presented at the nodes and well supported bootstraps (> 70%) are also highlighted. For species names refer to Table 3.

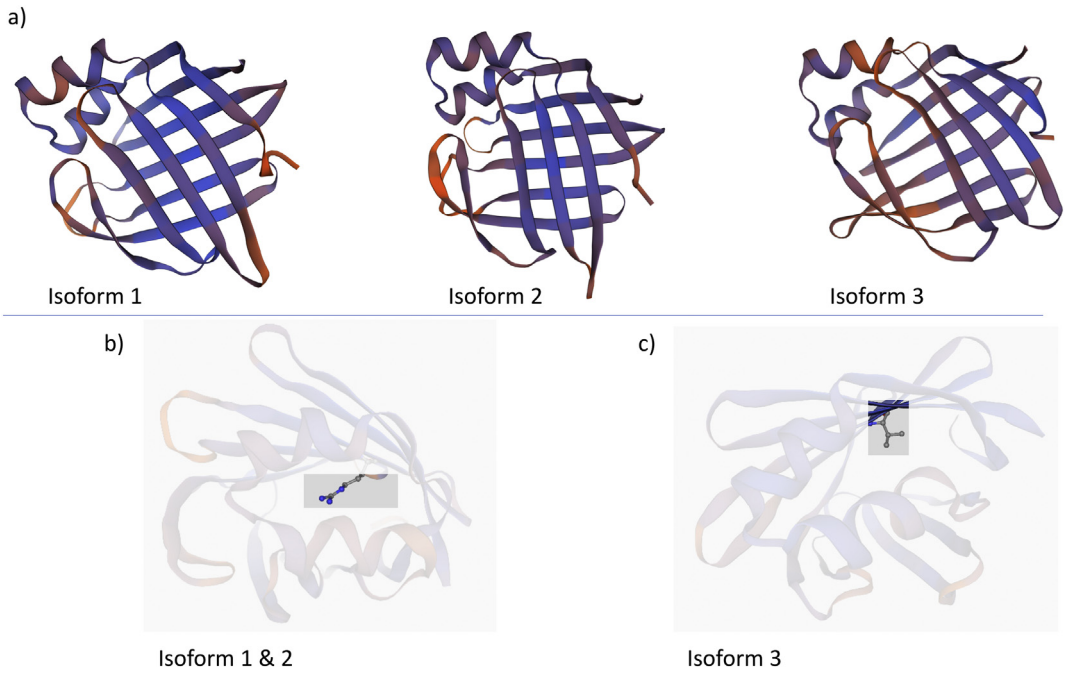

Fig. 5. 3D structure of the three FABP isoforms from the midgut gland of *Crangon crangon*, all of them present the α helix and β strands characteristic of FABPs (a). Representation of the arginine residue characteristic of the FABP family, which is present in isoforms 1 and 2 (b). Representation of the valine residue, which substitutes the arginine residue in the isoform 3 of *Crangon crangon* (c).

Table 6
Proteins involved in lipid metabolism and the numbers of their transcripts found in the transcriptome of *Crangon crangon* midgut glands (Martínez-Alarcón et al., 2019a).

	Name of protein	Abbreviation	Number of transcripts (n)
Transport	Lipophorin	LPP	31
	Microsomal triacylglycerol transfer protein	MTP	4
	Fatty acid binding protein	FABP	18
	Non-specific lipid-transfer proteins	LTP	4
Anabolism	Glycerol-3-phosphate acyltransferase	GPAT	22
	Acylglycerophosphate acyltransferase	AGPAT	1
	Phosphatidic acid phosphohydrolase	PAP	2
	Diacylglycerol acyltransferase	DGAT	9
Catabolism	Triacylglycerol lipase	TAG lipase	14
	Phospholipase A2	PLA_2	20

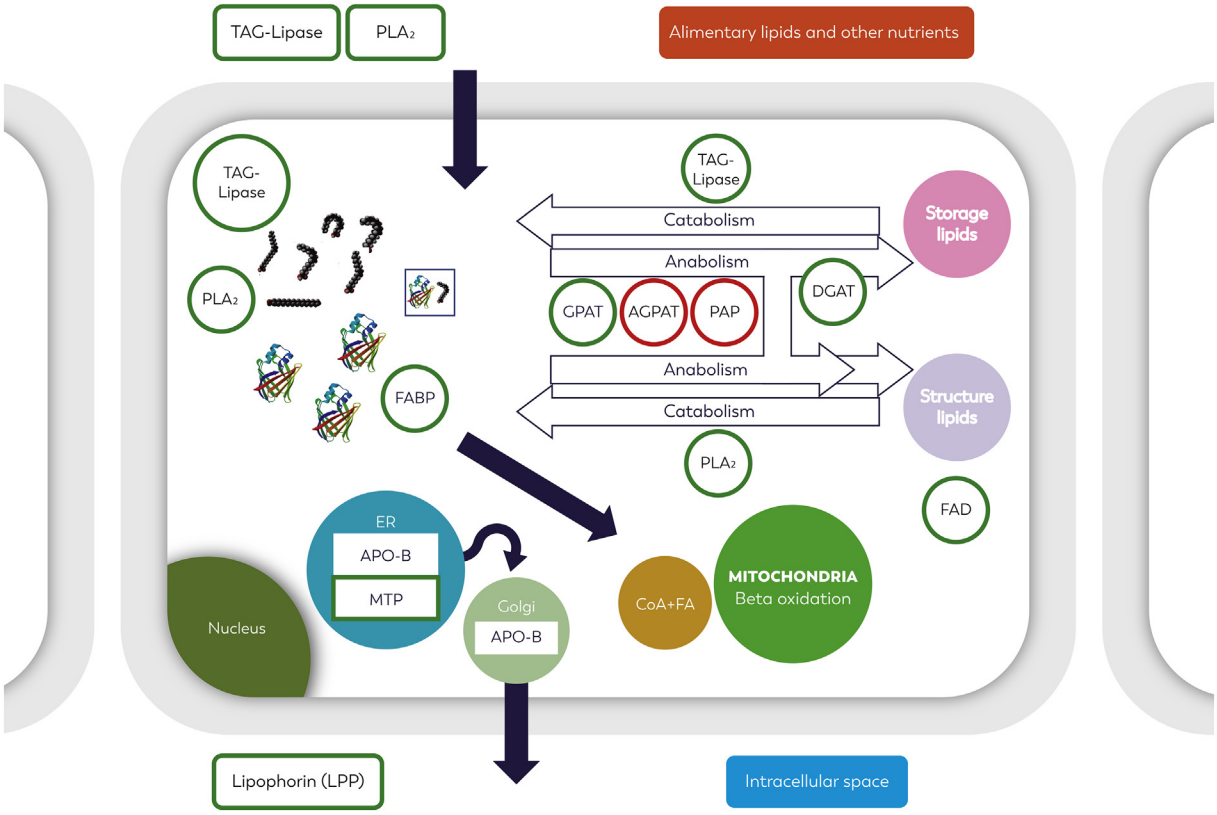

Fig. 6. Overview of relevant pathways and processes of lipid metabolism in the midgut gland of *Crangon crangon.* Proteins and enzymes found in the transcriptome of *C. crangon* are shown in green circles. Proteins found in very low numbers in the transcriptome of *C. crangon* are shown in red circles. LPP (Lipophorin), FABP (fatty acid binding protein), TAG lipase (triacylglycerol lipase), PLA_2 (phospholipase A_2), FAD (fatty acid desaturase), APO-B (apolipoprotein B), GPAT (glycerol-3-phosphate acyltransferase), AGPAT (acylglycerophosphate acyltransferase), PAP (phosphatidic acid phosphohydrolase), DGAT (diacylglycerol acyltransferase), MTP (microsomal triacylglycerol transfer protein). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during autumn and winter, but a negative correlation with the expression of phospholipase A_2. These results suggest that in autumn with still sufficient food availability, the midgut gland of *C. crangon* up-regulated the expression of triacylglycerol lipase to hydrolyze dietary TAG, and simultaneously, up-regulated the fatty acid desaturase. This enzyme is essential for the modulation and proper functioning of biomembranes, which are very rich in 20:5(n-3) and 22:6(n-3) polyunsaturated fatty acids (Lee et al., 2006). Fatty acid desaturases provide membranes with the necessary fluidity by introducing double bonds into the fatty acids (Los and Murata, 1998). In winter, when food becomes scarce, the midgut gland down-regulates triacylglycerol lipase and fatty acid desaturase and, instead, up-regulates the phospholipase A_2, which supports the utilization of fatty acids from biomembrane-derived phospholipids.

4.2. Cellular transport of lipids

The cytosolic fatty acid binding proteins (FABPs) bind non-covalently to hydrophobic ligands, mainly fatty acids, and facilitate their intracellular transport (Esteves and Ehrlich, 2006). Although FABPs show variable sequences, all of them have a conserved tertiary structure. This comprises ten anti-parallel β-strands forming a β-barrel and helix-turn-helix motifs, thus forming the binding cavity for the ligands (Esteves and Ehrlich, 2006). A triad of amino acids that are involved in the binding with the ligand remains is, however, highly conserved.

Few FABPs from invertebrates have been reported so far, showing low sequence similarities among each other (Esteves and Ehrlich, 2006). Information about crustacean FABPs is even scarcer. In the crayfish *Pacifastacus leniusculus* and the shrimp *Penaeus monodon*, two

FABPs from hemocytes were isolated and characterized. Both proteins show the binding triad residues involved in the interaction with the ligand (Söderhäll et al., 2006). We found at least three main FABP isoforms in *C. crangon*. All three *C. crangon* FABPs possess the characteristic ten anti-parallel β-strands forming a β-barrel and two α-helices. Surprisingly, two of these three isoforms in *C. crangon* show a substitution in one of the three typically conserved amino-acid residues of the P2 motif characteristic of RA-binding proteins and FABPs (Söderhäll et al., 2006). Since two of the three isoforms show mutations in one of those amino acids, the interaction with the ligands, i.e. fatty acids, may be hampered and, thus, lipid metabolism impaired.

4.3. Lipid anabolism and TAG storage

Alimentary lipids are digested by gastric TAG lipases, phospholipases, other unspecific digestive lipases, and esterases (Saborowski, 2015). The resulting fatty acids and glycerides interact with membrane proteins, which facilitate their vesicular or non-vesicular transfer into the cell. Once inside the cell, the molecules can follow different pathways, depending on the physiological requirements of the organism. Metabolic energy is gained from fatty acids via β-oxidation. Other products, such as glycerol, phosphate, choline or ethanolamine contribute to the cellular pool of metabolites. The anabolic formation of TAGs as storage lipids follows the Kennedy pathway (Kennedy and Weiss, 1956), which is the most important route to TAG synthesis (see Fig. 6).

In this pathway, the enzymes glycerol-3-phosphate acyltransferase (GPAT), acylglycerophosphate acyltransferase (AGPAT), phosphatidic acid phosphohydrolase (PAP) and diacylglycerol acyltransferase (DGAT) successively facilitate the synthesis of diacylglycerols (DAGs) and TAGs. The DAGs may be modified via the Lands cycle (Lands, 1958) and incorporated into cell membranes. The TAGs may be transported with the help of FABPs or LTPs and stored as lipid droplets. On demand, these lipid depots can be catabolized by phospholipases and TAG lipases, respectively.

The transcriptome data of *C. crangon* includes 22 transcripts of GPAT, the enzyme catalysing the first step of the Kennedy pathway forming a monoacylglycerol. AGPAT, the enzyme for the second step, which is the formation of phosphatidic acid, a key intermediate in the biosynthesis of glycerolipids, is present with only one transcript. The enzyme for the next step (PAP), which participated in the dephosphorylation of phosphatidic acid and formation of a diacylglycerol, appears with only two transcripts. DGAT is the key enzyme in the biosynthesis of TAGs (Yen et al., 2008; Turchetto-Zolet et al., 2011). No alternative pathways are known.

The transcriptome obtained by Martínez-Alarcón et al., (2019a) contains nine transcripts of DGAT. The alignment of the deduced amino acid sequences shows low similarity (< 50%) with primates and chelicerates and slightly higher similarity (66%) with the shrimp *Penaeus vannamei*. However, the obtained sequences are short and probably not suitable for an appropriate comparison.

In marine phytoplankton, it has been proposed that during the final step in storage lipid biosynthesis (the formation of TAG), the diacylglycerol (DAG), which is a precursor of TAG, PL and GL, may follow PL synthesis to membrane lipids instead of TAG synthesis (Jónasdóttir, 2019). If this is also the case in *C. crangon*, it would provide a reason for the low storage lipid (TAG) but high PL levels (Martínez-Alarcón et al., 2019b). It would, however, not explain the high number of transcripts of DGAT responsible for TAG synthesis (Table 6). Therefore, further analyses of the functionality of the DGAT sequences are required. Mutations that hamper functionality, as shown for the FABPs, would explain, why DAGs are directed towards phospholipid synthesis instead of TAG synthesis.

5. Conclusion

Gene expression of digestive enzymes reflects the seasonal food availability and feeding activity of *C. crangon*. Elevated expression of phospholipase enzymes indicates utilization of intracellular lipid reserves, in this case polar lipids of biomembranes, which may fulfill an extra function as energy storage compounds besides their structural function. The limited ability of *C. crangon* to store TAG remains unexplained. However, here we hypothesize that the mutation on the FABP binding site could have some consequences for intracellular lipid transport and lipid storage. Furthermore, we found the relevant transcripts of those enzymes facilitating the respective anabolic pathways, i.e. the so-called Kennedy pathway. However, the number of transcripts was low compared to other enzymes and the functionality of the enzymes could not be evaluated due to limited bioinformatics and database entries and the small size of the sequences. Future research will aim at elucidating the sequences, structures, and functionalities of the Kennedy pathway enzymes of *C. crangon* and other crustaceans with special focus on the key enzyme DGAT.

Data accessibility

The sequence data have been submitted to the GenBank databases under the following accession numbers: Fatty acid-binding protein, isoform 1 (**MT005540**), Fatty acid binding protein, isoform 2 (**MT005541**), Fatty acid binding protein, isoform 3 (**MT005542**).

Author contributions

The concept of the study was elaborated by DMA, RS and WH. DMA performed the molecular analyses under the advice of CH and RS. Data analysis and interpretation of the results were done by DMA and RS. DMA drafted the manuscript and RS assisted during the entire process. All co-authors participated in the revisions of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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