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Research paper

Lipid metabolism in *Rhodnius prolixus*: Lessons from the genome

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ABSTRACT

The kissing bug *Rhodnius prolixus* is both an important vector of Chagas' disease and an interesting model for investigation into the field of physiology, including lipid metabolism. The publication of this insect genome will bring a huge amount of new molecular biology data to be used in future experiments. Although this work represents a promising scenario, a preliminary analysis of the sequence data is necessary to identify and annotate the genes involved in lipid metabolism. Here, we used bioinformatics tools and gene expression analysis to explore genes from different genes families and pathways, including genes for fat breakdown, as lipases and phospholipases, and enzymes from β-oxidation, fatty acid metabolism, and acyl-CoA and glycerolipid synthesis. The *R. prolixus* genome encodes 31 putative lipase genes, including 21 neutral lipases and 5 acid lipases. The expression profiles of some of these genes were analyzed. We were able to identify nine phospholipase A2 genes. A variety of gene families that participate in fatty acid synthesis and modification were studied, including fatty acid synthase, elongase, desaturase and reductase. Concerning the synthesis of glycerolipids, we found a second isoform of glycerol-3-phosphate acyltransferase that was ubiquitously expressed throughout the organs. Finally, all genes involved in fatty acid β-oxidation were identified, but not a long-chain acyl-CoA dehydrogenase. These results provide fundamental data to be used in future research on insect lipid metabolism and its possible relevance to Chagas' disease transmission.

1. Introduction

Recent estimates by the World Health Organization indicate that more than 1 billion people are infected each year with pathogens transmitted by vectors and that over 3.5 billion occupy areas of risk (WHO, 2015). These diseases are among the most prevalent in tropical and subtropical areas.

In Latin America, attention has historically been focused on Chagas' disease, or American trypanosomiasis. Currently, 18 million people are infected with *Trypanosoma cruzi,* and another 40 million reside in hazardous areas. Over 80% of its transmission is attributed to insect vectors from the Triatominae family and to contamination by blood transfusions (Costa and Lorenzo, 2009).

Strategies to control these diseases vary according to the levels of endemicity, magnitude and distribution of potential insect vectors. Studies in fields associated with controlling the transmission of parasites, which involve vector control, drug development, and genetic and immunological approaches, are limited because the molecular aspects of parasite development within the vertebrate hosts and insect vectors are poorly understood and remain fragmented. For this reason, new data on basic vector and parasite biochemistry is crucial. To achieve this goal, our knowledge of the metabolic pathways of vectors is an aspect of research to be expanded. The publication of the kissing bug *Rhodnius prolixus* genome is an excellent opportunity to deepen our investigation into an insect model for which biochemical information is relatively abundant but molecular knowledge has remained incipient.

Lipid metabolism plays a crucial role in insects. As in other organisms, lipids perform various functions in insects, such as forming constituents of cellular structures, acting as signaling messengers, and serving as the most significant form of stored energy. These lipid reserves are fundamental in certain situations of high metabolic demand,

Abbreviations: 4CL, 4-coumarate:CoA ligase; ACS, acyl-CoA synthetases; ACSBG, ACS bubblegum; ACSL, ACS long-chain; ACSM, ACS medium-chain; ACSS, ACS short-chain; ACSVL, ACS very long-chain; Bmm, brummer; iPLA2, calcium-independent PLA2; CPT, carnitine palmitoyltransferase; cPLA2, cytosolic calcium-dependent PLA2; DAG, diacylglycerol; FAD, fatty acid desaturase; ELOVL, fatty acid elongase; FAS, fatty acid synthase; FAR, fatty acyl-CoA reductase; FA, free fatty acids; G3P, glycerol-3-phosphate; GPAT, G3P acyltransferase; HSL, hormone-sensitive lipase; PLA2, phospholipase A2; PL, phospholipid; PAF-AH, platelet activating factor acetylhydrolase; sPLA2, secreted PLA2; TAG, triglyceride; VLCFA, very long-chain fatty acids.

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such as flight and egg production (Arrese et al., 2001). Also, the cuticle lipids, a complex mixture of hydrocarbons, wax esters and acylglycerols, play an essential role in insect fitness, both as a waterproof barrier and a means to regulate the penetration of pesticides and microorganisms; moreover, they also participate in chemical communication events (Juárez and Calderón-Fernandez, 2007).

In insects, as in other arthropods, nutrients obtained by digestion are used for a variety of processes, such as molting, locomotion and oogenesis. In *R. prolixus* adult females, for example, approximately 40 eggs are produced after each blood meal, representing a huge metabolic demand. During digestion in the intestinal lumen, dietary complex lipids, such as triglycerides (TAGs) and phospholipids (PLs), are hydrolyzed, and the released free fatty acids (FA) are absorbed by the intestinal epithelium (Canavoso et al., 2001). TAG-lipase activity and fatty acid absorption by midgut cells were already described in *R. prolixus* (Grillo et al., 2007). These fatty acids are used as a substrate to synthesize complex lipids, as diacylglycerol (DAG), TAG and PL. In the kissing bug, DAG and TAG are produced by the glycerol-3-phosphate (G3P) pathway, and the genes encoding the enzymes of this pathway have been characterized (Alves-Bezerra and Gondim, 2012). The lipids obtained during digestion are transferred to lipophorin, a major hemolymphatic lipoprotein in insects (Atella et al., 1995). The lipid-loaded lipophorin then interacts with the fat body (Pontes et al., 2008) and the ovaries (Santos et al., 2011), and transfers lipids to be stored or used for membrane synthesis. Lipophorin binds to a specific receptor on the cell surface (Entringer et al., 2013; Grillo et al., 2003; Pontes et al., 2002); however, the identity of this receptor remains unknown. The dynamics of TAG mobilization was also investigated in the fat body (Pontes et al., 2008) and in oocytes (Santos et al., 2011) of *R. prolixus*.

In addition to the insect fat body, a major role in lipid biosynthesis has long been proposed for the oenocytes localized in the integument of *R. prolixus* (Wigglesworth, 1933, 1975). It has now been established that interplay between the fat body and oenocytes is essential to regulate lipid metabolism in insects; the oenocytes also regulate growth, development and feeding behavior (Gutierrez et al., 2007).

However, other parts of kissing bug lipid metabolism remain nearly unknown. There is no information available on *de novo* fatty acid synthesis and degradation through β-oxidation or on the regulation of these pathways; fatty acid metabolism, including elongation and desaturation, has not been described, and the genes encoding lipases and phospholipase are unknown.

Despite the availability of information on *R. prolixus* genes provided by VectorBase (Megy et al., 2012), a complete analysis of gene function regarding the vector lipid metabolism is not yet available.

In this study, we describe the annotation, homology analysis and expression profile of genes associated with lipid metabolism pathways, including the lipase and phospholipase families, fatty acid metabolism, acyl-CoA and glycerolipid synthesis, and β-oxidation. The possible functions of these genes are discussed. It is possible that these new data will be used in the future to predict candidate genes that can be manipulated to prevent vectorial transmission of Chagas' disease.

2. Material and methods

2.1. Ethical statement

All animal care and experimental protocols were conducted following the guidelines of the institutional animal care and use committee (Committee for Evaluation of Animal Use for Research from the Universidade Federal do Rio de Janeiro, CEUA-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by CEUA-UFRJ. The technicians at the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) conducted all aspects of rabbit husbandry under strict guidelines to ensure the careful and consistent handling of animals.

2.2. Insects

The insects were kept in a colony at 28 °C and relative humidity of 70–75%, at the Universidade Federal do Rio de Janeiro. Experiments were performed using adult females or males on the third feeding cycle as adults, after being mated and fed rabbit blood at intervals of three weeks. According to our experience, the insects at that age are metabolically stable, which reduces experimental variability and facilitates the analysis of the results.

2.3. Gene annotation

The contig sequences of the *R. prolixus* genome (RproC1 assembly) were obtained from VectorBase (Megy et al., 2012). The present work analyzed two preliminary gene annotation: (1) the RproC1.3 gene set (available at Vectorbase), and (2) RPAL – *Rhodnius prolixus* alternative annotation - gene set (Mesquita et al., 2015). Two strategies were used to identify genes in the *R. prolixus* preliminary genome annotation:

(1) Gene families were searched within the preliminary annotation using the software FAT (Seabra-Junior et al., 2011; Brazil patent 11083-6), with the consensus sequence of various domains from the Pfam database (Finn et al., 2014), which are described in Table S1. This program uses the indicated Pfam domains consensus sequences and searches the annotated protein database for proteins containing sequences similar to the Pfam family used. Thus, it is not necessary to use a protein sequence of a related insect as query, as in a search for Blast, in a first moment. This strategy was used to find genes that encode putative lipases, phospholipases, glicerolipids metabolism genes and beta-oxidation pathway genes. FAT software is available upon request. Alternatively, a second round of searches was performed using proteins of each studied family, from *Drosophila melanogaster*. CG7367 and Lipase 4 (CG6113) were chosen as prototypes of neutral lipase (Pfam motif PF00151) and acid lipase (Pfam motif PF04083) families, respectively. These proteins were obtained from the FlyBase database (McQuilton et al., 2012) and used as queries in a search against the *R. prolixus* genome using the TBlastN algorithm (Altschul et al., 1997). If an unannotated gene was found, the respective contig sequence was re-analyzed using the GeneWise algorithm (McWilliam et al., 2013) and the most similar protein sequence from the pea aphid *Acyrthosiphon pisum* (Richards et al., 2010), attempting to correct the preliminary annotation. These newly found genes do not have a VectorBase accession number, and they are described in the text as *SuperContigNumber_GeneFamilyandNumber*. These genes and proteins sequences will be published in a next genome annotation gene set at Vectorbase. They are available as support information (File S1).

(2) Alternatively, the genes were directly searched within the preliminary annotation using the BlastP algorithm (Altschul et al., 1997), with protein sequences from *D. melanogaster* and *A. pisum* usedas the queries. The proteins used were (GenBank accession numbers): AAF51148, EAA46042, NP_647613, XP_001945190, and XP_008187029, for fatty acid synthase search; NP_730843, XP_003240836, NP_732761, NP_648909, NP_001156725, NP_001280394, XP_008184040, and AAF54461, for fatty acid elongase (ELOVL) search; XP_001948947, NP_652731, NP_651781, NP_001156221, NP_651780, NP_001119674, NP_651779, and AAF52318, for fatty acid desaturase (FAD) search; and XP_008183884, NP_611143, XP_001948821, XP_001948060, NP_726498, NP_611140, XP_001949683, XP_001950244, NP_572276, and AAF56838, for fatty acid reductase (FAR) search.

2.4. Homology analysis

Protein sequences were aligned using ClustalW 2.0 (Larkin et al., 2007), and dendrograms were constructed using the maximum likelihood method (Felsenstein, 1981) with 500 bootstrap replications in MEGA 6.0 software (Tamura et al., 2013). The alignments used for the dendrograms are available as supplementary material (File S2).

2.5. Gene expression analysis

Insects were dissected on the fourth day after a blood meal. The obtained organs were washed in 0.15 M NaCl, homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and the total RNA was extracted according to the manufacturer's protocol. Total RNA concentrations were determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), and all RNA samples included in further analysis had an A260/A280 ratio between 1.8 and 2.0. RNA integrity was checked using native agarose gel electrophoresis. The RNA samples were considered intact when an 18S band was clearly observed. The band corresponding to 28S rRNA could not be identified because of the 'hidden break' present in insects (Ishikawa, 1977; Winnebeck et al., 2010). A 1 μg sample of RNA was treated with 1 U of RNase-free DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C in a final volume of 10 μl. The DNAse I reaction was stopped by the addition of 50 nmol of EDTA and incubation at 65 °C for 10 min. Then, the treated RNA was used to synthesize cDNA samples using M-MLV Reverse Transcriptase (Sigma-Aldrich) in a final reaction volume of 20 μl. Each reaction mixture contained 200 U of reverse transcriptase, and cDNA synthesis was carried out with random nonamers primers (Sigma-Aldrich). Reactions were incubated at 37 °C for 50 min. The PCR reactions were performed using PCR Master Mix (Thermo Fisher Scientific) and 3.0 pmol of each primer (Integrated DNA Technologies, Coralville, IA, USA). The primers used for each gene were designed using the Primer3 algorithm (Rozen and Skaletsky, 2000) and are shown in Table S2. We analyzed the expression of eight genes; the Brummer/ATGL lipase, five neutral lipases and one acidic lipase randomly chosen, and two glycerol-3-phosphate acyltransferase (GPAT). The temperature variation program included the following steps: 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; followed 72 °C for 10 min. *RpEF-1* gene (Majerowicz et al., 2011) amplification was used as a positive control. PCR products were then visualized using native agarose gel electrophoresis.

3. Results and discussion

3.1. Lipase genes

Lipases hydrolyze the ester links of neutral lipids and PLs and they act mainly at the hydrophilic-hydrophobic interface (Derewenda, 1994). These enzymes have many metabolic functions in insects, including dietary lipid digestion and lipid store mobilization (Canavoso et al., 2001). The number of lipase coding genes in the genome of analyzed insects varies from 26 in the honey bee *A. mellifera* to 56 in *D. melanogaster* (Horne et al., 2009). In the search on the *R. prolixus* genome, we were able to identify and annotate 31 lipase genes (Table S3). In order to infer the phylogenetic origin of the genes, we generated different dendrograms using *R. prolixus* and lipases sequences from other insects. The lipase sequences of the water flea *Daphnia pulex* were used as an outgroup. All the trees are available in the supplementary figures (neutral lipase family, Fig. S1; acidic lipase family, Fig. S2; lipase 3 family, Fig. S3; GDSL-like lipase family, Fig. S4; GDSL-like lipase 2 family, Fig. S5; patatin domain containing lipase, Fig. S6; and hormone-sensitive lipase family, Fig. S7). We chose to focus our discussion on the neutral and acidic lipases families, as they are the most numerous lipases families. However, the full dendrograms are too large to fit suitably on a page. In that way, we constructed smaller dendrograms with proteins sequences from *R. prolixus*, *D. melanogaster* and *A. pisum*. Fig. 1 shows the dendrogram of neutral lipases, while Fig. 2 shows the dendrogram of acid lipases. Our analyses and discussions were based in both dendrograms.

As was already observed (Horne et al., 2009), a considerable number of genes do not have a traceable common origin between insects and may have appeared through independent duplications. This phenomenon also seems to hold true in the comparison between *R. prolixus* and other insects (Figs. 1 and 2). However, certain genes had clear orthologues. For example, *RPRC000003* and *A. pisum ACYPI002250* are orthologues (Fig. 1). Similarly, *RPRC000246* and *D. melanogaster CG13282* are also orthologues (Fig. 1). Interestingly, the *Drosophila* gene is expressed in the crop (Chintapalli et al., 2007), and the *R. prolixus* gene is highly expressed in the posterior midgut (Table S3), according to a transcriptome published elsewhere (Ribeiro et al., 2014). A digestive lipase activity was characterized in the kissing bug gut (Grillo et al., 2007) and these results may indicate that *RPRC000246* is involved in the digestive process, although this hypothesis needs to be tested in the future. In another example, *RPRC000836* and *KQ034265_Lip2* are paralogous and closely related to *Drosophila CG7367*. However, this gene seems to have suffered a huge expansion in *A. pisum* genome and four of its neutral lipases clustered in this same branch (Fig. 1). Lastly, the *R. prolixus* genes *RPRC004324*, *RPAL003531*, *RPRC001451*, and *RPRC013099* are closely related and probably paralogous, but these duplication events may have happened after the rise of the Triatominae lineage, as no related gene is present in the aphid genome (Fig. 1).

Other phylogenetic relations can be drawn analyzing the dendrogram of the neutral lipase family and we can speculate about functions of *R. prolixus* lipases (Fig. 1 and Fig. S1). The *R. prolixus* gene *RPAL001874* has orthologues in all species (eight insects and one crustacean) included in the full dendrogram. However, data about their functions are scarce. Horne et al. (2009) hypothesized that the *Drosophila* orthologue *CG6847* may be a midgut intracellular TAG-lipase or a lipoprotein lipase involved in TAG mobilization from hemolymphatic lipophorin, present in the ovary membrane. We investigated *RPAL001874* gene expression in different tissues four days after meal and we could not detected its mRNA neither in the midgut or ovary, although the gene was expressed in the fat body, flight muscle, and testis (Fig. 3). The lower molecular weight amplicons seen in the midgut and ovary are due to unspecific amplification, probably primer dimers, as they can also be found in the PCR negative control, which did not contain cDNA sample (data not shown). This result indicated that RPAL001874 may not be involved in TAG metabolism in the gut or ovary and that its function needs to be investigated in the future.

We also analyzed the expression of *RPAL010098* gene by RT-PCR. This gene was expressed in the midgut and flight muscle, suggesting that this enzyme can be involved in the lipid digestion process. According to the neutral lipase dendrogram, *RPAL010098* has a paralogue, *RPRC006121* (Fig. 1). It is noteworthy that *RPRC006121* was also shown to be highly expressed in the posterior midgut (Ribeiro et al., 2014). The role of both lipases in the kissing bug lipid digestion is currently unknown and needs to be studied in the future.

The *R. prolixus* genes *RPRC001451* and *RPRC13099* are closely related and share a tree branch with genes from *A. pisum*, the body louse *Pediculus humanus*, *D. melanogaster* and *A. mellifera* (Fig. S1). Both *R. prolixus* genes showed a very low expression in the published transcriptome (Ribeiro et al., 2014). The *Drosophila* orthologue *GC10357* is moderately active in the adult fly head (Chintapalli et al., 2007). There is no data concerning this lipase functions, but only *R. prolixus* has two copies of it. It would be interesting to investigate if both *R. prolixus* genes are expressed in a similar profile and have the same role in metabolism or if they have already acquired different functions.

The *Drosophila* gene *CG5966* has orthologues in all species studied here. Moreover, it is noteworthy that this gene seems to have been duplicated in the termite *Zootermopsis nevadensis* (Fig. S1). Many data on the *CG5966* gene are available in the literature. This gene is highly expressed in the fat body (Chintapalli et al., 2007) and its activity is modulated by AMPK (Tohyama and Yamaguchi, 2010) and up-regulated upon starvation (Fujikawa et al., 2009; Grönke et al., 2005). These results indicate that this lipase may be involved in the mobilization of stored TAG in the fat body, probably working together with Brummer/ATGL lipase and hormone-sensitive lipase. However, the actual function of this protein has not been investigated. Due to the dynamic TAG metabolism in fat body (Pontes et al., 2008), *R. prolixus* can be a good

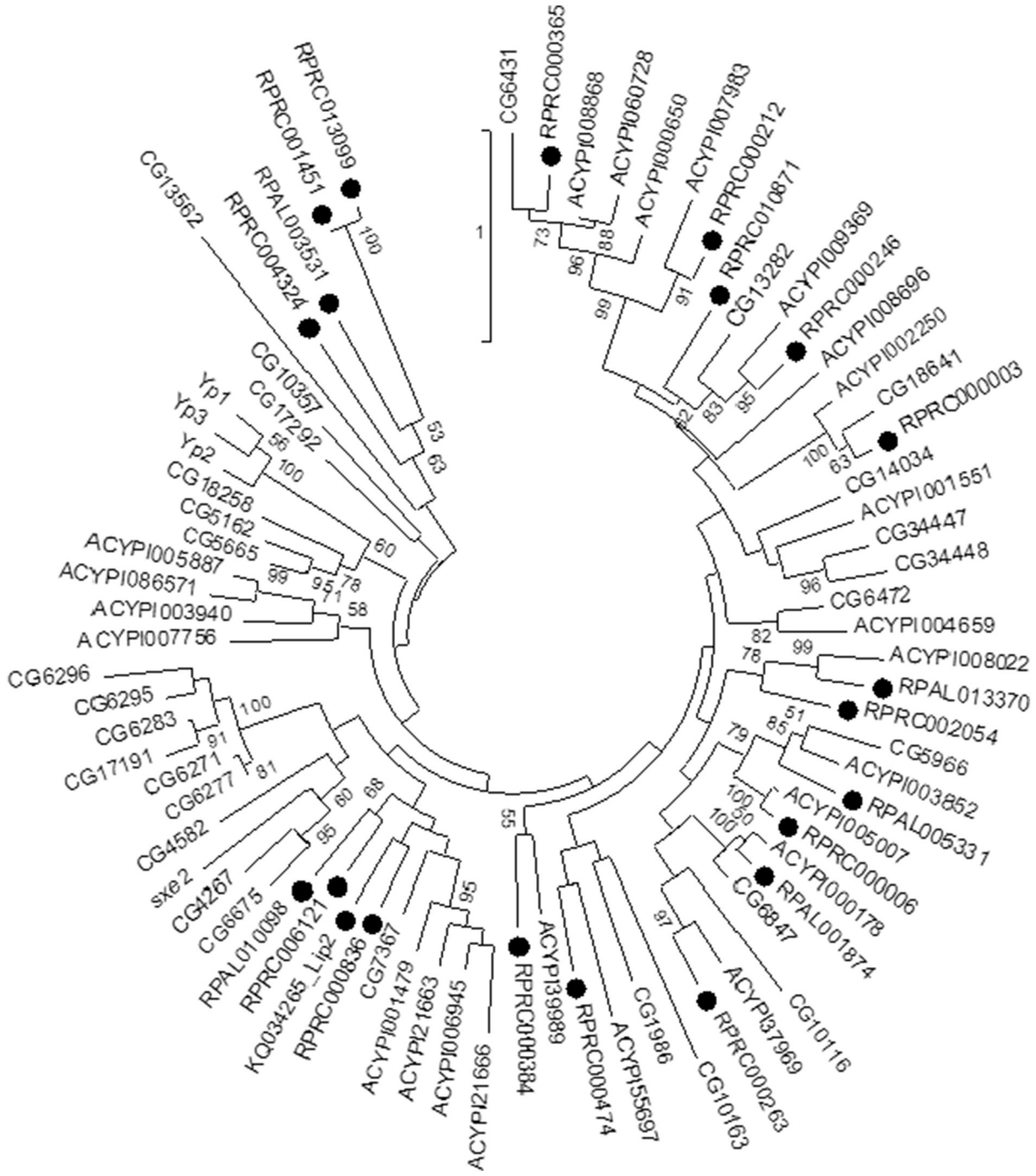


Fig. 1. Dendrogram tree comparing neutral lipases from *R. prolixus*, *D. melanogaster*, *and A. pisum*. Predicted protein sequences from *D. melanogaster*, *A. pisum*, and *R. prolixus* neutral lipases were aligned, and the dendrogram was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. *R. prolixus* genes are marked with black dots. Genes represented by names or codes starting with "CG" are from *D. melanogaster*. Codes starting with "ACYPI" represent genes from *A. pisum*.

model for experiments on the role of this lipase (*RPAL005331*). Unfortunately, we have no information about the level of expression of the gene in the published transcriptome (Ribeiro et al., 2014).

The gene *RPRC000006* was expressed in all analyzed organs (Fig. 3). This gene clustered in a tree branch containing genes from *A. mellifera*, *A. pisum*, *P. humanus*, *T. castaneum*, the postman butterfly *Heliconius melpomene* (one gene for each specie), and *D. pulex* (five genes) (Fig. S1). However, no information about its function is known. Similarly to *RPRC000006*, *RPRC000384* was also active in all studied organs (Fig. 3). *RPRC000384* is closely related to a tree branch containing six *Drosophila* neutral lipases (Fig. S1). Of these six genes, five (*CG6296*, *CG6295*, *CG6277*, *CG6271*, and *CG6283*) were shown to be at least moderately expressed in the fly midgut (Chintapalli et al., 2007), indicating that these related lipases may have a role in lipid digestion or lipid metabolism in the enterocytes. Moreover, some indirect evidences linked TAG originated from diet digestion and *CG6295* expression, regulated by the sterol-responsive element binding protein (SREBP) transcription factor (Kunte et al., 2006). The role of *RPRC000384* on *R. prolixus* lipid digestion and the way this gene expression can be regulated are interesting topics for a future investigation.

The dendrogram of neutral lipases also allowed us to try to find genes that may have suffered recent duplication. We have identified

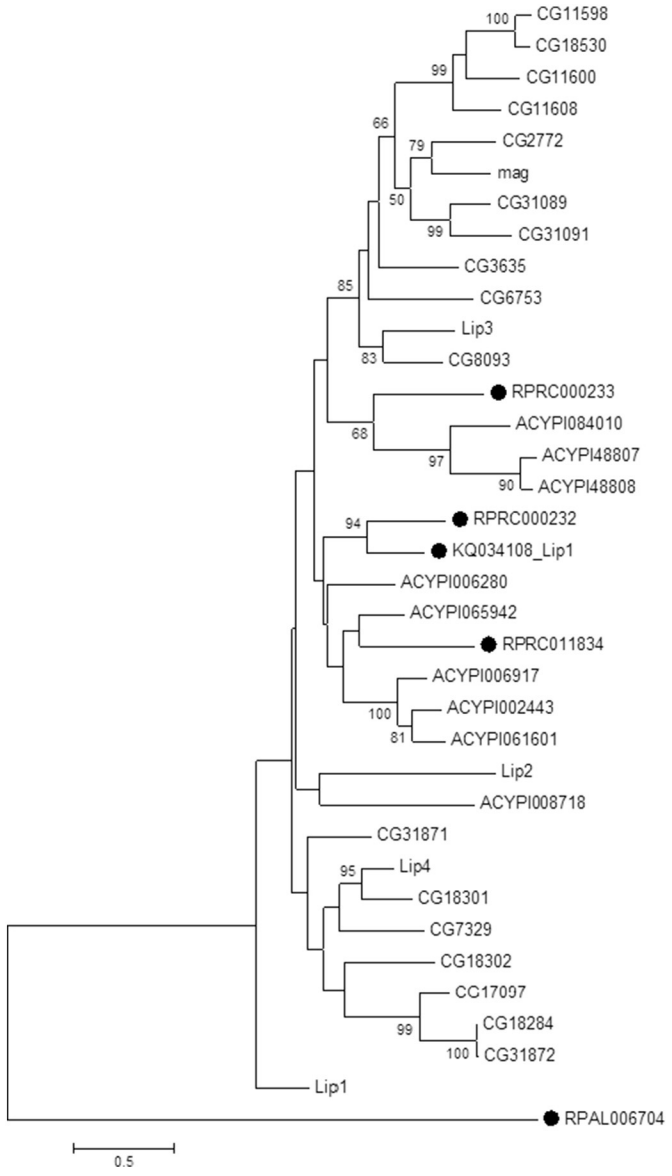


Fig. 2. Dendrogram tree comparing acidic lipases from *R. prolixus*, *D. melanogaster*, and *A. pisum*. Predicted protein sequences from *D. melanogaster*, *A. pisum*, and *R. prolixus* acidic lipases were aligned, and the dendrogram was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. *R. prolixus* genes are marked with black dots. Genes represented by names or codes starting with "CG" are from *D. melanogaster*. Codes starting with "ACYPI" represent genes from *A. pisum*.

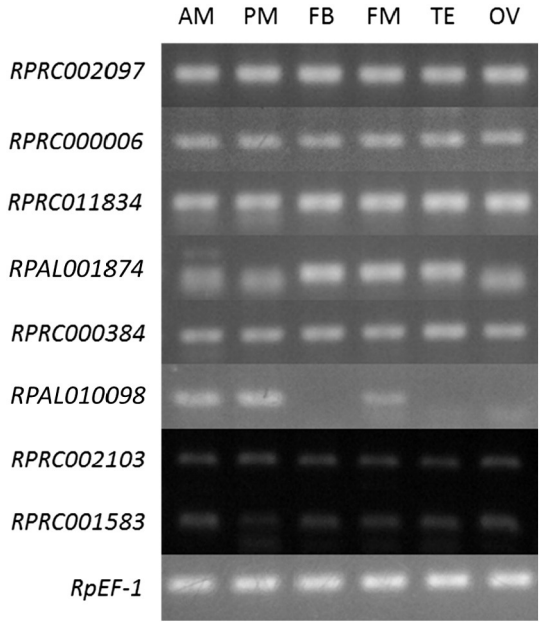


Fig. 3. Expression of some annotated genes in various adult insect organs. The organs were obtained from females and males on the fourth day after a blood meal. Total RNA was extracted from organs and treated with DNase I, and RT-PCR was carried out using specific primers designed for each gene. *RpEF-1* amplification was used as a positive control. RT-PCR products were separated for evaluation using agarose gel electrophoresis. The image shown is representative of three independent experiments. AM: anterior midgut; PM: posterior midgut; FB: fat body; FM: flight muscle; TE: testis; OV: ovary.

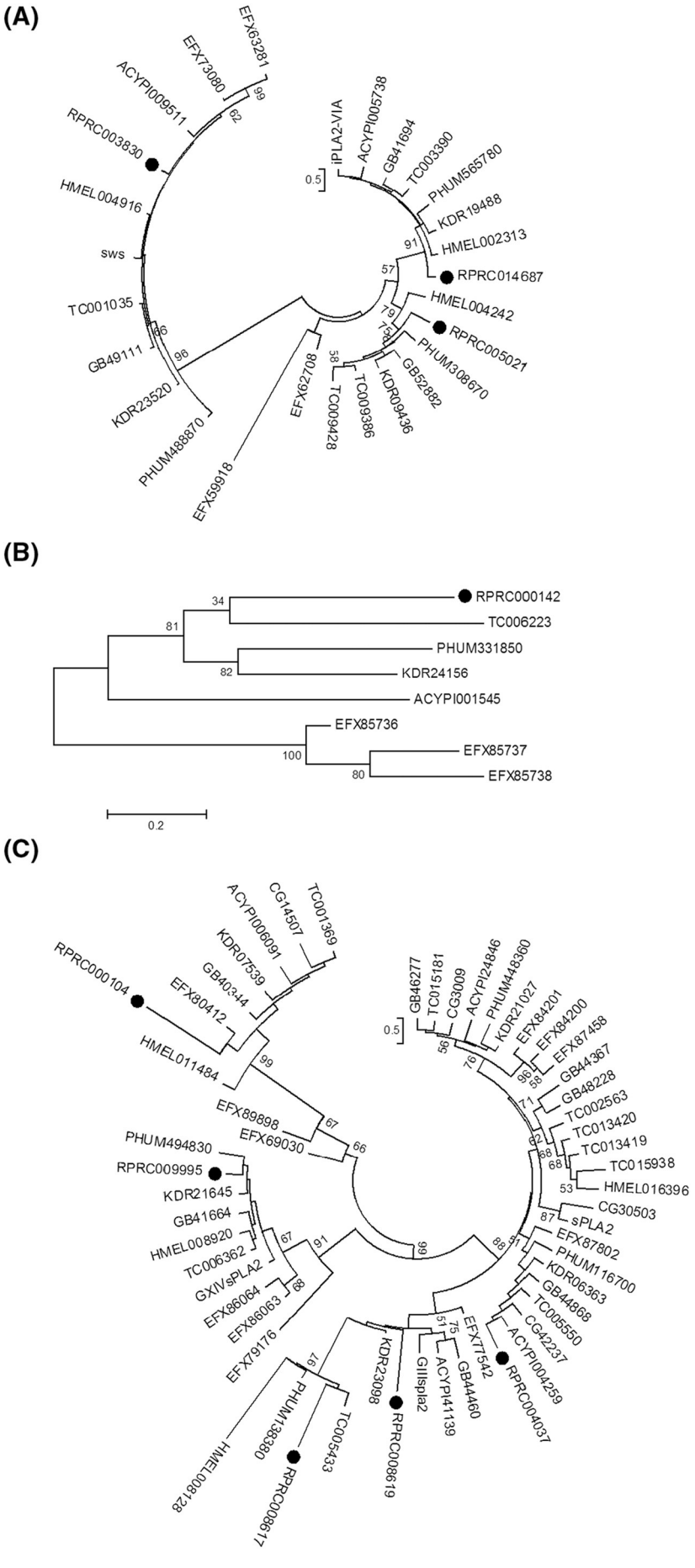
three possible gene pairs that are in the same contig or nearby contigs and whose coded proteins have a recent evolutionary divergence point (KQ034265_Lip2 and RPRC000836; RPRC001451 and RPRC013099; and RPRC004324 and RPAL003531). In addition, we could identify a tandem repeat cluster of neutral lipases (KQ034265_Lip2, RPAL010098, RPRC000246 and RPRC000863) in the contig KQ034265. None of these genes show any pseudogene features. Moreover, their intron structure did not show clear signs of conservation and, thus, they are not informative about the evolution of these genes.

Unlike the neutral lipases, the dendrogram of acidic lipases did not allow obtaining many data about *R. prolixus* genes (Fig. 2 and Fig. S2). The expression of *RPRC011834* was studied as an example of acidic lipase (Fig. 3). The gene mRNA could be detected in all organs analyzed. This gene is closely related to four genes of *Z. nevadensis*, one of *T. castaneum*, and one of *H. melpomene* (Fig. S2). However, these lipase functions are totally unknown. The genes *RPRC000232* and *GL563039_Lip1* are paralogues (Fig. 2). The other two genes (*RPRC000233* and *RPRC006704*) are also paralogues and seem to have suffered a recent duplication as they are located in the same contig, near to each other (Fig. S2). They share a tree branch with three genes from *A. pisum*, from which no information concerning function is available. Interestingly, certain important acidic lipases with known roles in *D. melanogaster* lipid metabolism seem to be absent in the genomes of the kissing bug and other insects. For example, *lipase 4*, which is associated with fat body lipid metabolism (Vihervaara and Puig, 2008), and *magro*, a lipase involved in lipid digestion and essential to cholesterol and TAG homeostasis in the fly (Sieber and Thummel, 2009, 2012), do not show similarity with any gene in the *R. prolixus* genome. Whether any other lipases assumed their roles is currently unknown, and it is an important question to be answered in future experiments.

The kissing bug genome encodes only one gene from the lipase 3 family (Fig. S3). The *R. prolixus* gene *RPRC000586* is the orthologue of the *D. melanogaster* gene *inactivation no afterpotential E* (*inaE*). This protein was shown to act as a DAG-lipase *in vitro* (Leung et al., 2008). Expression of *inaE* is found in the nervous system (Chintapalli et al., 2007), and its activity is essential for signal transduction in fly photoreceptors (Leung et al., 2008).

The gene *RPRC000271* is the only one from GDSL-like lipase family in *R. prolixus* genome (Fig. S4). The pea aphid genome shows two paralogues (Waterhouse et al., 2011), which may indicate that the Triatominae lineage had lost one of these genes. However, these lipases functions are still unknown. The GDSL-like lipase 2 family also consists of only one gene, *RPRC000295* (Fig. S5). Although the *Drosophila* orthologue was annotated as a phospholipase A2, its actual activity has not yet been demonstrated (Sheffield et al., 2000).

The *Drosophila brummer* lipase gene (*bmm*) is ubiquitously expressed, but highest levels are found in the gut and fat body (Chintapalli et al., 2007). Transcription of this gene is induced in the fat body during starvation (Bi et al., 2012) through a FOXO-regulated mechanism (Wang et al., 2011). Furthermore, *bmm*-knockout fruit


(A)
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EFX73080
ACYPI009511
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sws
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EFX62708
TC009428
TC009386
KDR09436
GB52882
PHUM308670
RPRC005021
HMEL004242
RPRC014687
HMEL002313
KDR19488
PHUM565780
TC003390
GB41694
ACYPI005738
iPLA2-VIA
0.5
(B)
RPRC000142
TC006223
PHUM331850
KDR24156
ACYPI001545
EFX85736
EFX85737
EFX85738
0.2
(C)
ACYPI006091
CG14507
TC001369
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EFX80412
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GB44460
EFX77542
ACYPI004259
RPRC004037
CG42237
TC005550
GB44868
KDR06363
PHUM116700
EFX87802
sPLA2
CG30503
HMEL016396
TC015938
TC013419
TC013420
TC002563
GB48228
GB44367
EFX87458
EFX84200
EFX84201
KDR21027
PHUM448360
ACYPI24846
CG3009
TC015181
GB46277
0.5

flies showed lipid accumulation in the fat body (Grönke et al., 2005, 2007; Wang et al., 2012), whereas *bmm* overexpression reduced fat body TAG levels and longevity (Chien et al., 2012; Grönke et al., 2005) with increased lipid accumulation in the oenocytes (Tian et al., 2011). These results clearly indicate that this lipase is involved in lipid stores mobilization. The *R. prolixus* gene *RPRC002097* is the *bmm* orthologue (Fig. S6). Expression analysis using RT-PCR revealed that this gene is transcribed in all tested organs (Fig. 3), as expected. A second lipase involved in fat body lipid mobilization is the Hormone-Sensitive Lipase ortholog (HSL). In *D. melanogaster* under starvation, HSL is present at the lipid droplet, in a LSD-1-dependent mechanism (Bi et al., 2012). The *hsl* gene is conserved through evolution, and the *R. prolixus* gene *RPRC000497* is its orthologue (Fig. S7). Finally, it was shown that a phospholipase A1 have triacylglycerol lipase activity in the fat body of the tobacco hornworm *Manduca sexta* (Arrese et al., 2006). This enzyme is homologous to PAPLA1 of *D. melanogaster* and mutant flies to this gene are sensitive to prolonged fasting (Kunduri et al., 2014). This result indicates that PAPLA1 may play a role in mobilizing the stock of lipids in fat body. However, in our analyzes, we could not find a clear orthologue gene to *papla1* (*cg8552*) in the genome of *R. prolixus* (Fig. S8). The genes that are closest phylogenetically are *RPRC000465* and *RPRC001232*, but we cannot state that these genes have some lipase activity without biochemical analyzes. Future studies should examine *bmm* and *hsl* genes in *R. prolixus* to define the contribution of each to the lipid mobilization process, and if *RPRC00465* and *RPRC001232* have any role in this pathway.

3.2. Phospholipase genes

Phospholipases A2 (PLA2s) are characterized by the ability to specifically hydrolyze the ester bond at the sn-2 position of PLs, generating FA and lysophospholipids. In insects, in addition to their role in poisons, PLA2s are also involved in digestion, pathogen defense, reproduction and lipid metabolism (Stanley, 2006). PLA2s are classified into groups based on amino acid sequence, molecular weight, disulfide bonds, calcium loop and other features. There are five major classes of PLA2: secreted (sPLA2), cytosolic calcium-dependent (cPLA2), calcium independent (iPLA2), platelet activating factor acetylhydrolase (PAF-AH) and lysosomal (Murakami et al., 2011).

Prostaglandins and leukotrienes play an important role in inflammation and immunity. They are the result of enzymatic modifications of arachidonic acid, which is released by cPLA2. This enzyme is only present in vertebrates, and it seems to have evolved simultaneously with eicosanoid receptors. As in other invertebrates, these enzymes are not present in the *R. prolixus* genome (data not shown). In contrast, the iPLA2 and sPLA2 families are found in many organisms, including yeasts, nematodes, amebae, flies and even plants. This conservation suggests that the iPLA2 and sPLA2 families play fundamental roles in membrane and energy metabolism of all eukaryotes (Murakami et al., 2011). We found nine PLA2 genes in the data analysis of *R. prolixus* genome (*RPR004037*, *RPR008617*, *RPR009995*, *RPR000104*, *RPR008619*, *RPRC000142*, *RPRC014687*, *RPRC005021*, and *RPRC003830*).

The iPLA2 family includes six groups: GVIA, GVIB, GVIC, GVID, GVIE and GVIF. Besides the phospholipase activity, the iPLA2s display lysophospholipase and transacylase activities. Moreover, iPLA2 GVIA also features acyl-CoA thioesterase activity (Lei et al., 2010). In the *R. prolixus* genome, we identified three genes from the iPLA2 family (*RPRC014687*, *RPRC005021*, and *RPRC003830*) each one belonging to one type of iPLA2, VIA, VIB and VIC, respectively (Fig. S9A). The *R. prolixus* gene *RPRC014687* has an orthologue in *D. melanogaster*, the gene *calcium-independent phospholipase A2 VIA* (*iPLA2-VIA*). Knockdown of *iPLA2-VIA* gene expression in flies caused a dramatic impact on store-operated Ca^{2+} entry activation (Vig et al., 2006) and also a change in the lipids droplets morphology, that became smaller and more dispersed (Guo et al., 2008). Moreover, this gene is highly expressed in the adult fat body (Chintapalli et al., 2007), which may indicate a role in lipid storage or mobilization. However, *RPRC014687* functions need to be investigated. The gene *RPRC005021* has orthologues in all studied insects, but *D. melanogaster* and *A. pisum* (Fig. 4A). Noteworthy, two paralogues could be found in the *T. castaneum* genome. This result may indicate that this gene do not have an essential function, although this hypotheses needs to be further studied. However, because of the association of iPLA2 GVIB with mitochondrial and peroxisomal membranes, it can be anticipated that these enzymes may be involved in integrating lipid and energy metabolism (Murakami et al., 2011). We also identified an iPLA2 GVIC gene, *RPRC003830* (Fig. 4A). It is orthologue to *Drosophila swiss cheese* (*sws*). Fruit flies carrying mutations in this gene exhibit progressive degeneration of the adult nervous system, as well as glial and neuronal hyperwrapping. However, overexpression of *sws* causes the formation of abnormal intracellular membranous structures and cell death. Moreover, this phenotype involved an increase in esterase activity and a reduction in the levels of phosphatidylcholine. Thus, SWS is essential for membrane lipid homeostasis and cell survival in both neurons and glia of the adult *Drosophila* brain, and this gene may play an analogous role in other animals (Muhlig-Versen et al., 2005).

Since the first iPLA2 was described (Wolf and Gross, 1985), other six groups were identified in mammals (Schaloske and Dennis, 2006). In *R. prolixus* genome, as we mentioned before, only groups VIA, VIB and VIC seems to be represented. In mammals these enzymes have different roles in a large number of biological process, as phospholipid remodeling, arachidonic acid release and eicosanoid formation, cell proliferation, brain development, and triacylglycerol homeostasis (Schaloske and Dennis, 2006), but the function of each enzyme described in *R. prolixus* still needs to be investigated.

The PAF-AH comprises two groups of PLA2 (VII and VIII), both of which are able to hydrolyze the acetyl group of the sn-2 position of platelet activating factor (Marathe et al., 2014). In the *R. prolixus* genome, one gene that encodes a putative PAF-AH was found (*RPRC000142*). To evaluate the group with which the enzyme is associated, we performed a phylogenetic analysis using the sequences of mammalian phospholipases A2 from the VII and VIII groups (Fig. S9B). Our analysis indicated that *R. prolixus* PAF-AH is related to PLA2 VII, as it has been observed in other insects. This gene orthologues could be found in *T. castaneum*, *A. pisum*, *P. humanus*, and *Z. nevadensis*, but not in *D. melanogaster*, *A. mellifera*, and *H. melpomene* (Fig. 4B), which indicate that this gene may have been lost after the divergence of the Premecoptera lineage. The activity of this enzyme was already confirmed in *R. prolixus* hemolymph, and its role in innate immunity was discussed (Figueiredo et al., 2008a, 2008b). Furthermore, PAF-AH activity was also studied in salivary glands, and it has been hypothesized that this enzyme may play a role in the inhibition of platelet aggregation during the insect feeding (Corte-Real et al., 2011).

To date, 12 sPLA2s groups (IB, IIA, IIC, IID, IIE, IIF, III, V, X, otoconin, XIIA and XIIB) have been identified in mammals (Murakami and Kudo, 2001). Group III PLA2s have been characterized from various organisms, including insects, parasites, scorpions, reptiles and mammals (Hariprasad et al., 2013). In the *D. melanogaster* genome, there are five identified sPLA2 III genes (Murakami et al., 2011), whereas in the *R. prolixus* genome, we were able to find only three genes

Fig. 4. Dendrograms trees of the main PLA_2 groups found in *R. prolixus*: $iPLA_2$ (A); PAF-AH (B); $sPLA_2$ (C). Predicted protein sequences from *D. melanogaster*, *A. mellifera*, *T. castaneum*, *A. pisum*, *R. prolixus*, *P. humanus*, *Z. nevadensis*, and *H. melpomene* phospholipases A2 (PLA2) were aligned, and the dendrograms were constructed using the maximum likelihood method with 500 bootstrap replications. PLA2 from *D. pulex* were used as outgroup. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. *R. prolixus* genes are marked with black dots. Genes represented by names or codes starting with "CG" are from *D. melanogaster*. Codes starting with "ACYPI", "EFX", "GB", "HMEL", "KDR", "PHUM", and "TC" represent genes from *A. pisum*, *D. pulex*, *A. mellifera*, *H. melpomene*, *Z. nevadensis*, *P. humanus*, and *T. castaneum*, respectively.

(*RPRC004037*, *RPRC008617*, and *RPRC008619*) (Fig. S9C). *RPRC008617* and *RPRC008619* are paralogues and closely related to *Drosophila GIIIspla2* (Fig. 4C). This gene is highly expressed in the fly fat body, from both larvae and adults (Chintapalli et al., 2007), however its metabolic function is unknown. As this gene is duplicated in the *R. prolixus* genome, the kissing bug seems to be an interesting model to investigate this question. *RPRC004037* gene expression was observed in most tissues at relatively low levels, except for the central nervous system, salivary glands and hindgut (Defferrari et al., 2014). The high expression in salivary glands is interesting due to the evolutionary convergence of poison and saliva composition in hematophagous animals (Fry et al., 2009). However, the function of the PLA2 III in saliva is still unknown. The gene *RPRC000104* is closely related to group X of secretory PLA2s (Fig. S8C). However, unfortunately, the obtained gene sequence is truncated and is not possible to confirm that prediction. At last, the *RPRC009995* gene is closely related to mammalian sPLA2 from group XIIA (Fig. S8C) and contained an active site domain with the conserved sequence CCXXHDXC (data not shown). It was recently shown that this gene is expressed ubiquitously in *R. prolixus* (Defferrari et al., 2014). *RPRC009995*-knockdown insects exhibit a 50% decrease in jack bean urease toxicity, due to modulation of eicosanoid release.

Many questions are still open about the sPLA2 after that genome analysis. Is there PLA2 any belonging to other groups that we were not able to find? Is the gene *RPRC000104* a true sPLA2 X? What is the role of each sPLA2 III transcript? In which tissues each sPLA2 form is expressed? What is the role of each sPLA2 in metabolism? These issues will be address in a near future.

3.3. Fatty acid metabolism

Insect microsomal and cytosolic fatty acid synthases (FASs) were reported in the oenocyte-rich integument of insects (Juárez et al., 1992, 1996). Only the microsomal FAS (mFAS) incorporates efficiently methylmalonyl-CoA units producing methyl-branched fatty acids, which serve as precursors to complex mixtures of branched hydrocarbons, as those reported in *R. prolixus* (Juárez and Calderón-Fernandez, 2007). Three distinct type I FAS (FAS I) orthologues were reported in the *D. melanogaster* genome: *CG3523*, *CG3524* and *CG17374* (McQuilton et al., 2012). After RNA *in situ* hybridization of these three *Drosophila* FAS genes in adult males, it was shown that *CG3523* is expressed only in the adult fat body, whereas *CG17374* and *CG3524* are both exclusively expressed in oenocytes. Furthermore, the *CG3524* gene is responsible for methyl-branch formation (Chung et al., 2014). Here, we report three FAS I genes in *R. prolixus* genome: *RPRC000269*, *RPRC000123* and *RPRC002909*. Eleven FAS genes of selected insect species, representing several orders, were used to construct the dendrogram. FAS sequences were grouped in two clades supported by high bootstrap values (Fig. 5). The large clade included the human and *Caenorhabditis elegans* FAS genes used as outgroups, together with two *Rhodnius* FAS genes (*RPRC00269* and *RPRC002909*), two *Drosophila* FAS orthologues (*CG3523* and *CG3524*) and other insect FAS-like proteins. The other clade grouped the FAS genes exclusively detected in *Drosophila* oenocytes (*CG17374*), the corresponding *R. prolixus* orthologue, *RPRC000123*, together with other insect FAS genes. This clade might group only putative oenocyte-specific genes, all of which lack functional characterization.

It was rather interesting that the fruit fly *mFAS* of the oenocytes clusters closely to the cytosolic fat body FAS gene and more distantly to the other FAS gene expressed in the oenocytes.

However, an intriguing observation comes out from gene location in the supercontig KQ034222. The putative *R. prolixus* mFAS gene (*RPRC002909*) is located immediately downstream from the putative fat body FAS gene (*RPRC000269*) in the supercontig sequence. Furthermore, the genome of *Drosophila* shows the same feature, with the genes *CG3523* (fat body FAS) and *CG3524* (mFAS) next to each other into the chromosome 2L. This suggests that the *mFAS* gene, responsible for the synthesis of methyl-branched fatty acids, might have arisen as result of duplication of the fat body FAS gene, which is orthologue of the FASN gene of mammals and other groups.

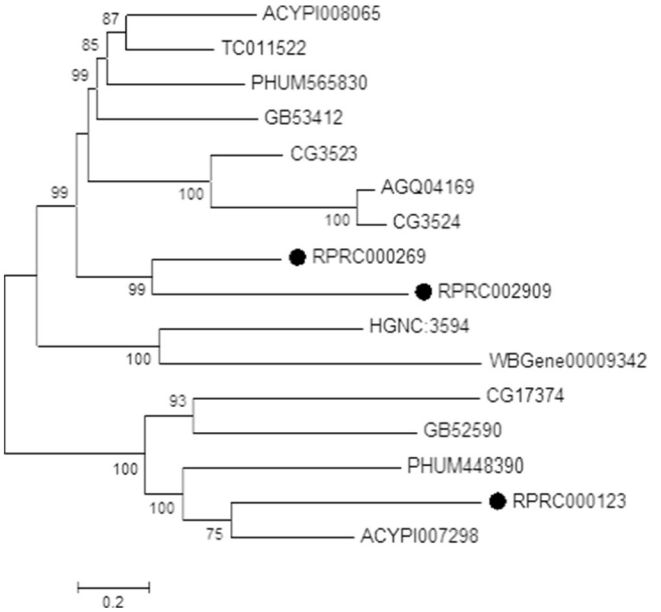


Fig. 5. Dendogram tree of *R. prolixus* fatty acid synthase (FAS) genes. Predicted protein sequences of FAS from *R. prolixus* were identified from the RproC1.3 gene set. Predicted protein sequences encoding these enzymes in other species were downloaded from NCBI GenBank. The sequences were aligned and the tree was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. Human and *C. elegans* FAS genes were used as outgroups. *R. prolixus* genes are marked with black dots. Codes starting with "ACYPI", "AGQ", "CG", "GB", "HGNC", "PHUM", "TC", and "WBGene" represent genes from *A. pisum*, *D. serrata*, *D. melanogaster*, *A. mellifera*, *H. sapiens*, *P. humanus*, *T. castaneum*, and *C. elegans*, respectively.

Increasing evidence supports that methyl-branched lipids, which have melting points above ambient temperature, help maintain a barrier against evaporative water loss (Chung et al., 2014; Gibbs and Pomonis, 1995). The cuticle of *R. prolixus* and other triatomines has 2-fold more branched chain lipids than straight chain lipids, mostly in the form of hydrocarbons with 1 up to 4 methyl ramifications (Juárez and Calderón-Fernandez, 2007). This cuticle lipid composition might explain the success of triatomines to tolerate a wide range of temperatures, relative humidity and environmental conditions along their large geographic distribution (Calderón-Fernández et al., 2011, 2012; Calderón-Fernández and Juárez, 2013; Gibbs, 2002).

Functional characterization of these genes is necessary to clarify their roles and relevance to insect fitness. Over the last decade, the correlation between FAS overexpression and human cancer has become evident, causing interest in FAS as a potential therapeutic target in cancer to grow steeply (Orita et al., 2007). Gaining additional knowledge of these fatty acids synthases in model organisms might help unravel the mode of action of potential FAS inhibitors.

Both FAS products and fatty acids derived from the diet are further elongated into very long-chain fatty acids (VLCFA) by successive addition of two-carbon units. There are seven fatty acid elongases (ELOVLs) known in mammals that perform the initial and rate-controlling condensation reaction in the elongation cycle. In insects, the biochemistry of fatty acid elongation was studied in the fly *Musca domestica*, kissing bug *Triatoma infestans* and cockroach *Blatella germanica* (Juárez, 2004; Juárez and Brenner, 1989; Vaz et al., 1988). A large number of ELOVL genes have been reported in insect genomes, although their functions remain mostly unknown. In *Drosophila*, twenty ELOVLs were reported, but only two of them were fully characterized, indicating involvement in male and female pheromone production (Chertemps et al.,

2005, 2007). Ten ELOVL putative genes were found in the genome of *R. prolixus*. The dendrogram (Fig. 6) showed that the ELOVL genes included in the analysis were distributed in six clades. The "ELOVL 1/7 clade" contains the *R. prolixus* genes *RPRC003507* and *RPRC009588*, orthologous to the human *ELOVL1* and *ELOVL7* genes. ELOVL7 family members elongate acyl-CoAs ranging in length from 16 to 20 carbons (Naganuma et al., 2011). This clade also includes an ELOVL gene of the mosquito *Aedes albopictus* (GenBank accession number ACS37245), which is involved in controlling dehydration resistance in diapause eggs through regulation of the formation of hydrocarbons from VLCFA precursors (Urbanski et al., 2010). *RPRC015196* was included in the "ELOVL 4 clade" containing the insect and *Daphnia* genes orthologous to the human gene ELOVL4. ELOVL4s are the only mammalian enzymes known to synthesize C28-C36 fatty acids (Leonard et al., 2004), which are similar in chain length to the VLCFA described in *T. infestans* and serve as precursors to the major straight chain hydrocarbons and fatty alcohols (Juárez and Brenner, 1989). The "ELOVL 6 clade" includes the phylogenetically related *Homo sapiens* genes *ELOVL3* and *ELOVL6*; the latter is involved in the elongation of long-chain saturated and monounsaturated fatty acyl-CoAs (Naganuma et al., 2011). Among insect genes orthologous to human ELOVL6, the *R. prolixus gene RPRC013853* and the fruit fly gene *baldspot* (*CG3971*) were included. The *baldspot* gene was shown to be essential for insect viability and is expressed in several tissues (Jung et al., 2007).

Five ELOVL genes of *R. prolixus* (*RPRC003658*, *RPRC014612*, *RPRC00545*, *RPRC002656* and *RPRC000121*), were included within three insect-specific ELOVL clusters "ELOVL X clades," which are phylogenetically related to both the ELOVL 1/7 and ELOVL 4 clades (Fig. 6). Within these clades, functional characterization is available only for the fruit fly *elongase F* (*CG16905*) and *james bond* (*CG6921*) genes, which play a critical role in the production of hydrocarbon pheromone in females (Chertemps et al., 2007) and during cytokinesis in male spermatocytes (Szafer-Glusman et al., 2008).

The structure of fatty acids is also regulated by membrane-bound fatty acid desaturases (FADs). They play essential roles in fatty acid metabolism and in the regulation of homeostasis by producing unsaturated chains that help maintain the structure and function of biological membranes. The insect FADs exhibit high functional diversity. Each member includes three characteristic histidine box motifs essential for their

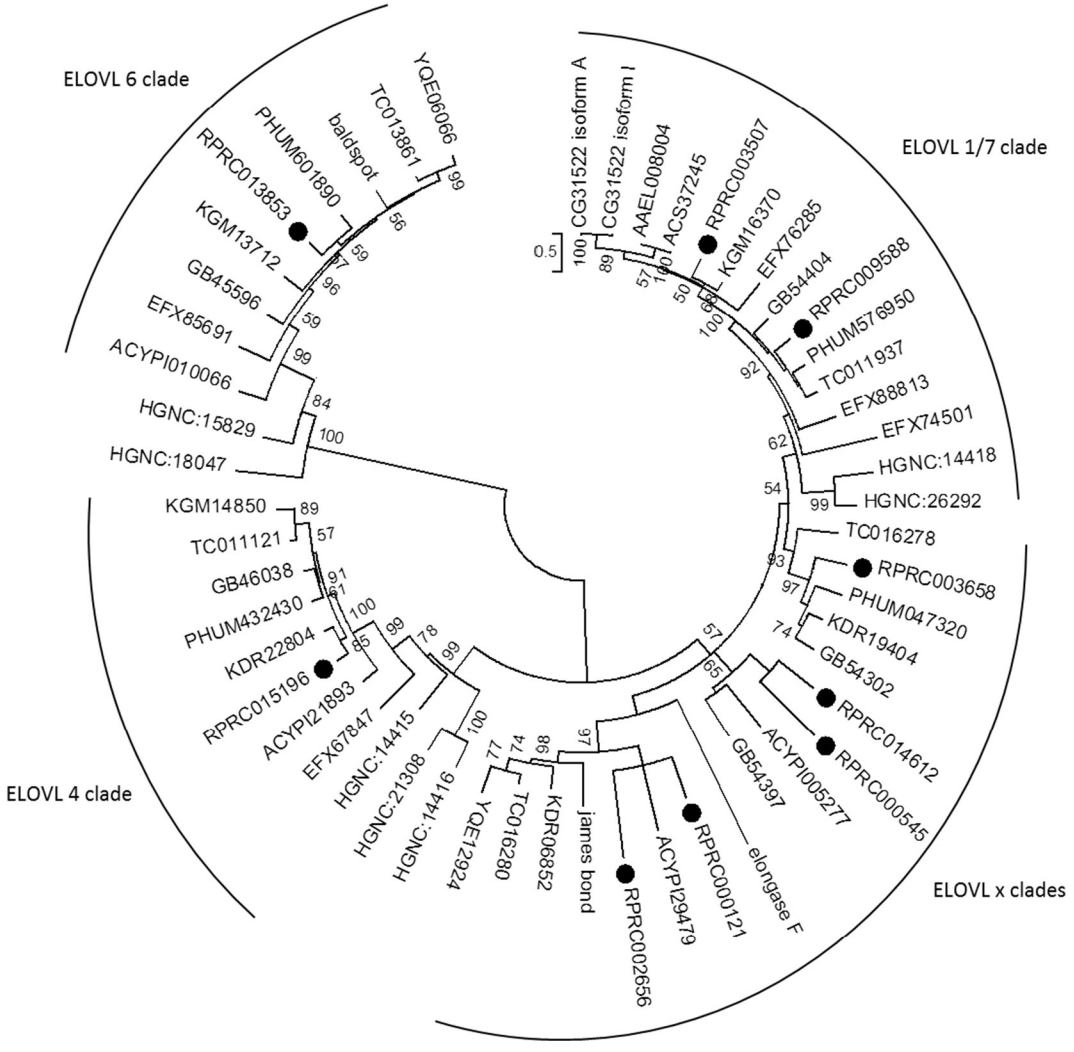


Fig. 6. Dendogram tree of *R. prolixus* fatty acid elongase (ELOVL) genes. Predicted protein sequences of ELOVL of *R. prolixus* were identified from the RproC1.3 gene set. Predicted protein sequences encoding these enzymes in other species were downloaded from the NCBI GenBank. The sequences were aligned, and the tree was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. The clades associated with human ELOVL1/7, ELOVL4 and ELOVL6 are labeled, together with three ELOVL1/7-related clades only present in insects (ELOVL X Clades). Human sequences of ELOVL1 to 7 genes and their corresponding *D. pulex* orthologues were used as outgroups. *R. prolixus* genes are marked with black dots; only complete sequences (9 out of 10) were used in the phylogenetic analysis. Genes represented by names or codes starting with "CG" are from *D. melanogaster*. Codes starting with "AAEL", "ACS", "ACYPI", "EFX", "GB", "HGNC", "KDR", "KGM", "PHUM", "TC", and "YQE" represent genes from *Aedes aegypti*, *A. albopictus*, *A. pisum*, *D. pulex*, *A. mellifera*, *H. sapiens*, *Z. nevadensis*, *Danaus plexippus*, *P. humanus*, *T. castaneum*, and *Dendroctonus pondeosae*, respectively.

activity (Shanklin et al., 1994) but show variations that are predicted to produce various types of unsaturated fatty acids. The evolution of these enzymes, with distinct regiospecificities and stereospecificities, has been linked to the production of species-specific pheromone components (Roelofs et al., 2002). Nine putative *R. prolixus* desaturase genes were identified. The dendrogram indicates that two genes (*RPRC000617* and *RPRC6553*) were orthologous to other insect Δ9-desaturase genes, *Daphnia* and the human Δ9-desaturase gene (Fig. 7). These genes encode highly regulated enzymes that catalyze the formation of a carbon-carbon double bond at the nineth position from the carboxyl end of a saturated fatty acid. These enzymes contribute to the formation of the major monounsaturated components of most insect acylglycerols, which are essential for fat storage. Using *Daphnia* sequences as an outgroup for Δ9 and Δ11 clades, the Δ11 clade remained as an insect-specific family. Within this clade, six *R. prolixus* genes were grouped into four clusters (Fig. 7). Although closely related to the Δ9-desaturases, Δ11 enzymes exhibit a wider substrate range and are involved in the production of sex pheromones in Lepidoptera (Blomquist et al., 2005). In addition, one *R. prolixus* gene (*RPRC013818*) encodes a putative member of the Δ4-desaturase family, which is a less diverse family. These enzymes are mostly involved in sphingolipid Δ4 desaturation, a relevant process in cell cycle control during *Drosophila* spermatogenesis (Ternes et al., 2002).

Fatty acid reductases (FARs) convert fatty acids into their corresponding fatty alcohols. Insect fatty alcohols are components of the cuticle lipid blend and participate in chemical communication (Juárez and Calderón-Fernandez, 2007; Zhu et al., 1996). Fatty alcohols are also major components of the wax secretion of honey bee (Blomquist et al., 1980). However, few FAR genes have been cloned and characterized from insects, among them those involved in pheromone biosynthesis in the silkworm *Bombyx mori* (*pgFAR*), in *A. mellifera* (*FAR1*) and in the European corn borer *Ostrinia nubilalis* (*pgFAR*) (Antony et al., 2009; Moto et al., 2003; Teerawanichpan et al., 2010). FARs can also lead to the formation of intermediate aldehydes that serve as substrates for the last oxidative decarbonylation step in the formation of hydrocarbons *via* a P-450 oxidative decarbonylase system (Qiu et al., 2012). Fifteen FAR genes were identified in *R. prolixus*, all of which share three characteristic conserved domains: the NADB-Rossmann superfamily domain, the FAR_C superfamily domain and a transmembrane domain that is absent in plant FARs (Hellenbrand et al., 2011). These genes clustered into several insect-specific FAR clades (Fig. 8). The FAR sequences of *Daphnia* used as outgroups clustered into a distinct clade closely related to human and chicken orthologues involved in the reduction of long-chain fatty acids (C16-C18), together with three insect FAR genes. One of the insect-specific clades contains six sequences (*RPRC013997*, *RPRC013998*, *RPRC000880*, *RPRC014002*, *RPRC014004* and *RPRC006662*)

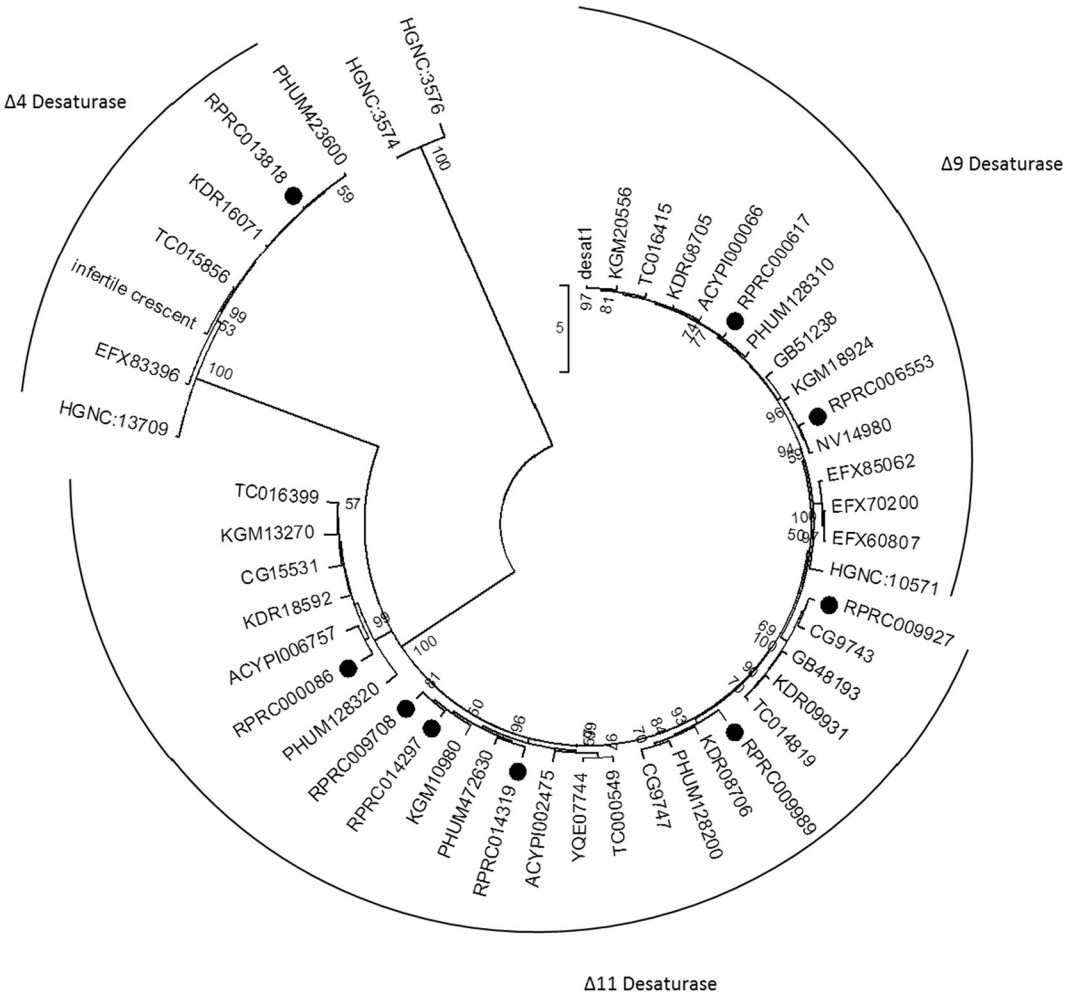


Fig. 7. Dendrogram tree of *R. prolixus* fatty acid desaturase (FAD) genes. Predicted protein sequences of FAD of *R. prolixus* were identified from the RproC1.3 gene set. Predicted protein sequences encoding these enzymes in other species were downloaded from the NCBI GenBank. The sequences were aligned, and the tree was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. Human FAD genes of Δ4, Δ5, Δ6 and Δ9 families and the corresponding *D. pulex* orthologues of the Δ4 and Δ9 families were used as outgroups. *R. prolixus* genes are marked with black dots. Genes represented by names or codes starting with "CG" are from *D. melanogaster*. Codes starting with "ACYPI", "EFX", "GB", "HGNC", "KDR", "KGM", "NV", "PHUM", "TC", and "YQE" represent genes from *A. pisum*, *D. pulex*, *A. mellifera*, *H. sapiens*, *Z. nevadensis*, *D. plexippus*, *N. vitripennis*, *P. humanus*, *T. castaneum*, and *D. pondeosae*, respectively.

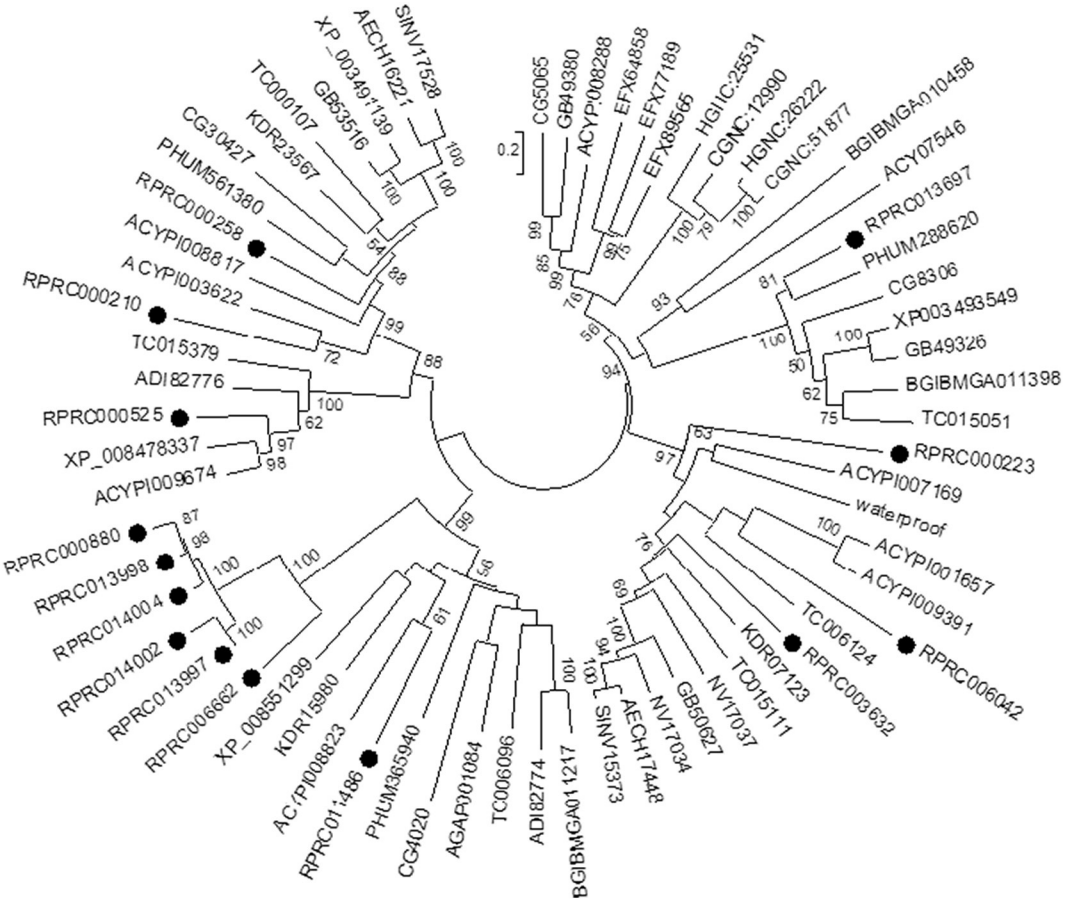


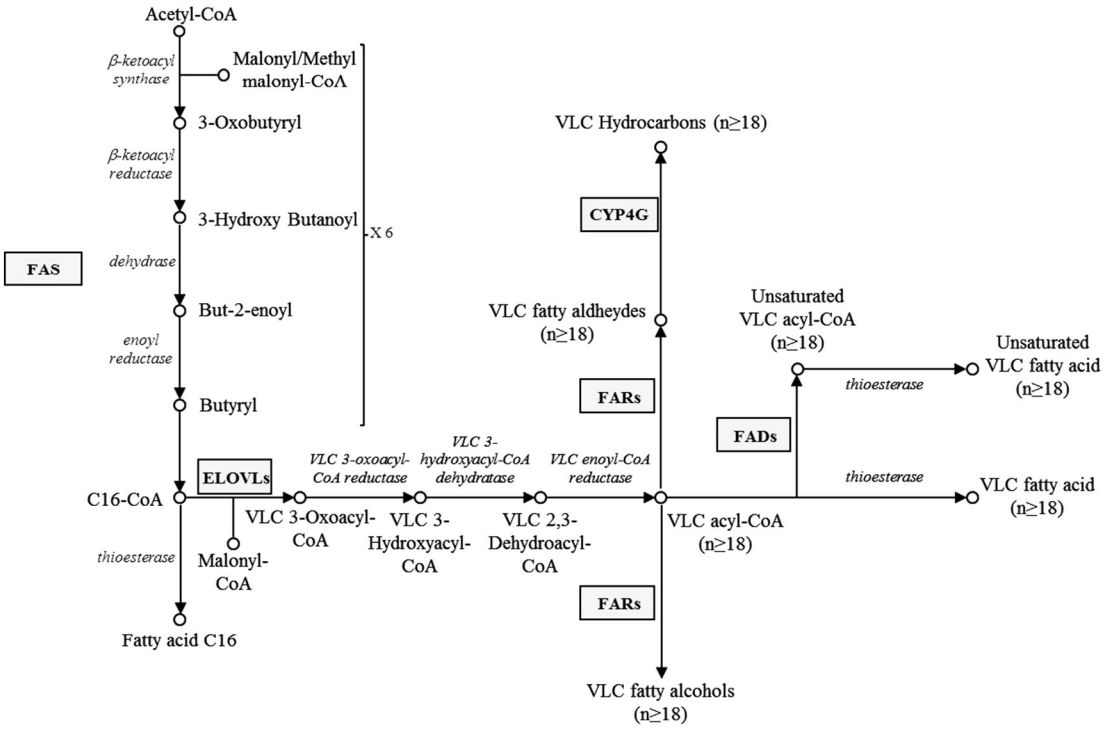
Fig. 8. Dendrogram tree of *R. prolixus* fatty acid reductase (FAR) genes. Predicted protein sequences of FAR of *R. prolixus* were identified from the RproC1.3 gene set. Predicted protein sequences encoding these enzymes in other species were downloaded from the NCBI GenBank. The sequences were aligned, and the tree was constructed using the maximum likelihood method with 500 bootstrap replications. The numbers indicate the bootstrap support. The branch length scale denotes the number of substitutions per site. Human, chicken and *Daphnia* FAR genes were used as outgroups. *R. prolixus* genes are marked with black dots; only complete sequences (14 out of 15) were used in the phylogenetic analysis. Genes represented by names or codes starting with "CG" are of *D. melanogaster*. Codes starting with "ACYPI", "ADI", "AECH", "AGAP", "BGIBMGA", "CGNC", "EFX", "GB", "HGNC", "KDR", "NV", "PHUM", "SINV", and "TC" represent genes from *A. pisum*, *O. nubilalis*, *Acromyrmex echinatior*, *Anopheles gambiae*, *B. mori*, *Gallus gallus*, *D. pulex*, *A. mellifera*, *H. sapiens*, *Z. nevadensis*, *N. vitripennis*, *P. humanus*, *Solenopsis invicta*, and *T. castaneum* respectively. XP_003491139 and XP_003493549 are genes from *Bombus impatiens*. XP_008478337 is a gene from *Diaphorina citri* and XP_008551299 is a gene from *Microplitis demolitor*.

that form a *R. prolixus*-specific gene expansion (Fig. 8). This gene expansion might be associated with a higher biosynthesis demand. Very long-chain fatty alcohols can reach up to 42% of the cuticle lipids of triatomines, and together with hydrocarbons, they are the major lipid components in the cuticle (Juárez and Calderón-Fernandez, 2007). *R. prolixus* fatty alcohols have not yet been characterized, but the role of long-chain fatty alcohols as contact sex pheromones has been shown in the triatomine *T. infestans* (Cocchiararo-Bastias et al., 2011). Recently, a gene controlling the reduction of VLCFA to fatty alcohols of 24–26 carbons was characterized in *D. melanogaster* (Jaspers et al., 2014). This gene, called *waterproof*, was shown to mediate airway clearance in the tracheal tubes by providing a hydrophobic lining. Thus, along with their well-known function as pheromones, fatty alcohols could serve, together with branched lipids, as waterproofing agents of the cuticle allowing triatomines to inhabit a wide range of environments. The lack of information about FAR genes and the scarce functional studies preclude further analyses on the relationships depicted in the dendrogram. A scheme with different metabolic pathways of fatty acids is shown in Fig. 9, indicating functions of the genes described above.

Given the relevance of the cuticle lipids in the physiology and barrier properties of the cuticle by regulating water loss by transpiration, participating in a wide variety of chemical communication events and also in the regulation of chemical and microbial attack, this study is a first step towards transcriptomic, metabolomic and proteomic studies to further identify specific pathways relevant to insect fitness. *R. prolixus* will serve as a model for triatomine physiology research; experiments should be performed in a variety of species (among them those of relevance as Chagas' disease vectors), and phenotypes (based on evidence of the cuticular hydrocarbon pattern diversity over different geographical areas). Also, the first evidence of the relationship between insecticide resistance and cuticle lipids was obtained in a triatomine, thus focusing on genes related to the biosynthesis and metabolism of lipids deserves further attention.

3.4. Acyl-CoA and glycerolipid synthesis genes

For use in metabolic routes, such as the synthesis of glycerolipids and cholesteryl-esters, β-oxidation and protein acylation, FA chains are first activated by thioesterification to Coenzyme A (CoA) to form acyl-CoA in a reaction catalyzed by acyl-CoA synthetases (ACS). These enzymes are members of the fatty acid:CoA ligase [AMP forming] superfamily, which also includes aryl-CoA synthetases, insect luciferases, and the adenylation domains of the modular non-ribosomal peptide synthetases (NRPSs) based on the similarities of their mechanism of catalysis (Gulick, 2009). According to their preferred acyl-chain length, mammalian ACS isoforms can be divided into five subfamilies: (i) ACS short-chain (ACSS; C2 to C4), (ii) ACS medium-chain (ACSM; C4 to C12), (iii) ACS long-chain (ACSL; C12 to C20), (iv) ACS bubblegum (ACSBG; C14 to C24), and (v) ACS very long-chain (ACSVL, also known as solute



Protein symbol	Kegg orthology	E.C.	*Rhodnius prolixus* matches
CPY4G	Cytochrome P450, family 4, subfamily G	1.14.-.-	RPRC003227, RPRC013847
ELOVL	Fatty acid elongase	2.3.1.199	RPRC000121, RPRC000545, RPRC002656, RPRC003507, RPRC003658, RPRC009588, RPRC013853, RPRC014612, RPRC015196
FAD	Fatty acid desaturase	1.14.19.5	RPRC000086, RPRC000617, RPRC006553, RPRC009708, RPRC009927, RPRC009989, RPRC013818, RPRC014297, RPRC014319
FAR	Fatty acid reductase	1.2.1.84	RPRC000210, RPRC000223, RPRC000258, RPRC000525, RPRC000880, RPRC003632, RPRC006042, RPRC006662, RPRC011486, RPRC013697, RPRC013997, RPRC013998, RPRC014002, RPRC014004
FAS	Fatty acid synthase	2.3.1.85; 2.3.1.41 (beta-ketoacyl synthase); 2.3.1.38, 2.3.1.39 (acyl transferase domain); 1.1.1.100 (beta-ketoacyl reductase); 4.2.1.59 (polyketide synthase dehydratase); 1.3.1.39 (enoyl reductase); 3.1.2.14 (thioesterase)	RPRC000123, RPRC000269, RPRC002909

Fig. 9. Fatty acid metabolism pathways. Gray boxes indicate gene presence. CYP4G: Cytochrome P450, family 4, subfamily G; ELOVL: Fatty acid elongase; FAD: Fatty acid desaturase; FAR: Fatty acid reductase; FAS: Fatty acid synthase.

carrier family 27A, SLC27A; C18 to C26) (Soupene and Kuypers, 2008). In humans, the ACS protein family comprises 26 members: 3 ACSSs, 6 ACSMs, 5 ACSLs, 2 ACSBGs, 6 ACSVLs, and 4 not assigned ACS isoforms (Watkins et al., 2007), which differ in expression pattern, subcellular localization and acyl substrate preference (Grevengoed et al., 2014). Twenty putative ACS members were found in the *R. prolixus* genome: 2 ACSSs (*RPRC013075* and *RPRC000574*), 2 ACSLs (*RPRC009774* and *RPRC000439*), 5 ACSVLs (*RPRC004287*, *RPRC005284*, *RPRC009983*, *RPRC004368*, and *RPRC006560*), and 11 unassigned ACS (*RPRC000595*, RPRC001107, RPRC001108, RPRC001190, RPRC014409, RPRC011278, *RPRC000110*, *RPRC000533*, *RPRC004384*, *RPRC008381*, *and RPRC011528*) (Alves-Bezerra et al., 2016).

The relevance of ACS enzymes resides not only in their role in FA activation but also in the suggestion that various ACS isoforms are responsible for the formation of specific acyl-CoA pools for different metabolic fates (Grevengoed et al., 2014). The overexpression of ACSL5 in rat hepatoma cells resulted in increased incorporation of exogenous fatty acid into triacylglycerol but not into phospholipids (Mashekm et al., 2006). Also in hepatoma and primary rat hepatocytes, *Acsl1* gene was shown to be a target of PPARα, which induces

the transcription of genes involved in β-oxidation, thus suggesting a catabolic role of this ACS isoform in those cells (Schoonjans et al., 1995, 1996). In *R. prolixus*, ACSL2 but not ACSL1 is required for fatty acid β-oxidation in the fat body and for oocyte maturation (Alves-Bezerra et al., 2016).

Identification of orthologues in other insect genomes for comparative purposes has been difficult due to mistakes generated during automatic gene annotation. One example of these types of errors occurs in the firefly luciferase enzymes, which are also part of the fatty acid:CoA ligases [AMP forming] superfamily. Luciferases are ATP-dependent monooxygenases that catalyze not only the bioluminescence reaction but also the fatty acyl-CoA synthetic reaction (Oba et al., 2003, 2005). Accordingly, firefly luciferases share high sequence homology with mammalian ACS (Oba et al., 2005), although the primary function of these enzymes involves a bioluminescent process. The presence of enzymes named as luciferase-like proteins has been reported in non-bioluminescent beetle species, where these enzymes act exclusively as an ACS, and it was suggested that luciferases evolved from a duplication and subsequent specialization of an ancestral ACS gene (Day et al., 2009). Genomic automatic annotation has misassigned luciferase genes in several non-bioluminescent arthropods, such as the tick *Ixodes scapularis* (GenBank accession number XP_002436092.1), *P. humanus* (GenBank accession number EEB16371.1), *T. castaneum* (GenBank accession number XP_967226.1), the ant *Camponotus floridanus* (GenBank accession number EFN69681.1), and the mosquito *Anopheles darlingi* (GenBank accession number ETN63630.1).

A similar mistake was observed with the plant enzyme 4-coumarate:CoA ligase (4CL), another member of fatty acid:CoA ligase [AMP forming] superfamily (Stuible et al., 2000). Some of the 4CL isoforms showed high activities using medium- and long-chain FA as substrates, suggesting that the function of ACS could be conserved from an ancestral ACS protein (Kienow et al., 2008; Souza et al., 2008). Although 4CL seems to be specific for phenylpropanoid metabolism in plants and, to our knowledge, there is no evidence for this catalytic activity in animals, its similarity to ACSs and luciferases led to the misannotation of several genes among insect species. For example, 4CL putative enzymes are present in the genomes of the wasp *Nasonia vitripennis* (GenBank accession number XP_001604903.1), of *A. pisum* (GenBank accession number XP_003243588.1), of *B. mori* (GenBank accession number XP_004928344.1), and of the ants *Harpegnathos saltator* (GenBank accession number EFN81838.1) and *C. floridanus* (GenBank accession number EFN62390.1). Unless further experimental evidence is provided, we understand that luciferases (in non-bioluminescent animals) and 4CL-related sequences should be annotated as ACS proteins. Thus, the automatic annotation resulted in the incorrect nomenclature of some fatty acid:CoA ligases [AMP forming] superfamily members that should be corrected in future genome projects.

In *R. prolixus*, acyl-CoA generated by ACSs are used for TAG synthesis *via* the G3P (or Kennedy) pathway (Alves-Bezerra and Gondim, 2012), a *de novo* pathway that may also produce DAG and non-sphingomyelin-derived PL. The first step of this pathway is catalyzed by the enzyme GPAT, which acylates G3P (Wendel et al., 2009). Although four isoforms of this enzyme are found in mammals (two mitochondrial and two microsomal forms), only one putative protein (RPRC002103) had been described in *R. prolixus*. The amino acid sequence and structural organization of conserved motifs of this protein are closely related to the mammalian mitochondrial GPAT isoform (Alves-Bezerra and Gondim, 2012). In *D. melanogaster*, besides the homolog of the mammalian mitochondrial isoforms (*CG5508*), two other genes similar to the mammalian microsomal isoforms were identified: *CG3209* and *CG15450* (Kühnlein, 2012). DmGPAT4 was demonstrated to migrate from the endoplasmic reticulum to the lipid droplet during lipid accumulation, and its knockdown affects the formation of these organelles (Wilfling et al., 2013). In the present study, a microsomal GPAT-related sequence (*RPRC001583*) was also identified in the genome of *R. prolixus*. In adult insects, these genes were expressed in all analyzed organs (anterior and posterior midgut, fat body, flight muscles, testis and ovary) (Fig. 3). Although two GPAT isoforms were identified in *R. prolixus*, more studies are required to understand the specific role of each one in insect lipid metabolism.

A diagram for glycerolipid metabolism pathways is shown in Fig. 10, indicating the genes that were found in the genome of *R. prolixus*. A member of the DAGAT family (PF03982), which includes the acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2), acyl-CoA:monoacylglycerol acyltransferase 1-3 (MGAT1-3), and wax monoester synthases (Yen et al., 2008) is present in the genome. The assignment of the predicted transcript (RPRC000305) into a specific subclass of DAGAT family is difficult due to the high similarity among family members and the lack of biochemical information from Arthropoda orthologues. Because a MGAT activity has not been detected in the organs of *R. prolixus* whereas the G3P pathway is active, this transcript was previously suggested to encode a putative DGAT2 enzyme (Alves-Bezerra and Gondim, 2012). However, it remains unclear whether *RPRC000305* encodes a functional DGAT2, a MGAT enzyme that is a negligible contributor for TAG synthesis in this insect, or another member of DAGAT family.

Moreover, we were not able to find genes encoding monoacylglycerol lipases, indicating that this insect is not able to hydrolyze mono acylglycerol into glycerol and free fatty acid. However, the *R. prolixus* genome encodes a putative monoacylglycerol kinase (*RPRC0037632*), which catalyzes the phosphorylation of this lipid into phosphatidic acid. Thus, according to the data obtained, the only metabolic pathway that monoacylglycerol can follow is the TAG synthesis through the glycerol-3-phosphate pathway. However, this hypothesis needs to be confronted experimentally.

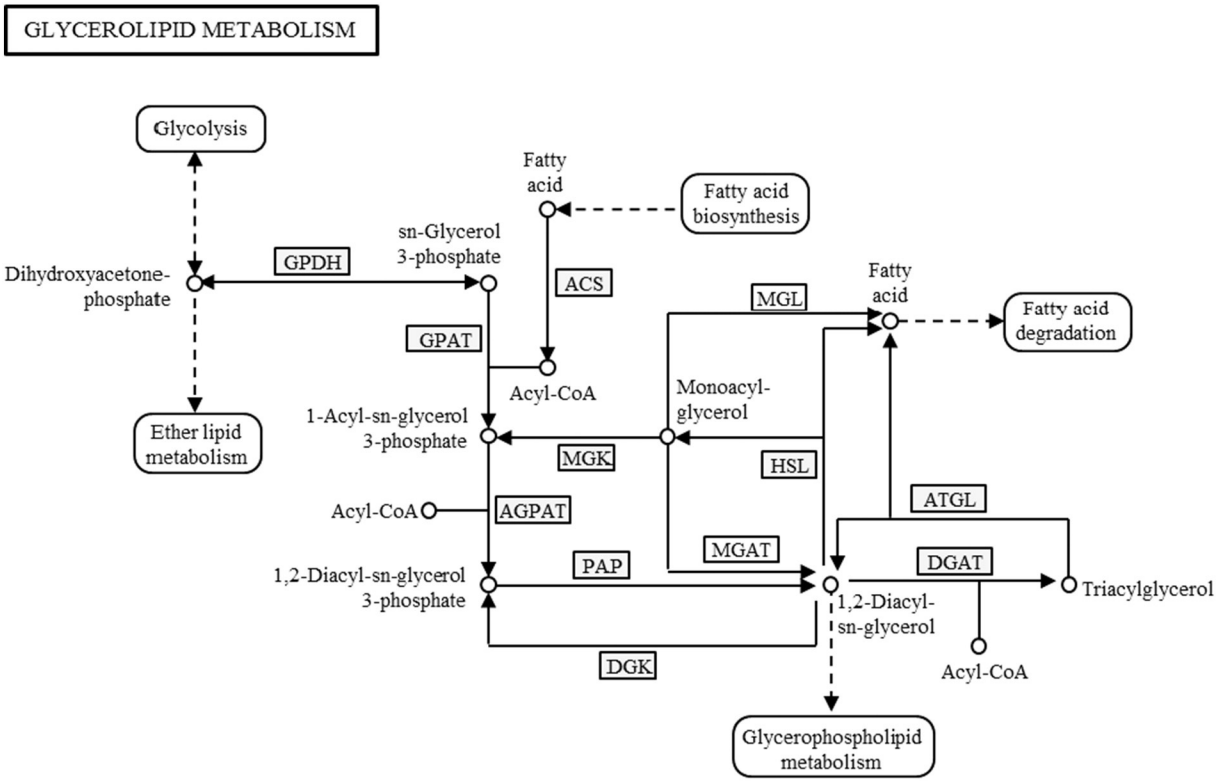
3.5. β-oxidation genes

The *R. prolixus* genome shows all the genes related to mitochondrial fatty acid β-oxidation, including the gene *RPRC011131*, which encodes a putative organic cation transporter responsible for the transport of carnitine to the intracellular medium. This transporter is present in other insect genomes, exhibiting roughly 60% sequence similarity (data not shown).

All the components of the carnitine shuttle, namely carnitine palmitoyltransferase I and II (*RPRC005639* and *RPRC008379*, respectively), as well as the carnitine/acylcarnitine translocase (*RPRC005034*), were found in the genome. Each gene is present in a single copy. Analysis of RPRC005034 protein sequence revealed that the predicted protein contains three repeated domains of 100 amino acids (data not shown), which are typically found in mitochondrial carrier proteins (Palmieri, 1994), and the sequence is similar to that of the *D. melanogaster* gene *congested-like trachea* (Oey et al., 2005).

At least in *Drosophila*, there is also only one copy of the *carnitine palmitoyltransferase-1* (*CPT-1*) orthologue (*withered*) in the genome (Jackson et al., 1999); however, this feature is not yet certain for other insect genomes due to variation in the function of the carnitine shuttle and in the β-oxidation rates among insects (Childress et al., 1967; Crabtree and Newsholme, 1972; Stevenson, 1968).

The mitochondrial acyl-CoA dehydrogenases are all present in their various acyl-CoA length-specific isoforms, with the notable exception of long-chain acyl-CoA dehydrogenase (LCAD), which was not found in any of the insect or crustacean genomes (Table S3). When mammalian sequences were used as query, the closest hit was a short-chain acyl-CoA dehydrogenase (SCAD), fact that was confirmed by checking the protein function motif similarity against the best hit of other proteins from annotated insect genomes. In each and every case, all protein hits showed similarity with SCAD (CDD accession number: cd01158) (Marchler-Bauer et al., 2015). Noteworthy, LCAD was also not found in the nematode *Caenorhabditis elegans* or bacterial genomes; instead, just a "LCAD-like" gene is present (Mohsen and Vockley, 2009).



Protein symbol	Kegg orthology	E.C.	*Rhodnius prolixus* matches
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase	2.3.1.51	KQ034389_Agpat1, KQ034126_Agpat2 KQ034389_Agpat3, KQ034389_Agpat4
ATGL	Patatin-like phospholipase domain-containing protein, Triacylglycerol lipase, Brummer lipase	3.1.1.3	RPRC002097
DGAT	Diacylglycerol O-acyltransferase 1	2.3.1.20	RPRC003803
MGAT/DGAT	DAGAT family member	2.3.1.20/22	RPRC000305
DGK	Diacylglycerol kinase	2.7.1.107	RPRC008855
GPAT	Glycerol-3-phosphate O-acyltransferase	2.3.1.15	RPRC002103, RPRC001583
GPDH	Glycerol-3-phosphate dehydrogenase (NAD+)	1.1.1.8	RPRC005747
GPDH	Glycerol-3-phosphate dehydrogenase	1.1.5.3	RPRC011461
HSL	Hormone-sensitive lipase, Diacylglycerol lipase	3.1.1.79	RPRC000497
MGK	Monoacylglycerol kinase	2.7.1.94	RPRC003762
MGL	Monoacylglycerol lipase	3.1.1.23	Not found
PAP	Phosphatidate phosphatase	3.1.3.4	RPRC010599

Fig. 10. Glycerolipids metabolism pathways. Gray boxes indicate gene presence and white boxes indicate gene absence. ACS: acyl-CoA synthetase; AGPAT: 1-acylglycerol-3-phosphate O-acyltransferase; ATGL: adipose triglyceride lipase (Brummer); DGAT: diacylglycerol O-acyltransferase; DGK: diacylclycerol kinase; GPAT: glycerol-3-phosphate O-acyltransferase; GPDH: glycerol-3-phosphate dehydrogenase; HSL: hormone-sensitive lipase (diacylglycerol lipase); MGAT: 2-acylglycerol O-acyltransferase; MGK: monoaclyglycerol kinase; MGL: monoacylglycerol lipase; PAP: phophatidate phosphatase.

LCADs show highly different levels of expression and importance for β-oxidation in mice compared with humans, being very important in mice (despite some overlap in function with VLCAD) but not at all in humans, with low levels of expression and VLCAD being essential instead (Chegary et al., 2009; Yamaguchi et al., 1993). Some groups demonstrated that LCAD seems to be involved with branched chain fatty acid oxidation and α-oxidation instead, at least in humans (Mao et al., 1995; Wanders et al., 1998). So, considering these evidences, there seems to be some space for controversy regarding the function of LCAD, or at least it seems to take on very different roles as evolution unfolds. We consider that its absence in *R. prolixus* genome (and in the other analyzed insect genomes) is not worrisome. Perhaps it is an expected byproduct of the genomic evolution, with other genes taking on the role of branched chain dehydrogenases, and the insects VLCAD

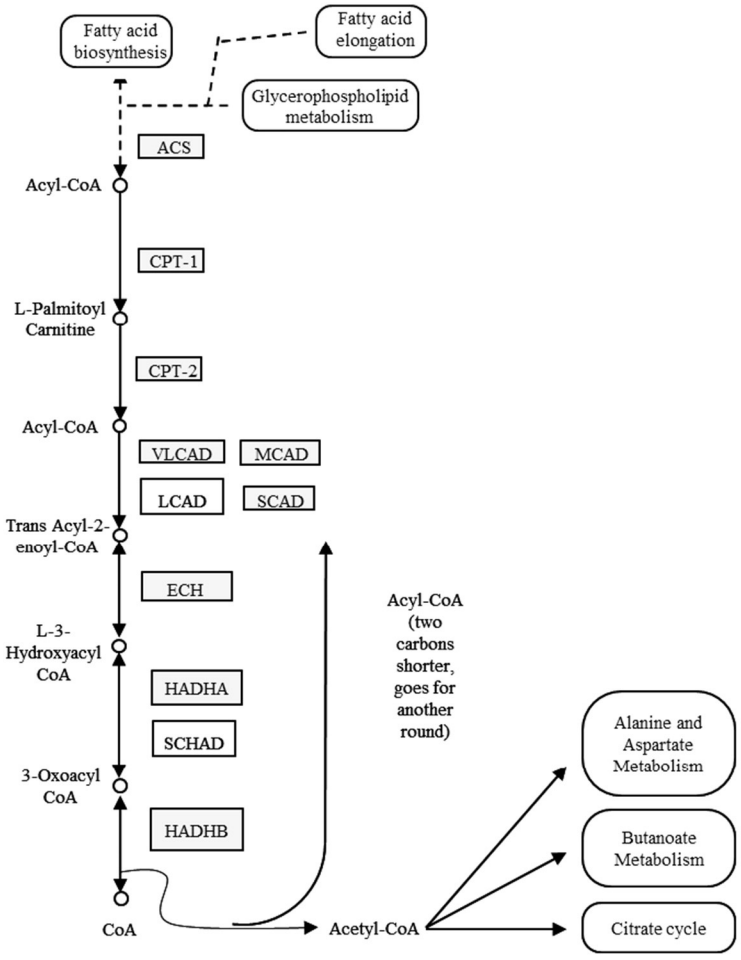
sequences acting as the only group of enzymes responsible for oxidation of long chain fatty acids (16 carbon chain lengths and longer).

The *R. prolixus* genome also possesses genes encoding α and β subunits of the mitochondrial trifunctional protein (*RPRC006225* and *RPRC007372*, respectively). Curiously, there is also no orthologue of a α subunit of the mitochondrial trifunctional protein for short chain 3-hydroxyacyl-CoA. The fatty acid degradation pathway is diagrammed in Fig. 11, and the genes found in the genome of *R. prolixus* are indicated.

4. Conclusion

Here, we analyzed the *R. prolixus* genome and annotated genes involved in various pathways of lipid metabolism. Different families of lipases and phospholipases were identified and its genes identified. Genes involved in fatty acid synthesis and modification and those encoding fatty acid synthases, desaturases, reductases and elongases were extensively annotated. Furthermore, a second GPAT was identified, and its expression profile was investigated. Finally, all genes involved in fatty acid β-oxidation were found, with the exception of the long-chain acyl-CoA dehydrogenase, which is absent in all insects investigated to date. These results will form an important basis for future experiments using a molecular approach to better understand the metabolism of lipids in insects and its relationship to parasite-vector interaction.

Some questions raised by these results will be investigated in the near future:



Protein symbol	Kegg orthology	E.C.	*Rhodnius prolixus* matches
CPT-1	Carnitine O-palmitoyltransferase I	2.3.1.21	RPRC005639
CPT-2	Carnitine O-palmitoyltransferase II	2.3.1.21	RPRC008379
ECH	Enoyl-CoA hydratase	4.2.1.17	RPRC003563, RPRC005720
HADHA	3-Hydroxyacyl-CoA dehydrogenase	1.1.1.35	RPRC011764, RPRC006225
HADHB	Acetyl-CoA C-acyltransferase; Beta-ketothiolase; 3-ketoacyl-CoA thiolase;	2.3.1.16	RPRC007372
LCAD	Long chain Acyl-CoA dehydrogenase	1.3.8.8	Not found
MCAD	Medium chain Acyl-CoA dehydrogenase	1.3.99.-	RPRC012869
SCAD	Short chain Acyl-CoA dehydrogenase	1.3.8.1	RPRC004042, RPR004622
VLCAD	Very long chain Acyl-CoA dehydrogenase	1.3.8.9	RPRC003803

Fig. 11. β-oxidation pathway. Gray boxes indicate gene presence and white boxes indicate gene absence. CPT-1: carnitine O-palmitoyltransferase I; CPT-2: carnitine O-palmitoyltransferase II; ECH: enoyl-CoA hydratase; HADHA: 3-Hydroxyacyl-CoA dehydrogenase; HADHB: acetyl-CoA C-acyltransferase, β-ketothiolase, 3-ketoacyl-CoA thiolase; LCAD: long chain acyl-CoA dehydrogenase; MCAD: medium chain acyl-CoA dehydrogenase; SCAD: short chain Acyl-CoA dehydrogenase; VLCAD: very long chain Acyl-CoA dehydrogenase.

1) *R. prolixus* is an excellent model for the study of mobilization of lipids in fat body, due to the dynamic accumulation and degradation of TAG in this organ (Pontes et al., 2008). The results presented here indicate at least five genes encoding putative lipases possibly involved in this process (*RPRC002097*, homologous to *bmm*; *RPRC000497*, homologous to *hsl*; RPAL005331, homologous to *CG5966* from *Drosophila*, and *RPRC000465* and *RPRC001232*, related to the fat body lipase from *M. sexta*). The study of the expression of these genes in the fat body and analysis of phenotypes resulting from the use of RNAi will generate new information to help understand the role of each gene in the kissing-bug and other insects.
2) *R. prolixus*' saliva contains a large amount of lysophosphatidylcholine (Golodne et al., 2003) and this lipid plays an important role in the transmission of Chagas' disease (Mesquita et al., 2008). Understanding `which PLA2 are involved in the synthesis of lysophosphatidylcholine will be important for the development of possible innovative ways of control of this disease transmission.
3) Our results showed that genes encoding FARs underwent a major expansion in the genome of *R. prolixus*. This makes this insect a unique model for the study of the functions and the biological importance of these enzymes in the evolutionary process of these animals.
4) Previous results clearly showed that, in *R. prolixus*, TAG is synthesized by the G3P pathway (Alves-Bezerra and Gondim, 2012), whose first step is catalyzed by GPAT. In this study, we identified a second putative GPAT encoded by the genome of *R. prolixus*. It would be extremely interesting to investigate the role of each of these genes in lipid synthesis in the kissing-bug.
5) The study on β-oxidation of insects is basically neglected. The annotation of the genes in this pathway in *R. prolixus*, added to the study on TAG mobilization dynamics in the fat body (Pontes et al., 2008), makes this insect an interesting model to fill this gap in knowledge on insect physiology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2016.09.045.

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Each author's contributions were as follow: DM analyzed the genes encoding lipases, performed the analysis of gene expression and wrote the final version of the manuscript, LSC and GCA analyzed the gene encoding phospholipase, GMCF and MPJ analyzed the genes involved in the fatty acids metabolism, MAB and KCG analyzed genes involved in the synthesis of acyl-CoA and glycerolipids and performed the analysis of gene expression, and IFP analyzed β-oxidation pathway genes.

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