Published in final edited form as:

Biochim Biophys Acta. 2016 July ; 1861(7): 650–662. doi:10.1016/j.bbali.2016.04.007.

Long-chain acyl-CoA synthetase 2 knockdown leads to decreased fatty acid oxidation in fat body and reduced reproductive capacity in the insect *Rhodnius prolixus*

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Abstract

Long-chain acyl-CoA esters are important intermediates in lipid metabolism and are synthesized from fatty acids by long-chain acyl-CoA synthetases (ACSL). The hematophagous insect *Rhodnius prolixus*, a vector of Chagas' disease, produces glycerolipids in the midgut after a blood meal, which are stored as triacylglycerol in the fat body and eggs. We identified twenty acyl-CoA synthetase genes in *R. prolixus*, two encoding ACSL isoforms (*RhoprAcs1* and *RhoprAcs2*). *RhoprAcs1* transcripts increased in posterior midgut on the second day after feeding, and *RhoprAcs2* was highly transcribed on the tenth day. Both enzymes were expressed in *Escherichia coli*. Recombinant RhoprACSL1 and RhoprACSL2 had broad pH optima (7.5–9.5 and 6.5–9.5, respectively), were inhibited by triacsin C, and were rosiglitazone-insensitive. Both showed similar apparent K_m for palmitic and oleic acid (2–6 μ M), but different K_m for arachidonic acid (0.5 and 6 μ M for RhoprACSL1-Flag and RhoprACSL2-Flag, respectively). The knockdown of *RhoprAcs1* did not result in noticeable phenotypes. However, RhoprACSL2 deficient insects exhibited 2.5-fold increase in triacylglycerol content in the fat body, and 90% decrease in fatty acid β -oxidation. *RhoprAcs2* knockdown also resulted in 20% increase in lifespan, delayed digestion, 30% reduced oviposition, and 50% reduction in egg hatching. Laid eggs and hatched nymphs showed remarkable alterations in morphology. In summary, *R. prolixus* ACSL isoforms have distinct roles on lipid metabolism. Although RhoprACSL1 functions remain unclear, we propose that RhoprACSL2 is the main contributor for the formation of the intracellular acyl-CoA pool channeled for β -oxidation in the fat body, and is also required for normal reproduction.

Keywords

Acyl-CoA metabolism; lipid synthesis; β -oxidation; insect reproduction; oogenesis

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1. Introduction

Fatty acids are required for the synthesis of a variety of complex lipids, including stored triacylglycerol (TAG), membrane phospholipids and bioactive lipids. Alternatively, fatty acids can be oxidized in the mitochondria for energy production. The use of a fatty acid for metabolism requires its activation to an acyl-CoA intermediate by a thioesterification reaction catalyzed by one of the acyl-CoA synthetase family members (ACS; EC 6.2.1.x). Long-chain acyl-CoA synthetases (ACSL) activate long-chain fatty acids (12–20 carbons) to acyl-CoA, allowing acyl chains to enter metabolic pathways. The five mammalian isoforms have specific tissue distributions, subcellular locations and substrate preferences [1]. Additionally, the ACSL isoforms appear to partition their acyl-CoA products into different metabolic pathways [1, 2].

The blood-feeding insect *Rhodnius prolixus* is a vector of Chagas' disease. This disease is caused by the protozoan *Trypanosoma cruzi* and currently affects about 7 – 8 million people in Central and South America [3]. Apart from its medical relevance, *R. prolixus* has also been used as a model to study lipid metabolism in arthropods. Mechanisms involved in lipid synthesis [4–7], transport [8–11], mobilization [12, 13] and the endocrine regulation of lipolysis [14] have been described.

In *R. prolixus*, dietary TAG is hydrolyzed in the midgut lumen by TAG lipase activity. The midgut epithelium absorbs the released fatty acids, which are used via the glycerol-3-phosphate pathway for the synthesis of glycerolipids, such as phospholipids (PL), diacylglycerol (DAG) and TAG [5, 7]. These newly synthesized lipids are distributed to other tissues by the shuttle system mediated by lipophorin, a major circulating lipoprotein present in the hemolymph [5, 10, 15]. Lipids carried by lipophorin are stored mainly as TAG in the fat body [12], an organ functionally analogous to both mammalian liver and adipose tissue [16]. During prolonged starvation, which may be as long as five months for *R. prolixus* [17], TAG is mobilized to sustain the energy needs of the insect [12, 17], probably by the oxidation of released fatty acids. TAG, DAG and PL are also synthesized by the ovaries during oogenesis [5, 8, 13]. In this organ, TAG accumulation is required for egg growth and nymph development [13]. In addition, arachidonic acid may potentially be used for the synthesis of eicosanoids, which act as mediators for the nutrient endocytosis in growing eggs [18]. Because ACSL activity could control the formation of acyl-CoA pools used in most of these pathways, we asked whether different ACSL isoforms are required for specific physiological purposes.

ACSL isoforms have been characterized in arthropods. A microsomal ACS activity for palmitate (16:0) activation was identified in the *Macrobrachium borelli* shrimp [19]. In *Drosophila melanogaster*, an ACSL isoform (dACSL) is required for normal embryonic segmentation and normal TAG accumulation in larvae [20, 21]. However, the presence of additional isoforms and their specific physiological roles remain unclear. To our knowledge, none of the ACSL enzymes have been previously studied in vectors of human diseases. Here we show that *R. prolixus* genome encodes at least twenty acyl-CoA synthetases (ACS). Among them, two isoforms are ACSL and exhibit tissue-specific transcriptional regulation. To determine whether specific characteristics could result in differential roles for

RhoprACSL1 and 2 isoforms, we investigated biochemical properties from recombinant enzymes and analyzed the phenotypes generated by dsRNA-mediated knockdown of each gene. We found that RhoprACSL2 is required for fatty acid β -oxidation in the fat body, and for oviposition and egg viability. These data provide compelling evidence for the central role of RhoprACSL2 in the insect energetic and reproductive physiology.

2. Material and methods

2.1. Insects and ethical statement

Insects were taken from a colony of *Rhodnius prolixus* maintained at 28 °C and 70 – 75 % relative humidity. Adult insects were fed with live rabbit blood at 3-week intervals, beginning 15 days after the moult from 5th instar to adult. Adult females were studied after the second or third meal (fed condition), or three weeks after the first or second blood meal (unfed condition). All animal care and experimental protocols were conducted following the guidelines of the Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro (CAUAP-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by CAUAP-UFRJ. Technicians dedicated to the animal facility at the Institute of Medical Biochemistry (UFRJ) conducted all aspects related to rabbit husbandry under strict guidelines to ensure the careful and consistent animal handling.

2.2. Gene identification

Genes were searched in the *R. prolixus* genome assembly (VectorBase, <http://www.vectorbase.org>, *R. prolixus* CDC annotation, RproC1) [22] by similarity to the AMP-binding family consensus sequence (Pfam number PF00501) using FAT software [23]. Retrieved sequences were manually analyzed to confirm the presence of additional ACS conserved motifs [24, 25]. Identified supercontigs were compared to ACS protein sequences from different organisms available in GenBank by tBlastn [6]. Coding region prediction, protein primary sequence and phylogenetic analyses were performed as described [14].

2.3. RNA isolation and cDNA synthesis

For gene expression analysis in different organs, anterior and posterior midguts, abdominal fat bodies and ovaries were obtained from females on the fourth day after a blood meal. To analyze the time-dependent response, organs were dissected before feeding (day 0) and on different days after a blood meal. To analyze gene expression in follicles at different stages of growth, ovaries were dissected from females on the fourth day after a blood meal, and follicles were removed in 0.15 M NaCl under a stereomicroscope and grouped according to their length into four categories: 0.5, 1.0, 1.5 and 2.0 mm. Total RNA was isolated from samples (pools from 3 to 5 organs), treated with RNase-free DNase I and used to synthesize cDNA as described [14].

2.4. Rapid amplification of cDNA ends (RACE)

The 3' end of the *RhoprAcs11* cDNA and the 5' end of the *RhoprAcs11* and *RhoprAcs12* cDNAs were amplified using 3'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, USA) and the GeneRacer kit for full-length, RNA ligase-mediated

rapid amplification of 5' and 3' cDNA ends (RLM-RACE, Invitrogen), respectively, according to the manufacturers' recommendations. Amplification of the full-length cDNA was performed using primers designed for specific amplification of target genes based in *in silico* predictions and the information obtained using RACE (Supplementary Table 1). PCR reactions were carried out using iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, USA). PCR products were separated by electrophoresis in agarose gel and isolated with the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA). The full length CDS sequences obtained for *RhoprAcs11* or *RhoprAcs12* were registered in the NCBI GenBank database under the accession numbers KY328601 and KT328602, respectively.

2.5. Quantitative PCR (qPCR)

qPCR was performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR Green PCR Master Mix (Applied Biosystems) under the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. qPCR amplification was performed using specific primers for the target genes (Supplementary Table 1), and were designed using the Primer3 software [26]. *Rhopr18S* gene amplification was used for normalization [27]. Primer efficiencies and qPCR inhibition were determined as described [28]. Amplification specificity analysis and qPCR controls to detect contaminations were carried out following the MIQE guidelines [29].

2.6. Construction of recombinant plasmids

cDNA was synthesized from posterior midgut RNA and used as template to amplify the *RhoprAcs11* and *RhoprAcs12* open reading frames by PCR using the iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, USA). Primers were designed for *RhoprAcs11* or *RhoprAcs12* specific amplification with the addition of specific restriction sites (Supplementary Table 1). The amplified PCR product was digested with restriction enzymes (XhoI, EcoRI or HindIII; New England Biolabs, Ipswich, USA) and ligated into pFlag-CTS vector (Sigma-Aldrich Co., St. Louis, USA). The sequences of RhoprACSL-Flag fusion constructs were verified by the University of North Carolina Automated Sequencing Facility.

2.7. Expression of recombinant proteins in *Escherichia coli*

Recombinant RhoprACSL1-Flag and RhoprACSL2-Flag were expressed in *E. coli* JM109 strain after induction with 0.5 mM IPTG (Thermo Fisher Scientific, Waltham, USA) at an A600 of 0.5. Induced bacteria was grown in Terrific Broth (1.2% triptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄, pH 7) supplemented with ampicillin (50 µg/mL) at 15 °C and shaken at 150 rpm. After a 6-h induction, cells were harvested by centrifuging at 3,200 *g* for 15 min at 4 °C. The cell pellet was resuspended in 8.5 mL of lysis buffer (10 mM HEPES, pH 7.8, 1 mM ATP, 0.5 mM EDTA, 10 µM PMSF, 5% glycerol, 1% protease inhibitor cocktail (Sigma), 10 U/mL benzonase and 0.2 mg/mL lysozyme). The resuspended cells were incubated for 30 min on ice and then sonicated with ten 10-s bursts, each followed by a 10-s rest on ice. Cellular fractions (Suppl. Fig 1) were obtained as described [30]. Protein concentrations were determined by the BCA method (Thermo Fisher Scientific).

2.8. Purification of the recombinant proteins

RhoprACSL1- and RhoprACSL2-Flag were purified by column chromatography using a Flag M2 antibody affinity matrix (Sigma) [30]. Eluates were analyzed by electrophoretic separation on an 8% polyacrylamide gel containing 1% SDS and stained with Coomassie Brilliant Blue R-250 dye (Thermo Fisher Scientific). Alternatively, proteins separated by electrophoresis were transferred to a nitrocellulose membrane (Bio-Rad), and incubated with monoclonal antibody against the Flag M2 epitope (Sigma). The immunoreactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-mouse IgG and Supersignal PicoWest Chemiluminescent Substrate (Thermo Fisher Scientific). RhoprACSL1- and RhoprACSL2-Flag were purified to near homogeneity (Suppl. Fig. 2). Compared with the membrane fractions, the specific activities of the purified proteins increased 16-fold for RhoprACSL1-Flag and 4-fold for RhoprACSL2-Flag.

2.9. ACS assay

ACS specific activity was assayed in a 200 μ L mixture containing 0.1 μ Ci [14 C]-palmitate (0.1 μ Ci/ μ L; Perkin-Elmer, Waltham, USA), supplemented with 50 μ M palmitate in 0.5 mM Triton X-100, 200 mM Tris (pH 7.4), 12 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM ATP, 250 mM CoA and 30 μ g of micelles composed by phosphatidylcholine and phosphatidylserine (2:1; w/w) [31, 32]. The reaction was initiated by adding 0.1–6 μ g of protein to the assay mixture. ACS activity was measured after 10 min incubation at room temperature. Other assay conditions are indicated in the figure legends. Substrate concentrations and incubation time used in the assays allowed measurement of enzymatic activity initial rates, and 3 technical replicates were done for each condition. The specific activities of recombinant RhoprACSL1- and RhoprACSL2-Flag proteins from different preparations were in the ranges of 4.6–9.8 and 53.2–101.8 nmol acyl-CoA $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$, respectively (Suppl. Fig 3 A and B)

2.10. Gene knockdown using double-stranded RNA (dsRNA)

dsRNA was synthesized by MEGAScript RNAi Kit (Ambion Inc., Austin, USA) using primers for *RhoprAcs11* or *RhoprAcs12* specific gene amplification (Supplementary Table 1) [14]. Unfed adult females were injected with dsRNA [14], and fed 3 days later. Knockdown efficiency was confirmed by qPCR. The bacterial *MalE* gene was used as a control dsRNA [33].

2.11. Phenotypic analyses

Adult females injected with dsRNA were fed with live-rabbit blood and immediately transferred to individual vials. The mortality rates and the number of eggs laid were recorded daily. The rate of egg eclosion was determined 21 days after oviposition. Laid eggs were visually inspected and classified into two categories: (i) normal morphology, for those with size, shape and color similar to the control group; (ii) abnormal morphology, when any alteration was observed.

2.12. Protein digestion analysis

Total midguts (epithelium and content) were obtained from dsRNA injected insects on different days after feeding and homogenized in cold PBS buffer (10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl) in 500 µl (final volume) and the protein content of the samples was determined [34].

2.13. Determination of TAG content

Abdominal fat body and total flight muscle were dissected from dsRNA injected insects, unfed or at days 5, 10 or 16 after feeding, and the organs were subjected to enzymatic TAG determination [14].

2.14. De novo lipid synthesis assay

At the 16th day after a blood meal, insects were carefully dissected, midgut and ovary were removed, and abdominal fat body was kept intact, associated with the ventral cuticle [9]. The organs were rinsed in cold 0.15 M NaCl and incubated *ex vivo* in humid chambers in the presence of 0.1 µCi [³H]acetate (0.1 µCi/µL; Perkin-Elmer) and Medium 199 (Sigma) supplemented with sodium phosphate (10 mM, pH 7.4) and sodium acetate (10 mM) (20 µL final volume) for 1h at room temperature. After incubation, the organs were collected, washed and homogenized in 250 µL of 0.15 M NaCl. Lipids were extracted [35] and the radioactivity present in organic phase was determined by scintillation counting.

2.15. Fatty acid oxidation assay

Fat bodies were obtained from five insects on day 16 after a blood meal, washed in 0.15 M NaCl and homogenized with a Potter-Elvehjem tube (15 strokes) in 200 µL of cold buffer H (10 mM Hepes-KOH, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT) with 0.002% v/v protease inhibitor cocktail (Sigma) and the total protein content was determined [34]. The homogenate (150 µg protein) was incubated with 8 µCi ³H-palmitate (0.1 µCi/µl; Perkin-Elmer) in the presence of 75 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 2 mg/mL fatty acid free albumin, 5 mM ATP, 5 mM DTT, 0.2 mM CoASH, 10 mM L-carnitine, 20 mM palmitate (200 µL final volume) at 28 °C for 30 min. Reactions were stopped with 200 µL cold perchloric acid (24%) and incubated at 4°C for at least 16h to precipitate palmitate-bound albumin. Supernatants (300 µL) were centrifuged at 14,000 rpm and 4°C for 5 min and lipids were extracted into CHCl₃ [35]. Radioactivity of the oxidized products in the aqueous phase was measured using a liquid scintillation counter.

2. 16. Transmission electron microscopy

Opercula from oocytes and eggs were detached and the samples were fixed, post-fixed and dehydrated as described [36]. Samples were embedded in Polybed 812 resin and thin sections were contrasted with lead citrate and uranyl acetate, followed by observation in a Zeiss 900 transmission electron microscope (Carl Zeiss Microscopy, Jena, Germany) operating at 80 kV.

2.17. Statistical analyses

For the statistical analysis of the qPCR results, the relative expression and C_t values were calculated from obtained C_t (cycle threshold) values [37]. The C_t mean values obtained from the experiments were submitted to Grubb's test to detect outliers [38], and the comparison among different conditions was made using One-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$. The relative expression values (2^{-C_t}) were used only for graph construction.

Other results were analyzed by Student's t-Test for the comparison of two different conditions, One-way ANOVA followed by Tukey's test or Two-way ANOVA followed by Bonferroni correction for the comparison among more than two conditions. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the Prism 5.0 software (GraphPad Software, San Diego, USA).

3. Results

Gene identification and sequence analyses

Twenty genes encoding ACS-like enzymes were identified in *R. prolixus* genome supercontigs. All identified sequences were also present in the predicted peptides dataset available at the VectorBase databank (Supplementary Table 2), in which the molecular functions of sequences are not given. A phylogenetic analysis of the ACS family members revealed that the insect predicted proteins are distributed among different ACS subfamilies (Fig. 1). Two *R. prolixus* sequences were similar to the short-chain ACS isoforms (ACSS), two sequences were closely related to long-chain ACS (ACSL) isoforms, and five sequences were similar to the very long-chain ACS (ACSVL)/fatty acid transport protein (FATP). Remaining sequences were grouped into the minor ACS subfamilies, ACSF1–5 (Fig. 1). Among these, ACSF5 clustered with a high number of sequences from lower animals, including *Caenorhabditis elegans*, *Drosophila melanogaster* and *R. prolixus*. None of the analyzed insect proteins were in the medium-chain ACS (ACSM) or ACS *bubblegum* (ACSBG) subfamilies (Fig. 1). It was unclear whether RhoprACS6 and RhoprACS8 represented a sister group of the ACSL proteins (Fig. 1).

Long-chain fatty acids are the most abundant fatty acid species in animal cells and are required for multiple processes, including the synthesis of complex lipids, energy production, and signaling. Different ACSL isoforms appear to determine distinct metabolic fates of their long-chain acyl-CoA products [1]. To address the roles of *R. prolixus* ACSL isoforms in lipid metabolism, we confirmed *RhoprAcs11* and *RhoprAcs12* CDS sequences by cloning (NCBI accession numbers: KY328601 and KT328602, respectively). Both genes showed the five conserved motifs (I-V) found in the ACSL subfamily members (Fig. 2A and Table 1). In spite of the high similarity found in these motifs, phylogenetic analysis indicated that RhoprACSL1 and 2 belong to two distinct ACSL groups (Fig. 2B). One ACSL group contains mammalian ACSL1, 5 and 6 isoforms and an arthropod isoform (here referred to as ACSL1) (Fig. 2B). The second ACSL branch was comprised of the mammalian ACSL3 and 4 proteins and a second arthropod isoform (ACSL2) (Fig. 2B).

***RhoprAcs1* and 2 transcripts were differentially regulated**

Mammalian ACSL isoforms exhibit tissue-specific transcriptional patterns [1]. To determine whether *R. prolixus* ACSL isoforms are similarly regulated, mRNA levels were measured in different organs under varying physiological conditions. The *RhoprAcs2* transcript was more abundant than that of *RhoprAcs1* in all the analyzed organs at the 4th day after a blood meal, when most organs are metabolically very active (Fig. 3A).

R. prolixus is a hematophagous insect with a very particular feeding behavior: in the laboratory, adults feed only once during periods as long as three weeks. To determine the influence of nutritional status in gene expression, transcripts were measured in the anterior midgut (stomach), posterior midgut (intestine) and fat body. Organs were obtained from unfed insects or insects at different days after feeding. The first week after feeding comprises the initial stage of digestion, intense lipid synthesis in the midgut, and the onset of lipid accumulation in the fat body. During the second week after feeding, the digestive process is almost complete and the mobilization of lipid reserves in the fat body is initiated. *RhoprAcs1* gene expression was constant in the anterior midgut along all the analyzed days, while *RhoprAcs2* transcription showed a 2-fold increase on the 10th day after feeding in comparison to fasted insects (Fig. 3B). In the posterior midgut, the expression of *RhoprAcs1* was induced after a meal and reached its maximal level on the 2nd day after feeding, when transcription level was ~50-times higher than in unfed insects (Fig. 3C). In this same organ, *RhoprAcs2* gene achieved its highest transcriptional levels (~4.5-fold increase) on the 10th day after feeding (Fig. 3C). A 3-fold increase was observed for *RhoprAcs2* in the fat body on the 16th day after feeding, and no variation in *RhoprAcs1* gene expression levels was detected in this organ (Fig. 3D).

Because ACSL is required for the TAG synthesis associated with egg growth and insect reproduction, we questioned whether *R. prolixus* ACSL genes would be modulated during the oogenesis cycle. Follicles were separated according to their length into four classes: 0.5 mm length (pre-vitellogenic oocytes, which represent an initial developmental phase); 1.0 and 1.5 mm (vitellogenic oocytes) and 2.0 mm (chorionated oocytes, which are fully grown) [8, 40, 41]. The expression of *RhoprAcs1* was constant during oocyte growth, and was very diminished in 2.0 mm follicles. *RhoprAcs2* mRNA level was higher at the initial phases of oocyte development, and gradually decreased as follicle matured (Fig. 3E).

RhoprACSL1 and 2 activated long-chain fatty acid species

To measure the ACSL activity of the *R. prolixus* enzymes, both isoforms were expressed in JM109 *E. coli* bacteria as Flag-tagged proteins (Suppl. Fig. 1) and purified to near homogeneity by immunoaffinity chromatography (Suppl. Fig. 2).

Mammalian ACSL proteins are able to activate a variety of long-chain fatty acids [30, 42–44]. *R. prolixus* recombinant isoforms activated long-chain fatty acids with different chain lengths and degrees of unsaturation. RhoprACSL1- and RhoprACSL2-Flag enzymes had similar apparent K_m values for palmitate (16:0; 4 and 6 μ M, respectively) and oleate (18:1; 2 μ M) (Fig. 4). However, RhoprACSL1-Flag had an apparent K_m value for arachidonate (20:4) that was 12-fold lower than that of RhoprACSL2-Flag (0.5 μ M versus 6 μ M) (Fig. 4).

RhoprACSL2-Flag showed a lower maximal specific activity for arachidonate than for palmitate and oleate (Fig. 4B). RhoprACSL1- and RhoprACSL2-Flag had broad pH optima between pH 7.5 – 9.5 and 6.5 – 9.5, respectively (Suppl. Fig. 3C).

Previous studies reported the ability of triacsin C, an alkenyl-N-hydroxytriazene fungal metabolite, and members of the oral antidiabetic drug thiazolidinedione class (rosiglitazone, pioglitazone and troglitazone) to inhibit specific isoforms of rat ACSLs [30, 44–48]. Triacsin C, but not rosiglitazone, inhibited both *R. prolixus* isoforms ($IC_{50} = 0.94 \pm 0.26 \mu M$ and $0.10 \pm 0.05 \mu M$, respectively) (Fig. 5).

Loss of RhoprACSL2 impaired fatty acid oxidation

Insects were injected with dsRNA designed for *RhoprAcs11* or *RhoprAcs12* to silence mRNA. Transcription of target genes in all the analyzed organs was reduced 70–90% (Suppl. Fig. 4) for at least two weeks (data not shown). Phenotypic alterations were not detected in insects with knockdown of *RhoprAcs11*. However, reduced transcription of *RhoprAcs12* increased the average insect lifespan upon starvation by 20% (39 days versus 33 days in control animals) (Fig. 6A). *RhoprAcs12* deficiency also resulted in delayed dietary protein digestion. On the 10th day after feeding, protein content of the midgut lumen was about 5.5-times higher in *RhoprAcs12*-silenced insects than in control animals (Fig. 6B).

Because the fat body is a primary site of lipid accumulation [12], we questioned whether the loss of ACSL would affect the content of TAG, the main lipid class stored in the fat body [12]. The reduction of *RhoprAcs12* transcripts resulted in a 2.5-fold increase in TAG (2.5-fold increase) and a 70% increase in protein content on the 16th day after feeding (Figs. 6C and D).

To determine whether the increased TAG content in the fat body of RhoprACSL2-deficient insects was due to altered lipid metabolism, we examined the rates of *de novo* lipid synthesis and fatty acid β -oxidation. Lipid synthesis from acetate was not affected by dsRNA treatment (Fig. 6E), but the rate of fatty acid β -oxidation was decreased by 90% in fat body samples from RpACSL2 deficient females (Fig. 6F).

RhoprACSL2 is required for reproduction

In comparison to controls, RhoprACSL1 deficient animals did not show significant differences in the total number of eggs laid during the reproductive cycle induced after the blood meal. In contrast, this number decreased by 20% in RhoprACSL2 deficient insects (Fig. 7A). In addition, during the oviposition cycle, which starts around the 5th day after feeding, the eggs laid by these insects showed remarkable reduction in their viability, and the eggs laid after day six of the cycle were unable to hatch (Fig. 7B).

Eggs laid by insects with reduced expression of *RhoprAcs12* showed morphological changes, such as abnormal size, shape and color patterns (Fig. 7C). Moreover, some of the hatched nymphs were born dead and were physically associated with egg structures (Fig. 7C). The frequency of aberrant egg morphologies increased at later days of the oviposition cycle (Fig. 7D), coinciding with the observed decrease in viability rates (Fig. 7B).

Morphological alterations were also detected in oocytes (Fig. 8A). Transmission electron microscopy of oocytes and laid eggs showed lack of the plasma membrane and numerous aberrant lipid inclusions (Fig. 8B). Because ACSL is required for normal lipid homeostasis, we questioned whether the eggs with abnormal morphology could reflect alterations in cellular lipid content. Females injected with dsRNA against *RhoprAcs12* showed a 55% decrease in TAG content and a 45% reduction in protein content in eggs laid on the 8th day of the oviposition cycle (Figs. 8C and D).

4. Discussion

The synthesis of acyl-CoA from fatty acid and CoA is a critical and highly conserved metabolic process [49]. Here, we identified 20 putative genes coding for ACS proteins in *R. prolixus* (Fig. 1, Supplementary Table 2). The insect genome contained members from the major ACS subfamilies reported [24, 50] with the exception of ACSM and ACSBG. ACSM protein sequences were found only in vertebrates, while ACSBG sequences were found in vertebrates and *D. melanogaster* (Fig. 1).

Numerous sequences from *R. prolixus*, *D. melanogaster*, *C. elegans* and *S. cerevisiae* matched an ACSBG sister group (Fig. 1). This ACS group was previously reported as a “worm/fly clade” by Watkins, Maignel, Jia and Pevsner [24] and is renamed in the present work as ACS family 5 (ACSF5). Two of the *D. melanogaster* proteins from ACSF5 clade – Drome10 (Pudgy) and Drome14 (CG6178) – show medium- and long-chain ACS activity [51, 52] and are functionally equivalent to ACSBG proteins. Based on substrate preference, it is possible that ACSF5 subfamily members supplanted ACSM proteins to activate medium-chain fatty acids in yeast and non-chordate animals.

Because they use fatty acid substrates of 12 to 20 carbons, ACSL proteins would be expected to synthesize most of the acyl-CoA species within animal cells [50]. Based on primary sequence analysis, we found that the two *R. prolixus* ACSL proteins are members of distinct subgroups. RhoprACSL1 is closely related to *S. cerevisiae* Faa2p, and mammalian ACSL1, 5 and 6, as well as to two uncharacterized *D. melanogaster* sequences (Drome 8 and 9) (Fig. 2B). A second ACSL subgroup is comprised of *S. cerevisiae* Faa1p, Faa3p and Faa4p, the mammalian ACSL3 and 4 proteins, and two sequences from fly (Drome 15 and 16) (Fig. 2B).

Except for *D. melanogaster*, which had four ACSL isoforms, all the Arthropoda species analyzed in the present study showed two ACSL isoforms which had the same phylogenetic pattern as *R. prolixus*. Based on gene structure and protein homologies, the existence of two ACSL subgroups was previously proposed, although a phylogenetic analysis was not performed to test this hypothesis [50]. Our data suggest an ancient origin of ACSL subgroups that could result in specialized functions gained during their evolution. This idea is in strong agreement with the current hypothesis that different ACSL isoforms drive the partitioning of different pools of acyl-CoA toward specific metabolic pathways [1].

The transcription of the *RhoprAcs1* genes was affected by cell type, nutrient status of the animal and developmental oocyte phase (Fig. 3). Both transcripts were induced during early

stages of oogenesis (Fig. 3E). However, *RhoprAcs11* showed a highly controlled transcriptional pattern in the posterior midgut, with mRNA levels being induced at the onset of the digestive process when the organ is committed to lipid biosynthesis (Fig. 3C) [5]. In contrast, *RhoprAcs12* was highly expressed after prolonged starvation both in the midgut and in the fat body (Figs. 3B, C and D). This change in gene expression suggests an association of RhoprACSL2 with fatty acid oxidation.

A relationship between ACSL and lipid catabolism has been proposed in flies. In fed *D. melanogaster*, insulin release and the consequent FOXO inactivation result in repression of *Pudgy* gene transcription, increased TAG accumulation and reduced lipid oxidation. This effect is reversed in flies after starvation, when increased *Pudgy* expression culminates in higher rates of lipid catabolism. It is expected to be facilitated by *Pudgy*-mediated channeling of fatty acids derived from lipolysis towards mitochondrial oxidation [52].

Purified RhoprACSL1- and RhoprACSL2-Flag proteins exhibited similar apparent K_m values (2 to 6 μM) when palmitate and oleate were provided as substrates (Fig. 4). However, the apparent K_m observed for arachidonate was 12-times lower in RhoprACSL1-Flag (0.5 μM) than in RhoprACSL2-Flag (6 μM), suggesting a higher affinity of RhoprACSL1-Flag for this substrate (Fig. 4).

To our knowledge, this is the first report of enzymatic parameters of an ACSL isoform from arthropod species. In the present study, the K_m values obtained from the different long-chain fatty acid substrates were similar to those reported for rat ACSL1 and ACSL3–6 [30, 44]. A much higher palmitate K_m (100 μM) was reported for the murine recombinant ACSL4 when an spectrophotometric assay was used to measure enzyme activity [43].

The effect of ACS inhibitors on invertebrate ACSL isoforms remains poorly investigated. In *X. laevis*, oocytes incubated with triacsin C matured more rapidly, suggesting that ACSL proteins might be required for oocyte arrest [53]. However, this report did not show direct inhibition of ACSL activity by triacsin C. As observed for most of mammalian isoforms [30, 44], *R. prolixus* ACSL1 and RhoprACSL2 recombinant proteins were not inhibited by rosiglitazone, however both proteins were highly sensitive to triacsin C inhibition (Fig. 5). The IC_{50} values were 0.9 and 0.1 μM for RhoprACSL1- and RhoprACSL2-Flag, respectively, while the IC_{50} determined for murine triacsin C-sensitive isoforms ranges from 4 to 7.5 μM [30, 44].

In *R. prolixus*, the knockdown of *RhoprAcs12* but not *RhoprAcs11* extended the insect's lifespan upon starvation by 20% (Fig. 6A). Although several studies have reported an interaction between lipid stores and longevity in animals [54–59], the mechanisms underlying this effect are not well understood. In flies, genetic induction of β -oxidation rates increases the average lifespan [60]. Conversely, in *C. elegans* increased lifespan is associated with a decrease in energy metabolism, possibly due to a reduction in oxidative stress [61].

Insects with reduced *RhoprAcs12* expression had delayed digestion (Fig. 6B). ACS proteins are expected to affect the uptake of exogenous fatty acids via vectorial acylation, which assures the unidirectional transport of fatty acids into the cells [25, 62, 63]. It is possible that the lack of RhoprACSL2 limits the acyl-chain substrates available for the synthesis of the

lipids [5]. After a blood meal, *R. prolixus* and other hemipteran insects produce a phospholipidic bilayer (perimicrovillar membrane) that covers the midgut epithelial cells and guarantees compartmentalization of the luminal content [64]. This structure also allows anchoring of digestive enzymes [65]. It is likely that RhoprACSL2 activates and channels dietary fatty acids into pathways of intracellular PL synthesis that constitute the perimicrovillar membranes [66], thereby directly affecting digestion rates.

The knockdown of *RhoprAcs12* also affected lipid homeostasis in the fat body. During the second week after feeding, TAG mobilization was impaired (Fig. 6C). TAG constitutes the main lipid reserve in this organ and is expected to be consumed during prolonged starvation [12], possibly providing acyl substrates for the mitochondrial β -oxidation. In fact, the ablation of RhoprACSL2 strongly reduced β -oxidation rates but not *de novo* lipid synthesis (Figs. 6E and F), supporting a role for this protein in fat body lipid catabolism.

The ACSL contribution to β -oxidation has also been reported in mice. Animals lacking ACSL1 showed 50% reduction in liver acyl-carnitines [67]. ACSL1 is correlated to catabolic function in highly oxidative tissues, and the knockout of this gene in adipose tissue, heart, or skeletal muscle dramatically reduced fatty acid oxidation [68–70].

The deficiency of RhoprACSL2 also affected insect reproduction, with reduced rates of oviposition and egg hatching (Figs. 7A and B). Moreover, oocytes and laid eggs exhibited remarkable alterations in external morphology and cellular ultrastructure (Figs. 7C, 8A and 8B), and in nutrient composition, with decreased content of TAG and protein (Figs. 8C and D). These effects were more evident at the onset of the second week after feeding when almost all laid eggs were defective and unable to hatch (Figs. 7C, 7B, 8C and 8D). Apparently, RhoprACSL2 deficient oocytes had severely altered maturation, and impaired embryo development (Figs. 7 and 8).

During oogenesis, the oocytes incorporate lipids from lipophorin [8, 13, 71]. These lipids are used to synthesize a variety of glycerolipids that are important for both oocytes and embryos [13]. RhoprACSL2 could be required to activate and channel acyl substrates used in TAG and PL synthesis, resulting in the formation of oocyte lipid stores and cellular membranes. PL membranes are essential not only for cellular division, but they are also required to delineate yolk organelles and, consequently, to aid in nutrient storage in the embryos [72].

In oocytes of *M. musculus* and *X. laevis*, the chemical or genetic inhibition of ACSL affects the meiotic cycle, causing early cell maturation and impaired embryo development [53]. In mouse heart, *Acs11* gene expression is induced starting on the first post-natal day. This change coincides with the increase after birth in fatty acid oxidative pathways for energy production, corroborating the role of cardiac ACSL1 in channeling acyl-chains for mitochondrial β -oxidation [73]. In flies, an ACSL4 homologue is involved in the transcriptional regulation of *gap* genes and determination of body axes. Although the mutation in *Acs14* gene causes defective embryonic segmentation, the complete mechanism underlying this effect remains unknown [21].

In summary, our data support the idea that the different ACSL isoforms are functionally distinct. Although the role of RhoprACSL1 remains unclear, RhoprACSL2 was shown to be

essential for the channeling of substrates towards lipid oxidation in the fat body and also for successful reproduction. It will be important to determine whether the effects of RhoprACSL2 on reproduction are a consequence of a direct impact on energetic homeostasis or a result of regulatory functions played by acyl-CoA and/or their derivatives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Jorge M. Medina, Lilian S. C. Gomes and Heloisa S. L. Coelho for excellent technical assistance and José de S. Lima Junior and Gustavo Ali for insect care. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM) (to K.C.G.) and U.S. National Institutes of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases Grants DK59935 (to R.A.C.).

Abbreviations:

ACS	acyl-CoA synthetase
ACSS	short-chain ACS
ACSM	medium-chain ACS
ACSBG	<i>bubblegum</i> ACS
ACSL	long-chain ACS
ACSVL	very long-chain ACS
DAG	diacylglycerol
FATP	fatty acid transport protein
PL	phospholipid
TAG	triacylglycerol

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Figure 1: Phylogenetic analysis of the ACS family.

Amino acid sequences of ACS from different organisms (145 taxa) were aligned and the dendrogram was constructed using MEGA 4.0 software with the Maximum Parsimony algorithm and a Bootstrap of 1000 replications. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). The final dataset contained 48 informative positions. The accession numbers for protein sequences used in these analyses are presented in Supplementary Table 3. The protein RhoprACS5 was excluded from the dataset due to the lack of the positions used for phylogenetic analysis. Sacce, *Saccharomyces cerevisiae*; Caeel, *Caenorhabditis elegans*; Drome, *Drosophila melanogaster*; Rhopr, *Rhodnius prolixus*; Danre, *Danio rerio*; Musmu, *Mus musculus*; Homsa, *Homo sapiens*. ACS, acyl-CoA synthetase; ACSS, short-chain ACS; ACSM, medium-chain ACS; ACSL, long-chain ACS; ACSBG, bubblegum ACS; ACSVL/FATP, very long-chain ACS/fatty acid transport protein; ACSF1–5, ACS families 1–5.

Figure 2: Domain structure and phylogenetic analysis of ACSL proteins.

(A) *R. prolixus* ACSL proteins: red, yellow, blue, green and purple boxes, motifs I–V, respectively. Numbers indicate amino acid positions. (B) Amino acid sequences of ACSL from different organisms (45 taxa) were aligned and the dendrogram was constructed using MEGA 4.0 software with the Maximum Parsimony algorithm and a Bootstrap of 1000 replications. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). The final dataset contained 351 informative positions. The numbers at the branching points are Bootstrap values. The accession numbers of the protein sequences used in these analyses are presented in Supplementary Table 4. Acypi, *Acyrtosiphon pisum*; Aedae, *Aedes aegypti*; Anoga, *Anopheles gambiae*; Apime, *Apis mellifera*; Bommo, *Bombyx mori*; Drome, *Drosophila melanogaster*; Camfl, *Camponotus floridanus*; Danre, *Danio rerio*; Homsa, *Homo sapiens*; Musmu, *Mus musculus*; Nasvi,

Nasonia vitripennis; Rhopr, *Rhodnius prolixus*; Sacce, *Saccharomyces cerevisiae*; Trica, *Tribolium castaneum*; Xenla, *Xenopus laevis*.

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Figure 3: *RhoprAcsl1* and *RhoprAcsl2* are differentially expressed in *R. prolixus* organs.

(A) Anterior and posterior midgut, fat body and ovary were obtained from adult females four days after blood feeding. Alternatively, anterior midgut (B), posterior midgut (C) and fat body (D) were obtained from adult females before (day 0) or at different days after feeding. (E) Follicles of different lengths were isolated from females at day 4 after a blood meal. Differences between cDNA levels were determined by qPCR analysis showing mRNA expression levels relative to anterior midgut values (A), unfed insects (B-D) or 0.5 mm follicles (E), set as 1.0. The results represent the means $\pm$ S.E.M. of 3–4 independent experiments. Different letters above bars indicate significant differences ($P < 0.05$). (*) and (**): $P < 0.05$ and $P < 0.001$, respectively, compared to reference values. AM: anterior midgut; PM: posterior midgut; FB: fat body; OV: ovary.

Figure 4: RhoprACSL1-Flag and RhoprACSL2-Flag activate long-chain fatty acids. Flag-purified RpACSL1- (0.25 – 1 μg) (A) and RpACSL2-FLAG (0.1 – 0.5 μg) (B) were assayed for ACS activity. Palmitate (16:0), oleate (18:1) or arachidonate (20:4) concentrations varied as indicated. Apparent K_m values for palmitate, oleate and arachidonate were: 4 ± 1 , 2 ± 0.5 and 0.5 ± 0.1 μM, respectively, for RpACSL1-FLAG, and 6 ± 0.7 , 2 ± 0.3 and 6 ± 3 μM for RpACSL2-FLAG. . Results are representative for 3 independent experiments.

Figure 5: Triacsin C but not rosiglitazone inhibits RpACSL1- and RpACSL2-FLAG. Flag-purified RpACSL1- (0.25 – 1 μg) (A) and RpACSL2-FLAG (0.1 – 0.5 μg) (B) were assayed for ACS activity in the presence of varying concentrations of triacsin C or rosiglitazone in Me₂SO (2.5% of final assay reaction). Activities in the absence of drugs were 5 nmol acyl-CoA · min⁻¹ · mg protein⁻¹ for RpACSL1-FLAG, and 52 nmol acyl-CoA · min⁻¹ · mg protein⁻¹ for RpACSL2-FLAG. Results are representative for 3 independent experiments.

Figure 6: *RhoprAcs12* knockdown affects lifespan, digestion and fat body lipid metabolism.

Fasted adult females were injected with 1 μg of dsRNA for *RhoprAcs11*, *RhoprAcs12* or *Mal* (control) genes. Insects were fed on the third day after injection. (A) Insect mortality was followed ($n = 13$). (*): $P < 0.05$ when compared to dsMal control, by the Log-Rank test. (B) The amount of total protein in the midgut was determined at different days after the blood meal. Results are means $\pm$ S.E.M. for $n = 8$. (**): $P < 0.01$ when compared to dsMal values by two-way ANOVA. (C-F) Insects were dissected before feeding (day 0) or at different days after the blood meal. The amount of triacylglycerol (C) and protein (D) was determined. Fat bodies obtained at the 16th day after feeding were incubated with 10 mM [^3H]-acetate to measure lipid synthesis (E). Alternatively, fat bodies were homogenized and incubated in the presence of 20 mM [^3H]-palmitate to measure lipid oxidation (F). Results are means $\pm$ S.E.M. $N = 9$ for (C) and (D). $N = 3$ for (E) and (F). (***) : $P < 0.001$ when compared to dsMal, by two-way (C and D) or one-way (F) ANOVA.

Figure 7: *RhoprAcsl2* knockdown affects the production, hatching and morphology of eggs. Fasted adult females were injected with 1 μ g of dsRNA for *RhoprAcsl1*, *RhoprAcsl2* or *Mal* (control) genes. Insects were fed on the third day after injection. (A) Cumulative oviposition was followed (n = 21). (*): $P < 0.05$ when compared to dsMal control, by two-way ANOVA. (B) The eggs were collected at different days after the onset of oviposition and the egg hatching percentage was determined (n = 929). (C) Representative egg and nymph morphologies. Scale bar = 2 mm. (D) Frequency of morphological alterations among eggs laid by females injected with dsRNA against *RhoprAcsl2* (n = 929).

Figure 8: *RhoprAcs12* knockdown affects composition and ultrastructure of oocytes and laid eggs. Fasted adult females were injected with 1 μg of dsRNA for *RhoprAcs11*, *RhoprAcs12* or *Mal* (control) genes. Insects were fed on the third day after injection. Ovarioles were dissected from females on the 8th day of the oviposition cycle. (A) Representative ovariole morphology. Scale bars = 1 mm. (B) The ultrastructure of fully grown oocytes (2 mm in length) and eggs laid on the 8th day of the oviposition cycle was analyzed by transmission electron microscopy. Ch, chorion; Ld, lipid droplet; Pm, plasma membrane; Rib, ribosomes; YG, yolk granule. Scale bars = 1 μm . Alternatively, eggs were collected at different days of the oviposition cycle. The amount of triacylglycerol (C) and protein (D) was determined. Results are means $\pm$ S.E.M. for 3 independent determinations. (*), (**) and (***): $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, when compared to dsMal, by two-way ANOVA.

Table 1:

ACSL motifs.

Protein	Motif I	Motif II	Motif III	Motif IV	Motif V
SacceFaa1p	Y T SGT T GL P KS A	-TVFTAM P LFHSTA	FQ-Q T LVR	DAWYRC G DL L KAD E YGLWYFL D RM G	WL K N
SacceFaa2p	F T SGT T GL P KG V	-YDMC F L P L A H I F E	QG-Y G L T E	DGW F STGDVAFIDGKGRISVIDRV K	K L K R
SacceFaa3p	Y T SGSISAPKG V	-RVLS F L P L A H I F E	IG-Y G L T E	DGW F RTGDIVEWTPKGQV K I D R K K	K L K R
SacceFaa4p	Y T SGS T GT P KG V	-RIIA F L P L A H I F E	IG-Y G L T E	DGW F RTGDIAEWTPKGQV K I D R K K	K L K R
RhoprACSL1	Y T SGT T GN P KG V	DIMIS F L P L A H M L E	VEGY G Q T E	LG W HH T GDIGQWLPNGTL K I D R K K	K S K R
DromeACSL1	Y T SGT T GN P KG V	DIMIS F L P L A H M L E	LEGY G Q T E	LG W HH T GDIGQWLPNGTL K I D R K K	K A K R
HomsaACSL1	F T SGT T GN P KG A	DTLIS F L P L A H M F E	YEGY G Q T E	DG W L H TGDIGKWLPNGTL K I D R K K	K A K R
MusmuACSL1	F T SGT T GN P KG A	DVLIS F L P L A H M F E	YEGY G Q T E	DG W L H TGDIGKWLPNGTL K I D R K K	K A K R
HomsaACSL5	F T SGT T GD P KG A	DVAIS Y L P L A H M F E	YEAY G Q T E	DG W L H TGDIGRWLPNGTL K I D R K K	K S K R
MusmuACSL5	F T SGT T GD P KG A	DVTIS Y L P L A H M F E	FEAY G Q T E	DG W L H TGDIGRWLPNGTL K I V D R K K	K A K R
HomsaACSL6	F T SGT T GN P KG A	DVHIS Y L P L A H M F E	YEGY G Q T E	DG W L H TGDIGKWLPAGTL K I D R K K	K A K R
MusmuACSL6	F T SGT T GN P KG A	DVLIS F L P L A H M F E	YEGY G Q T E	DG W L H TGDIGKWLPAGTL K I D R K K	K A K R
RhoprACSL2	Y T SGS T GV P KG V	-VFLG F L P L A H V F E	IQGY G L T E	RR W FR T GDIGECHPDGV I K I D R K K	K L K R
DromeACSL2	Y T SGS T GT P KG V	-VLIG F L P L A H V F E	IQGY G L T E	RR W FR T GDIGECHPDGV I K I D R K K	K L K R
HomsaACSL3	Y T SGS T GL P KG V	-VYIG Y L P L A H V L E	GQGY G L T E	QR W LC T GDIGEFEPD G CL K I D R K K	K L K R
MusmuACSL3	Y T SGS T GI P KG V	-VYIG Y L P L A H V L E	GQGY G L T E	QR W LC T GDIGEFDPD G CL K I D R K K	K L K R
HomsaACSL4	Y T SGS T GR P KG V	-TYIG Y L P L A H V L E	GQGY G L T E	QR W FC T GDIGEFHPD G CL Q I D R K K	K L K R
MusmuACSL4	Y T SGS T GR P KG V	-TYIG Y L P L A H V L E	GQGY G L T E	QR W FC T GDIGEFHPD G CL Q I D R K K	K L K R

ACSL sequences were aligned using the ClustalW algorithm. Grey boxes: highly conserved residues function (based on Watkins et al. [24]). Black boxes: aromatic residues required for catalytic function (based on Hisanaga et al. [39]). Accession numbers of analyzed sequences are provided in Supplementary Table 3. Sacce, *Saccharomyces cerevisiae*; Rhopr, *Rhodnius prolixus*; Drome, *Drosophila melanogaster*; Homsa, *Homo sapiens*; Musmu, *Mus musculus*.