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Differential expression of trypsin-3 and phosrestin II genes in the main malaria vector, *Anopheles darlingi*, from the Brazilian Amazon region

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Genet. Mol. Res. 16 (4): gmr16039852

Received October 14, 2017

Accepted November 08, 2017

Published December 15, 2017

DOI http://dx.doi.org/10.4238/gmr16039852

ABSTRACT. Anopheles darlingi is the most anthropophilic mosquito related to Plasmodium infection of malaria, causing significant morbidity and mortality in South America. Pyrethroid chemical has been used to control mosquitos. We analyzed the expression of trypsin-3 and phosrestin II genes implicated to feeding and resistance to insecticides, immune response and sensory antenna mechanisms, respectively, of larvae and adult of A. darlingi, through quantitative reverse transcription polymerase chain reaction (qRT-PCR). We aimed to validate the similarity in nucleotide sequences of A. darlingi RNA sequencing libraries by *in silico*, and qRT- PCR, owing to their possible effects on the ability to spread disease. The expression of trypsin-3 and phosrestin II was higher in the first and second instar larvae as compared with that in adults. These differentially expressed trypsin-3 and phosrestin II genes do not provide us evidence that both genes participate in pyrethroid resistance. The signaling pathway involving both genes requires further study. Preliminary phylogenetic relationships and the accumulation of mutations analysis in both genes were also compared with trypsin and phosrestin sequences of 15 and 17 other anopheline species, respectively, to obtain a mutational rate of 0.02 on phylogenetic trees. Trypsin gene of A. darlingi and A. albimanus clustered into the same group and was distinct from the species of A. gambiae complex and other anopheline. For phosrestin II, A. darlingi

was separated from the remaining species from Africa, Asia, and Europe. Although the groups showed low to moderate support, it is possible to infer that both genes may belong to two evolutionary groups: one presents in the anopheline species of New World and other in the anopheline species of Old World, and be useful for future studies.

Key words: qRT-PCR; Immune response; Resistance to insecticides; Phylogenetic relationship; Malaria.

INTRODUCTION

Malaria affects more than 90 countries around the world. In 2013, about 789 cases of malaria were reported in the Brazilian Amazon region, 26% less than those reported in 2012; of these, 67% cases occurred only in the state of Amazonas, Brazil (SIVEP, 2014). *Anopheles darlingi* transmits the *Plasmodium* species of human malaria across South America, but the species is especially important as vector of malaria in the Amazonian countries. In the Brazilian Amazon region, the variation in the population density of *Anopheles darlingi* and records of malaria cases are correlated with the annual hydrological cycle (periods of rains and flooding) and various anthropic activities (deforestation, road construction, agriculture, and mining). These factors, in combination with biological and ecological aspects, anthropophily, and susceptibility of infection by *Plasmodium* of *A. darlingi* have contributed to the proliferation of this species and occurrence of this parasitosis (Deane, 1(989; Tadei et al., 1998, 2016).

Malaria cases are directly related to the resistance of mosquitoes to synthetic insecticides. There are four main groups of neurotoxic insecticides: organochlorines, organophosphates, carbamates and pyrethroids, one of the most relevant advances occurred in the 20th century for controlling insects (Mellanby, 1992). Mosquito resistance to at least one insecticide used for malaria control has been identified in 64 countries (WHO, 2012). Pyrethroids, such as DDT and lambda-cyhalothrin have caused resistance in *A. darlingi* populations from Quibdo locality, Colombia, with a mortality rate of 65-75% (Quiñones et al. 1987; Suarez et al. 1990; Fonseca-González et al. 2009).

Insecticide resistance and phylogenetic studies on gene sequences in *A. darlingi* are still scarce. The glutathione S-transferase (GST) gene is a good indicator of resistance to the insecticide deltamethrin, once GST gene family is known to be primarily involved in oxidative stress metabolism. In a recente study of *A. darlingi* from Coari city, State of Amazonas, Brazil, Azevedo-Junior et al. (2014) obtained the GST sequence of cDNA libraries of *A. darlingi* from the Brazilian Amazon region (Rafael et al. 2010) for differential expression level by quantitative Real Time–Polymerase Chain Reaction (qRT-PCR) after exposure to insecticide of deltamethrin. The authors suggested that the GST gene was a good indicator of resistance to deltamethrin in *A. darlingi*, because of its high level of expression in relation to the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Azevedo-Junior et al. (2014). The authors argue, it is possible that other mechanisms (e.g., metabolic resistance) are involved with the tolerance to pyrethroid in the *A. darlingi*, as has already been reported in Colombia (Quiñones et al. 1987; Fonseca-González et al. 2009).

Studies related to biology, genome, physical map, karyotype, polytene chromosome mapping of repetitive DNA, HSP70 (Rafael et al. 2003, 2004, 2010), and actin (Bridi et al. 2013) genes, as well as the evolution of *A. darlingi* (Tadei et al., 2016) have provided important data for comparative genomics and to establish chromosome homologies relative to other primary anopheline vectors of malaria in South and Central America and Africa (Rafael et al. 2010; Bridi et al. 2013). Studies on population genetics of *A. darlingi* from the Brazilian Amazon with respect to isoenzymes (Santos et al. 1999) and microsatellites (Scarpassa and Conn 2007; Lima et al. 2010) have demonstrated low to moderate genetic structure. On the other hand, Emerson et al. (2015) studied 12 populations of *A. darlingi* from across the complex Brazilian landscape by single nucleotide polymorphisms (SNPs) and showed that *A. darlingi* likely represents a species complex in Brazil.

The geographical distribution of *A. darlingi*, the main malaria vector in South America, spans from northern Argentina to the southern United States of America. There are two related species of *A. albimanus*, the main malaria vector in Central America (Harbach and Kitching, 2016). Studies have compared the evolutionary divergence between *A. darlingi* and others anophelines such as *A. gambiae*, *A. funestus*, and *A. quadrimaculatus* as well as culicines such as *Aedes aegypti* and *Culex quinquefasciatus* (Moreno et al. 2010). The separation between anophelines and culicines occur at the beginning of the Cretaceous and the radiation among *A. gambiae, A. funestus, A. quadrimaculatus*, and *A. darlingi* occurred approximately 79 million years ago.

Phylogenetic analyses of cDNA sequences from several mosquito species have allowed determination of patterns and mechanisms of chromosome evolution and reconstruction of ancestral genes among related species (Rafael et al. 2010). *Anopheles* species exhibited a dynamic genomic evolutionary profile; some determinants of vectorial capacity such as chemosensory genes failed to show elevated turnover but diversified through protein sequence changes (Neafsey et al. 2015). There is an evidence of variation in genes controlling immunity to *Plasmodium* parasites, such as those encoding cuticular and salivary proteins as well as that conferring metabolic insecticide resistance. The phosrestin II and trypsin genes are involved in the immune response and resistance of *Anopheles* mosquitoes to insecticides. Mohammed et al. (2015) inferred that the resistance to insecticides is the ability of an organism to tolerate high doses of toxic substances, which could be lethal to other individuals from the population of the same species. Phosrestin II, a member of the arrestin-A family, is a protein that has a signal transduction function, a process wherein a signal is transmitted to induce a change in the activity or state of a cell, such as the phosphorylation of proteins (Hardin et al. 2016). Arrestin-A in the fly *Calliphora* regulates and controls phosphorylation and dephosphorylation of a visual pigment activated by light. Arrestin-A can be associated with resistance to insecticides in *Culex pipiens pallens*, as the gene arrestin acts in the process of resistance through opsin G-protein-coupled receptors **(**GPCRs), which regulate signal transduction pathways and have various roles in the physiology of insects (Moreno et al. 2010).

Trypsin or serine protease is a proteolytic enzyme that acts on chymotrypsinogen and peptidases. In mosquitoes, the regulation of immune response occurs through the involvement of classes of antimicrobial peptides (AMPs) such as serine proteases, which can be found in viruses, bacteria, and even eukaryotes (Biessmann et al. 2005). These enzymes recognize and cleave peptide bonds by triggering a cascade of defense reactions through the synthesis of AMPs against microorganisms (Biessmann et al. 2005). Trypsin is the principal enzyme responsible for the digestion of the blood meal in anophelines (Müller et al. 1995), as seven different genes (*antryp*1 to 7) of digestive trypsin have been described in an adult *A. gambiae* (Müller et al. 1995).

In this work, we studied the differential expression of the target genes trypsin-3 and phosrestin II in two stages of development of *A. darlingi*: first and second instar larvae and adults. We compared trypsin-3 and phosrestin II genes of *A. darlingi* transcriptome through multiple alignment of the supercontigs from 17 other species of *Anopheles* to understand the phylogenetic evolutionary relationships of both *A. darlingi* genes as compared to those of the homologous genes from other *Anopheles* species. In the light of limited genomic and transcriptomic data for *A. darlingi*, this study may be important in evolution of this mosquito and its role in malaria transmission.

MATERIALS AND METHODS

Adult female *A. darlingi* were collected from Puraquequara neighborhood (3º 3′ 6.95″ S and 59º 52′ 31.38″ W), situated in the suburbs of Manaus, Amazonas State, Brazil. Specimens were identified according to the taxonomic keys of Forattini (1962) and Consoli and Lourenço-de-Oliveira (1994). After oviposition and eclosion of the eggs, a group of first and second instar larvae were stored -80 ^{o}C, and other larvae samples were maintained in an insectary until their adult stage. Total RNA (100 µg) was extracted in triplicates from the first and second instar larvae (n, 360 × 3 = 1080) and adults (n, 30 × 3 = 90) using the extraction and purification kit from QIAGEN (Biotechnology Brazil). RNA was quantified with a Nano Drop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Life Technology Brazil). We used the Ribo Green RNA reagent to quantify RNA, as it is far more precise as compared with the direct quantification of RNA by measuring absorbance at 230 nm wavelength. However, the relative amount of RNA was also determined using denaturing electrophoresis. Moreover, the expression of a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization.

The similarity in contigs 637 (phosrestin II [arrestin-A]), 517 (trypsin-3), and 433 (GAPDH, housekeeping gene to normalize expression levels) was evaluated using the databank (http://sysbiol.cbmeg.unicamp.br.adarlingi/) for *A. gambiae* (https://www.vectorbase.orgdatabase). The primers for phosrestin II, trypsin-3, and GAPDH (Table 1) were designed using the programs Gene Runner and Primer 3 (Tm of 58-60°C and 60% GC content) and synthesized by Integrated DNA Technologies IDT.

Table 1. Primers specific for phosrestin II, trypsin-3, and GAPDH of *Anopheles darlingi* for qRT-PCR.

Genes	Sequences (5' – 3')	References
Trypsin - 3 of *A. darlingi* (contig 517)	Fw: 5'-TTCGAAAGGAAACCGTATCG-3' Rv: 5'-ATCCGTTGGAGATGATCGAG-3'	Database of the cDNA library *An.darlingi* of INPA<https://valine.cenargen.embrapa.br/*Anopheles*20080 7/>
Phosrestin II (Arrestin A) of *A. darlingi* (Contig 637)	Fw: 5'-CTTGCTCGGGTCCTTGATG-3' Rv: 5'-ATACCCGACGCTGGCTTC-3'	https://sysbiol.cbmeg.unicamp.br.Adarlingi/ Vector Base: https://www.vectorbase.org/blast
GAPDH gene reference of *A. darlingi* (Contig433)	Fw: 5'-CGAGTACGGCTACTCCAACC-3' Rv: 5'-CTGGCACACAAGTGAGGCTA-3'	

The cDNA fragments of phosrestin II (arrestin-A, contig 637), trypsin-3 (contig 517), and GAPDH (contig 433) were checked for similarity with the sequences from GenBank (http://www.ncbi.nlm.nih.gov). The complementary strand of mRNA (cDNA) was obtained using a kit from Promega. cDNA amplification reactions were performed using an ABI-7500™ Real-Time PCR System with SYBR® Green (Life Technology, Brazil). The conditions for the gene amplification reactions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Samples were analyzed at each stage in biological triplicates.

The cDNAs of larvae (first and second instar) were utilized as control samples for the comparison among different developmental stages of *A. darlingi*, as it showed little variation in the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) between biological replicates; hence, it was reasonable to compare the gene expression between non-fed adults and fed larvae, thereby allowing validation of expression levels of phosrestin II, trypsin-3, and GAPDH genes in qRT-PCR assays using mean and standard deviation.The gene expression level of trypsin-3, phosrestin II, and GAPDH was calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Statistically significant differences ($p < 0.05$) in the gene expression level was calculated using the Student's *t*-test (Zar 1984), which enabled us to determine the differences between the means of each factor in adults and larvae.

The sequence of trypsin-3 gene of *A. darlingi* (accession number ADAC007736) was aligned with those of the ortholog genes obtained from the vector database https://www.vectorbase.orgdatabase, which included 15 trypsin supercontigs (scaffold) for other *Anopheles* species. For phosrestin II sequence of *A. darlingi* (accession number ADAC002761), 18 ortholog sequences were found. For both supercontigs of *A. darlingi*, we failed to find corresponding sequences for *A. aquasalis*, another member of the *Nyssorhynchus* subgenus.

The phylogenetic tree was generated using trypsin-3 supercontig of *A. darlingi* and compared with the trypsin sequences of 15 additional anopheline species, which were obtained from VectorBase (https://www.vectorbase.orgdatabase). The cDNA sequences downloaded were of *A. albimanus*, *A. arabiensis. A. atroparvus*, *A. christyi*, *A. coluzzii*, *A. culicifacies*, *A. epiroticus*, *A. funestus*, *A. gambiae*, *A. maculatus*, *A. melas*, *A. merus*, *A. quadriannulatus*, *A. sinensis* and *A. stephensi* species. In this analysis, cDNA sequences of *Culex quinquefasciatus* were downloaded and used as an outgroup (Table 2).

Phylogenetic analysis of phosrestin gene sequences was performed using the criteria applied for trypsin-3 supercontig of *A. darlingi* and included sequences from 18 other anopheline species retrieved from VectorBase (www.vectorbase.orgdatabase). These included *A. albimanus*, *A. arabiensis*. *A. atroparvus*, *A. christyi*, *A. coluzzii*, *A. culicifacies*, *A. diru*, *A. epiroticus*, *A. faraute*, *A. funestus*, *A. gambiae*, *A. maculatus*, *A. melas*, *A. merus*, *A. minimus*, *A. quadriannulatus*, *A. sinensis* and *A. stephensi* Trypsin and phosrestin sequences of *C. quinquefasciatus* (outgroup) were also analyzed (Table 3). Considering the alignment of trypsin-3 and phosrestin II supercontigs from *A. darlingi*, we also queried gene sequences for *Aedes aegypti*, *A. albimanus* and *C. quinquefasciatus.* Sequences of *C. quinquefasciatus* were used as outgroup to facilitate the interpretation of the phylogenetic relationships, as trypsin-3 and phosrestin II supercontigs of *A. darlingi* were phylogenetically distant from those of *A. albopictus.* These criteria were established to avoid any bias related with the presence of different sequences.

Table 2. Trypsin-3 supercontig of *Anopheles darlingi* (ADAC007736 - GO: 0006508) was compared with trypsin genes from 15 *Anopheles* species and *Aedes albopictus* (outgroup) to estimate sequence similarity and generate phylogeny tree of trypsin gene.

Mosquito species	Sequence access number	Target id%	Query id%	Gene Ontology: biological process
Anopheles albimanus	AALB001881	85.77%	84.86%	0006508
Anopheles arabiensis	AARA016534	61.68%	59.51%	
Anopheles atroparvus	AATE013252	66.12%	57.04%	
Anopheles christyi	ACHR010215	62.04%	59.86%	
Anopheles coluzzii	ACOM030207	61.82%	59.86%	
Anopheles culicifacies	ACUA012421	59.12%	57.04%	
Anopheles epiroticus	AEPI015411	62.77%	60.56%	
Anopheles funestus	AFUN016449	60.56%	53.52%	
Anopheles gambiae	AGAP008296	61.68%	59.51%	
Anopheles maculatus	AMAM020996	63.14%	60.92%	
Anopheles melas	AMEC003978	61.68%	59.51%	
Anopheles merus	AMEM014426	61.31%	59.15%	
Anopheles quadriannulatus	AQUA016663	61.73%	60.21%	
Anopheles sinensis	ASIS001831	63.10%	60.21%	
Anopheles stephensi	ASTE016302	64.23%	61.97%	
Aedes aegypti	AAEL006425	58.55%	56.69%	
Aedes albopictus	AALF005199	58.82%	56.34%	
Culex quinquefasciatus	CPIJ007079	56.93%	54.93%	

Table 3. Phosrestin II supercontig of Anopheles darlingi (ADAC002761 - GO: 0007165) was compared with phosrestin II (Arrestin-A) sequences from 18 Anopheles species and Aedes albopictus (outgroup) to estimate sequence similarity and generate phylogenetic tree of Arrestin gene family.

Mosquito species	Sequence access number	Target id%	Query id%	Gene Ontology: biological process
Anopheles albimanus	AALB007920	25.50%	99.74%	0007165
Anopheles arabiensis	AARA003662	93.85%	95.56%	
Anopheles atroparvus	AATE021140	93.21%	93.21%	
Anopheles christyi	ACHR005623	96.87%	96.87%	
Anopheles coluzzii	ACOM022471	91.29%	95.82%	
Anopheles culicifacies	ACUA015243	97.13%	97.13%	
Anopheles dirus	ADIR009271	97.13%	97.13%	
Anopheles epiroticus	AEPI011115	43.85%	96.87%	
Anopheles farauti	AFAF007746	96.34%	96.34%	
Anopheles funestus	AFUN002187	97.13%	97.13%	
Anopheles gambiae	AGAP010134	95.82%	95.82%	
Anopheles maculatus	AMAM019456	97.65%	97.65%	
Anopheles melas	AMEC009122	96.08%	96.08%	
Anopheles merus	AMEM003932	96.08%	96.08%	
Anopheles minimus	AMIN007215	96.87%	96.87%	
Anopheles quadriannulatus	AQUA002255	96.08%	96.08%	
Anopheles sinensis	ASIS018985	94.26%	94.26%	
Anopheles stephensi	ASTE007929	97.65%	97.65%	
Aedes aegypti	AAEL013535	92.17%	92.17%	
Aedes albopictus	AALF000534	91.64%	91.64%	
Culex quinquefasciatus	CPIJ003101	91.15%	91.38%	

Site: www.vectorbase.org, access: September 17, 2017

Trypsin-3 and phosrestin II supercontigs were aligned in the AliView program (Larsson, 2014). The AliView program automatically aligns the sequences using MUSCLE (Multiple Sequence Comparison by Log-Expectation), as shown by Edgar (2004). MrBayes v.3.2.6 program (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck 2003) was used to perform the preliminary phylogenetic evolution analyses based on the Bayesian Inference (BI) algorithm. After the analyses, the trees were edited in the program FigTree v. 1.4.2 (Rambaut, 2014). Values in each cluster represent the bootstrap supports, a statistical re-sampling test (Efron, 1979), which highlights the reliability of the groupings generated in the phylogenetic evolutionary analysis.

RESULTS

In *A. darlingi*, GAPDH expression was constant and no variation was observed between samples; therefore, this gene was chosen as the constitutive gene (used as a control for the expression). In contrast, phosrestin II and trypsin-3 super contigs were differentially expressed and hence, selected as the target genes. qRT-PCR analysis showed that GAPDH gene in larvae (first and second instar) and adults of *A. darlingi* was differentially expressed, with relatively low variation (*t*-test = 0.41, $p > 0.05$).

The Ct values for trypsin analyses performed in plates were not constant for both larvae and adults of *A. darlingi*. Results showed variations between the biological replicates of larvae, with one of these replicates showing a Ct value over the maximum measurable level. The trypsin gene showed significant overexpression in the first and second instars larvae (SD =1.0) as compared with that in the adults (SD $=5.5^{-5}$) (t=0.02, $p < 0.05$; Figure 1).

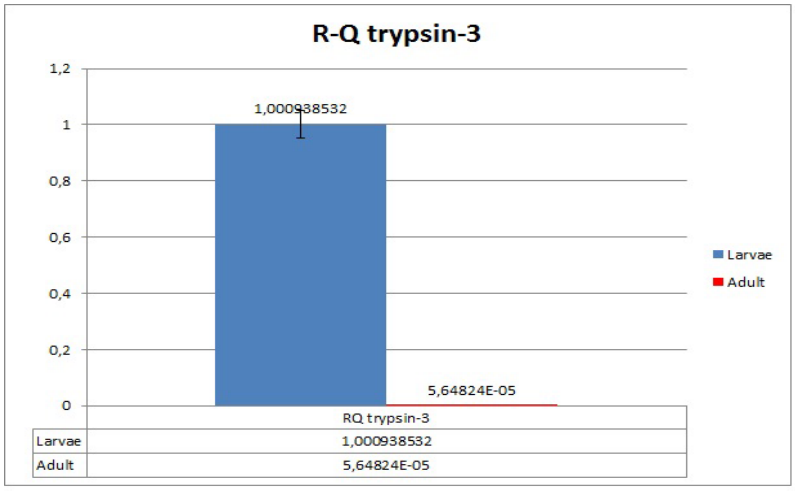

Figure 1. Relative quantification (R-Q) of trypsin-3 in *A. darlingi* showed that it is preferentially expressed in larvae (blue color) as compared with that in the adults (red color); data are presented as mean ± standard deviation.

The *in-silico* analysis of the expressed sequence tag (EST) database of *A. darlingi* showed that the expression level of trypsin-3 was higher in larvae (6.2%) as compared with that in adults (3.1%; Figure

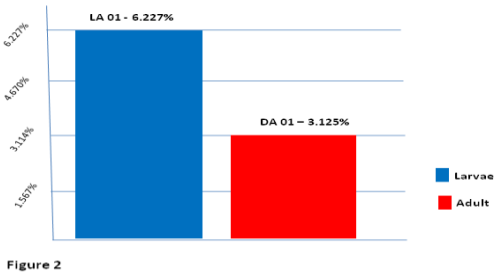

Figure 2. *In silico* normalization of trypsin-3 for the most expressed clusters (gi|157113343|ref|XP_001657786.1|trypsin[Aedesaegypti]gi|108877776|gb|EAT42001.1| trypsin [*Aedes aegypti*]) in A. darlingi(https://valine.cenargen.embrapa.br/Anopheles200807/) –A. darlingi/adult (DA) and larvae (LA). The genetic expression of trypsin-3 decreases during the development of *A. darlingi*.

The differential expression of phosrestin II (arrestin-A) was statistically significant in larvae, which showed a greater level of expression (t=0.06) as compared with the expression observed in adults (0.7%) (Figure 3), thereby corroborating the *in-silico* analysis of the expression of phosrestin II (arrestin-A) from the EST library of *A. darlingi.* The percentage expression of phosrestin II in adults (0.7%) was lower than that observed in larvae (7.18%; Figure 4). These results are consistent with our initial hypothesis in that trypsin-3 and phosrestin (arrestin-A) of *A. darlingi* are a functional orthologs to 15 trypsin and 18 phosrestin sequences for other anopheline species retrieved from Vector Base.

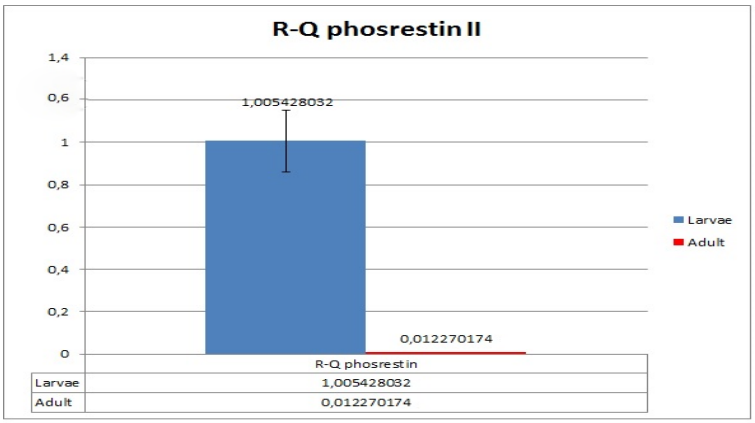

Figure 3. Relative quantification (R-Q) of phosrestin II during different stages of development in *A. darlingi* (larvae and adults); data are presented as mean ± standard deviation. Phosrestin II is preferentially expressed in larvae of *A. darlingi.*

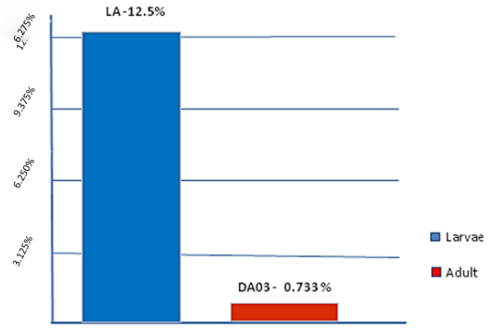

Figure 4.

Figure 4. *In silico* normalization of phosrestin II for the most expressed clusters (gi|157113343|ref|XP_001657786.1|trypsin [*Aedes aegypti*]gi|108877776|gb|EAT42001.1| trypsin *A. aegypti*) in each library of *A. darlingi*

(https://valine.cenargen.embrapa.br/Anopheles200807/) -*A. darlingi*/adult (DA) and larvae (LA). The genetic expression of phosrestin II decreases during the development of *A. darlingi.*

The phylogenetic relationships and accumulation of mutations over time in trypsin-3 supercontig of *A. darlingi* were compared with those in the trypsin sequences of *A. albimanus*, *A. funestus*, *A. culicifacies*, *A. epiroticus*, *A. quadriannulatus*, *A. melas*, *A. christyi*, *A. coluzzii*, *A. gambiae*, *A.* arabiensis, *A. merus*, *A. stephensi*, *A. maculatus*, *A. atroparvus* and *A. sinensis.* Sequences of *C. quinquefasciatus* were included as outgroup. The BI analysis generated a topology comprising three main clusters (Figure 5). The *A. aegypti* and *A. albopictus* clusters were also used in this analysis so that the topology generated a higher resolution. The anophelines species were grouped into the other two clusters, being one grouped into *A. darlingi* and *A. albimanus*; another one, major grouping consisted of sequences of all other *Anopheles* species, and was subdivided into four main subgroups: first subgroup clustered sequences of *A. funestus, A. culicifacies, A. epiroticus, A. melas* and *A. christyi*; second subgroup clustered sequences of *A. coluzzii, A. gambiae, A. arabiensis* and *A. merus*; third subgroup clustered sequences of *A. stephensi* and *A. maculatus*, and fourth subgroup clustered sequences of *A. atroparvus* and *A. sinensis*. All the clusters were strongly supported by high bootstrapping support (95% - 100%).

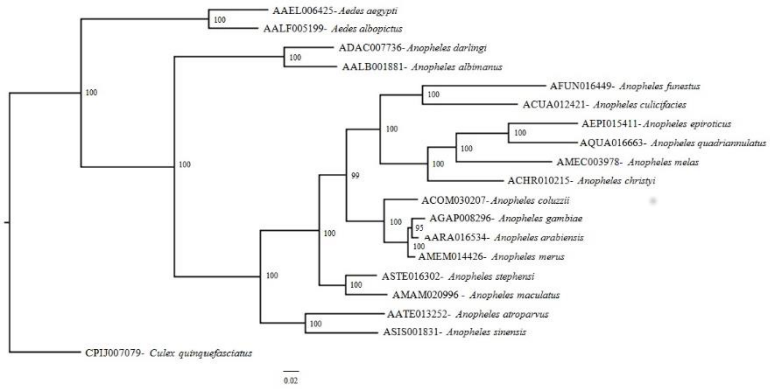

Figure 5. The phylogenetic tree of trypsin-3 supercontig (ADAC007736) of *A. darlingi* and 15 other anopheline species.The sequence CPIJ007079 of *Culex quinquefasciatus* was used as an outgroup. The sequences were aligned with AliView (Larsson, 2014) and the phylogeny of the gene constructed by Bayesian Inference program (MrBayes v.3.2.6) according to Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003.The bar indicates a genetic distance.

The phylogenetic tree of phosrestin II supercontig of *A. darlingi* was compared with those of 21 others species being 18 *Anopheles* and two *Aedes* species and *C. quinquefasciatus* (outgroup). The topology generated is divided in two clusters, in the first and more basal group appeared *A. albopictus* and *A. aegypti*. The second cluster contain all species of the *Anopheles* genus composed in five subgroups: first subgroup clustered sequences of *A. darlingi* and *A. albimanus*; second subgroup clustered sequences of *A. atroparvus* and *A. sinensis*; third subgroup clustered sequences of *A. epiroticus*, *A. christyi*, *A. merus*, *A. quadriannulatus A. arabiensis*, *A. gambiae*, *A. coluzzii*, *A. melas;* and fourth subgroup clustered sequences of *A. maculatus*, *A. stephensi*, *A. funestus*, *A. minimus*, *A. culicifacies* and fifth subgroup clustered sequences of *A. dirus* and *A. farauti*. The topology was generated with a high degree of support (bootstrapping support of 66-100% (Figure 6).

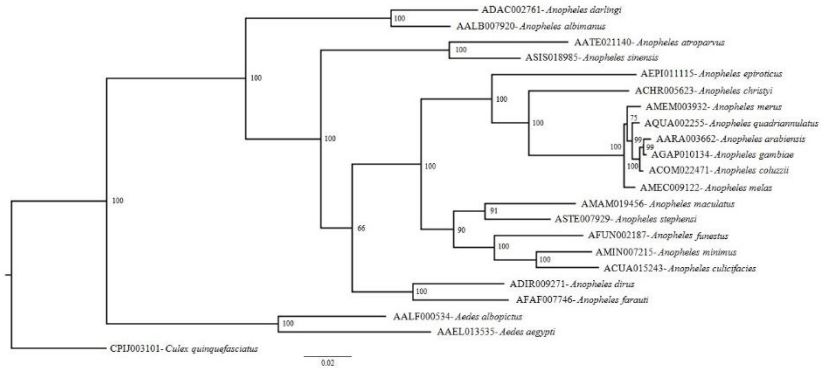

Figure 6. The phylogenetic tree of Phosrestin II (ADAC002761) of *A. darlingi* and 18 others anopheline species. The sequence CPIJ003101 of *Culex quinquefasciatus* was used as an outgroup. The sequences were aligned with AliView (Larsson, 2014) and the phylogeny of the gene constructed by Bayesian Inference program (MrBayes v.3.2.6) according to Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003.The bar indicates a genetic distance.

DISCUSSION

In the transcriptome of the salivary gland of *A. darlingi*, the conserved housekeeping proteins are associated with metabolic processes, protein synthesis, and signal transduction. Comparative analyses of this transcriptome in *A. darlingi* and *A. gambiae* have revealed that the housekeeping proteins are 86% identical between the two species (Calvo et al. 2004). Trypsin or serine protease is a class of AMPs found in viruses, bacteria, and even eukaryotes (Biessmann et al. 2005). AMPs have evolved specifically to fight microbial flora or malaria parasites, as these are highly expressed in response to infections by bacteria or species of *Plasmodium* (Dimopoulos et al. 1997; Dimopoulos 2003). This response of the immune system in mosquitoes against microorganisms is regulated by Toll and Imd (immunodeficiency) pathways (Christophides

et al. 2002) as well as through two transcription factors (NF-kappaB Rel1 and Rel2). The signal transduction pathways communicate for the recognition of pathogens and transcription activation causes amplification of these signals. In mosquitoes, a third pathway, Janus kinase/signal transducers and activators of transcription (JAK/STAT), is also involved in immune response and gets activated after infection by bacteria (Hoffmann and Reichhart, 2002; Michell and Kafatos, 2005; Biessmann et al. 2005). The signal receptors for the initiation of the Imd pathway in response to microbial infection is still unknown. Although the digestive trypsins in insects interact differently with protein inhibitors as compared to those in mammals, there is a great similarity between the Imd pathway of insects and the tumor necrosis factor alpha (TNF-α) pathway of mammals.

Seven genes for digestive trypsins (AnTryp1 to 7) have been described in *A. gambiae* (Müller et al. 1995). Public databanks of NCBI revealed that the gene product trypsin-3 of *A. darlingi* has higher similarity to trypsin from *A. aegypti*. Trypsin of *A. aegypti* is related to digestion in adults and third and fourth instars larvae. In this study, qRT-PCR of trypsin-3 gene showed that its expression was significantly higher in the first and second instar larvae as compared with that in mosquito adults.

Among the trypsin family, seven digestive enzymes have been determined in *A. gambiae* (Müller et al. 1995); of these, the expression of trypsin-3 is higher in larvae and adults unfed with blood. In the present study, *A. darlingi* adults were unfed with blood and comparative analysis of trypsin-3 of *A. darlingi* larvae against public databanks revealed that the expression of trypsin-3 in larvae was higher as compared with the expression of trypsins (AnTryp1 to 7) in adult *A. gambiae* (Müller et al. 1995). Bacteria are commonly found in the intestinal flora of *A. darlingi* collected from the field, which is in line with the findings of the bacterial genome in the complete genome of *A. darlingi* (Marinotti et al. 2013). This observation suggests the presence of a catalytic mechanism of AMPs such as serine protease-endopeptidase in the intestine of *A. darlingi* (Marinotti et al. 2013). Transcriptome analysis of *A. aquasalis* females fed with sugar showed the expression of trypsin-type peptidases (Costa-da-Silva et al. 2014). Muller et al. (1995) reported that *A. gambiae* expressed a set of seven trypsin genes, including *antryp1* and *antryp2*, which are induced after feeding with blood, while *antryp3*, *antryp4*, and *antryp7* are constitutively expressed and undetectable after feeding with blood (Borges-Veloso et al. 2015). Understanding of the physiology and presence of specific trypsins in mosquito tissues that are involved in host-pathogen interactions may provide relevant findings for the development of alternative and more effective control measures for the vectors (Shahabuddin and Costero, 2001; Chertemps et al. 2010).

Insecticides resistance studies of *A. darlingi* are scarce. The glutathione S-transferase (GST) gene (KC890767) obtained from cDNA libraries of larvae and adults of *A. darlingi* (Rafael et al., 2010) was compared by *in silico* mapping (Azevedo-Junior et al. 2014). The GST sequence of *A. darlingi* clustered with *A. gambiae* on the same branch, with an identity of greater than 70%, suggesting that the sequence evolved from a common ancestor. These authors also used the GST of *A. darlingi* for phylogenetic analysis to understand the base composition of the most recent common ancestor between *A. darlingi*, *A. gambiae* (Q8MUR9), *A. aegypti* (Q16P78) and *C. quinquefasciatus* (B0WFX0), and showed divergence between *A. gambiae*, *A. Aegpti* and *C. quinquefasciatus*, and a homology relationship between GSTS1-1 genes of *A. darlingi* and *A. gambiae*.

Little is known about the expression of phosrestin gene in larvae of mosquitoes. Although originally characterized as visual system proteins, arrestin 1 of *Drosophila melanogaster* (DmArr1) and *A. gambiae* (AgARR1) was shown to localize to sub sensillar olfactory neurons of the third antennal segment and function in peripheral olfactory signaling and cell biology (Merrill et al. 2002). In addition, phosrestin II (arrestin 1) gene has been identified with olfactory sensory receptors related to transcription in *A. gambiae* (Biessmann et al. 2005). The expressed genes coding for olfactory sensory proteins are thought to be preferentially expressed in male *A. gambiae* and probably involved in the localization of sources of nectar for food or attracting females for mating. Furthermore, differences such as habitat, climatic conditions, behavior, and genetic variability between populations of *Anopheles* species may influence the level of gene expression (Matsumoto et al. 1994). In our study, the gene expression level of phosrestin II was significantly higher in larvae (1.0%) as compared with that in adults in *A. darlingi* (0.01%). These results are in agreement with the *in-silico* analysis of the expression of the enzyme phosrestin II of *A. darlingi*, wherein the percentage expression in adults (0.70%) was approximately ten times lower than that in larvae (7.18%). Further investigations should be conducted on the expression of this gene in blood-fed adults (females) of *A. darlingi* to compare the results with those obtained from larvae of this study.

The expression of trypsin gene occurred mainly in the larvae and was reduced in adults, which may be attributed to the non-blood feeding, corroborating the *in-silico* data of the EST library of this anopheline.

Further investigations are necessary to test these larvae against fed adults to confirm this hypothesis.

In *A. darlingi*, the expression of phosrestin II was significantly higher in first and second instar larvae (t=0.06) as compared with that in adults (0.7%), with GAPDH used as a housekeeping gene. According to Tristan et al. (2011), the housekeeping gene used as a control showed no significant variation in its expression level. From a biological point of view, this observation may be related to the cellular processes associated with resistance to insecticides, as discussed for *Culex pipiens pallens*. The arrestin gene acts in the process of resistance and has been associated to various functions in the physiology of insects (Sun et al. 2012), including *A. aegypti*, the vector of the arboviruses, yellow fever, dengue, Zika, and chikungunya (Matsumoto and Yamada, 1991).

The enhanced expression level of trypsin-3 and phosrestin II genes in *A. darlingi* larvae (first and second instar) as compared with that in adults may be related to responses to stimuli, as observed for gene expression of trypsin-3 and phosrestin II in *A. aegypti* and *A. gambi*ae, respectively. This point should be taken into consideration while performing studies with these organisms.

Molecular phylogeny of *Anopheles* mosquito's studies are scarce (Neafsey et al., 2015). These authors generated a molecular phylogenetic tree of 16 *Anopheles* species and other Diptera, as outgroup, according to Maximum Likelihood method. In addition, Harbach and Kitching (2016) studied the phylogenetic relationships between species of anophelines by cladistic methods. Although there are studies of *Anopheles* phylogenetic trees, there are no records on the evolutionary behavior of a single gene or contigs among these mosquitoes. The topologies recovered from the trypsin-3 and phosrestin II sequences of *A. darlingi* showed some coincidences regarding the findings on the phylogenetic trees of some species of anophelines (Neafsey et al., 2015; Harbach and Kitching, 2016).

In our study, we validated the expression of trypsin-3 and phosrestin II of *A. darlingi* transcriptome (first and second instar larvae and unfed adults) through the multiple alignment of the supercontigs from 18 other species of anophelines. According to Neafsey et al. (2013, 2015), sets of genes in functional categories such as odorant and taste receptors linked among vector or species-specific traits show high evolutionary rates; e.g., chemosensory genes, which are diversified by changes in the protein sequence. The evolutionary origin and advantage of this observation was explained by a scenario in which these genes duplicated from a common ancestor before the anopheline species diverged and each one evolved with a different digestive function. In support of this hypothesis, Muller et al. (1995) showed that AnTryp 1 of *A. gambiae* from Africa was equally active to hydrolyze albumin and hemoglobin, while AnTryp 2 preferentially hydrolyzed hemoglobin. Two trypsins (AnTryp 1 and AnTryp 2) are also induced in the midguts after the ingestion of a protein meal such as observed in *A. albimanus* from Central America (Hörler and Briegel, 1995), *A. aquasalis*, *A. albitarsis* (Caroci et al., 2003) and *A. darlingi* from South America (Okuda et al., 2005).

In the phylogenetic tree, trypsin-3 and phosrestin II supercontigs of *A. darlingi* showed close similarity to that of *A. albimanus*, both belonging to *Nyssorhynchus* subgenus — an efficient malaria vector in the New World. In addition, another cluster comprised *A. gambiae* sibling species such as *A. gambiae* s.s., *A. arabiensis*, and *A. quadriannulatus*, which include the most important malaria vectors in the Old World. Although the high support values, it is possible to infer that trypsin-3 gene of *A. darlingi* may belong to two evolutionary groups or gene families—one present in the *Anopheles* species of the New World and another, in the species of the Old World. Similar situation was observed with phosrestin II gene. The phylogenetic tree also revealed that most of *Anopheles* species from Africa and Asia evolved into subgroups 1 and 2 for the trypsin-3 and subgroups 2 and 3 for the topology of phosrestin II, respectively. High-level gene conservation between first and second instar larvae and adults of *A. darlingi* for trypsin and phosrestin II clusters was also partially related, indicating some level of evolutionary conservation between them in both trees. These findings have to be viewed with caution, as they may represent evolutionary divergence in trypsin-3 and phosrestin II sequences of *A. darlingi.*

CONCLUSION

Overall, these findings suggest that the different groups or families of both genes observed in the phylogenetic analyses may reflect distinct selective pressures acting physiologically in these anopheline species. Neafsey et al. (2013) suggest that this dynamism of genes in *Anopheles* species may contribute to their flexible ability to take advantage of new ecological niches, including adaptation to humans as primary hosts.

Indeed, our results has a great meaning in evolution of *A. darlingi*, and may contribute mainly to its remarkable adaptability in taking advantage of new ecological niches and association with landscape changes. This mosquito has been observed frequently at the modified sites, especially in and around houses (Tadei et al., 1998, 2016) in several localities in many countries, confirming its high degree of association with human wellings. The eradication of malaria has been a challenge or years, and it is important to increase the knowledge for better understanding of the evolutionary relationships between trypsin and phosrestin genes and their roles in immune response to bacteria, fungi, and *Plasmodium* species of *Anopheles* mosquitoes.

ACKNOWLEDGEMENTS

This study was supported by Project PAPAC, Edital 020/2013, process No. 1570/2013/FAPEAM, as well as, EDITAL FAPEAM/MCT/CNPq/CT-INFRA N°. 012/2009, process N°. 2680/2009 – FAPEAM, both awarded to MSR, project ADAPTA II- MCTI-INPA/CNPq-2017 headed by Dr. Adalberto Luiz Val. We would like to thank Dr. Michel Vincentz and Renato Vicentini, UNICAMP, Campinas, São Paulo State, Brazil, and Dr. Vera Maria Fonseca de Almeida e Val, INPA, Manaus, Amazonas State, Brazil for technical help. We would also like to thank Dr. A. Leyva for his help with English translation and editing of the manuscript.

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