GMR
The Original by FUNPEC-RP

# Differential expression of genes related to the immune response of *Anopheles* (*Nyssorhynchus*) *darlingi* in the Brazilian Amazon Basin

**L.R.L. Fernandes[1] and M.S. Rafael[2]**

[1,2] Instituto Nacional de Pesquisas da Amazônia (INPA), Coordenação de Sociedade, Ambiente e Saúde (COSAS), e Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva (PPG-GCBEv), Manaus, Amazonas, Brasil

Corresponding author: M.S. Rafael
E-mail: msrafael@inpa.gov.br
All three authors contributed equally to this work

Genet. Mol. Res. 17 (4): gmr18076
Received July 09, 2018
Accepted October 22, 2018
Published December 18, 2018
DOI http://dx.doi.org/10.4238/gmr18076

**ABSTRACT.** *Anopheles* (*Nyssorhynchus*) *darlingi* is the primary vector of human malaria in South America. Immune responses in mosquito vectors of malaria are mainly regulated by genes of the Toll and IMD pathways through the transcription factors NF-kappa-β, *Rel1* and *Rel2*, which are controlled by the negative regulatory genes *Cactus* and *Caspar*. We measured the expression levels of *Rel1*, *Rel2*, *Caspar* and *Cactus* genes, which are related to the immune system, in adult females of *A. darlingi* after blood feeding compared to adult females without blood feeding (controls) due to their possible effects on the ability of becoming infected with species of *Plasmodium* and spreading malaria. Quantitative expression was determined by real-time PCR, using the reference genes *GAPDH* and *β-actin*. The expression levels of *Rel1*, *Rel2*, *Caspar* and *Cactus* varied significantly at 4, 8, 14 and 24 h in mosquitoes that had fed on blood compared to control insects (0 h), with significantly greater expression at 24 h after blood feeding. Relative expression levels among these genes varied at the different post blood feeding times. This information adds to our understanding of the insect immune

response system and related questions involved in understanding the biology and control of this mosquito.

**Key words**: Gene expression; qRT-PCR: *Rel1*; *Rel2*; *Caspar*; *Cactus*

## INTRODUCTION

Malaria is one of the main public health problems of the poor regions of tropical and subtropical countries (Who, 2014). It is caused by five species of the genus *Plasmodium* (*Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*), with the first four species being transmitted to humans exclusively through the bite of infected *Anopheles* mosquitoes, according to Deane (1988). It is estimated that about 3.4 billion people in the world live in areas at risk of contracting malaria, due to environmental conditions in lakes and reservoirs with clear, shaded water, which is favorable for the development of species of the genus *Plasmodium* and its mosquito hosts (Forattini, 1962; Deane, 1988; WHO, 2014). In 2013, records showed 198 million cases of the disease, with about 584,000 deaths, most of which were children in Africa (WHO, 2014).

In Brazil, 99.6% of records of malaria are concentrated in the Amazon (Tadei et al., 1998; Tadei et al., 2016), where in 2014 there were over 143,250 cases of this disease (Ministério da Saúde, 2015). *Anopheles darlingi* is the principal vector of malaria parasites in South America (Rachou, 1958; Hiwat and Bretas, 2011). It shows high rates of biting, anthropophily and susceptibility to infection by species of the genus *Plasmodium* (Deane, 1988; Tadei et al., 1998; Hiwat and Bretas, 2011). Another important local feature is the variety and size of breeding sites of *A. darlingi*, ranging from large basins (lakes and backwaters) to puddles, tire tracks and ditches during the rainy season (Galvão and Damasceno, 1944). These factors, along with its strong preference for feeding on human blood, contribute to *A. darlingi* being the principal vector of malaria in this country, because even though there has been a decrease in population density, the efficiency of transmission of plasmodia remains high (Tadei et al., 1998; Hiwat and Bretas, 2011).

In the last decade, advances in the understanding of the immune system of insect vectors of disease have resulted in the identification of many genes that play important roles in their immune responses (Dimopoulos et al., 2000; Barillas et al., 2000; Bladin and Levashina, 2004; Riehle et al., 2006). The immune response system of insects is not specific as in vertebrates; it maintains the same mechanism of immune response against bacteria, fungi or parasites, including cellular and humoral defenses (da Silva, 2002). Cellular defenses are based on hemocytes, such as phagocytic cells, granulocytes, plasma cells and coagulocytes. Also, humoral defenses consist of soluble proteins in the hemolymph, which take hours or days to achieve an effective concentration to combat invading organisms, most of them being inhibitors of fungi and bacteria (da Silva, 2002).

In some insects, injury or infiltration of bacteria induces the synthesis of antimicrobial peptides (AMPs) (Cociancich et al., 1994). The cecropins, a class of such AMPs, are amphiphilic molecules with cylindrical structures, and they have two poles that bind negative peripheral proteins of the bacterial phospholipid membrane, weakening the lipid bilayer and causing leakage of the cytoplasm and cell apoptosis (da Silva, 2002). In anophelines infected with malaria plasmodia, injection of cecropins has been shown to

cause the formation of pores in the cell membrane of *Plasmodium* oocysts, arresting development at the sporozoite stage (Gwadz et al., 1989).

Defensins are another class of antimicrobial peptides. They are cationic defense proteins rich in cysteine and capable of forming pores in the membranes of bacteria and fungi. A study of silencing of the expression of the defensin gene in *Anopheles gambiae* demonstrated the occurrence of this protein in antimicrobial defenses against gram-positive bacteria (Blandin et al., 2002).

Another important protein in the immune defense system of insects is the AMP gambicin. This AMP has evolved specifically to combat microbial flora or malaria parasites, because it is highly expressed in bacteria or infections by *Plasmodium* species (Dimopoulos, 2003).

Serine proteases are also important proteins in the immune defense system and form the largest superfamily of peptidases. They are enzymes that break peptide bonds of proteins using serine at the catalytic site, and are found in a wide range of organisms, from viruses and bacteria to eukaryotes (Rawlings and Barrett, 1993). Serine proteases are required for recognition of *Plasmodium* species, against which they trigger amplification cascades of immune defense reactions, causing the synthesis of AMPs (Dong et al., 2006).

The regulation of the immune response system of *Anopheles* mosquitoes is performed by the Toll and IMD (immunodeficiency) pathways and two transcription factors (NF-kappaB *Rel1* and *Rel2*), which are controlled by the negative regulators *Cactus* and *Caspar* (a homolog of the Fas-associated factor of mammals), which are connected to *Rel1* and Dredd (a homolog of caspase-8), respectively (Garver et al., 2009; Cirimotich et al., 2010). The Toll and IMD pathways send a pathogen recognition signal to inhibit the negative regulators *Cactus* and *Caspar*, activating the nuclear translocation of transcription factors NF-kappaB, *Rel1* and *Rel2*, which induce the transcription of effector genes such as those encoding AMPs (Garver et al., 2009).

In *A. gambiae*, the transcription factor *Rel1*is controlled by the Toll pathway, in which Toll is an analogue of Dif factor in *Drosophila melanogaster*, being *Rel2* controlled by the IMD pathway, which is an ortholog of the factor Relish of *D. melanogaster* (Lemaitre et al., 1995; Meister et al., 2005). In both insects, *Cactus* is a negative regulator of the Toll pathway, which under normal conditions is bound to Dif/*Rel1*, retaining the transcription factor in the cytoplasm (Belvin and Anderson, 1996; Christophides et al., 2002). When *Toll* is activated, the signal is transmitted through adapter proteins (Pelle and Tube), triggering the phosphorylation of *Cactus*, marking it for degradation. The degradation of *Cactus* releases Dif/*Rel1*, which migrates to the cell nucleus, activating the transcription of AMP genes (Belvin et at., 1996).

The activity of IMD/*Rel2* (Relish/*Rel2*) in *Drosophila* is controlled by its own inhibitory factors (repetitions of ankyrin), which can be cleaved by Dredd (catalytic protein), which causes the activation of Relish/*Rel2* (Stoven et al., 2000; Stoven et al., 2003). Negative regulation of this factor (Relish/*Rel2*) is controlled by the regulator *Caspar*, specific for the IMD pathway, which binds to Dredd, preventing the cleavage of Relish/*Rel2* (Kim et al., 2006). After observing an increase in resistance to exposure to Gram-negative bacteria and increased constitutive position of AMPs, Kim *et al.* (2006) determined that *Caspar* blocks the nuclear translocation of Relish and Dredd (a necessary prerequisite for Relish to enter the nucleus) and cleaves Relish; this is the target of suppression by *Caspar*

in the IMD pathway (Kim et al., 2006). Silencing of *Caspar* prevents the development of *P. falciparum* in various *Anopheles* species (Garver et al., 2009).

We analyzed the expression of the genes *Rel1*, *Rel2, Caspar* and *Cactus* of females after blood feeding, compared to non-fed females, because of possible effects on the ability of *A. darlingi* to become infected with *Plasmodium* species and spread malaria. This may be useful for future inquiries about these genes in the control of this mosquito.

## MATERIAL AND METHODS

Larvae of *A. darlingi* were collected in Bairro Puraquequara (03º 03' 06.14'' S and 59º 53' 38.56'' W), in the municipality of Manaus, Amazonas State, Brazil, with permission from the Ministry of the Environment (IBAMA), No. 17524. Larvae of *A. darlingi* were maintained in the Laboratory of Cytogenetics, Genomics and Evolution of Mosquito Vectors - Coordination of Society, Environment and Health (CSAS), INPA, Manaus, Amazonas State, Brazil. The samples were identified according to taxonomic keys (Forattini, 1962; Consoli and Lourenço, 1994). They were fed commercial fish food, Tetra Cichlid flakes, up to adulthood, and then fed with 10% sugar solution for 24 h (Ohse et al., 2017). Blood feeding was conducted with domestic ducks (*Anas platyrhynchos*) in groups of 20 female subjects (pooled), and samples were afterwards collected at 4, 8, 14 and 24 h, with each pool being in biological triplicate. Non-fed females (0 h) were also included, for a total of 300 samples, which were frozen at - 80 $^{o}$C.

Total RNA was extracted from adult females of *A. darlingi* (100 mg) fed with blood and from non-fed females (0 h), all in triplicate, using the extraction and purification kit from QIAGEN Biotechnology Brasil Ltda (São Paulo, SP, Brazil). RNA was quantified with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific - Life Technology) São Paulo, SP, Brazil. We used the RiboGreen RNA reagent to quantify RNA because it is more precise compared with direct quantification of RNA using a 230 nm wavelength. The integrity of total RNA was verified using a 1% agarose denaturing gel. The reference gene should normalize small discrepancies between samples. The similarity of contigs 83970 (*Rel1*), 17805 (*Rel2*), 103357 (*Caspar*), 83470 (*Cactus*), 68 (*GAPDH*), and 433 (*β-actin*) of *A. darlingi* was evaluated in the databank (http://sysbiol.cbmeg.unicamp.br.adarlingi/) against *A. gambiae* (http//www.ncbi.nlm.nih.gov). The primers for *Rel1* (Fw: 5 GAAACACCTGGAAGCACAAC3' and Rv: 5'TTAAGCAGCGACTGGAAATC3'), *Rel2* (Fw: 5'GCTCGCATCGCTCGTATT3' and Rv: 5-TTCGCCTTCTTCGTCGTC3'), *Caspar* (Fw 5'CACATTATCAATAGCCGTTATGC3' and Rv:5'TTGCTGTCGTCGCTTCTAC3'), *Cactus* (Fw: 5'GCCCGATTGCGACATCA3' and Rv: 5'ACCAGTTTCCTTACCAATTCC3'), *GAPDH* (Fw: 5'CGAGTACGGCTACTCCAACC3' and Rv: 5'CTGGCACACAAGTGAGGCTA3'), and *β-actin* (Fw: 5'TCGTGCGTGACATTAAGGAG3' and Rv: 5'GCAGCTCGTACGACTTTTCC3') were designed with the help of the programs Gene Runner and Primer 3, according to Tm of 60% GC, and synthesized by Integrated DNA Technologies IDT.

The complementary strand of mRNA (cDNA) was obtained using a kit from Promega, processed according to the manufacturer's instructions. The cDNA amplifications were done in a 7500 Real-Time PCR System thermocycler, with the SYBR Green system (Applied Biosystems® - Life Technology Brazil, São Paulo, SP, Brazil). The conditions for the amplification reactions of *Rel1, Rel2*, *Caspar* and *Cactus* were: 95°C for 10 min,

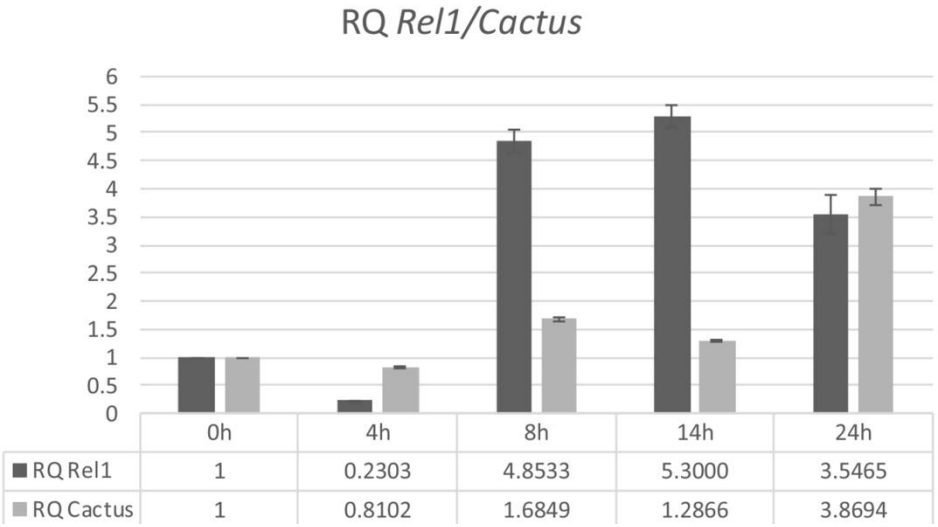
followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The samples of each stage were analyzed in biological triplicate to optimize the reliability of the results.

cDNA of non-blood-fed adult females of *A. darlingi* was used as a calibrator (control samples) for comparison with fed adult females because of little variation in Ct between biological replicates, allowing comparison of gene expression of these samples. This allowed the validation of expression levels of *Rel1*, *Rel2*, *Caspar*, and *Cactus* in the qRT-PCR assays, according to the mean and standard deviation. The constitutive genes *GAPDH* and *β-actin* were used as controls (endogenous) for normalization of the reactions.

The relative expression levels of *Rel1, Rel2, Caspar, Cactus*, *GAPDH*, and *β-actin* genes in *A. darlingi* were calculated by the comparative Ct (2-ΔΔCt) method (Livak and Schmittgen, 2001).

## RESULTS

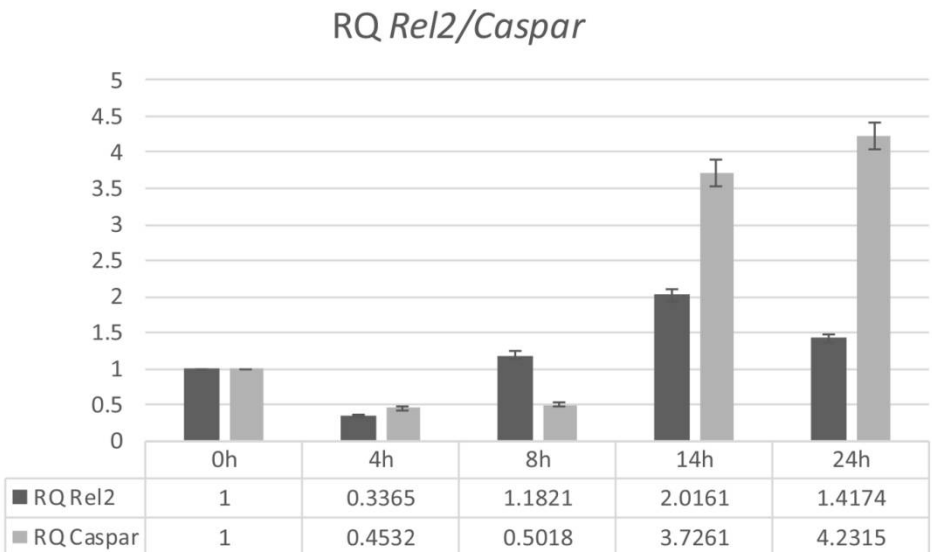
In the comparison of expression between the *Rel1* and *Cactus* genes, expression of the *Rel1* gene was greater than that of the *Cactus* gene at times 8 and14 h, while the expression of *Cactus* was greater at 4 and 24 h. That is, when *Rel1* showed higher expression, *Cactus* showed lower expression and vice versa (Figure 1).




| | 0h | 4h | 8h | 14h | 24h |
|---|---|---|---|---|---|
| RQ Rel1 | 1 | 0.2303 | 4.8533 | 5.3000 | 3.5465 |
| RQ Cactus | 1 | 0.8102 | 1.6849 | 1.2866 | 3.8694 |

**Figure 1**. Expression of the gene *Rel1* and *Cactus* in *Anopheles darlingi* not fed (0 h) and after blood feeding at 4, 8, 14 and 24 h.

The expression of *Caspar* was greater than that of *Rel2* at times 4, 14 and 24 h, while the expression of *Rel2* was greater than that of *Caspar* at 8 h (Figure 2).




| | 0h | 4h | 8h | 14h | 24h |
|---|---|---|---|---|---|
| RQ Rel2 | 1 | 0.3365 | 1.1821 | 2.0161 | 1.4174 |
| RQ Caspar | 1 | 0.4532 | 0.5018 | 3.7261 | 4.2315 |

**Figure 2**. Expression of the genes *Rel2* and *Caspar* in *Anopheles darlingi* not fed (0 h) and after blood feeding at 4, 8, 14 and 24 h.

The expression of the *Rel1* and *Rel2* genes showed a gradual increase at 8 h after blood feeding, with peak expression levels at 14 h. After 24 h of blood feeding, the expression levels of *Rel1* and *Rel2* decreased in the females. The *Cactus* and *Caspar* genes, on the contrary, had peak expression at 24 h after the blood meal, contrasting with the other times (4, 8 and 14 h), during which expression varied.

## DISCUSSION

Studies of immune response in mosquito vectors of pathogens to humans, such as *A. darlingi*, the primary vector of malaria in South America, are still scarce (Lehmann et al., 2009). In our study, expression of the genes *Rel1, Rel2, Caspar* and *Cactus*, related to the immune system, was determined in adult females of *A. darlingi* with and without blood feeding, using the reference genes *GAPDH* and *β-actin*.

In studies of gene expression in adult females of *A. gambiae* after a blood meal, using the microarray method, gene expression has been quite variable (Marinotti et al., 2005), with an increase and decrease in gene expression (TNF receptor of the Toll pathway) related to immune function 24 h after the blood meal. In our study in *A. darlingi* females, 24 h after blood feeding, there was an increase in the expression of the genes *Cactus* and *Caspar*, whose products are inhibitors of the transcription factors of the Toll and IMD pathways. Another study of gene expression patterns in *A. gambiae* through microassays (Dana et al., 2005) used 2000 transcripts of abdomens of adults from insectarium colonies fed on three foods (glucose, uninfected blood and blood infected by *P. berghei*). These authors found variation in expression of transcripts at 1, 3, 5, 12, 16, 24 and 48 h.

The immune response in *Anopheles* mosquitoes is regulated by the Toll and IMD pathways and the transcription factors NF-kappaB *Rel1* and *Rel2*. These two factors are

controlled by the negative regulators *Cactus* and *Caspar*. Rel1 acts as a transcription factor of the Toll pathway and has *Cactus* as its negative regulatory protein (Belvin and Anderson, 1996; Christophides et al., 2002). These authors argue that *Cactus* under normal conditions binds to and suppresses the transcription factor *Rel1* in the cytoplasm. However, when the Toll receptor is activated, *Cactus* is degraded by adapter proteins (Pelle and Tube) releasing *Rel1*, which then enters the nucleus and regulates the transcription of effector AMP genes (Belvin et al., 1995).

Accordingly, the level of transcription of AMP genes in *A. gambiae*, compared to the findings for *A. darlingi* in our study (Figure 1), suggests an antagonistic relationship between the expressions of the *Rel1* and *Cactus* genes in blood-fed mosquitoes after 4, 8, 14 and 24 h. *Rel1* showed higher expression, while *Cactus* had a decreased expression and vice versa. Nevertheless, we noticed a difference in gene expression level in *A. darlingi* of approximately 0.3 relative quantification units at 24 h after the blood meal. This suggests that at this time, the expression of *Rel1* started to decrease, while the expression of *Cactus* increased, since at 14 h, the expression of *Rel1* was greater than that of *Cactus*, a difference of on average of 4 relative expression units. As early as 4 h after the blood meal, the expression of *Rel1* was lower than that of *Cactus*, suggesting that at this time the transcription level of AMP genes was lower than at 8 and 14 h. But at 24 h, gene transcription level decreased further, which showed that the Toll pathway regulates the transcription of effector genes such as AMPs (Christophides et al., 2002).

The *Rel2* gene is translated into *Rel2* protein, which is a transcription factor of the IMD pathway, while the *Caspar* gene is translated into the *Caspar* protein, which is an inhibitor of *Rel2* (Kim et al., 2006). *Rel2* is controlled by inhibitory factors, while it can be cleaved by *Dredd*, resulting in its activation. Activated *Rel2* can cross the nuclear membrane and stimulate the transcription of *AMP* genes (Stoven et al., 2000; Stoven et al., 2003). *Caspar* under normal conditions binds to Dredd, preventing it from cleaving *Rel2*. Once the receptor IMD is activated, *Caspar* separates from Dredd, leaving it free to cleave *Rel2* (Kim et al., 2006).

In our study, the expression of the *Rel2* gene and of its inhibitor *Caspar* in adult females of *A. darlingi* not fed with blood (0 h) and individuals after blood feeding (4, 8, 14 and 24 h) were similar to the above findings, showing an antagonistic relationship between the *Rel2* and *Caspar* genes (Figure 2). In this interaction, the expression of *Caspar* was greater than that of *Rel2*, with a difference of 0.1, 1.7 and 2.8 at 4, 14 and 24 h, respectively. On the contrary, only at 8 h was the expression of *Caspar* less than that of *Rel2*. It is therefore inferred that at 4, 14 and 24 h, *Caspar* gene transcription increased, indicating that there could have been a greater inhibition of *Dredd*, preventing cleavage of *Rel2*, which promotes the transcription of effector genes.

Because of the difficulty of mating and effective reproduction of *A. darlingi* in the laboratory, we used samples of this mosquito caught in natural breeding sites, which have uncontrolled environmental variables (volume, pH, turbidity and temperature of water, besides environmental contaminants (Tadei et al., 1998). The expression of specific genes can be taken into account when varying the mechanisms of adaptation of organisms to various environmental conditions (Desroche et al., 2005). Sequences of bacteria have been detected in the genome of *A. darlingi*; these could be derived from environmental contamination or from microorganisms associated with this mosquito (Marinotti, 2013). *Anopheles darlingi* in our study was obtained in the field, and it is emphasized that it is not

feasible to control for possible infections of this mosquito with bacteria, fungi and viruses, for example. Thus, differences in the expression of the genes *Rel1*, *Rel2*, *Cactus* and *Caspar* may be related to the immune status of these samples of *A. darlingi* caught in the field. These results are similar to the findings of gene expression of *A. gambiae* from laboratory colonies, which also did not achieve control the exposure of the mosquitoes to bacteria and fungi (Marinotti et al., 2005).

In a study of *A. gambiae* fed with blood, Bryant and Michel (2014) analyzed the proliferation and activation of hemocytes of this mosquito. They found significant changes in hemocytes, with higher levels of components and added melanization in the immune response 24 h after feeding; the blood meal induced the activation of the innate response of the specimens. Our results also showed this immune response in *A. darlingi*, since the expression levels of *Rel1*, *Rel2*, *Caspar* and *Cactus* were higher at 24h after blood feeding, compared to the control (0 h).

In a study of *A. gambiae* fed with blood, the proliferation and activation of hemocytes of this mosquito was analyzed (Bonizzoni et al., 2011). The authors found that over 30% of the 18,000 transcripts were expressed differentially at 5 h after the blood meal. Our data are similar to what was found with *A. gambiae*, as there was an increase in the accumulation of mRNA of *Rel1*, *Rel2*, *Caspar* and *Cactus* at 8, 14 and 24 h compared to 4 h after blood feeding.

The findings of this study adds to our understanding that the mRNA of *Rel1*, *Rel2*, *Caspar* and *Cactus* are able to affect the ability of *A. darlingi* to become infected with *Plasmodium* species and spread malaria.

## CONCLUSION

Differences in gene expression between adult females of *A. darlingi* after blood feeding compared to control insects (0 h) affected the *A. darlingi* immune response. Further expression studies are needed to define immune responses and related questions that may be involved in understanding how expression of *Rel1* and *Cactus*, *Rel2* and *Caspar* genes and affects malaria vector biology.

## 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 No. 012/2009, process No. 2680/2009 – FAPEAM, both awarded to MSR, and project INCT-ADAPTA II (CNPq / FAPEAM), awarded by Dr. Adalberto Luis Val. L.R. Fernandes performed the research; M.S. Rafael designed the research study and wrote the paper with help of W.P. Tadei. We thank Dr. Cleverson C. Mattiolli who helped to perform this study and analyzed the data. We thank Dr. Vera Maria Fonseca de Almeida and Luciana Mara Lopes Fé of the Laboratório de Ecofisiologia e Evolução Molecular (LEEM), INPA, Manaus, Amazonas State, Brazil for technical help. Dr. A. Leyva helped with English translation and editing of the manuscript.

## CONFLICT OF INTEREST

All authors declare they have no conflicts of interest

## REFERENCES


Barillas-Mury C, Wizel B, Han YS, (2000). Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrate innate immunity and vaccine development. *Insect Biochem Mol Biol:*; 30:429-42. https://doi.org/10.1016/S0965-1748(00)00018-7.

Belvin MP, Jin Y, Anderson KV, et al. (1995). *Cactus* protein degradation mediates *Drosophila* dorsal-ventral signaling. *Genes Dev*.: 9:783-93.

Belvin MP, Anderson KV. (1996). A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. *Annu Rev Cell Dev Biol*.: 12:393-416. doi: 10.1146/annurev.cellbio.12.1.393.

Blandin S, Moita LF, Kocher T, Wilm M, et al. (2002). Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. *EMBO Rep*. 3:852-6. https://doi.org/ 10.1093/embo-reports/kvf180.

Bonizzoni M, Dunn WA, Campbell CL, Olson KE, et al. (2011). RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, *Aedes aegypti*. *BMC Genomics:* 12:82.. https://doi.org/10.1186/1471-2164-12-82.

Bryant WB, Michel K. (2014). Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, *Anopheles gambiae* Giles. *J Exp Biol*. 217(Pt 8):1238-45. . https://doi.org/ 10.1242/jeb.094573.

Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, et al. (2002). Immunity-related genes and gene families in *Anopheles gambiae*. *Science:* 298(5591):159-65. https://doi.org/ 10.1126/science.1077136.

Cirimotich CM, Dong Y, Garver LS, Sim S, et al. (2010). Mosquito immune defenses against *Plasmodium* infection. *Dev Comp Immunol:*34:387-95. . https://doi.org/ 10.1016/j.dci.2009.12.005.

Cociancich S, Bulet P, Hetru C, Hoffmann JA (1994). The inducible antibacterial peptides of insects. *Parasitol Today*: 10:132-9. https://doi.org/10.1016/0169-4758(94)90260-7.

Consoli RAGB, Lourenço de Oliveira R. (1994). Principais Mosquitos de Importância Sanitária no Brasil. Rio de Janeiro. 228 p.

Da Silva CCA. (2002). Aspectos do sistema imunológico dos insetos. *Biotecnologia Ciência & Desenvolvimento*: 68-72.

Dana AN, Hong YS, Kern MK, Hillenmeyer ME, et al. (2005). Gene expression patterns associated with blood-feeding in the malaria mosquito *Anopheles gambiae*. *BMC Genomics*: 6:5. https://doi.org/ 10.1186/1471-2164-6-5.

Deane LM. (1988). Malaria studies and control in Brazil. *Am J Trop Med Hyg:* 38:223-30.

Desroche N, Beltramo C, Guzzo J. (2005). Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. *J Microbiol Methods*: 60:325-33. https://doi.org/10.1016/j.mimet.2004.10.010.

Dimopoulos G, Casavant TL, Chang S, Scheetz T, et al. (2000). *Anopheles gambiae* pilot gene discovery project: identification of mosquito innate immunity genes from expressed sequence tags generated from immune-competent cell lines. *Proc Natl Acad Sci U S A*: 97:6619-24.

Dimopoulos G. (2003). Insect immunity and its implication in mosquito-malaria interactions. *Cell Microbiol*: 5:3-14.

Dong Y, Aguilar R, Xi Z, Warr E, et al. (2006). *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog:* 2: e52. https://doi.org/ 10.1371/journal.ppat.0020052.

Forattini OP (1962). Entomologia Médica. Faculdade de Saúde e Higiene Pública da USP; 662 p.

Galvão ALA, Damasceno R. (1944). Observações sobre anofelinos do complexo *Albitarsis* (Diptera, Culicidae). *An Fac Med*: 73-87.

Garver LS, Dong Y, Dimopoulos G. (2009). *Caspar* controls resistance to *Plasmodium* falciparum in diverse anopheline species. *PLoS Pathog*: 5: e1000335. https://doi.org/ 10.1371/journal.ppat.1000335.

Gwadz RW, Kaslow D, Lee JY, Maloy WL, et al. (1989). Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infect Immun.:* 57:2628-33. https://doi.org/ 0019-9567/89/092628-06$02.00/0.

Hiwat H, Bretas G. (2011). Ecology of *Anopheles darlingi* Root with respect to vector importance: a review. *Parasit Vectors:* 4:177. https://doi.org/10.1186/1756-3305-4-177.

Ohse KC, Matiolli CC, Scarpassa VM, Santos VS, et al. (2017). Differential expression of trypsin-3 and phosrestin II genes in the main malaria vector, *Anopheles darlingi*, from the Brazilian Amazon region. Genet Mol Res. Genet Mol Res: 16: 1-12. http://dx.doi.org/10.4238/gmr16039852.

Kim M, Lee JH, Lee SY, Kim E, et al. (2006). *Caspar*, a suppressor of antibacterial immunity in *Drosophila*. *Proc Natl Acad Sci U S A:* 103(44):16358-63. https://doi.org/ 10.1073/pnas.0603238103.

Lehmann T, Hume JC, Licht M, Burns CS, et al. (2009). Molecular evolution of immune genes in the malaria mosquito *Anopheles gambiae*. *PLoS One*: 4: e4549. https://doi.org/ 10.1371/journal.pone.0004549.

Lemaitre B, Meister M, Govind S, Georgel P, et al. (1995). Functional analysis and regulation of nuclear import of dorsal during the immune response in *Drosophila*. *EMBO J.*:14: 536-45.

Livak KJ; Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. *Methods:* 25: 402-408. https://doi.org/10.1006/meth.2001.1262.

Marinotti O, Nguyen QK, Calvo E, James AA, et al. (2005). Microarray analysis of genes showing variable expression following a blood meal in *Anopheles gambiae*. *Insect Mol Biol*: 14:365-73. https://doi.org/10.1111/j.1365-2583.2005.00567.x.

Marinotti O, Cerqueira GC, de Almeida LG, Ferro MI, et al. (2013). The genome of *Anopheles darlingi*, the main neotropical malaria vector. *Nucleic Acids Res*: 41:7387-400. https://doi.org/ 10.1093/nar/gkt484.

Ministério da Saúde. (2015). Malária: Monitoramento dos casos no Brasil em 2014. Secretaria de Vigilância em Saúde Ministério da Saúde.

Meister S, Kanzok SM, Zheng XL, Luna C, et al. (2005). Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A.*: 102:11420-5. https://doi.org/10.1073/pnas.0504950102.

Rachou RG. (1958). Anofelinos do Brasil: comportamento das espécies vetoras de malária.*Revista Brasileira de Malariologia:*145-81.

Rawlings ND, Barrett AJ. (1993). Evolutionary families of peptidases. *Biochem J.:* 290( Pt 1):205-18. https://doi.org/10.1042/bj2900205.

Riehle MM, Markianos K, Niare O, Xu J, et al. (2006). Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science:* 312(5773):577-9. https://doi.org/10.1126/science.1124153.

Stoven S, Ando I, Kadalayil L, Engstrom Y, et al. (2000). Activation of the *Drosophila* NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep.*: 1:347-52. https://doi.org/ 10.1093/embo-reports/kvd072.

Stoven S, Silverman N, Junell A, Hedengren-Olcott M, et al. (2003). Caspase-mediated processing of the *Drosophila* NF-kappaB factor Relish. *Proc Natl Acad Sci U S A.:* 100:5991-6. https://doi.org/10.1073/pnas.1035902100.

Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, et al. (1998). Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon.*Am J Trop Med Hyg*: 59:325-35.

Tadei WP, Rodrigues IB, Rafael MS, Sampaio RTM, et al. (2016). Adaptative processes, control measures, genetic background, and resilience of malaria vectors and environmental changes in the Amazon region. *Hydrobiologia* (The Hague. Print), v. 000, p. 1-18. https://doi.org/10.107/s10750-016-2960-y.

World Health Organization (2014). World Malaria Report.