PRISCILA GONÇALVES FERREIRA

EFEITO DAS VARIAÇÕES DE TEMPERATURA NA EXPRESSÃO GÊNICA NO INTESTINO MÉDIO DE *Aedes aegypti* E SUAS IMPLICAÇÕES PARA A INTERAÇÃO COM *Zika virus*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada para obtenção do título de *Doctor Scientiae*.

Orientador: Tiago Antônio de O. Mendes

Coorientador: Gustavo Ferreira Martins

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RESUMO

FERREIRA, Priscila Gonçalves, D.Sc., Universidade Federal de Viçosa, janeiro de 2022. **Efeito das variações de temperatura na expressão gênica no intestino médio de *Aedes aegypti* e suas implicações para a interação com *Zika virus*.** Orientador: Tiago Antônio Mendes. Coorientador: Gustavo Ferreira Martins.

A descoberta contínua de novos patógenos e o surgimento de novas formas de transmissão revelam uma ameaça constante à saúde pública. Somado a isso, as mudanças climáticas podem ser um fator agravante na transmissão de patógenos. Os patógenos transmitidos por vetores representam um grande desafio à saúde pública e, no entanto, estamos apenas começando a entender a complexidade subjacente às interações mosquito-patógeno e como os fatores abióticos, como a temperatura, afetam essas interações. Para entender melhor os efeitos da temperatura na fisiologia do vetor e seus efeitos na interação mosquito-patógeno, comparamos o transcriptoma de amostras do intestino médio de mosquitos expostos ao *Zika virus* (ZIKV) e mosquitos não expostos alojados em três temperaturas diferentes (20, 28 e 36°C) durante 24 e 48 horas de exposição. Inicialmente, a quantificação do número de cópias de RNA viral não mostrou diferença entre 24 e 48h pós-exposição a 20°C, sugerindo que a replicação do ZIKV é limitada por alterações induzidas por temperaturas mais baixas no ambiente do intestino médio. Genes que codificam proteínas envolvidas com a digestão de sangue, metabolismo de ROS e imunidade do mosquito foram os que apresentaram maior alteração de expressão pela temperatura de 20ºC. A partir desses resultados, hipotetizamos que a variação de temperatura afeta o processo de digestão do sangue, modulando a geração de espécies reativas de oxigênio (ROS) no intestino médio. Mostramos que a variação de temperatura afeta tanto a expressão de genes envolvidos na digestão do sangue quanto a taxa de digestão. Além disso, devido aos efeitos da temperatura nos níveis de ROS e à expressão de proteínas antioxidantes no intestino médio, temperaturas mais baixas (20°C) resultam em um estado de estresse oxidativo mais longo quando comparado a outras temperaturas (28 e 36°C). Finalmente, eventos de *splicing* alternativos foram investigados sob diferentes temperaturas. Curiosamente, entre os genes listados, encontramos Tripsina, Ferritina, Tiorredoxina e a proteína de reconhecimento de Peptidoglicano LC, genes previamente demonstrados ter sua

expressão modulada pela temperatura. Nossos resultados revelam os processos fisiológicos que são impactados pela variação de temperatura e sugerem como a resposta imunológica é modulada frente a infecção pelo *Zika virus.*

Palavras-chave: *Aedes aegypti*. Temperatura. *Zika virus*. Transcriptoma. *Splicing* Alternativo

ABSTRACT

FERREIRA, Priscila Gonçalves, D.Sc., Universidade Federal de Viçosa, January, 2022. **Effect of temperature variations on gene expression in the midgut of *Aedes aegypti* and its implications for the interaction with *Zika virus*.** Adviser: Tiago Antônio Mendes. Co-adviser: Gustavo Ferreira Martins.

The continuous discovery of new pathogens and the emergence of new transmission forms reveal a constant threat to public health. Added to that, climate change can to be an aggravating factor on pathogen transmission. Vector-borne infections represent a major public health challenge and however, we are only just beginning to understand the complexity underlying mosquito–pathogen interactions and how abiotic factors, as temperature affect these interactions. To understand better the temperature effects on vector physiology and its effects on mosquito-pathogen, we compared the transcriptome of mosquito midgut samples from mosquitoes exposed to *Zika virus* (ZIKV) and non-exposed mosquitoes housed at three different temperatures (20, 28, and 36°C) during 24 and 48 hours of exposure. Initially, the quantification of viral RNA copy number does not show difference between 24 and 48 h post-exposure at 20°C, suggesting that ZIKV replication is limited by cold-induced changes to the mosquito midgut environment. Genes encoding proteins involved in blood digestion, ROS metabolism and mosquito immunity were the ones that showed the greatest change in expression at 20ºC. From these results, we hypothesize that temperature variation affects the blood digestion process, modulating the generation of reactive oxygen species (ROS) in the midgut. We show that temperature variation affects both the expression of genes involved in blood digestion and the rate of digestion. Additionally, due to temperature effects on ROS levels and the expression of antioxidant proteins in the midgut, lower temperatures (20°C) result in a longer state of oxidative stress when compared to other temperatures (28 and 36°C). Finally, alternative splicing events were investigate under different temperatures. Interestingly, among listed genes, we found Trypsin (TRY), Ferritin (FER), Thioredoxin (TRX) and Peptidoglycan recognition protein LC (PGRP-LC), genes previously shown to have their expression modulated by temperature. Our results reveal the physiological processes that are impacted by temperature variation and suggest how the immune response is modulated against Zika virus infection.

Keywords: *Aedes aegypti*. Temperature. *Zika virus*. Transcriptome. Alternative Splicing.

SUMÁRIO

INTRODUÇÃO

Infecções transmitidas por vetores representam um grande desafio à saúde pública, tanto em termos de abundância e diversidade de vetores e patógenos quanto em mortalidade humana e animal. Além disso, a descoberta contínua de novos patógenos e o surgimento de novas formas de transmissão, revelam sua constante ameaça. Segundo a Organização Mundial de Saúde, doenças transmitidas por vetores representam mais de 17% de todas as doenças infecciosas, causando mais de 700.000 mortes anualmente (World Health Organization).

Compreender completamente as interações moleculares entre patógenos e vetor e como elas determinam a competência vetorial é importante para elucidar os fatores nessa interação complexa a fim de encontrar novas abordagens para o controle de doenças. A competência do vetor é definida como a capacidade intrínseca de se tornar infectado e subsequentemente transmitir um patógeno para um hospedeiro susceptível. Nosso entendimento a respeito dessas interações fez um progresso considerável nas últimas duas décadas identificando as principais vias e genes de imunidade que limitam o desenvolvimento do patógeno (Lemaitre and Hoffmann, 2007; Fragkoudis et al., 2009; Souza-Neto et al., 2009; Cirimotich et al., 2010; Green et al., 2014; McFarlane et al., 2014; Myllymäki et al., 2014; Kumar et al., 2018). No entanto, a competência envolve aspectos amplos da fisiologia do vetor, que são moldados por diversos fatores bióticos e abióticos, incluindo limitação de alimento, competição, variação de temperatura, umidade, entre outros (Schulenburg et al., 2009; Wolinska and King, 2009; Lefèvre et al., 2013; Murdock et al., 2013). Em particular, muitos estudos têm enfatizado o papel dessas variáveis ambientais na modulação da competência de mosquitos para patógenos (Pridgeon et al., 2008;

Murdock et al., 2012a; Lefèvre et al., 2013; Moller-Jacobs et al., 2014; Zouache et al., 2014).

Dentre as variáveis ambientais que potencialmente afetam a competência de mosquitos à patógenos, a temperatura desempenha um papel central. Mosquitos são organismos pecilotérmicos e a temperatura afeta profundamente seu ciclo de vida, incluindo o desenvolvimento larval, sobrevivência de adultos e reprodução (Delatte et al., 2009; Padmanabha et al., 2012; Carrington et al., 2013c). Existem evidências abundantes de que pequenas mudanças na temperatura ambiente podem influenciar significativamente a resistência à patógenos. Da mesma forma, as taxas de desenvolvimento dos principais patógenos transmitidos por mosquitos (Dengue (Lambrechts et al., 2011; Carrington et al., 2013a, 2013b), Zika (Tesla et al., 2018), Nilo Ocidental (Kilpatrick et al., 2008), malária (Mordecai et al., 2013)) são fortemente dependentes da temperatura. Portanto, a temperatura pode moldar o fenótipo de resistência e o crescimento do patógeno de duas maneiras: (i) efeitos diretos da temperatura corporal do hospedeiro no crescimento do patógeno (que são independentes do hospedeiro) e (ii) os efeitos indiretos, menos estudados, no crescimento do patógeno, que são mediadas pelos efeitos da temperatura nos mecanismos imunológicos inatos dos mosquitos.

O sistema imunológico de mosquitos consiste em respostas celulares e humorais que interagem para controlar a propagação de uma infecção (Schmid-Hempel, 2005). Há diversas evidências do impacto da variação da temperatura na modulação das principais mecanismos de resposta imune (melanização (Suwanchaichinda and Paskewitz, 1998; Murdock et al., 2012b), RNAi (Adelman et al., 2013), fagocitose (Murdock et al., 2012b), immune deficiency (IMD) (Muturi et al., 2012) e resposta mediada por receptors toll (Muturi et al., 2012)).

Kramer e colaboradores demonstraram que a infecção por vírus da encefalomielite equina ocidental (Alphavirus) em *Culex tarsalis* diminuiu em função do aumento da temperatura (Kramer et al., 1983). Achados semelhantes foram feitos por Kay e Jennings usando o vírus Ross River (Alphavirus) em *Aedes vigilax* criado a 18°C (Kay and Jennings, 2002). Turell também encontrou taxas significativamente mais altas de infecções disseminadas para vírus da encefalomielite equina venezuelana (Alphavirus) após uma ingestão de uma refeição de sangue infecciosa por *Aedes taeniorhynchus* a 19°C em comparação com 26°C (Turell, 1993). Mais recentemente, Westbrook e colaboradores descobriram que a infectividade do Chikungunya para *Aedes albopictus* aumentou com uma diminuição da temperatura de criação (18°C > 24°C > 32°C) (Westbrook et al., 2010).

Contrariamente, ao que visto para Alphavirus, as temperaturas mais quentes são mais permissivas para a infecção pelo vírus Zika (gênero Flavivirus) em mosquitos *Aedes aegypti*, enquanto que as temperaturas mais frias limitam a fuga e a disseminação do vírus do intestino (Tesla et al., 2018). Interessantemente, a mesma tendência é observada para outros Flavivirus, como Dengue (Watts et al., 1987), Febre do Nilo Ocidental (Dohm et al., 2002; Reisen et al., 2006) e Encefalite Saint Louis (Reisen et al., 1993).

Recentemente esse efeito da temperatura foi ilustrado durante emergência de Zika nas Américas no ano de 2015. Naquele ano, o El Niño promoveu condições climáticas excepcionais durante o inverno (Paz and Semenza, 2016). De acordo com a Administração Nacional Oceânica e Atmosférica dos EUA, temperaturas mais altas foram registradas no norte e leste da América do Sul, acompanhadas por uma seca intensa, durante o segundo semestre de 2015 (National Oceanic and Atmospheric Administration. Global analysis—annual 2015). Essas condições extremas podem ser

uma manifestação da mudança climática que contribuiu para a rápida dispersão do vírus Zika.

A relação entre temperatura e transmissão de patógenos tem recebido grande atenção devido ao debate de mudanças climáticas. No entanto, os mecanismos subjacentes através dos quais a temperatura afeta a competência do vetor, permanecem desconhecidos. Neste estudo nós estudamos os mecanismos pelos quais a temperatura modela a resistência de mosquitos *Ae. aegypti* à infecção por Zika. O presente trabalho envolveu; 1. O estudo do transcriptoma do intestino médio de fêmeas adultas de *Ae. aegypti* infectadas oralmente por *Zika virus* em diferentes temperaturas; 2. Avaliação do impacto da variação da temperatura na digestão do sangue e homeostase redox e; 3. Impacto da temperatura nos eventos de *splicing* alternativo no intestino médio de fêmeas adultas de *Ae. aegypti*.

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Temperature Dramatically Shapes Mosquito Gene Expression With Consequences for Mosquito–Zika Virus Interactions

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Vector-borne flaviviruses are emerging threats to human health. For successful transmission, the virus needs to efficiently enter mosquito cells and replicate within and escape several tissue barriers while mosquitoes elicit major transcriptional responses to flavivirus infection. This process will be affected not only by the specific mosquito-pathogen pairing but also by variation in key environmental variables such as temperature. Thus far, few studies have examined the molecular responses triggered by temperature and how these responses modify infection outcomes, despite substantial evidence showing strong relationships between temperature and transmission in a diversity of systems. To define the host transcriptional changes associated with temperature variation during the early infection process, we compared the transcriptome of mosquito midgut samples from mosquitoes exposed to Zika virus (ZIKV) and non-exposed mosquitoes housed at three different temperatures (20, 28, and 36°C). While the high-temperature samples did not show significant changes from those with standard rearing conditions (28°C) 48 h post-exposure, the transcriptome profile of mosquitoes housed at 20°C was dramatically different. The expression of genes most altered by the cooler temperature involved aspects of blood-meal digestion, ROS metabolism, and mosquito innate immunity. Further, we did not find significant differences in the viral RNA copy number between 24 and 48 h post-exposure at 20°C, suggesting that ZIKV replication is limited by cold-induced changes to the mosquito midgut environment. In ZIKV-exposed mosquitoes, vitellogenin, a lipid carrier protein, was most up-regulated at 20°C. Our results provide a deeper understanding of the temperature-triggered transcriptional changes in *Aedes aegypti* and can be used to further define the molecular mechanisms driven by environmental temperature variation.

Keywords: temperature, *Aedes aegypti*, Zika virus, RNA-seq, transcriptome, immune response

INTRODUCTION

Over the past three decades, there have been significant advances in our understanding of the physiological and molecular interactions between pathogens and mosquito vectors (Bartholomay and Michel, 2018; Kumar et al., 2018; Simões et al., 2018). Research has provided substantial insights into the immune genes and pathways that shape resistance to vector-borne pathogens and has revealed many promising targets for genetic manipulation (Wilke and Marrelli, 2015; Yen et al., 2018; Shaw and Catteruccia, 2019). However, we are only just beginning to understand the complexity underlying mosquito–pathogen interactions. The response mosquitoes mount toward a given pathogen is a dynamic phenotype that is dependent upon both the specific mosquito–pathogen pairing (Lambrechts et al., 2013; Zouache et al., 2014; Duggal et al., 2015; Chouin-Carneiro et al., 2016) and the variation in key environmental factors (Okech et al., 2007; Alto et al., 2008; Parham and Michael, 2010; Parham et al., 2015; Cohen et al., 2016; Gloria-Soria et al., 2017; Murdock et al., 2017; Shragai et al., 2017; Siraj et al., 2018). Knowledge of how vectors respond to environmental variation is especially relevant for understanding how vector-borne pathogens emerge, defining the biological constraints on transmission, and anticipating the robustness of novel vector-control approaches (that manipulate mosquito physiological and immunological mechanisms to limit virus infection) in field settings.

Vector-borne flaviviruses are emerging threats to human health. Yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), and most recently, Zika virus (ZIKV) can be found throughout tropical and subtropical zones. For successful transmission, flaviviruses are taken up through the bite of a mosquito vector when it takes a blood meal from an infectious host. To complete infection within the mosquito vector, the virus needs to efficiently enter, replicate within, and escape several tissue barriers, primarily the midgut and salivary glands (Franz et al., 2015; Kumar et al., 2018). A number of studies have demonstrated that mosquitoes elicit major transcriptional changes in response to flavivirus infection, which could play an important role in limiting infection (Xi et al., 2008; Sim and Dimopoulos, 2010; Colpitts et al., 2011; Bonizzoni et al., 2012; Chauhan et al., 2012; Angleró-Rodríguez et al., 2017; Etebari et al., 2017; Saldaña et al., 2017a). These include differential regulation of genes involved in RNA interference (RNAi), classical immune pathways (e.g., JAK-STAT, Toll), production and transport of energy, and metabolism, as well as the production of non-coding RNAs (small and long) and microRNAs that could be involved in targeted gene regulation. Currently, it is unclear how conserved these responses to infection are across different mosquito–flavivirus combinations (Etebari et al., 2017) and how relevant environmental variation shapes the nature, magnitude, and timing of these responses (Murdock et al., 2012b, 2014; Adelman et al., 2013).

One of the major environmental variables that influence mosquito–pathogen interactions, as well as vector-borne transmission, is variation in environmental temperature. Mosquitoes are small ectothermic organisms, and many studies have already demonstrated that temperature can markedly affect diverse aspects of mosquito physiology, ecology, and pathogen replication. This, in turn, will shape the proportion of mosquitoes that become infected and infectious, the overall transmission potential of mosquito populations (Christofferson and Mores, 2016; Murdock et al., 2017; Tesla et al., 2018a; Mordecai et al., 2019), and how the distributions of mosquito vectors and vector-borne pathogens will shift in response to climate change (Siraj et al., 2014; Campbell et al., 2015; Ryan et al., 2015, 2018; Misslin et al., 2016). The extent to which temperature shapes transmission directly, through effects on pathogen biology, or indirectly, through effects on mosquito immunity and physiology, remains largely unexplored (Murdock et al., 2012a).

In previous work (Tesla et al., 2018a), we have demonstrated that ZIKV transmission by *Aedes aegypti* is optimized at a mean temperature of 29°C and has a thermal range from 22.7 to 34.7°C. Further, we observed constraints on ZIKV transmission at cool and warm temperatures to be mediated by different factors. To overcome the midgut barrier, a minimal viral load is necessary (Vazeille et al., 2019). Cool temperatures inhibited ZIKV transmission due to slow virus replication and escape from the midgut, resulting in a decrease in metrics of vector competence (e.g., the proportion of mosquitoes that became infected, disseminated infection, and became infectious) and lengthening the extrinsic incubation period (e.g., the interval of time before ZIKV can be detected in the saliva and mosquitoes become infectious [Tesla et al., 2018a; see **Figures 1**, **2**)]. In contrast, increased mosquito mortality at hotter temperatures constrained transmission despite efficient ZIKV infection and rapid dissemination.

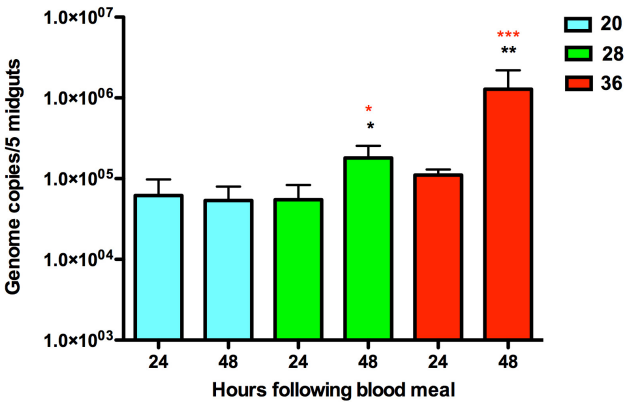

FIGURE 1 | Quantification of ZIKV RNA in infected *Aedes aegypti* mosquitoes housed at 20, 28, and 36°C at two time points, 24 and 48 h. The average number of ZIKV genome copies present in pools of five midgut samples and standard error are shown. The *y*-axis is log-transformed. Black asterisks represent *p*-value < 0.05 calculated by *T*-test between the time points of 24 and 48 h post-infection for the same temperature. Red asterisks represent *p*-value < 0.05 calculated by ANOVA with Tukey correction for multiple hypotheses between the three different temperatures for the same time point after blood meal.

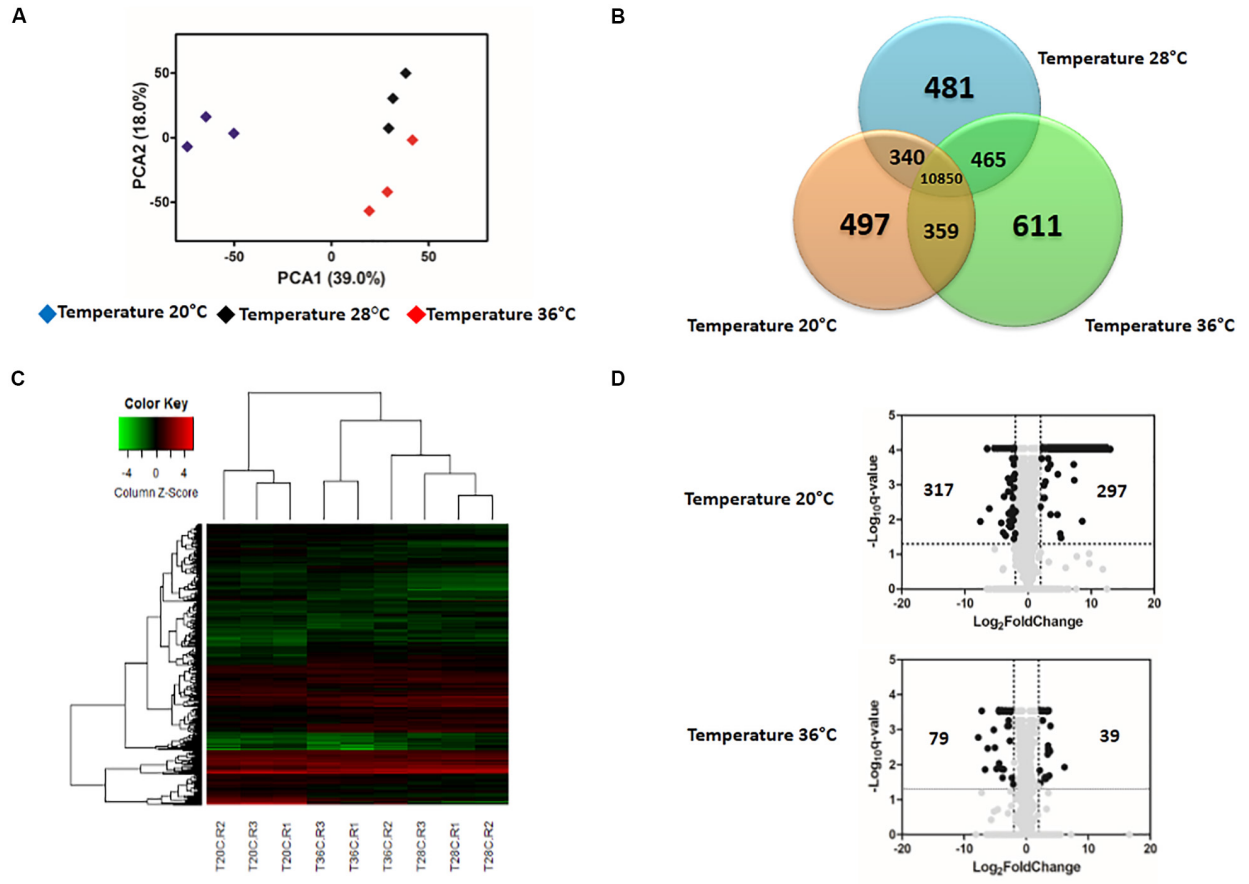

FIGURE 2 | Effect of temperature in the expression profile of uninfected blood-fed *Aedes aegypti.* **(A)** Principal Component Analysis (PCA) plot showing the global gene expression profiles. **(B)** Venn Diagram reporting the number of specific and shared genes. **(C)** Heatmap plot showing local differences (naming scheme: TC20R1 = replicate 1 for the temperature 20°C). **(D)** Volcano plot representing the differential gene expression in RNAseq samples from *Ae. aegypti* exposed to three different constant temperatures (20, 28, and 36°C) at 48 h.

These temperature constraints on infection are likely regulated through different mechanisms. Temperature variation in general could profoundly impact arbovirus infection and replication early in infection due to shifts in the balance and dynamics of the midgut environment, the first host environment encountered. This environment is fairly complex, as arboviruses encounter oxidative and nitration stress associated with digestion of the blood meal (Luckhart et al., 1998; Graça-Souza et al., 2006; Xi et al., 2008), the presence [e.g., *Wolbachia* (Bian et al., 2010)] and proliferation of microbial flora (Xi et al., 2008; Carissimo et al., 2014; Hegde et al., 2015; Saraiva et al., 2016; Barletta et al., 2017; Saldaña et al., 2017b), and key immune factors (Campbell et al., 2008; Xi et al., 2008; Cirimotich et al., 2009; Sánchez-Vargas et al., 2009; Souza-Neto et al., 2009). To better understand the effects of temperature on the ZIKV-mosquito interaction, we used RNA sequencing to describe the transcriptional response of *Ae. aegypti* midguts to ZIKV during the early infection process at three different temperatures (20, 28, and 36°C) previously shown to impact ZIKV infection, dissemination, and transmission rates (Tesla et al., 2018a).

MATERIALS AND METHODS

Ethics Statement

All mosquito infection work with ZIKV was approved by the University of Georgia, Athens Institutional Biosafety Committee (reference number 2015-0039).

Virus Production

Zika virus MEX1-44 was isolated from *Ae. aegypti* mosquitoes from Tapachula, Chiapas, Mexico in January 2016, kindly provided by the University of Texas Medical Branch Arbovirus Reference Collection. ZIKV stocks were propagated in Vero cells cultured in DMEM (Dulbeco's Modified Eagle Medium), 5% fetal bovine serum (FBS) at 37°C and 5% CO_2. Four days following inoculation, when cells showed visible cytopathic effect (>90%), supernatant containing virus was collected, cell debris was cleared by centrifugation (3,500 × *g* for 5 min), and virus was aliquoted and frozen at −80°C. The stock was titrated using standard plaque assays on Vero cells (Willard et al., 2017) and expressed in plaque-forming units per milliliter (PFU/mL).

Mosquito Husbandry

Aedes aegypti eggs collected in Chiapas, Mexico, were hatched in ddH_2O under reduced pressure in a vacuum desiccator. Larvae were grown in trays, with 200 larvae in 1L ddH_2O and four fish food pellets (Hikari Cichlid Cod Fish Pellets). Emerging adults were kept in rearing cages and fed with 10% sucrose solution *ad libitum*. Colonies were maintained on O-positive human blood (Interstate Blood Bank, male donors between 30 and 35 year). Both larvae and adults were maintained under the standard insectary conditions (27°C ± 0.5°C, 80% ± 5% relative humidity, and a 12:12 light: dark diurnal cycle) (Percival Scientific) that replicate rearing and experimental conditions used in Tesla et al. (2018a).

Experimental Mosquito Infection

Briefly, 3 to 4-day-old female mosquitoes (F5 generation; n = 600) were separated using a vacuum aspirator, transferred to an arthropod containment level three (ACL-3) facility at the University of Georgia and housed at 28°C ± 0.5°C, 80% ± 5% relative humidity, 12 h:12 h light:dark cycle (Percival Scientific). Mosquitoes were fed with a non-infectious or ZIKV-containing blood-meal (10^6 PFU/mL) after a 12-h period of starvation in a manner previously described (Tesla et al., 2018b). After the feed, engorged ZIKV-exposed (n = 120) and unexposed mosquitoes (n = 120) were randomly allocated across 12 paper cups (six with ZIKV-exposed and six with ZIKV-unexposed mosquitoes, n = 20 mosquitoes per cup). Forty engorged females that fed on either infectious blood or non-infectious blood were randomly distributed across one of three temperature treatments, 20°C, 28°C, and 36°C (±0.5°C) at 80% ± 5% relative humidity and with a 12 h:12 h light:dark cycle. Mosquitoes were provided 10% sucrose *ad libitum* throughout the duration of the experiment (48 h), and three full biological replicates were performed.

Viral Genome Quantification

Viral genomes were detected as previously described (Willard et al., 2019). Briefly, dissected mosquito midguts were collected in pools of five, and viral RNA was isolated using the Zymo Quick-RNA Viral Kit (Zymo, Irvine, CA, United States). Viral RNA samples were reverse-transcribed (RT) to cDNA (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, CA, United States). The F5 generation was used to maintain genetic proximity with field populations of *Ae. aegypti*. To quantify the number of ZIKV genomes, we used the cDNA in a quantitative PCR (qPCR) reaction assay using TaqMan Gene Expression Master Mix (Applied Biosystems, ThermoFisher, Waltham, MA, United States), primers, and probes (F: ZIKV 1086, R: ZIKV 1162c, ZIKV 1107-FAM; TaqMan MGB Probe; Invitrogen Custom Primers) (Lanciotti et al., 2008). Each sample was analyzed in duplicate, and each plate contained a DNA plasmid standard curve (ZIKV molecular clone), no template, and no primer controls. ZIKV copy numbers were extrapolated from the generated standard curve using the Applied Biosystems protocol. The limit of detection was experimentally established to be 30 copies. Final copy numbers were adjusted by back-calculations to the total RNA and cDNA volume and expressed as copies per five-midgut pool. Outliers were identified by Grubbs test, implemented in Graphpad QuickCalcs[1], and removed. The results were analyzed in GraphPad Prism 5.0. An unpaired T-test was applied to compare the two different times (24 and 48) post-infection for the same temperature. ANOVA with Tukey as the multiple hypothesis correction was used to compare the data from the three different temperatures (20, 28, and 36) at the same time points. A p-value below 0.05 was considered statistically significant.

Midgut Dissection and RNA Isolation

Mosquito responses to blood meal digestion, midgut microbial proliferation, and arbovirus midgut infection contribute significantly to viral bottlenecks during infection, and these processes could dynamically differ with temperature (Xi et al., 2008; Bonizzoni et al., 2011; Oliveira et al., 2011; Ramirez et al., 2012). Further, these responses likely occur early on during the infection process (<3 days post-infection) at optimal temperatures for ZIKV transmission (e.g., 29°C), as the proportion of mosquitoes with ZIKV-infected midguts plateaus by day three post-infection at temperatures above 28°C (Tesla et al., 2018a). To capture gene expression changes in the mosquito midgut early during the infection process, we isolated total RNA from midgut tissues. Fifteen ZIKV-exposed and 15 non-exposed mosquitoes were removed from each temperature at 24 and 48 h post-infection for RNA sequencing. The mosquitoes were killed with cold ethanol and washed in cold PBS containing 0.2% RNase inhibitor (Sigma-Aldrich) and 0.1% DEPC (Amresco). Midguts were carefully dissected and stored immediately in RNAlater (Invitrogen) at 4°C for 24 h, after which they were transferred to −80°C conditions. Total RNA was isolated from pools of 15 midguts using the Qiagen RNeasy Mini Kit as per the manufacturer's instructions.

Library Preparation and Sequencing

Extracted RNA was sent to the University of Georgia Genomics Core for cDNA library preparation and RNA sequencing. The quality of the total RNA was analyzed using an Agilent Bioanalyzer RNA 6000 Pico Kit (Agilent Technologies). Poly(A) mRNA from total RNA was captured by magnetic oligo-dT beads and fragmented by heating (94°C, 6 min) in the presence of divalent cations (Mg^{2+}) using the KAPA Stranded mRNA-Seq Kit for Illumina®. Fragmented poly(A) mRNA samples were converted to cDNA using random priming. After second-strand synthesis, an adenine residue was added to 3′-end of dscDNA fragments, and Illumina TruSeqLT adapter ligation to 3′-dAMP library fragments was performed. Adapter-ligated DNA was amplified by PCR using the KAPA Library Amplification Primer Mix. After creating the library of DNA templates, the fragment size distribution of these libraries was confirmed by electrophoresis and the library concentration was determined by qPCR. Sequencing was completed on an Illumina NextSeq 500 using the PE75bp (75 bp sequencing reads) settings on a High-Output 150-cycle kit using Illumina standard protocols for loading. Raw sequencing files were demultiplexed using the

[1]https://www.graphpad.com/quickcalcs/Grubbs1.cfm

Illumina BaseSpace cloud platform demultiplexing pipeline. Four technical replicates were run per sample.

RNA Sequence Analysis in Response to Temperature and Zika Virus Exposure

The Tuxedo suite of tools was used to analyze the RNA-seq data (Trapnell et al., 2013). At each stage of the analysis pipeline, we were careful to identify and correct possible sources of bias in the study (Conesa et al., 2016). Read quality was assessed using FastQC (Andrews, 2010). Poor quality reads (quality score below 20), short reads (less than 25 bases), and adapter sequences (Bolger et al., 2014) were removed using Trimmomatic (v 0.36).

We aligned and mapped clean reads up to two different loci to the *Ae. aegypti* genome (NCBI ID: GCA_002204515.1) obtained from NCBI[2] using TopHat (v 2.1.1) (Trapnell et al., 2009). We then performed differential gene expression analysis on RNAseq reads using Cuffdiff (Trapnell et al., 2013), which calculates expression levels in response to experimental treatments of interest (e.g., temperature and ZIKV-exposure) and determines whether observed variations in expression levels across groups are significantly different. The *Ae. aegypti* genome and its annotation file (NCBI ID: GCA_002204515.1) were used to run bias detection and determine the transcripts examined for differential expression, respectively. The relative expression levels were produced as fragments per kilobase of transcript per million fragments mapped (FPKM values), which normalized read count based on gene length and the total number of mapped reads. After quantification and normalization of expression values, differential expression analysis was carried out on the experimental data from 24 and 48 h following the blood meal. To characterize changes in the RNA transcriptome in response to temperature, we compared the RNA expression profiles of unexposed mosquitoes maintained at standard conditions and near the predicted thermal optimum for ZIKV transmission (28°C) to those of mosquitoes housed under cool (20°C) and hot (36°C) conditions. To determine whether temperature modified global RNA expression in ZIKV-exposed individuals, we compared whether the variation in expression profiles of ZIKV-exposed individuals across temperature treatments was similar to that of their unexposed counterparts. Finally, to evaluate the effects of ZIKV infection on global RNA expression, we compared RNA expression between unexposed and ZIKV-exposed mosquitoes within a given temperature treatment. The data quality assessment from the RNAseq analysis was performed using R package (v 3.4.4) (R Development Core Team, 2008) and plotted using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego, CA, United States)[3]. Perl scripts were written to extract specific information from RNAseq analysis output files necessary for each assessment of the results obtained. Principal-component analysis (PCA) was employed to examine the quality of replicates and the overall differences between samples and to determine the proximity among the experimental groups.

[2]https://www.ncbi.nlm.nih.gov/

[3]www.graphpad.com

Gene Ontology (GO) Analysis

We employed BiNGO (v 3.0.3) (Maere et al., 2005) to determine which GO terms were significantly over-represented in a set of genes. First, the FASTA sequences of all differentially expressed genes were recovered using the protein database on the NCBI's Batch Entrez[4]. The sequences were inputted into STRING (v 10.5) (Szklarczyk et al., 2017) to predict protein–protein interactions. GO functional annotations were provided by AgBase-Goanna (v 2.00) (Mccarthy et al., 2006), using the *Ae. aegypti* protein sequences in FASTA format from the STRING database as an input file. The similarity search for GO annotations was performed by Blastp using the UniProt Database with *E*-value: $10e^{-5}$, Matrix: BLOSUM62, and word size: 3. This file annotation was employed as a reference set for enrichment analysis. We used the default mode, employing Hypergeometric tests for assessing the enrichment of a GO term in the test set, and *p*-values were corrected by Benjamini and Hochberg False Discovery Rate (FDR) correction to control for type I error. Corrected *p*-values less than 0.05 were considered significant.

RESULTS

From a total of 36 RNA samples that were sequenced using the Illumina platform, the number of clean paired reads varied between 2,063,917 and 3,028,305 for each library. All libraries resulted in a concordant pair alignment rate higher than 70%.

Cool Temperatures Restrict ZIKV Replication in the Mosquito Midgut

Viral RNA quantification of exposed mosquitoes revealed levels of ZIKV RNA in midgut samples from mosquitoes that imbibed a blood meal containing ZIKV (**Figure 1**). ZIKV replication was evident in mosquitoes housed at 28°C and 36°C, with increases in mosquito RNA copy number between 24 and 48 h post-feed (hpf). Further, the efficiency of ZIKV replication was maximized at the warmest temperature, with mosquitoes housed at 36°C having higher ZIKV RNA levels in their midguts than those housed at 28°C. Although the presence of ZIKV RNA was observed 24 and 48 h following the infectious blood meal in mosquitoes housed in the cool environment (20°C), we did not find significant increases in the viral copy number (**Figure 1**). This result suggests that cooler temperatures constrain ZIKV replication in the midgut.

The Effect of Temperature on Gene Expression in Unexposed Mosquitoes

Gene-expression patterns were influenced by both temperature and time post-blood feed. In general, the principal component analysis (PCA) plot showed a high degree of reproducibility among the replicate samples within each temperature treatment. At 24 hpf, temperature clearly distinguished gene expression profiles (**Supplementary Figure S1A**). However, by 48 hpf, mosquitoes housed at 28°C and 36°C had gene expression

[4]https://www.ncbi.nlm.nih.gov/sites/batchentrez

profiles that were more similar to each other than to mosquitoes housed at 20°C (**Figure 2A**). According to the Venn diagram, the vast majority of genes expressed in the midgut were similar for mosquitoes housed across the three temperature treatments (10,459 at 24 hpf, **Supplementary Figure S1B**; 10,850 at 48 hpf, **Figure 2B**). When concentrating on the differentially expressed genes, Euclidean distance heatmap analysis demonstrated that samples at 24 hpf were separated by temperature treatment in three distinct groups (**Supplementary Figure S1C**), while for samples at 48 hpf, 28 and 36°C samples cluster more closely with one another than with the 20°C (**Figure 2C**). This is the same profile as was seen in the PCA analysis, which employed all transcriptome data. Among these genes, 1665 differentially expressed genes (q-value < 0.05) were seen between mosquitoes held at 20 and 28°C for 24 h, and 3634 were seen by 48 h (**Supplementary Table S1**). In contrast, when comparing mosquitoes held at 36 and 28°C, we identified 1697 and 1695 differentially expressed genes after 24 and 48 h, respectively (**Supplementary Table S2**). In order to perform a more stringent analysis, we produced a volcano plot, which indicated a total of 70 and 297 upregulated genes [$\log_2$(Fold Change) >2 and q-value < 0.05] in mosquitoes housed at 20°C for 24 and 48 h, respectively, compared to those housed at 28°C. For downregulated genes [$\log_2$(Fold Change) <-2 and q-value < 0.05], we found 53 and 317 after 24 and 48 h, respectively (**Supplementary Figure S1D** and **Figure 2D**). We did not find as strong an effect of exposure time when mosquitoes were housed at 36°C, with only 19 and 39 genes up-regulated and 89 and 79 genes down-regulated after 24 and 48 h, respectively (**Supplementary Figure S1D** and **Figure 2D**).

To further define the effect of temperature on mosquito cellular and physiological responses, we sorted the top 20 up- or down-regulated genes at both the cool and hot temperatures, using mosquitoes maintained at 28°C as our standard (**Table 1**). The cool temperature produced a larger response, up-regulating genes hundreds of times compared to standard conditions, whereas the 36°C treatment had a more moderate effect. The transcript with the highest enrichment at 20°C was protein-G12 (1459-fold), whereas a serine protease easter was the most down-regulated (187-fold). For mosquitoes housed at 36°C, heat shock protein 70 (HSP 70) was the transcript most enriched (71-fold) relative to 28°C, while facilitated trehalose transporter Tret1 was the most down-regulated gene (212-fold). Surprisingly, both cool and warm temperature treatments induced some of the same genes to be differentially regulated. Six genes (protein G12, serine protease SP24D, and chymotrypsin-2) were up-regulated in mosquitoes housed at both 20°C and 36°C when compared to those at 28°C, while six genes (serine protease easter, facilitated trehalose transporter Tret1, venom protease, solute carrier family 2 facilitated glucose transporter member 3, and tryptase) were down-regulated.

All differentially expressed genes in the midgut at 48 hpf were submitted for gene ontology (GO) analysis to identify cellular processes that were most perturbed in response to temperature treatment, as revealed by the transcriptome profile. GO analysis of the enriched and depleted transcripts from mosquitoes housed at 20°C revealed that 20 enriched GO terms were related to oxidation–reduction processes and 111 depleted GO terms were involved in gene expression, RNA processing, metabolic processes, and generation of energy (**Figure 3**). Mosquitoes housed at 36°C displayed up-regulated expression related to amine metabolism and cell redox homeostasis processes and down-regulated expression of genes associated with metabolic processes, cellular respiration, and energy derivation by oxidation of organic compounds (**Figure 3**).

The Effect of Temperature on Gene Expression in ZIKV-Exposed Mosquitoes

The gene expression profiles in ZIKV-exposed mosquitoes were also significantly affected by environmental temperature and time post-blood feed. In general, the effect of temperature on differential gene expression was similar to patterns observed in non-ZIKV-exposed blood-fed control mosquitoes outlined above. For example, PCA and heatmap analyses on differential gene expression from mosquito midguts at 24 hpf illustrated three distinct groups separated by temperature treatment (**Supplementary Figures S2A,C**). At 48 hpf, gene expression in mosquitoes housed at 20°C was more distinct than those housed at 28 and 36°C (**Figures 4A,C**). Further, Venn diagrams demonstrate that 1,416 and 10,786 genes were expressed across all temperatures at 24 hpf (**Supplementary Figure S2B**) and 48 hpf (**Figure 4B**), respectively, with the highest overlap in gene expression occurring in mosquitoes housed at 28°C and 36°C at both time points (**Supplementary Figure S2B** and **Figure 4B**). Finally, as seen in the absence of ZIKV infection, at 24 hpf, only 1669 and 1797 genes were differentially expressed in mosquitoes housed at 20 and 36°C, respectively, relative to those housed at 28°C. By 48 hpf, we observed a general increase in the number of genes differentially expressed at 20°C (3056, **Supplementary Table S3**) and a general decrease at 36°C (1518, **Supplementary Table S4**) relative to mosquitoes housed at 28°C. When we evaluated differential expression through a volcano plot, the profile was similar to that of mosquitoes that were not exposed to ZIKV (**Supplementary Figure S2D** and **Figure 4D**).

Several genes that strongly increased at 20°C remained among the top 20 differentially expressed after ZIKV exposure. Lysosomal alpha-mannosidase (XP_021703511.1), two vitellogenins (XP_001660818.2 and XP_001657509.1), phosphoenolpyruvate carboxykinase (XP_001647937.2), protein G12 isoforms (XP_021712126.1, XP_021701760.1, XP_001660827.1, XP_021701761.1, XP_001656377.1, and XP_001656375.1), beta-galactosidase (XP_021705369.1), alpha-N-acetylgalactosaminidase (XP_001650490.2), serine protease SP24D (XP_001659962.1), and chymotrypsin-2 (XP_021698904.1) are some of the genes that remained among the top 20 genes changed by cold temperature in the ZIKV-infection condition. We also saw that although glutamine synthetase (XP_001654186.1) and trypsin (XP_001663002.1) were not listed among the 20 most differentially expressed, they showed enrichment of 50- and 74-fold, respectively. Further, facilitated trehalose transporter Tret1, found to be the most down-regulated in unexposed mosquitoes kept at 36°C, was also the most down-regulated (25-fold) in ZIKV-exposed mosquitoes

TABLE 1 | Top 20 up- and down-regulated genes of uninfected *Aedes aegypti* in response to low (20°C) and to high (36°C) temperature for 48 h post-blood-feeding (relative to standard rearing temperature of 28°C).

GENES UP-REGULATED		TEMPERATURE 20°C		TEMPERATURE 36°C	
Gene ID	**Gene Description**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_021701760.1	Protein G12	1459.541989	0.0000886602	11.11657146	0.000290131
XP_021698904.1	Chymotrypsin-2	857.284352	0.0000886602		
XP_001660827.1	Protein G12	603.3619885	0.0000886602	7.4143488679	0.000290131
XP_001650490.2	Alpha-N-acetylgalactosaminidase	548.7746389	0.0000886602		
XP_001659962.1	Serine protease SP24D	473.2635493	0.0000886602	11.29286782	0.000290131
XP_021712126.1	Protein G12-like	442.0421098	0.0000886602		
XP_021701761.1	Protein G12	402.0329675	0.0000886602	6.3523041907	0.000548702
XP_001663002.1	Trypsin 3A1-like	384.5293743	0.0110252		
XP_021705369.1	Beta-galactosidase	315.6714553	0.0000886602		
XP_001656375.1	Protein G12	245.4048583	0.0000886602		
XP_001656377.1	Protein G12 isoform X2	221.8114454	0.0000886602	5.0806039301	0.000290131
XP_021703511.1	Lysosomal alpha-mannosidase isoform X2	217.023882	0.0000886602		
XP_001647937.2	Phosphoenolpyruvate carboxykinase [GTP]	180.2043466	0.0000886602		
XP_001659796.1	Beta-1,3-glucan-binding protein	159.2092116	0.000730421		
XP_021712858.1	Protein G12 isoform X2	149.8743826	0.000256108		
XP_001657509.1	Vitellogenin-A1-like	138.4047839	0.0000886602		
XP_001659961.1	Chymotrypsin-2	110.7367288	0.0000886602	8.596677982	0.000290131
XP_001652194.1	Protein singed wings 2	84.66536466	0.0000886602		
XP_001660818.2	Vitellogenin-A1	67.79655212	0.0000886602		
XP_001654186.1	Glutamine synthetase 1 mitochondrial	65.69082595	0.0000886602		
XP_021693649.1	Heat shock protein 70 A1			71.53314665	0.0118095
XP_001660673.2	Trypsin 5G1-like			14.98866514	0.0007852
XP_001663776.1	Protein G12			14.35856831	0.00416417
XP_001658359.2	Brachyurin			12.24869336	0.000290131
XP_021701762.1	Protein G12-like			12.07440339	0.00379042
XP_001663895.2	Trypsin 5G1			11.04851064	0.00505811
XP_001658471.2	Mite group 2 allergen Gly			10.38200486	0,000290131
XP_011493274.2	Extensin			9.222769233	0.000290131
XP_001659492.2	Serine protease SP24D			7.5082672369	0.000290131
XP_001661186.2	Protein G2			6.9220050786	0.000290131
XP_001651623.2	Surface antigen CRP170			6.151678044	0.000290131
XP-021712126.1	Protein G12-like			5.6022641153	0.000548702
XP_001660908.1	Maltase 1			5.3506716535	0.000290131
XP_011492940.1	Peptidoglycan-recognition protein SC-2			5.030075309	0.000290131
GENES DOWN-REGULATED		**TEMPERATURE 20°C**		**TEMPERATURE 36°C**	
Gene ID	**Gene Description**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_001652078.2	Serine protease easter	187.8360285	0.0112238	33.06859855	0.0032544
XP_001658147.1	Facilitated trehalose transporter Tret1	39.72595509	0.0000886602	212.5163457	0.00166701
XP_001652075.2	Venom protease	31.87315659	0.0000886602	21.42212957	0.000290131
XP_001663064.2	UDP-glucuronosyltransferase 2B18	28.70798168	0.0000886602		
XP_001652079.1	Serine protease easter	18.68203877	0.0124204	15.08894466	0.0134155
XP_001655069.1	Queuine tRNA-ribosyltransferase accessory subunit 2	15.34875785	0.0000886602		
XP_001656516.2	Xaa-Pro aminopeptidase ApepP	15.23840501	0.0000886602		
XP_001649987.2	Synaptic vesicle glycoprotein 2B	13.81630566	0.00215644		
XP_021706766.1	Glycerol-3-phosphate dehydrogenase mitochondrial isoform X1	13.39803945	0.0000886602		
XP_021699787.1	Solute carrier family 2 facilitated glucose transporter member 3	13.34678167	0.0000886602	99.27028299	0.0138613
XP_001655305.2	Protein MAK16 homolog A	12.43254717	0.0000886602		
XP_001649768.1	Exosome complex component RRP43	11.98593043	0.0000886602		
XP_001653029.1	Protein takeout	11.66609644	0.0000886602		

(Continued)

TABLE 1 | Continued

GENES DOWN-REGULATED		TEMPERATURE 20°C		TEMPERATURE 36°C	
Gene ID	**Gene Description**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_021698953.1	CAD protein	11.58599466	0.0291848		
XP_001656680.1	NHP2-like protein 1 homolog	10.79948043	0.0000886602		
XP_001655729.2	Tryptase	10.69882162	0.0000886602	13.05191869	0.000290131
XP_001662512.1	Nucleoside diphosphate kinase	10.5849313	0.0000886602		
XP_001663497.1	Protein lethal(2)essential for life	10.23543137	0.0000886602		
XP_001655105.1	Sorbitol dehydrogenase	10.21211653	0.0000886602		
XP_021699336.1	Nucleoside diphosphate kinase-like	10.07585248	0.0000886602		
XP_001652056.2	Vitellogenic carboxypeptidase			37.57757336	0.00101347
XP_001655104.1	Sorbitol dehydrogenase			27.67324652	0.013266
XP_001663173.2	Solute carrier family 22 member 8			19.16072174	0.000290131
XP_021702456.1	Sodium-coupled monocarboxylate transporter 1 isoform X1			18.47599301	0.000290131
XP_001651077.2	Synaptic vesicle glycoprotein 2B			17.7140231	0.000290131
XP_001656519.2	Solute carrier family 22 member 21 isoform X2			14.01519585	0.000290131
XP_001662495.2	Lipase 1			13.73780875	0.000290131
XP_021706833.1	Sodium-coupled monocarboxylate transporter 1 isoform X2			13.50049126	0.000290131
XP_021698236.1	Solute carrier organic anion transporter family member 2A1			11.93072658	0.000290131
XP_021702682.1	Acidic amino acid decarboxylase GADL1 isoform X3			11.91023521	0.000290131
XP_001649855.2	Sodium/potassium/calcium exchanger 4			11.67450924	0.000290131
XP_021710339.1	Synaptic vesicle glycoprotein 2C			10.26811889	0.000290131
XP_001662720.1	Putative transporter SVOPL			10.08241963	0.000290131
XP_021713275.1	Synaptic vesicle glycoprotein 2A			8.744949179	0.000290131

kept at the same temperature. Finally, serine protease SP24D (XP_001659962.1) and some G12 proteins (XP_021701760.1, XP_021701761.1, XP_001660827.1, and XP_001656377.1) that were enriched at both cool and warm temperatures remained enriched at these temperatures in ZIKV-exposed mosquitoes (**Table 2**).

Despite these similarities, we did note some key differences between the top 20 genes most differentially expressed in unexposed (**Table 1**) and ZIKV-exposed mosquitoes (**Table 2**), with the greatest change in expression reflected in mosquitoes housed at 20°C and 36°C at 48 h relative to those housed at 28°C. In unexposed mosquitoes kept at 20°C, vitellogenin-A1-like (XP_001657509.1) and vitellogenin-A1 (XP_001660818.2) were up-regulated, yet ZIKV exposure amplified the enrichment from 138- and 68-fold to 3748- and 2644-fold (**Tables 1**, **2**). Surprisingly, in ZIKV-exposed mosquitoes, we did not detect a large up-regulation of Hsp70 at 36°C like we did in the unexposed population.

The GO analysis of differentially expressed genes at 48 hpf in ZIKV-exposed mosquitoes demonstrated distinct effects of cool and warm temperatures on cellular and metabolic function relative to mosquitoes housed at 28°C (**Figure 5**). Further, the functions of these differentially expressed genes were, in part, different from those in unexposed mosquitoes housed at these temperatures (**Figure 3**). For example, when maintained at 20°C, oxidative-reduction processes were no longer enriched, as was seen in unexposed mosquitoes. Instead, ZIKV-exposed mosquitoes housed at 20°C had significant enrichment of the Toll signaling pathway, a known anti-dengue pathway in *Ae. aegypti* (Tchankouo-Nguetcheu et al., 2010). Additionally, endosome transport, Ras protein signal transduction, actin cytoskeleton organization, epithelial tube morphogenesis, pH reduction, and proteolysis were enriched. In contrast, nuclear transport, regulation of viral reproduction, "*de novo*" protein folding, generation of energy, gene expression, RNA processing, and metabolic processes were down-regulated in addition to the expression associated with gene expression, RNA processing, and metabolic processes observed in unexposed counterparts at this temperature (**Figure 5**). ZIKV-exposed mosquitoes housed at 36°C no longer had significant enrichment in genes associated with cellular amine processes and had significant depletion in genes associated with hexose and phosphate metabolic processes and with the generation of precursor metabolites and energy (**Figure 5**).

Effects of Temperature on Oxidative Stress and Innate Immune Mechanisms

To further explore the effects of temperature on uninfected and ZIKV-exposed mosquitoes, we highlight differences in those genes involved in managing oxidative stress, innate immunity, and apoptosis at both 20 and 36°C compared to 28°C at 48 hpf (**Supplementary Tables S5**, **S6** and **Supplementary Figures S3**, **S4**). Virus infection has previously been shown to be modified by complex responses related to detoxification of the blood meal, metabolism, immunity, and apoptosis in some systems (Sanders et al., 2005; Girard et al., 2010; Tchankouo-Nguetcheu et al., 2010; Colpitts et al., 2011; Wang et al., 2012; Neill et al., 2015; Eng et al., 2016). Further, these responses need not respond

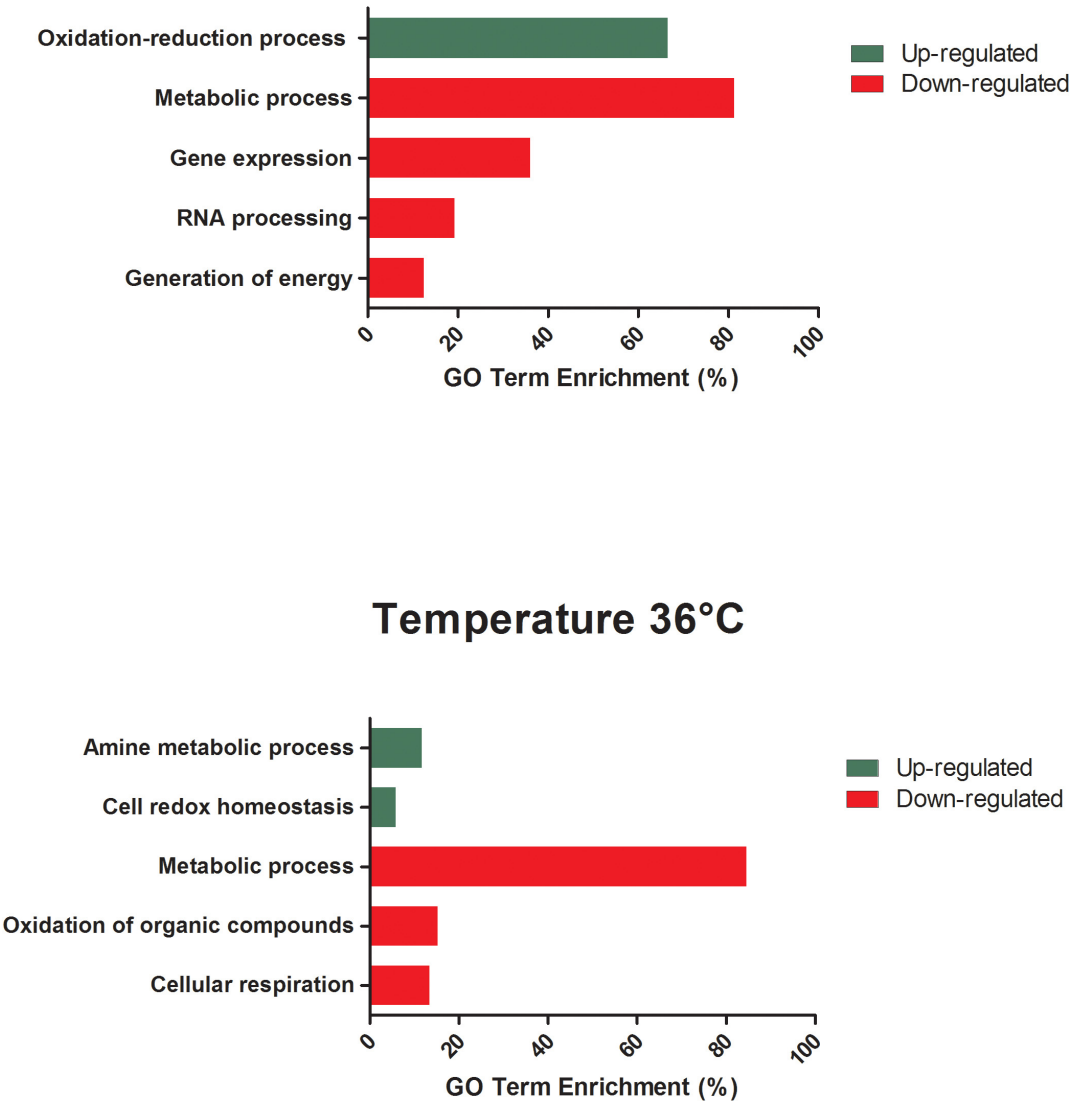

FIGURE 3 | GO term enrichment analysis of differentially expressed genes of unexposed *Aedes aegypti* kept under the lowest (20°C) and the highest (36°C) temperature conditions in the category of biological processes for up- and down-regulated genes at 48 h.

equivalently to temperature variation, as shown in previous work, which demonstrated that mosquito immune responses differed qualitatively and quantitatively across a range of environmental temperatures (Murdock et al., 2012b). The majority of genes involved in managing oxidative stress, innate immunity, and apoptosis exhibited qualitatively different patterns in gene expression in response to cool and warm temperatures in uninfected and ZIKV-exposed mosquitoes. However, while significant, the majority of these differences were very subtle (<2.0-fold; **Supplementary Figures S3**, **S4**). We did observe components of the melanization and Toll pathways to be modestly expressed (>2.0 fold; **Supplementary Figures S3**, **S4**) in response to temperature. An isoform of phenoloxidase (XP_021699380.1), a c-type lectin (XP_001661643.1), and the Toll receptor 6 (TLR6) (XP_021712805.1) were significantly enriched in both uninfected and ZIKV-exposed mosquitoes housed at 20°C.

Mosquito Responses to ZIKV Infection Are Remarkably Modulated by Environmental Temperature

To investigate the effects of ZIKV-exposure on global gene-expression patterns from mosquito midguts early on in the

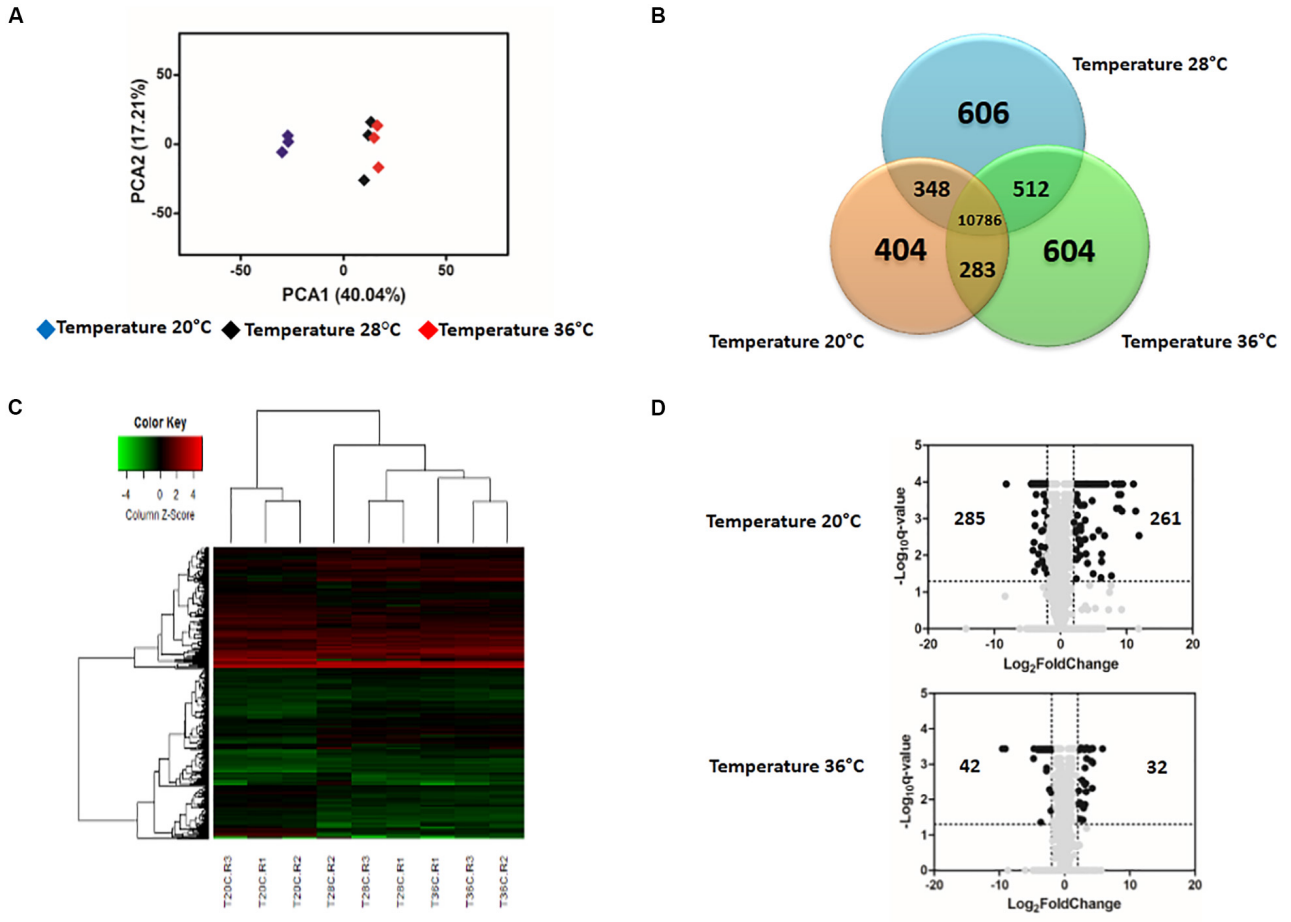

FIGURE 4 | Effect of temperature on the expression profile of Zika-exposed *Aedes aegypti.* **(A)** Principal Component Analysis (PCA) plot showing the global gene expression profiles. **(B)** Venn Diagram reporting the number of specific and shared genes. **(C)** Heatmap plot showing local differences (naming scheme: TC20R1 = replicate 1 for the temperature 20°C). **(D)** Volcano plot representing the differential gene expression in RNAseq samples from infected *Ae. aegypti* exposed to three different constant temperatures (20, 28, and 36°C) at 48 h.

infection process, we compared gene expression between ZIKV-exposed and unexposed mosquitoes within each temperature treatment. The PCA plots demonstrate that ZIKV exposure does not alter mosquito transcription at 24 hpf (**Supplementary Figure S5**) within a given temperature treatment. However, 48-hpf ZIKV-exposed and unexposed samples under the cold temperature condition were distributed in two distinct groups, although one of the ZIKV-exposed biological replicates was relatively close to the control group (**Figure 6A**). Also, the overall number of differentially expressed genes between ZIKV-exposed and unexposed mosquitoes varied across temperature treatments. For example, at 24 hpf, we observed a total of 225, 154, and 161 genes differentially expressed between ZIKV-exposed and unexposed mosquitoes at 20°C, 28°C, and 36°C, respectively (**Supplementary Tables S7–S9**). We identified only two proteins – a sodium/potassium/calcium exchanger 4 (XP_001649855.2) and an uncharacterized protein (XP_001654261.2) – that were up-regulated in ZIKV-exposed mosquitoes at all temperatures (**Supplementary Figure S6A**) and one protein – chymotrypsin-2 (XP_021698609.1) that was down-regulated (**Supplementary Figure S6B**). At 48 hpf, we observed more genes to be differentially expressed (1188 genes) in mosquitoes housed at 20°C between ZIKV-exposed and unexposed mosquitoes, versus only 180 and 50 genes at 28 and 36°C, respectively (**Supplementary Tables S7–S9**). Only one uncharacterized protein (XP_001658660.2) was up-regulated in the ZIKV-exposed mosquitoes at all temperatures (**Figures 7A,B**) at this sampling time point. These results indicate that while the physiological responses of mosquito midguts to ZIKV exposure early in the infection process are similar within a given temperature treatment, these responses are significantly distinct across different environmental temperatures.

When concentrating on the top 10 most differentially expressed genes between ZIKV-exposed and unexposed mosquitoes at each temperature treatment at 48 hpf (**Table 3**), only at 20°C did we observe genes with altered expression (enrichment or depletion) of 10-fold or more. GO analysis

TABLE 2 | Top 20 up- and down-regulated genes of Zika-exposed *Aedes aegypti* in response to low (20°C) and to high (36°C) temperature for 48 h post-blood-feeding relative to standard insectary conditions (28°C).

GENES UP-REGULATED		TEMPERATURE 20°C		TEMPERATURE 36°C	
Gene ID	**Gene Description**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_001657509.1	Vitellogenin-A1-like	3748.76585	0.00288439		
XP_001660818.2	Vitellogenin-A1	2644.35472	0.000624083		
XP_021701760.1	Protein G12	2081.92156	0.000113659	18.9560964	0.00036736
XP_001660827.1	Protein G12	668.331471	0.000113659	9.8533206	0.00036736
XP_021701762.1	Protein G12-like	626.881959	0.000624083	9.79462325	0.00599791
XP_021712126.1	Protein G12-like	580.732514	0.000113659	8.68875819	0.00126501
XP_001663776.1	Protein G12	519.780968	0.000113659	10.3753637	0.000690526
XP_021701761.1	Protein G12	498.942585	0.000113659	9.76777521	0.00036736
XP_021698904.1	Chymotrypsin-2	476.004037	0.000113659		
XP_001657506.2	Vitellogenin-A1-like	467.414928	0.000526591		
XP_001656377.1	Protein G12 isoform X2	400.561513	0.000113659	8.94238711	0.00036736
XP_021702099.1	Probable nuclear hormone receptor HR3 isoform X5	356.184271	0.000526591		
XP_001656375.1	Protein G12	315.925373	0.000113659		
XP_001659962.1	Serine protease SP24D	315.50958	0.000113659	8.63508273	0.00036736
XP_021705369.1	Beta-galactosidase	310.614205	0.000113659		
XP_001650490.2	Alpha-N-acetylgalactosaminidase	292.584595	0.000113659		
XP_001652055.1	Vitellogenic carboxypeptidase-like	206.875645	0.0359751		
XP_001652056.2	Vitellogenic carboxypeptidase	123.386436	0.000113659		
XP_001647937.2	Phosphoenolpyruvate carboxykinase [GTP]	106.495599	0.000113659		
XP_021703511.1	Lysosomal alpha-mannosidase isoform X2	103.072096	0.000113659		
XP_001652358.2	Peritrophin-1			55.8211523	0.00036736
XP_021697715.1	Peritrophin-1-like			16.3831402	0.00036736
XP_001658471.2	Mite group 2 allergen Gly d 2.01			15.940003	0.00036736
XP_001663895.2	Trypsin 5G1			9.16859073	0.00365688
XP_001660673.2	Trypsin 5G1-like			8.69411995	0.0136618
XP_001661186.2	Protein G12			7.2863687	0.00036736
XP_001663102.2	Malate synthase			6.86576273	0.00036736
XP_001663439.2	Collagenase			6.39130263	0.00279659
XP_021707618.1	Probable chitinase 2			6.27684681	0.00036736
XP_001651623.2	Surface antigen CRP170			5.38042449	0.00036736
XP_001653091.2	Trypsin alpha-3			5.26940475	0.00036736
XP_001659961.1	Chymotrypsin-2			5.00475692	0.00036736
GENES DOWN-REGULATED		**TEMPERATURE 20°C**		**TEMPERATURE 36°C**	
	Gene Description	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_001663064.2	UDP-glucuronosyltransferase 2B18	22.0442425	0.000113659	5.62311675	0.00036736
XP_011493503.2	H/ACA ribonucleoprotein complex subunit 1	20.5197322	0.000113659		
XP_001654398.2	rRNA 2'-O-methyltransferase fibrillarin	19.1980783	0.000113659		
XP_001659197.1	DNA-directed RNA polymerase III subunit RPC10	18.0109204	0.00732014		
XP_001658147.1	Facilitated trehalose transporter Tret1	17.354405	0.000113659	25.7984056	0.00036736
XP_001663005.2	Periodic tryptophan protein 1 homolog	16.363733	0.000113659		
XP_021698953.1	CAD protein	16.0097625	0.00443189		
XP_001655305.2	Protein MAK16 homolog A	15.3024421	0.000113659		
XP_001656228.2	COX assembly mitochondrial protein 2 homolog	14.7057657	0.000721477		
XP_021706766.1	Glycerol-3-phosphate dehydrogenase mitochondrial isoform X1	14.6844772	0.000113659		
XP_001649209.1	RRP15-like protein isoform X2	14.5214136	0.00156177		
XP_001652303.1	mRNA turnover protein 4 homolog	14.3570755	0.000113659		
XP_001662723.2	Titin homolog	14.3017521	0.000113659		

(Continued)

TABLE 2 | Continued

GENES DOWN-REGULATED		TEMPERATURE 20°C		TEMPERATURE 36°C	
	Gene Description	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_021699084.1	Proton-coupled amino acid transporter 1-like	14.2666035	0.000113659		
XP_001658562.1	Activator of basal transcription 1	13.971742	0.000113659		
XP_021695012.1	46 kDa FK506-binding nuclear protein	13.8346093	0.000113659		
XP_001660583.2	Glutamate-rich WD repeat-containing protein 1	13.2594589	0.000113659		
XP_021703839.1	Protein Notchless	13.0245353	0.000113659		
XP_001656680.1	NHP2-like protein 1 homolog	12.0716418	0.000113659		
XP_001655996.2	H/ACA ribonucleoprotein complex non-core subunit NAF1	11.9572191	0.000113659		
XP_001662495.2	Lipase 1			16.3418567	0.00036736
XP_021698905.1	Chymotrypsin-2			13.9310298	0.00036736
XP_001649987.2	Synaptic vesicle glycoprotein 2B			12.6667105	0.0434966
XP_021702682.1	Acidic amino acid decarboxylase GADL1 isoform X3			11.6866538	0.00036736
XP_001649098.2	Probable cytochrome P450 9f2			8.68297843	0.00036736
XP_021699787.1	Solute carrier family 2 facilitated glucose transporter member 3			7.85777033	0.00036736
XP_001652075.2	Venom protease			6.9627634	0.00153067
XP_021702456.1	Sodium-coupled monocarboxylate transporter 1 isoform X1			6.31621377	0.00036736
XP_001651954.2	Trypsin			6.12067175	0.00036736
XP_001649855.2	Sodium/potassium/calcium exchanger 4			5.8573577	0.00036736
XP_021704942.1	Excitatory amino acid transporter 1			5.56974007	0.00036736
XP_021708608.1	Putative helicase MOV-10			5.4055447	0.00036736
XP_001656046.1	Alanine–glyoxylate aminotransferase 2-like			5.35883724	0.00036736
XP_021698609.1	Chymotrypsin-2			5.27740974	0.00036736
XP_001661015.2	Putative alpha-L-fucosidase			5.02108797	0.00036736
XP_001658000.3	Glutathione S-transferase 1			5.0085396	0.00517339
XP_021704288.1	Phosphotriesterase-related protein			4.96319797	0.00036736
XP_001661388.1	Chymotrypsin-1			4.7758913	0.00036736

demonstrated that functions associated with these genes included metallopeptidases (angiotensin-converting enzyme and vitellogenic carboxypeptidase), ammonia/nitrogen metabolism (argininosuccinate lyase and alanine aminotransferase 1), and iron ion binding (a member of the cytochrome P450 family, 4g15).

Interestingly, two vitellogenins (XP_001660818.2 and XP_001657509.1) that were strongly up-regulated in ZIKV-exposed and unexposed mosquitoes housed at 20°C relative to those housed at 28°C were the genes most enriched by ZIKV exposure at the cold temperature (**Table 3**). The expression of these genes did not change in response to ZIKV exposure at 28°C (**Supplementary Table S8**) and 36°C (**Supplementary Table S9**), suggesting that cold stress alters the midgut vitellogenin expression and may be more significant during a viral infection. ZIKV exposure induced a depletion of beta-1,3-glucan-binding protein (GNBP), which binds to β−1,3-glucan and lipopolysaccharide on the surface of pathogens (Dimopoulos et al., 1997), when mosquitoes were maintained at 20°C. Finally, among the most down-regulated genes in ZIKV-exposed mosquitoes at 28°C, solute carrier family 22 (XP_001656519.2), synaptic vesicle glycoprotein (XP_001651077.2), and vitellogenic carboxypeptidase (XP_001652056.2) (**Table 3**) were also among the most down-regulated genes in unexposed mosquitoes housed at 36°C relative to 28°C (**Table 1**).

DISCUSSION

The dynamics and distribution of vector-borne diseases depend on the interplay between the pathogen, the mosquito, and the environment. Temperature is a strong driver of vector-borne disease transmission (Kilpatrick et al., 2008; Lambrechts et al., 2011; Carrington et al., 2013a,b; Mordecai et al., 2013, 2017; Johnson et al., 2015; Shocket et al., 2018; Tesla et al., 2018a). Despite the strong effects of temperature on mosquito-borne pathogens, little is known about the underlying mechanisms involved (Adelman et al., 2013). In this study, RNA sequencing of *Ae. aegypti* midguts unexposed or exposed to ZIKV, taken early in the infection process, revealed different transcriptional responses to variation in environmental temperature, with ZIKV infection modifying these responses to temperature.

Previously, we found that temperature significantly affected the efficiency of the establishment of ZIKV infection in *Ae. aegypti* midguts, with cool temperatures limiting ZIKV transmission primarily due to poor midgut infection, slow replication, and poor dissemination, while high mosquito mortality at warmer temperatures inhibited ZIKV transmission despite efficient ZIKV infection (Tesla et al., 2018a). Similar to our previous study, we were unable to detect ZIKV replication when mosquitoes were housed in cool conditions (**Figure 1**), which is similar to findings for other flavivirus systems in

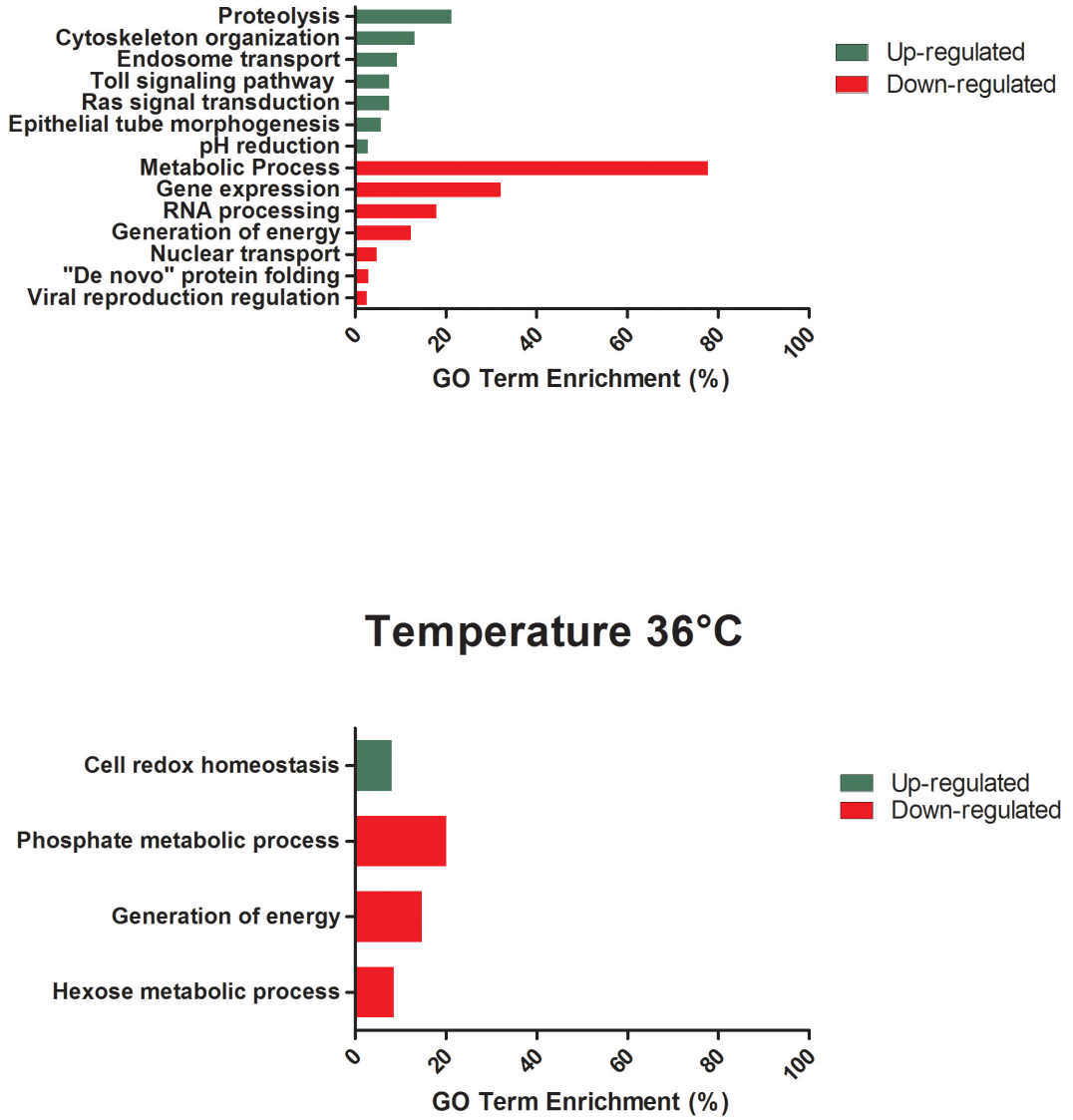

FIGURE 5 | GO term enrichment analysis of differentially expressed genes of *Aedes aegypti* exposed to Zika virus under the lowest (20°C) and in the highest (36°C) temperature conditions in the category of biological processes for up- and down-regulated genes at 48 h.

which infection rates were measured across different constant temperatures (Watts et al., 1987; Kilpatrick et al., 2008; Johansson et al., 2012; Xiao et al., 2014). There was no significant difference in the viral RNA levels quantified from the three temperatures at 24 hpf, reflecting the initial concentration of viral RNA ingested in the blood meal (**Figure 1**). Therefore, we can confirm that while all treatment groups obtained ZIKV in the blood meal, only the mosquitoes housed at standard (28°C) or warm (36°C) temperatures were actively replicating virus by 48 hpf.

We found that variation in temperature elicited strong expression responses in unexposed and ZIKV-exposed mosquitoes. We observed that mosquitoes housed at a cold temperature (20°C) had more genes differentially expressed at 48 hpf relative to mosquitoes housed in standard (28°C) and warm environments (36°C), which exhibited more similar patterns in gene expression (**Figures 2A**, **4A**). This is not entirely surprising, as metabolic theory predicts that low body temperatures will inevitably depress the rates of biochemical reactions (Angilletta et al., 2010). Our data support this hypothesis, as many of the genes drastically altered at 20°C participate in blood meal digestion, peritrophic membrane (PM) formation, metabolism, and managing oxidative stress associated

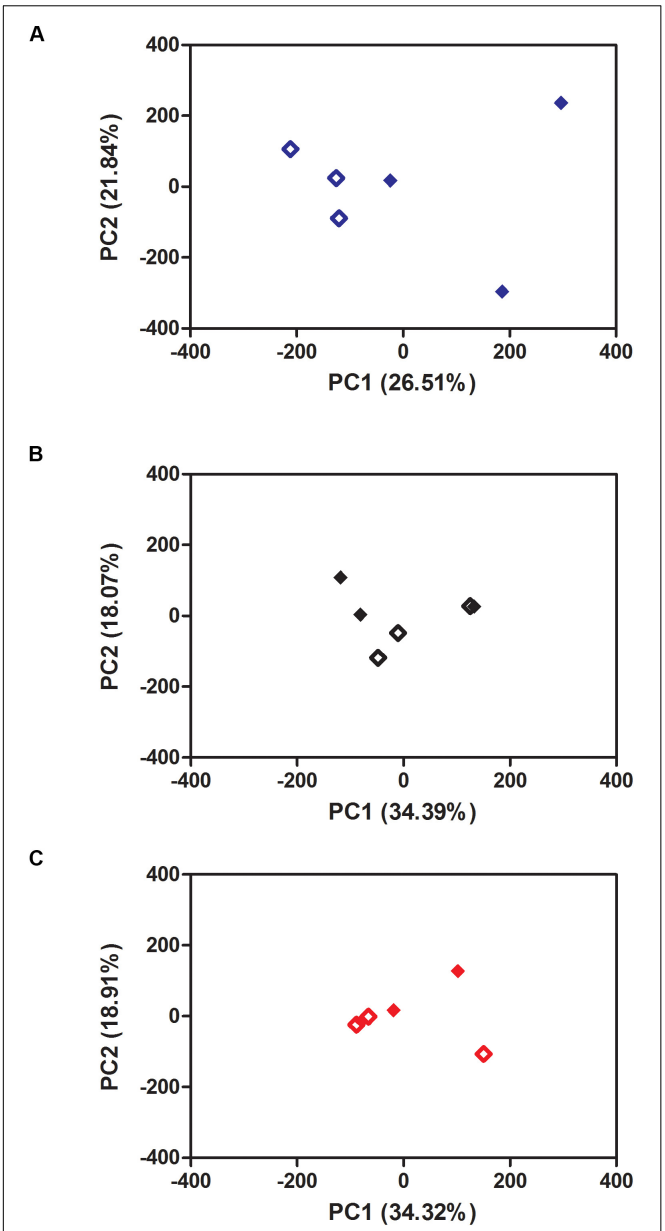

FIGURE 6 | Principal Component Analysis (PCA) of the effect of Zika exposure on *Aedes aegypti* gene expression at three different constant temperatures: **(A)** 20°C, **(B)** 28°C, **(C)** 36°C at 48 h. Open diamond: non-exposed mosquitoes. Closed diamond: Zika-exposed mosquitoes.

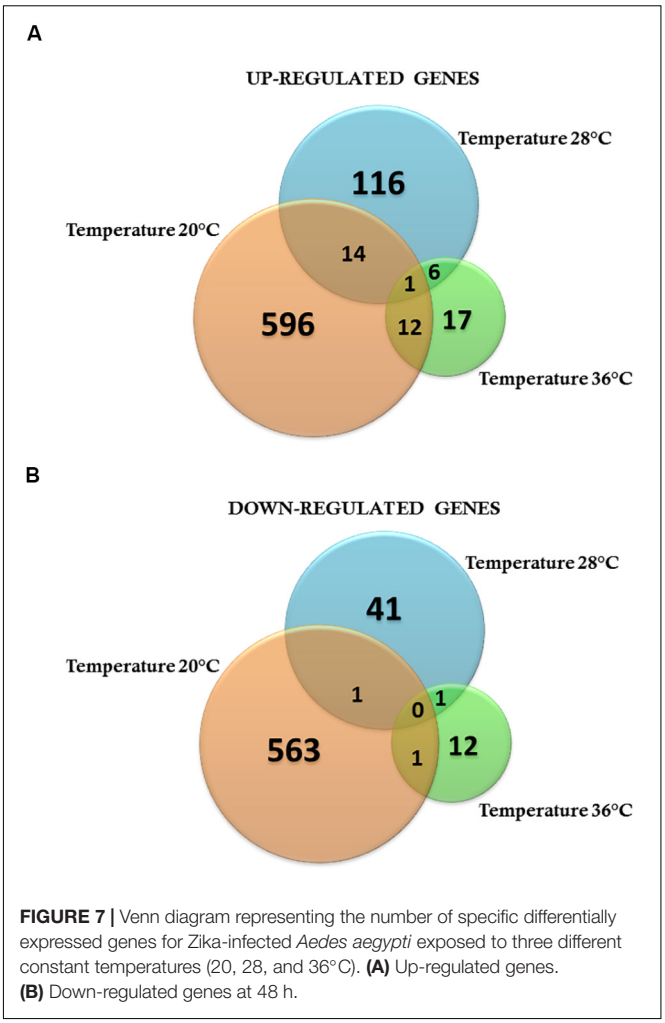

FIGURE 7 | Venn diagram representing the number of specific differentially expressed genes for Zika-infected *Aedes aegypti* exposed to three different constant temperatures (20, 28, and 36°C). **(A)** Up-regulated genes. **(B)** Down-regulated genes at 48 h.

with the breakdown of hemoglobin into heme, a cytotoxic product (**Tables 1**, **2**). Phosphoenolpyruvate carboxykinase and trypsin are upregulated in *Ae. aegypti* midgut during the first few hours after ingestion of a blood meal (Sanders et al., 2003) but were extraordinarily up-regulated in mosquitoes 48 hpf when housed in a cool environment in our study. Additionally, protein G12, which has previously been associated with blood meal digestion and nitrile-specific detoxification (Morlais et al., 2003; Fischer et al., 2008; Bonizzoni et al., 2011), was one of the most enriched transcripts. Further, two digestive proteases involved in glycoside hydrolysis, beta-galactosidase and alpha-N-acetylgalactosaminidase (Santamaría et al., 2015), were highly induced at 48 hpf. Glutamine synthetase, an enzyme that contributes to PM formation, was also highly induced, further demonstrating that cool temperatures delay blood meal digestion (**Supplementary Tables S1**, **S3**). Although the PM is semi-permeable, it is thought to form a barrier and protect the midgut from pathogens [e.g., viruses (Wang and Granados, 2000), bacteria (Kuraishi et al., 2011; Jin et al., 2019), malaria (Rodgers et al., 2017), and protozoa (Weiss et al., 2014)] and other harmful substances present in the insect gut after a blood meal (Wang et al., 2004; Shibata et al., 2015).

We also observed several genes involved in the innate immune response to be modestly upregulated in response to 20°C relative to their levels under warmer temperature conditions in both unexposed and ZIKV-exposed mosquitoes. Melanization is a major effector mechanism of the mosquito immune response and has been implicated in the defense against a diversity of pathogens [e.g., bacteria (Hillyer et al., 2003a,b), malaria (Kumar et al., 2003; Jaramillo-Gutierrez et al., 2009), filarial worms (Christensen et al., 2005; Huang et al., 2005), and viruses (Rodriguez-Andres et al., 2012)]. Phenoloxidase, a key enzyme

TABLE 3 | Top 10 up- and down-regulated genes of Zika-exposed *Aedes aegypti* relative to uninfected mosquitoes kept at low (20°C), standard (28°C), and high (36°C) temperatures 48 h post-blood-feeding.

GENES UP-REGULATED		TEMPERATURE 20°C		TEMPERATURE 28°C		TEMPERATURE 36°C	
Gene ID	**Gene Description**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_011493087.1	Angiotensin-converting enzyme	14.1809276	0.000429978				
XP_001652055.1	Vitellogenic carboxypeptidase-like	13.75772483	0.000429978				
XP_001657506.2	Vitellogenin-A1-like	11.96534425	0.000429978				
XP_001657509.1	Vitellogenin-A1-like	11.70010847	0.000429978				
XP_001660818.2	Vitellogenin-A1	10.9320344	0.000429978				
XP_001652056.2	Vitellogenic carboxypeptidase	10.74370729	0.000429978				
XP_001660472.2	Alanine aminotransferase 1	10.39479931	0.000429978				
XP_001656695.1	Argininosuccinate lyase	10.17572724	0.000429978				
XP_001648376.1	Cytochrome P450 4g15	10.00001321	0.000429978				
XP_001659164.1	Leucine-rich repeat transmembrane neuronal protein 3	9.612555348	0.000429978				
XP_001649098.2	Probable cytochrome P450 9f2			4.118816919	0.0027631		
XP_001659492.2	Serine protease SP24D			3.289321443	0.0027631		
XP_021698905.1	Chymotrypsin-2			3.165229262	0.0027631		
XP_001661721.2	Solute carrier family 45 member 4			3.060526797	0.0027631		
XP_001654886.2	Zinc carboxypeptidase A 1			2.871948741	0.0027631		
XP_001661388.1	Chymotrypsin-1			2.816727502	0.0027631		
XP_021698904.1	Chymotrypsin-2			2.669611858	0.0027631		
XP_021707253.1	Lipase member H			2.612389473	0.0027631		
XP_021697282.1	Multiple inositol polyphosphate phosphatase 1			2.446636883	0.0027631		
XP_001658491.2	Trypsin 5G1-like			2.279646301	0.0027631		
XP_001652358.2	Peritrophin-1					8.437408782	0.00630758
XP_001648381.1	UNC93-like protein					7.430915686	0.00630758
XP_021710339.1	Synaptic vesicle glycoprotein 2C					5.329166839	0.00630758
XP_021697715.1	Peritrophin-1-like					4.823164447	0.00630758
XP_011493129.2	Flocculation protein FLO11					2.282808753	0.00630758
XP_021706761.1	Cysteine sulfinic acid decarboxylase					2.236697262	0.00630758
XP_021707618.1	Probable chitinase 2					2.214805485	0.00630758
XP_001658086.2	Peptidoglycan recognition protein 1					2.009494011	0.00630758
XP_001649855.2	Sodium/potassium/calcium exchanger 4					1.947834362	0.00630758
XP_021709756.1	ATP-binding cassette sub-family A member 3 isoform X1					1.930421649	0.00630758

(Continued)

TABLE 3 | Continued

GENES DOWN-REGULATED		TEMPERATURE 20°C		TEMPERATURE 28°C		TEMPERATURE 36°C	
Gene ID	**Gene Description**	**Fold Change**	**q-value**	**Fold Change**	***q*-value**	**Fold Change**	***q*-value**
XP_001659797.2	Beta-1.3-glucan-binding protein	8.48073909	0.000429978				
XP_001657206.1	Cytochrome P450 9e2	4.355565374	0.000429978				
XP_001652358.2	Peritrophin-1	2.847448995	0.000429978				
XP_021699084.1	Proton-coupled amino acid transporter 1-like	2.551861447	0.000429978				
XP_001659796.1	Beta-1.3-glucan-binding protein	2.517270058	0.000429978				
XP_001649098.2	Probable cytochrome P450 9f2	2.415932254	0.000429978				
XP_001649745.1	Very long-chain specific acyl-CoA dehydrogenase mitochondrial	2.281021429	0.000429978				
XP_001654398.2	rRNA 2'-O-methyltransferase fibrillarin	2.204742243	0.000429978				
XP_001661250.1	Peroxiredoxin-6	1.971170346	0.000429978				
XP_001649797.1	Peptide methionine sulfoxide reductase	1.965683434	0.000429978				
XP_001652056.2	Vitellogenic carboxypeptidase			6.420385839	0.0027631		
XP_001655729.2	Tryptase			5.300762859	0.0027631		
XP_001647719.2	Transferrin			4.298907297	0.0027631		
XP_001655031.2	Carbonic anhydrase 2			3.707662693	0.0027631		
XP_011493147.1	Glycine-rich protein 5			3.626857343	0.0027631		
XP_001651077.2	Synaptic vesicle glycoprotein 2B			3.02148965	0.0027631		
XP_001659383.2	Angiopoietin-related protein 1			2.46044625	0.0027631		
XP_001651411.2	Lysosomal alpha-mannosidase			2.427598779	0.0027631		
XP_001656519.2	Solute carrier family 22 member 21 isoform X2			2.425681278	0.0027631		
XP_011493149.1	Hyphally-regulated protein			2.384835903	0.0049383		
XP_021696806.1	Pupal cuticle protein Edg-78E					18.1975207	0.00630758
XP_011493274.2	Extensin					3.699473591	0.00630758
XP_001661011.1	Protein lethal(2)essential for life					3.215359388	0.00630758
XP_001658359.2	Brachyurin					2.357013464	0.00630758
XP_001649783.1	Maltase 1					1.680632876	0.0148679
XP_001647586.2	Asparagine synthetase [glutamine-hydrolyzing] 1					1.637484216	0.00630758
XP_021694990.1	Myrosinase 1-like					1.626761779	0.00630758
XP_021693649.1	Heat shock protein 70 A1					1.55902061	0.02715
XP_001651895.1	Acyl-CoA:lysophosphatidylglycerol acyltransferase 1					1.523434001	0.00630758
XP_001649752.1	Heat shock protein 83					1.48484568	0.00630758

in the melanization pathway, was up-regulated in mosquito midguts at 20°C (**Supplementary Figures S3B**, **S4B**). Studies in both butterflies and *Anopheles stephensi* demonstrated that phenoloxidase activity was higher at cool temperatures and becomes less efficient at warmer temperatures (Suwanchaichinda and Paskewitz, 1998; Murdock et al., 2012b). The production of melanin is also essential for other physiological processes such as cold acclimatization in insects (Crill et al., 1996; Kutch et al., 2014), the formation of the hard protective layer around eggs, and wound healing (Lai et al., 2009). Our data also reveal that c-type lectin, reported to participate in the activation of the melanization cascade (Yu and Kanost, 2000; Christensen et al., 2005), was also up-regulated. Therefore, our results suggest that cold stress triggers numerous molecular changes in the mosquito, including modest changes in the levels of important immune effectors that could have important consequences for arboviral infection.

Contrary to what we observed at the cool temperature, exposure to hot conditions (36°C) does not trigger pronounced up- or down-regulation of genes relative to standard conditions (28°C). The heat shock protein 70 (HSP 70) transcript was most enriched in response to the hot environment (**Table 1**). The upregulation of HSP 70 is associated with reduced lifespans in other insect systems (Feder and Krebs, 1998; Feder, 1999), which may explain the rapid mosquito mortality we observed at this temperature in previous work (Tesla et al., 2018a). HSP70 has also been suggested to facilitate arbovirus infection in mosquitoes in terms of viral entry, viral RNA synthesis, and virion production (Kuadkitkan et al., 2010; Taguwa et al., 2015).

Zika virus exposure induced very modest effects when comparing ZIKV-exposed and unexposed mosquitoes reared at 28 and 36°C, while those that experienced the cool temperature exhibited a larger alteration in gene expression at 48 hpf (**Figure 6** and **Supplementary Figure S5**). This may not be entirely surprising, as ZIKV-induced transcriptional changes under standard rearing conditions (28°C) have previously been shown to be subtle 48 h post-infection (Murdock et al., 2012a) and ZIKV was only observed to be actively replicating at 28 and 36°C. When concentrating on differentially expressed genes between ZIKV-exposed and unexposed mosquitoes at each temperature treatment (**Table 3**), only at 20°C do we observe changes of 10-fold or more. Therefore, the presence of ZIKV in the blood meal did alter the response of mosquitoes to temperature variation, with the most pronounced differences occurring in mosquitoes housed at the cool temperature. In particular, the midguts of ZIKV-exposed mosquitoes experienced enhanced signal transduction processes, pH modification, midgut epithelial morphogenesis, and Toll pathway activation relative to ZIKV-exposed mosquitoes at 28°C (**Figure 5**), and this pattern was qualitatively different to a similar comparison in unexposed mosquitoes (**Figure 3**). These changes could be reflective of patterns observed in other studies demonstrating that mosquitoes infected with blood-borne pathogens actively modify ROS metabolism in midgut cells to control levels of hydrogen peroxide (H_2O_2), which in turn is an important modulator of downstream innate immune responses (e.g., Toll pathway), antimicrobial peptide production, and pathogen infection (Molina-Cruz et al., 2008; Herrera-Ortiz et al., 2011; Oliveira et al., 2011, 2012). Furthermore, the presence and abundance of particular microbial flora [e.g., *Wolbachia* (Pan et al., 2012)] and the proliferation of the midgut flora due to ingestion of the blood meal (Carissimo et al., 2014; Saraiva et al., 2016; Barletta et al., 2017) can also trigger ROS production, with temperature variation modifying these effects.

Additionally, vitellogenin proteins (Vg) were highly upregulated (>3000 fold) when ZIKV-exposed mosquitoes were housed at cool temperatures relative to ZIKV-exposed mosquitoes at 28°C (**Table 2**) and unexposed mosquitoes at 20°C (**Table 3**). Vg is a precursor egg-yolk protein but may also function by shielding cells from the negative effects of inflammation and infection (Corona et al., 2007; Azevedo et al., 2011). Work with honey bees suggests that Vg-incubated insect cells display enhanced tolerance against H_2O_2-induced oxidative stress (Fluri et al., 1977; Seehuus et al., 2006). Vg also binds to dying cells, suggesting that it may play a role in recognizing damaged cells and shielding healthy cells from toxic by-products (Havukainen et al., 2013). Both caspase dronc [an inhibitor caspase (Cooper et al., 2007)] and an effector caspase (Bryant et al., 2008), which are important components of the apoptotic pathway controlling mechanisms of cell death, were enriched in mosquitoes housed at 20°C (**Supplementary Figures S3E**, **S4E**). Thus, elevated Vg expression may combat cellular damage resulting from elevated heme toxicity and oxidative stress (Havukainen et al., 2013) associated with the delayed breakdown of hemoglobin in the blood meal (Bottino-Rojas et al., 2015) in mosquitoes housed at cool temperatures. There may also be a direct effect of Vg on ZIKV, as it has been associated with antiviral effects in some fish species (Garcia et al., 2010). Entry of ZIKV into mammalian cells is associated with apoptotic mimicry, with viral lipids interacting with phosphatidylserine receptors on cells gaining entry in a pathway similar to clearance of apoptotic cells (Hamel et al., 2015). Honey bee Vg binds to dying cells through interactions with lipids (Havukainen et al., 2013). Although the receptors ZIKV uses to interact and enter mosquito cells have not been identified, if Vg protein coats viral particles, it may impede normal cellular interaction and entry. Alternatively, ZIKV infection could be limited at the cooler temperature if infected mosquitoes balance ROS metabolism toward a higher state of oxidative stress, as shown in other systems (Molina-Cruz et al., 2008; Herrera-Ortiz et al., 2011; Pan et al., 2012; Bottino-Rojas et al., 2015; Wong et al., 2015), facilitating downstream innate immune mechanisms and virus killing. Whether overexpressions of Vg lipoproteins have direct effects on ZIKV infection or reflect a response to buffer ZIKV-exposed mosquitoes to a higher state of oxidative stress in the midgut remain open questions that will be explored in future experiments.

Finally, in ZIKV-exposed mosquitoes at 36°C, we observed peritrophin, one of the components of the peritrophic matrix, to be highly up-regulated relative to ZIKV-exposed mosquitoes housed at 28°C (**Table 2**) and unexposed mosquitoes housed at 36°C (**Table 3**). Although PM formation is highly induced 3–24 hpf, peritrophin can undergo positive modulation by pathogens in other vector-borne disease systems (e.g., *Le. major*) (Coutinho-Abreu et al., 2013). While we cannot confirm whether HSP70 or modulation of peritrophin play a role in ZIKV

infection, these could be potential mechanisms explaining why we detect higher viral RNA levels at 36°C in this study (**Figure 1**) and efficient viral dissemination and salivary gland invasion in previous work (Tesla et al., 2018a).

Although we estimated the effects of mean constant temperatures on the immune-physiological profiles of *Ae. aegypti* in response to ZIKV infection to maintain experimental tractability, mosquitoes and their pathogens live in a variable world where temperatures fluctuate daily and seasonally (easily encompassing the range of temperatures explored here). Both mosquito immunity and virus transmission have previously been shown to differ in fluctuating environments relative to constant temperature environments (Lambrechts et al., 2011; Carrington et al., 2013a,b; Murdock et al., 2013). There is also a substantial body of work demonstrating carry-over effects of environmental variation in the larval environment on adult mosquito phenotypes, fitness, and metrics of transmission (Muturi and Alto, 2011; Muturi et al., 2011a,b; Alto and Bettinardi, 2013; Buckner et al., 2016; Evans et al., 2018). While outside the scope of this study, future work should investigate how both fluctuating environmental conditions and the larval environment modify the physiological and immunological responses of adult mosquitoes to arbovirus infection and temperature variation in the adult environment. Finally, while there is currently limited evidence demonstrating that mosquitoes select microhabitats to optimize internal body temperatures for metabolic demands (Blanford et al., 2009), as in other ectothermic organisms (Huey, 1991; Hertz et al., 1994; Gvoždík, 2002; Dillon et al., 2009), if this did occur in the field, it would modify the range of field-relevant temperatures mosquitoes effectively experience.

In this study, we demonstrate profound effects of temperature on ZIKV viral replication and the transcriptional responses of mosquitoes. Temperature variation may alter the ZIKV infection process either through modifying the response of mosquitoes to ZIKV infection, altering the efficiencies of viral-specific processes, or, more likely, both. Our study focused on midgut responses early in the infection process. However, disentangling these effects will require the sampling of other immunological tissues and at later time points where high levels of ZIKV RNA can be detected. While further work is needed to determine the precise mechanisms at play, our results indicate that temperature shifts the balance and dynamics of the midgut environment, which could result in direct and indirect consequences for the ZIKV-infection process. These results do reinforce the assertion that the conventional approach of studying the mechanisms underpinning mosquito–pathogen interactions under a narrow set of laboratory conditions or across canonical innate immune pathways is likely missing important biological complexity. To move forward, we need to begin framing our mechanistic understanding of this dynamic phenotype in the ecologically variable world in which mosquitoes and pathogens associate. This study represents a key advance toward this objective.

DATA AVAILABILITY STATEMENT

The fast raw data were deposited in the NCBI SRA database under accession number PRJNA615972. This Sequence Read Archive (SRA) submission will be released on 2020–10–20 or upon publication, whichever is first.

ETHICS STATEMENT

All mosquito infection work with ZIKV was approved by the University of Georgia, Athens Institutional Biosafety Committee (reference number 2015-0039).

AUTHOR CONTRIBUTIONS

BT performed the experiments. PF and TO analyzed the RNAseq data. CM, MB, TO, and LN designed the experiments, acquired the funding, and supervised the project. All authors wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00901/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Redox homeostasis change in Aedes aegyptimidgut under different temperatures is associated to blood digestion and retention

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Abstract:	Temperature is an important abiotic factor for ectothermic organisms deeply affecting several facets of the physiology, life-cycle and susceptibility to pathogens. Several studies have demonstrated the temperature influence on mosquito vector competence, however, we still lack knowledge about the underlying mechanisms. Based on previous studies of the transcriptome of the midgut of Ae. aegypti mosquitoes maintained at different temperatures, we hypothesize that temperature variation impacts the blood digestion process, modulating the generation of reactive oxygen species (ROS) in the midgut. We show that temperature variation affects both the expression of genes involved in blood digestion and the rate of digestion, with cool temperatures (20°C) delaying this process. Additionally, due to temperature effects on ROS levels and the expression of antioxidant proteins in the midgut, lower temperatures (20°C) result in a longer state of oxidative stress when compared to other temperatures (28 and 36°C). We also demonstrate that temperature does not directly affect the oxidative state in the midgut, but is a consequence of the direct effects of temperature on blood retention in the midgut. Our results highlight the complexity of intestinal environment, and demonstrates how variation in relevant environmental conditions affect physiological processes that will in turn influence vector competence.

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The Editor-in-Chief
Insect Biochemistry and Molecular Biology

Dear Editor,

Enclosed is an original manuscript, titled "***Redox homeostasis change in Aedes aegypti midgut under different temperatures is associated to blood digestion and retention***" which I wish to be considered for publication in Insect Biochemistry and Molecular Biology.

In this manuscript, we described demonstrates how temperature variation, a relevant environmental conditions, affects physiological processes in *Aedes aegypti* that can influence its vector competence for pathogens. We show that temperature variation affects both the expression of genes involved in blood digestion and the rate of digestion with validation of phenotype by measurement of enzymatic activities and quantification of metabolite production. We also demonstrate that temperature does not directly affect the oxidative state in the midgut, but is a consequence of the direct effects of temperature on blood retention in the midgut.

The content of this manuscript is an original work. This work is not under consideration for publication elsewhere and has not been previously published.

Thank you.

Yours sincerely,

Prof. Dr. Tiago Antônio de Oliveira Mendes
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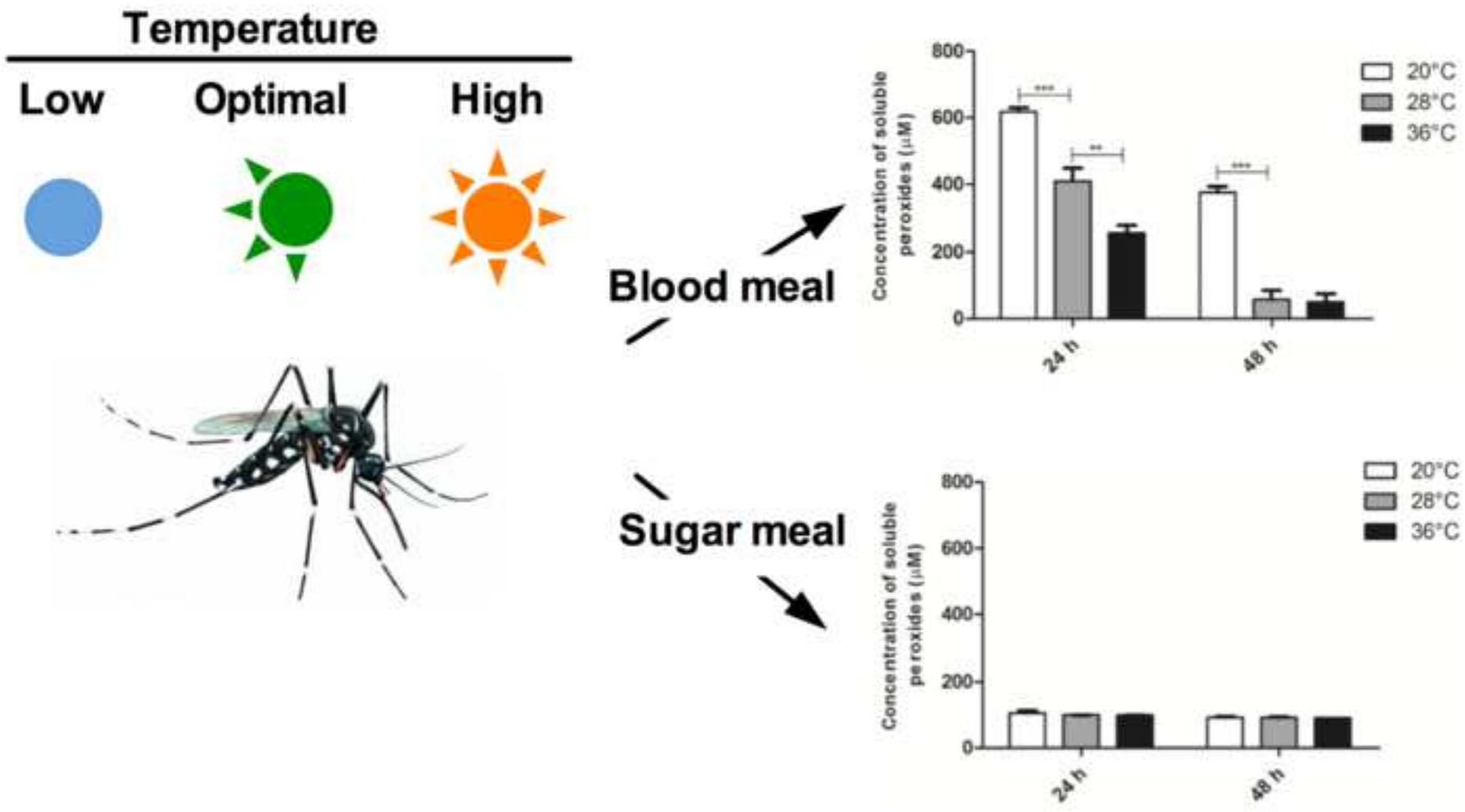
Highlights:

- Temperature variation impacts the *Aedes aegypti* blood digestion process.
- Changes in blood digestion modulate production of reactive oxygen species in the midgut.
- Variations in environmental conditions affect physiological processes that will in turn influence vector competence.



Redox homeostasis change in *Aedes aegypti* midgut under different temperatures is associated to blood digestion and retention

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Declarations of interest: none

Abstract

Temperature is an important abiotic factor for ectothermic organisms deeply affecting several facets of the physiology, life-cycle and susceptibility to pathogens. Several studies have demonstrated the temperature influence on mosquito vector competence, however, we still lack knowledge about the underlying mechanisms. Based on previous studies of the transcriptome of the midgut of *Ae. aegypti* mosquitoes maintained at different temperatures, we hypothesize that temperature variation impacts the blood digestion process, modulating the generation of reactive oxygen species (ROS) in the midgut. We show that temperature variation affects both the expression of genes involved in blood digestion and the rate of digestion, with cool temperatures (20°C) delaying this process. Additionally, due to temperature effects on ROS levels and the expression of antioxidant proteins in the midgut, lower temperatures (20°C) result in a longer state of oxidative stress when compared to other temperatures (28 and 36°C). We also demonstrate that temperature does not directly affect the oxidative state in the midgut, but is a consequence of the direct effects of temperature on blood retention in the midgut. Our results highlight the complexity of intestinal environment, and demonstrates how variation in relevant environmental conditions affect physiological processes that will in turn influence vector competence.

Keywords: Temperature, *Aedes aegypti*, blood digestion, oxidative stress

1. Introduction

Given the increasingly widespread geographical distribution of mosquito vector species, the severity of recent outbreaks, and the potential emergence of new pathogens (Campbell et al., 2015; Parham et al., 2015; Misslin et al., 2016; Caminade et al., 2019), extensive knowledge of the interactions mosquito-borne pathogens within their hosts has been acquired in past decades (Bartholomay and Michel, 2018; Kumar et al., 2018; Simões et al., 2018). Despite several scientific groups advance on different strategies to control these infectious mosquitoes-borne diseases (Jupatanakul et al., 2017; Jones et al., 2021), they just considerate mosquito proteins and cellular machinery involved in vector–pathogen interactions, discrediting that the response mosquitoes mount toward a given pathogen is a dynamic phenotype that also dependent of the variation in key environmental factors (Okech et al., 2007; Parham and Michael, 2010; Cohen et al., 2016; Murdock et al., 2017; Siraj et al., 2018).

Due to their ectothermic nature, their physiology, ecology, as well the pathogens replication depend on temperature in which they live (Rueda et al., 1990; Alto and Juliano, 2001; Briegel and Timmermann, 2001; Delatte et al., 2009). As a result, environmental temperatures can also affect the mosquito–pathogen interactions (Watts et al., 1987; Lambrechts et al., 2011; Carrington et al., 2013b; Ciota et al., 2014; Tesla et al., 2018). There are numerous examples that small changes in environmental temperature can significantly influence mosquito resistance to infections (Jodell E. Linder et al., 2008; Lazzaro et al., 2008; Hye Jin Chung et al., 2010; Yoshida, 2010; Adamo and Lovett, 2011; Ruiz-vega et al., 2011). Likewise, the development or replication rates of pathogens transmitted by mosquitoes, including: Malaria (Mordecai et al., 2013), Dengue virus (Lambrechts et

al., 2011; Carrington et al., 2013a, 2013b), West Nile virus (Kilpatrick et al., 2008) and Zika virus (Tesla et al., 2018), are strongly dependent on temperature.

Studies about the effects of temperature on ZIKV transmission by *Aedes aegypti* showed that constraints on ZIKV transmission at cool and warm temperatures are modulating by different mechanisms. Cool temperatures inhibited ZIKV transmission due to slow virus replication and escape from the midgut, while in warm temperatures it was due high mosquito mortality despite efficient ZIKV infection and rapid dissemination (Tesla et al., 2018). Although there are many evidences of the effects of temperature on the transmission of pathogens by mosquitoes (Murdock et al., 2012, 2014; Muturi et al., 2012; Adelman et al., 2013; Carrington et al., 2013a, 2013b; Zouache et al., 2014; Xiao et al., 2014; Gloria-Soria et al., 2017; Shocket et al., 2018; Tesla et al., 2018), little is known about the underlying mechanisms.

In previous work (Ferreira et al., 2020), we have demonstrated that cool temperatures restrict ZIKV replication in the *Ae. aegypti* midgut at the same time as is seen a remarkable delayed enrichment of transcripts involved in the process of blood digestion, oxidation and reduction in those mosquitoes. At cold temperature condition, both, ZIKV-exposed and unexposed had a significant enrichment of these process indicating that temperature shifts the balance and dynamics of the midgut environment, which could result in direct and indirect consequences for the ZIKV- infection process.

The intestinal lumen, the host's first environment encountered by the pathogen, is complex, containing a wide variety of immune factors, microbiota and nutrients derived from the blood meal. ROS are continuously produced by midgut epithelial cells of sugar-fed mosquitoes and released into the lumen to control bacterial growth (Oliveira et al., 2011). Upon acquisition of blood meal, the rate of ROS generation is increased (Kumar et al., 2003) due to increase metabolic activity. At the same time, digestion of hemoglobin, the main blood protein, releases large amounts of its prosthetic group,

heme. Heme converts lipid hydroperoxides (ROOH), produced during lipid oxidation, into peroxy ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals, which are highly reactive and induce pronounced cytotoxicity (Balla et al., 2007). Therefore, after a blood meal, hematophagous mosquitoes need to manage heme's pro-oxidant activity and the ROS generation in order to avoid heme-mediated oxidative stress. Thus, after blood feeding, protective mechanisms as the expression of ROS detoxifying enzymes (such as superoxide dismutase that detoxifies $O2^{-}$, and catalase, glutathione peroxidase, and thioredoxin peroxidase that detoxify H_2O_2) are activate (Molina-Cruz et al., 2008). Because the ROS production is one of the key players in gut immunity, the reduction on its levels, as well the increase in availability of nutrients favor the bacterial proliferation (Demaio et al., 1996; Pumpuni et al., 1996; Gusmão et al., 2010; Oliveira et al., 2011). This explosion in growth is accompanied by positive regulation of genes associated to immunological response (Oliveira et al., 2011), probably in an attempt to reduce tissue damage caused by high bacterial growth in the gut lumen. If the mosquito takes up an infectious blood, these immune responses can influence infection rates of pathogens present in the meal (Pumpuni et al., 1996). Additionally to responses microbiota-mediated, the malaria literature suggests that mosquitoes fine-tune their antioxidant responses to tolerate higher oxidative stress levels if they imbibe an infectious blood meal by a mechanism that consist in suppress of the catalase expression (Molina-Cruz et al., 2008). The accumulation of ROS may accelerate immune activation (Kumar et al., 2003) contributing to limit *Plasmodium* infection.

The mosquito immune system consists of cellular and humoral responses that interact to control the spread of an infection (Schmid-Hempel, 2005). In the midgut, the exogenous siRNA, Toll, and JAK-STAT pathways as well as melanization work together to restrict infection (Campbell et al., 2008; Xi et al., 2008; Cirimotich et al., 2009; Sánchez-Vargas et al., 2009; Souza-Neto et al., 2009; Ramirez et al., 2012; Jupatanakul et al., 2014). There are many studies that demonstrate the impact temperature can play on the modulation of mosquito immune response pathways

(melanization (Suwanchaichinda and Paskewitz, 1998; Murdock et al., 2012), RNAi (Adelman et al., 2013), phagocytosis (Murdock et al., 2012), IMD (Muturi et al., 2012) and response mediated by toll receptors (Muturi et al., 2012)), however, there are still gaps as to how temperature modulates these responses.

Considering the remarkable effects of temperature on digestion and oxidation-reduction process as well as on ZIKV replication in *Ae. aegypti* midguts previously seen in our studies, we hypothesize that cold temperatures slows down the blood digestion process, causing a state of prolonged oxidative stress, which, in turn promotes the activation of immune response pathways (Ferreira et al., 2020). To elucidate the mechanisms behind temperature-induced phenotypic alterations in mosquitoes, we mapped and quantified transcripts of genes that encode proteins involved in digestion, we measured blood digestion rates, protein content and the ROS levels in the midgut of *Ae. aegypti* mosquitoes exposed to three temperatures (20, 28 e 36°C) for 24 and 48 hr after blood meal.

2. Material and Methods

2.1. Transcript Abundance Estimation

First, RNASeq data from *Ae. aegypti* midgut exposed to three temperatures (20,28 e 36°C) for two time intervals (24 e 48 hr) were recovery (NCBI SRA ID: PRJNA615972) (Ferreira et al., 2020). Poor quality reads (quality score below 20), short reads (less than 25 bases), and adapter sequences (Bolger et al., 2014) were removed using Trimmomatic (v 0.36). Tophat (v 2.1.1) (Trapnell et al., 2009) was used to perform the alignment and mapping of the high quality reads to the *Ae aegypti* genome (NCBI ID: GCA_002204515.1) obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and the quantification of the transcripts was performed using Cuffdiff (v 2.2.1) (Trapnell et al., 2013). We

149 selected transcripts of genes involved in blood digestion (trypsin (Billingsley and Hecker, 1991),
150 phosphoenolpyruvate carboxykinase (PEPCK) (Sanders et al., 2003), alpha-N-
151 acetylgalactosaminidase (α-NaGalase) and beta-galactosidase (β-gal) (Dana et al., 2005)) in order to
152 assess the initial impact of temperature on the expression of these genes. The relative expression
153 levels were produced as fragments per kilobase of transcript per million fragments mapped (FPKM
154 values), which normalized read count based on gene length and the total number of mapped reads.

155

156 **2.2. Ethics Statement**

157 The approval for the use of mice (*Mus musculus*) in the feeding of mosquitoes was obtained
158 from the Ethics Committee at the Universidade Federal de Viçosa (UFV) (reference number
159 561/2016).

160

161 **2.3. Mosquito Husbandry**

162 *Ae. aegypti* mosquitoes (lineage PPCampos - Campos dos Goytacazes) were reared in an
163 insectary at the Department of General Biology at UFV (Viçosa, MG, Brazil) under controlled
164 temperature (25 ± 2°C), relative humidity (60 ± 10%), and photoperiod (12:12 light:dark). Eggs were
165 hatched in de-chlorinated tap water and larvae were fed with turtle food pellets (ReptoLife,
166 Camboriú, SC, Brazil). Pupae were transferred to cages and a 10% sucrose solution was provided *ad*
167 *libitum* for emerging adults. Egg production was stimulated after providing a blood meal on
168 anesthetized mice (0.8 mL (2%) xilazina and 0.2 mL (10%) ketamine in order to maintain the colony.

169

170 **2.4. Mosquito Feeding Regime**

171 Adult mosquitoes (3 to 4-day-old), previously deprived of food for 12 h, were anesthetized
172 with CO_2. Females were separated and kept in a Petri dish on ice, transferred to a plastic cage and

subsequently fed with mice blood. After 30 minutes, engorged mosquitoes were randomly allocated across six plastic cages and distributed across one of three temperature treatments (20, 28 and 36°C (± 2°C)) at 60 ± 10% relative humidity, with a 12:12 light:dark cycle. A 10% sucrose solution was provided *ad libitum* to mosquitoes throughout the duration of the experiment (48 h). Three full biological replicates were performed.

2.5. Protein Content and Trypsin Activity

Fifteen midguts were dissected from each temperature treatment group at 24 and 48 hours post feeding (hpf) and homogenized in 35 µL buffer (0.1 M sodium phosphate (pH 7.4)). The homogenate was cleared by centrifugation (10,000 rpm, 5 min) and protein was quantified using the Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as a reference. To provide a more accurate assessment of protein digestion rates at each temperature, albumin content and trypsin activity were determined. Albumin was measured using monoreagent albumin (Bioclin, K040) following the manufacturer's instructions. Trypsin activity was determined using N-alpha-benzoyl-DL-arginine-para-nitroanilide (BApNA) as substrate in the final concentration of 0.36 mM in 0.1 M Tris-HCl buffer in 20mM $CaCl_2$ (pH 8.0). The assay was performed by incubating 20 µl of the homogenate with 50 µl of Buffer A (20mM $CaCl_2$, 0.1 M Tris–HCl, pH 8.0) and 30 µl of 1.2 mM BApNA for 10 min at 25°C. The formation of p-nitroaniline was measured at 410 nm in a plate reader (Molecular Devices/VersaMax). Two controls were prepared, replacing the sample and the enzyme with Buffer A. A unit of enzymatic activity (U) was defined as the amount of enzyme capable of hydrolyzing 1 µmol de BApNA per minute under the established conditions, using a molar coefficient of 8,800 $M^{-1}cm^{-1}$ (Erlanger et al., 1961). Assays were performed in triplicate in three independent experiments.

197 **2.6. Quantification of Reactive Oxygen Species (ROS)**

198 In order to measure oxidative stress in blood fed mosquitoes exposed to different
199 temperatures, the concentrations of soluble peroxides and nitric oxide were measured in the midgut.
200 For the determination of soluble peroxides, fifteen midguts were dissected 24 and 48 hpf,
201 homogenized in 35 µL of catalase inhibitor (3-amino-1,2,4-triazole) and centrifuged at 10,000 rpm
202 for 5 minutes. Ten microliters of midgut homogenates were diluted 1:5 and added to 950 µL of FOX
203 reagent (100 mM sorbitol; 100 µM xylenol orange; 250 µM ferrous ammonium sulfate; 25 mM
204 sulfuric acid). Absorbance was measured at 596 nm after 30 minutes of incubation at room
205 temperature (Paris et al., 2017). Nitric oxide was also quantified using 15 midguts homogenized in 35
206 µL of buffer 0.1 M sodium phosphate (pH 7.4) and centrifuged at 10,000 rpm for 5 minutes. For
207 quantification, the homogenate was diluted 1:10 and 50 µL of this sample was added to 100 µL of
208 Griess reagent (0.5% sulfanilamide and 0.05% N-(1-Naphthyl)-ethylenediamine in 2.5% phosphoric
209 acid). The mixture was incubated for 10 minutes at room temperature in the absence of light and the
210 absorbance was measured at 560 nm (Eckmann et al., 2000). Soluble peroxides and nitric oxide were
211 quantified using a calibration curve of H_2O_2 (0-1,000 µM) and $NaNO_2$ (0–125 µM), respectively.
212 Finally, to check if the temperature affects the ROS levels in the *Ae. aegypti* midgut in the absence of
213 blood, soluble peroxides and nitric oxide were measured in the mosquito midguts 24 and 48 hr after a
214 sugar meal at each temperature as described above.
215
216 **2.7. RNA Extraction and RT-PCR**

217 To capture the changes in gene expression involved in the response to temperature, total RNA
218 was extracted from 20 dissected midguts 24 and 48 hr after blood ingestion using the RNeasy Mini
219 Kit as instructed by the manufacturer. One microgram of total RNA was treated with DNAse
220 (Promega). RT-qPCR was performed on the synthesized cDNA (High-Capacity cDNA Reverse

Trancription Kit - Applied Biosystems) using SYBER Green. Oligonucleotide primer sequences used in this study are shown in Table 1. The amplification and detection of specific products was performed on StepOne™ Real Time PCR system (Applied Biosystems, USA), using the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 ◦C for 30 s and 60 ◦C for 1 min and 1 cycle at 95°C for 30 s, 60°C for 1 min and 95°C for 30 s. Melting curves were analysed to confirm specificity of amplified products. All reactions were performed in triplicate on the three biological replicates. The calculation of the relative abundance of each transcript was performed using the relative standard curve method (de Oliveira Mendes et al., 2013; Souza et al., 2019), constructed by serial dilution of a cDNA pool starting at 25 ng/µL, ending at 1.56 ng/ µL and with a dilution factor of two. The gene expression data in each sample were normalized due to the expression levels of the ribosomal S7 gene used as a endogenous constitutive control (Taracena et al., 2018).

2.8. Statistical Analysis

Statistical analyses were performed using one-way or two-way ANOVA followed by Bonferroni correction for multiple comparisons to assess statistical differences between samples using the software*GraphPad Prism*® v. 7 (*GraphPad Software*, Inc. La Jolla, California, USA). All tests were considered statistically significant when *p-value* was less than 0.05.

3. Results

3.1. Effect of temperature on blood digestion

In order to investigate the effects of temperature on blood digestion rates in *Ae. aegypti* mosquitoes, transcripts of trypsin, phosphoenolpyruvate carboxykinase (PEPCK), alpha-N-acetylgalactosaminidase (α-NaGalase) and beta-galactosidase (β-gal) genes were quantified at each temperature (20, 28 and 36°C) at both 24 and 48 hpf from a previous transcriptome study by our

group (Ferreira et al., 2020). As shown in Figure 1, the count of transcripts was distinctly different between the temperature and time treatments. At 24 hpf, a significant number of fragments referring to PEPCK, α-NaGalase, and β-gal transcripts were identified at the coldest temperature when compared to the other treatments (Figure 1A). In contrast, a smaller number of Trypsin transcripts were quantified at this temperature. At 28 and 36°C, the count of the transcripts revealed a more similar profile, in which the number of fragments was found to be greater at 28°C (Figure 1A). Later in the digestive process (48 hpf), an increase in the number of transcripts was observed at 20°C, while at other temperatures there was almost no presence of them (Figure 1B).

Quantitative real-time PCR was used to assess the levels of the transcripts mentioned above. In accordance with the results obtained from the RNASeq count, the expression of trypsin occurred late in the condition of the coldest temperature (20°C), being found significantly increased by 48 hpf, whereas under warmer conditions expression was greater at 24 hpf (Figure 2A). Expression of PEPCK was significantly higher in mosquitoes at 28°C and decreased over time. A similar decrease was observed at 36°C. In contrast, the expression PEPCK significantly increased over the two day period in the mosquitoes maintained at 20°C (Figure 2B). In addition, α-NaGalase expression remained high in mosquitoes kept at the coldest temperature, yet declined in the warmer conditions (Figure 2C). Finally, real-time PCR analysis revealed that there was no significant difference in the expression of the β-gal gene among temperature treatments 24 hpf, and that only for mosquitoes exposed to 20°C was there a significant increase in expression (Figure 2D). In general, the expression profile found from the PCR analysis (Figure 2) was very close to that found in the transcriptome data (Figure 1), in which there is a late accumulation of transcripts involved in the blood digestion in mosquitoes that experienced low temperatures.

Blood is a nutritionally rich meal with a high protein content. In order to investigate the impact of temperature on the blood digestion process, total protein content of blood-fed mosquito

midguts was determined. As seen in Figure 3A, there was a difference in the protein content of the midguts between temperature treatments. Twenty-four hours after feeding, the protein content per midgut varied between 86.22 ± 11.09 (20°C), 76.40 ± 10.79 (28°C) and 35.12 ± 6.41 µg/midgut (36°C), with significantly less protein detected in mosquitoes maintained at 36°C. By 48 hpf, a large blood volume was still evident in the midguts of those mosquitoes housed at the lowest temperature, while at 28°C it was common to observe only a small amount of blood contents. In accordance with the presence of blood in the midguts of mosquitoes housed at 20°C, high protein levels (80.92 ± 15.31 µg/midgut) were also measured (Figure 3A) 48 hpf. In contrast, low protein levels were found at warm temperatures (2.24 ± 2.29 µg/midgut, 28°C and 2.79 ± 0.80 µg/midgut, 36°C), especially at 48 hpf.

Albumin, one of the main constituents of blood and with no production in mosquitoes, was also measured after the blood meal to validate that the levels of protein observed are associated to blood retention in the midgut. In accordance with the quantification of total proteins, a difference in the content of albumin in the midgut was found between treatments (Figure 3B). Within 24 hr, a significant difference was found between the levels of albumin, which varied between 108.11 ± 28.23, 72.55 ± 3.63 and 44.56 ± 4.66 µg/midgut for temperatures of 20, 28, and 36°C, respectively. Forty-eight hours after the blood meal, midguts showed significantly high levels of albumin (*p-value* < 0.001) in mosquitoes housed at 20°C (70.60 ± 7.78 µg/midgut) in relation to mosquitoes housed at the warmer temperature treatments (3.58 ± 3.11 µg/midgut, 28°C and 1.15 ± 1.21 µg/midgut, 36°C) confirming that blood was digested more slowly in this treatment (Figure 3B).

Serine proteases, including trypsins, are responsible for most of the digestion of blood proteins in *Ae. aegypti* (Dana et al., 2005). In order to measure trypsin activity in midguts of mosquitoes exposed to temperature treatments, we performed enzymatic assays using the substrate BApNA. As shown in Figure 3C, there was a significant difference in enzyme activity seen at the

293 coldest temperature (6.33 ± 5.66 mU/midgut), which was the lowest among all treatments (25.90 ±
294 2.29 mU/midgut, 28°C and 25.62 ± 7.35 mU/midgut, 36°C) after 24 h. An increase in trypsin
295 activity was observed late (48 h) at 20°C (34.35 ± 5.70 mU/midgut). For the other temperatures, it
296 was not possible to determine the enzymatic activity after 48 hr of the blood meal suggesting
297 reduction of production of enzyme after all blood is consumed.
298
299 **3.2. Impact of digestion delay on oxidative stress**
300 Subsequent to the investigation of the effect of temperature in blood digestion process,
301 experiments were carried out to evaluate the ROS levels in the midgut of mosquitoes exposed to the
302 three temperatures studied. The measurement of soluble peroxides in blood-fed mosquitoes revealed
303 that there was a significant difference between the concentrations of these species between treatments
304 after 24 hr (618.00 ± 21.63 µM, 20°C; 409.55 ± 66.64 µM, 28°C; 254.45 ± 38.59 µM, 36°C)
305 (Figure 4A). Later, the concentration of soluble peroxides in midgut of mosquitoes exposed to low
306 temperature remained high (375.92 ± 30.94 µM) when compared to other treatments (56.91 ± 47.31
307 µM, 28°C; 50.13 ± 42.58 µM, 36°C). We observed similar patterns for the concentration of nitric
308 oxide, with higher nitric oxide concentrations being present in midguts from mosquitoes housed at
309 20°C at both sampling time points (Figure 4B). In *An. gambiae*, the levels of hydrogen peroxide
310 (H_2O_2) increase dramatically after a blood meal, but not a sucrose meal (Herrera-Ortiz et al., 2011).
311 Therefore, we decided to investigate whether temperature variation affects ROS levels in midgut of
312 mosquitoes fed with 10% sucrose or if the ROS level was associated specifically to blood retention.
313 Overall, the concentration of soluble peroxides was significantly lower 24 hr after mosquitoes were
314 fed a sugar meal relative to mosquitoes fed a blood meal, especially in those housed at a cool ambient
315 temperature (20°C, Figure 4A and 4C). There was no effect of temperature on the concentration of
316 soluble peroxides among mosquitoes housed at different temperatures and sampled at different times.

317 Similarly, when assessing nitric oxide levels in mosquitoes that received a sugar meal, concentrations
318 were found to be lower than those were blood-fed. Likewise, there was no significant difference in
319 the concentrations of nitric oxide found among temperature treatments after a sugar meal (Figure
320 4D).

321 In order to reinforce the ROS measurement data, we analyzed the abundance of mRNA of
322 some genes involved in redox homeostasis by RT-qPCR in mosquito midguts exposed to temperature
323 treatments for 24 and 48 hpf. Xanthine dehydrogenase (xdh), an enzyme that catalyzes the production
324 of uric acid, which acts as an antioxidant (Souza et al., 1997) was most expressed when mosquitoes
325 experienced the lowest temperature. As shown in Figure 5A, the xdh1 isoform was significantly more
326 expressed at 20°C 24 hpf while the xdh2 isoform was differentially expressed between treatments at
327 48 hpf (Figure 5B). The TrxR/Trx system plays a central role in the detoxification of oxygen radicals
328 (Kanzok et al., 2001). Unlike what was seen for xdh1 and 2, its expression was greater in mosquitoes
329 exposed to warmer temperatures for 24 hr (Figure 5C). At 48 hpf the expression of Trx decreased at
330 36°C while it increased in expression in mosquitoes kept at 20 and 28°C. Finally, when we evaluated
331 the expression profile of ferritin, which accumulates in female mosquitoes after a blood meal and is
332 used to sequester and prevent oxidative damage from iron released from heme (Dunkov et al., 2002),
333 significantly higher expression was found in mosquitoes housed at the cool ambient temperature
334 (20°C) at 24 hpf. At 48 hpf, mosquitoes housed across all temperature treatments experienced
335 reduced expression in ferratin (Figure 5D).

336 **4. Discussion**

337 Temperature is one of the most important ecological factors for mosquitoes, affecting diverse
338 aspects of their physiology and ecology (Lyimo et al., 1992; Alto and Juliano, 2001; Lazzaro et al.,
339 2008; Delatte et al., 2009; Muturi et al., 2011, 2012; Padmanabha et al., 2012; Carrington et al.,
340 2013c; Ciota et al., 2014). Based on the strong dependence on temperature, it is not surprising that

investigating responses to temperature is a recurrent issue in ecological research (Padmanabha et al., 2012; Campbell et al., 2015; Parham et al., 2015). Although many studies have already demonstrated that temperature can markedly impact on humoral and cellular immune responses shaping the host resistance to pathogen (Muturi and Alto, 2011; Murdock et al., 2012; Muturi et al., 2012; Xiao et al., 2014; Gloria-Soria et al., 2017), several important gaps limit our ability to understand the mechanisms by which these phenotypes occur. In this work we proposed that the temperature slows down the digestion of the blood meal, which results in a prolonged state of oxidative stress in the midgut environment, that in turn could potentially explain why arbovirus infection is limited at cooler temperatures as seen before by Tesla (2018).

Previously we found that temperature significantly affected the gene expression profiles in ZIKV-exposed and unexposed *Ae. aegypti* mosquitoes, in which many of the genes drastically altered participate in blood meal digestion and managing oxidative stress associated with the breakdown of hemoglobin into heme (Ferreira et al., 2020). The increase of temperature resulted also in increase of infection of mosquitoes by ZIKV. Similar to our previous study, we found that temperature significantly affects the expression of enzymes involved in blood digestion (Figure 1 and 2). Trypsin, the main proteolytic enzymes for blood digestion, has its expression induced in *Ae. aegypti* midgut during the first few hours after ingestion of a blood meal in standard rearing conditions (28°C) (Sanders et al., 2003), but were highly expressed in mosquitoes 48 hpf when housed in a cool environment in our study (Figure 1B and 2A). PEPCK, a gluconeogenesis enzyme that respond by 3-24 hpf (Sanders et al., 2003), also showed highly elevated expression levels at a late time in those mosquitoes housed at 20°C (Figure 1B and 2B). Further, two digestive proteases required for the digestion of non-proteinaceous blood constituents, α-NaGalase and β-gal (Dana et al., 2005) were highly induced 48 hpf (Figure 1B and 2C and D). For mosquitoes housed under standard (28°C) and warm environments (36°C) the accumulation of transcripts involved in digestion occurred within 24

365 hpf (Figure 1 and 2), in concordance with what is observed for these temperature conditions (Sanders
366 et al., 2003).

367 Blood is nutritionally rich in proteins, which represent 20% of the dry weight. Hemoglobin,
368 albumin and immunoglobulins constitute 80% of the total protein. Complete digestion of a blood
369 meal in hematophagous insects under standard rearing conditions (28°C) is achieved within 48 hr
370 (Gaio et al., 2011). We found low protein contents in the midgut of mosquitoes kept at this
371 temperature 48 hpf (Figure 3A and B). Contrary to what we observed at 28 and 36°C, the protein
372 content in the gut of mosquitoes housed at 20°C remained significantly higher suggesting the rate of
373 blood meal digestion is slower at this temperature. It has been well documented that trypsin reaches
374 the highest enzyme activity between 18-36 hr after a blood feeding (Sanders et al., 2003; Dana et al.,
375 2005; Isoe et al., 2009). In agreement with total protein content, trypsin activity was affected by
376 temperature revealing that the digestion of blood proteins was delayed in mosquitoes housed at cold
377 environment (Figure 3C). This is not entirely surprising as metabolic theory predicts low body
378 temperatures will inevitably depress the rates of biochemical reactions (Angilletta et al., 2010). These
379 results corroborate with previous studies which demonstrated that the rate of digestive enzyme
380 synthesis and the kinetics of enzyme reactions are affected by temperature and this directly affects
381 the rate of digestion of the blood meal (Briegel and Lea, 1975; Service et al., 1986; Hatfield, 1988;
382 Billingsley and Hecker, 1991; Leighton, 2005).

383 It is well documented that blood intake results in increased oxidative stress due to increased
384 metabolic activity in the midgut (Souza et al., 1997; Kumar et al., 2003; DeJong et al., 2007; Molina-
385 Cruz et al., 2008; Herrera-Ortiz et al., 2011). In fact, high levels of ROS were observed 24 hpf under
386 standard insectary conditions (28°C). We also observed that the generation of ROS was affected by
387 variation in ambient temperature (Figure 4A and B). This may not be entirely surprising, because the
388 induction of the detoxification enzymes shown to be faster and more robust in *An. gambiae* housed at

27°C than at 21°C (Molina-Cruz et al., 2008). In addition, as the effects of temperature on nitric oxide synthase (NOS) gene expression, have previously been shown, where in mosquitoes (*Anopheles stephensi*) housed at cooler temperatures (18°C: 24 h; 22°C: 18 h), NOS expression peak later than in mosquitoes housed at optimal or warmer temperatures (26–34°C: 12 h) (Murdock et al., 2012). Similarly, our data revealed that at cold environments ROS levels are significantly higher at 24 and 48 hpf, probably associated with the delayed breakdown of hemoglobin in the blood meal (Bottino-Rojas et al., 2015) in mosquitoes housed at this temperature.

It has been suggested that H_2O_2 participates in mosquito defenses as an important modulator of the immune response via NF-κB (Gloire et al., 2006) and melanization (Kumar et al., 2003; Molina-Cruz et al., 2008). The involvement of NO and H_2O_2 in the induction of the mosquito systemic immune response during the infection with malaria parasites is well known (Herrera-Ortiz et al., 2011). In *An. gambiae*, levels of H_2O_2 significantly increases after a blood meal. (Kumar et al., 2003). When mosquitoes picks up a malaria infectious blood meal, H_2O_2 increases to even higher levels (Molina-Cruz et al., 2008). Similarly, for a refractory strain of *An. gambiae*, ROS levels are increased after a blood meal (uninfected and infected with *Plasmodium*), however, this strain lives in a chronic state of oxidative stress what seems like to limit the infection. Our previous studies revealed that *Ae. aegypti* mosquitoes in cold conditions constrained transmission due to slow virus replication and escape from the midgut (Tesla et al., 2018). Furthermore, in these mosquitoes the expression of genes managing oxidative stress were drastically altered (Ferreira et al., 2020), suggesting that a prolonged state of oxidative stress, promoted by cold temperatures, could also be potentially important in limiting arbovirus infection in the mosquito. As seen in other systems, a higher state of oxidative stress facilitate downstream innate immune mechanisms as activation of the Toll pathway (responsible for activation of antimicrobial peptides) (Herrera-Ortiz et al., 2011; Pan et al., 2012) and melanization (Kumar et al., 2003; Molina-Cruz et al., 2008). In contrast to that seen in

413 blood-fed mosquitoes, the generation of ROS was not affected by the temperature in mosquitoes fed
414 with sugar (Figure 4C and D) confirming the importance of blood retention in the immune response
415 induction.

416 Several genes involved in redox metabolism are upregulated after a blood meal (Bottino-
417 Rojas et al., 2015). Dana and colleagues (2005) investigating the expression profile in *An. gambiae*
418 during and post ingestion of a blood meal demonstrated that several genes encoding antioxidants
419 were up-regulated at 12-48 hpf (Dana et al., 2005). Additionally, Bottino-Rojas and colleagues
420 (2015) studying the transcriptional changes regulated by the heme in *Ae. aegypti*, found an
421 accumulation of transcripts that encode typical antioxidant proteins such as, ferritin, glutathione S-
422 transferase and cytochrome P-450. Ferritin, an iron responsive gene (Law, 2002), responded to the
423 temperature variation with the greatest expression occurring at 20°C. Thioredoxin and xanthine
424 dehydrogenase, involved in the uric acid cycle, respectively, also regulate redox metabolism and the
425 genes that encode these proteins are positively regulated in strains of *An. gambiae* with a state of
426 chronic oxidative stress (Herrera-Ortiz et al., 2011). Since exposure to low temperature promoted a
427 condition of prolonged stress (Figure 4A and B), we expected to find accumulation of the transcripts
428 encoding thioredoxin and xanthine dehydrogenase. A significant difference was found in the
429 expression of two xanthine dehydrogenase isoforms between temperatures, with the highest
430 expression found at the lowest temperature (Figure 5), in accordance with the findings for ROS
431 (Figure 4).

432 Our results provide for the first time evidence of the impact of temperature on the oxidative
433 balance in the intestinal environment of *Ae. aegypti* mosquitoes and to associate this phenomenon to
434 blood retention in the midgut. Although further investigation is needed to determine the precise
435 molecular mechanisms of this regulation, the results presented here indicate that temperature can
436 profoundly impact the expression of genes involved in blood digestion, as well as digestion rates with

a consequent impact on oxidative balance in the midgut of *Ae. aegypti* mosquitoes, which could result in the modulation of immune response pathways with important consequences on vector competence, the extrinsic incubation period of developing pathogens, and overall transmission. Blood feeding is the primary port of entry into mosquitoes for several pathogens that cause diseases in vertebrates, therefore, it would not be surprising if the modulation of the infection resistance phenotype promoted by temperature started with effects on the digestion process. Future research will include the investigation of temperature in infected mosquitoes in order to verify if the mechanisms of regulation of intestinal homeostasis triggered by cool temperature are preserved when the pathogen is present and if these explain the limitation of the infection.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1- Gene-specific primers used for Real-Time qPCR

GENE	ACESSION N°	PRIMER 5'-3'		PRODUCT SIZE (pb)
		FORWARD	REVERSE	
Ribosomal protein S7	AAEL009496	ACCGCCGTCTACGATGCCA	ATGGTGGTCTGCTGGTTCTT	131
Trypsin	AAEL012852	ACCAGTATGCTACGGGCAAC	CCAGGAAACCACTCCGATAA	192
Phosphoenolpyruvate carboxykinase	AAEL000080	CGGGTACAACTTCGGTGACT	CCAATCCATAACTCGGCAGT	169
Alpha-N-acetylgalactosaminidase	AAEL005188	ACATCGACGATTGTTGGTCA	CGGGTATCCAGCACAAGTTT	170
Beta-galactosidase	AAEL004582	CCACTCGGATCCCTTGACTA	TGATACGGCCTTGATTCTCC	176
Xanthine dehydrogenase isoform 1	AAEL002683	GCGATTGACATTGGACAGATCGA	CCAGGAATGTCGGCGAAACC	146
Xanthine dehydrogenase isoform 2	AAEL014493	GTTATGGACATTGGCTCTAGCCT	CCTGGACCTCTCGATAGCAGTGT	140

Thioredoxin reductase	AAEL010777	GACGAGTGCGAAGATCTGGC	CATCTCCAGCTTCTGGTCGTT	120
Ferritin	AAEL004335	GACGCGATAGCACTGATGAA	CTCGTACTCCATGGCCAACT	153

Figure captions

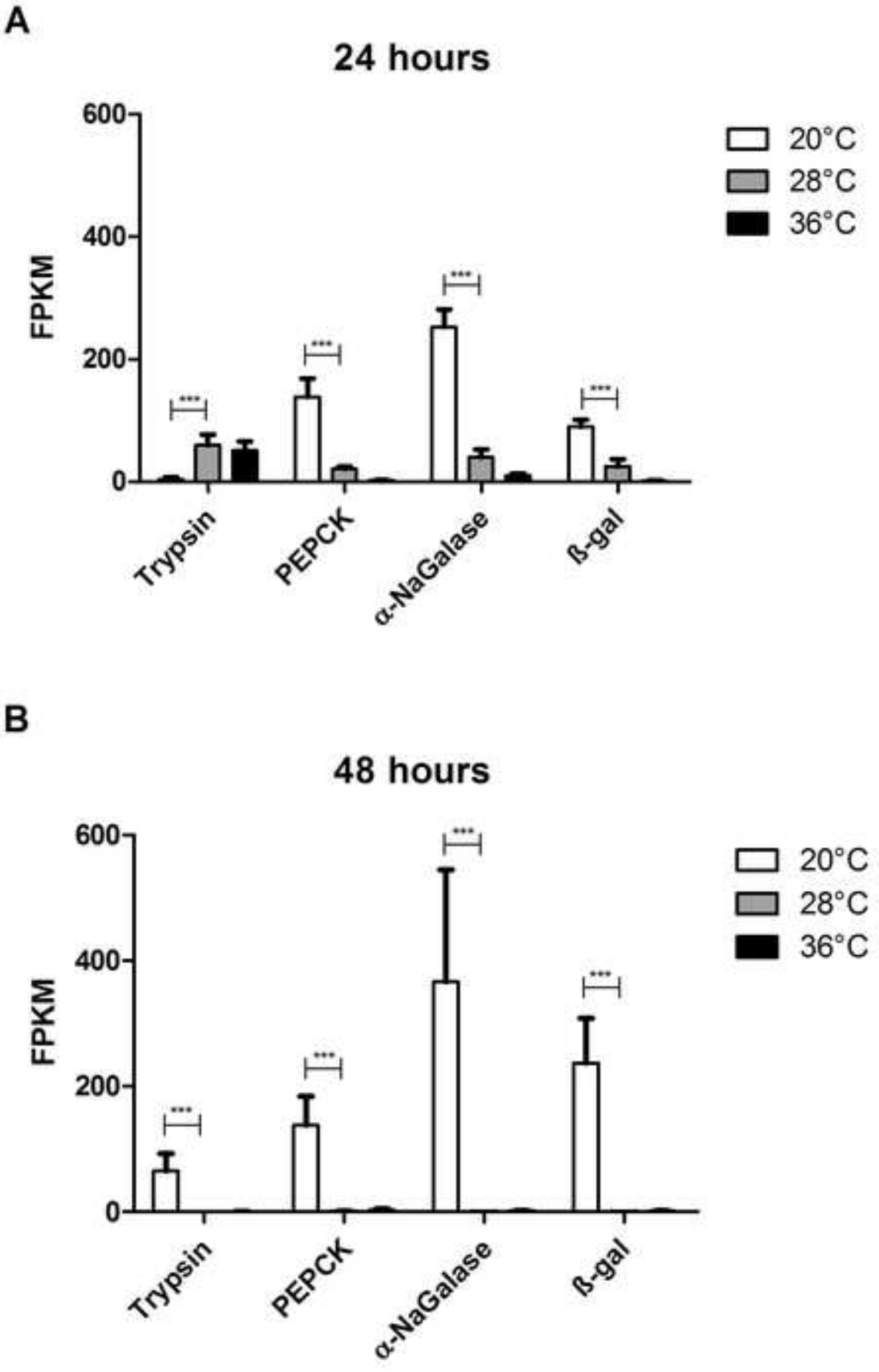
Figure 1. Fragments per kilobase of transcript per million mapped reads (FPKM) for respect genes; Tripsin, PEPCK – Phosphoenolpyruvate carboxykinase, α-NaGalase – alpha-N-acetyl-galactosaminidase and β-gal – Beta-galactosidase, in midguts of blood-fed *Ae. aegypti* mosquitoes exposed to three temperatures (20, 28 and 36°C) at 24 and 48 hpf. The data are the average of three independent experiments. P-value *< 0.05, **< 0.01 e ***< 0.001.

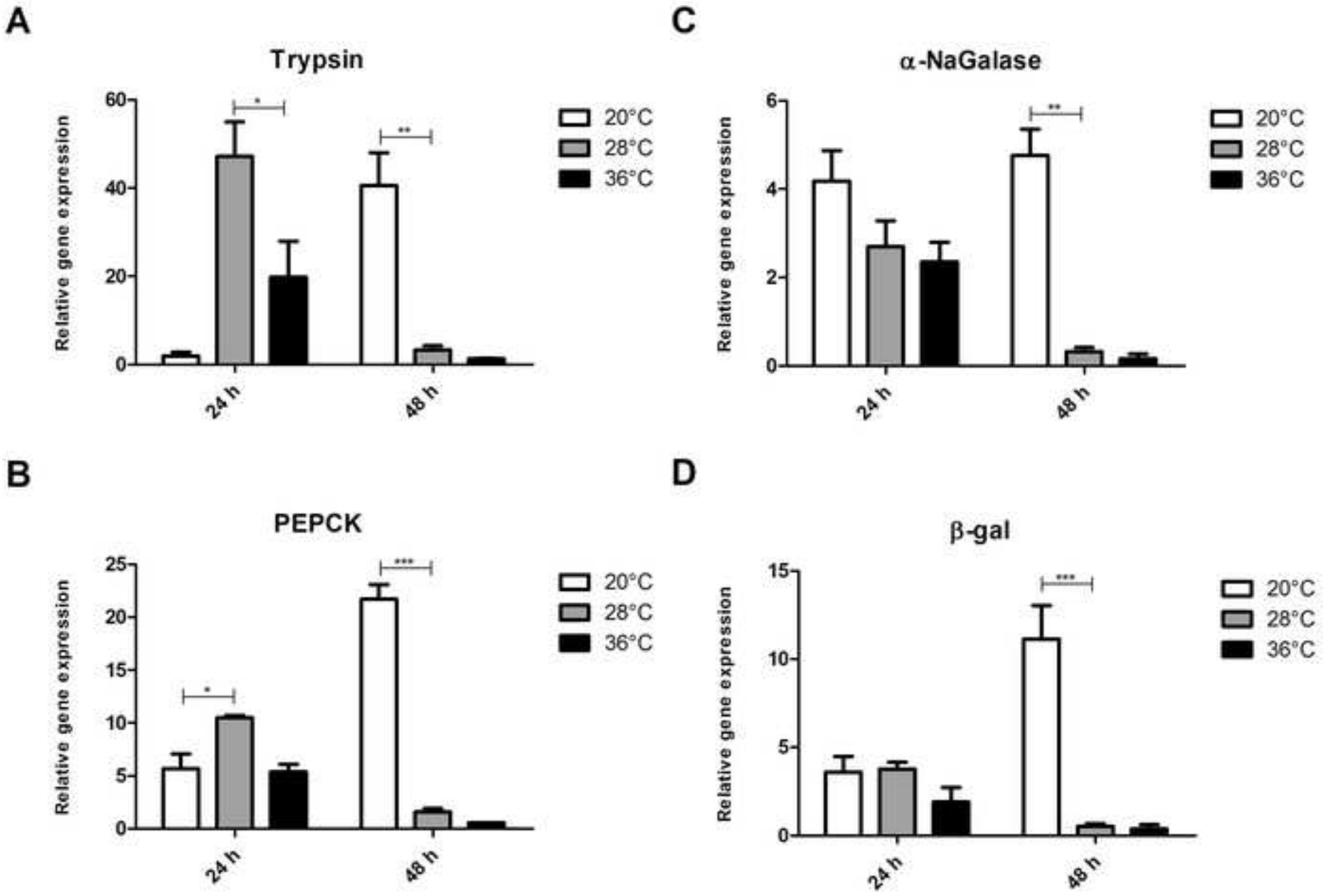
Figure 2. Abundance of genes involved in the blood digestion **A.** Trypsin, **B.** PEPCK, **C.** α-NaGalase, **D.** β-gal in midgut of blood-fed *Ae. aegypti* mosquitoes exposed to three temperatures (20, 28 and 36°C) at 24 and 48 hpf in relation to ribossomal R7 protein. The data are the average of three independent experiments. P-value *< 0.05, **< 0.01 e ***< 0.001.

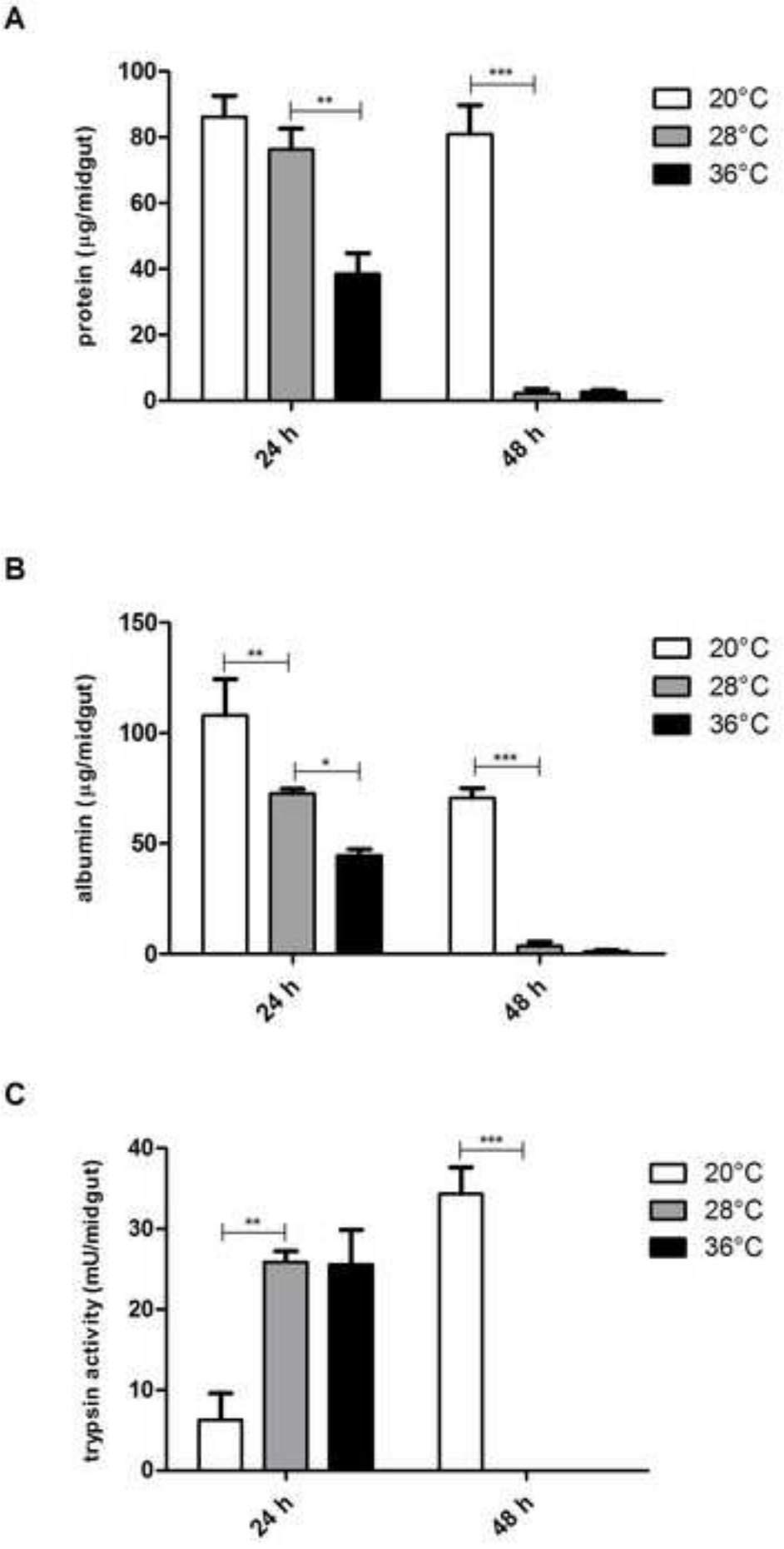
Figure 3. Effect of temperature on protein degradation in blood-fed *Ae. aegypti* mosquitoes at 24 and 48 hpf. **A.** Total protein**. B.** Albumin. **C.** Tripsin activity. The data are the average of three independent experiments. P-value *< 0.05, **< 0.01 e ***< 0.001.

Figure 4. Levels of **A.** soluble peroxides and **B.** nitric oxide in midgut of blood-fed *Ae. aegypti* mosquitoes, **C.** soluble peroxides and **D.** nitric oxide in midgut of sugar-fed *Ae. aegypti* mosquitoes exposed to three temperatures (20, 28 and 36°C) at 24 and 48 hpf. The data are the average of three independent experiments. P-value *< 0.05, **< 0.01 e ***< 0.001.

Figure 5. Abundance of genes involved in the oxidative stress **A.** xdh1, **B.** xdh2, **C.** trx and **D.** ferritin in midgut of blood-fed *Ae. aegypti* mosquitoes exposed to three temperatures (20, 28 and 36°C) at 24 and 48 hpf in relation to ribossomal R7 protein. The data are the average of three independent experiments. P-value *< 0.05, **< 0.01 e ***< 0.001.







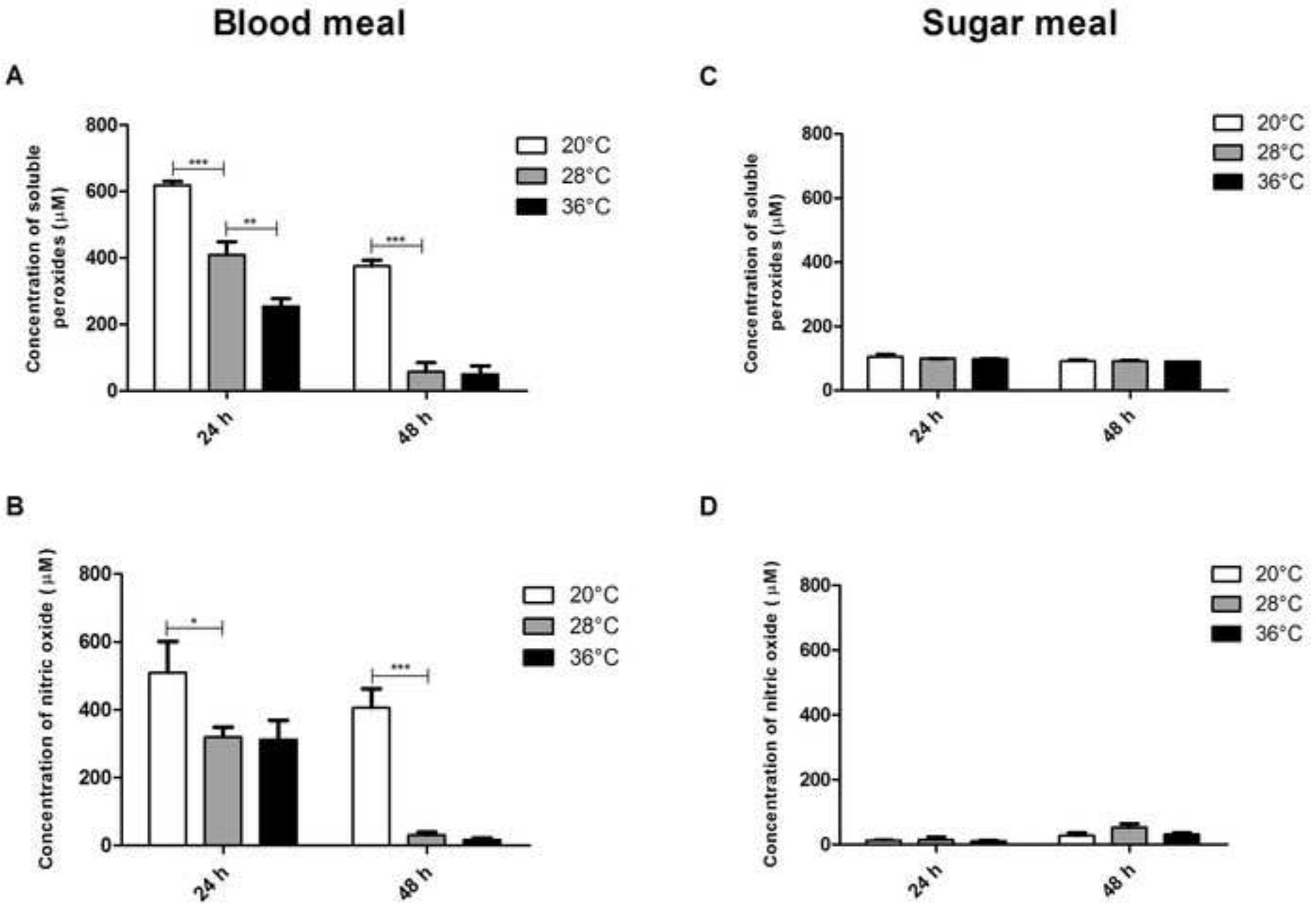


Figure 5

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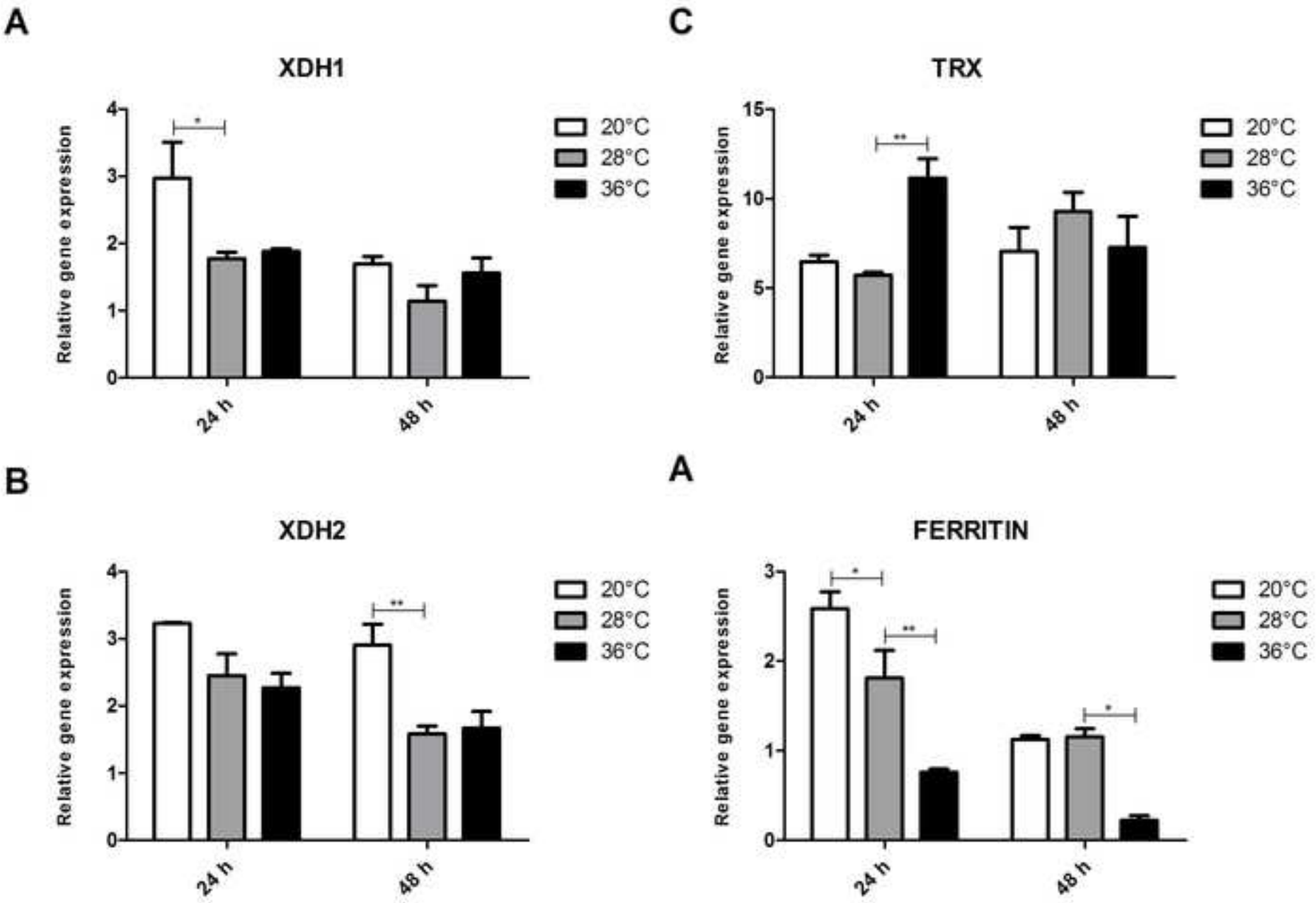

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Ribosomal protein S7	AAEL009496	ACCGCCGTCTACGATGCCA	ATGGTGGTCTGCTGGTTCTT	131
Trypsin	AAEL012852	ACCAGTATGCTACGGGCAAC	CCAGGAAACCACTCCGATAA	192
Phosphoenolpyruvate carboxykinase	AAEL000080	CGGGTACAACTTCGGTGACT	CCAATCCATAACTCGGCAGT	169
Alpha-N-acetylgalactosaminidase	AAEL005188	ACATCGACGATTGTTGGTCA	CGGGTATCCAGCACAAGTTT	170
Beta-galactosidase	AAEL004582	CCACTCGGATCCCTTGACTA	TGATACGGCCTTGATTCTCC	176
Xanthine dehydrogenase isoform 1	AAEL002683	GCGATTGACATTGGACAGATCGA	CCAGGAATGTCGGCGAAACC	146
Xanthine dehydrogenase isoform 2	AAEL014493	GTTATGGACATTGGCTCTAGCCT	CCTGGACCTCTCGATAGCAGTGT	140
Thioredoxin reductase	AAEL010777	GACGAGTGCGAAGATCTGGC	CATCTCCAGCTTCTGGTCGTT	120
Ferritin	AAEL004335	GACGCGATAGCACTGATGAA	CTCGTACTCCATGGCCAACT	153

Temperature-dependent alternative splicing affects gene expression in *Aedes aegypti* mosquitoes midgut

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Abstract

Temperature is one of the most significant abiotic factors that influence in vector resistance to pathogens. Research has provided substantial insights into the immunological and oxidation process that are impacted by temperature. However, we are only just beginning to understand the complexity underlying mosquito–pathogen interactions. Here we went further and investigated, firsthand, the impact of temperature on alternative splicing in *Aedes aegypti* mosquitoes midgut under different temperatures. Few genes were found with differential alternative splicing when comparing low (20°C) and high (36°C) temperatures with standard rearing conditions (28°C). Interestingly, between listed genes, we found Trypsin (TRY), Ferritin (FER), Thioredoxin (TRX) and Peptidoglycan recognition protein LC (PGRP-LC), genes previously shown to have their expression modulated by temperature. Experimental validation of TRX and PGRP-LC, genes already well known for their role in defending against pathogen establishment, confirmed the results that TRX have a differentially spliced exon in warmer conditions that can affect its activity. Differently, for PGRP-LC our experimental results do not showed differential alternative splicing between temperature treatments.

Keywords: Temperature, *Aedes aegypti*, Alternative splicing, Thioredoxin, PGRP-LC

1. Introduction

Mosquitoes are important vectors for an extensive range of pathogenic organisms. Due to their ectothermic nature, their survival and performance depend on temperature in which they live (Rueda et al., 1990; Alto and Juliano, 2001; Briegel and Timmermann, 2001; Delatte et al., 2009). As a result, environmental temperatures can also affect the spread of diseases transmitted by these vectors (Watts et al., 1987; Lambrechts et al., 2011; Carrington et al., 2013b; Ciota et al., 2014; Tesla et al., 2018a). There are abundant evidences that small changes in environmental temperature can significantly influence mosquitoes resistance to infections (Jodell E. Linder et al., 2008; Lazzaro et al., 2008; Hye Jin Chung et al., 2010; Yoshida, 2010; Adamo and Lovett, 2011; Ruiz-vega et al., 2011).

During the transmission cycle, these insects feed on volumes of blood that are 2–3 times their weight, and the digestion of this large meal results in several potentially damaging conditions (Graça-Souza et al., 2006). The digestion of blood meal requires intense proteolytic activity in the midgut and results in the formation of large amounts of potentially harmful substances such as heme, iron, amino acids and ammonia (Balla et al., 2007; Oliveira et al., 2011). In addition, a dramatic increase in intestinal microbiota occurs after a blood feeding (Gusmão et al., 2010). Consequently, several physiological processes are activated, such as formation of a peritrophic matrix (PM), that surrounds the food bolus to defend itself against these pathogens and harmful substances (Pascoa et al., 2002) and the expression of ROS detoxifying enzymes (Molina-Cruz et al., 2008).

The midgut epithelium is the first barrier that viruses must cross in the mosquito to achieve a successful viral cycle. In previous works, we verify that at higher temperatures, there is a greater efficiency in *Zika virus* (ZIKV) infection in the

Ae. aegypti (*Ae. aegypti*) midgut while that in lower one, ZIKV is not detected (Ferreira et al., 2020), which is also observed for other flaviviruses (Dohm et al., 2002; Reisen et al., 2006). In the same study, by evaluating of the transcriptome of ZIKV-exposed and unexposed mosquitoes, a remarkable enrichment of transcripts involved in blood digestion, oxidation and immunological process was seen in mosquitoes exposed to colder conditions (Ferreira et al., 2020). Among these genes are Trypsin (TRY), Ferritin (FER), Thioredoxin (TRX) and PGRP-LC.

Additionally to gene expression at the transcriptional level, alternative splicing is a fundamental mechanism for the functional diversification of the eukaryotic transcriptome. Some studies have already demonstrated the role of alternative splicing in the resistance of vectors to infections (Bauer et al., 2003; Lin et al., 2007; Meister et al., 2009; Smith et al., 2011). However, they use a reductionist concept evaluating only few targets. Genes involved in immunological and managing oxidation stress are processes recurrently found in literature (Bauer et al., 2003; Meister et al., 2009).

This report expands the temperature effects on alternative splicing in mosquitoes *Ae. aegypti* showing for the first time, that some important genes, already knowledge by its role in the mosquitoes resistance to infection, have splicing alternative events impacted by temperature environment.

2. Material and Methods

2.1. Data generation

First, RNASeq data from *Ae. aegypti* midgut exposed to three temperatures (20,28 e 36°C) for two time (24 e 48 hr) were recovery from NCBI SRA (ID: PRJNA615972) (Ferreira et al., 2020). Poor quality reads (Phred quality score below

20), short reads (less than 25 bases), and adapter sequences (Bolger et al., 2014) were removed using Trimmomatic (v 0.36). Tophat (v 2.1.1) (Trapnell et al., 2009) was used to perform the alignment and mapping of the high quality reads to the *Ae. aegypti* genome (NCBI ID: GCA_002204515.1) obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

2.2. Differential alternative splicing analysis

To identify differential alternative splicing events (ASE) between the temperature of 28°C and other temperatures (20, and 36°C), we used the statistical parameters of replicate multivariate analysis of transcript splicing (rMATS) (Shen et al., 2014). GTF file constructed from the NCBI Reference AaegL5.0 Primary Assembly: GCF_002204515.2_AaegL5.0_genomic.gff and the mapped bams were submitted on rMATS v4.0.3b with --cstat 0.0001 parameter. Then rMATS JCEC outputs were submitted to MASER package of Biocondutor (Huber et al., 2015) to filter significant events. Five main alternative splicing types, including skipping exon (SE), retention intron (RI), alternative 5′splice site (A5SS), alternative 3′splice site (A3SS) and mutually exclusive exons (MXE), which satisfied the criteria of FDR q-value <0.5, were screened out as differential alternative splicing genes. To visualize rMATS results and differential splicing events between treatments rmats2sashimiplot software (https://github.com/Xinglab/rmats2sashimiplot) was used. Bam files and rMATS output files were used to run the program.

2.3. Ethics statement

The approval for the use of mice (*Mus musculus*) in the feeding of mosquitoes was obtained from the Ethics Committee at the Universidade Federal de Viçosa (UFV) (reference number 561/2016).

2.4. Mosquito

Ae. aegypti mosquitoes (lineage PPCampos - Campos dos Goytacazes) were reared in an insectary at the Department of General Biology at UFV (Viçosa, MG, Brazil) under controlled temperature (25 ± 2°C), relative humidity (60 ± 10%), and photoperiod (12:12 light:dark). Eggs were hatched in de-chlorinated tap water and larvae were fed with turtle food pellets (ReptoLife, Camboriú, SC, Brazil). Pupae were transferred to cages and a 10% sucrose solution was provided *ad libitum* for emerging adults. Egg production was stimulated after providing a blood meal on anesthetized mice (0.8 mL (2%) xilazina and 0.2 mL (10%) ketamine in order to maintain the colony.

2.5. Mosquito feeding regime

Adult mosquitoes (3 to 4-day-old), previously deprived of food for 12 h, were anesthetized with CO_2. Females were separated and kept in a Petri dish on ice, transferred to a plastic cage and subsequently fed with mice blood. After 30 minutes, engorged mosquitoes were randomly allocated across six plastic cages and distributed across one of three different temperatures (20, 28, and 36°C (± 2°C)) at 60 ± 10% relative humidity, with a 12:12 light:dark cycle. A 10% sucrose solution was provided *ad libitum* to mosquitoes throughout the duration of the experiment (48 h). Three biological replicates were performed.

2.6. RNA extraction and RT-PCR

In order to validate differential ASE in the response to temperature, total RNA was extracted from 20 dissected midguts 24 h after blood ingestion using the RNeasy Mini Kit as instructed by the manufacturer. One microgram of total RNA was

173 treated with DNAse (Promega). RT-qPCR was performed on the synthesized cDNA
174 (High-Capacity cDNA Reverse Trancription Kit - Applied Biosystems) using SYBER
175 Green. Oligonucleotide primer sequences used in this study are shown in
176 Supplementary Table 1. The amplification and detection of specific products was
177 performed on StepOne™ Real Time PCR system (Applied Biosystems, USA), using
178 the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 ◦C for 30 s and
179 60 ◦C for 1 min and 1 cycle at 95°C for 30 s, 60°C for 1 min and 95°C for 30 s.
180 Melting curves were analysed to confirm specificity of amplified products. All
181 reactions were performed in triplicate. The calculation of the relative abundance of
182 each transcript was performed using the relative standard curve method (de Oliveira
183 Mendes et al., 2013; Souza et al., 2019), constructed by serial dilution of a cDNA
184 pool starting at 25 ng/µL, ending at 1.56 ng/ µL and with a dilution factor of two. The
185 gene expression data in each sample were normalized by the expression levels of
186 the ribosomal S7 gene used as a endogenous constitutive control (Taracena et al.,
187 2018).
188

189 **2.7. Statistical analyses**

190 Statistical analyses were performed using one-way ANOVA followed by
191 Tukey's Multiple Comparasion Test to assess statistical differences between samples
192 using the software*GraphPad Prism*® v. 7 (*GraphPad Software*, Inc. La Jolla,
193 California, USA). All tests were considered statistically significant when *p-value* was
194 less than 0.05.
195

196 **3. Results**

To identify mRNA isoforms expressed in de the midgut of adult *Ae. aegypti* kept under different temperatures, we performed paired-end RNA-seq from *Ae aegypti* midguts exposed to three temperatures (20, 28 and 36°C) at 24 and 48 hours post feeding (hpf) (NCBI SRA ID: PRJNA615972) (Ferreira et al., 2020). From a total of 36 RNA samples that were sequenced using the Illumina platform, more than 2 million reads of these libraries were mapped to the *Ae. aegypti* genome (NCBI ID: GCA_002204515.1). All libraries resulted in a concordant pair alignment rate higher than 70%.

Using splice junction counts as input, ASE were investigated using rMATs. Because rMATS works detecting differential alternative splicing between two RNA-Seq samples, bam files from six samples groups were submitted in four runs: 20 versus 28°C and 36 versus 28°C, at 24 and 48 hpf. Five basic and generally recognized alternative splicing models were identified, including skipped exon (SE), mutually exclusion exons (MXE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), and retained intron (RI). At 24 h, 2,655 ASEs were identified in mosquitoes kept at 20°C, and 2,753 at 36°C (Supplementary Table 2-5). Among them, 18 and 38 showed differential alternative splicing compared to 28°C (FDR < 0.05), respectively. In both conditions, SE was the most common event, while RI was the least common (Figure 1). Under the lowest temperature (20°C) were found 4 MXE events (1 up-regulated and 3 down-regulated), 4 A5SS (1 up-regulated and 3 down-regulated), 4 A3SS (2 up-regulated and 2 down-regulated), 5 SE (3 up-regulated and 2 down-regulated) and 1 RI (Figure 2A) for a total of 18 alternative splicing events in 18 genes. For the highest temperature (36°C), 3 MXE (3 down-regulated), 8 A5SS (5 up-regulated and 3 down-regulated), 9 A3SS (5 up-regulated

221 and 4 down-regulated), 17 SE (13 up-regulated and 4 down-regulated) and 1 RI
222 (Figure 2A) for a total of 38 alternative splicing events in 36 genes.

223 More ASEs were found (3,633 and 3,330 for 20 and 36°C, respectively) 48
224 hpf. In the same way, the number of events with differential alternative splicing
225 compared to 28°C was higher (51 for 20°C and 69 for 36°C) (Figure 1). At this
226 sampling time point 9 and 8 MXE, 13 A5SS, 5 and 11 A3SS, 19 and 31 SE and 5
227 and 6 RI were identified for 20 and 36°C temperature treatments, respectively (Figure
228 2B).

229 When we evaluated the set of genes that shown significant differential ASE
230 (Table 1), genes involved in managing oxidative stress, digestion process and innate
231 immunity were identified. Ferritin (XP_001652732.2) and Thioredoxin
232 (XP_001662665.1), related to the cellular redox balance, were differentially spliced in
233 36°C compared with 28°C at 24 hpf. For Ferritin down-regulated A3SS event, with 3
234 base pairs (bp) spliced, was identified at 36°C (ΔPSI = -0.034), while that Thioredoxin
235 showed up-regulated SE event, with 202 pb spliced, in this temperature (ΔPSI =
236 0.024). Curiously, only for Ferritin that event was noticed at 48 hpf. Alternative 3'
237 splice sites (with 6 bp spliced) also was seen for Trypsin, an important insect
238 digestive proteinase. In this case, the exon was up-regulated at 36°C (ΔPSI = 0.029)
239 and down-regulated at 20°C (ΔPSI = -0.036) at 24 hpf. Finally, between 94 genes
240 alternatively spliced, PGRP-LC, an innate immunity molecule that activate the Imd
241 pathway, showed up-regulated A5SS event, with 75 bp spliced, in the lowest
242 temperature (20°C), when compared to standard rearing conditions (28°C) (ΔPSI =
243 0.213).
244

245 **Table 1 –** Set of genes with significant differential ASE in mosquitoes housed at 20 and 36°C
246 compared to 36°C.

Gene ID	Gene Description	Gene ID	Gene Description
XP_001648292.2	eukaryotic translation initiation factor 4H isoform X4	XP_021697109.1	2-oxoglutarate dehydrogenase, mitochondrial isoform X6
XP_001649020.1	homeotic protein caudal isoform X2	XP_021698076.1	PDZ and LIM domain protein Zasp isoform X6
XP_001649058.1	60S acidic ribosomal protein P2	XP_021698096.1	matrix metalloproteinase-14 isoform X4
XP_001650333.1	tubulin beta chain isoform X2	XP_021698348.1	microtubule-associated protein RP/EB family member 1 isoform X4
XP_001651042.1	RNA-binding protein squid isoform X4	XP_021698460.1	zinc finger protein on ecdysone puffs isoform X4
XP_001651511.1	uncharacterized protein LOC5579836	XP_021698616.1	peptidoglycan-recognition protein LC isoform X4
XP_001651845.1	uncharacterized protein LOC5567650 isoform X2	XP_021698725.1	transcription factor CP2 isoform X4
XP_001652606.1	tumor protein D54 isoform X5	XP_021699115.1	uncharacterized protein LOC5576069
XP_001652671.1	cAMP-dependent protein kinase catalytic subunit	XP_021699196.1	uncharacterized protein LOC5580119 isoform X3
XP_001652732.2	ferritin subunit isoform X2	XP_021699235.1	uncharacterized protein LOC5577147 isoform X3
XP_001652944.1	trypsin 3A1 isoform X2	XP_021699489.1	coronin-6 isoform X2
XP_001653077.1	RNA-binding protein 1 isoform X2	XP_021699574.1	glucose-6-phosphate exchanger SLC37A2 isoform X2
XP_001654804.2	gamma-glutamyl hydrolase isoform X3	XP_021700066.1	transcription factor HNF-4 homolog isoform X4
XP_001655215.2	renin receptor	XP_021700093.1	myosin heavy chain 95F isoform X2
XP_001655223.1	troponin T, skeletal muscle isoform X6	XP_021700317.1	peroxisomal multifunctional enzyme type 2 isoform X2
XP_001655755.2	probable methylthioribulose-1-phosphate dehydratase isoform X4	XP_021700401.1	ribosome biogenesis protein NSA2 homolog
XP_001655810.1	minor histocompatibility antigen H13 isoform X2	XP_021700502.1	adenylyl cyclase-associated protein 1 isoform X2
XP_001655957.1	tropomyosin-1, isoforms 9A/A/B isoform X36	XP_021700621.1	sodium/hydrogen exchanger 3 isoform X13
XP_001655997.2	inositol-3-phosphate synthase	XP_021700629.1	exportin-1
XP_001656254.2	60S ribosomal protein L15	XP_021701557.1	alpha-protein kinase 1 isoform X7
XP_001656331.2	B-cell receptor-associated protein 31 isoform X4	XP_021702033.1	vascular endothelial growth factor receptor 1 isoform X6
XP_001656389.1	ras-like GTP-binding protein Rho1 isoform X2	XP_021702248.1	CD63 antigen
XP_001656505.2	60S ribosomal protein L10a isoform X2	XP_021702289.1	zinc-type alcohol dehydrogenase-like protein C1773.06c isoform X2
XP_001657237.1	RNA-binding protein lark	XP_021702485.1	protein tyrosine phosphatase type IVA 1 isoform X2
XP_001657492.2	clathrin light chain isoform X4	XP_021702520.1	trafficking kinesin-binding protein milt isoform X7
XP_001657924.1	uncharacterized protein LOC5568231	XP_021702612.1	uncharacterized protein LOC5576029 isoform X3
XP_001660861.1	glutamate dehydrogenase, mitochondrial isoform X2	XP_021702841.1	Niemann-Pick C1 protein isoform X5
XP_001661179.1	uncharacterized protein LOC5574109 isoform X2	XP_021703511.1	lysosomal alpha-mannosidase isoform X2
XP_001661720.2	proton-associated sugar transporter A isoform X2	XP_021703759.1	S-adenosylmethionine synthase isoform X1
XP_001661771.2	uncharacterized protein LOC5575041	XP_021704121.1	2-(3-amino-3-carboxypropyl)histidine synthase subunit 1
XP_001662312.1	rab-like protein 3 isoform X2	XP_021704674.1	serine/threonine-protein kinase MARK1 isoform X19
XP_001662665.1	thioredoxin reductase 1, mitochondrial isoform X4	XP_021705282.1	hrp65 protein isoform X3
XP_001663675.1	zinc transporter ZIP11	XP_021705538.1	pantothenate kinase 3 isoform X3
XP_001663974.1	glia maturation factor gamma	XP_021706052.1	lysosomal alpha-glucosidase
XP_011493322.1	protein jagunal isoform X2	XP_021706431.1	sodium-dependent nutrient amino acid transporter 1 isoform X2
XP_011493660.2	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	XP_021706661.1	cAMP-regulated phosphoprotein 19 isoform X2
XP_011493704.2	nucleolar and coiled-body phosphoprotein 1 isoform X4	XP_021708350.1	protein DEK isoform X2
XP_021692927.1	eukaryotic translation initiation factor 4B isoform X2	XP_021708424.1	uncharacterized protein LOC5576866 isoform X6
XP_021693296.1	heterogeneous nuclear ribonucleoprotein R isoform X19	XP_021708804.1	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 isoform X2

XP_021693313.1	uncharacterized protein LOC5576344	XP_021709017.1	casein kinase I isoform X8
XP_021693503.1	sodium/potassium-transporting ATPase subunit alpha isoform X7	XP_021709148.1	probable pseudouridine-5'-phosphatase isoform X2
XP_021693758.1	multiple coagulation factor deficiency protein 2 isoform X12	XP_021710706.1	putative sodium-dependent multivitamin transporter
XP_021694161.1	transmembrane protein 192 isoform X2	XP_021710738.1	uncharacterized protein LOC5568046
XP_021694820.1	programmed cell death protein 4	XP_021711524.1	putative polypeptide N-acetylgalactosaminyltransferase 9 isoform X3
XP_021695424.1	uncharacterized protein LOC5566116 isoform X10	XP_021711954.1	mitochondrial import inner membrane translocase subunit Tim17-A isoform X2
XP_021695999.1	14-3-3 protein zeta isoform X1	XP_021712219.1	fatty acid hydroxylase domain-containing protein 2 isoform X3
XP_021696670.1	synaptobrevin isoform X4 [Aedes aegypti]	XP_021712996.1	heterogeneous nuclear ribonucleoprotein 87F

TRX and PGRP-LC genes are differentially expressed when mosquitoes are exposed to different temperatures. At lower temperature, these genes are up-regulated and mosquitoes showed more resistance to Zika infections (Ferreira et al., 2020), therefore, we chose these targets for experimental evaluation. The Figure 3 shown sashimi plot for two genes, TRX and PGRP-LC. As seen, the exon inclusion level in all replicates at the transcript of TRX reveal a low rate skipped exon events occurring in both conditions, 28 and 36°C being, however, the long isoform more abundant at 36°C. Unlike what was seen for TRX, a high rate A5SS event at the transcripts of PGRP-LC was observed in the lower temperature, being the long isoform more abundant at 20°C.

To detect and validate the occurrence of these events, SE and A5SS, in the transcripts of, TRX and PGRP-LC, respectively, primers were designed. The relative positions of theses primers are shown in Figure 4 and their sequences are listed in Supplementary Table 1.

Initially, we verify the presence e/or absence of the alternative exon by RT-PCR. For TRX, the presence of alternative exon (TRX1) was mainly detected in the higher temperature (36°C), which was detected in both replicates. On the order hand, we detected the presence of the short isoform (TRX2) in all samples (Figure 5).

Similarly, both PGRP-LC isoforms, short and long, were detected at three temperatures (Figure 5). The long isoform expression evaluated by RT-PCR confirmed the isoform differential expression in the temperature of 28 and 36°C early pointed in rMATS software. Differently, we do not seen the temperature result in exons differently spliced when we test PGRP-LC (Figure 6).

4. Discussion

Innumerous studies have already demonstrated the impact of temperature environment on mosquitoes physiology and behavior (Rueda et al., 1990; Alto and Juliano, 2001; Briegel and Timmermann, 2001; Carrington et al., 2013a, 2013c; Ciota et al., 2014; Tesla et al., 2018b). Genes involved in digestion (Ferreira et al., 2020), oxidation (Muturi et al., 2012), immunity (Murdock et al., 2012; Muturi et al., 2012), and reproduction (Delatte et al., 2009) are some examples found recurrently in studies involving variation of temperature and arthropod vectors. To our knowledge, this is the first study exploring ASE in mosquitoes exposed at different temperature.

Previously we found that temperature significantly affected the gene expression profiles in *Ae. aegypti* mosquitoes with genes involved in the blood digestion, oxidation and immunological process the most drastically altered (Ferreira et al., 2020). Differently, temperature seems do not affect as profoundly on ASE. We identified only 18 ASE occurring in 18 genes for mosquitoes kept at 20°C for 24hr compared to rearing standard conditions. In warmer conditions, 38 ASE in 36 genes were identified. At last sampling time point (48 h), a greater number of events were revealed. In this time point, a total of 51 ASE in 49 genes and 69 ASE in 56 genes, respectively for 20 and 36°C. Skipping exon, the most common alternative splicing, was the main event in all conditions and RI the least one.

Among genes identified with alternative splicing, we highlighted those involved in managing oxidative stress, digestion process and innate immunity; ferritin, thioredoxin, trypsin and PGRP-LC. These genes were markedly impacted by temperature variation when we evaluated transcriptome profile (Ferreira et al., 2020). Like vertebrate ferritins, insect ferritins are heteromultimers composed by a heavy (HCH) and light (LCH) chains. It is known that LCH gene present skipping event in exon 2 and 3 and as the cassette exon is not part of the coding region; its removal will not alter the protein, but significantly increases its expression in mosquitoes *Ae. aegypti* (Geiser et al., 2003). In our previous studies, ferritin was up regulated in colder temperature, but rMATS did not identify the SE event that is knowledge by increase expression. Instead, an A3SS event was found.

The thioredoxin system, including NADPH, thioredoxin and thioredoxin reductase, plays significant roles in maintaining intracellular redox homeostasis and protecting organisms against oxidative damage (Juan and Arne, 2014). In *Anopheles sp.* mosquitoes, one thioredoxin reductase gene is present, which occurs in three splice variants. The isoforms varying in the sequence of the first exon only, therefore there are three alternative transcription start sites. Exon 1 encodes the N-terminal sequence of an abundant cytosolic, exon 2 of a mitochondrial, and exon 3 of a minor cytosolic thioredoxin reductase form (Bauer et al., 2003). While *Anopheles* thioredoxin reductase gene presents 4 exons, in *Ae aegypti* mosquitoes there are only 3 exons in that gene. Differently, here was identified a SE event in exon 2, so that transcription start sites remains the same for both isoforms. In our transcriptome study (Ferreira et al., 2020), TRX was down-regulated in mosquitoes kept at 36°C, condition where we found a differential alternative splicing compared to 28°C.

316 Mosquitoes require a blood meal to develop their eggs and consequently
317 synthesize trypsin, its main protease, in few hours in order to digest the blood meal
318 into free amino acids, that are used for vitellogenin biosynthesis (Borovsky, 2003).
319 This expression and activity showed to be dramatically impacted by temperature
320 environment, occurring more lately at cool temperatures and earlier at warmer
321 compared to standard conditions. In the midgut of *Ae. aegypti*, exon also was
322 differently spliced at 20 and 36°C in relation to 28°C, suggesting that the long isoform
323 (XP_001652943), that includes 6 bp, can may play a role in increased translation
324 and/or enzyme activity.

325 PGRPs constitute the primary means of bacterial recognition in insects and its
326 downstream responses also control the numbers of symbiotic bacteria that are mostly
327 found in the mosquito (Rodgers et al., 2020). PGRP-LC is required for the activation
328 of the Imd pathway in response to Gram (-) bacteria (Goto and Kurata, 2006).
329 Alternative splicing of PGRP-LC transcripts produces three main protein isoforms
330 (LC1, LC2, and LC3), each having a different PGRP domain and an optional 75-
331 nucleotide cassette at the 3′ end of the common exon 3. Thus, each isoform exists in
332 two versions; the long one (a) is 25 amino acids (aa) longer than the short version
333 (b), which utilizes a cryptic splice acceptor in exon 3. In our study, the variant LC3
334 showed differential splicing event in exon 3 (Figure 4), being the isoform LC3a more
335 abundant at 20°C. Lin *et al* (2007) studying the impact of PGRP-LC variants on
336 *Plasmodium* infection in *Anopheles gambiae* shown that over-expression of LC3a
337 induces the expression of cecropin1 (CEC1), while that for others splice forms (LC2b
338 and LC3b) a minimal effect on the expression of CEC1 was seen. In concordance, at
339 lower temperatures, *Ae. aegypti* mosquitoes have been showed more resistant to
340 *Zika virus* infections.

To our knowledge, this study comprises the first investigation of temperature effects on ASE in *Ae. aegypti* mosquitoes. Added to our previous research, these results revealed some promising targets for future studies of its role in *Ae. aegypti* competence to transmit infection diseases, and therefore, a better understanding of the mechanisms activated by temperature change and how they affect vectorial competence and consequently the spread of diseases.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure captions

Figure 1 - Global analysis of alternative splicing events. Number of events detected for five classes of alternative splicing: i) mutually exclusive exons, ii) alternative 5' splice sites, iii) alternative 3' splice sites, iv) skipped exon and v) retained intron, in analysis comparing mosquitoes kept at 20 to 28°C and 36 to 28°C for 24 and 48 hr.

Figure 2 - Volcano plot representing transcripts expression profile from *Ae. aegypti* mosquitoes exposed to three different temperatures (20, 28 and 36°C) for 24 and 48 hr. Red and blue points represented up and down transcripts isoforms ($p < 0.05$), respectively. Gray points refers not significant data.

Figure 3 – Sashimi plot showing **A.** exon skipping in TRX transcript in mosquitoes kept at 36°C and 28°C and **B.** A5SS in PGRP-LC transcript in mosquitoes kept at 20°C and 28°C for 24 hr. The tracks represent warmer (36°) or cold (20°C) conditions (red) and standard (28°C) conditions (orange) samples. The number on curved lines indicates continuous (top number) and differentially spliced (bottom number) exon-exon junction read counts. The x-axis depicts genomic coordinates. Inc level represents the exon inclusion level in warmer or cold and standard conditions.

Figure 4 - Gene structure of TRX and PGRP-LC, targets selected for experimental validation. Primers flanking skipped exons and retained introns are indicated by arrows. Green arrows – primers for amplification of both isoforms, long and short, in order to assess the presence/absence (PCR) of the event in different temperature treatments. Yellow arrows - primers for amplification of the long isoform, which contain the alternative exon, Red arrows - primers for short isoform amplification aiming to evaluate its expression (Real Time PCR) under different temperature conditions.

Figure 5 – PCR analysis for presence and absence of splice variants of TRX (long – TRX1 and short – TRX2) and PGRP-LC (long – 1 and short – 2) in mosquitoes kept at three temperatures treatment for 24 hr. Two replicates were evaluated for each temperature treatment.

Figure 6 – Real Time PCR analysis of TRX and PGRP-LC long isoform expression in mosquitoes kept at three temperatures treatment for 24 hr. The data are the average of three independent experiments. P-value **< 0.01

Figures

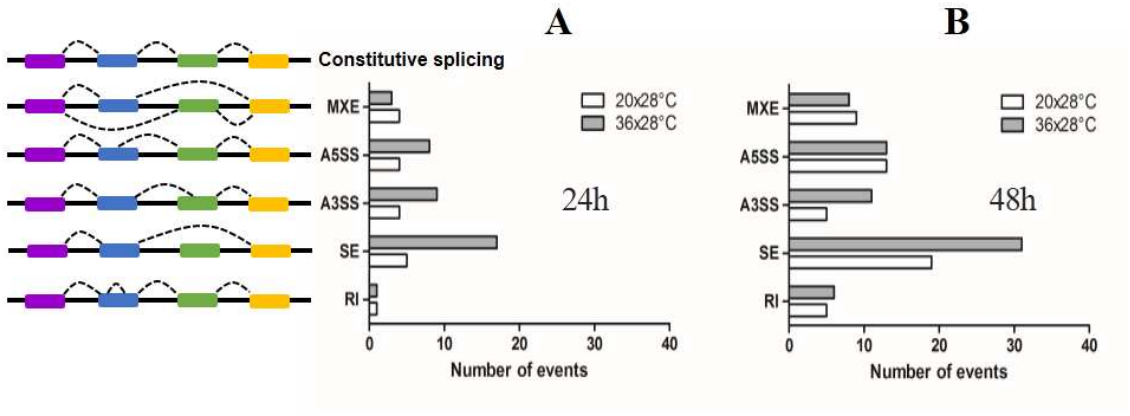


Figure 1

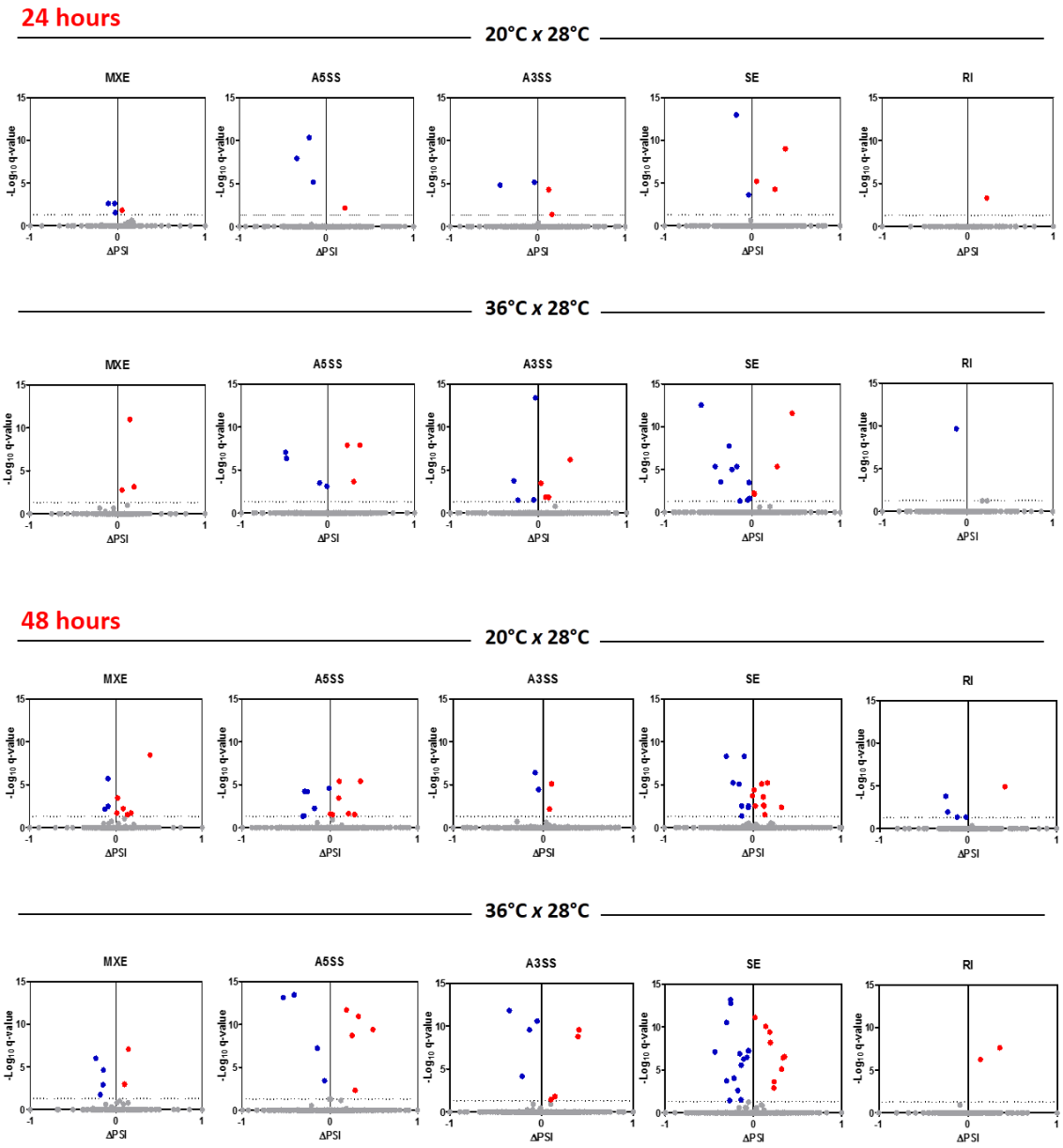
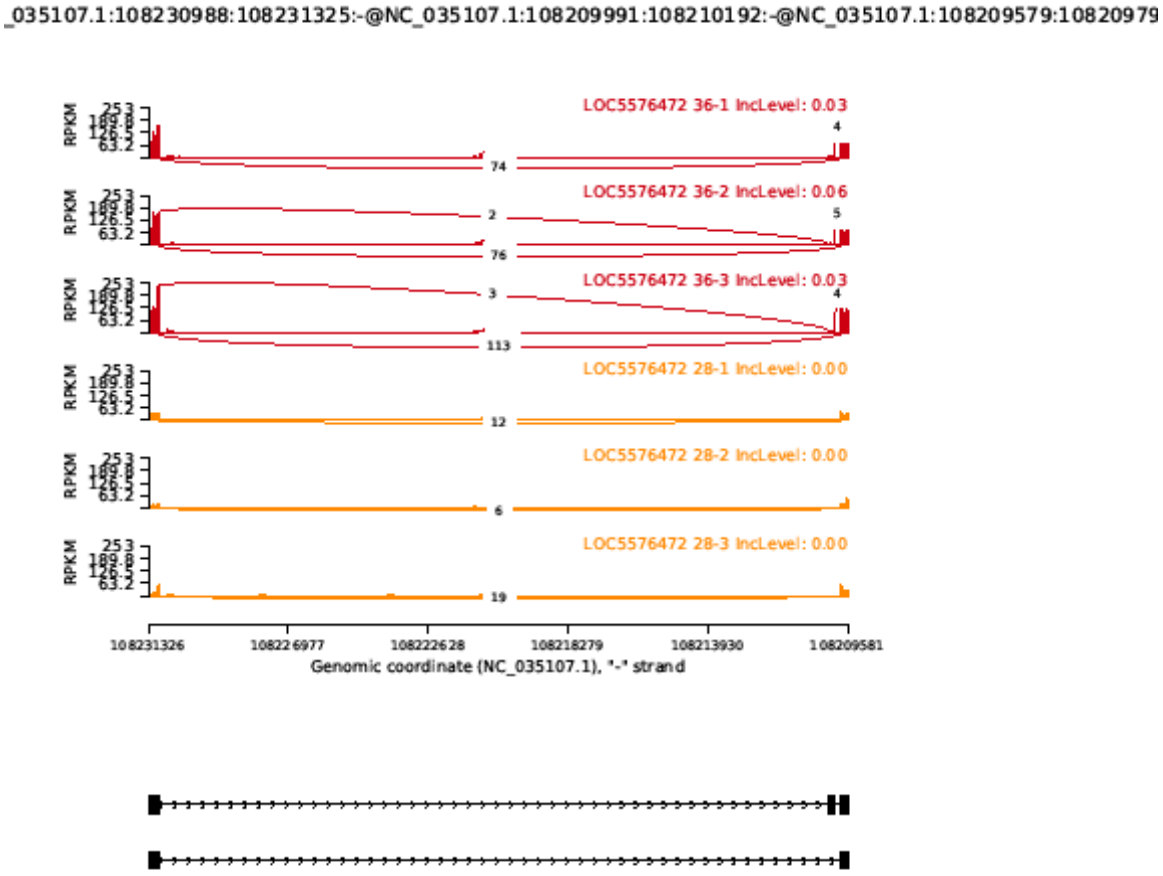

24 hours
20°C x 28°C
MXE
A5SS
A3SS
SE
RI
-Log10 q-value
ΔPSI
36°C x 28°C
48 hours
20°C x 28°C
36°C x 28°C

Figure 2



631

632 ***Figure 3A***

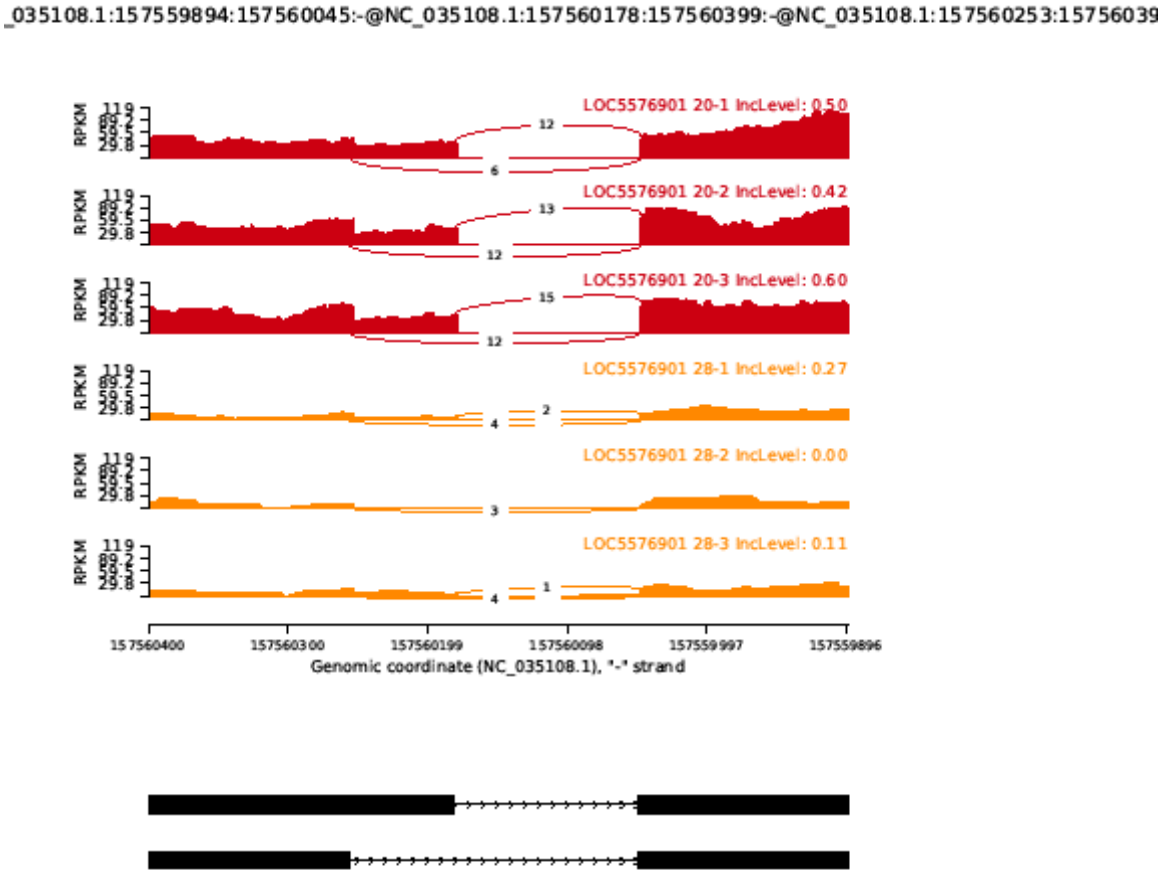

_035108.1:157559894:157560045:-@NC_035108.1:157560178:157560399:-@NC_035108.1:157560253:15756039
RPKM
LOC5576901 20-1 IncLevel: 0.50
LOC5576901 20-2 IncLevel: 0.42
LOC5576901 20-3 IncLevel: 0.60
LOC5576901 28-1 IncLevel: 0.27
LOC5576901 28-2 IncLevel: 0.00
LOC5576901 28-3 IncLevel: 0.11
157560400
157560300
157560199
157560098
157559997
157559896
Genomic coordinate (NC_035108.1), "-" strand

Figure 3B

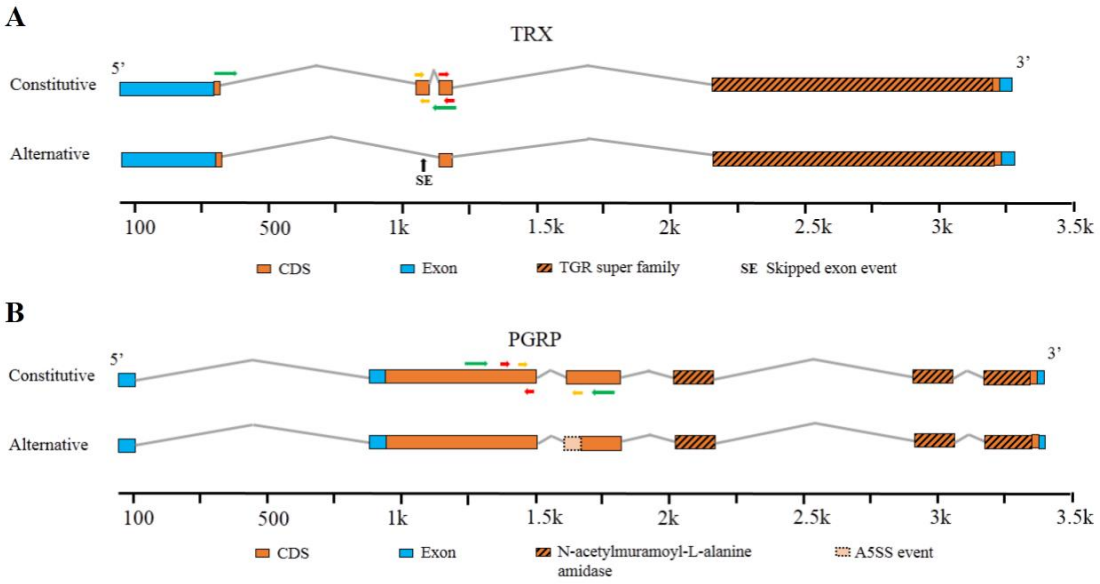

A
TRX
Constitutive
5'
3'
Alternative
SE
100
500
1k
1.5k
2k
2.5k
3k
3.5k
CDS
Exon
TGR super family
SE Skipped exon event
B
PGRP
Constitutive
5'
3'
Alternative
100
500
1k
1.5k
2k
2.5k
3k
3.5k
CDS
Exon
N-acetylmuramoyl-L-alanine amidase
A5SS event

Figure 4

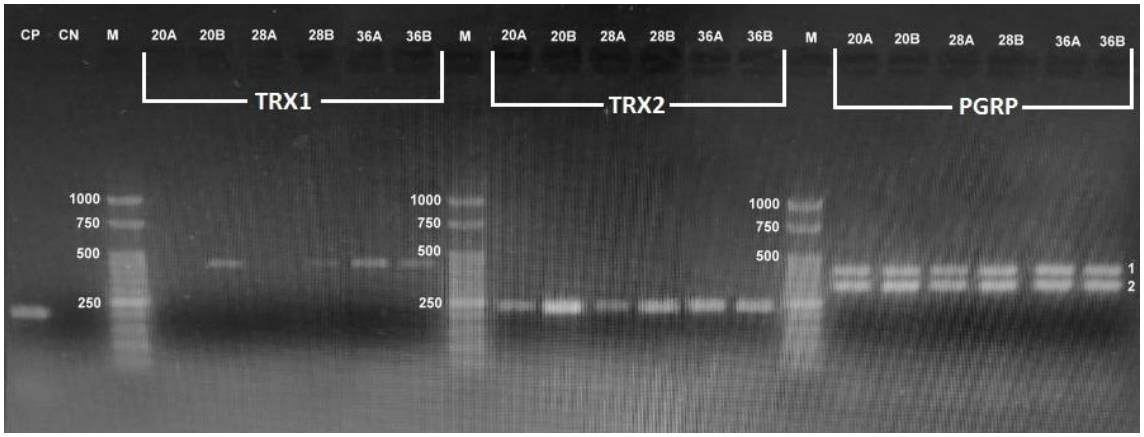

CP
CN
M
20A
20B
28A
28B
36A
36B
TRX1
TRX2
PGRP
1000
750
500
250
1
2

Figure 5

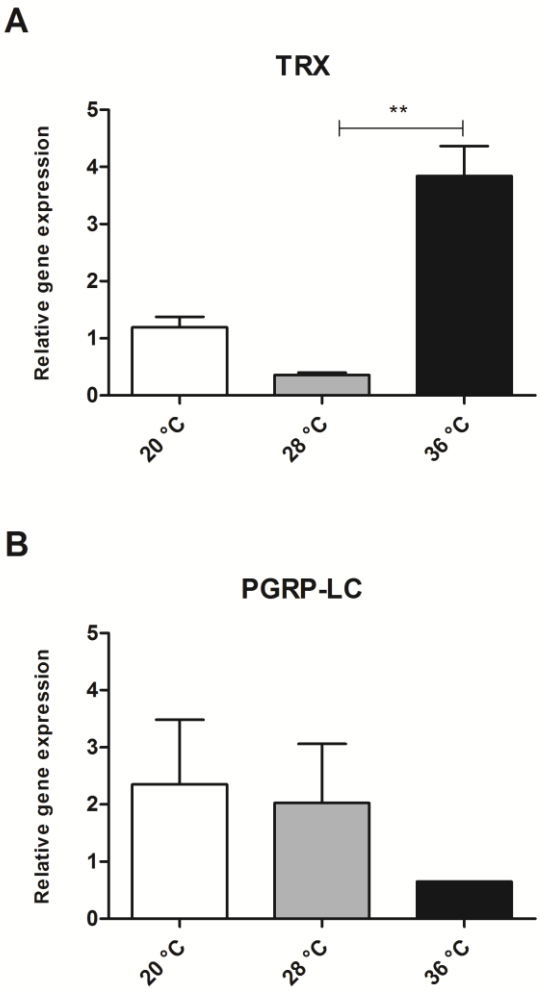

A
TRX
**
Relative gene expression
5
4
3
2
1
0
20 °C
28 °C
36 °C
B
PGRP-LC
Relative gene expression
5
4
3
2
1
0
20 °C
28 °C
36 °C

Figure 6

691 **Supplementary Material**

692

693 **Table 1 -** Gene-specific primers used for Real-Time qPCR.

Transcript	Primers (5'-3')		Product size (pb)	Goal
	Forward	Reverse		
PGRP-LC	TAAACAGAATGGTGCCGTCAA	AACAGGCAACTTCAGTTTGGT	400 or 325	Show size difference
PGRP-LC	ATTCTTCGAAAGCCAGCAAA	GACGAATGCTACGATCACGA	137	Quantify both isoforms
PGRP-LC	ATTCTTCGAAAGCCAGCAAA	GTCACCATCGCCGTAGTTTC	185	Quantify long isoform
TRX	GCCCATCAATCCAAAACAAA	ATGGATAGCTTCTCCCAGCA	434	Show size difference
TRX	GCCCATCAATCAGGAAAACT	ATGGATAGCTTCTCCCAGCA	232	Show size difference
TRX	CCAAGGAAGCCGTACAATTC	ATGGATAGCTTCTCCCAGCA	161	Quantify both isoforms
TRX	GCCCATCAATCCAAAACAAA	TCAGCGGGCATATATTTGAA	183	Quantify long isoform

694

CONCLUSÃO

O estudo do transcriptoma de fêmeas adultas de *Ae. aegypti* infectadas oralmente por *Zika virus* sob diferentes temperaturas revelou que a variação de temperatura impactou drasticamente a expressão de genes em mosquitos expostos e não expostos ao ZIKV. Mosquitos mantidos a 20°C apresentaram mais genes diferencialmente expressos em relação aos mosquitos mantidos a 28°C e 36°C 48 h após a ingestão de sangue. Dos genes com maiores alterações foram aqueles envolvidos na digestão de sangue, formação da matriz peritrófica (PM), metabolismo e gerenciamento do estresse oxidativo associado à quebra da hemoglobina em heme. Vários genes envolvidos na imunidade inata foram moderadamente regulados a 20°C em relação aos seus níveis sob condições de temperatura mais quente em ambos mosquitos, não expostos e expostos ao ZIKV. Adicionalmente, a temperatura afetou significativamente a eficiência do estabelecimento da infecção pelo ZIKV em temperaturas mais baixas limitando, a infecção do intestino médio pelo vírus.

Nossos resultados também sustentaram a hipótese de que a variação de temperatura afeta o processo de digestão do sangue, modulando a geração de espécies reativas de oxigênio (ROS) no intestino médio. Tripsina, Fosfoenolpiruvato carboxiquinase, alfa-N acetilgalactosaminidase e beta-galactosidase, genes envolvidos na digestão de sangue mostraram níveis de expressão elevados em um momento tardio em mosquitos mantidos a 20°C quando comparado com 28 e 36°C. Tardiamente também foi visto o acúmulo de ROS quando mosquitos foram mantidos a 20°C. Níveis de ROS não foram impactados pela variação de temperatura quando mosquitos foram alimentados somente com solução de sacarose, revelando que a temperatura não impacta diretamente a geração dessas espécies reativas.

Eventos de *splicing* alternativo foram diferencialmente modulados conforme a temperatura. Entre os genes impactados, Tripsina, Ferritina, Tioredoxina e Peptidoglicano LC tiveram a expressão influenciada pela variação de temperatura. Tioredoxina, que desempenha um papel significativo na manutenção da homeostase redox intracelular e na proteção contra o dano oxidativo, mostrou *splicing* diferencial no exon 2 em mosquitos mantidos a 36°C comparado aqueles à 28°C.

Neste estudo nós demonstramos efeitos profundos da temperatura na replicação do ZIKV, nas respostas transcricionais e de *splicing* alternativo no intestino médio de fêmeas de *Ae. aegypti*. Nosso estudo, embora focado nas respostas do intestino médio (início do processo de infecção), indicam que a temperatura muda o equilíbrio e dinâmica do ambiente do órgão, o que poderia resultar em consequências diretas e indiretas para a infecção pelo ZIKV. Esses resultados reforçam a afirmação de que a abordagem convencional de estudar os mecanismos sustentando as interações mosquito-patógeno sob um estreito conjunto de condições de laboratório ou através de vias imunes canônicas, ignora a complexidade biológica inata dos organismos.