Increased insulin signaling in the *Anopheles stephensi* fat body regulates metabolism and enhances the host response to both bacterial challenge and *Plasmodium falciparum* infection

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Abstract

In vertebrates and invertebrates, the insulin/insulin-like growth factor 1 (IGF1) signaling (IIS) cascade is highly conserved and plays a vital role in many different physiological processes. Among the many tissues that respond to IIS in mosquitoes, the fat body has a central role in metabolism, lifespan, reproduction, and innate immunity. We previously demonstrated that fat body specific expression of active Akt, a key IIS signaling molecule, in adult *Anopheles stephensi* and *Aedes aegypti* activated the IIS cascade and extended lifespan. Additionally, we found that transgenic females produced more vitellogenin (Vg) protein than non-transgenic mosquitoes, although this did not translate into increased fecundity. These results prompted us to further examine how IIS impacts immunity, metabolism, growth and development of these transgenic mosquitoes. We observed significant changes in glycogen, trehalose, triglycerides, glucose, and protein in young (3–5 d) transgenic mosquitoes relative to non-transgenic sibling controls, while only triglycerides were significantly changed in older (18 d) transgenic mosquitoes. More importantly, we demonstrated that enhanced fat body IIS decreased both the prevalence and intensity of *Plasmodium falciparum* infection in transgenic *An. stephensi*. Additionally, challenging transgenic *An. stephensi* with Gram-positive and Gram-negative bacteria altered the expression of several antimicrobial peptides (*AMPs*) and two anti-*Plasmodium* genes, *nitric oxide synthase* (*NOS*) and *thioester complement-like protein* (*TEP1*), relative to non-transgenic controls. Increased IIS in the fat body of adult female *An. stephensi* had little to no impact on body size, growth or development of progeny from transgenic mosquitoes relative to non-transgenic controls.

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This study both confirms and expands our understanding of the critical roles insulin signaling plays in regulating the diverse functions of the mosquito fat body.

Graphical Abstract
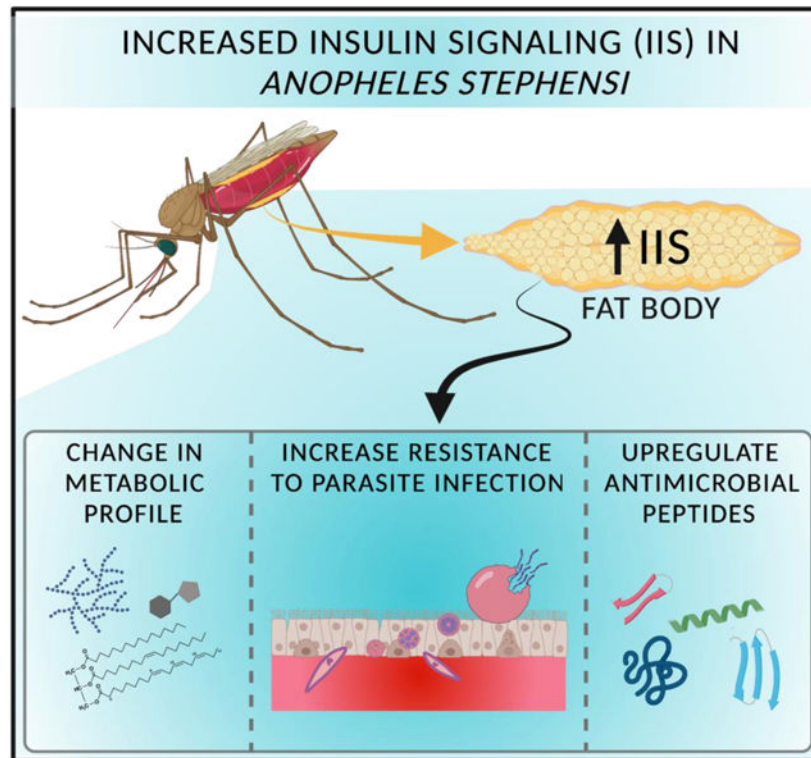
Keywords

insulin signaling; *Anopheles stephensi* ; mosquito; metabolism; innate immunity

1. Introduction

Malaria continues to be a major problem worldwide with an estimated 229 million cases and more than 400,000 deaths annually (World Health Organization, 2020). While the widespread distribution of long-lasting insecticide treated bednets has improved the situation, increasing drug and insecticide resistance will likely limit these gains unless new control strategies can be identified. The insulin/insulin growth factor 1 signaling (IIS) cascade serves as a central regulator of a variety of physiologies critical to vectorial capacity, including innate immunity, reproduction, growth, metabolism and lifespan (Ahlers et al., 2019; Antonova et al., 2012; Hun et al., 2019; Ling et al., 2017; Ling and Raikhel, 2021; Sharma et al., 2019). As such, IIS is an attractive target for reducing parasite infections in the mosquito vector and limiting parasite transmission. IIS functions in a tissue-specific manner to control cellular metabolism based on the requirements of each tissue (Antonova et al., 2012; Li et al., 2020; Nandi et al., 2004; Saltiel and Kahn, 2001). In insects, insulin-like peptides (ILPs) are primarily expressed in the medial neurosecretory cells (MNCs) located

in the pars intercerebralis of the head. However, the expression of ILPs has also been characterized in a variety of other insect tissues, including the midgut, ovary, glial cells, fat body and imaginal discs (Das and Arur, 2017; Lin and Smagghe, 2019; Marquez et al., 2011; Nässel et al., 2013; Riehle et al., 2006). When secreted, ILPs activate the IIS cascade in a range of tissues, including the midgut, ovaries and fat body, to elicit specific physiological effects. The insect fat body is particularly interesting as IIS in this tissue has been shown to play a fundamental role in energy metabolism, reproduction, and lifespan (Arrese and Soulages, 2010; Bai et al., 2012; Li et al., 2019). The insect fat body is also the primary site of transcriptionally induced humoral immune responses against a variety of pathogens (Ali Mohammadie Kojour et al., 2020; Lemaitre and Hoffmann, 2007; Wang et al., 2018), although the role of IIS on fat body immunity remains unclear.

Prior studies in *Drosophila melanogaster* revealed IIS regulation of immune pathways (DiAngelo et al., 2009; Dionne, 2014; Dionne et al., 2006; Hotamisligil, 2017; Rynes et al., 2012). For example, activation of FOXO in a non-tissue specific manner resulted in strong induction of antimicrobial peptides in both larvae and adults (Becker et al., 2010; Guo et al., 2014). Mutations in chico, the *Drosophila* insulin receptor substrate orthologue, were associated with increased infection resistance and a reduced bacterial load when flies were challenged (Libert et al., 2008; McCormack et al., 2016). *Drosophila* FOXO regulates antimicrobial peptide (*AMP*) expression downstream of target of rapamycin (TOR) and is essential for controlling *AMP* gene expression during starvation (Becker et al., 2010; Varma et al., 2014). Activation of the Toll signaling pathway in larval *Drosophila* fat body has been shown to disrupt IIS by preventing the phosphorylation of Akt and limiting fat body expression of *dilp6* (Roth et al., 2018; Suzawa et al., 2019). In mosquitoes, the IIS cascade similarly contributes to innate defenses against pathogens. Pakpour et al. (2012) and Surachetpong et al. (2011) determined that components of the blood meal including transforming growth factor-β1 and insulin activated the IIS and MAPK signaling pathways in *Anopheles stephensi*, a key vector of *Plasmodium* that has become an emerging threat in Africa (Sinka et al., 2020). This led to increased mosquito susceptibility to infection with the human malaria parasite *Plasmodium falciparum*. Conversely, mosquitoes provisioned with human insulin-like growth factor (IGF)-1 in the bloodmeal exhibited reduced susceptibility to *P. falciparum* (Drexler et al., 2013). Moreover, overexpression of an active form of Akt, a key activator of the IIS pathway, in the midgut of *An. stephensi* completely blocked *P. falciparum* development (Corby-Harris et al., 2010). Further work revealed that this parasite-blocking phenotype resulted from increased synthesis of mitochondrially-derived reactive oxygen and nitrogen species and an inhospitable environment for developing parasites (Luckhart et al., 2013). Surprisingly, midgut expression of the IIS inhibitor PTEN was also associated with reduced susceptibility of *An. stephensi* to *P. falciparum*, although in this case improved midgut epithelial integrity and possibly the depletion of pantothenate, the precursor of coenzyme A required by the parasite, led to parasite death (Hauck et al., 2013; Souvannaseng et al., 2018). In addition to the effects of IIS manipulation on parasite infection, manipulation of *An. stephensi* ILP levels altered mosquito susceptibility to *P. falciparum* infection. Specifically, knockdown of *An. stephensi ilp3* or *ilp4* decreased the prevalence and intensity of *P. falciparum* infection and increased anti-parasite gene expression (Pietri et al., 2015). When *An. stephensi* were provisioned

with ILP4, both prevalence and intensity of parasite infection were significantly increased, whereas provisioning of ILP3 significantly decreased infection prevalence (Pietri et al., 2016).

In addition to its role in insect host defense, IIS is an important regulator of nutrient metabolism in the fat body where a majority of insect energy regulation and nutrient storage occurs. ILPs and IIS induce fat body cells to convert glucose to glycogen, and to switch from catabolic to anabolic lipid and protein metabolism. In *D. melanogaster*, knockout of DILPs, individually or in combination, suggested that DILPs 2, 3, 5, 6 and 7 work together to regulate carbohydrate and lipid metabolism (Grönke et al., 2010; Semaniuk et al., 2018; Zhang et al., 2018). Slaidina et al. (2009) showed that under larval starvation conditions, expression of *dilps* 2, 3 and 5 were suppressed, whereas the fat body specific *dilp6* was induced. In adult *Drosophila*, increased activity of the insulin receptor in the fat body led to significantly increased triglyceride levels and fat cell numbers, whereas increased activity of dFOXO led to reduced adipocyte numbers (DiAngelo et al., 2009). In the kissing bug *Rhodnius prolixus*, knockdown of *Rhopr-ILP* led to increased circulating lipids and carbohydrates, but decreased fat body carbohydrates (Defferrari et al., 2016). In the mosquito *Aedes aegypti*, ILP3 injection following decapitation was associated with significantly increased glycogen and lipid stores and reduced circulating trehalose levels (Brown et al., 2008). Disruption of ILP7 in *Ae. aegypti* via CRISPR/Cas9 led to increased triglyceride levels and decreased glycogen levels, while disruption of ILP8 had the opposite effects (Ling et al., 2017). The complexity of these phenotypes highlight the fact that additional studies on the role of mosquito IIS regulation of nutrient metabolism are needed.

In our previous studies, we genetically engineered an active form of the IIS kinase Akt into *An. stephensi* and *Ae. aegypti* mosquitoes under the regulation of the fat body and bloodmeal specific vitellogenin (Vg) promoter (Arik et al., 2015). We found that these TG mosquitoes survived significantly longer than NTG sibling controls, likely due to decreased expression of *ilp2* in the brain and increased expression of the putative insulin binding protein *imp-l2*. We also found that TG mosquitoes produced significantly more Vg transcript and protein than NTG controls, although we surprisingly did not observe any changes to female fecundity (Arik et al., 2015; Hun et al., 2019). Although egg production was not increased, female mosquitoes may have provisioned their progeny with additional reserves, in turn providing them with increased resources during embryogenesis or they may have simply reallocated this excess protein to other uses. In this study, we attempted to address the role of fat body IIS on innate immunity, metabolism and progeny fitness using Vg-Akt transgenic *An. stephensi*.

2. Methods:

2.1. Mosquito culture

An. stephensi (Indian strain) mosquitoes were reared as described previously (Corby-Harris et al. 2010). Hemizygous transgenic (TG) mosquito lines were outcrossed with wild type colony mosquitoes each generation to enhance genetic diversity, resulting in a 50:50 mix of hemizygous TG and non-transgenic (NTG) sibling mosquitoes reared under identical conditions. TG and NTG mosquitoes were separated at the pupal stage using an

Olympus SZX10 fluorescent stereomicroscope (Olympus, Tokyo, Japan) and appropriate filters for detecting enhanced green fluorescent protein (EGFP). For line maintenance and experiments, female mosquitoes were provided human blood (American Red Cross; IBC protocol 2010–014) via artificial membrane feeders.

2.2. Metabolic assays

To determine nutrient reserves for TG and NTG mosquitoes, both young (3–5 d) and old (18 d) mosquitoes were collected and allowed to mate for 48 h. Mosquitoes were then provided a blood meal via an artificial membrane feeder for 45 minutes. Whole bodies of two blood fed female TG and NTG *An. stephensi* mosquitoes were collected at various time points (non-blood fed or NBF, 24, 36, 48 and 72 h) post blood meal (PBM) and homogenized in phosphate buffered saline (PBS; 50 mM NaCl, 50 mM sodium phosphate buffer, pH 7.2). For the 18 d mosquito metabolic assays, all females were provided with a blood meal every 2 d until 18 d post-emergence. Samples were then collected as described for young mosquitoes.

Glucose, glycogen, trehalose, triglycerides and proteins were extracted from two mosquitoes following the modified micro-separation protocol (Telang and Wells, 2004; Zhou et al., 2004). Once fractionated, the glucose, glycogen, trehalose, triglyceride, and protein extracts were frozen at −80°C until the nutrients were quantified as described below. Three biological replicates of each experiment using unique cohorts of mosquitoes were conducted. To quantify triglycerides, a vanillin reagent assay (Van Handel, 1985a; Van Handel, 1985b) was used with the following modifications. A stock solution of triolein (1.0 mg/ml, Sigma-Aldrich, St. Louis, MO) was used to generate a standard curve from 0–200 μg. Frozen lipid fractions were dried by chloroform removal at 65°C. Next, 100 μl of 95% sulfuric acid was added to the dried sample and the resuspended sample vortexed and heated at 100°C for 10 min. Samples were cooled for 10 min at room temperature, 2 ml of vanillin reagent was added, and samples vortexed. The samples were developed in darkness for 15 min, and 100 μl of sample was added to a 96 well plate in triplicate. Absorbance at 525 nm was recorded using a spectrophotometer (Multiskan Go by Thermo Scientific, Waltham, MA) against a blank reagent control.

Glycogen was quantified using a modified anthrone-based assay (Van Handel, 1985a; Van Handel, 1985b). A 1.0 mg/ml glycogen stock (Thermo Scientific, Waltham, MA) was diluted to generate a 0–200 μg standard curve. At the final step of the micro-separation the glycogen sample was precipitated to form a pellet and dried. The glycogen pellet was solubilized with 200 μl of 25% ethanol and vortexed. Subsequently, 2 ml of anthrone reagent was added, the sample vortexed again, and then incubated for 20 min at 90°C in the dark to protect the reaction from light. Samples were cooled at 4°C for 15 min followed by vortexing. Finally, 100 μl of each sample or standard was added to a 96 well plate in triplicate. Absorbance at 625 nm was recorded spectrophotometrically (Multiskan Go by Thermo Scientific, Waltham, MA).

For glucose determination, solvent from the glucose fraction was evaporated in a heating block and the resulting residue was heated with anthrone reagent (Van Handel, 1985a; Van Handel, 1985b). Subsequently, 2 ml of anthrone reagent was added, the samples vortexed

and heated at 90°C for 20 min This step releases glucose units, which react with anthrone to generate a green substrate in the dark. Samples were cooled for 15 min at 4°C, vortexed, and 100 μl of each solution was added to a 96 well plate in triplicate. Absorbance at 625 nm was recorded spectrophotometrically (Multiskan Go by Thermo Scientific, Waltham, MA) against a blank reagent control and glucose concentrations were determined against the standard curve.

To determine trehalose levels, 25% ethanol (0.2 ml) and 1M HCl (25 μl) were added to the solvent tube, the samples vortexed and then heated at 90°C for 7 min. Next, 75 μl of 1M NaOH was added to each tube, vortexed, and then heated at 90°C for 7 min. This ensured that free glucose and fructose would not lead to an overestimation of trehalose concentration. Finally, 2 ml of anthrone reagent was added, vortexed, and the sample heated at 90°C for 20 min. Samples and standards were cooled at 4°C for 15 min, vortexed and 100 μl of each solution was added to a 96 well plate in triplicate. Absorbance at 625 nm was recorded spectrophotometrically (Multiskan Go by Thermo Scientific, Waltham, MA).

To quantify protein, we used the BCA protein assay reagent kit (Thermo Scientific, Waltham, MA). A 1.0 mg/ml solution of bovine serum albumin was diluted to a 0–200 μg final concentration to generate the standard curve. Both samples and standards were treated according to the manufacturer's protocol. Tubes were heated at 60°C for 30 min and then cooled to room temperature. Absorbance at 562 nm was recorded spectrophotometrically (Multiskan Go by Thermo Scientific, Waltham, MA) against a blank reagent control. All nutrient assays were reported as μg per individual mosquito.

2.3. Growth rate measurement

To determine the impact of fat body IIS and increased Vg titers (Arik et al., 2015) on the growth and development of *An. stephensi* progeny we performed three crosses: (1) TG female crossed with wild type male (F-TG), (2) TG male crossed with wild type female (M-TG), and (3) wild type female crossed with wild type male (WT). The F-TG cross was performed to examine how increased Vg protein titers in TG females affected progeny fitness. The M-TG and WT crosses were controls where no additional Vg was provided to the developing embryos by the female. Previously, we reported that engineered, myristoylated (activated) *An. stephensi* Akt (myrAkt) was expressed in the fat body at 24 h PBM, reaching its maximal expression at 48 h and decreasing by 72 h (Arik et al., 2015). We also showed that TG *An. stephensi* had significantly higher Vg protein levels at 24 h PBM relative to the NTG controls (Arik et al., 2015; Hun et al., 2019). Although we did not observe significant differences in egg production during the first and second reproductive cycles (Arik et al. 2015), the resulting offspring may have been provisioned with additional Vg, leading to improved larval fitness. To determine whether increased Vg abundance influenced progeny fitness during the first and second reproductive cycles, fifty 3–5 d old adult female mosquitoes were allowed to mate for 4 h and then separated into two groups. One group was provided with a single blood meal, while the other group received two blood meals. The second blood meal was provided at 48 h following the first blood meal to ensure that myrAkt was expressed in the fat body at the beginning of the second reproductive cycle. Mosquitoes were offered blood via an artificial membrane

feeder for 45 min and fully engorged females were separated into individual cages (10 cm × 8.5 cm × 14.8 cm) with an oviposition cup. Non-fed or partially fed females were discarded. All cages were checked for eggs 72 h PBM. Eggs from individual females were hatched and mosquitoes reared to the pupal stage. Pupae were collected daily until no surviving progeny remained. TG and NTG pupae were separated using an Olympus SZX10 fluorescent stereomicroscope (Olympus, Waltham, MA) using EGFP filters and the number of TG and NTG pupae recorded. Emerging adults were collected daily, and the number of male and female mosquitoes recorded at the time of collection.

2.4. Weight and body size measurement

A total of 20 TG and 20 NTG F1 progeny were frozen overnight at −80°C in 50 ml conical tubes (VWR) and wet weight was recorded (Satorius: ED1245, Vernon Hills, IL). The same mosquitoes were subsequently dried overnight in an incubator at 50°C and weighed again to determine dry weight. As a proxy for body size we measured the wing length of both male and female progeny. The wings were mounted on slides and its length (from the axillary incision to the wing tip) and maximum width were measured using a light microscope (Olympus, Waltham, MA) and an ocular micrometer calibrated with a micrometer scale at a fixed optical zoom.

2.5. Bacterial challenge assay

To test for inducible expression of key antimicrobial genes, five TG and five NTG female mosquitoes were collected at various time points after a blood meal (NBF, 24 h, 36 h, 48 h PBM). To induce an immune response, we challenged mosquitoes from each time point against Gram-negative *Escherichia coli* or Gram-positive *Bacillis subtilis* bacteria by pricking each mosquito in the thorax with a needle dipped in bacterial culture (OD600 = 0.5). Sham inoculation (buffer only) and no inoculation controls were also included. To minimize temporal or human bias, mosquitoes were alternately inoculated with different treatments. Following inoculation, mosquitoes were given 4 h to develop a response to the bacterial challenge prior to total RNA isolation. The assay was replicated with three unique cohorts of mosquitoes.

2.6. RNA extraction and qPCR assays

We performed qPCR to assess antimicrobial peptide transcript expression in TG and NTG mosquitoes. Total RNA was isolated from pools of five whole bodies or abdominal body walls from female *An. stephensi* mosquitoes using the RNeasy kit and QIAcube (Qiagen, Germantown, MD) per the manufacturer's protocol. Following insolation, total RNA samples were treated with RNAse-free DNAse 1 (Thermo Scientific Waltham, MA) to eliminate genomic DNA contamination. Total RNA concentrations were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and diluted to a final concentration of 250 ng/μl for cDNA synthesis using the cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) per the manufacturer's protocol. The integrity of the cDNA samples was validated by performing a reverse-transcriptase PCR amplification of actin using Gotaq Green Master Mix (Promega, Madison, WI) and *An. stephensi* actin-specific primers.

The cDNA samples were used as templates for quantitative real time PCR (qPCR) on a Bio-Rad CFX Real-Time System Thermocycler (Bio-Rad, Hercules, CA) to assess the transcript abundance of various immune genes using specific primer pairs as shown in Table S1. A 20 µl qPCR reaction was assembled using 2X Sybr Green reaction mixture prepared according to the manufacturer's protocol. Samples were loaded in triplicate onto 96 well plates and the following cycling parameters were used: 95°C for 10min, [95°C for 15 sec, 55°C for 30 sec, 72°C for 40 sec] (36 cycles), 95°C for 60 sec. qPCR data for target genes were normalized to the S7 ribosomal control (Ct). Fold change expression levels between TG and NTG mosquitoes were calculated using the 2^(– Ct) method. Three biological replicates from unique cohorts of mosquitoes were used for these assays. Data were analyzed by post-hoc two-way ANOVA to determine differences between TG and NTG control groups. P-values were deemed significant at P   0.05.

2.7. Bacterial challenge mortality assay

TG and NTG female mosquitoes (n=150) were allowed to mate with wild type males for 48 h before receiving a blood meal. Engorged females were separated into new containers for each group (TG and NTG). Prior to the bloodmeal (NBF) and 12 and 24 h PBM, TG and NTG female mosquitoes (n=25 each) were challenged against *E. coli* or *B. subtilis* cultures at 0.5 OD600. Challenged mosquitoes included *E. coli*- or *B. subtilis*-inoculated, PBS-inoculated and non-injected controls. To eliminate any potential time bias, pricking was performed by two people alternating between TG and NTG mosquitoes and bacteria or buffer control. Once pricked, mosquitoes were allowed to recover for 4 h, provided sugar water *ad libitum*, and maintained in a temperature and humidity-controlled chamber to minimize desiccation. Dead mosquitoes were counted and removed daily to determine TG and NTG mosquito mortality. Survival curves were analyzed using the Log- Rank (Mantel-Cox) test. These experiments were replicated with three biologically distinct cohorts of mosquitoes.

2.8. *Plasmodium falciparum* infection studies

Cultures of *P. falciparum* NF54 were initiated at 1% parasitemia in 10% heat-inactivated human serum, and 6% washed human red blood cells (RBCs) in RPMI 1640 with HEPES (Gibco, Thermo Scientific Waltham, MA) and hypoxanthine. Stage V gametocytes were evident by day 15 and exflagellation was evaluated on the day prior to and the day of mosquito feeding. For our assays, 5-day old female TG and NTG *An. stephensi* were fed on a mature gametocyte culture diluted with human erythrocytes and heat-inactivated serum. On day 10, midguts from infected females were dissected in PBS and stained with 1% mercurochrome/PBS to visualize *P. falciparum* oocysts. Oocysts were counted for each midgut. The mean number of oocysts per midgut (infection intensity) and percentages of infected mosquitoes (infection prevalence or presence of at least one oocyst) were calculated from all dissected mosquitoes. Experiments were independently replicated with three cohorts of 60–120 mosquitoes. Data were pooled for analysis as one-way ANOVA determined that infections in control groups were not significantly different among replicates. Infection prevalence and intensity data were analyzed by Fisher's exact test, Mann–Whitney test, or nonparametric ANOVA (Kruskal–Wallis) followed by Dunn's

multiple comparisons test to determine differences between TG and NTG control. P-values were deemed significant when P   0.05.

3. Results

3.1. Increased IIS in the fat body increased early glucose levels and later nutrient storage following the blood meal in young *An. stephensi*

We previously demonstrated that myrAkt protein expression in the *An. stephensi* fat body was initially observed at 24 h PBM, with FOXO phosphorylation evident at 36 h PBM, and myrAkt expression reaching maximal levels at 48 h PBM (Arik et al., 2014). In this work we assessed the impact of increased fat body IIS on *An. stephensi* nutrient metabolism by quantifying glucose, glycogen, triglycerides, trehalose and protein levels in young (3–5 days) and older (18 days) mosquitoes at various time points during a reproductive cycle. Young TG mosquitoes exhibited significantly increased glucose levels early in the reproductive cycle (24 h PBM, $P<0.01$; 36 h PBM, $P<0.0008$) relative to NTG controls (Fig. 1A). Metabolic storage molecules were significantly increased later in the reproductive cycle. Specifically, glycogen levels were significantly elevated in TG mosquitoes at 48 h ($P<0.01$) and 72 h ($P<0.005$) PBM relative to NTG controls (Fig. 1B), while triglyceride levels were significantly higher in TG mosquitoes at 48 h ($P<0.001$) and 96 h ($P<0.0003$) PBM (Fig. 1D). Interestingly, levels of trehalose ($P<0.01$) and protein ($P<0.003$) in TG mosquitoes were both significantly higher at 72 h PBM (Fig. 1C and E) relative to NTG controls but did not differ at other timepoints. In contrast to younger mosquitoes where dramatic energy changes were observed in TG mosquitoes, in older mosquitoes (18 days), only triglyceride levels were significantly higher in TG mosquitoes at 24 h ($P<0.03$) and 36 h ($P<0.002$) PBM (Fig. S1D). No significant differences in glucose, glycogen, trehalose, or protein were observed between TG and NTG controls at any time points (Fig. S1A–C, E). It should be noted that older mosquitoes, both TG and NTG, trended towards reduced levels of glucose, trehalose, and glycogen, suggesting modest but not significant reductions in energy reserves as mosquitoes aged.

3.2. Increased fat body IIS did not affect the growth and development of *An. stephensi* progeny

Previously, we demonstrated that increased IIS in the fat body led to increased synthesis of Vg proteins in TG *An. stephensi* and *Ae. aegypti*, but this did not result in an increase in egg production during the first two reproductive cycles (Arik et al., 2015). To assess whether increased Vg production leads to increased provisioning of developing eggs and in turn increased progeny fitness, we performed three different crosses (F-TG, M-TG, and WT per above Methods). We examined the number of eggs, pupae and the number of adults (males and females) from all three crosses that received either a single blood meal or a second meal 48 h after the first ensuring transgene expression throughout the second reproductive cycle (Arik et al., 2015). Female *An. stephensi* provided a single blood meal showed no differences in either the total number of eggs or average number of eggs per female (Fig. 2A–B). While F-TG ($P<0.03$) and M-TG ($P<0.03$) crosses generated significantly higher numbers of pupae per female relative to WT controls (Fig. 2C), this did not translate into significantly increased numbers of emerged adults per female (Fig. 2D). Furthermore, there

were no differences in the percentage of eggs from each individual female that survived to adulthood (Fig. 2E), in the total number of males or females produced among these three crosses, or in the observed sex ratios (Fig. 2F and G).

In our previous study, we demonstrated that myrAkt transgene transcript expression was first observed at 12 h after a blood meal, with initial protein expression occurring at 24 h and maximal expression observed by 48 h (Arik et al., 2015). Thus, during the first blood feeding of transgenic mosquitoes, IIS is not increased during the first third of the reproductive cycle, which includes the initiation of vitellogenesis. To ensure that myrAkt protein was present at the beginning of the second reproductive cycle, we provided a second blood meal 48 h after the initial bloodmeal to the F-TG cross. This allowed for the initiation of the next reproductive cycle while significant levels of myrAkt protein were present. We again observed a significant increase in that the number of pupae produced by the F-TG cross (P<0.03) relative to the WT control (Fig. S2C). However, we did not observe any differences in the total number of eggs (Fig. S2A), the number of eggs per female (Fig. S2B), the number of adults per mosquito (Fig. S2D), or the total number of females and males (Fig. S2A and B) among the three crosses. Furthermore, there were no differences in males and females produced nor were there any differences in sex ratios (Fig. S2E and F) among the three crosses. In summary, the expression of myrAkt in the *An. stephensi* fat body did not affect the number of progeny that successfully developed to adulthood or their sex ratios.

We also considered the possibility that increased nutrient availability during embryo provisioning could lead to increased growth and adult body size. To assess the impact on progeny body size, we measured wing length as a proxy for body size, as well as wet and dry weights from all three crosses. We observed no significant differences in wing length, wet weight or dry weight among TG, NTG and WT mosquitoes for any of the three crosses (Fig. S3).

3.3. Bacterial challenge upregulated immune gene expression in transgenic *An. stephensi* relative to non-transgenic controls

The mosquito fat body plays a key role in innate immunity as the primary source of antimicrobial peptides (Bian et al., 2005; Kokoza et al., 2010). To assess whether fat body IIS functions in the regulation of innate immunity in *An. stephensi*, we measured transcript expression of various immune genes including *Defensin (Def), Gambicin (Gam), Cercropin (Cec), Thioester-containing protein 1 (TEP1), Anopheles Plasmodium-responsive leucine-rich repeat 1 (APL1)*, and the transmembrane *peptidoglycan (PGN) Recognition Protein LC (PGRP-Lc)* following three different treatments: (1) unmanipulated (no inoculation), (2) injured (saline control), and (3) Gram-positive or Gram-negative bacterial challenge at various time points prior to and following a blood meal (NBF, 24, 36, 48 h PBM) in TG and NTG mosquitoes. When challenged with *E. coli*, TG mosquitoes exhibited increased transcript expression of *Def* at 24 h PBM (Fig. 3M; P< 0.001), *Gam* in NBF (Fig. 3N; P<0.0007) and at 24 h PBM (Fig. 3N; P<0.02), *Cec* in NBF, 24, 36 and 48 h PBM (Fig. 3O; P<0.001, P< 0.02, P<0.02 and P<0.01) and *TEP1* at 36 h PBM (Fig. 3P; P<0.02) relative to NTG controls. We did not observe any differences in *PGRP-Lc* (Fig. 3Q)

and *APL1* (Fig. 3R) expression levels in *E. coli*-challenged TG relative to NTG controls. Importantly, there were no significant differences in *Def*, *Gam, Cec, TEP1, APL1*, and *PGRP-Lc* transcript levels between TG and NTG mosquitoes in unmanipulated (Fig. 3A–F) and injured (Fig. 3G–L) *An. stephensi* demonstrating that increased antimicrobial peptide expression was specific to the presence of bacteria. When TG and NTG mosquitoes were challenged with *B. subtilis*, we observed significantly increased expression of *Def* at 24 h PBM (Fig. 4M; P<0.01), *Gam* at 36 h PBM (Fig. 4N; P<0.01), *TEP1* at 48 h PBM (Fig. 4P; P<0.02), and *PGRP-Lc* at 48 h PBM (Fig. 4Q; P<0.04) in TG mosquitoes relative to NTG controls. No significant differences were observed for *Cec* (Fig. 4O) and *APL1* (Fig. 4R) expression. As with *E. coli*, there were no significant differences in expression between unmanipulated (Fig. 4A–F) or injured (Fig. 4G–L) TG and NTG at any time point PBM. Finally, we also report the Ct values relative to S7 ribosomal control to demonstrate the relative abundance of antimicrobial peptide transcript in the mosquito (Fig. S4 – *E. coli*; Fig. S5 - *B. subtilis*). Notably, *E. coli* challenge resulted in increased expression of *Gam* in both TG and NTG mosquitoes relative to the sham injected controls, while *Cec* levels were only increased relative to the sham injected controls in TG mosquitoes (Fig. S4). In contrast, *B. subtilis* challenge led to significant increases in *Def* and *Cec* levels in both TG and NTG mosquitoes (Fig. S5). Importantly, Ct values greater than 10, as seen for many *TEP1, APL1* and *PGPLC* samples in the non-injected and sham injected treatments, suggest minimal transcript expression.

3.4. Increased fat body IIS partially rescued *An. stephensi* survival following bacteria challenge

The marked increase in antimicrobial peptide transcript expression in myrAKT transgenic *An. stephensi* led us to test whether survivorship following bacterial challenge might be improved in TG relative to NTG mosquitoes. Survivorship was examined under three treatments: unmanipulated, injured, or *E. coli-* or *B. subtilis*-challenged, with these treatments delivered prior to blood feeding (NBF) or at 12 h or 24 h PBM. These time points were selected because, in our previous work, we found that myrAKT transgene expression in *An. stephensi* was initiated at 12 h PBM and increased in expression up to 48 h PBM. As would be expected, unmanipulated mosquitoes survived longer than injured mosquitoes, which in turn survived longer than mosquitoes challenged with *E. coli* or *B. subtilis* (Figs. 5A, 5B and 5C; S6A, B and C). Survivorship of TG and NTG mosquitoes challenged with *E. coli* or *B. subtilis* prior to blood feeding was not different in any of the three biological replicates (Figs. 5B and S6B). This was expected since the transgene is not expressed in mosquitoes in the absence of a bloodmeal. However, at 12 h and 24 h PBM, when myrAKT expression was increasing, survival of TG mosquitoes challenged with *E. coli* was significantly increased (P<0.03 to P<0.0006) relative to NTG controls (Fig.5B and 5C and S6B and C). As with *E. coli* challenge, TG and NTG NBF mosquitoes challenged with *B. subtilis* showed no differences in survival (Figs. 5B and S6B). However, survival of TG mosquitoes was significantly increased following challenge with *B. subtilis* at 24 h PBM (P<0.021 to P<0.011) in 2 out of 3 independent experiments (Fig. 5C and Fig. S6C), and marginally increased (P<0.1) at both 12 h and 24 h for all other biological replicates of the *B. subtilis* treatments (Fig.5B and C; S6B and C). Collectively, the increased survival of TG mosquitoes challenged with both Gram-negative and Gram-positive bacteria, coupled with

the increased expression of antimicrobial peptides, provides strong evidence that fat body IIS plays an important role in regulating host defenses that are critical to survival following immune challenge.

3.5.   Increased fat body IIS reduced the prevalence and intensity of *P. falciparum* infection

Increased fat body myrAKT activity and IIS in TG mosquitoes relative to NTG mosquitoes resulted in significant reductions in both the percentage of mosquitoes infected with *P. falciparum* (infection prevalence) as well as the number of oocysts per infected mosquito midgut (infection intensity, zeroes not included; Fig. 6). Specifically, the percentage of mosquitoes with one or more oocysts was significantly decreased, from an average of 55% (n=127) in NTG mosquitoes to 22% (n=115) in TG mosquitoes (P<0.0001; Fig. 6A). Furthermore, infection intensity was reduced by 33.5%, from an average of 1.71 oocysts/midgut (n=70) in NTG controls to 1.22 oocysts/midgut (n=23) in TG mosquitoes (P<0.0001; Fig. 6B).

3.6.   Increased fat body IIS was associated with increased *NOS* mRNA expression

Studies have shown that *NOS* expression in *An. stephensi* is transcriptionally activated following infection by *Plasmodium*, thereby limiting infection with both mouse malaria parasites and the human parasite *P. falciparum* (Drexler et al., 2014; Lim et al., 2005; Luckhart et al., 2013; Luckhart et al., 1998; Peterson et al., 2007; Surachetpong et al., 2011). Based on the reduction of the *P. falciparum* above in TG relative to NTG *An. stephensi*, we measured *NOS* transcript expression in whole bodies and abdominal walls of TG and NTG NBF mosquitoes and at 24, 36, and 48 h PBM (Fig. 7). In these experiments, TG mosquitoes exhibited significantly increased *NOS* expression in the abdomen at 24 h (P=0.03) and 36 h (P=0.03) PBM relative to NTG controls (Fig. 7D). Similarly, in whole body, *NOS* expression was significantly higher in TG mosquitoes at 24 h (P=0.003) and 36 h (P=0.0008) PBM as compared to NTG controls (Fig. 7B). These results are consistent with a variety of studies demonstrating that manipulation of mosquito IIS leads to increased *AsNOS* gene expression in *An. stephensi* (Drexler et al., 2014; Luckhart et al., 2013; Pietri et al., 2015). Collectively, these data suggest that NO, in addition to increased antimicrobial peptides, contribute to significantly reduced *P. falciparum* infection observed in TG relative to NTG mosquitoes.

4.   Discussion

The IIS cascade is present in all multicellular organisms and regulates diverse physiological functions including growth, development, fecundity, metabolic homeostasis, immunity and lifespan in a tissue dependent manner (Antonova-Koch et al., 2013; Sharma et al., 2019). In our previous work, we demonstrated that myrAkt-dependent IIS activation in the mosquito fat body led to a significant extension of lifespan relative to NTG controls (Arik et al., 2015). We reported that this lifespan extension was likely due to reduced expression of *ILP2* in the head and increased expression of *imp-L2* (Hun et al., 2019). Furthermore, we observed that adult female mosquitoes produced significantly more Vg protein, although this did not translate to an increase in fecundity (Arik et al., 2015). In this study, we explored the impact of fat body specific IIS on mosquito metabolism, innate immune response,

and whether increased Vg expression had any impact on the growth and development of progeny.

As expected, IIS in the mosquito fat body was a key regulator of metabolism in the mosquito. During the first half of the reproductive cycle in young TG mosquitoes, glucose levels were increased relative to NTG controls, but metabolic stores of glycogen and triglycerides were minimally changed. During the second half of the reproductive cycle, in contrast, we observed increased levels of both storage molecules. In *D. melanogaster*, ablation of MNCs in early larval stages resulted in increased hemolymph sugar levels (Rulifson et al., 2002). However, this phenotype could be abolished by ubiquitously overexpressing *dilp2* (Rulifson et al., 2002). Another study in *D. melanogaster* showed that overexpression of *dilp6* in the adult fat body suppressed *dilp2* expression in the head (Bai et al. 2012). This ultimately extended lifespan and increased levels of triglycerides, glycogen and trehalose, similar to our observations in the latter half of the reproductive cycle. Increased fat storage and lipid turnover have been shown to positively impact lifespan in *D. melanogaster* (Katewa et al., 2012; Longo and Mattson, 2014). In our previous study, we observed that mosquitoes with increased fat body IIS exhibited significantly reduced *ILP2* expression in the head (Hun et al., 2019), which could explain the metabolic changes we observed in TG mosquitoes in a manner consistent with observations in *D. melanogaster*. While the high level of triglycerides observed in older mosquitoes could result from the conversion of carbohydrates into stored fat and improved lipid turnover, this metabolism could also contribute to extended lifespans of TG mosquitoes (Arik et al., 2015). In contrast to younger mosquitoes, we did not see marked changes in the metabolism of older TG and NTG mosquitoes with the exception of 24 h to 36 h PBM when triglyceride levels were increased in TG mosquitoes. These older mosquitoes had modestly reduced levels of glucose, trehalose, and triglycerides and markedly reduced levels of glycogen compared to younger mosquitoes, which likely represents the ongoing depletion of these resources as mosquitoes aged. Thus, increased fat body IIS in the TG mosquitoes may not have sufficient capacity to influence resource levels in older mosquitoes.

In addition to controlling metabolism, fat body IIS plays an important role in regulating the innate immune responses. Specifically, *E. coli* challenge induced *Def* and *Gam* expression in both TG and NTG mosquitoes relative to non-injected and sham-injected controls (Fig. 4S), whereas *B. subtilis* challenge induced the expression of both *Def* and *Cec* (Fig. 5S). Since AMPs are synthesized on demand in response to an immune challenge, it is likely that transcript titers provide a reasonable estimate of the AMP immune response (Jovanovic et al., 2015; Liu et al., 2016). Importantly, increased fat body IIS in TG mosquitoes further induced expression of AMPs and *TEP1* following bacterial challenge (Figs. 3 and 4) relative to NTG controls. TG mosquitoes challenged with *E. coli* increased *Def, Gam Cec* and *TEP1* expression relative to NTG controls, while TG mosquitoes challenged with *B. subtilis* had greater expression of *Def, Gam, TEP1*, and *PGRP-Lc* compared to NTG controls. These inductions occurred after TG mosquitoes blood fed (24–48 h PBM), which corresponded to expression of the myrAkt under control of the Vg promoter (Arik et al., 2015). Previous studies in *D. melanogaster* indicated that the Toll pathway antagonized insulin signaling downstream of phosphoinositide 3-kinase (PI3K) as evidenced by a reduction in Akt phosphorylation (DiAngelo et al., 2009). In TG mosquitoes Akt myristylation

circumvents the need for PI3K phosphorylation of phosphatidylinositol (3,4,5)-trisphosphate and thus may evade this inhibition by the Toll pathway. In *Bombyx mori*, it was also demonstrated that increased FOXO expression or decreased insulin receptor expression led to an increase in defensin transcript (*BmDefB*) in the fat body, although no immune challenge was performed (Zhang et al., 2018). We did not observe significant differences in immune gene expression between TG and NTG control groups (i.e., non- or sham-injected), only in bacterially challenged mosquitoes, which may explain this difference. Further, our IIS manipulation was fat body specific, whereas in *B. mori* overexpression of FOXO (baculovirus) and suppression of the insulin receptor (RNAi) were systemic. Surprisingly, *Gam* and *Cec* transcript levels were abundant in NBF TG mosquitoes relative to NTG controls following *E. coli* challenge, suggesting a low level of transgene expression in NBF TG mosquitoes. In support of this, we previously detected very low levels of *myrAkt* transcript in carcasses prior to blood feeding, although no protein was detected (Arik et al., 2015).

Because TG mosquitoes exhibited significantly increased expression of AMPs and other immune genes, we expected that this would increase the survival of bacterially challenged TG mosquitoes relative to NTG controls. Indeed, our survival analysis of challenged TG and NTG mosquitoes indicated that TG mosquitoes had significantly increased survival following challenge with *E. coli* or *B. subtilis* at 12 h and 24 h PBM. As anticipated, mosquitoes that did not receive a bloodmeal, and thus no transgene expression or increased fat body IIS, did not survive any better than NTG sibling controls. This provides compelling evidence that the significantly improved survival of TG mosquitoes was in fact due to increased fat body IIS. In *D. melanogaster*, both the Toll and Imd pathways are required to maximize resistance against Gram-negative *Pseudomonas aeruginosa*, which suppresses AMP gene expression (Apidianakis et al., 2005; Lau et al., 2003). *Ae. aegypti* engineered to overexpress *Cec-A* and *Def-A* under the Vg promoter were highly resistant to *P. aeruginosa* infection (Kokoza et al., 2010). in this light, our data suggest that increased fat body IIS is linked to improved innate immune responses against both Gram-positive and Gram-negative bacteria, likely through increased expression of AMPs in response to immune challenge.

In addition to enhancing survival in response to bacterial challenge, we also demonstrated that increased fat body IIS had a profound effect on *P. falciparum* infection. Our previous work on midgut IIS in *An. stephensi* has demonstrated that IIS and the various signaling pathways it interacts with in this tissue can significantly reduce *P. falciparum* infection prevalence and intensity (Luckhart and Riehle, 2020). For example, increased expression of active Akt in the midgut of *An. stephensi* completely blocked *P. falciparum* infection (Corby-Harris et al., 2010) by disrupting mitochondrial function (Luckhart et al., 2013). Conversely, midgut overexpression of the IIS inhibitor PTEN also increased resistance to *P. falciparum,* although via a different mechanism (Hauck et al., 2013). More recently, we demonstrated that IIS and JNK signaling can influence the synthesis of coenzyme A through the rate limiting enzyme pantothenate kinase (Simão-Gurge et al., 2021; Souvannaseng et al., 2018), presumably limiting the availability of endogenous pantothenate essential for *P. falciparum* sporogony. However, these studies focused on the mosquito midgut, the first barrier the parasite must physically cross and interact with for an extended period. It was unclear whether IIS in this fat body would have an impact on *P. falciparum* development.

Here we observed significant reductions in both the prevalence and intensity of *P. falciparum* infection in our TG mosquitoes, indicates that IIS can influence parasite development both locally (midgut) and systemically (fat body). While increase AMP expression in the fat body could disrupt parasite development, other mechanism may also be at play. We observed that TG mosquitoes exhibited significantly higher levels of *NOS* expression in both the whole body and the abdominal body wall at 24 h and 36 h PBM. Moreover, we found that *TEP1* mRNA expression was significantly higher in TG mosquitoes at 36 h and 48 h PBM when challenged with *E. coli* and *B. subtilis*, respectively. Since TEP1 binds and kills *P. berghei* and *P. falciparum* during the midgut stages (Blandin and Levashina, 2004), it is has been suggested that *TEP1* expression is induced in the fat body to replenish circulating titers (Gupta et al., 2009; Volohonsky et al., 2017). Combined, our results are consistent with published studies associating *NOS* and *TEP1* with reduced *Plasmodium* infection (Drexler et al., 2014; Luckhart et al., 2013).

ILPs and IIS are key regulators of reproduction, both in ovarian follicle cells for the synthesis of 20-hydroxyecdysone and in the fat body for the synthesis of Vg (Sharma et al., 2019; Arik et al., 2009). While we observed significant increases in both *Vg* transcript and protein levels in TG mosquitoes (Arik et al., 2015), this did not appear to directly affect mosquito fecundity. In this work we explored whether observed increases in Vg titers in TG mosquitoes conferred a fitness advantage to the progeny of male or female mosquitoes. Despite the fact that we observed a significant increase in pupae from TG mosquitoes, there were no significant increases in the number of male or female adults resulting from these crosses. Similar outcomes have been observed in *Caenorhabditis elegans*, *D. melanogaster*, and several species of ants and bees. In *C. elegans*, mutation of the *age-1* gene resulted in lifespan extension with no associated fitness cost or loss of fecundity (Johnson, 2003; Walker et al., 2000). Mutation of *Indy* and *daf-2* genes in *C. elegans* extended lifespan without any associated change in reproduction (Gems et al., 1998; Kenyon et al., 1993; Marden et al., 2003). Similarly, overexpression of dFOXO in the pericerebral fat body in *D. melanogaster* extended lifespan without reducing fecundity (Hwangbo et al., 2004). Moreover, a study by Buch et al. (2008) demonstrated that fruit flies with ablated insulin-producing cells at the post-larval stage are long-lived and have normal fecundity when kept on a protein-rich diet (Buch et al., 2008). Our data also showed no differences between the wet weights, dry weights and wing lengths of male or female progeny among all three crosses. Thus, even though TG female mosquitoes synthesized additional Vg, this did not confer a fitness advantage to the progeny, suggesting that excess Vg was likely reallocated by the female mosquito for other uses. Interestingly, Vg, lipophorin and Apolipophorin III have been implicated in the innate immune response against *Plasmodium* parasites (Mendes et al., 2008; Rono et al., 2010). Rono et al. (2010) demonstrated that RNAi knockdown of Vg led to a reduction in the infection intensity of *Plasmodium berghei* in *Anopheles gambiae* mosquitoes. Although these data are counter to our observations of reduced oocyst numbers in the presence of increased Vg, other factors, including timing of the Vg increase, increased fat body IIS and increased AMP synthesis likely play a role in these complex interactions. Nevertheless, additional studies to elucidate the interactions between Vg, IIS on anti-*Plasmodium* responses in the mosquito are warranted.

In this study, we demonstrated that increased fat body IIS in *An. stephensi* plays central and conserved roles in maintaining energy balance, resulting in higher levels of stored energy while changing the profile of circulating carbohydrates. We also demonstrated that fat body IIS is an important regulator of mosquito innate immunity conferring increased resistance to bacterial challenge and *P. falciparum* infection. This is accomplished through the upregulation of AMPs and other components of the innate immune response. Combined with our previous studies demonstrating the key role fat body IIS plays in adult survival we have begun to unravel the complex signaling regulations of diverse physiologies in the fat body of adult mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AMP	Antimicrobial Peptide
DILPs	*Drosophila* Insulin-Like Peptides
EGFP	Enhanced Green Fluorescent Protein
FOXO	Forkhead Box O
IGF-1	Insulin-Like Growth Factor-1
INR	Insulin Receptor
ILP	Insulin-Like Peptide
IIS	Insulin/Insulin Growth Factor 1 Signaling
MNC	Medial Neurosecretory Cells
Myr	Myristoylation
NOS	Niitric Oxide Synthase
NTG	Non-Transgenic
PBM	Post-Bloodmeal
PI3K	Phosphoinositide 3-Kinase

PTEN	Phosphatase and Tensin Homolog
PTTH	Prothoracicotropic Hormone
TEP1	Thioester Complement-Like Protein 1
TG	Transgenic
VG	Vitellogenin
WT	Wild-Type

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Highlights

- Fat body IIS increased mosquito survival and AMP levels after bacterial challenge.

- Fat body IIS significantly reduced malaria parasite infection prevalence and intensity.

- Mosquitoes with increased fat body IIS had increased nutrient storage.
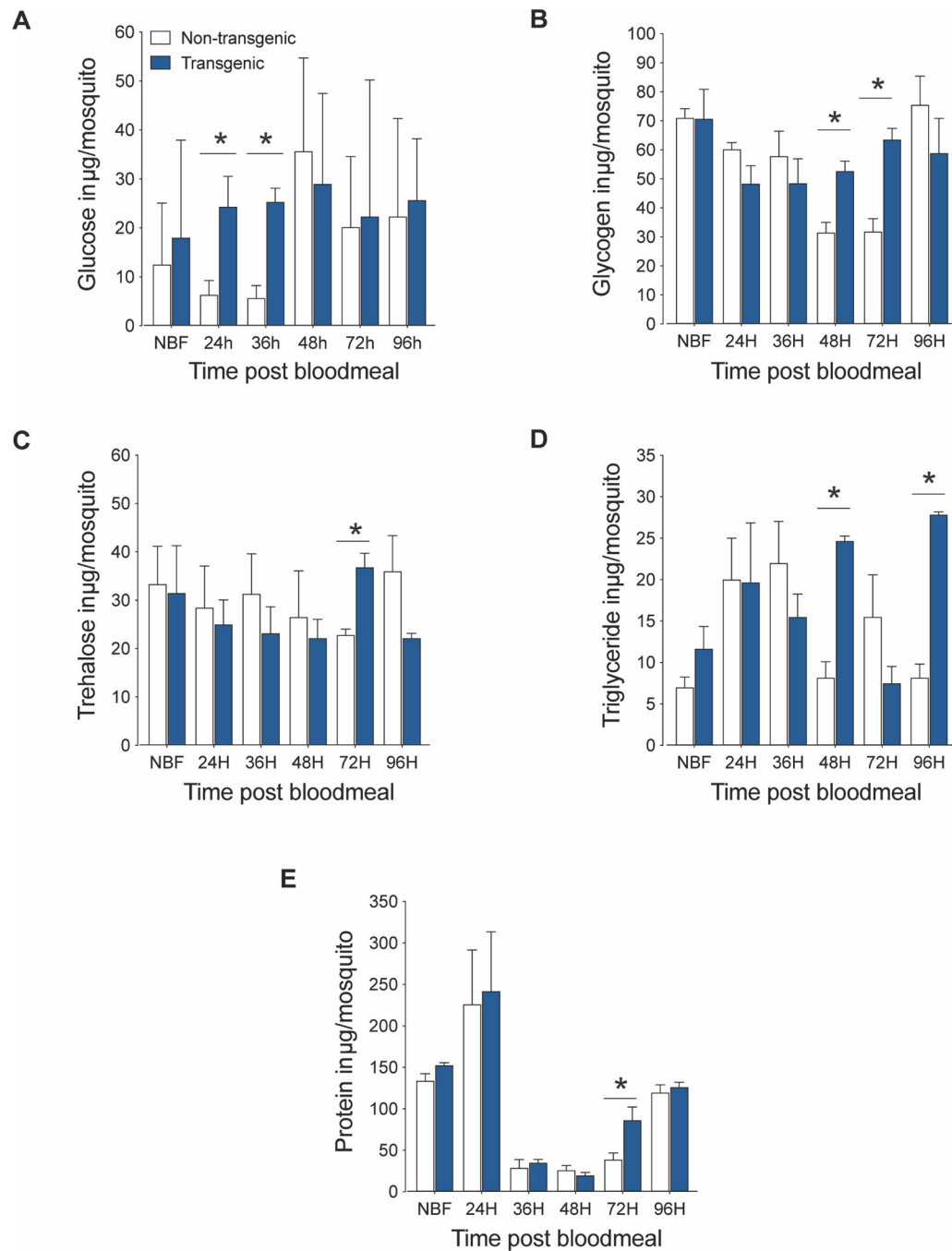
Fig.1. Impact of increased fat body IIS on the nutrient stores of young mosquitoes.
Increased IIS in the fat body during a reproductive cycle significantly increased glucose, glycogen, trehalose, and triglyceride levels in young (3–5 days) TG female *An. stephensi.* Glucose (A), glycogen (B), trehalose (C), and triglycerides (D) were extracted and assayed from at least three unique cohorts of mosquitoes. Asterisks (*) indicate a significant difference (P<0.05) versus NTG controls.
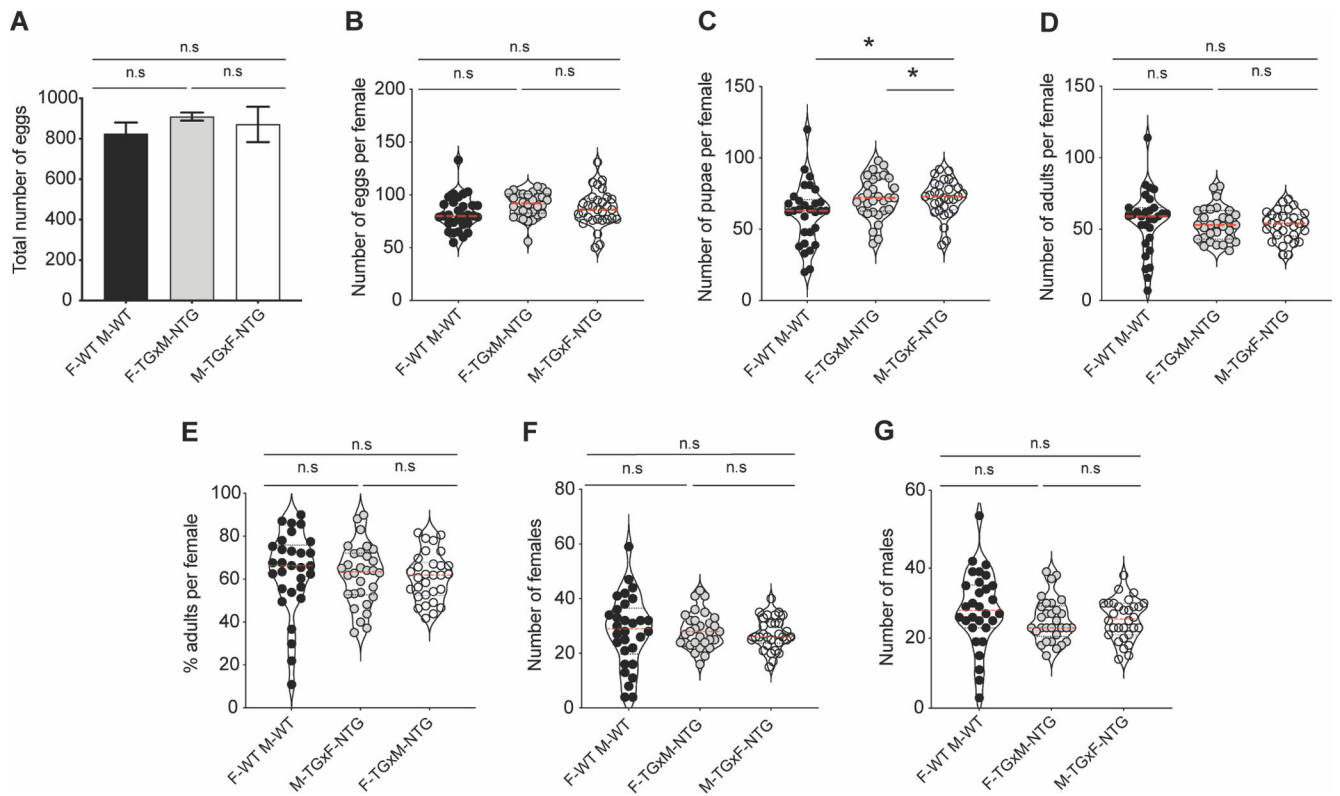
Fig.2. Increased fat body IIS does not affect the development of progeny.
Three crosses were (F-WT X M-WT; F-TG X M-WT and F-WT X M-TG) and the impact
on their progeny examined. The total number of eggs (A), number of eggs per female (B),
number of progeny successfully pupating per female (C), number of progeny reaching adult
emergence (D), percentage of eggs from each individual female that survived to adulthood
(E) and the ratio of females (F) and males (G) were assayed from three different cohorts of
ten females per replicate (n=3). Asterisks (*) indicate a significant difference from the NTG
controls (P<0.05), while n.s indicates no (significant) difference.
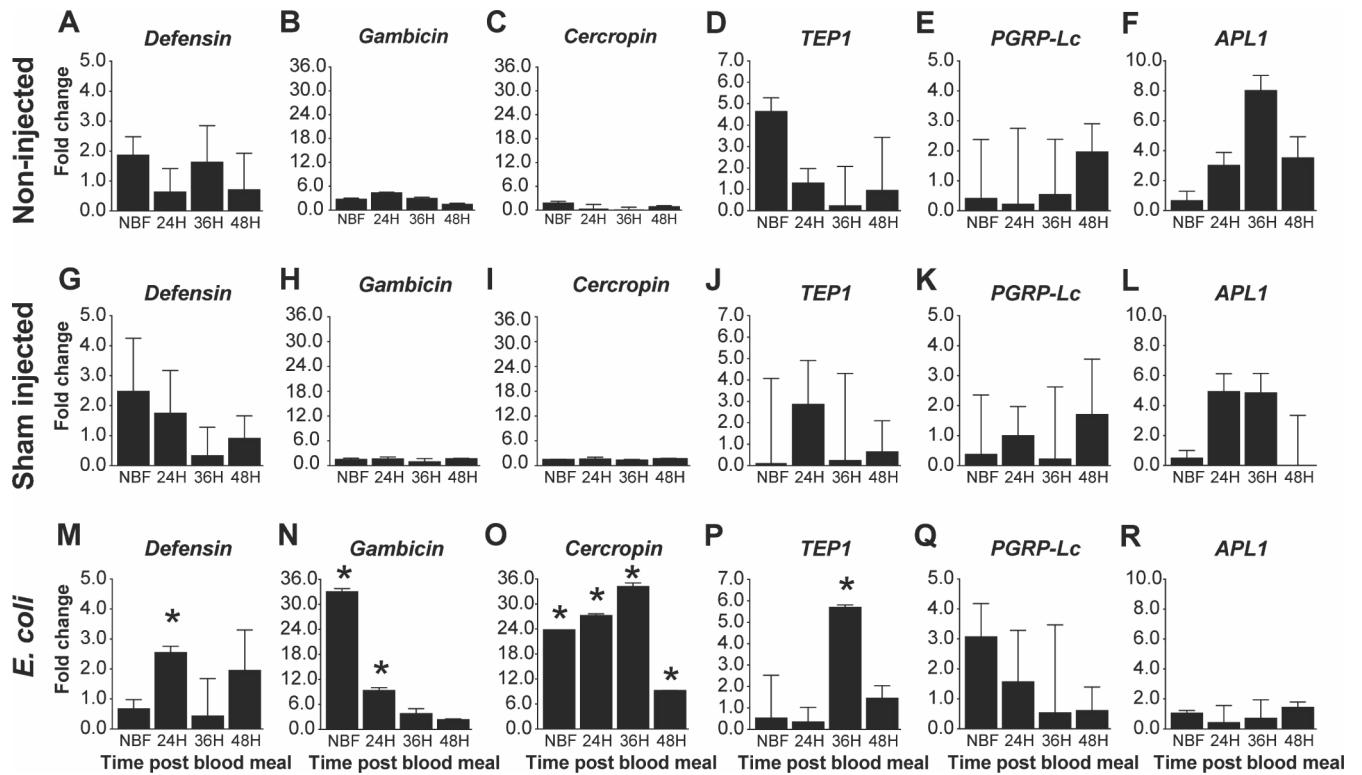
Fig. 3. Impact of increased fat body IIS on antimicrobial peptide expression following an *E. coli* challenge.

Graphs represent fold-change ($2^{\wedge}(-\quad Ct)$) of TG *An. stephensi* transcript expression for five antimicrobial peptides (AMPs) and the thioester containing protein 1 (TEP1) relative to NTG sibling controls. Total RNA was isolated from TG and NTG *An. stephensi* pools (n=5 females per pool) that were either not inoculated (non-injected), inoculated with buffer only (sham injected), or inoculated with *E. coli* at various time points after a bloodmeal (0, 24, 36 or 48 h) to stimulate transgene expression. Transcript expression in TG mosquitoes were normalized against NTG sibling controls at each specific time points to calculate the fold change. Three biological replicates were conducted. Error bars indicate SEM and an asterisks (*) represents a significant difference (P<0.05) between the TG and NTG samples.
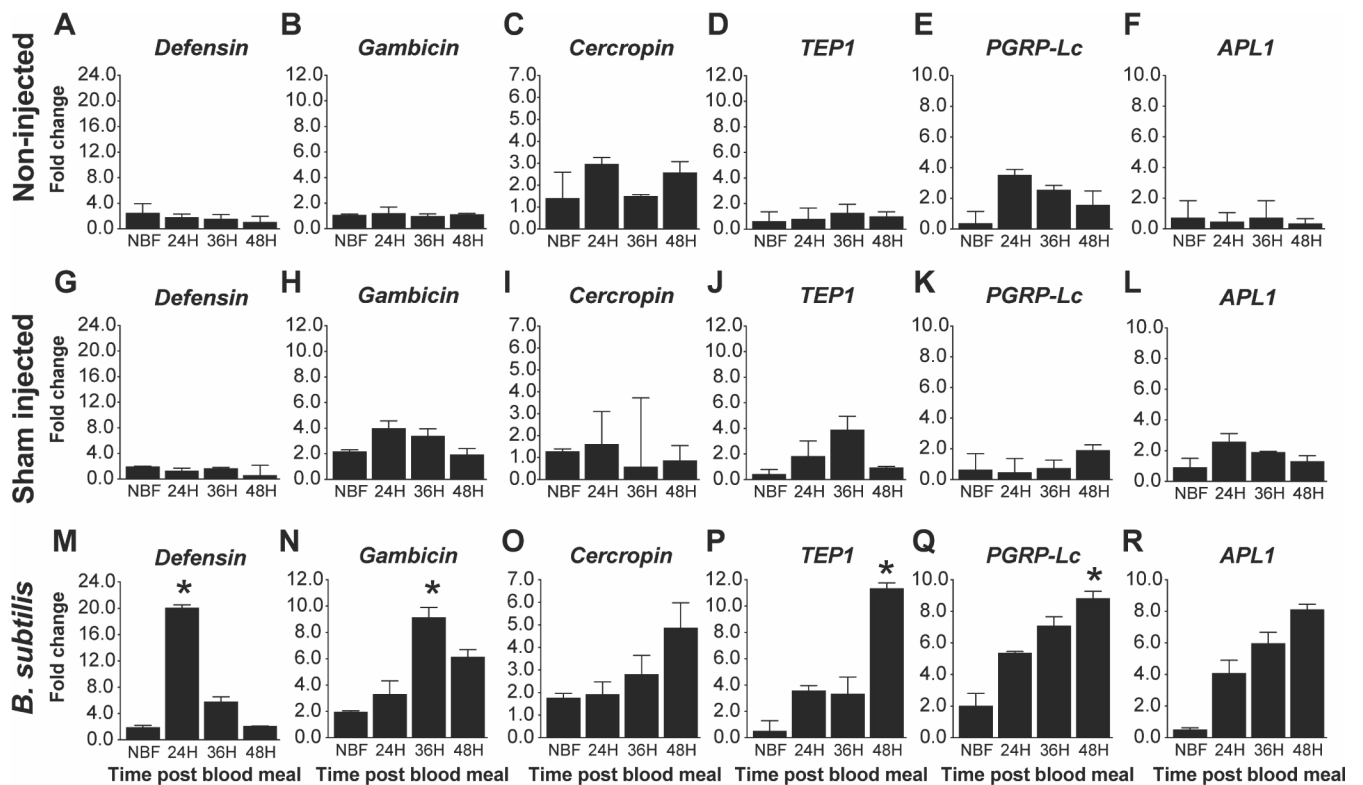
Fig. 4. Impact of increased fat body IIS on antimicrobial peptide expression following a *B. subtilis* challenge.

Graphs represent fold-change ($2^{-\Delta\Delta Ct}$) of TG *An. stephensi* transcript expression for five antimicrobial peptides (AMPs) and the thioester containing protein 1 (TEP1) relative to NTG sibling controls. Total RNA was isolated from TG and NTG *An. stephensi* pools (n=5 females per pool) that were either not inoculated (non-injected), inoculated with buffer only (sham injected), or inoculated with *B. subtilis* at various time points after a bloodmeal (0, 24, 36 or 48 h) to stimulate transgene expression. Transcript expression in TG mosquitoes were normalized against NTG sibling controls at each specific time points to calculate the fold change. Three biological replicates were conducted. Error bars indicate SEM and an asterisks (*) represents a significant difference (P<0.05) between the TG and NTG samples.
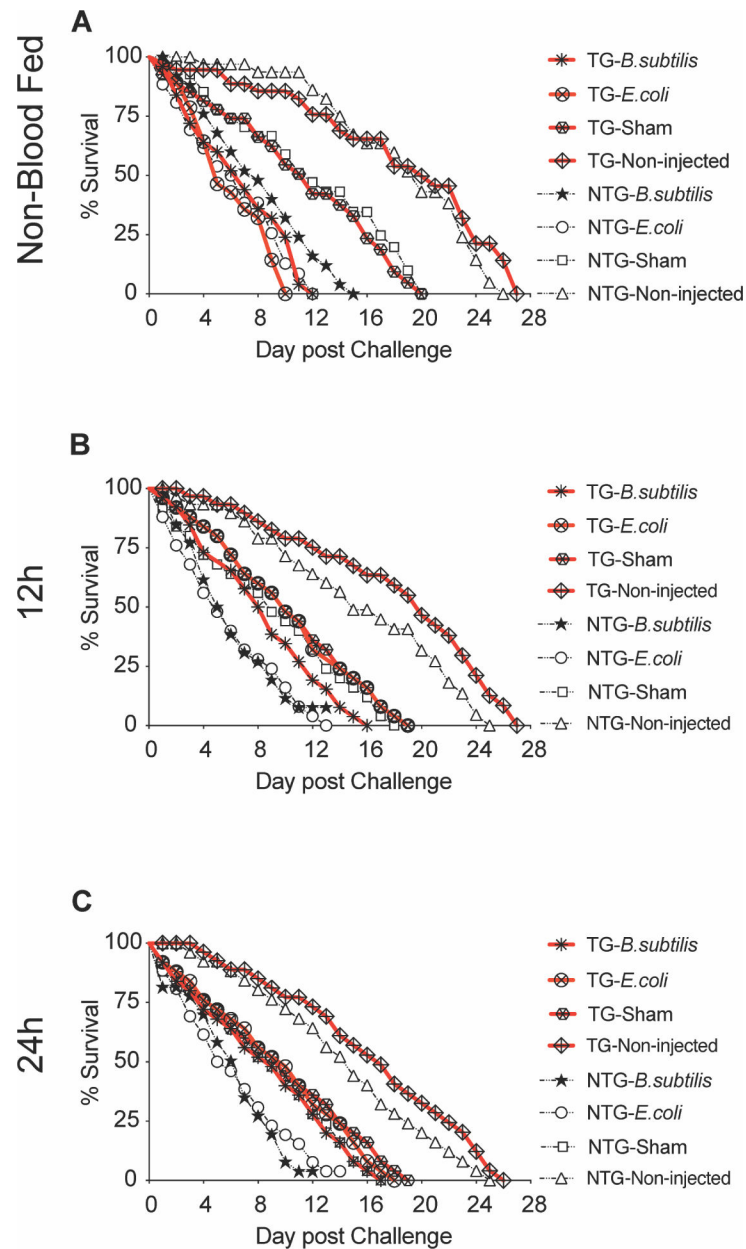
Fig. 5. Survival of TG and NTG mosquitoes following *E. coli* or *B. subtilis* infection.
TG and NTG sibling mosquitoes (n=25/treatment) were challenged with either *E. coli* or *B. subtilis* and maintained until the final mosquito perished to establish a survival curve. Non-injected and sham injected mosquitoes were used as controls. Mosquitoes were challenged prior to blood feeding, when no transgene was present and 12 and 24 h post-bloodmeal after transgene induction. All treatments are shown on the graphs (A, B and C). This figure is a representative assay and survival curves from two additional biological replicates can be found in supplementary figures (Supp. Figs 6). *P* values were calculated using the Log-Rank (Mantel-Cox) test and reflect comparisons between matched TG and NTG controls at alpha = 0.05.
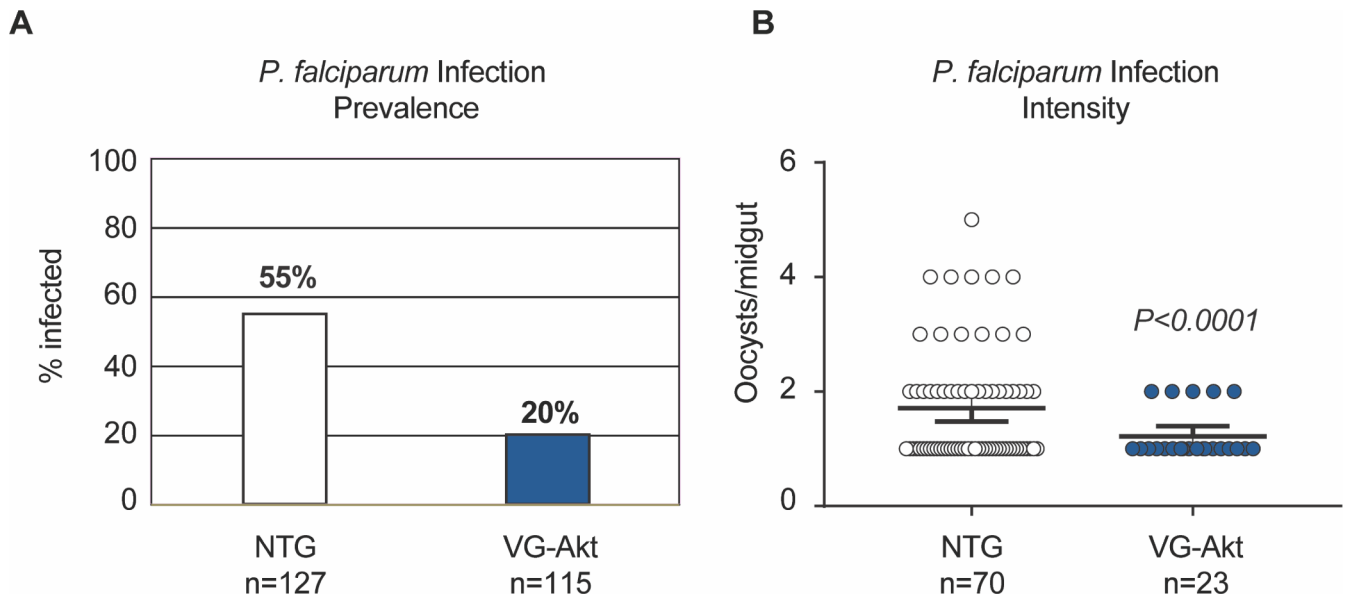
A

B

Fig. 6. Resistance of TG and NTG mosquitoes to *P. falciparum* infection.
TG and NTG mosquitoes were provided with an artificial blood meal containing *P. falciparum* NF54 gametocytes. Ten days after infection, the midguts were dissected and the numbers of *P. falciparum* oocysts were counted. A) Infection prevalence was defined as the percentage of mosquitoes that had at least one oocyst in the midgut. B) Infection intensity was defined as the total number of oocysts per midgut. The black line represents the mean number of oocysts per midgut ± SEM. Three replicates were conducted using unique biological cohorts.
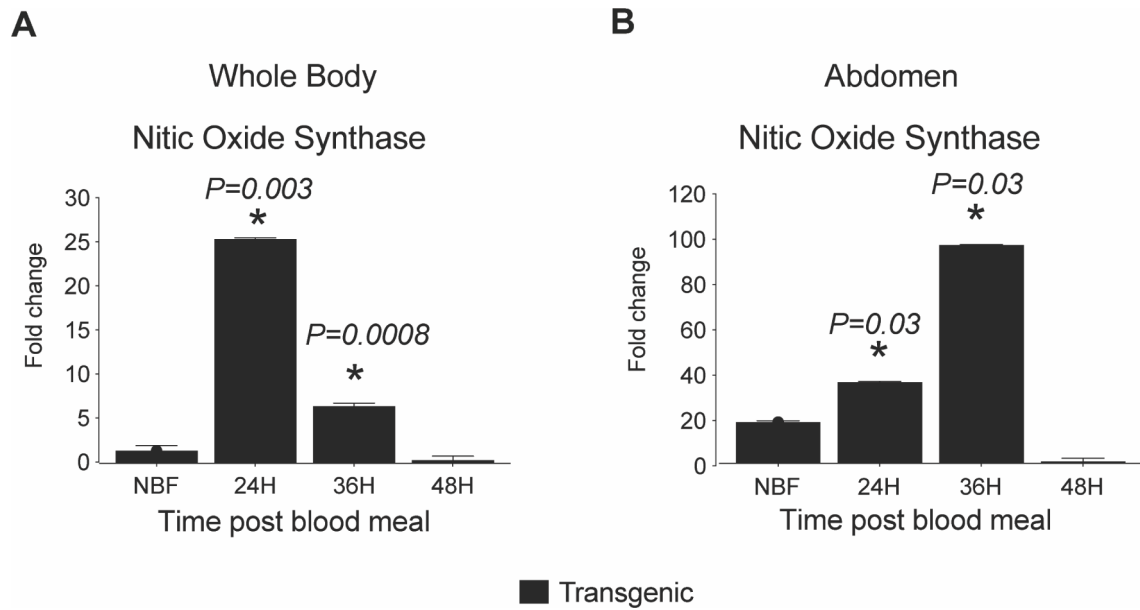
Fig. 7. Fat body IIS increased *AsNOS* gene expression in *An. stephensi.*
Graph represents the mean (Ct) mRNA expression of selected immune genes in RNA samples prepared from a pool of 5: (A) whole body mosquitoes, and (B) abdominal wall (of mosquito abdomen) at different time points for both TG and NTG mosquitoes. Graphs represent means ± SEMs of fold change in the expression of *AsNOS* for: (C) whole body mosquitoes, and (D) abdominal wall (of mosquito abdomen). Pairwise comparisons of treatments and matched controls were analyzed by Student's t-test; significant and marginally significant p-values are shown, n = 5.