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Overexpression of phosphatase and tensin homolog improves fitness and decreases *Plasmodium falciparum* development in *Anopheles stephensi*

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Abstract

The insulin/insulin-like growth factor signaling (IIS) cascade is highly conserved and regulates diverse physiological processes such as metabolism, lifespan, reproduction and immunity. Transgenic overexpression of Akt, a critical regulator of IIS, was previously shown to shorten mosquito lifespan and increase resistance to the human malaria parasite *Plasmodium falciparum*. To further understand how IIS controls mosquito physiology and resistance to malaria parasite infection, we overexpressed an inhibitor of IIS, phosphatase and tensin homolog (PTEN), in the *Anopheles stephensi* midgut. PTEN overexpression inhibited phosphorylation of the IIS protein FOXO, an expected target for PTEN, in the midgut of *A. stephensi*. Further, PTEN overexpression extended mosquito lifespan and increased resistance to *P. falciparum* development. The reduction in parasite development did not appear to be due to alterations in an innate immune response, but rather was associated with increased expression of genes regulating autophagy and stem cell maintenance in the midgut and with enhanced midgut barrier integrity. In light of previous success in genetically targeting the IIS pathway to alter mosquito lifespan and malaria parasite transmission, these data confirm that multiple strategies to genetically manipulate IIS can be leveraged to generate fit, resistant mosquitoes for malaria control.

Keywords

phosphatase and tensin homolog (PTEN); *Plasmodium falciparum*; mosquito; insulin/insulin-like growth factor signaling (IIS); malaria; *Anopheles stephensi*

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1. Introduction

More than half of the world's population is at risk for malaria [1]. Heavy use of antimalarials has led to the emergence of drug resistant parasites, resulting in a surge in malaria cases [2]. Furthermore, mosquito resistance to the most widely used insecticides has increased [3]. These issues highlight the urgent need for novel control strategies against malaria. One such strategy is to genetically modify mosquito vectors to make them unable to transmit malaria parasites [4]. A number of different approaches have been used to develop malaria parasite resistant mosquitoes. Of these approaches, both the use of single chain antibodies [5] and manipulation of the insulin/insulin growth factor signaling (IIS) cascade [6] have yielded *Anopheles* mosquitoes that are incapable of supporting *Plasmodium falciparum* development.

The IIS cascade is highly conserved and all IIS pathway proteins are functional in the midgut epithelium of *Anopheles stephensi*, a primary vector of *P. falciparum* and *Plasmodium vivax* in India and parts of Asia. IIS in the mosquito midgut can be activated by *P. falciparum* glycosylphosphatidylinositols, parasite factors that mimic the effects of insulin [7]. In addition, we have shown that human insulin ingested in a blood meal can activate IIS in midgut epithelial cells within 30 min of feeding and that continued IIS activation via weekly supplemented blood meals reduces mosquito lifespan [8]. These effects of insulin were expected in light of observations of increased lifespan following IIS inhibition in a variety of model organisms [9,10]. In addition to lifespan, IIS regulates immunity and pathogen resistance [11,12]. In *A. stephensi*, expression of an active form of the key IIS regulator Akt in the midgut completely inhibited *P. falciparum* infection [6]. Collectively, our work affirms that the midgut is central to IIS regulation of multiple physiologies in the mosquito.

IIS regulates immunity through a variety of mechanisms. For example, insulin can suppress lipopolysaccharide (LPS)- and tumor necrosis factor alpha (TNF- α)-induced nuclear factor (NF)- κ B activation in human cell lines [13,14]. IIS activation can also attenuate degradation of the negative regulators of NF- κ B, resulting in a general decrease in NF- κ B signaling [15]. Human insulin similarly inhibits NF- κ B-dependent immunity in *A. stephensi* cells *in vitro* and in the midgut epithelium *in vivo*, effects that underlie enhanced parasite development in insulin-fed mosquitoes [16]. In addition to direct effects of IIS on immunity, IIS regulates autophagy, an evolutionarily conserved process for the segregation and disposal of damaged organelles that can control pathogen resistance. In particular, overexpression of Akt inhibits autophagy [17], while activation of the IIS inhibitor phosphatase and tensin homolog (PTEN) positively regulates autophagy [18]. The most compelling connection between IIS-dependent autophagy and immunity comes from *Caenorhabditis elegans*, in which the *daf2* (insulin receptor) phenotype of infection resistance is reversed by mutations that inhibit autophagy [19]. Hence, upregulation of autophagy can enhance pathogen resistance, a phenomenon that has also been associated with resistance of the fruit fly *Drosophila melanogaster* to bacterial challenge [20] and of mammalian cells to bacteria, viruses and parasites such as *Toxoplasma gondii* [21,22]. Intriguingly, *P. falciparum* infection of the African malaria mosquito *Anopheles gambiae* induced translation of 5 of 16 autophagy (ATG) protein mRNAs, including those for key regulators ATG6 and ATG8, in the midgut epithelium at 24 hr after infection [23], suggesting that autophagy is induced early during sporogonic development in the mosquito host. While autophagy induction can control resistance – perhaps contributing to the largescale death of parasites in the midgut – it is possible that highly conserved regulation of stem cell renewal and differentiation by autophagy could also impact the midgut response to parasite infection. Apoptotic cells in the mosquito midgut have been observed following parasite invasion and are sloughed off into the midgut lumen [24], a process that would necessitate epithelial recovery by regenerative

cells. Stem cells in the posterior midgut of *D. melanogaster* [25] regulate tissue regeneration and maintenance [26,27] and are the only dividing cell type in the midgut [27]. Analogous cells are thought to play a similar role in *A. stephensi* [28]. Hence, given the conserved role for autophagy in regulation of stem cell maintenance and differentiation from insects to humans [29,30], autophagy in the mosquito midgut may be essential for epithelial barrier repair and responsiveness to malaria parasite invasion.

PTEN is a lipid and protein phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5) P_3) to phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5) P_2). This removes a critical docking site for IIS pleckstrin homology domain proteins such as Akt and phosphoinositide-dependent kinase 1 (PDK1). Thus, PTEN acts as a direct antagonist of phosphoinositide 3-kinase (PI3K) in regulating the activity of Akt substrates forkhead box protein O (FOXO), the E3 ubiquitin ligase MDM2, and BCL2 antagonist of cell death (BAD) to control cell proliferation and apoptosis [31]. The lipid phosphatase activity of PTEN regulates self-renewal and differentiation of human embryonic stem cells and hematopoietic stem cells as well as the chemotaxis of neutrophils [32,33]. PTEN lipid phosphatase-independent activities include inhibition of cell growth, invasion, and migration [34] as well as control of DNA-damaged cell size by regulation of actin remodeling [35].

Molecular regulation of PTEN – from invertebrates to humans – is complex, with invertebrates providing significant understanding of PTEN biology in normal, non-cancerous phenotypes. In particular, the yellow fever mosquito *Aedes aegypti*, generates six PTEN splice variants, of which three arise from improper splicing of one of seven introns resulting in truncated, and likely rapidly degraded, proteins lacking portions of the conserved C2 domain and C-terminal tail [36]. The remaining three splice variants encode full length proteins. Only one splice variant, *AaegPTEN6*, however, encodes the C-terminal PDZ binding motif and knockdown of this splice variant significantly decreased egg production in *A. aegypti* [37]. Here we have taken a forward approach – via overexpression of an analogous PTEN with PDZ motif – in *A. stephensi* to reveal PTEN-dependent biology arising from the signaling center for IIS, the midgut, as a complement to our studies of Akt in the same tissue. Here, we show that PTEN overexpression in *A. stephensi* regulates the fundamental physiologies of lifespan and infection resistance, providing significant new insights into the biology of PTEN and genetic strategies for malaria transmission blocking.

2. Materials and Methods

2.1 Mosquito rearing and experimental treatments

Non-transgenic (NTG) *A. stephensi* Liston (Indian wild-type strain) and PTEN transgenic (TG) siblings were reared and maintained at 27°C and 75% humidity. Laboratory-reared 3–5 day-old female mosquitoes were maintained on water for 24–48 hr prior to any experiment and then allowed to feed for 30 min on artificial blood meals provided through a Hemotek Insect Feeding System (IFS; Discovery Workshops, Accrington, UK). For midgut signaling and lifespan studies, mosquitoes were fed artificial blood meals containing washed human red blood cells (RBCs) and saline (10 mM NaHCO₃, 15 mM NaCl, pH 7.0) with or without recombinant human insulin (1.7×10⁻⁴ μM in saline) and midguts dissected at 2 hr post feeding. Human RBCs for these studies were purchased as anonymously donated samples from Interstate Blood Bank (Memphis, TN). For midgut gene expression analyses, mosquitoes were fed artificial blood meals containing freeze thaw parasite product (FTPP; see 2.7 below) or a control blood meal and then dissected at 24 hr post feeding. For detection of PTEN mRNA and protein in TG adult female *A. stephensi* and to assess reproduction relative to NTG *A. stephensi*, mosquitoes were fed on whole human blood acquired as anonymously donated, expired human blood from the American Red Cross.

2.2 Identification of the *A. stephensi* PTEN ortholog

Rapid amplification of cDNA ends using a gene fragment was used to obtain a full-length *A. stephensi* PTEN clone (Firstchoice RLM RACE kit; Life Technologies, Grand Island, NY) with the primers 5' Outer 5'-CACTTTGTACTTGCCCTCGTGCTT-3', 5' Inner 5'-TTGCGGTAGATGGCCTCCATGTTT-3', 3' Outer 5'-ATCGGTGGAGCTTGTCTGCTGTAT-3', and 3' Inner 5'-CAACAAGCTGGACAAAGGATGGCA-3'. We identified the complete 5' UTR and the 3' UTR with a single splice variant corresponding to AegPTEN3. The full-length PTEN sequence was amplified with forward (5'-ACGCTTCGAAAGAGGTGACAAGGA-3') and reverse (5'-TTTCGTCGTGCGTCTACACCTCAA-3') primers and a proofreading enzyme (Klentaq; Clontech, Mountain View, CA) and sequenced twice to verify the complete open reading frame.

2.3 Generation and maintenance of TG *A. stephensi* that overexpressed HA-AstePTEN

The *A. gambiae* carboxypeptidase (CP) promoter (provided by Dr. Luciano Moreira [38]) was modified by PCR to eliminate the signal peptide, start methionine and Kozak consensus sequence, and to add XhoI and NotI restriction sites at the 5' and 3' ends, respectively. The modified CP promoter was ligated into the pSL1180fa shuttle vector [39] using XhoI and NotI. An SV40 3' UTR was ligated into the EcoRI site of pSL1180fa. A Kozak consensus sequence (CCAACCATGG), HA epitope (YPYDVPDYA) and NdeI restriction site were added to the 5' end and a PDZ binding motif and XmaI restriction site were added to the 3' end using custom primers (5' primer 5' TAGGCATATGCCACCATGGCTTACCCATACGATGTCCCAGATTACGCTAATCC AACG AATATTATC-3'; 3' primer 5' -GATCCCCGGGTCATAGATGCGTCGATTACCCGATTCCCAATCTTCATC-3'). This construct was inserted into the pSL1180fa shuttle containing the CP promoter and SV40 3' UTR using the NdeI and XmaI restriction sites. Finally, the CP-HA-PTEN construct was ligated into the pBac[3XP3-EGFPafm] construct using the AscI restriction sites to generate the pBac[3XP3-EGFPafm]CP-HA-PTEN plasmid for injection into *A. stephensi* embryos. Donor and helper (phsp-pBac) plasmids were sent to the University of Maryland Biotechnology Institute Insect Transformation Facility (UMBI-ITF) to be genetically engineered into *A. stephensi*. Four stable lines expressing EGFP under the control of the 3XP3 promoter and HA-PTEN regulated by the CP promoter were generated (F2, M7, M8 and INT). Crosses between heterozygous TG females and NTG males produced a 50/50 ratio of TG to NTG siblings, and these crosses were used for colony maintenance. For the F2 line, crosses between TG males and NTG females produced progeny in which all females were TG and all males were NTG, indicating an insertion into the X-chromosome. Experiments were not initiated until six generations of outcrossing with our NTG lab strain were completed. After 16 generations, a homozygous line was created and used to assess the role of the PTEN transgene on reproduction, longevity, and anti-*Plasmodium* resistance.

2.4 Genomic insertion site identification

Genomic DNA was extracted from homozygous PTEN TG pupae (DNeasy Tissue kit; Qiagen, Germantown, MD) then treated with RNase A following the manufacturer's instructions. Insertion analysis was performed following the protocol of Buchholz et al. ([40]; Exelixis, Inc) with modifications (Fig. S3).

2.5 Transcript and protein expression analysis

To examine *HA-AstePTEN* transcript expression during development and a reproductive cycle, whole bodies of 4th instar larvae, early and late pupae, 1–3 day-old adult females prior to a blood meal, and blood fed females at 6, 24, and 48 hr post-blood meal were collected

for RNA extraction (RNeasy kit; Qiagen). Contaminating genomic DNA was removed with Dnase1 (Fermentas, Waltham, MA), and cDNA was synthesized (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA) with random hexamer primers. RT-PCR was performed (GoTaq master mix; Promega, Madison, WI) using a forward primer complementary to the HA-tag 5' - ACCATGTACCCATACGATGTTCCAGATTACGCT-3' and the reverse primer 5' - CACTTTGTACTTGCCCTCGTGCTT-3'. NTG *A. stephensi* females at 24 hr post-blood meal were used as a negative control. Ten mosquitoes were used at each time point and the experiment was repeated three times with independent cohorts of the mosquitoes.

To examine tissue specific expression of HA-AstePTEN protein, midguts and carcasses (total body minus midgut) were collected from 3–5 day-old previtellogenic (PV, non-bloodfed) females and from females at 24 hr post-blood meal. For the expression of HA-AstePTEN protein during a reproductive cycle, midguts were dissected from PV and bloodfed females at 5, 12, 24, 36, 48, and 72 hr post-blood meal into 1X TBS buffer with 10X protease inhibitor cocktail (Complete Ultra protease inhibitor; Roche Applied Science, Indianapolis, IN). Blood was removed by washing midguts in 1X TBS buffer with 10X protease inhibitor cocktail and the midguts were frozen at -80°C . Two midgut tissue equivalents or 1/20 carcass equivalent was used per lane. Immunoblots were performed as described [41] with anti-HA antibody (1:5000, Roche Applied Science, Indianapolis, IN) and with anti-GAPDH antibody (1:30000; Cell Signaling Technologies, Danvers, MA) to assess loading. Densitometry analysis of HA-AstePTEN relative to the GAPDH was performed with ImageJ software (<http://rsb.info.nih.gov/ij>). All experiments were repeated three times with independent cohorts of mosquitoes.

2.6 Phosphorylation analysis

Protocols for analysis of phosphorylation levels have been described previously [42]. In brief, protein lysates from 40 bloodfed mosquito midguts were probed using primary and secondary antibodies at the following dilutions: 1:10,000 phospho-ERK (1° ; Sigma Aldrich, St. Louis, MO) and 1:20,000 rabbit anti-mouse IgG (2°); 1:1,000 phospho-FOXO (1° ; Millipore, Billerica, MA) and 1:2,000 goat anti-rabbit IgG (2°); 1:5,000 phospho-p38 (1° ; Millipore) and 1:10,000 goat anti-rabbit IgG (2°); 1:1,250 phospho-JNK (1° ; Biosource-Life Technologies, Grand Island, NY) and 1:10,000 goat anti-rabbit IgG (2°); 1:10,000 GAPDH (1° ; Abcam, Cambridge, MA) and 1:20,000 goat anti-rabbit IgG (2°). All secondary antibodies were purchased from Sigma Aldrich.

2.7 Preparation of *P. falciparum* freeze/thaw parasite product (FTPP)

Mosquito infection with malaria parasites is variable and dependent, in part, on the total number of gametocytes ingested with the blood meal [43,44]. To minimize effects of this variation, we generated freeze/thaw parasite product (FTPP) of *P. falciparum* to induce uniform signaling events in midguts of fully-engorged *A. stephensi*. FTPP was prepared from 15 day-old cultures (or when stage V gametocytes were evident) of *P. falciparum*-infected red blood cells (RBCs) subjected to three freeze/thaw cycles of -80°C for 10 min followed by 37°C for 10 min. To mimic parasite infection, FTPP was diluted with uninfected human RBCs and heat-inactivated human serum. As a control, an equal volume of uninfected RBCs was similarly frozen and thawed, diluted with intact uninfected RBCs, and fed to mosquitoes. To further correct for possible differences in the quantity of parasite products consumed among the treatment groups, proteins from pooled midguts were diluted 1:100 (vol:vol), subjected to electrophoresis and membrane transfer, and incubated with a 1:2,000 dilution of mouse monoclonal anti-*P. falciparum* endoplasmic reticulum-associated protein Pf39 (MRA-87, MR4, ATCC), a protein marker that is positively correlated with total parasite levels [45]. To reveal antibodies bound to Pf39, membranes were incubated

with 1:20,000 HRP-conjugated rabbit anti-mouse IgG, then with SuperSignal West Dura chemiluminescent reagent and visualized using the Kodak Image Station 4000MM Pro and Molecular Imaging software (Carestream Health). No significant differences in Pf39 protein levels from *A. stephensi* midguts were apparent in any of the FPHP feeding studies (data not shown).

2.8 Reproductive assays

To study egg production over multiple reproductive cycles, 3–5 day-old female NTG and PTEN TG *A. stephensi* were provided with a blood meal. Nonfed and partially fed mosquitoes were discarded while fully engorged females were provided with individual oviposition cups at 48 hr post-blood meal. Eggs were collected from individual female mosquitoes from 72–96 hr post-blood meal and counted under a stereomicroscope. Only females that laid eggs in the first reproductive cycle were pooled and provided with second blood meal. Following the second blood meal, females that did not take blood were once again discarded, and individual oviposition cups were provided to the fully engorged females as above. After collecting and counting eggs, the procedure was repeated for the third and fourth reproductive cycles. The cycles of blood feeding and egg counting were repeated once per week for 4 wk. Females had access to 10% dextrose *ad libitum*.

2.9 Lifespan studies

PTEN TG and NTG female *A. stephensi* were maintained in 5 gal cartons and provided with an artificial blood meal once per week for the duration of their lifespans. Blood meals consisted of either washed human RBCs in saline supplemented with insulin (1.7×10^{-4} μ M) or RBCs in saline only and 300 mosquitoes were used per treatment. Dead mosquitoes were counted three times per week and oviposition cups provided once per week after blood feeding. This experiment was replicated three times with separate cohorts of mosquitoes.

2.10 Malaria parasite culture and mosquito infection

Cultures of *P. falciparum* strain NF54 were grown in 10% heat-inactivated human serum and 6% washed human RBCs in RPMI 1640 with HEPES (Gibco/Invitrogen) and hypoxanthine for 15 days or when stage V gametocytes were evident. Mosquitoes were fed on mature gametocyte cultures ($n = 125$ per treatment group) diluted with uninfected human RBCs and heat-inactivated human serum. After 10 days, midguts from gravid females were dissected and stained with 0.1% mercurochrome to visualize *P. falciparum* oocysts. Mean oocysts per midgut (infection intensity) and percentage of infected mosquitoes (infection prevalence; infection defined as at least one oocyst on a dissected midgut) were calculated. The experiment was replicated three times with separate cohorts of mosquitoes.

2.11 RNA extraction and qRT-PCR assays

For these assays, 20–30 midguts were dissected from 3 day-old and 18 day-old NTG and PTEN TG *A. stephensi* maintained under normal colony conditions (see 2.1 above) or from 3 day-old NTG and PTEN TG *A. stephensi* at 24 hr after provision of FPHP-supplemented or control blood meals. The 24 hr timepoint was selected based on timing of peak levels of PTEN protein in the midgut epithelium following blood feeding (see below) and a recent study showing that key cellular regulators for innate immune defenses are translationally upregulated in the midgut epithelium at 22–26 hr post-infection with *P. falciparum* [23]. The 3 day and 18 day timepoints, in contrast, were selected to discern direct effects of PTEN activity, in the absence of confounding immune stimuli (FFPP), on expression of autophagy (*ATG6*, *ATG8*) and stem cell (*esg*, *prospero*) biomarkers at times consistent with first blood-seeking behavior (3 days) and with completion of parasite development (18 days). Dissected midguts were homogenized in TriZOL reagent (Invitrogen) for RNA extraction. cDNA was

synthesized from RNA samples using the SuperScript® III First-Strand Synthesis System (Invitrogen). For *ATG6*, *ATG8*, *prospero* (PROS), and *escargot* (ESG) expression analysis, cDNA samples were pre-amplified by PCR using gene specific primers. Cycling conditions for pre-amplification were as follows: 95°C for 5 min, 20 cycles at 95°C for 30 sec, 55°C for 30 sec, 70°C for 30 sec, followed by 70°C for 5 min and 4°C for 5 min. Relative expression levels for *LRIM1*, *LRRD7*, *nitric oxide synthase* (*NOS*), *TEP1*, *defensin* (*DEF*) and *APL1* were analyzed with Maxima SYBR green/ROX qPCR Master Mix (Fermentas ThermoScientific, Waltham, MA) on an ABI 7300 Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: 95°C for 5 min, 50°C for 2 min, 35 cycles at 95°C for 15 sec, 60°C for 1 min. Expression levels were calculated using the 2^{-Ct} method relative to the ribosomal protein *s7* (*RPS7*) gene.

2.12 Functional assay of midgut permeability in NTG and homozygous PTEN TG *A. stephensi*

Laboratory reared 3–5 day-old female NTG or PTEN TG *A. stephensi* were kept on water for 48 hr and then allowed to feed for 30 min on artificial human blood meals [1:1 washed human RBCs (Interstate Blood Bank) in PBS (Cellgro)] with 1×10^6 fluorescent beads/ml (3.0–3.4 μ m, Sphero™ Rainbow Calibration particles RCP-30-5A-2; Spherotech, Lake Forest, IL) as described [46]. Nonfed mosquitoes were removed and, at 72 hr post blood feeding (equivalent to 8–10 days post-emergence), samples of three whole mosquitoes or three dissected midguts were placed in PBS, pulse sonicated, and filtered through a 35 μ m nylon mesh to remove tissue debris. A timepoint of 72 hr post blood feeding was selected to maximize the likelihood that remaining beads in the midgut would be excreted with the completion of blood digestion and, thereby, contribute little to no signal to whole body bead counts. Sample data were acquired with a FACScan flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 6.4.1; Tree Star, Ashland, OR). Beads per three midguts were quantified and subtracted from each analyzed sample of five replicates of three whole mosquitoes to remove the contribution of beads remaining in the midgut to whole body bead counts.

2.13 Statistical analyses

Data were tested for normality using Kolmogorov-Smirnov, D'Agostino-Pearson omnibus, and Shapiro-Wilk methods. Relative levels of HA-AstePTEN, MAPKs or FOXO proteins normalized to GAPDH were analyzed using one-way ANOVA and the Tukey-Kramer HSD test. qRT-PCR data that were normally distributed were analyzed using Fisher's exact test to determine if fold induction differed between treatment conditions. Non-normally distributed data were analyzed by Friedman's test for overall significance followed by Dunn's Multiple Comparison tests for pairwise comparisons. Infection data were first analyzed by ANOVA to determine whether NTG *A. stephensi* oocyst intensity differed among replicates. No significant differences were evident, so data were pooled across replicates and distributions were compared with a Mann-Whitney test for group differences. Infection prevalence was analyzed by Fisher's exact test to identify differences between treatment conditions. Survival analyses were performed using the Kaplan Meier method and differences between curves calculated using the Log-Rank test. Egg count data were analyzed using a factorial model followed by Tukey-Kramer HSD. Differences in bead counts between NTG and PTEN TG groups were determined by Student's t-test. Tests were performed using JMP 9.0 (SAS Institute Inc. 2010, Cary, NC 27513) or Prism 5.02 (GraphPad, LaJolla, CA). All differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Identification of the *A. stephensi* PTEN ortholog

The *A. stephensi* PTEN ortholog was identified using a combination of degenerate PCR and 5' and 3' RACE to identify the full length mRNA transcript from total RNA isolated from mosquito heads and whole bodies. The identified transcript was 2,332 bp long and encoded an open reading frame of 588 amino acids that most closely matches the predicted amino acid sequence for the *AaegPTEN3* splice variant (Fig. S1). The *AstePTEN* transcript encoded putative phosphatase (amino acids 17-187) and C2 (amino acids 190-316) domains at the N terminus. A conserved catalytic signature motif was identified in the putative encoded phosphatase domain (HCKAGKGR; amino acids 125-132). The encoded C terminus included a 120 amino acid extension following the final splice site and lacked a PDZ binding motif, which was consistent with *AaegPTEN3*. As with other mosquito PTENs, the predicted sequence for *AstePTEN* possesses stretches of glutamine repeats with unknown function in the C-terminal tail. *AstePTEN* shares 83% sequence identity to *A. gambiae* PTEN, 47–58% sequence identity to *A. aegypti* PTEN variants and 46% identity to *D. melanogaster* PTEN. We were unable to identify an *AaegPTEN6* splice variant ortholog in *A. stephensi* that encoded a PDZ binding domain following the final splice site. It is almost certain, however, that this *A. stephensi* splice variant exists based on the fact that the prominent PTEN variants in all other organisms, including those in *A. gambiae* and *A. aegypti*, encode PDZ binding domains.

3.2 Generation and characterization of PTEN TG *A. stephensi*

We generated TG *A. stephensi* to overexpress PTEN under the control of the midgut-specific CP promoter. An HA epitope was added at the encoded N terminus (HA-*AstePTEN*) to facilitate protein identification and the construct was inserted into the pBac[3XP3-EGFPafm] vector for transformation (Fig. 1A). The pBac[3XP3-EGFPafm]HA-*AstePTEN* and phsp-pBac helper plasmids were inoculated into 1,010 *A. stephensi* embryos resulting in 176 larvae (17.4%) and 101 adults (10%). Progeny from these G0 mosquitoes yielded four independent transgenic lines with EGFP expression (Fig. 1B and not shown), of which one (M8) failed to express HA-*AstePTEN* and a second had minimal expression (INT; Fig. S2). The M7 line produced significant quantities of the transgene protein, but also had a large degree of degradation that was notable as a dark, lower molecular mass band (Fig. S2). The F2 line had strong, consistent expression of the transgene (Fig. S2) and was selected for our studies. A homozygous F2 line was established through a series of self-crosses.

Inverse PCR was used to identify a 463 bp amplicon from the F2 line that included the putative transgene insertion site in the *A. stephensi* genome (Fig. S3). Using Basic Local Alignment Search Tool (BLAST) we identified an identical sequence from the *A. stephensi* genomic contig assembly that was accompanied by a predicted transcript sequence (www.vectorbase.org). The search identified a perfect match to *A. stephensi* contig 35541 and a predicted *A. stephensi* gene (ASTM000660-RA) of unknown function. However, a search of *A. stephensi* assembled RNA transcripts did not return any matches nor did searches of the *A. gambiae* and *A. aegypti* transcript and protein databases. A search of the non-redundant NCBI database also did not return any strong matches. The absence of a matching transcript in the *A. stephensi* transcriptome as well as the lack of orthologs in the genomes of *A. gambiae*, *A. aegypti* and other organisms strongly suggested that the pBac[3XP3-EGFPafm]CP-HA-PTEN transgene did not disrupt any transcriptionally active genes.

3.3 HA-AstePTEN transgene expression was localized to the midgut and enhanced by blood feeding

The *HA-AstePTEN* transcript was not detected in larval *A. stephensi*, but was detected in early and late pupae, 1–3 day-old previtellogenic adult females, and throughout the reproductive cycle (Fig. 1C). Expression of the HA-AstePTEN protein in adult females was midgut specific, with no protein detected in the carcass (whole body minus midgut; Fig. 1D). Consistent with myrAsteAkt protein expression driven by the CP promoter [6], HA-AstePTEN protein was detected in non-bloodfed mosquitoes (Fig. 1E). However, ingestion of a blood meal significantly increased expression of HA-AstePTEN protein in the midgut from 12–24 hr post-blood meal, with expression levels returning to non-bloodfed levels at 36–48 hr post-blood meal (Figs. 1E and 1F).

3.4 Repression of IIS activation in the midgut of homozygous PTEN TG *A. stephensi*

PTEN inhibits the PI3K/Akt branch of the IIS pathway. Therefore, we anticipated that overexpression of PTEN would result in decreased phosphorylation of proteins downstream of PI3K/Akt (e.g., FOXO). To address this assumption, we probed isolated midgut proteins from bloodfed PTEN TG and NTG *A. stephensi* for phosphorylated FOXO and for phosphorylated ERK, p38 MAPK, and JNK to assess other IIS proteins (ERK) and proteins associated with stress signaling (p38 MAPK, JNK). NTG and PTEN TG mosquitoes were fed blood meals of RBCs in saline with human insulin (1.7×10^{-4} μ M) or RBCs with saline only as a control. There was a trend toward increased phosphorylation of both FOXO and ERK in NTG mosquitoes in response to insulin relative to untreated NTG controls, but both basal and insulin-induced phosphorylation of FOXO were significantly inhibited in the midgut of PTEN TG mosquitoes (Fig. 2). Insulin did not increase phosphorylation of p38 MAPK or JNK in NTG females and phosphorylation levels of these proteins in the midguts of PTEN TG mosquitoes were not significantly different from those in insulin-fed NTG and untreated PTEN TG controls (Fig. 2).

3.5 Minimal impact of HA-AstePTEN overexpression on homozygous TG *A. stephensi* fecundity

Activation of IIS stimulates the production of 20-hydroxyecdysone in ovarian follicle cells and vitellogenin synthesis in the mosquito fat body [47,48] and, thereby, ultimately affects reproductive output. To determine whether HA-AstePTEN overexpression in the midgut affected reproductive capacity of TG females, we assessed the number of eggs laid by NTG and homozygous PTEN TG females over four consecutive reproductive cycles. There were no significant differences between the number of eggs laid by PTEN TG and NTG females during any of the four reproductive cycles (Fig. 3). Across the four reproductive cycles for each genotype, there were no significant changes in the number of eggs produced by NTG females, although the number of eggs produced did decrease with time. In contrast, a significant decrease in the number of eggs laid by PTEN TG females was observed in the fourth reproductive cycle relative to earlier reproductive cycles of these females (Fig. 3).

3.6 Reduction of homozygous TG *A. stephensi* lifespan by HA-AstePTEN overexpression

We hypothesized that HA-AstePTEN overexpression should increase lifespan based on observations from a variety of model organisms. To understand effects of transgene expression more completely, however, we analyzed lifespan of homozygous PTEN TG mosquitoes relative to NTG mosquitoes in both the presence and absence of insulin in the blood meal (Fig. 4). In all three experiments, insulin reduced the median lifespan of NTG *A. stephensi* relative to non-supplemented NTG controls (Fig. 4), confirming previous observations of lifespan reduction by insulin [8]. In the absence of insulin supplementation, PTEN TG *A. stephensi* survived longer than NTG controls, with median lifespan extension

of 16 to 26% ($P < 0.0001$; Fig. 4). Insulin supplementation reduced lifespan of PTEN TG *A. stephensi* relative to unsupplemented PTEN TG females, but insulin-fed PTEN TG females still survived 9 to 17% longer than unsupplemented NTG mosquitoes ($P < 0.0002$; Fig. 4).

3.7 HA-AstePTEN overexpression reduced prevalence and intensity of *P. falciparum* infection in homozygous TG *A. stephensi*

Given that decreased IIS activation in *D. melanogaster* and *C. elegans* has been associated with resistance to infection, we hypothesized that HA-AstePTEN overexpression would enhance resistance to *P. falciparum* in homozygous TG *A. stephensi*. Indeed, HA-AstePTEN overexpression in the midgut epithelium reduced both the prevalence of mosquitoes infected with *P. falciparum* and the intensity of infection relative to NTG controls. Ten days after the infectious blood meal, the percentage of mosquitoes with one or more oocysts decreased from an average of 73% in NTG controls to 39% in PTEN TG *A. stephensi* (Fig. 5A, $P = 0.0024$). In addition, the intensity of infection was significantly reduced ($P < 0.0001$) from an average of 2.0 oocysts/midgut (0–24; $n = 134$) in NTG controls to 0.95 (0–16; $n = 141$) in PTEN mosquitoes (Fig. 5B).

3.8 Parasite product-inducible immune gene expression was unchanged by HA-AstePTEN overexpression in homozygous TG *A. stephensi*

In *D. melanogaster*, mutations that inhibit IIS and overexpression of active FOXO in the absence of NF- κ B-dependent signaling result in enhanced synthesis of antimicrobial peptides in the midgut and other tissues [49], facilitating enhanced resistance in long-lived IIS mutants. These observations suggested that enhanced immune gene activity could be responsible for reduced intensity and prevalence of *P. falciparum* infection in PTEN TG *A. stephensi*. To test this hypothesis, we examined midgut expression levels of known anti-parasite immune genes (*NOS*, *LRIM1*, *TEP1*, *APL1*, *LRRD7*) and an immune gene marker (*DEF*) that are regulated by the principle immune signaling cascades defined by NF- κ B-dependent Toll, NF- κ B-dependent Immune deficiency (IMD) and/or Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT) [50] in the presence and absence of FPHP. Midgut expression levels were increased in response to FPHP in NTG females for *APL1*, *LRRD7*, *LRIM1*, and *TEP1* and for *LRIM1*, *NOS*, and *TEP1* in PTEN TG females (Fig. 5C). However, there were no significant differences between NTG and PTEN TG *A. stephensi* for FPHP-dependent expression for any gene. Hence, while FOXO activation has been shown to enhance immune gene activity in *D. melanogaster*, HA-AstePTEN-associated reduction in FOXO phosphorylation (Fig. 2B) was not consistent with immune gene activation and infection resistance in PTEN TG *A. stephensi*.

3.9 Overexpression of HA-AstePTEN increased expression of autophagy and stem cell biomarkers and enhanced midgut barrier integrity in homozygous TG *A. stephensi*

As discussed above, activated PTEN can enhance autophagy, which can contribute to enhanced pathogen resistance as well as enhanced epithelial barrier integrity and repair resulting from optimal stem cell maintenance and differentiation. Collectively, these mechanisms could contribute to enhanced resistance of PTEN TG *A. stephensi* to *P. falciparum* infection in the absence of prototypical NF- κ B-dependent immunity (Fig. 5C). To begin to examine these possibilities, we quantified expression of *escargot* and *prospero*, two genes associated with midgut stem cell development in *D. melanogaster* [51], as well as that of key autophagy genes *ATG6* and *ATG8*. In the fly, midgut stem cells (MSCs) divide to produce daughter enteroblasts (EBs) that then differentiate into digestive/absorptive cells (DCs) or peptidergic endocrine (ECs) cells. MSCs and EBs express *escargot*, while ECs secrete different peptide hormones and express the transcription factor *prospero*. At the initiation of autophagy, ATG6 is associated with nucleation of ATG proteins on the isolation membrane, which ultimately forms the autophagic membrane. ATG8 is associated with the

nascent autophagic membrane, is involved with elongation of this membrane, and remains associated with the mature autophagosome until it is trafficked to the lysosome to form the autolysosome. Although there were no significant differences in midgut expression for any biomarkers between 3 day-old homozygous PTEN TG and NTG females, we did see trends toward increased expression in some PTEN TG samples (Figs. 6A–D). In contrast, by 18 days post-emergence, midgut expression levels of all biomarkers were significantly increased in PTEN TG *A. stephensi* relative to NTG females (Figs. 6E–H), suggesting that autophagy and stem cell maintenance and differentiation in the midgut were enhanced by HA-AstePTEN overexpression. Because of the association of these processes with improved epithelial barrier integrity, we examined midgut permeability in PTEN TG and NTG females following ingestion of fluorescent nanoparticles in a supplemented blood meal [46]. In contrast to significantly enhanced permeability in midguts from *A. stephensi* that overexpressed HA-AsteAkt relative to NTG controls [46], homozygous PTEN TG *A. stephensi* were defined by extreme barrier integrity (Fig. 6I), with nearly complete lack of bead passage through the epithelium over the course of blood digestion relative to NTG females.

4. Discussion

When a mosquito takes a blood meal, she is exposed to a diversity of proteins in blood, including insulin that can activate IIS through an insulin receptor in the mosquito midgut [8,52]. In the present work, we overexpressed PTEN, an inhibitor of PI3K/Akt-dependent signaling, in the midgut epithelium of *A. stephensi* to examine effects that were predicted to be complementary to Akt overexpression in the same tissue [46]. As expected, HA-AstePTEN overexpression inhibited FOXO activation in the midgut (Fig. 2) and extended the lifespan of homozygous TG females relative to NTG controls (Fig. 4). In contrast, overexpression of HA-AsteAkt in the midgut significantly reduced *A. stephensi* lifespan [46], indicating that effects on lifespan by these IIS manipulations are indeed complementary. Intriguingly, lifespan extension by HA-AstePTEN expression was enhanced in the presence of insulin relative to unsupplemented NTG females, suggesting that some effects of PTEN are independent of IIS or perhaps that a reduction in lifespan by insulin stimulation is due, in part, to signaling that is independent of PI3K/Akt activation. Although the mechanism of lifespan extension is unclear as yet, our results were consistent with PTEN-dependent lifespan extension in multiple organisms [53].

As expected from model phenotypes of enhanced lifespan and resistance in both *D. melanogaster* and *C. elegans*, overexpression of HA-AstePTEN in the midgut resulted in enhanced resistance to *P. falciparum* infection. Unexpectedly, complete resistance to infection was also observed when HA-AsteAkt was overexpressed in the same tissue [46]. In the latter, Akt-induced changes in mitochondrial dynamics perturbed midgut homeostasis to dictate resistance that was directly attributable to parasite killing by very high levels of NO and reactive oxygen species [46]. While this response was protective against infection, the resulting stress combined with Akt-induced mitochondrial dysfunction degraded midgut barrier integrity and fitness of *A. stephensi*. Hence, quality control of mitochondrial function in the midgut is necessary for the maintenance of midgut health as reflected in energy homeostasis and tissue repair and renewal for barrier integrity.

In the present work, enhanced resistance due to overexpression of HA-AstePTEN was not associated with increased NF- κ B-dependent immunity in the midgut. While our panel of immune genes reflected gene products and/or signaling pathways with robust effects on modulation of *Plasmodium* infection, it is possible that unrepresented NF- κ B-dependent defenses account for observed effects of HA-AstePTEN overexpression on parasite development. Infection resistance, however, was also positively associated with increased

signatures for autophagy and epithelial regeneration and maintenance as well as a functionally improved midgut barrier (Fig. 6) in a manner analogous to positive PTEN regulation of junctional integrity and barrier function in mammalian intestinal epithelia [54]. Of relevance, proper functioning of tight junctions is dependent on actin polymerization [55] and the latter process – interrogated by RNAi in *A. gambiae in vivo* – was strongly inhibitory to midgut invasion of the murine parasite *Plasmodium berghei* [56]. Taken together, these observations affirm that transit of malaria parasites across a midgut barrier with enhanced epithelial junctions should be impaired. Further examination of this biology will be necessary to fully define the underlying mechanism(s) of resistance.

The phenotypes of reduced parasite infection and comparable reproductive output over an extended lifespan relative to NTG *A. stephensi* could be leveraged to develop transgenic, parasite-resistant mosquitoes that are competitive under natural conditions. Indeed, we can envision the use of PTEN TG *A. stephensi* as a base to which we can engineer additional transgenes (e.g., single chain antibodies) to achieve complete resistance in desirable genetic background. Further, such resistance – mediated by multiple, independent effector mechanisms – would be predicted to minimize the emergence of resistant strains of *P. falciparum*, a highly useful addition to the armamentarium for malaria control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HA-AstePTEN mRNA and protein expression was midgut-specific and induced by blood feeding in the F2 line of TG *A. stephensi*

A. Cartoon of the pBac[3XP3-EGFPafm]CP-HA-PTEN plasmid transformed into the F2 *A. stephensi* line. Below is the phsp-pBac helper plasmid source for *piggybac* transposase driven by the hsp70 constitutive promoter. **B.** EGFP is expressed in the eyes of F2 PTEN TG larvae. The top panel is a white light image of three 4th instar larvae. The first is a TG F2 individual showing the ventral side, the center larva is NTG from the dorsal side and the right larva is a PTEN TG larva from the dorsal side. The center panel shows the same mosquitoes under UV light and visualized with an EGFP filter. The PTEN TG larvae both show intense signal. The bottom panel is a merge of the top two panels demonstrating the localization of the EGFP signal in the eyes and thoracic ganglia. **C.** Expression analysis of the *HA-AstePTEN* transcript in larvae (L), early pupae (EP), late pupae (LP), previtellegenic adult females 1–3 d post emergence (PV Adults 1d, 2d, and 3d), adult females 6–24 hr after a blood meal (PBM 6h, 24h, and 48h), NTG control mosquitoes and a no-template control (NTC). The final lane in the No-RT actin control is a positive control. **D.** Expression of HA-AstePTEN protein is midgut specific. Protein was not detected in the midgut (MG) or carcass (Car) of NTG controls or in the carcass of PTEN TG mosquitoes. The protein was detected only in the midgut of PTEN TG mosquitoes. **E.** Expression profile of the HA-AstePTEN protein during a reproductive cycle. Non-bloodfed (NBF) and bloodfed (6hr, 12hr, 24hr, 36hr, 48hr, and 72hr) in heterozygous and homozygous PTEN TG females. **F.** Densitometry analysis comparing the ratio of HA-AstePTEN to the GAPDH loading control in homozygous PTEN TG mosquitoes. All expression studies were replicated a minimum of three times with independent cohorts of mosquitoes.

Figure 2. Phosphorylation of FOXO – but not ERK, p38 MAPK, or JNK – was significantly reduced in the midgut of homozygous PTEN TG *A. stephensi*

A. Total proteins were isolated from the midguts of NTG and PTEN TG mosquitoes at 2 hr post-blood meal. Proteins were blotted and probed with phospho-specific antibodies or with anti-GAPDH antibodies to assess protein loading. Representative western blots showing insulin (ins)-induced changes in protein phosphorylation in NTG and PTEN TG midguts relative to matched controls (ctl) fed an identical blood meal without insulin. Fold changes indicated below were calculated by dividing GAPDH-normalized phospho-protein levels in insulin-treated samples by GAPDH-normalized phospho-protein levels in matched controls (ctl). Control levels, set at 1.0, are indicated by a dash. **B.** Average expression of transgenic protein normalized to GAPDH loading controls and shown relative to levels in bloodfed

mosquitoes. Data are represented as means $\pm$ SEMs from five replicates with independent cohorts of mosquitoes.

Figure 3. Egg production declined over successive reproductive cycles in homozygous PTEN TG *A. stephensi*, but was not significantly different from that of NTG females

Eggs produced during the first four reproductive cycles are indicated for PTEN TG *A. stephensi* and NTG controls. The numbers below the bars (n) indicate the number of individual mosquitoes for which eggs were counted during each cycle. The numbers within the bars indicate the mean number of eggs produced. Significant differences are indicated by different upper case letters above the bars. * = $P < 0.05$.

Figure 4. Overexpression of HA-AstePTEN extended lifespan of homozygous TG *A. stephensi* relative to NTG females

For these studies, 3–5 day-old *A. stephensi* NTG or PTEN TG females were fed weekly blood meals of human RBCs and saline supplemented with 1.7×10^{-4} μ M insulin (NTG-Insulin or NTG Ins, PTEN-Insulin or PTEN Ins) or with RBCs and saline (NTG-Buffer or NTG B, PTEN-Buffer or PTEN B) alone. A total of 300–400 mosquitoes were used per treatment and experiments were replicated three times with separate cohorts of mosquitoes.

A. Survivorship curves from Experiment 3 showing the effects of insulin and HA-AstePTEN overexpression on *A. stephensi* lifespan. **B.** Summary of the sample size medians and significance for all treatment groups for Experiments 1–3. *P* values were calculated using the Log-Rank test and reflect comparison with matched controls at alpha = 0.05.

Figure 5. Overexpression of HA-AstePTEN in the midgut of homozygous TG *A. stephensi* reduced *P. falciparum* development without altering immune gene expression
 NTG and PTEN TG mosquitoes were fed blood meals with mature gametocytes of *P. falciparum* strain NF54. Midguts were dissected and oocysts counted at 10 days after infection. **A.** Prevalence of *P. falciparum* infection (percentage of midguts with at least one oocyst out of the total number of midguts dissected) in PTEN TG *A. stephensi* was reduced relative to the same in NTG females. Data were analyzed by Fisher's exact test to assess differences between groups and *P* values are noted on the graphs. **B.** PTEN TG mosquitoes had reduced numbers of oocysts per midgut relative to NTG controls. This experiment was replicated four times with independent cohorts of mosquitoes. There were no significant differences among oocysts in NTG controls from these replicates, so the data were pooled across replicates and the distributions compared with a Mann-Whitney non-parametric test. **C.** NTG and PTEN TG *A. stephensi* were provided with a blood meal plus FTPP or a blood meal with frozen and thawed uninfected RBCs as a control. At 24 hr post-feeding, midguts were dissected for RNA isolation for qRT-PCR. These assays were replicated 3–6 times with separate cohorts of mosquitoes. Data are represented as fold-change in immune gene expression in FTPP-treated *A. stephensi* relative to matched controls of the same genotype. No significant differences in FTPP-dependent changes in expression for any immune gene between PTEN TG and NTG *A. stephensi* were detected (two-tailed Student's *t*-test; $\alpha = 0.05$). Primers for *defensin* (DEF), *TEP1*, *LRIM1*, *APL1*, *LRRD7*, *nitric oxide synthase* (NOS), and *ribosomal protein S7* (RPS7) were as follows: DEF1F 5' - AGTCGTGGTCCTGGCGGCTCT-3', DEF1R 5' - ACGAGCGATGCAATGCGCGGCA-3', TEP1F 5' - TCAGATGCGCTATCGCCAGT-3', TEP1R 5' - GCTCAGATAGGCCATTGCATT-3', LRIM1F 5' -

GCGTCGGTTCGGAAAAGGAGCGG-3 , LRIM1R 5 -
TACATATCCCAATCGCGGATGGC-3 , APL1F 5 -
CGTATCGAGGACGAAACGTTCC-3 , APL1R 5 -
TGATACGTACAGTCGCTCCAGA-3 , LRRD7F 5 -
AAGCTGATCACACTCGATCTGT-3 , LRRD7R 5 -
TACGCACCATCACCGGGAACGA-3 , NOSF 5 -GACCAAACCGGTCATCCTGAT-3 ,
NOSR 5 -GGAATCTTGCAGTCAACCATTTC-3 , RPS7F 5 -
GAAGGCCTTCCAGAAGGTACAGA-3 , RPS7R 5 -CATCGGTTTGGGCAGAATG-3 .

Figure 6. Overexpression of HA-AstePTEN increased midgut expression of autophagy and stem cells biomarkers and enhanced midgut barrier integrity in homozygous TG *A. stephensi* Midgut expression levels of genes associated with autophagy (*ATG6*, *ATG8*) and stem cell maintenance and differentiation (*escargot*, *prospero*) were not different in 3 day-old PTEN TG and NTG *A. stephensi* (A–D), but were significantly increased in PTEN TG relative to NTG females at 18 days post-emergence (E–H). These analyses were performed on midgut RNAs from at least five independent cohorts of *A. stephensi*. Each data point represents target gene expression from one biological replicate. Values were normalized to NTG levels (indicated as 1.0) and means are indicated as bars for each treatment. Data were analyzed by Student's t-test (alpha = 0.05) or Mann-Whitney test (alpha=0.05). *P* values are noted on the graph. Primers for *ATG6*, *ATG8*, *escargot*, *prospero* and *ribosomal protein S7* (RPS7) were as follows: *ATG6F* 5'-GCGCGAGTATACGAAGCAT-3', *ATG6R* 5'-

GCTTCTCTAGCTGGCTCTGG-3 , ATG8F 5'-GCCATCATTCTTTGGAGAGC-3 ,
ATG8R 5'-TGCTATTAAAATGCGTAGAATGG-3 , ESGF 5'-
GAGAAGCCGTTTCGTGTG-3 , ESGR 5'-CACGTCGGACACTTGAAGC3 , PROSF 5'-
TGCGAAATGTGATGATTTGC-3 , PROSR 5'-GATCACGTCGCTCTTCTCCT-3 ,
RPS7F 5'-GAAGGCCTTCCAGAAGGTACAGA-3 , and RPS7R 5'-
CATCGGTTTGGGCAGAATG-3 . **I.** Bead numbers per three whole mosquitoes minus
bead numbers in three paired midguts from the same groups at 72 hr post-feeding are
represented as individual dots (means are indicated as bars). These numbers averaged
 341 ± 60 for bloodfed NTG and 0.6 ± 1 for bloodfed PTEN TG at 72 hr post-feeding. Midgut
beads (not shown) averaged 15 for NTG and 10 for PTEN TG at 72 hr post-feeding, so
midgut beads accounted for less than 4% of NTG whole body bead counts. Data were
analyzed by Student's t-test, *P* value is shown.