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Increased Akt signaling in the fat body of *Anopheles stephensi* extends lifespan and increases lifetime fecundity through modulation of insulin-like peptides

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Abstract

Insulin-like peptides (ILPs) and the insulin/insulin-like growth factor 1 signaling (IIS) cascade regulate numerous physiological functions, including lifespan, reproduction, immunity, and metabolism, in diverse eukaryotes. We previously demonstrated that in female *Anopheles stephensi* and *Aedes aegypti* mosquitoes, activation of the IIS cascade in the fat body led to a significant increase in lifespan. In this work, we elucidated two putative mechanisms in *A. stephensi* behind the observed lifespan extension and assessed whether this lifespan extension confers an overall fitness advantage to the mosquito. Specifically, we demonstrated that increased Akt signaling in the mosquito fat body following a blood meal significantly suppressed the expression of *ILP2* in the head. Moreover, overexpression of active Akt in the fat body altered the expression of a putative insulin binding protein ortholog, Imaginal morphogenesis protein-Late 2 (*Imp-L2*), in response to transgene expression. Combined, these two factors may act to reduce overall levels of circulating ILP2 or other ILPs in the mosquito, in turn conferring increased survival. We also examined the impact increased fat body IIS had on lifetime fecundity and demonstrated that transgenic female mosquito populations had higher lifetime fecundity relative to non-transgenic sibling controls. These studies provide new insights into the complex hormonal and molecular mechanisms regulating the interplay between IIS, aging, and reproduction in this important vector of human malaria parasites.

Graphical Abstract

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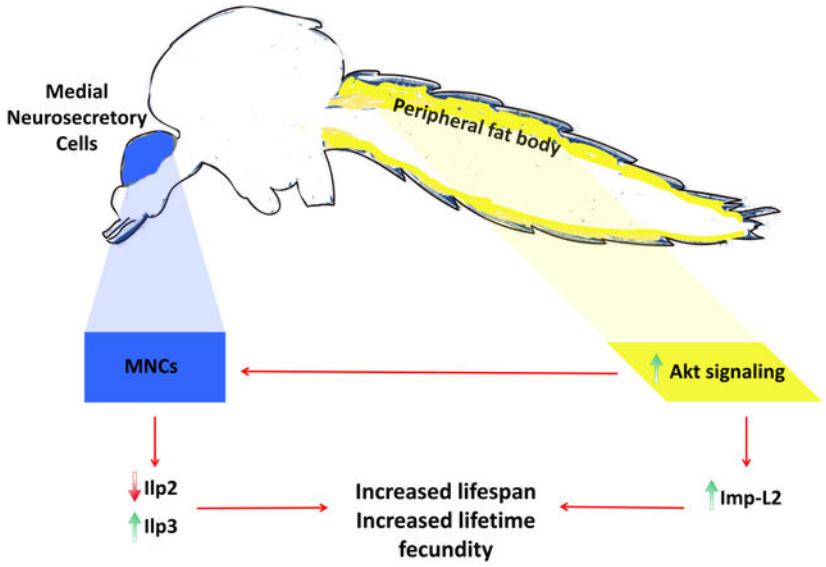

Keywords

Insulin-like peptide; Imaginal morphogenesis protein-Late 2; Imp-L2; insulin binding protein; insulin signaling; mosquito

1. Introduction

Malaria is a devastating disease afflicting over 200 million people annually and is a leading cause of death among children under the age of five in sub-Saharan Africa (World Health Organization 2018). *Plasmodium* parasites, the causative agent of malaria, are transmitted by *Anopheles* spp. mosquitoes including *Anopheles stephensi*, a key malaria vector in the Indian subcontinent that recently expanded its geographic range into East Africa (Faulde et al., 2014). In an effort to combat malaria, a combination of vector control and anti-malarial therapies resulted in a dramatic reduction in malaria cases and malaria-related mortality. Globally, from 2000 and 2015, there was a 37% decrease in malaria cases and a 60% decline in malaria-related deaths, with approximately 440,000 deaths in 2015 as compared to 839,000 in 2004 (Bhatt et al., 2015). Despite these successes, insecticide-based tools are threatened by the rapid spread of resistance to critical insecticides such as pyrethroids and *Plasmodium* parasites have developed resistance against the most effective anti-malarial drugs (Thomsen et al., 2014; Toe et al., 2014). These issues highlight the urgent need for novel malaria control strategies. One strategy is to genetically modify key *Anopheles* spp. vectors and render them unable to transmit human *Plasmodium* parasites (Marshall and Taylor 2009). While numerous anti-*Plasmodium* effector molecules have been identified, the majority of these incur fitness costs when engineered into the mosquito vector. Genetic drive strategies such as maternal effect dominant embryonic arrest (MEDEA) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) may be able to overcome some of these fitness costs, but other approaches to minimize fitness load should be explored. An alternative approach is to manipulate key signaling cascades, such as the

insulin/insulin growth factor 1 signaling (IIS) cascade, to optimize critical life history traits that impact both fitness and pathogen resistance.

The IIS cascade is an evolutionarily conserved pathway that regulates diverse physiologies in a broad range of eukaryotes including insects, nematodes, and mammals (Antonova-Koch et al., 2013; Okamoto and Yamanaka 2015). IIS functions in a tissue-specific manner, with important signaling centers located in the midgut, brain and fat body affecting both local and systemic signaling networks (Antonova-Koch et al., 2013). Fat body IIS in insects, including mosquitoes, has been implicated in the regulation of lifespan, reproduction, and innate immunity (Antonova-Koch et al., 2013). In this work, we explored how fat body IIS influences the balance between lifespan and fecundity, two key life history traits critical to mosquito transmission of pathogens.

Lifespan is one of the most complex physiologies regulated by the IIS cascade (Antonova-Koch et al., 2013; Okamoto and Yamanaka 2015). Numerous studies in a variety of organisms implicate both insulin-like peptides (ILPs) and IIS as important regulators of senescence (Okamoto and Yamanaka 2015). However, the molecular mechanisms of lifespan control are not yet fully elucidated. In *D. melanogaster*, suppression of the IIS cascade, either through mutation of the insulin receptor (InR), knockdown of the insulin receptor substrate ortholog chico or ectopic expression of phosphatase and tensin homolog (PTEN) resulted in increased lifespan (Tatar et al., 2003; Slack et al., 2011; Tu et al., 2002; Clancy et al., 2001; Hwangbo et al., 2004). In *C. elegans*, genetic changes in daf-2 (InR), age-1 (phosphatidylinositol 3-kinase) and daf-16 (forkhead box protein O) all led to dramatic changes in nematode lifespan. In *D. melanogaster*, ablation of the medial neurosecretory cells (MNCs) reduced levels of ILPs, the activating ligands for the IIS cascade, and increased fly survival (Wessels and Perez-Pomares 2004; Broughton et al., 2005). Identifying the impact of individual ILPs on lifespan has been challenging due to that fact that many invertebrates have multiple ILPs that have both discreet and overlapping roles and can exhibit compensatory expression (Broughton et al., 2008b; Min et al., 2008). Success in knocking out each of the seven individual *dilp* genes in *D. melanogaster*, however, using homologous recombination revealed that the absence of *dilp2* was sufficient to significantly increase survival (Grönke et al., 2010). A subsequent study demonstrated that overexpression of *dilp6* in the adult fat body suppressed *dilp2* and *dilp5* mRNA in the brain, which in turn, also extended lifespan (Bai et al., 2012).

The IIS cascade also plays a critical role in the hormonal regulation of reproduction during both steroidogenesis in the ovary and vitellogenesis in the fat body. In mosquitoes, ILPs synthesized by the brain activate IIS in the follicle cells surrounding the developing oocyte to stimulate the synthesis of 20-hydroxyecdysone (Brown et al., 2008). In the fat body of *Ae. aegypti*, the IIS cascade, along with amino acids and ecdysteroids, is essential for the complete activation of vitellogenin synthesis (Hansen et al., 2014). Notably, systemic activation of *Ae. aegypti* IIS through knockdown of the IIS inhibitor PTEN via RNAi significantly increased egg production (Arik et al., 2009). Further, both *Aaeg*ILP3 and *Aaeg*ILP4 activate ovarian steroidogenesis and yolk uptake by the developing oocytes (Brown et al., 2008; Wen et al., 2010) and disruption of *Aaeg*ILP7 and 8 by CRISPR-Cas9 resulted in defects in the development of primary oocytes (Ling et al., 2017). In *D.*

melanogaster, reduced IIS activity can decrease or even prevent female reproduction. Specifically, ablation of insulin-producing cells (IPCs) in the female brain (Broughton et al., 2005) and knockout of *dilp2*, *dilp3*, and *dilp6* led to significant reductions in fecundity (Grönke et al., 2010). Inhibition of IIS through overexpression of forkhead transcription factor (dFOXO) in the fruit fly peripheral fat body resulted in reduced fecundity, while dFOXO overexpression in the pericerebral fat body showed normal fecundity (Hwangbo et al., 2004), suggesting tissue-specific regulation of the balance between lifespan and reproduction. Finally, loss-of-function mutations in *InR* and the insulin receptor substrate *chico* resulted in increased female sterility in *D. melanogaster*, primarily due to the failure of vitellogenesis (Tatar et al., 2001; Drummond-Barbosa and Spradling 2001).

In vertebrates, the activities of circulating insulin and insulin growth factor 1 (IGF-1) are titrated through binding to IGF-1 binding proteins (IGFBPs) that prevent receptor activation by IGFBP-bound hormones. An insulin binding protein was recently characterized in the fruit fly and is known as Imaginal morphogenesis protein-Late 2 (Imp-L2) (Flatt et al., 2008; Honegger et al., 2008). Imp-L2 is a putative ortholog of human IGFBP7, which unlike most IGFBPs, binds insulin with high affinity and IGF-1 with low affinity (Yamanaka et al., 1997). In *D. melanogaster*, Imp-L2 acts as a negative regulator of IIS during development, controlling cell growth and antagonizing the activity of dilp2 (Honegger et al., 2008). Notably, removal of the germline late in fruit fly development resulted in extended lifespan and increased *Imp-L2* transcript levels (Flatt et al., 2008), suggesting that Imp-L2 reduces IIS activity by binding to circulating dilps. In this context, *D. melanogaster* Imp-L2 has been shown to bind dilp2 and dilp5 in the adult fly and enhanced expression of *Imp-L2* resulted in increased *dilp2*, *dilp3*, and *dilp5* transcript levels. More importantly, induced expression of *Imp-L2* in the midgut/fat body increased *dilp2* mRNA levels, suggesting that a feedback loop occurs between the fly's head and fat body. Finally, overexpression of *Imp-L2* in both *dilp*-producing cells and in the fat body resulted in extended lifespan (Alic et al., 2011). Collectively, these data suggest that Imp-L2 negatively regulates IIS in insects by binding and inactivating circulating ILPs, which extends lifespan.

Previously we demonstrated that overexpression of Akt in the fat body of *A. stephensi* resulted in a 20% increase in the survival of female transgenic (TG) mosquitoes as compared to non-transgenic (NTG) sibling controls reared under identical conditions (Arik et al., 2014). We also observed a significant increase in the expression of vitellogenin proteins in the fat bodies of TG females relative to controls, although we did not observe changes in overall egg production or viability during the first or second reproductive cycles (Arik et al., 2014). The extension of lifespan through increased fat body IIS was surprising and the mechanism that contributed to the extended lifespan was unclear. Thus, in this study, we have attempted to elucidate possible mechanisms for the extended lifespan phenotype that we observed in TG *A. stephensi*. In addition, we examined the impact of increased fat body IIS on vitellogenesis and lifetime fecundity to define the balance or tradeoffs between lifespan and fecundity in this important malaria vector species.

2. Materials and Methods

2.1 Mosquito rearing

A. stephensi (Indian) mosquitoes were reared at 27°C and 75% relative humidity in a 16/8 h light/dark photoperiod as previously described (Arik et al., 2014). During each generation, the Vg-myrAsteAkt-HA TG line was outcrossed with wild type (WT) colony mosquitoes to maximize genetic diversity and minimize fitness effects. For experiments, TG mosquitoes were crossed with WT mosquitoes resulting in a 50:50 mix of TG and NTG sibling mosquitoes reared in the same pan (identical crowding, food availability, etc). TG and NTG mosquitoes were separated based on eye fluorescence at the pupal stage using an Olympus SZX10 fluorescent stereomicroscope with EGFP filters and allowed to emerge as adults into two separate 3.8 L cartons. For line maintenance and experiments, female mosquitoes were provided human blood (American Red Cross; IBC protocol #2010–014) via artificial membrane feeders.

2.2 RNA extraction and cDNA synthesis

A total of five *A. stephensi* females were dissected per biological replicate to obtain various tissues, including the head, midgut, and abdominal wall for total RNA isolation. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD) per manufacturer's instructions and the concentration of total RNA in each sample determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, West Palm Beach, FL). Total RNA was DNase-treated (Fermentas, Thermo Scientific, West Palm Beach, FL) to eliminate genomic DNA contamination and cDNA was prepared from the DNase-treated RNA using the High-Capacity cDNA Reverse Transcription Kit per manufacturer's instructions (Applied Biosystems, Wobum, MA).

2.3 Identification of *A. stephensi* Imp-L2

To identify the *A. stephensi Imp-L2* ortholog, sequences of conserved regions of the immunoglobulin (Ig) superfamily containing two Ig C2-like domains of Imp-L2 from *Anopheles gambiae* (CP2953) and *D. melanogaster* (CG15009-RB) were used to search all six reading frames of the *A. stephensi* genome and predicted transcript database (http://vectorbase.org) using tblastn. The upstream and downstream sequences of significant matches were translated and examined for additional encoded conserved regions of Imp-L2 to identify ASTE008413-RA as the *A. stephensi Imp-L2* ortholog.

2.4 *A. stephensi ILPs*, *vitellogenin* and *Imp-L2* transcript analysis

A total of 100 female TG and NTG *A. stephensi* (3–5 days post-emergence) from a single generational cohort were provided with a single blood meal and 10% sucrose *ad libitum* through soaked cotton pads that were replaced every other day. Mosquito heads, midguts and abdominal body walls (after removal of other tissues) were dissected from five females at various time points prior to and following a blood meal (0 hour (h) or non-bloodfed (NBF), 6h, 24h, 36h, and 48h) for total RNA extraction and cDNA synthesis. Optimal annealing temperatures for each primer set were determined using gradient PCR with an annealing range of 55°C to 65°C. Transcript expression patterns for the five *A. stephensi ILPs,*

vitellogenin (this primer set amplifies both *AsteVg1* and *AsteVg2*, but the high sequence identity makes it impossible to differentiate between the two) and *Imp-L2* were determined by qPCR using a Bio-Rad CFX Real-Time System Thermocycler (Bio-Rad, Hercules, CA). Each sample was assayed in triplicate using Maxima SYBR Green/ROX qPCR Master Mix (2X; Thermo Scientific, Waltham, MA). Expression levels of these transcripts were compared between TG and NTG mosquitoes to determine the effect of increased fat body IIS on expression. Transcript levels were normalized to ribosomal protein S7 control, with fold changes in mRNA expression between TGs and NTGs calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ method described by Livak et al 2001. The qPCR assays were performed using a minimum of three separate cohorts (biological replicates) of *A. stephensi* at each time point.

2.5 Lifetime fecundity assay

Approximately 120 to 130 TG and NTG adult female *A. stephensi* mosquitoes from a heterozygous TG and WT cross were separated into two cages. Mosquitoes were provided with human blood daily via membrane feeders and allowed to feed on 10% dextrose *ad libitum*. Oviposition substrates were placed into each cage 48 h after the initial blood meal and changed daily until the final female mosquito perished. Eggs were counted using Image-J 1.42 software (NIH) following the protocol described in (Mains et al., 2008). In addition, dead females were counted and removed daily to establish survivorship curves as previously described(Arik et al., 2014)These experiments were replicated a minimum of three times using unique biological cohorts.

2.6 Statistical analysis

For lifespan studies, the survival curves were analyzed in JMP 13 software using Log-rank (Mantel-Cox) analysis. To assess differences between survivorship and egg clutch size we performed linear regression. For all other data sets, significance was determined by Student's t-test ($p<0.05$) using Graphpad Prism 7.

3. Results

3.1 Overexpression of myr-AsteAkt-HA in the mosquito fat body suppressed *ILP2* and induced *ILP3* transcript expression in the head

Transcript expression of all five *ILP*s prior to and throughout the reproductive cycle (NBF, 6h, 24h, 36h, 48h) was measured to establish relative expression between TG mosquitoes and NTG sibling controls (Figures 1, 2 and S1). While expression levels of *ILP1* in the head were unchanged (Figure 1A), expression of myr-AsteAkt-HA in the fat body significantly reduced *ILP*2 transcript expression in the head of female TG *A. stephensi* relative to NTG siblings. Specifically, suppression of *ILP*2 expression in the head was observed at 24h and 36h post blood meals (Figure1B; $p=0.014$ *and* $p=0.004$ respectively). These timepoints are consistent with myr-AsteAkt-HA expression that begins at 12–24h post feeding and rises to maximal levels in the fat body at 36–48h after the blood meal (Arik et al., 2014). In contrast to *ILP2*, expression of *ILP*3 in the head was significantly increased at 24h post blood meal (Figure 1C; $p=0.002$) relative to NTG controls and returned to comparable NTG control levels by 36h post blood meal. We did not observe significant differences in the expression of *ILP*4 in the head between TG and NTG siblings, although a modest increase was

observed at 24h (Figure 1D). Moreover, as with *ILP1* (Figure 1A), we detected only minimal expression of *ILP5* in the heads of female TG and NTG *A. stephensi* mosquitoes (Figures 1E and S4).

In contrast to the head samples, we observed that overexpression of myr-AsteAkt-HA in the fat body had no impact on abdominal *ILP1*, *ILP2, ILP3 and ILP5* transcript levels at the five time points examined (Figures 2A, B, C and E). We did observe, however, significantly increased levels of *ILP*4 transcript at 48h post blood meal (Figure1D; $p=0.011$), but not during earlier time points. Similar to the head, we detected only minimal expression of *ILP1* and *ILP5* in the abdominal body wall at the five different time points. Only minimal expression levels of *ILP*s were observed in midgut samples from both TG and their NTG siblings (Supplemental Figures S1A–E).

3.2 Activation of Akt signaling in the fat body induced *Imp-L2* transcript expression

Prior to *myr*-AsteAkt-HA transgene expression (NBF and 6h) we observed no significant differences between expression of *Imp-L2* in the whole body or abdominal body wall of TG and NTG mosquitoes (Figures 3A–B and C–D). However, following expression of *myr*-AsteAkt-HA and activation of the Akt signaling pathway (24h and 36h) we observed a significant decrease in *Imp-L2* expression (~5 fold, $p=0.03$) in TG relative to NTG *A. stephensi* mosquitoes at 24h, buta large significant increase (~60 fold, $p<0.001$) at 36h. We observed a similar, but non-significant, trend of *Imp-L2* mRNA expression in the whole body between TG and NTG mosquitoes (Figure 3A–B). Combined, these data suggest that Imp-L2 could moderate systemic IIS through the binding and inactivation of circulating ILPs, resulting in compensatory transcription, in a manner similar to that observed in *D. melanogaster* and *C. elegans* (Alic et al., 2011).

3.3 Effect of myr-AsteAkt-HA expression on *vitellogenin* transcript expression

IIS in the fat body plays a critical role in vitellogenin synthesis and ultimately egg production. We previously demonstrated that increased fat body IIS led to enhanced vitellogenin protein levels in both *A. stephensi* and *Ae. aegypti* (Arik et al., 2014). To confirm whether overexpression of Akt signaling in the fat body also impacts *Vg1*/*2* mRNA expression in *A. stephensi*, we examined fat body *Vg1*/*2* transcript expression in both TG and NTG *A. stephensi* at various time points (NBF, 6h, 24h, 36h and 48h) after the blood meal. In TG *A. stephensi* we observed a significant increase in *Vg1/2* expression in the abdominal wall body at 24, 36, and 48h (10 to 65 fold; $p<0.05$) after the blood meal relative to NTG mosquitoes (Figure 4C and D), consistent with myr-AsteAkt-HA transgene expression and our previous vitellogenin protein expression results (Arik et al., 2014). In assays of whole body samples, we observed a significant increase in *Vg1/2* expression in TG mosquitoes at 36h post-blood meal (~70 fold; $p<0.05$), but and no significant change in the absence of a blood meal (NBF) or at 6h, 24h, or 48h after a blood meal (Figure 4A and B).

3.4 Impact of myr-AsteAkt-HA on lifetime fecundity

Even though vitellogenin transcript and protein levels were significantly increased in TG *A. stephensi*, we did not observe significantly increased egg production during the first or second reproductive cycles (Arik et al., 2014). The lack of effect on the first reproductive

cycle was not surprising, however, since myr-AsteAkt-HA transgene expression and the corresponding increase in IIS occurred late in the reproductive cycle after the critical window of IIS regulation of reproduction had passed (Arik et al., 2014). However, the observed increases in vitellogenin protein (Arik et al., 2014) and transcript levels (Figure 4) coupled with a lack of effect of transgene expression on fecundity during the second reproductive cycle was unexpected. To examine this surprising result in more detail, we examined the lifetime fecundity of TG and NTG mosquitoes provided access to daily blood meals, which ensured ample opportunities for acquiring blood meals and sustained expression of the transgene. Consistent with our previous study (Arik et al., 2014) survival curves from our three new lifetime fecundity assays with distinct cohorts of TG and NTG mosquitoes again revealed a significant lifespan extension in two of the three replicates (Figures 5A–C). In replicates represented in Figures 5A and 5B, TG females survived for an average of 22 and 21 days as compared to 19 and 13 days respectively for the NTG controls, resulting in a significant increase in survivorship of 15% ($p=0.013$) to 47% ($p=0.0067$). In the replicate represented in Figure 5C, TG mosquitoes displayed a similar trend of increased survivorship relative to NTG controls (26 days TG vs 19 days NTG), but the difference in survival curves was not significant ($p=0.432$). These survival assays, in combination with all four significant assays in our previous work (Arik et al., 2014), reconfirm that increased fat body IIS improves adult survival, although some variation is observed. Analyses of three independent lifetime fecundity experiments, we found that caged populations of TG *A. stephensi* laid more eggs relative to NTG siblings (Figures 5D–F). We then compared the three combined lifetime fecundity assays using paired t-test and found that caged populations of TG *A. stephensi* laid significantly more eggs relative to NTG siblings ($p=0.049$; Figure 5G). However, the number of eggs laid by TG and NTG mosquitoes within individual gonotrophic cycles were not significantly different, even during later cycles when longer lived mosquitoes might be expected to have reduced egg production to offset their extended lifespans (Figures S2A–C). Notably, cumulative egg production from the caged TG and NTG populations revealed that TG mosquitoes start producing more eggs by approximately the fifth reproductive cycle when survival curve separation is taking place (Figures S2D–F). This observation suggests that the increased lifetime fecundity observed in TG mosquitoes was due to the increased survivorship of the TG line and not to an increase in egg production per mosquito. In fact, when total egg production is divided by the number of surviving mosquitoes in the caged population (to estimate average eggs produced per female) we did not observe a significant difference in egg production between TG and NTG mosquitoes (Figures S3A–C), again suggesting that the observed increases in the lifetime fecundity of TG caged populations were due to the increased survival of the TG line. Finally, when we compared egg production per reproductive cycle against percent survivorship (Figure S3D) we did not see a significant difference in the TG and NTG linear regression slopes of two of the three slopes (replicate 2 (p=0.806) and 3 (p=0.148)), and only a modestly significant difference in the slope of replicate 1 (0.0473), again indicating that increased survival was responsible for the observed increase in lifetime fecundity. Importantly, these data also demonstrate that a tradeoff between lifespan and fecundity is not absolute and that these two physiologies can be uncoupled.

4. Discussion

In our previous work, we demonstrated that overexpression of active Akt in the fat body of *A. stephensi* and *Ae. aegypti* significantly extended mosquito lifespan (Arik et al., 2014). However, the mechanism regulating this increased lifespan was unknown, as was the impact that lifespan extension had on overall reproductive capacity. For the lifespan effects we observed, studies in *D. melanogaster* suggested a possible explanation. *D. melanogaster* encodes dilps that have distinct temporal and tissue specific expression patterns (Brogiolo et al., 2001; Grönke et al., 2010; Garelli et al., 2012; Broughton et al., 2005; Grönke et al., 2010; Garelli et al., 2012). Of these, only *dilp2* has been shown to have a marked impact on lifespan when knocked out via ends-out homologous recombination (Grönke et al., 2010). Knockout of *dilp2* led to significant lifespan extensions in both male and female flies, although partial knockdown via RNAi did not elicit this phenotype (Broughton et al., 2008a). More recently it was demonstrated that increased IIS in the fat body of *D. melanogaster*, induced through the fat body specific overexpression of *dilp6*, led to a significant decrease in *dilp2* expression in the head (Bai et al., 2012). These results led us to explore the impact of increased fat body Akt signaling on *ILPs* expression in *A. stephensi*.

The five *ILP*s are predominantly expressed in the *A. stephensi* brain, but can also be found in various other tissues such as the midgut and fat body (Marquez et al., 2011). In this work, we demonstrated that when myr-AsteAkt-HA transgene expression in the fat body was maximal (24–36h post blood meal) and a corresponding increase in fat body IIS was observed (Arik et al., 2014) transcript expression of *ILP2* was significantly downregulated in the head (Figure 1B). Based on phylogenetic analysis of the complete ILP coding regions, *A. stephensi* ILP2 is most closely related to dilp3, but is also quite similar to dilp2. A closer examination of the biologically active domains (i.e. the A and B chains) of *A. stephensi* ILP2/3 and dilp2/3, A-chain homology suggests that *A. stephensi* ILP2 is at least as similar to dilp2, if not more so, than dilp3 (Figure 5S). This makes it is difficult to infer physiological functions of the *A. stephensi* ILPs simply by phylogenetic relatedness to the dilps. With that said it is certainly feasible, considering the extended lifespan and decreased expression of *ILP2* in the TG line, that *A. stephensi* ILP2 shares many of the lifespan regulatory functions attributed to dilp2. We also observed a significant increase in *ILP3* transcript levels in the head when fat body Akt signaling was increased (24h post blood feeding; Figure 1C), but not at other time points. In *D. melanogaster*, when individual dilps were knocked out, compensatory expression of other *dilps* has been observed (Grönke et al., 2010). For example, the knockout of dilp2 led to compensatory expression of *dilp3* in the brain, which may explain the observed increase in *ILP3* expression. Until a functional analysis of *A. stephensi* ILP2 is conducted it will be difficult to attribute the observed lifespan extension in this line directly to the reduced expression of *ILP2*.

In addition to directly regulating expression of *A. stephensi ILPs* in the head, increased IIS in the fat body may indirectly regulate ILP activity via the expression of the putative insulin/IGF-1 binding protein ortholog *Imp-L2*. In *D. melanogaster*, overexpression of *Imp-L2*, either ubiquitously or in specific tissues (fat body or *dilp*-producing cells) led to an extension of lifespan, (Alic et al., 2011) and Imp-L2 directly bound and inactivated dilp2 (Honegger et al., 2008; Alic et al., 2011; Grönke et al., 2010). In this work, we identified the

Imp-L2 ortholog in *A. stephensi* and demonstrated that increased fat body IIS through myr-AsteAkt-HA expression influenced *Imp-L2* expression in *A. stephensi* fat body. Importantly, we observed a nearly 60-fold increase in *Imp-L2* transcript expression in the abdominal body wall at 36h post blood meal in TG mosquitoes relative to NTG controls (Figure 3C–D) corresponding to high levels of fat body IIS in TG *A. stephensi*. This suggests a second mechanism for the unexpected lifespan extension in which circulating ILP levels are reduced due to increased binding and inactivation by ImpL2, which would dampen systemic IIS and extend *A. stephensi* lifespan. We recognize that transcript expression may not necessarily correlate with biological function of ILPs, which are stored as peptides in the MNCs and secreted as needed. At this time, however, experimental tools to address ILP regulation of IIS in *A. stephensi* are not available. Nevertheless, the dramatic changes in the transcript levels of *ILP2* (~20–100 fold reduction), *ILP3* (~70 fold increase) and *Imp-L2* (~60 fold increase) in response to increased Akt signaling in the *A. stephensi* fat body are strongly suggestive of a role in lifespan regulation. Collectively, results from our studies in mosquitoes and others in *D. melanogaster* suggest that lifespan regulation by fat body IIS is likely controlled systemically by at least two mechanisms: the suppression of ILPs, particularly ILP2, in the brain and the enhanced expression of the IGF binding protein *Imp-L2* to inactivate circulating ILPs.

The evolutionary theory of aging suggests that a selective advantage exists for species capable of responding to reproductive opportunities by increasing reproductive output, even when this increased fecundity shortens lifespan (Partridge and Harvey 1988). This tradeoff between reproduction and lifespan has been observed in organisms ranging from nematodes to mammals (Nasiri Moghadam et al., 2015). However, this tradeoff is not absolute and there are multiple examples where these physiologies are uncoupled. For example, *C. elegans age-1* mutants are long-lived, yet do not have a corresponding loss of fecundity (Johnson et al., 1994). Similarly, certain long-lived *C. elegans daf-2* mutants do not exhibit reduced reproduction (Gems et al., 1998; Kenyon et al., 1993). The influence of IIS on the balance between lifespan and reproduction, however, is both tissue- and stage-specific. For example, silencing *daf-2* with RNAi in pre-adult *C. elegans* extends lifespan but decreases fertility, while knockdown of *daf-2* in adults promotes longevity without impairing reproduction (Dillin et al., 2002). In *D. melanogaster*, overexpression of dFOXO in the pericerebral fat body extends life span but does not reduce fecundity (Hwangbo et al., 2004), in some cases, lifespan extension is even associated with increased reproductive output (Partridge and Fowler 1992). Fruit flies with mutations in *Indy*, a gene that encodes a transporter protein for Krebs cycle intermediates, are long-lived and exhibit increased fecundity relative to wild type flies (Marden et al., 2003). Similarly, female *D. melanogaster* with a mutation in the ecdysone receptor (*EcR*) show increased lifespan as well as greater age-specific fecundity and fertility relative to control flies (Simon et al., 2003).

In this study, we compared lifetime fecundity of both TG and NTG mosquitoes and found that even though TG mosquitoes survived longer than NTG mosquitoes, egg production did not differ significantly during each reproductive cycle (Figures 2SA–C and 3SA–D). In other words, TG mosquitoes do not appear to compensate for their increased lifespans by reducing egg production. Because of this, we found that the lifetime fecundity of caged populations of TG mosquitoes was significantly higher than their NTG counterparts due to the fact that

their increased survivorship increased their potential for additional reproductive cycles later in life (Figures 5D–G). These results suggest that, under certain conditions and manipulations, lifespan and reproduction can be uncoupled in *A. stephensi*. It is important to note that most studies demonstrating uncoupling of lifespan and reproduction, including ours, were conducted under optimal conditions with abundant resources. Under suboptimal conditions, the physiological tradeoff between lifespan and reproduction may be more intractable. For example, long-lived *C. elegans age-1* mutants had lower fitness than wild type nematodes when they were nutritionally stressed (Walker et al., 2000). Similarly, under optimal nutritional conditions long-lived *D. melanogaster Indy* mutants produced more eggs than control flies, however *Indy* mutant females produced fewer eggs on a decreased calorie diet, suggesting that Indy mediates a conditional tradeoff between survival and reproduction (Marden et al., 2003).

In summary, we identified two possible mechanisms to explain the unexpected lifespan extensions we observed in *A. stephensi* and *Ae. aegypti* engineered for increased abdominal fat body IIS. First, we established that overexpression of Akt in the *A. stephensi* fat body following blood feeding led to the suppression of *ILP2*, a potential ortholog for *D. melanogaster dilp2* that regulates lifespan (Grönke et al., 2010). Furthermore, we found that fat body specific expression of *Imp-L2* was increased in long-lived TG *A. stephensi*, suggesting further suppression of ILP2 activity in these mosquitoes. We also demonstrated that sustained overexpression of Akt in the *A. stephensi* fat body resulted in increased lifetime fecundity in caged populations. Importantly, this was not due to an increase in egg production during any individual reproductive cycle, but rather sustained reproduction throughout the extended lifespan of TG populations. Thus, TG *A. stephensi* did not display the typical tradeoff in reproduction associated with extended lifespan. These findings open new avenues to explore the impact of Akt signaling on lifespan and reproduction, allowing us to further elucidate the molecular mechanisms regulating fitness in mosquitoes. Furthermore, we have developed a strategy that may be useful in offsetting fitness costs associated with the engineering of pathogen-resistant mosquitoes for use in population replacement strategies to combat vector-borne pathogen transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

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
Myr	MYRISTOYLATION
NTG	NON-TRANSGENIC
PI3K	PHOSPHOINOSITIDE 3-KINASE
PTEN	PHOSPHATASE AND TENSIN HOMOLOG
PTTH	PROTHORACICOTROPIC HORMONE
TG	TRANSGENIC
VG	VITELLOGENIN
WHO	WORLD HEALTH ORGANIZATION

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Highlights

- Akt signaling was increased in the *Anopheles stephensi* fat body using the vitellogenin promoter.
- Increased fat body Akt activity increased *ILP2* and decreased *ILP3* transcript levels in the head.
- Increased fat body Akt activity greatly increased transcript levels of the putative insulin binding protein *Imp-L2*.
- Akt transgenic mosquitoes had a significantly higher lifetime fecundity than non-transgenic sibling controls

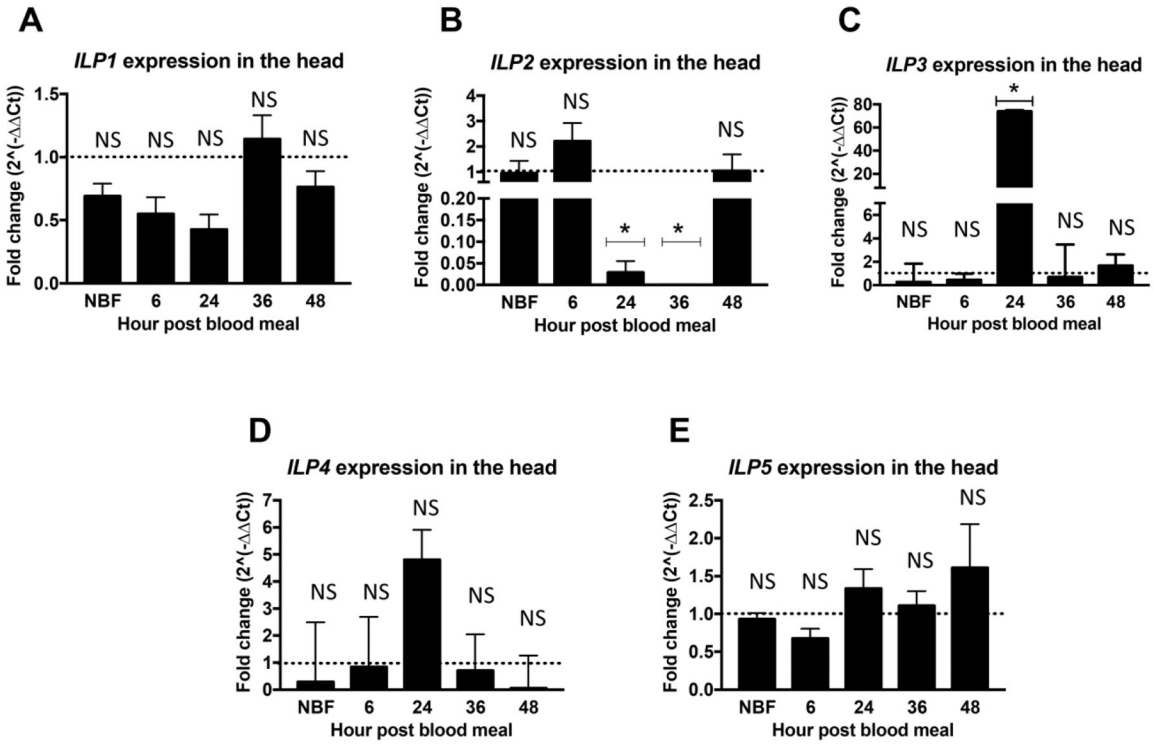

Figure 1: Overexpression of myr-AsteAkt-HA in the abdominal fat body suppressed *ILP2* but increased *ILP3* transcript expression in the head.

Heads from 3 to 5 day old TG and NTG *A. stephensi* females were dissected to assess mRNA expression of (A) *ILP1*, (B) *ILP2*, (C) ILP3, (D) *ILP4*, and (E) *ILP5*, at 0h (non-blood fed, NBF), 6h, 24h, 36h, and 48h post blood meal. *ILP* expression was quantified by qPCR. NTG samples were used as a control 2for each time points for the relative transcript expression determination. The dotted line on the graphs represents normalized transcript levels in the NTG samples (control). The Y-axis represents the $\log_2$ of fold changes which were calculated by the $2^{\wedge-\Delta\Delta Ct}$ method in which the C_t values of TG gene were normalized to the level of NTG control within each time point. Each value is the mean ± SEM of three independent experiments from five pooled mosquito heads. Results were analyzed using Student's paired t-test (* = $p<0.05$; Graphpad Prism 7) to determine differences between TG mosquitoes and NTG sibling controls. All mRNA expression studies were replicated a minimum of three times.

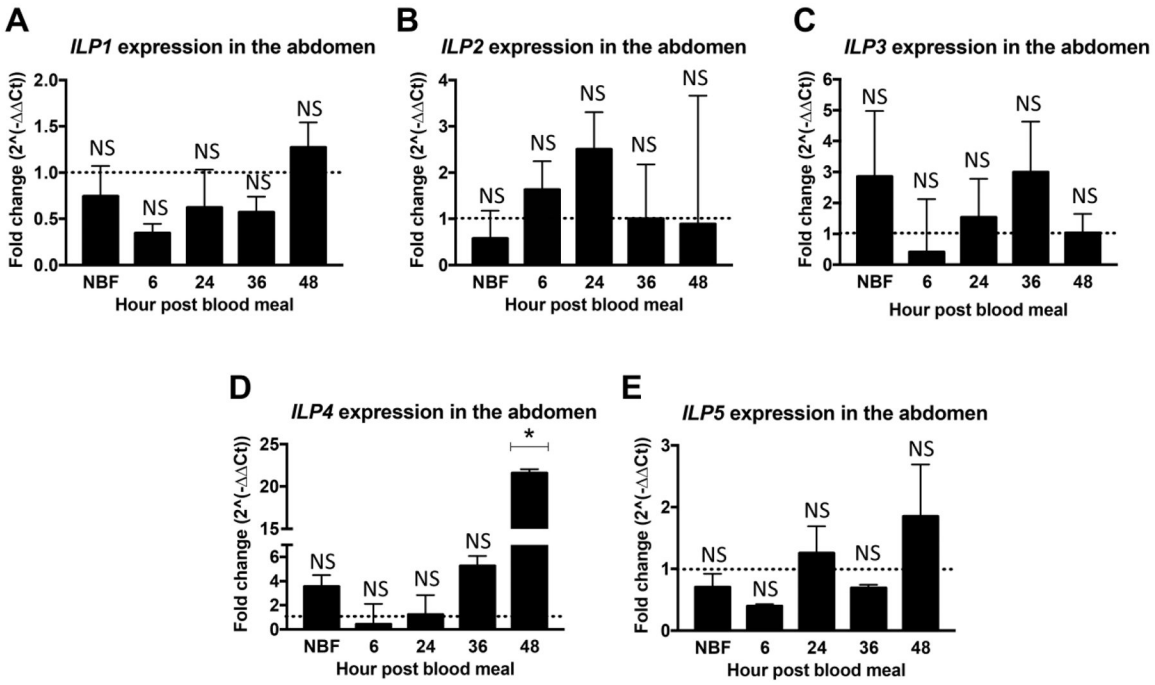

Figure 2: Overexpression of myr-AsteAkt-HA in the abdominal fat body induced *ILP4* transcript expression in the abdominal body wall.

Abdominal body walls from 3 to 5 day old TG and NTG *A. stephensi* females were dissected to assess the transcript expression of (A) *ILP1*, (B) *ILP2*, (C) *ILP3*, (D) *ILP4*, and (E) *ILP5*, at 0h (non-blood fed, NBF), 6h, 24h, 36h, and 48h post blood meal. *ILP* expression was quantified by qPCR. NTG samples were used as a control for each time points for the relative transcript expression determination. The dotted line on the graphs represents transcript levels in the NTG control samples normalized to one. The Y-axis represents the $\log_2$ of fold changes which were calculated by the $2^{\wedge-\Delta\Delta Ct}$ method in which the C_t values of TG gene were normalized to the level of NTG control within each time point. Each value is the mean ± SEM of three independent experiments from five pooled mosquito abdominal body walls. Results were analyzed using Student's paired t-test (* = $p<0.05$) to determine differences between TG mosquitoes and NTG sibling controls. All mRNA expression studies were replicated a minimum of three times.

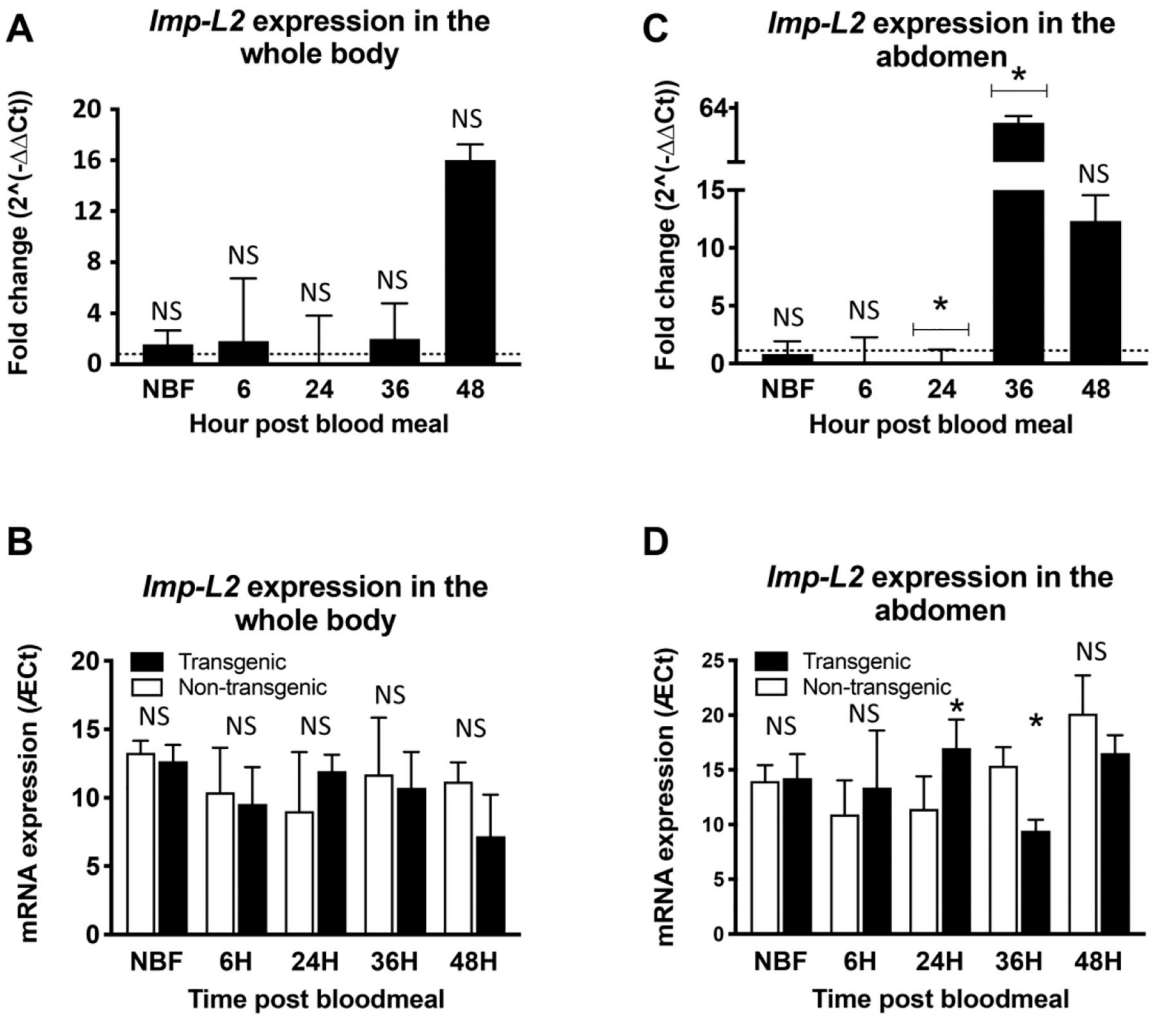

Figure 3: Overexpression of myr-AsteAkt-HA in the abdominal fat body impacts *Imp-L2* transcript expression.

Abdominal body walls from 3 to 5 days old TG and NTG *A. stephensi* females were dissected to assess the mRNA expression of *Imp-L2* at 0h (non-blood fed, NBF), 6h, 24h, 36h, and 48h after a blood meal. *Imp-L2* mRNA expression was quantified by qPCR. NTG samples were used as a control for each time points for the relative transcript expression determination. The dotted line on the upper graphs (A and C) represents transcript levels in the NTG control samples normalized to one. The Y-axis represents the $\log_2$ of fold changes which were calculated by the $2^{\wedge-\Delta\Delta Ct}$ method in which the C_t values of TG gene were normalized to the level of NTG control within each time point. Each value is the mean ± SEM of three independent experiments from five pooled mosquito (A and B = whole mosquito; C and D = abdominal body wall). The lower graphs (B and D) represent the ΔC_t of *Imp-L2* relative to the RP17 loading control for both TG and NTG mosquitoes. Results were analyzed using Student's paired t-test (* = $p<0.05$) to determine differences between TG mosquitoes and NTG sibling controls. (Note: while *Imp-L2* TG expression showed a large fold increase at 48 h, this change was marginally insignificant (p=0.07 (A) and 0.127 (C)) due to variation in C_t values (see B and D) between replicates. It should also be noted

that ΔCT values in Fig. 3B and D are the inverse of the $2^{\wedge -\Delta\Delta Ct}$ values presented in A and C.) All expression studies were replicated a minimum of three times with distinct cohorts of mosquitoes.

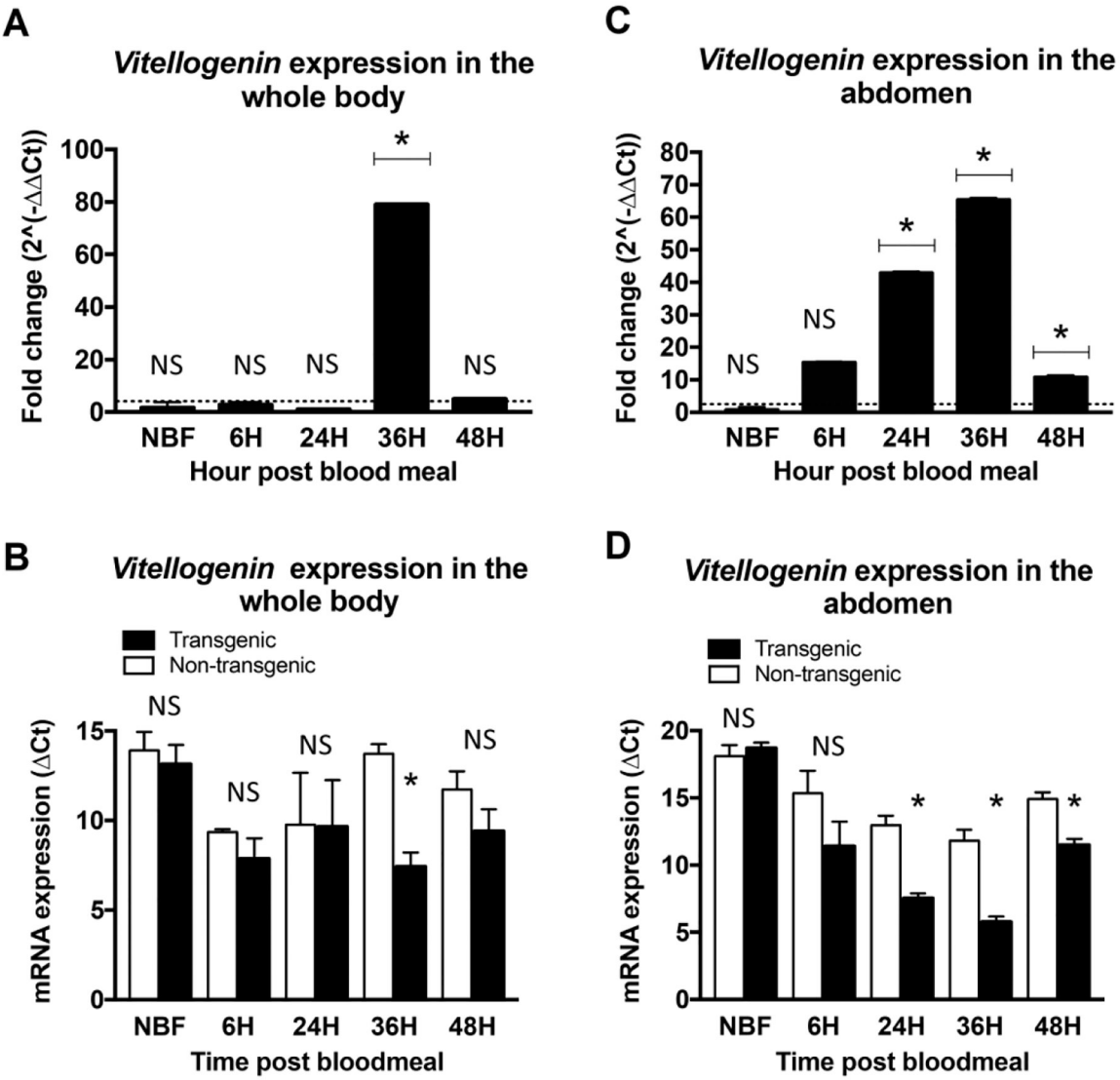

Figure 4: Overexpression of myr-AsteAkt-HA expression in the abdominal fat body induced *vitellogenin 1/2* (*Vg1/2*) transcript expression.

Whole bodies (A and B) and Abdominal body walls (C and D) from 3 to 5 days old TG and NTG *A. stephensi* females were used to assess transcript expression of *Vg1/2* at 0h (non-blood fed, NBF), 6h, 24h, 36h and 48h after a blood meal. *Vg1/2* expression was quantified by qPCR. NTG samples were used as a control for each time points for the relative transcript expression determination. The dotted line on the graphs represents transcript levels in the NTG control samples normalized to one. The Y-axis represents the $\log_2$ of fold changes, which were calculated by the $2^{\wedge-\Delta\Delta Ct}$ method in which the C_t values of TG gene were normalized to the level of NTG control within each time point. Each value is the mean ± SEM of three independent experiments from five pooled mosquito heads. Results were analyzed using Student's paired t-test (* = $p<0.05$) to determine differences between TG mosquitoes and NTG sibling controls. All expression studies were replicated a minimum of three times.

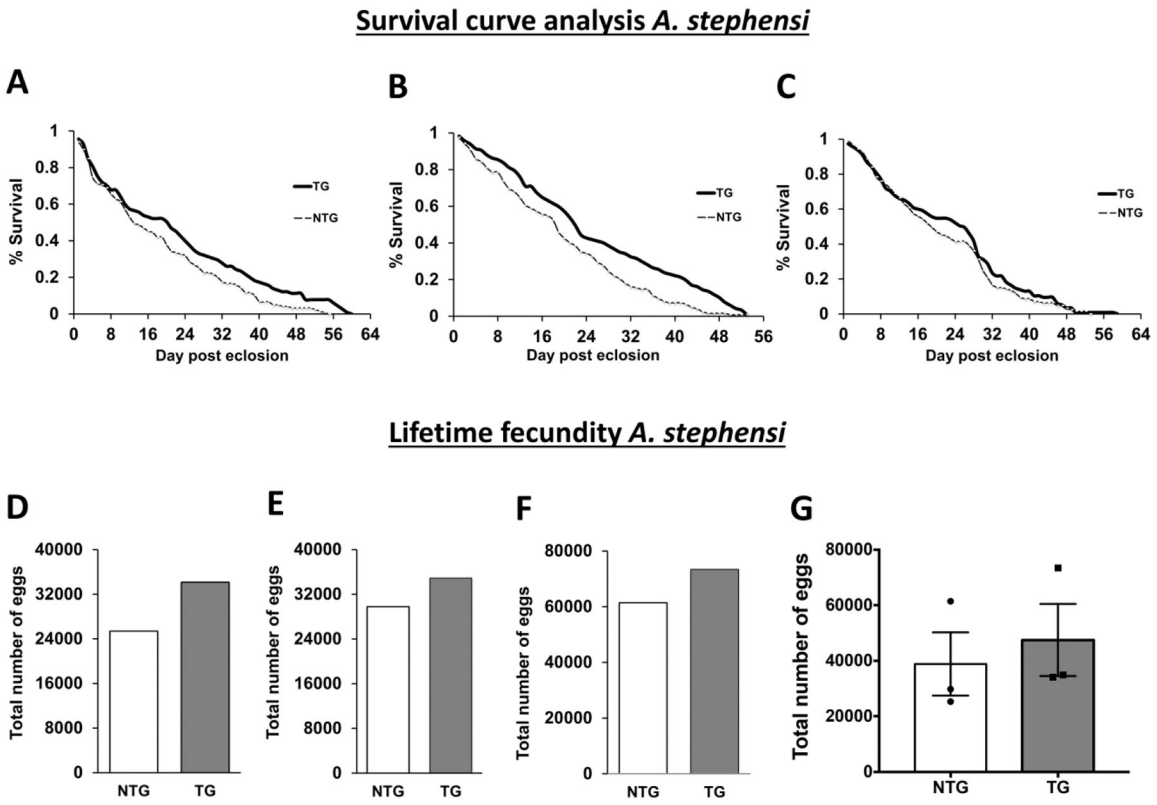

Figure 5: Impact of myr-AsteAkt-HA overexpression in the abdominal fat body on lifetime fecundity.

A-C) Survivorship curves of *A. stephensi* NTG and TG mosquitoes reared under identical conditions and provided with a blood meal daily in addition to 10% dextrose solution *ad libitum*. Each survivorship curve represents the data from a single biological replicate using a minimum of 100 TG and NTG female mosquitoes. Significant differences between TG and NTG survival curves were assessed using Log-rank (Mantel-Cox) analysis and were observed in replicates 1 (**A** p=0.013) and 2 (**B** p=0.0067). Replicate 3 was not significant (**C** = =0.3161) **D-F**) Graphs represent total egg production of each cage of TG and NTG sibling mosquitoes throughout their lifespan and are matched to the survival curves in A-C. **G)** Graph represent total egg production from all three independent experiments between TG and NTG sibling mosquitoes throughout their lifespan. Significant differences were observed in the lifetime fecundity between TG and NTG using Student's paired t-test ($p<0.05$; Graphpad Prism 7). Each graph represents the total number of eggs collected from an independent experimental caged population.