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Knockdown of mitogen-activated protein kinase (MAPK) signaling in the midgut of *Anopheles stephensi* mosquitoes using antisense morpholinos

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Abstract

Arthropod-borne infectious diseases are responsible for nearly 1.5 million deaths annually across the globe, with malaria responsible for over 50% of these deaths. Recent efforts to enhance malaria control have focused on developing genetically modified *Anopheles* mosquitoes that are resistant to malaria parasite infection by manipulating proteins that are essential to the immune response. Though this approach has shown promise, the lack of efficient genetic tools in the mosquito makes it difficult to investigate innate immunity using reverse genetics. Current gene knockdown strategies based on small interfering RNAs (siRNA) are typically laborious, inefficient, and require extensive training. Here, we describe the use of morpholino anti-sense oligomers to knockdown MEK-ERK signaling in the midgut of *Anopheles stephensi* through a simple feeding protocol. Anti-MEK morpholino provided in a saline meal was readily ingested by female mosquitoes with minimal toxicity and resulted in knockdown of total MEK protein levels 3-4 days after morpholino feeding. Further, anti-MEK morpholino feeding attenuated inducible phosphorylation of the downstream kinase ERK and, as predicted by previous work, reduced parasite burden in mosquitoes infected with *P. falciparum*. To our knowledge, this is the first example of morpholino use for target protein knockdown via feeding in an insect vector. Our results suggest this method is not only efficient for studies of individual proteins, but also for studies of phenotypic control by complex cell signaling networks. As such, our protocol is an effective alternative to current methods for gene knockdown in arthropods.

Keywords

Mosquito; insect; morpholino; knockdown; malaria; mitogen-activated protein kinase; MAPK; *Anopheles*; *Plasmodium*

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INTRODUCTION

The World Health Organization (WHO) now estimates that malaria causes over 200 million clinical episodes and over 650,000 deaths annually (WHO, 2012). While infection and mortality rates in much of the world have declined within the last decade, the failure of many common control methods has sparked the need for novel strategies to combat the disease. Among these are the development and improvement of anti-malarial drugs (Noedl, 2013), vaccine design (Birkett et al., 2013), and the enhancement of infrastructure in at-risk areas (Barclay et al., 2013; Klein, 2013). Additional efforts have focused on the development of genetically modified mosquitoes that are resistant to infection (Fuchs et al., 2013; Marshall & Taylor, 2009). Several groups, including our own, have now generated genetically modified mosquitoes that are refractory to *Plasmodium* infection (Corby-Harris et al., 2010; de Lara Capurro et al., 2000; Hauck et al., 2013; Isaacs et al., 2012; Kim et al., 2004) and similar methods are being used to combat other mosquito-borne infections such as dengue and yellow fever (Franz et al., 2006; Kokoza et al., 2000; Mathur et al., 2010; Travanty et al., 2004).

While the generation of stably transformed, pathogen-resistant mosquitoes has shown clear promise, the development and improvement of associated genetic techniques for use in the mosquito would greatly enhance research progress. Engineering pathogen resistance in a vector requires not only a detailed understanding of the complex mechanisms underlying natural immunity, but also the genetic tools to properly dissect these mechanisms in the lab. Readily available molecular methods to query the effects of mosquito immune genes and signaling pathways on pathogen infection include RNA interference (RNAi)-mediated knockdown (Boisson et al., 2006; Gulia-Nuss et al., 2011; Lamaccia et al., 2011), plasmid-based overexpression (Beumer et al., 2008; Peng et al., 2011), and provision of chemical inhibitors (Pakpour et al., 2012; Surachetpong et al., 2009). Virus-based expression has also been utilized with some success (de Lara Capurro et al., 2000). Although these techniques have been essential to ongoing progress in vector molecular biology, they each possess significant pitfalls. For example, large scale screens of chemical inhibitors against over 400 human kinases indicate that significant care must be taken to optimize inhibitor dose to minimize toxicity and off-target effects (Davis et al., 2011; Karaman et al., 2008). Further, studies using microinjection-based overexpression of gene or hairpin RNA-encoding sequences may result in higher mortality rates when compared to feeding based methods (Walshe et al., 2009) and may require multiple injections or rearing of transformed larvae to obtain adults with the desired genetic modification (Beumer et al., 2008; Peng et al., 2011). Efficient gene knockdown has been achieved through feeding of dsRNA in a variety of insects (Huvenne & Smagghe, 2010), including disease vectors such as the tsetse fly *Glossina morsitans* (Walshe et al., 2009), the triatomine bug *Rhodnius prolixus* (Araujo et al., 2006), and the deer tick *Ixodes scapularis* (Soares et al., 2005). Feeding of dsRNA to *Anopheles* mosquito larvae also yielded systemic target knockdown (Zhang et al., 2010). However, orally delivered dsRNA elicits a lower level of target knockdown when compared to injection in the tsetse fly (Walshe et al., 2009) and may be subject to degradation in the gut (Luo et al., 2013), suggesting that rapid methods for gene knockdown via feeding can be improved.

Anti-sense morpholino (MO) technology is an established method for gene knockdown that provides several key advantages over the aforementioned techniques (Heasman, 2012), including lower costs of materials and production (Summerton & Weller, 1997). Anti-sense MOs are small synthetic oligonucleotides, chemically modified to contain morpholine rings in place of a deoxyribose backbone for increased stability and can be conjugated to a cell-permeating moiety for *in vivo* uptake. MO oligomers reduce target protein levels by binding target transcript at the 5-prime untranslated region to prevent the initiation of translation (Summerton & Weller, 1997). Further, MOs are highly target specific due to their RNAse H-independent mechanism of action and inability to form small, transient RNA duplexes (Summerton, 2007). Previously, MOs have been used in a variety of vertebrate and invertebrate organisms to study gene function, though the method of delivery has been largely restricted to microinjection (Layden et al., 2013; McMahon et al., 2010; Melvin et al., 2013) or electroporation (Peng et al., 2012). However, MOs have also shown high bioavailability and efficiency for target knockdown when administered orally to adult rats (Arora et al., 2002).

The goal of this study, therefore, was to determine whether oral delivery of anti-sense MOs is a viable alternative for gene knockdown in mosquitoes using *Anopheles stephensi* as a model. In this study, we assayed the efficiency of inhibiting the mitogen-activated protein kinase (MAPK) MEK-ERK signaling pathway, a known regulator of immunity in the *A. stephensi* midgut (Surachetpong et al., 2009), via saline meal delivery of an anti-sense MO against MEK.

RESULTS

Efficiency of morpholino delivery

Female *A. stephensi* mosquitoes showed no significant aversion to feeding on a meal containing *As*MEK-MO relative to feeding on a saline meal control. Of mosquitoes provided with a meal of saline alone, an average of 92% completely engorged within 30 minutes, while of mosquitoes provided a meal of 10μM *As*MEK-MO, an average of 87.8% fed to completion within the same timeframe (Fig. 1A). Additionally, ingestion of *As*MEK-MO had no significant effect on short-term mosquito survival relative to ingestion of the saline meal control. Of mosquitoes initially provided a control meal, an average of 93.9% survived to day 3 post-feeding, while of mosquitoes initially fed 10μM *As*MEK-MO, 93.8% survived to the same time point (Fig. 1B).

Knockdown of total MEK levels in the midgut

Changes in midgut total MEK protein levels following feeding of *As*MEK-MO were observed in replicated assays (Fig. 2). As early as 2 days post-feeding, a modest (22%) reduction in total MEK was noted, although this was not significantly different from levels in saline-fed controls ($p > 0.1$). However, by day 3 post-feeding, total MEK levels were reduced by 59% relative to control (Fig. 2; $p = 0.06$); this knockdown was the largest achieved during the time course. MEK knockdown persisted through day 4 post-feeding, with a reduction of 43% relative to control ($p = 0.03$). By days 5 and 6 post-feeding, MEK

levels were increased relative to control by 61% and 19%, respectively, although neither increase was different from control levels ($p > 0.1$) at the same time points.

Knockdown of downstream ERK signaling

To determine the functional effects of MEK knockdown, changes in phosphorylation of ERK, a MEK-dependent kinase, were quantified in response to uninfected blood meals (Fig. 3A) and to *P. falciparum*-infected blood meals (Fig. 3B). For these experiments, mosquitoes were fed either saline or *As*MEK-MO in saline 3 days prior to blood-feeding; ERK phosphorylation in *As*MEK-MO-fed mosquitoes was normalized to levels in saline-fed controls (white column, Fig. 3A; dotted line, Fig. 3B). At 1 hour following an uninfected blood meal, phospho-ERK levels in mosquitoes previously fed 10μM or 50μM *As*MEK-MO were reduced relative to mosquitoes previously fed saline ($p < 0.001$; Fig. 3A). Inhibition of ERK phosphorylation was not dependent on *As*MEK-MO concentration, as provision of 10μM and 50μM *As*MEK-MO prior to blood-feeding resulted in 40% and 49% reductions, respectively, in ERK phosphorylation (Fig. 3A). Analyses of the effect of 10μM *As*MEK-MO on ERK phosphorylation in the context of a *P. falciparum*-infected blood meal provided 3 days post-*As*MEK-MO feeding included a 1, 6, and 24 hour post-infection timepoints. Midgut ERK phosphorylation levels at 1 hour post-infection in *As*MEK-MO-fed and saline-fed controls were not different ($p >0.1$; Fig. 3B). At 6 hours post-infection, midgut phospho-ERK levels in *As*MEK-MO-fed mosquitoes were reduced by 52% relative to levels in saline-fed controls ($p = 0.09$; Fig. 3B). By 24 hours post-infection, phospho-ERK levels in *As*MEK-MO fed mosquitoes trended towards increased ERK phosphorylation, although these levels were not different from levels in mosquitoes previously fed saline ($p > 0.1$; Fig. 3B).

Effects of MEK knockdown on *P. falciparum* infectivity

*As*MEK-MO-mediated knockdown of MEK, a known regulator of malaria parasite development in the midgut of female *A. stephensi* (Surachetpong et al., 2009), reduced *P. falciparum* infectivity. Specifically, 65% of mosquitoes fed *As*MEK-MO developed an infection (at least one oocyst per dissected midgut), whereas 75% of mosquitoes fed control-MO were infected ($p = 0.08$; Fig. 4A). In addition to a reduced prevalence of infection, treatment with *As*MEK-MO was associated with a reduced intensity of infection in *A. stephensi*. Specifically, the mean intensity of infection in *As*MEK-MO-fed *A. stephensi* was 2.37 oocysts per midgut compared to a mean of 3.19 oocysts per midgut in control-MO-fed mosquitoes ($p = 0.04$; Fig. 4B). This reduction was consistent with previous studies of chemical inhibition of MEK with PD98059. In particular, infection was reduced in two biological replicates by PD98059 treatment of *A. stephensi* from a mean of 4.75 oocysts per midgut in controls to mean of 0.20 oocysts per midgut and from a mean of 8.14 oocysts per midgut in controls to 4.56 oocysts per midgut (Surachetpong et al., 2009).

DISCUSSION

In the present study, we validated the use of anti-sense MOs for knockdown in *A. stephensi* using the MEK-ERK signaling pathway, which we had previously implicated in the control of *P. falciparum* development in this mosquito host (Surachetpong et al., 2009). To avoid

potential confounding effects of blood digestion, we delivered MOs in a saline meal using ATP as a feeding stimulant. The feeding rates of mosquitoes provided MO in saline (Fig. 1A) were comparable to rates observed for mosquitoes fed on blood (Andreasen et al., 2004; unpublished data). Further, rates of mosquito survival following ingestion of MOs in saline (Fig. 1B) were comparable to those observed after dsRNA feeding (Walshe et al., 2009) and substantially higher than in studies using microinjection (Beumer et al., 2008; Peng et al., 2011). Together, these data demonstrate the suitability of direct feeding as a delivery method for anti-sense MOs.

Current gene knockdown strategies are based on small interfering RNA (siRNA) and plasmid microinjection. Overexpression of hairpin RNAs or zinc finger nucleases can provide stable, long-lasting knockdown; however, these strategies often require 1-2 generations of post-injection rearing to achieve significant knockdown of the target protein (Beumer et al., 2008; Peng et al., 2011). Feeding or injection of dsRNA typically requires 24-48 hours for maximal effect, but may only last for a period of several days (Araujo et al., 2006; Welshe et al., 2009). In comparison, knockdown of target protein levels in the *A. stephensi* midgut was first observed 3 days after feeding of *As*MEK-MO, with the effect lasting through 4 days post-MO feeding (Fig. 2). Therefore, the use of MOs would be advisable when a strong and rapid, yet transient (2-3 days) effect is desired.

Interestingly, on days 5-6 post-*As*MEK-MO feeding, total MEK levels in the midgut appeared to increase above control levels. Chemical inhibition of MEK is known to increase its phosphorylation, likely due to a decrease in negative feedback from activated ERK to upstream signaling proteins such as Son of Sevenless (SOS) and Rat Sarcoma (Ras) (Dougherty et al., 2005). MEK inhibition has also been shown to increase ERK gene expression (Gioeli et al., 2011). Thus, this pathway appears to be controlled by a complex balance of both positive and negative feedback signals that may account for increased MEK levels observed following prolonged knockdown by *As*MEK-MO. The possibility of feedback regulation should be taken into consideration during MO assay design. For example, it may be possible to prolong knockdown with multiple, sequential MO treatments to block compensatory effects of knockdown as the MO effect declines over time.

In order to determine the effects of *As*MEK-MO-mediated MEK knockdown on downstream signaling, we examined phosphorylation of ERK, the downstream target of MEK, following blood-feeding (Fig. 3). Ingestion of blood containing mammalian cytokines and growth factors (e.g., transforming growth factor-β1, insulin) or *P. falciparum* signaling factors (e.g., hemozoin, glycosylphosphatadylinositols) is known to activate MEK-ERK signaling in the *A. stephensi* midgut (Akman-Anderson et al., 2007; Lim et al., 2005; Pakpour et al., 2012; Surachetpong et al., 2009; Surachetpong et al., 2011). In our studies, when mosquitoes were provided *As*MEK-MO 3 days prior to feeding on uninfected human RBCs, ERK phosphorylation was reduced by 40-49% relative to controls (Fig. 3). Intriguingly, *As*MEK-MO-fed mosquitoes subsequently provided a *P. falciparum*-infected blood meal also showed a decrease in ERK signaling compared to controls (Fig. 3B), but signaling knockdown was delayed, perhaps due to alternative parasite stimuli that can also function to activate ERK signaling (Akman-Anderson et al., 2007; Lim et al., 2005). Although it may be difficult to achieve total knockdown of target protein levels, the level of knockdown we observed was

comparable to that observed with RNAi in mosquitoes and other insects (Araujo et al., 2006; Gulia-Nuss et al., 2011; Lamacchia et al., 2013; Soares et al., 2005; Walshe et al., 2009) and impacted both signaling and parasite infection. These data suggest that because MOs directly inhibit mRNA translation, a lower level of efficiency may be sufficient to produce a biological effect when compared to RNAi-mediated knockdown of transcript levels. For example, Diekmann et al. (2009) reported that injection of brain-derived neurotrophic factor (BDNF)-MO produced only a 50% knockdown in BDNF protein levels in zebrafish, but this knockdown produced a more than 6-fold increase in caspase-3 activity that was associated with significant effects on zebrafish brain development. Another advantage to this methodology is that MOs can be combined with the use of small molecule inhibitors to target kinase enzymatic activity within the same pathway (Surachetpong et al., 2009).

Based on these observations, MO-mediated knockdown showed expected phenotypic effects. In particular, treatment with *As*MEK-MO phenocopied chemical inhibition of MEK – that is, treatment with MO or with small molecule inhibitors (Surachetpong et al., 2009) reduced the intensity of *P. falciparum* infection to a similar degree (Fig. 4). These data support a highly relevant application of our protocol and prove its usefulness in studies of complex mechanisms such as innate immunity of insect-pathogen interactions.

CONCLUSIONS

The feeding protocol presented here provides several advantages over current methods for gene knockdown in insects. The method is easy to apply, requires no special skills for delivery, and is highly target specific. Provision of *As*MEK-MO via feeding reduced MEK-ERK signaling by more than 50% relative to control treatment for a period of days with no notable toxicity. Furthermore, *As*MEK-MO-mediated inhibition of the MEK-ERK pathway, a known regulator of anti-parasite immunity in *A. stephensi*, produced the expected decrease in *P. falciparum* infectivity in the mosquito (Surachetpong et al., 2009). Therefore, this protocol should be considered as an efficient alternative in studies requiring target-specific protein knockdown in mosquitoes and other arthropods.

EXPERIMENTAL PROCEDURES

Rearing and care of mosquitoes

The Indian wild type strain of *A. stephensi* was maintained in environmental chambers at 27°C and 80% relative humidity with a L12:D12 cycle. Mice were used as a blood source for colony maintenance in compliance with federal and institutional guidelines for Animal Use and Care. Larvae were provided with a 2% solution of 2:1 Sera Micron® powdered fish-fry food (Sera North America, Montgomeryville, PA) through day 4 post-hatching and then were reared on high protein, low fat Game Fish Chow pellets (Purina Mills, St. Louis, MO) until pupation. Emerged adults were maintained on cotton pads soaked in a 10% sucrose solution.

Preparation of morpholino solutions

Anti-*As*MEK (5’-TTACAAGAAATGTGTCCTTGGGTGT-3’, Supplementary Fig.1) vivo-MO (*As*MEK-MO; GeneTools LLC, Philomath, OR) at a stock concentration of 500 μM

was diluted to 1:50 (10μM) or 1:10 (50μM) in saline (15 mmol l^{-1} NaCl, 10 mmol l^{-1} $NaHCO_3$, pH 7.0). ATP (Sigma Aldrich, St. Louis, MO) was added to a final concentration 0.33mmol l^{-1}as a feeding stimulant. Immediately prior to feeding, MO solutions were warmed in a water bath at 37°C for 10 minutes. Standard MO control (5-CCTCTTACCTCAGTTACAATTTATA-3) targeting a human beta-globin intron mutation (control-MO; GeneTools LLC) in saline at equal concentrations was used as a matched control meal for infection experiments while meals of saline were used as controls in all other assays (Supplementary Fig. 2).

Feeding of morpholino solutions

Four separate cohorts of newly emerged female *A. stephensi* were provided with water only (no sucrose) for 24 hours and then provided a meal of either control-MO or *As*MEK-MO in saline via a Hemotek circulation system (Discovery Workshops, Accrington, UK; n=50-60 mosquitoes per control and treatment groups). After 30 minutes, any unfed mosquitoes were counted and discarded from experimental cohorts. Mosquitoes were maintained on cotton pads soaked in 10% sucrose through day 3 post-feeding and any dead mosquitoes were counted daily and removed.

Mosquito blood-feeding

Female *A. stephensi* previously fed MO meals on day 0 were starved starting on day 2 for 24 hours and then provided with a meal of washed human red blood cells (RBCs) in saline via a Hemotek circulation system (Discovery Workshops) on day 3. Mosquitoes were allowed to feed for 30 minutes and were then maintained on cotton pads soaked in 10% sucrose.

Plasmodium falciparum culture and *A. stephensi* infections

The NF54 strain of *P. falciparum* was initiated at 1% parasitemia in 10% heat-inactivated human serum and 6% washed RBCs in RPMI 1640 with HEPES (Gibco/Invitrogen, Carlsbad, CA) and hypoxanthine. At days 15-17, stage V gametocytes were evident and exflagellation was evaluated the day before and day of feeding by observation of blood smears before addition of fresh media at 200X magnification with phase-contrast or modified brightfield microscopy. Female *A. stephensi* previously fed MO meals on day 0 were starved starting on day 2 for 24 hours then provided an infected blood meal on day 3 and maintained on cotton pads soaked in 10% sucrose until day 10 post infection. To quantify infection levels, midguts were dissected on day 10 post-infection and stained with 1% mercurochrome for visualization of *P. falciparum* oocysts by microscopy. Infections were replicated with four separate cohorts and 20-30 mosquitoes per control and treatment groups.

Western blot analyses

Protein extracts for western blotting were prepared by collecting *A. stephensi* midguts in cell extraction buffer (Invitrogen, Carlsbad, CA). In brief, midguts were dissected into phosphate-buffered saline (PBS; Cellgro, Manassas, VA) with protease inhibitor (Sigma Aldrich), centrifuged at 10,000 g for 5 minutes, re-suspended in 200μL cell lysis buffer for 1 hour and vortexed periodically. Cell lysates were cleared at 16,000 g for 10 min and protein

supernatants were mixed with sample loading buffer (125 mM Tris-HCl pH 6.8, 10% glycerol, 10% SDS,0.006% bromophenol blue, 130 mM dithiothreitol) and boiled for 5 minutes. Proteins were separated on 10% SDS-PAGE polyacrylamide gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; pH 7.0) containing 0.1% Triton-100 (TBS-T) for 1 hour at room temperature. For total MEK detection, membranes were incubated at 4°C overnight with a 1:5,000 dilution of rabbit anti-MEK1/2 antibody (Cell Signaling, Danvers, MA) in 5% nonfat dry milk TBS-T. Membranes were washed three times for 5 minutes in TBS-T and incubated overnight with a 1:10000 dilution of HRP-conjugated goat anti-rabbit antibody. Detection of phospho-ERK and GAPDH followed the same protocol with a 1:10,000 dilution of mouse anti-phospho-ERK antibody (Cell Signaling) and 1:20,000 dilution of HRP-conjugated rabbit anti-mouse antibody or 1:10,000 dilution of rabbit anti-GAPDH and 1:20,000 dilution of HRP-conjugated goat anti-rabbit antibody, respectively. Proteins were visualized by incubating membranes with SuperSignal West Dura chemiluminescent reagent (Pierce, Rockford, IL) for 3 minutes and exposing on an Image Station 4000 Pro digital imager (Kodak, Rochester, NY) for 1-5 minutes. Analyses of total MEK levels (Fig. 2) were replicated three times with pooled samples of 12-15 midguts per replicate, while analyses of ERK phosphorylation were replicated three times (Fig. 3A) or four times (Fig. 3B) with pooled samples of 12-15 midguts per replicate. Representative western blots are shown for each experiment (Supplementary Fig. 3, Supplementary Fig. 4)

Data analysis and statistics

Densitometry of western blots was performed using the ImageJ (http://rsbweb.nih.gov/ij/) gel analysis tool. Densitometry data were normalized to appropriate control groups as well as a GAPDH loading control and analyzed by either Wilcoxon signed-ranked test or ANOVA for overall significance followed by Tukey's multiple comparison post-test for all pairwise comparisons of means from significant data sets (GraphPad Prism version 5.02). Infection prevalence and intensity data for four replicates were combined (control intensities did not differ among replicates; ANOVA, $p>0.1$) and analyzed using goodness of fit (chi-square) and two-sample t-tests, respectively. Feeding and survival data were analyzed by goodness of fit (chi-square) test. For all analyses, confidence levels greater than 90% ($p < 0.1$) are reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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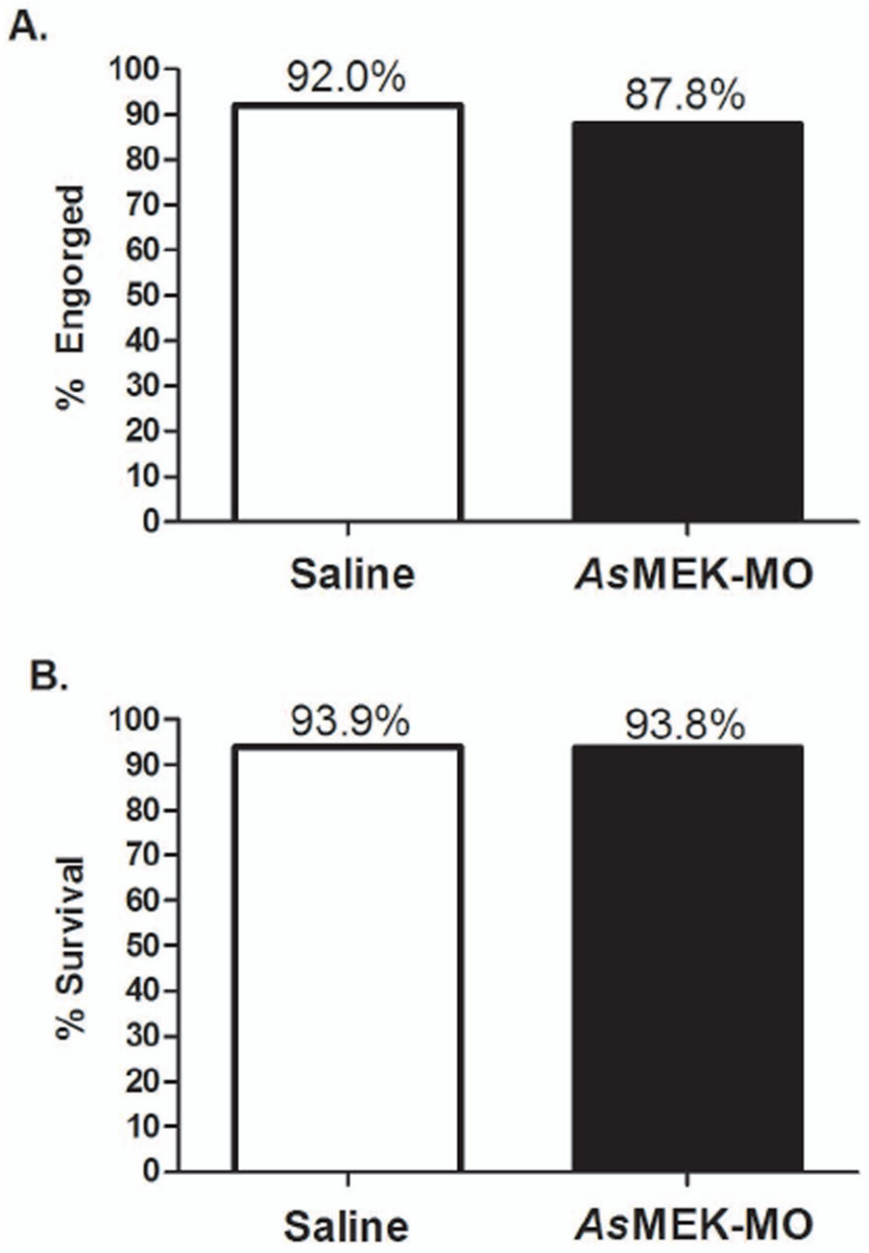

Figure 1. Provision of *As*MEK-MO in saline does not affect *A. stephensi* engorgement success or survivorship

Mosquitoes were provided with a saline meal (white bar) or saline with *As*MEK-MO (black bar) and monitored for **(A)** 30 minutes to determine engorgement success or **(B)** 3 days to assess survivorship. Bars represent the mean of three biological replicates comprised of 40-50 mosquitoes per saline and *As*MEK-MO groups. Data were analyzed by goodness of fit (chi-square) test ($p>0.1$).

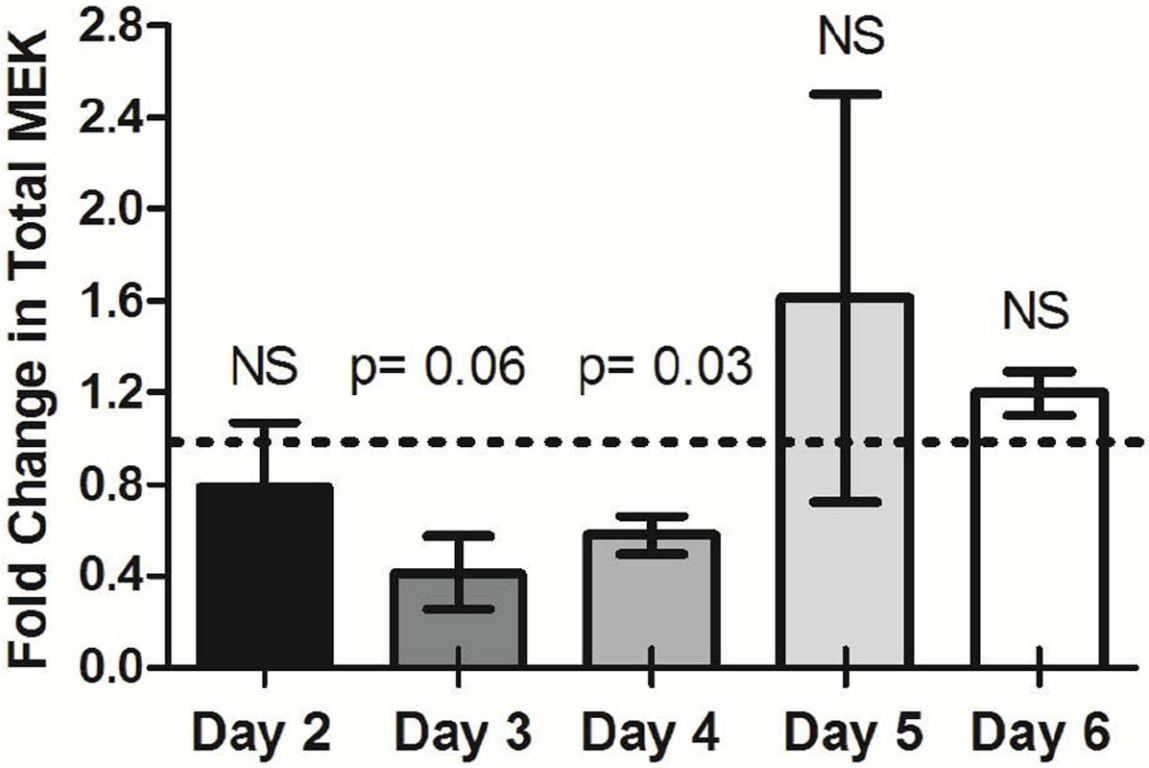

Figure 2. Provision of *As* MEK-MO by feeding reduces total MEK levels in the midgut of *A. stephensi* on days 3-4 post-treatment relative to control

Mosquitoes were provided with a saline meal or saline with *As*MEK-MO on day 0 and midgut tissues were dissected on days 2-6 post-feeding. Electrophoretically separated midgut proteins were probed for total MEK via western blot to determine knockdown efficiency relative to controls. Data are represented as mean fold inductions ± SEMs relative to day-matched controls (set at 1, dotted line) for three biological replicates of 12-15 midguts per timepoint. Densitometry data were normalized to total MEK in controls as well as to a GAPDH loading control and analyzed by Wilcoxon signed-rank test. NS = not significant, $p>0.1$.

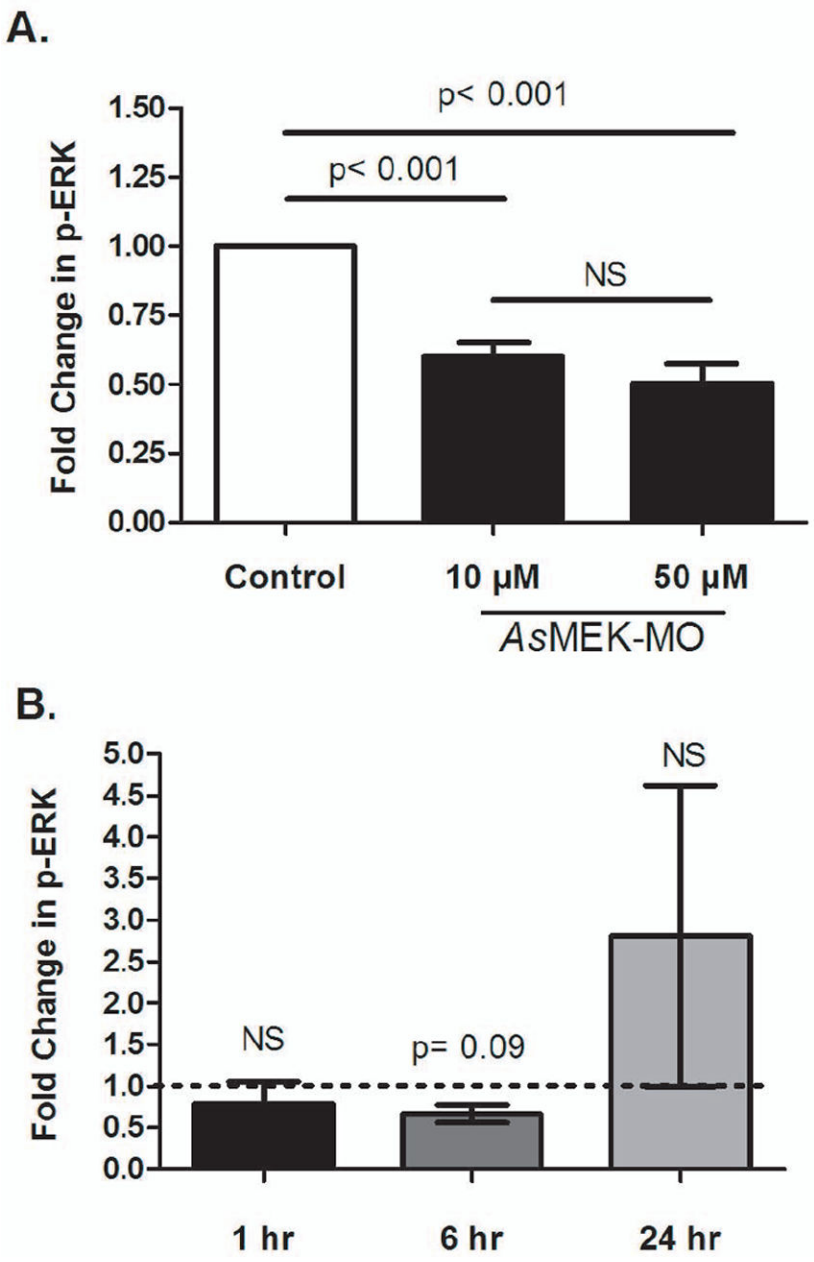

Figure 3. Provision of *As*MEK-MO by feeding inhibits blood- and *P. falciparum*-induced phosphorylation of the downstream kinase ERK in the midgut for up to 6 hours relative to control

Female *A. stephensi* were provided a control saline meal or saline with *As*MEK-MO (10µM and 50µM in A; 10µM in B) meal on day 0. On day 3, control and *As*MEK-MO-treated mosquitoes were given a meal of either **(A)** uninfected red blood cells or **(B)** *P. falciparum*-infected red blood cells. Midguts were dissected at 1, 6, or 24 hours post-feeding and proteins were probed for phospho-ERK via western blot. Bars represent the mean fold changes ± SEMs relative to time-matched controls (set at 1, dotted line in B) for four (A) and three (B) biological replicates of 12-15 midguts. Densitometry data were normalized to ERK phosphorylation in controls as well as to a GAPDH loading control and analyzed

ANOVA for overall significance followed by Tukey's multiple comparison post-test for all pairwise comparisons of means from significant data sets (A) or Wilcoxon signed-rank test (B). NS = not significant, $p>0.1$.

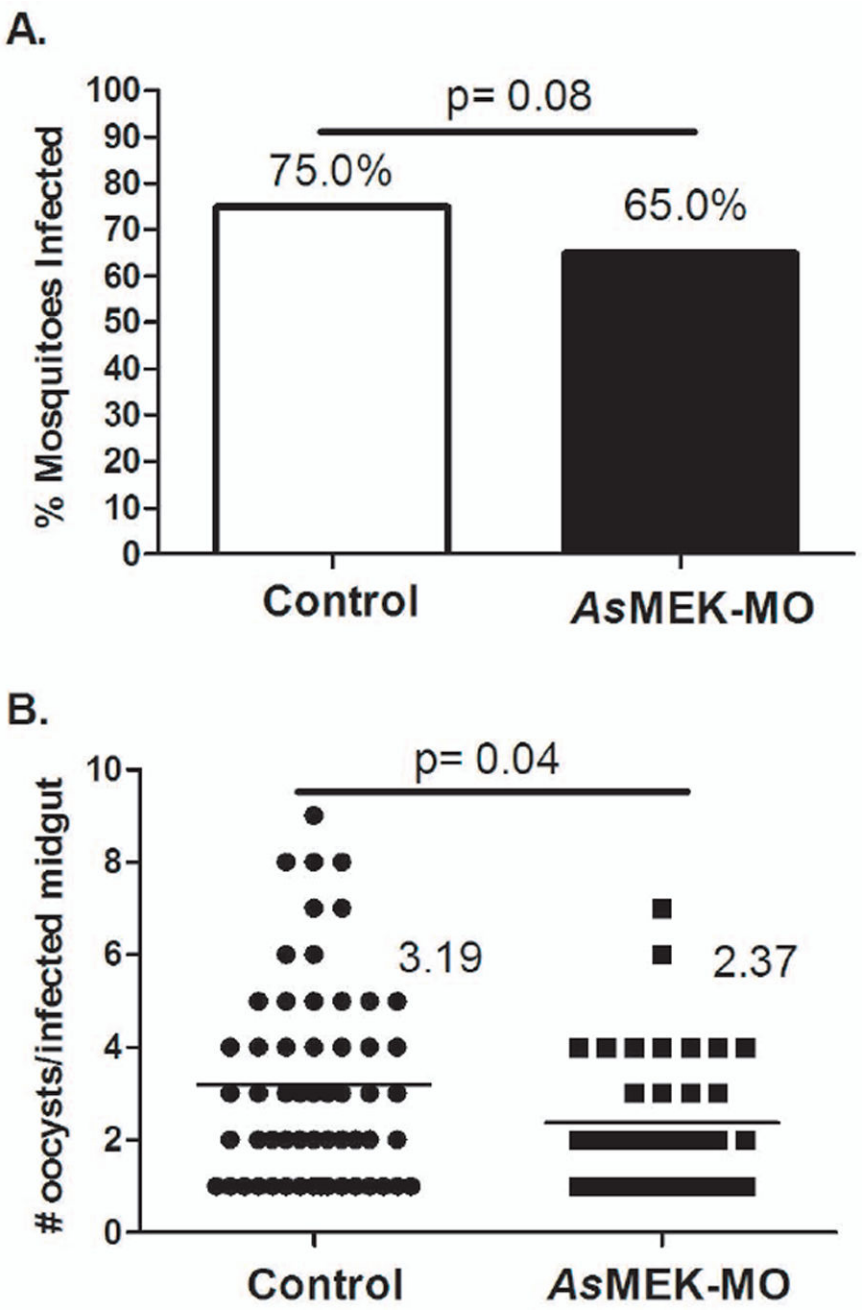

Figure 4. Provision of *As*MEK-MO reduces *P. falciparum* infection in *A. stephensi* relative to control

Female *A. stephensi* were fed *As*MEK-MO or control-MO in saline on day 0 and infected with *P. falciparum* on day 3 post-MO feeding. On day 10, midguts were dissected and stained with mercurochrome to determine **(A)** infection prevalence (percentage of mosquitoes infected with at least one oocyst) and **(B)** infection intensity (average number of oocysts per infected midgut, indicated numerically to the right of the data). Data are represented as means for four biological replicates comprised of 20-30 mosquitoes per

treatment group. Infection prevalence and intensity data were analyzed using goodness of fit (chi-square) and two-sample t-tests, respectively.