Effects of abscisic acid on life history traits and malaria parasite infection in the Indian malaria mosquito *Anopheles stephensi* Liston

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Authorization to Submit Thesis

This thesis of Dean M. Taylor, submitted for the degree of masters of science with a Major in Entomology and titled "The effects of abscisic acid on life history traits and malaria parasite infection in the Indian malaria mosquito *Anopheles stephensi* Liston" has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Larval conditions of holometabolous insects can have a substantial impact on adult life history traits including mortality, fecundity, and pathogen infection. For example, in *Anopheles stephensi,* overcrowded larval environments has been shown to reduce adult size, decrease fecundity, and increase *Plasmodium berghei* development. Presently, we are interested in the carryover effects of larval exposure to abscisic acid (ABA) on *A. stephensi*. ABA was first described as a plant hormone, but is now known for its effects as a stress hormone across animal taxa. We have previously shown ABA transiently alters adult mosquito metabolism through phosphorylation of metabolic mediators in the midgut following a supplemented blood meal. In this study, *A. stephensi* larvae were exposed to ABA for the entirety of their development and adult females derived from treated or control larvae were evaluated for fecundity, nutrient stores, and life span. Treatment of ABA in larval water significantly increased their development speed, while increasing pupal mortality. We also found that ABA supplementation during larval development has significant carryover effects on adult females, reducing their reproductive output and shortening their lifespan through reduced nutrient acquisition of larvae. Adult mosquitoes exposed to ABA during larval development also have reduced expression of insulin-like peptides (*ilp*s), thus supporting our findings of reduced fecundity and nutrient storage. These findings suggest larval exposure to ABA has the capacity to affect numerous adult life history traits related to vector biology, which could benefit areas with endemic mosquito-borne diseases.

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Dedication

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Chapter 1: Introduction

Overview and mosquito life cycle

Mosquito-borne diseases present one of the greatest challenges of the 21st century, with 700 million new clinical cases and 1 million deaths each year (WHO 2017). In particular, over half of the world's population is at risk of malaria, disproportionately affecting developing nations in Africa. Between 2000 and 2013, malaria-related mortality was reduced by 47% worldwide and it is estimated that malaria can be eliminated by 2050 or 2060 (Newby et al., 2016). Anopheline mosquitoes are the only mosquito genus that can transmit the *Plasmodium* spp. that cause human malaria. With growing concerns of *Anopheles stephensi* invasion and establishment in urban areas within Africa, it is imperative to understand transmission biology of this efficient vector (Takken and Lindsay, 2019).

Mosquitoes are holometabolous insects with four distinct life stages: egg, larva, pupa, and adult. Adult Anopheline females are anautogenous and require a blood meal for every reproductive cycle making them excellent vectors for human pathogens. After acquiring a blood meal, mosquitoes digest host-derived proteins into amino acids, stimulating the synthesis of yolk protein precursors (YPPs) in the fat body that are transported to the ovaries for the developing eggs (Dhadialla and Raikhels, 1990). Approximately 90% of energy stored in eggs is derived from lipids, which are transported to the eggs by the lipid carrying protein lipophorin (Lp), (Kawyoya and Law, 1987; Atella et al., 2006). At 72 hours after blood feeding, eggs are fully developed and ready to be oviposited.

Gravid female mosquitoes spend a considerable amount of energy in locating a suitable aquatic site for oviposition (Day, 2016). Females assess sites based on sufficient nutrient availability, presence of other mosquito larvae, sufficient water and many other considerations (Day, 2016). Females assess oviposition sites through chemical cues and each genus of mosquito has its own unique markers to identify the ideal oviposition site. For example the yellow fever mosquito *Aedes aegypti* prefers artificial containers, while *A. stephensi* prefers natural brackish shallow water and, in urban areas, these mosquitoes will lay eggs in manmade containers (Barrera et al., 2006; Soleimani-Ahmadi et al., 2014; Takken and Lindsay 2019). Recent work has shown changes to natural flora and the addition of artificial breeding containers in urban areas can increase oviposition (Stone et al., 2018).

Once a site has been chosen and the female mosquito oviposited, eggs will begin to hatch within 24 hours.

Larval development can be rapid in Anopheline mosquitoes and is typically complete in 6-12 days. Although relatively short compared to adult lifespan, the larval stage determines many adult mosquito life history traits, such as reproductive output, vulnerability to pathogens, and survival among many others (Joy et al., 2010 and Alto et al., 2005; Moller-Jacobs et al., 2014; Dickson et al., 2017). During the larval stage, over-crowding and under feeding can result in reduced fecundity and increased susceptibility of adult females to pathogens (Tripet et al., 2008). It is also important to note that, as adults, mosquitoes that were calorie-restricted during the larval stage live longer than adult mosquitoes derived from overfed larvae, which can increase the proportion of mosquitoes that survive the extrinsic incubation period (EIP) or time required for the completion of parasite development in the mosquito host (Joy et al., 2010). Increasing the proportion of mosquitoes surviving EIP can significantly increase pathogen transmission and disease burden. The suite of environmental factors that influence mosquito larval development can be exploited to block parasite transmission in the fight against mosquito-borne diseases.

Malaria parasite host manipulation

The parasites that cause human malaria undergo a complex life cycle between their human and mosquito hosts. When an infected mosquito bites a human she injects salivary proteins to aid in her feeding process. She also injects salivary gland sporozoites into human host blood, which travel to the liver, invade hepatocytes and undergo many rounds of asexual division. After 10-12 days, the infected hepatocytes will begin to bleb off small packets of merozoites (Burda et al., 2017). These merozoites will then initiate the erythrocytic stage of the parasite life cycle, invading red blood cells and causing clinical symptoms of malaria to first appear.

During the erythrocytic stage a small subset of parasites, usually fewer than 10%, commit to sexual differentiation or gametocytogenesis (Josling et al., 2018). This commitment initiates before schizogony, so every merozoite within the asexual schizont will form a gametocyte rather than a combination of sexual and asexual parasites (Josling et al., 2018). When a mosquito bites an infected human she ingests sexual stage parasites and once

in the midgut male parasites begin rapid cellular division, creating eight flagellated microgametes in response to a drop in temperature, change in CO_2, an increase in pH, and mosquito-derived xanthurenic acid (Garcia et al., 1998). A male microgamete unites with a female macrogamete to form a single diploid zygote. The zygote then develops into a mobile ookinete within the blood meal at 20 hours following feeding. Ookinetes are able to traverse the peritrophic membrane, a noncellular membrane formed to encapsulate blood within the mosquito midgut, and then cross the midgut epithelium. Once at the basal lamina the ookinete develops into an oocyst, a non-mobile stage undergoing ten rounds of division to create thousands of haploid sporozoites. After 12-14 days of growth, the oocyst wall breaks down, releasing these thousands of sporozoites into the hemolymph to travel to the salivary glands of the mosquito. Once in the salivary glands, the sporozoites are ready to be transmitted to new host.

Female mosquitoes use olfactory cues, such as CO_2 plumes, odorants released through the skin, and heat to locate suitable hosts (Zwiebel and Takken, 2004). Once she manages to approach a host she uses a direct visual cues, such as dark areas, to assist in landing. Interestingly, *Plasmodium* spp. parasites can manipulate the physiology and behavior of their insect host. The presence of gametocytes can alter the odor profile of humans, making them more attractive to mosquitoes than an uninfected person (Robinson et al., 2018). This increases the likelihood a mosquito will choose an infected host at just the right time for parasite sexual reproduction to occur within mosquitoes. Patients can also be differentiated between symptomatic and asymptomatic malaria based on volatile biomarkers collected from their skin (De Moraes et al., 2017). The significance of altering host volatile biomarkers to excite mosquito olfactory neurons has potential in exploiting vector behavior for disease control.

In mosquitoes, the parasite exerts control of the host from the level of altered gene expression to behavior (Reviewed in Murdock et al., 2017; Stanczyk et al,. 2017; Thomas et al., 2010). Following a blood meal, mosquitoes will rest on vertical surfaces for a few days to digest this large protein rich meal. However, when mosquitoes are infected with the human parasite *Plasmodium falciparum*, they rest for longer periods than uninfected mosquitoes (Anderson et al., 1999). Once sporozoites have reached the salivary glands, infected

mosquitoes display more aggressive feeding behavior compared to uninfected mosquitoes (Rossingol et al., 1984). Changes in behavior that temporarily reduce blood feeding is a risky reproductive strategy, but is obviously beneficial to the parasite. Given that calorie restriction can increase mosquito (Jia et al., 2004; Kapahi et al., 2003; Luckhart and Riehle, 2007), decreased feeding would increase the likelihood of an infected mosquito surviving parasite EIP.

At a genetic level, parasite infection changes the expression pattern of mosquito genes related to immunity and resource allocation. Ingestion of a *P. falciparum*-infected blood meal increases the expression of insulin like peptides (ILPs) in the midgut and head of *A. stephensi* in the first 24 hours, a critical period of parasite development (Pietri et al., 2015; Smith et al., 2014). Increased synthesis of ILPs activates the insulin/insulin like growth factor signaling (IIS) pathway which in turn down regulates immune genes, leaving mosquitoes more susceptible to parasite establishment in the midgut (Pietri et al., 2016).

Once established in the midgut wall the oocyst is non-mobile, so it relies completely on nutrients provided by the surrounding host tissue. *Plasmodium* parasites ingest host lipophorin (Lp), a family of lipid carrying proteins synthesized primarily in the fat body (Atella et al., 2009). It has been shown parasites manipulate lipid availability in the midgut by increasing mosquito synthesis of apolipophorin III (ApoLp-III), but not anywhere else in the mosquito body (Dhawan et al., 2017). In *A. stephensi*, ApoLp-III knockdown results in decreased parasite establishment after feeding on *Plasmodium berghei*-infected mice, possibly due to reduced trafficking of lipids to the midgut to feed the growing parasites. It has also been shown that in *A. stephensi* knockdown of ApoLp-III increases the expression of nitric oxide synthase (NOS), a protein that catalyzes the production of nitric oxide (NO), a strong reactive molecule that damages DNA, protein and lipids, resulting in cell death (Dhawan et al., 2017). Increased production of NO in *A. stephensi* has been shown to reduce establishment of both *P. falciparum* and mouse malaria parasites (Glennon et al., 2016; Drexler et al., 2014; Price et al., 2013; Luckhart et al., 2013; Surachetpong et al., 2009). Understanding co-evolved interactions between parasites and mosquitoes and the parasite's ability to exert control over their host's behavior and immune functions can aid our development of novel control strategies.

Abscisic acid

Abscisic acid (ABA) was discovered simultaneously in two different plant families with different functions (Cutler et al., 2010). In cotton (*Gossypium* spp.), ABA was found to control leaf abscission, and in maple (*Acer pseudoplatanus*), it was shown to maintain bud dormancy (Cutler et al., 2010). The name "abscisic acid" was adopted for both compounds when they were shown to be the same molecule. ABA is an isoprenoid and can be synthesized through the mevalonate pathway, as demonstrated by fungi, or though the non-mevalonate pathway, as in plants (Lange et al., 2000).

ABA is an essential regulator of stress responses across plants, sponges, hydroids, insects and mammals. In plants, for example, ABA regulates many functions related to stress tolerance across life stages. In seeds, ABA concentrations remain elevated in response to drought or high soil salinity, maintaining seed dormancy until optimal conditions for germination have been met (Vishwakarma et al., 2017). In mature plants under drought stress, ABA is transported from the roots to leaves and the increased concentration of ABA signals stomata closure, reducing water loss (Zhang et al., 2006). Stomata closure can also be induced by ABA in response to pathogen-associated molecular patterns (PAMPs) (Melotto, 2006). There is some evidence ABA promotes other defense-related responses to pathogens; however, much of this research is contradictory, so no clear pattern has been established (reviewed in Olds et al., 2018). ABA also has both negative and positive effects on the jasmonic acid pathway, a signaling pathway activated in response to herbivory (Bodenhausen and Reymond, 2007).

As with plant defense pathways, ABA signaling in other organisms appears to be species-dependent. In apicomplexans, ABA either has direct positive effects on growth, as for the protozoan parasite *Toxoplasma gondii*, or it has no effects on parasite growth, as shown for *P. falciparum* (Nagamune et al., 2008; Glennon et al., 2016). However, ABA supplementation to mice prior to infection with *Plasmodium yoelii* reduces liver and spleen pathology associated with parasite infection (Glennon et al., 2016). Moreover, oral ABA supplementation can reduce parasitemia, gametocytemia and transmission of parasites to *A. stephensi* (Glennon et al., 2016). Provision of ABA with a parasite-infected blood meal to *A. stephensi* results in phosphorylation (activation) of positive regulators of nuclear factor (NF)-

κB as well as the synthesis of NO within the first 24 hours of infection, significantly reducing infection prevalence relative to untreated infected mosquitoes (Glennon et al., 2017).

Mammalian response to exogenous ABA is expansive and includes alterations in the inflammatory response, cell proliferation, and glucose tolerance. For example, ABA has been shown to reduce the rate of undifferentiated cell growth (Suzuki et al., 1998). However, ABA isolated from bone marrow has been shown to increase the expansion of mesenchymal and hematopoietic stem cells (Scarfi et al., 2008; Scarfi et al., 2009). Inflammatory responses to ABA also appear to be context dependent. For example, oral supplementation with ABA reduces gut inflammation in animal models of inflammatory bowel disease and type II diabetes, but stimulates the inflammatory response of granulocytes by increasing synthesis of reactive oxygen species and NO (Guri et al., 2007; Guri et al., 2010; Bruzzone et al., 2007). Across taxa, ABA appears to have context- and species-specific activities in response to a myriad of stress-related conditions.

ABA biology in insects is most well-studied in the European honey bee (*Apis mellifera*), which can both synthesize and utilize plant-derived ABA. Evidence of the ability of *A. mellifera* to synthesize ABA is based on relatively low levels of ABA detected in L5 larvae, a non-feeding stage, followed by 7-fold increase in new emerged adults before feeding (Negri et al., 2015). The Luckhart lab has also detected ABA at low levels in adult male and female *A. stephensi* (not shown). In *A. mellifera*, ABA has been shown to increase wound healing, tolerance to pesticides and overwintering survival by increasing overall colony health (Negri et al., 2015; Ramirez et al., 2017). Interestingly, ABA does not appear to have such an overwhelmingly positive effect in other insects. For example, injection of ABA in adult female flesh flies, *Sarcophaga bullata* following feeding on liver reduces protein uptake and delays the rapid synthesis of vitellogenin proteins for egg development (De Man et al., 1981). Similarly, when the big headed grasshopper *Aulocara elliotti* is fed with grass soaked in high concentrations of ABA, females had reduced fecundity (Visscher, 1980).

Understanding complex interactions between parasites and their host biology is essential in the fight to eradicate malaria in the 21st century. Herein we want to understand

the effects of ABA, an ancient stress hormone with diverse functions in many species, on the development, reproduction and survival of the important malaria vector *A. stephensi*.

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Chapter 2: Comprehensive and durable modulation of growth, development, lifespan and fecundity in *Anopheles stephensi* following larval treatment with the stress signaling molecule and novel antimalarial abscisic acid

Abstract

The larval environment of holometabolous insects determines many adult life history traits including, but not limited to, rate and success of development and adult lifespan and fecundity. The ancient stress signaling hormone abscisic acid (ABA), released by plants inundated with water and by leaf and root fragments in water, is likely ubiquitous in the mosquito larval environment and is well known for its wide ranging effects on invertebrate biology. Accordingly, ABA is a relevant stimulus and signal for mosquito development. In our studies, the addition of ABA at biologically relevant levels to larval rearing containers accelerated the time to pupation and increased death of *A. stephensi* pupae. We could not attribute these effects, however, to ABA-dependent changes in JH biosynthesis-associated gene expression, 20E titers or transcript patterns of *insulin-like peptide* genes. Adult females derived from ABA-treated larvae had reduced total protein content and significantly reduced post blood meal transcript expression of *vitellogenin*, effects that were consistent with variably reduced egg clutch sizes and oviposition success from the first through the third gonotrophic cycles. Adult female *A. stephensi* derived from ABA-treated larvae also exhibited reduced lifespans relative to controls. Collectively, these effects of ABA on *A. stephensi* life history traits are robust, durable and predictive of multiple impacts on an important malaria vector spreading to new malaria endemic regions.

Introduction

Mosquitoes are the deadliest animals on the planet: in 2017, mosquito bites resulted in 219 million new cases of malaria, 435,000 of which were fatal (WHO 2017). A large effort has been made to estimate the burden of malaria in endemic areas using defined parameters including abiotic factors, human clearance of malaria parasites, and mosquito life history traits, such as survival, population density, reproductive output, biting rate, and parasite development (Smith et al., 2018). Many important mosquito species contribute to parasite transmission, increasing the complexity of both surveillance and control efforts. Our

focus is *Anopheles stephensi*, the Indian malaria mosquito, an aggressive malaria vector mosquito that has invaded and become established in Sri Lanka, Djibouti and Ethiopia, with significant risk for range expansion into Somalia, Eritrea and Sudan (Takken and Lindsay, 2019; Seyfarth et al., 2019; Surendran et al., 2018; Faulde et al., 2014). In Djibouti, *A. stephensi* has been linked to a resurgence of severe infection with the human malaria parasite *Plasmodium falciparum* (Seyfarth et al., 2019), so increased focus on this species is relevant and timely for control.

Larval development in *A. stephensi* is rapid and, under favorable conditions, may be completed within a week from egg hatching. On the other hand, less than ideal larval environments, for example, over-crowding, have been shown to alter life history traits, significantly reducing parasite transmission (Moller-Jacobs et al., 2014; Murdock et al., 2017). Larval development as well as timing of pupation, eclosion and subsequent adult female reproduction are tightly regulated by juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Jindra et al., 2013). In the first three instars, pulses of 20E occur when larvae reach critical mass and, in the presence of JH, 20E-dependent transcriptional regulators are inhibited and the larval structures are maintained (Brutis et al., 1990; Liu et al., 2009). Late in the final instar, JH synthesis decreases and JH esterase increases in activity, catalyzing the conversion of active JH to inactive JH acid (Vince and Gilbert, 1997), which triggers a 20E-dependent transcriptional cascade that induces the larval to pupal molt (Riddiford et al., 2000). Nutrient acquisition during the final larval instar determines adult body size (Boulan et al., 2015). Further, nutrient status of newly emerged adults modulates the effects of insulin/insulin-like growth factor signaling (IIS) on JH synthesis, which remodels the fat body in preparation for a bloodmeal (Raikhel & Lea, 1983; Perez-Hedo et. al, 2014).

Proteins and lipids stored from larval diet also determine proper ovarian development, with approximately half of the lipid stores carried forward to the adult stage (Zhou et al., 2004), such that mosquitoes with lower larval protein reserves develop smaller follicles (Caroci et al., 2004). During the first gonotrophic cycle, 80% of the lipids within the ovaries are derived solely from sugar meals acquired as adults (Ziegler et al., 2001). Following the blood meal, amino acids derived from blood proteins activate the target of rapamycin (TOR) signaling pathway, triggering a 20E cascade (Zhu et al., 2003). This cascade is initiated by 20E binding to the heterodimeric receptor comprised of the ecdysone receptor (EcR) and

ultraspiracle (USP) to upregulate the synthesis of vitellogenin (Vg) that is transported to the developing eggs (Martín et al., 2001). In the context of these changes, 20E delivered from the male during mating in *Anopheles gambiae* interacts with the EcR, together with a novel protein Mating-Dependent Regulator of Oogenesis or MISO, to regulate oogenesis and the post-mating switch to monandry and oviposition (Baldini et al., 2013; Gabrieli et al., 2014). While this physiology is presumably conserved in *A. stephensi* based on the presence of an orthologous *miso* gene, both post-feeding and post-mating physiology likely also modulate the switch to physiological sensitivity to oviposition site attractants (Davis and Takahashi, 1980).

Anopheles stephensi females show a breeding habitat preference for natural bodies of water, ranging in size from small puddles to larger calm riverbeds (Manouchehri et al., 1976). Flood and ditch irrigation can impact the ecology of mosquitoes by creating new breeding sites and larval habitats, which are more attractive to gravid female mosquitoes than natural habitats (Mwangangi et al., 2010; Wondwosen et al., 2016). Initial flooding and fast water currents are destructive to larval survival, causing physical harm and reducing critical oxygen tension (Soleimani-Ahmadi et al., 2014). However, following flooding there is an increase in mosquito density and diversity, due to increased temporary breeding habitats (Rodrigures et al., 2017). During flooding, inundated plants can experience high levels of stress, which can result in the release of plant stress hormones into aquatic environments. For example, research has shown flooding of tomato plants increases the concentration of the stress hormone abscisic acid (ABA) in soil water to ~1.7 µM (Else et al., 1995).

ABA was first identified in plants; however, it is now recognized as a universal signaling molecule which acts as an effective regulator of stress responses and pathogen biology in plants, parasitic protozoa, sponges, hydroids, insects, and mammals (reviewed in Olds et al., 2018). The interaction of ABA and insects has been studied in several contexts. In the big-headed grasshopper, *Aulocara elliotti,* ingested ABA increased the number of eggs per female; however, eggs derived from ABA-treated females exhibited decreased viability (Visscher et al., 1980). Injection of ABA reduced protein uptake and Vg concentrations following consumption of a liver meal in the flesh fly *Sarcophaga bullata* (De Man et al., 1981). In addition, ABA injection appeared to act negatively on 20E signaling by delaying the peak of 20E by 16 hours (De Man et al., 1981). ABA from nectar and pollen ingested by

honeybees (*Apis mellifera*) can be detected in honey (Lipp, 1990) and ingestion of ABA by honeybee larvae can increase cold tolerance and cellular immunity (Negri et al., 2017; Ramirez et al., 2017).

In our previous studies, we observed that ingestion of an ABA-supplemented blood meal by female *A. stephensi* induced signaling kinases associated with a transient metabolic shift in the midgut, fueling immune-mediated killing of *P. falciparum* prior to completion of oocyst development (Glennon et al., 2016; Glennon et al., 2017). Interestingly, ingested ABA did not decrease *A. stephensi* fecundity in the first gonotrophic cycle in contrast to our predictions based on the effects of ABA in *A. elliotti* and in *S. bullata* (Glennon et al., 2017). However, given the effects of ABA on metabolism and homeostasis of the *A. stephensi* midgut, on nutrient stores and 20E levels in other insects, and the potential that ABA in water used for oviposition could impact larval growth, we sought to understand whether ABA, at levels consistent with those released by inundated plants, could affect *A. stephensi* larval development, pupation and fitness of adult females emerging from treated larvae.

Our data show that, at concentrations in water as low as 1μM, ABA accelerated *A. stephensi* larval development with varying effects on larval 20E levels and increased mosquito death at the time of pupation. Adult females derived from ABA-treated larvae exhibited significantly reduced fecundity over multiple gonotrophic cycles and significantly reduced lifespan, which was not altered by additional treatment of adult females with ABA. Accordingly, the effects of exposure of larval *A. stephensi* to ABA were both striking and durable, suggesting that manipulation of ABA levels in breeding sites, perhaps through nanoparticle release of this natural compound, could be used to reduce mosquito development and reproduction as well as adult survival that is required for completion of the extrinsic incubation period of malaria parasite development.

Materials and Methods

Mosquito rearing

Anopheles stephensi Liston (Indian wild-type strain) were reared and maintained 27°C and 80% humidity with a 16/8-hour light/dark cycle. Adult mosquitoes were housed in 1 ft^3 wire mesh cages and provided continuous access to 10% sucrose-soaked cotton pads. Three days after eclosion, adult female mosquitoes were allowed to feed on live mice sedated

with ketamine (50 mg/kg) and xylazine (5 mg/kg) in sterile saline. All animal procedures were approved by the University of Idaho Animal Care and Use Committee. Mosquitoes were provided shallow cups of water to oviposit at 48 hours after blood feeding. Eggs were gently washed into 5 L Nalgene pans with shallow water. Larvae were maintained in 5 L Nalgene pans on a solution of 2% powdered fish food (Sera Micron) and baker's yeast in a 2:1 ratio for the first 3 days followed by Game Fish Chow pellet food (Purina) until pupation. Adult mosquitoes were collected for experiments within 12 hours post-eclosion and housed in screened, 500 mL polypropylene Nalgene containers.

Effects of ABA on A. stephensi larval development and pupation

Larvae were collected at 36 hours following transfer of eggs into Nalgene pans to reduce variability in the starting age among larvae used for these studies. For each treatment group, 100 larvae were placed in 500 mL polypropylene Nalgene containers with 200 mL water with or without 1, 10 or 100 μM ABA (Caisson Labs). Due to the light sensitivity of ABA, 50 mL of water from each container was removed daily and replaced with freshly made ABA-supplemented water to yield a final concentration of 1, 10 or 100 μM ABA. Low concentrations of ABA (1 and 10 μM) were based on published soil water concentrations of flooded tomatoes (Else et al., 1995) and on our data from submerged tomato leaves and roots in water (Table S1); the highest concentration (100 μM) was based on ABA treatment of *A. ellioti* from previous studies (Visscher et al., 1980). Larvae were fed as above and maintained through pupation and eclosion to adults. After the first pupae were observed, pupae were collected every 12 hours until no larvae remained. The numbers of pupae collected each day were recorded as "time to pupation" in days. Collected pupae were placed into cups with untreated water within cartons for adult eclosion; pupal survival was monitored until all adults had emerged and these data were recorded as the proportion of total pupae surviving through to adult eclosion. Based on this design, *A. stephensi* were exposed to ABA during the larval stage only. Five separate cohorts were used to complete biological replicates of this study.

Effects of ABA on adult A. stephensi lifespan

Female mosquitoes derived from untreated, control larvae or from larvae treated with 1, 10 or 100 μM ABA were maintained in separate cartons with 10% sucrose-soaked cotton

pads. At 3-5 days following eclosion, each group of mosquitoes was offered a "human blood meal" of washed human type O+ erythrocytes (Interstate Blood Bank) suspended 1:1 (vol:vol) in heat-inactivated human type A+ serum (Interstate Blood Bank). A similarly prepared blood meal was offered once weekly via a Hemotek feeder (Hemotek Ltd) until no mosquitoes remained alive. For one lifespan study, emerged adult female *A. stephensi* from each larval control and treatment group were split into two groups and treated as follows. One group of adults received an unsupplemented human blood meal each week whereas the other group was provided a weekly human blood meal supplemented with 100 nM ABA, which approximates the concentration of ABA present in blood in mice and humans with malaria (Glennon et al., 2016; Glennon et al., 2018). Two days following blood feeding, females were given the opportunity to oviposit in a shallow water dish. Dead females were counted and removed from each group every 48 hours. Two separate cohorts were used to complete biological replicates of the lifespan studies.

Effects of ABA on A. stephensi fecundity

At 3-5 days after adult eclosion from groups prepared as in 2.3, female *A. stephensi* were allowed to feed on a human blood meal. Following feeding, engorged females were carefully removed and placed into individual 50 mL conical tubes with moist filter paper and allowed to oviposit. Following oviposition, the number of eggs were counted and the females were returned to their respective control or treatment cartons. All females were held until they were fed again the following week. This process of feeding followed by separation and oviposition was repeated until females were no longer receptive to blood feeding. Four separate cohorts of mosquitoes were used to complete biological replicates of these studies.

qRT-PCR assays for relative gene expression

Relative transcript levels of *A. stephensi 3-hydroxy-3-methylglutaryl-coa reductase (hmg-r), juvenile hormone acid methyltransferase (jhamt), insulin-like peptides 1-5 (ilp1-5*; Marquez et al. 2011*),* and *Vg* were determined by qRT-PCR (Table S2). All data were normalized to transcript levels of *A. stephensi* housekeeping genes *ribosomal protein s7 (rps7)* and *rps17*. For larval gene expression analyses, five larvae from control and ABA-treated water were collected and pooled for RNA isolation at 1, 2, 4 and 6 hours following daily replacement of rearing water as described in 2.2. For *Vg* expression analyses, five adult

female *A. stephensi* were collected from control and ABA-treated larvae and pooled at 6, 12, 24, and 48 hours post-blood feeding for RNA isolation. Adults were killed by briefly freezing at -20°C and pools of larvae or adults were placed in 500 µL Trizol (Invitrogen). RNA was extracted using the phenol-chloroform method according to manufacturer's instructions. cDNA was synthesized using the QuantiTect reverse transcriptase kit (Qiagen) according to manufacturer's instructions. cDNA concentrations were adjusted to 500 ng/µL with molecular grade water. Data were normalized to housekeeping genes and reported as $Log_2(2^{-\Delta\Delta Ct})$. For each treatment group 4-5 replicates were completed, each with 3 technical replicates.

Effects of ABA on 20-hydroxyecdysone (20E) titer

20E titers were measured during larval and pupal development and in adult female *A. stephensi* derived from control and ABA-treated larvae. For these analyses, 40 larvae, 20 pupae or 20 adult female mosquitoes were collected and pooled for each time point from each group. Larval collections started at 3 days post-hatching and continued until pupae were detected at ~7 days post-hatching. Larvae were collected once a day at 8 hours following daily replacement of rearing water as described in 2.2. The pupal stage of *A. stephensi* lasts ~36 hours; pupae were collected every 8 hours for the duration of this stage. Adult female mosquitoes were collected within the first 8 hours following eclosion. Samples were prepared for analysis by adding 500 µL of 100% chilled methanol to pooled insects, then sonicating on ice (Fisher Scientific Model 100) at level 4 for 5 second intervals. Samples were centrifuged at 5000 *x g* for 5 minutes and the resulting supernatant transferred to a new tube. Methanol extraction was performed a second time and supernatants were pooled. Pooled supernatants were dried under N_2 stream and stored at -30°C until the 20E titers were measured using 20-hydroxyecdysone EIA kit (Arbor Assays), following manufacturer's instructions. Three separate cohorts of mosquitoes were used to complete biological replicates of these studies.

Effects of ABA on adult female A. stephensi protein content

To measure total protein content of adult female *A. stephensi* derived from control or ABA-treated larvae, mosquitoes within 8 hours of eclosion were homogenized in 10 mM dithiotheitol with 1 mM protease inhibitor cocktail (Sigma) in 100 µL loading buffer

(BioRad). Samples were boiled for 5 min and then centrifuged at 10,000 *x g* for 10 min at 4°C. Supernatants were transferred to new tubes and stored at -80°C. Total protein content was determined using Bradford reagent (Alfa Aesar) using bovine albumin serum (BSA) as a standard. Three separate cohorts of mosquitoes were used to complete biological replicates of these studies, each with three technical replicates.

Statistical analyses

All statistical analyses were performed using R statistical software version 3.5.3. Time to pupation and clutch sizes were analyzed by ANOVA and post hoc Tukey's test. Proportions of *A. stephensi* laying eggs and dying as pupae were analyzed using likelihood ratio test of independence (GTest). 20E titers by day were analyzed by Student's t-test. Data from qRT-PCR assays were normalized by $2^{-\Delta\Delta CT}$, $\log_2$ transformed and analyzed by ANOVA. Lifespan data were analyzed by two stage hazard rate analysis, in which the first stage is a log-rank test, and the second stage is used in cases where the hazard rates are not proportional and cross each other (Qiu and Sheng, 2008). Data across biological replicates within treatments were analyzed by ANOVA; if differences across replicates were not significant, replicate data were combined for analysis. Differences were considered significant at $\alpha \leq 0.05$. All figures were created using the ggplot2 package within R.

Results

ABA treatment of A. stephensi larvae reduced time to pupation, but not in association with rising 20E titers

ABA treatment of larval *A. stephensi* reduced mean time to pupation from 7.22 ± 0.77 days in untreated controls to 7.03 ± 0.67 days (1 μM ABA), 7.01 ± 0.76 days (10 μM ABA) and 7.11 ± 0.68 days (100 μM ABA) in treated larvae (**Figure 1**; ANOVA $p < 0.001$). Times to pupation in larvae treated with 1 and 10 μM ABA were not different (Tukey $p = 0.877$), but exhibited the shortest mean time to pupation relative to control. Larvae treated with 100 μM ABA had higher mean time to pupation relative to larvae treated with 1 μM ABA (Tukey $p < 0.001$) and 10 μM ABA (Tukey $p < 0.001$), but still pupated faster than untreated control larvae (Tukey $p < 0.001$). Although accelerated larval development would be expected to produce smaller adults (Lyimo et al., 1992), larval treatment with 10 and 100 μM ABA was

associated with increased size of emerged adult female *A. stephensi* relative to females derived from control untreated larvae (**Table S3**).

20E regulates the timing of larval molts and, during the fourth and final instar, 20E titers rise to induce a cascade of transcriptional responses resulting in the physiological changes during the larval-pupal molt (White and Ewer, 2014). Accordingly, we hypothesized that treatment of larvae with ABA might increase 20E titers earlier than in control larvae, resulting in reduced time to pupation in ABA-treated larvae. Based on the patterns observed in Figure 1, we analyzed 20E titers in larvae treated with the lowest (1 μM) and highest concentrations of ABA (100 μM) with untreated controls (**Figure 2**). Larvae exposed to 1 μM ABA showed no differences relative to controls in 20E titers over the course of larval development (days 1-7), during the pupal stage (days 8-9) or in newly emerged adult females (day 10). Larvae exposed to 100 μM ABA, however, had significantly reduced 20E titers on day 6 of larval development relative to controls (t-test $p = 0.017$), but no differences at any other timepoints. These results suggested that the observed reduction in time to pupation in larvae exposed to ABA did not result from significantly elevated 20E titers in the final days of larval development.

ABA did not alter transcript expression of JH-associated genes in larval A. stephensi

Since ABA treatment was not associated with increased 20E titers, we examined the expression of key genes in the mevalonate pathway and the branch pathway that synthesizes juvenile hormone (JH). During larval-larval molts JH titers remain elevated to suppress expression of 20E target genes and, in the final instar, JH titers decrease to undetectable levels, at which point 20E gene targets are upregulated and the pupal molt is initiated (Boulan et al., 2015). In the insect mevalonate pathway, 3-Hydroxy-3-Methylglutaryl-*CoA Reductase* (HMGCR) converts HMG-CoA to mevalonate, the precursor of farnesyl-pyrophosphate of the JH pathway. In the latter pathway, JH acid methyl transferase (JHAMT) methylates farnesoic acid to methylfarnesoate and is the rate-limiting enzyme in JH biosynthesis (Shinoda & Itoyama, 2003). The gene expression profiles of *hmgcr* and *jhamt* were measured hourly for 6 hours after daily replacement of rearing water as described in 2.2. If expression levels of *hmgcr* and *jhamt* were down regulated in 4th instar larvae following exposure to ABA this could translate to reduced JH titers and earlier expression of 20E gene targets. However, we did not observe any significant changes in *hmgcr* expression

relative to control in 4th instar larvae exposed to 1, 10 or with 100 μM ABA at 1 hr (ANOVA p = 0.070), 2 hr (ANOVA p = 0.275), 4 hr (ANOVA p = 0.683) or 6 hr post ABA treatment (ANOVA p = 0.239) relative to control (**Figure 3**). There were also no significant changes in expression of *jhamt* in 4th instar larvae through the same 6 hour period (ANOVA p = 0.144 for 1 hr, p = 0.462 for 2 hr, p = 0.054 for 4 hr, p = 0.690 for 6 hr post ABA treatment relative to control) (**Figure 3**). Based on these data, it is unlikely that ABA treatment of *A. stephensi* 4th instar larvae alters JH titers in a pattern consistent with the effects of ABA on larval development.

ABA did not alter expression of ilp genes in A. stephensi larvae

We previously demonstrated that ABA supplementation in a blood meal reduced the expression of *ilp3* and *ilp4* in adult female *A. stephensi* (Glennon et al., 2017), suggesting that ABA might also alter *ilp* expression in the larval stage. In *Drosophila melanogaster*, silencing of *ilp4, ilp5* and *ilp7* led to significantly increased food consumption by flies on almost all diets tested (Semaniuk et al., 2018). Since we saw no increase in 20E titers or changes in transcript expression of *hmgcr* or *jhamt*, we reasoned that ABA might decrease *ilp* expression in *A. stephensi* larvae in the 4th instar, which could increase food intake and trigger earlier pupation. Exposure of *A. stephensi* 4th instar larvae to ABA, however, through 6 hr after daily replacement of rearing water as described in 2.2 had no effect on transcript expression of *ilp4* (ANOVA p = 0.362 for 1 hr, p = 0.088 for 2 hr, p = 0.276 for 4 hr, p = 0.199 for 6 hr post ABA treatment relative to control) or *ilp5* (ANOVA p = 0.733 for 1 hr, p = 0.876 for 2 hr, p = 0.692 for 4 hr, p = 0.397 for 6 hr post ABA treatment relative to control) (**Figure 4)**. We also examined expression of *ilp1-3* through the same 6 hr period, but no significant differences relative to control were observed (not shown).

ABA increased the percentage of A. stephensi pupae dying prior to adult eclosion

In addition to reducing the time to pupation, treatment of *A. stephensi* larvae with ABA resulted in a higher percentage of dead pupae (out of the total for each treatment group) relative to control. In the absence of ABA treatment, 3.4% of pupae died prior to adult eclosion. However, treatment with 10 and 100μM ABA increased the percentage of dead pupae out of the total for each treatment group to 8.2% and 10.6 % relative to control (GTest $p < 0.001$ and $p < 0.001$, respectively). Accordingly, decreased time to pupation in ABA-

treated larvae (Figure 1) was associated with increased pupal death prior to adult *A. stephensi* eclosion.

ABA reduced the protein content in newly eclosed female A. stephensi

Female mosquitoes break down proteins derived from blood meals to amino acids, activating the TOR pathway, which signals fat body 20E synthesis and the upregulation of yolk protein precursor (YPP) genes in this tissue (Hansen et al., 2005). The nutrients stored during the larval stage are essential for JH-regulated fat body competency to respond to TOR activation such that malnourished mosquitoes exhibit reduced and delayed *Vg* transcript expression (Shiao et al., 2008). Based on these observations, we sought to examine the protein content of newly eclosed female *A. stephensi* to better understand the potential effects of ABA larval treatment on adult life history traits. Protein content of adult female *A. stephensi* derived from untreated control larvae was 14.6 μg/g (**Figure 6**). In comparison, protein content of adult females derived from larvae treated with 1 μM ABA trended downward relative to control but was not significantly different (**Figure 6**). However, protein content of females derived from larvae treated with 100 μM ABA was significantly lower than that in controls, but not significantly different from that of females derived from larvae treated with 1 μM ABA (**Figure 6**). Accordingly, increased body size in adult females derived from larvae treated with 10 and 100 μM ABA (Table S3) was associated with a trend towards decreased protein content (10 μM ABA) and significantly reduced protein content (100 μM ABA) relative to controls.

Adult female A. stephensi derived from ABA-treated larvae exhibited reduced fecundity

Given the reduction in protein content of newly eclosed female *A. stephensi* derived from ABA-treated larvae and the impact of nutritional status on JH-regulated fat body competency, we examined the fecundity of female *A. stephensi* derived from ABA-treated control larvae. In replicated assays, the percentages of females ovipositing and clutch sizes of female *A. stephensi* derived from ABA-treated larvae compared to females derived from untreated control larvae were variably reduced across three gonotrophic cycles.

For the first gonotrophic cycle adult females derived from larvae treated with 1 and 100 μM ABA had reduced clutch sizes (ANOVA $F = 15.195$, $p < 0.001$; Tukey $p < 0.001$) relative to controls (**Figure 7A**). In addition to smaller clutch sizes in the first gonotrophic

cycle, females derived from larvae treated with 100 µM ABA treatment were less likely to oviposit compared to controls (GTest p = 0.013) (**Figure 7B**). There was no effect of larval ABA treatment on clutch sizes in the second gonotrophic cycle (ANOVA F = 0.009, p = 0.99; **Figure 7C**) and an increase in the percentage of ovipositing females derived from larvae treated with 1 µM ABA mosquitoes compared to controls (GTest p = 0.038). As in the first gonotrophic cycle, however, there was a decrease in the percentage of ovipositing females derived from larvae treated with 100 µM ABA compared to controls (GTest p < 0.001; **Figure 7D).** By the third gonotrophic cycle, clutch sizes from females derived from larvae treated with ABA were again reduced relative to controls (ANOVA F = 4.49, p = 0.012; Tukey p = 0.017 for 1 µM ABA, Tukey p = 0.034 for 100 µM ABA; **Figure 7E**). However, in contrast to both prior gonotrophic cycles, there were no differences in the percentages of ovipositing females among the groups (GTest p = 0.62; **Figure 7F**).

Post-blood meal Vg transcript expression in adult female A. stephensi derived from ABA-treated larvae was delayed relative to controls

Based on the effects of ABA larval treatment on adult fecundity, we sought to quantify the expression of *Vg*, the major YPP gene, in adult female *A. stephensi* within the first 48 hours following a bloodmeal. The pattern of *Vg* expression in control females derived from untreated larvae followed the expected pattern of expression rising to a peak at 24 hours, then declining (**Figure 8**). Adult females derived from larvae treated with 1 and 100 µM ABA showed significantly reduced *Vg* expression at 24 hours post blood meal (ANOVA p < 0.001, Tukey p < 0.001 for 1 µM ABA; Tukey p < 0.001 for 100 µM ABA) (**Figure 8**), suggesting that the effects of ABA larval treatment on adult female fecundity are at least partially explained by a significant reduction in *Vg* expression post-blood meal.

Adult female A. stephensi derived from ABA-treated larvae exhibited reduced lifespan

Based on our observations of reduced protein content (Figure 6) and reduced fecundity (Figure 7) in adult female *A. stephensi* derived from ABA-treated larvae and evidence for tradeoffs between mosquito lifespan and reproduction (Harshman and Zera, 2007; Fairman et al., 2017), we predicted that ABA treatment of larvae might extend the lifespan of adult female *A. stephensi*. For this study, females derived from untreated control larvae and from larvae treated with 1 and 100 µM ABA received a blood meal once a week

with maintenance on 10% sucrose between blood meals. For an additional study, adult females derived from control and treated larvae were each split into two groups, with one group receiving no ABA in the weekly blood meals and the other group receiving 100 nM ABA in the weekly blood meals. In contrast to our prediction, median survival in adult females derived from larvae treated with ABA was reduced to 28 ± 10.78 days (1 μM ABA; log rank p = 0.028) and to 30 ± 14.22 days (100 μM ABA; two-stage p = 0.025) relative to the untreated control median lifespan of 34 ± 14.2 days. **(Figure 11)**. Consistent with previous observations of no effect of ABA in blood on adult female lifespan (Glennon et al., 2017), the addition of 100 nM ABA in the blood meal had no effect on adult lifespan nor did it alter the effects of larval treatment with ABA on adult lifespan (**Table S4**).

Discussion

Taken together, our data demonstrate that the effects of treating *A. stephensi* larvae with ABA are durable, starting with accelerated pupation and increased pupal death and lasting through multiple gonotrophic cycles to reduce fecundity and adult female survivorship. These effects indicate the potential for multiple population level impacts on mosquito density, biting and pathogen transmission through combined reductions in immature stages, fecundity and lifespan at ABA concentrations that could occur under natural conditions. In trying to understand these effects of ABA, we examined some obvious developmental cues in our studies. Accelerated pupation in holometabolous insects can result from blocking JH synthesis or activity. For example, removal of the corpora allata, which generates JH, results in precocious pupation in *Manduca sexta* due to a reduction in the critical weight threshold (Nijhout and Williams, 1974). Despite the obvious similarities in accelerated pupation, ABA had no effect on the expression of the JH synthesis-associated genes *hmgcr* and *jhamt*. Further, ABA affected 20E titers on only a single day of larval development (day 6) at the highest concentration of ABA (100 μM), affirming minimal to no effect of ABA on the interacting effects of JH and 20E. Our data contrast with the reported effects of ABA on 20E in *S. bullata* (De Man et al., 1981), suggesting the likely possibility that the effects of ABA vary to some degree across insect species. Among other cues that regulate growth and development, expression of *ilp* genes in 4th instar *A. stephensi* larvae was not altered by ABA treatment. While the current state of technology is not sufficient to

quantify ILPs in *A. stephensi*, we can reasonably conclude that JH, 20E and changes in ILP levels are not mediating the acceleration of development by ABA.

The protein content of newly eclosed adult females derived from larvae treated with ABA was reduced, perhaps as a consequence of accelerated development and increased adult size. Following a blood meal, malnourished mosquitoes have both delayed and reduced *Vg* transcript abundance and may require a second blood meal for proper egg development (Shiao et al., 2008). The reduction in *Vg* expression that we observed is consistent with reduced Vg levels in *S. bullata* injected with ABA and reduced Vg levels reported for malnourished *A. aegypti* (Shiao et al., 2008). Reduced protein content has been shown to result in smaller follicle size and increased resorption of oocytes (Caroci et al., 2004; Clifton & Noriega, 2011). In our studies, reduced protein content of newly eclosed adult females likely contributed to lower clutch sizes and reduced oviposition in the first gonotrophic cycle (following a blood meal at 4 days in week 1). Patterns of oviposition and fecundity in the second and third gonotrophic cycles (weeks 2-3) could reflect the fact that nutrients for optimizing reproduction and preserving the soma are limited. In fact, the inflection points in our lifespan data – which occur at 21 days (Figure 9) for 1 μM ABA and 26 days for 100 μM ABA – are consistent with the possibility that the cost of reproduction, even at reduced levels, outweighs any further investment in the soma of females derived from treated larvae relative to controls, which have consistently higher survivorship after these timepoints.

While dietary restriction and the resulting impacts on nutrient stores have been associated with lifespan extension in *Anopheles* mosquitoes (Fairman et al., 2017), we observed reduced survivorship with reduced body protein levels in *A. stephensi* derived from larvae treated with ABA. Reduced survival of ABA-treated mosquitoes was unexpected as weekly supplementation of adults with ABA in blood meals did not change adult female survival (Glennon et al., 2017). Our data indicate that the substantial effect of ABA on adult lifespan carries over from the larval stage and across not one, but two developmental transitions.

Study of the role of plants and plant biology in regulating mosquito life history has focused on the characteristics of oviposition sites that are shaped by both wild and cultivated plant species (Asmare et al., 2010; Wondwosen et al., 2018; Eneh et al., 2016, Wondwosen et al., 2017; Wondwosen et al., 2016; 2016; Omlin et al., 2007; Overgaard, 2007), on nectar

feeding and its effects on mosquito physiology (Jacob et al., 2018; Nikbakhtzadeh et al., 2014; Nyasembe et al., 2014), the potential role of invasive plants in promoting malaria parasite transmission (Stone et al., 2018), and the utility of plant-derived compounds as novel insecticides (Elango et al., 2009; Eliman et al., 2009; Govindarajan et al., 2008; Nathan et al., 2008). Here, we have taken the relationship between plant biology and mosquito biology a step further, connecting the effects of ABA, a universal signaling molecule first described in and well known from plants (Olds et al., 2018), at concentrations detected in water with submerged plant tissue that can alter substantial features of mosquito growth, development and survivorship across immature and adult stages. Together with our previous studies on the effects of ABA on *P. falciparum* development in *A. stephensi* (Glennon et al., 2017), the association of elevated blood levels of ABA with asymptomatic malaria in humans and reduced infection and disease pathology in our animal model of malaria (Glennon et al., 2018), the effects of ABA on the life cycle of malaria are both comprehensive and complex and will become undoubtedly more so with continued studies of mechanisms underlying this biology.

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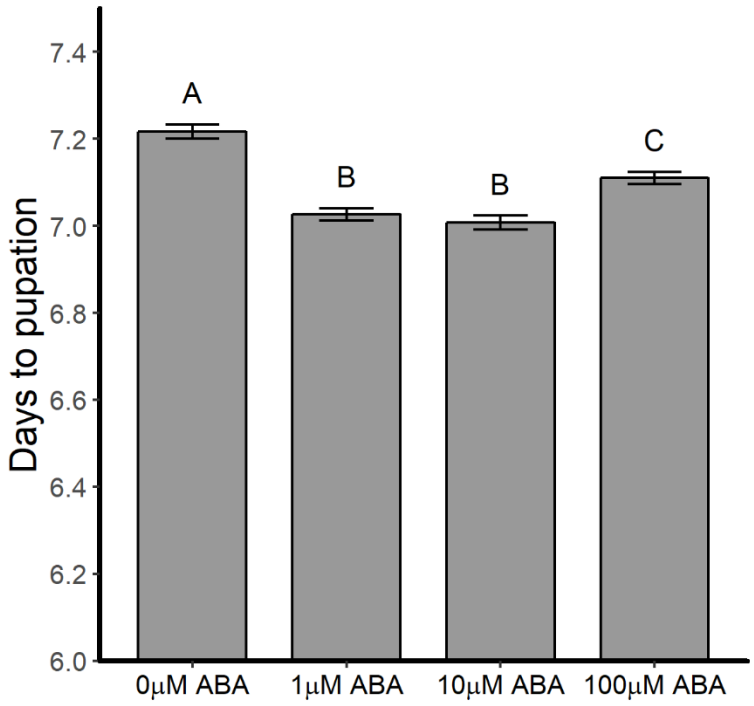

Figure 1. ABA treatment of larval *A. stephensi* reduced time to pupation. Larvae were exposed to 1, 10 or 100 µM ABA in rearing water as described in Methods 2.2. Time to pupation was reduced at all concentrations of ABA compared to control larvae (ANOVA $p < 0.001$). Data from five biological replicates were combined for analysis and are shown as mean ± SEM. Different capitalized letters indicate significant differences among controls and treatments. Control = 1456 larvae, 1 µM ABA = 1403 larvae, 10 µM ABA = 1382 larvae and 100 µM ABA = 1287 larvae.

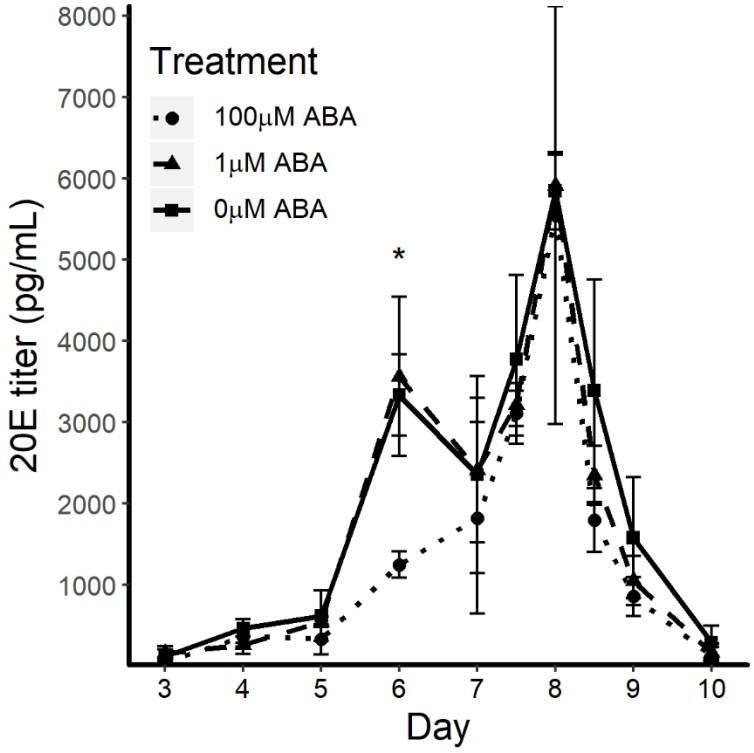

Figure 2. Effects of ABA on 20E titers through larval and pupal development and newly eclosed females. Measurement of 20-hydroxyecdysone (20E) starting 3-days post egg hatch through 8 hours post-eclosion of adult female mosquitoes. 1 μM ABA had no effect on 20E titers at any stage of development. 100 μM ABA reduces 20E titers during day 6 of the larval stage compared to control levels (t-test p = 0.017). However, 20E titers return to control levels during the pupal stage (Days 8-9) and the adult stage (Day 10). Data represents three biological replicates (20-40 individuals at each collection point), each biological replicate had three technical replicates, shown as mean ± SEM.

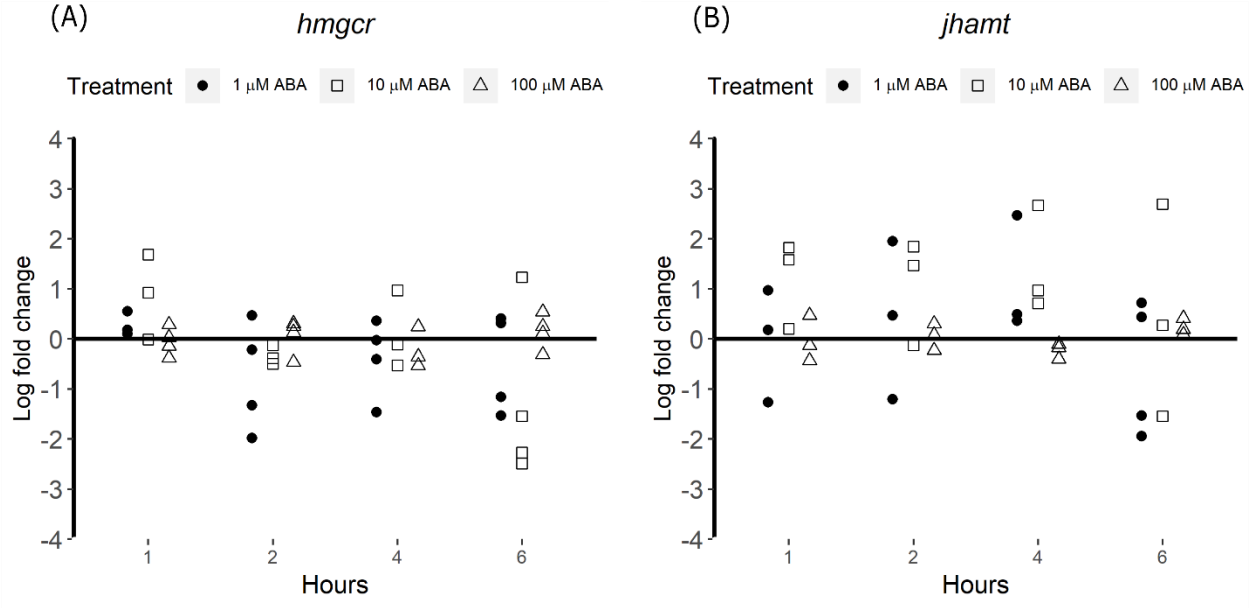

Figure 3. ABA treatment had no effects on transcript expression of *3-hydroxy-3-methylglutaryl-coa reductase* (*hmgcr*; **A**) and *juvenile hormone acid methyltransferase* (*jhamt*; **B**) in 4th instar *A. stephensi* larvae relative to controls through 6 hr following daily replacement of rearing water as described in Methods 2.2. Each data point represents a pool of five 4th instar mosquito larvae that were collected from five separate cohorts of *A. stephensi*. Three technical replicates were performed with each biological replicate sample to ensure assay reproducibility. Data are shown as Log_2-fold change expression over control, which is represented as the black line at "0".

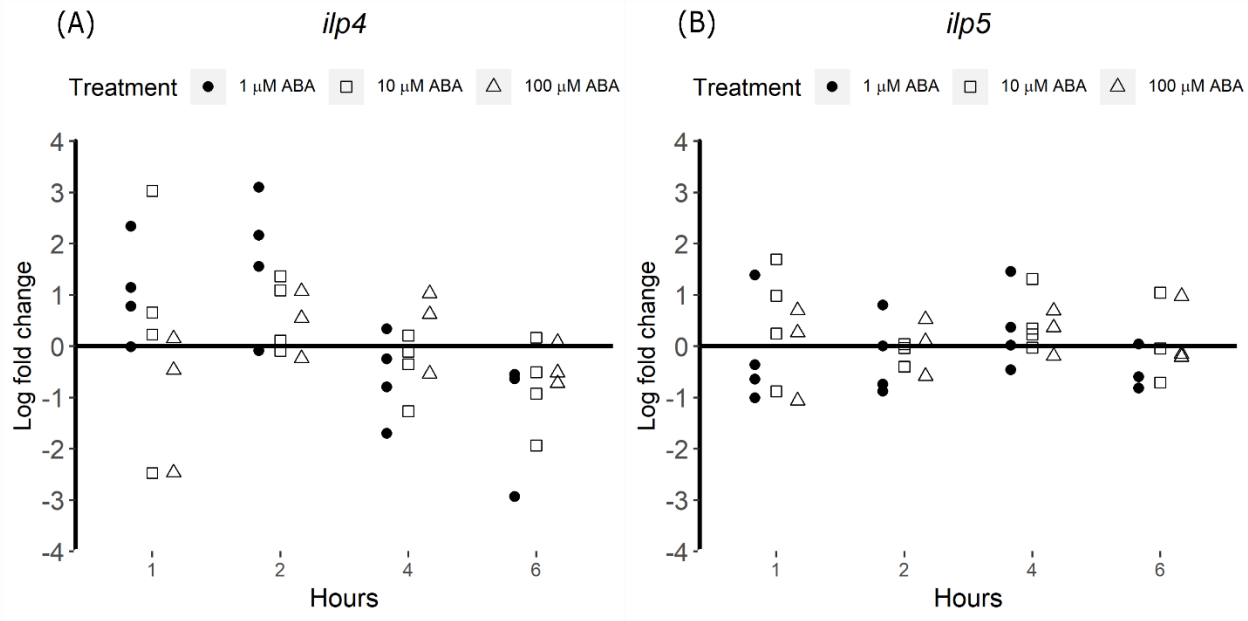

Figure 4. ABA treatment had no effects on transcript expression of ilp4 (A) and ilp5 (B) in 4th instar A. stephensi larvae relative to controls through 6 hr following daily replacement of rearing water as described in Methods 2.2. Each data point represents a pool of five 4th instar mosquito larvae that were collected from five separate cohorts of A. stephensi. Three technical replicates were performed with each biological replicate sample to ensure assay reproducibility. Data are shown as Log_2-fold change expression over control, which is represented as the black line at "0".

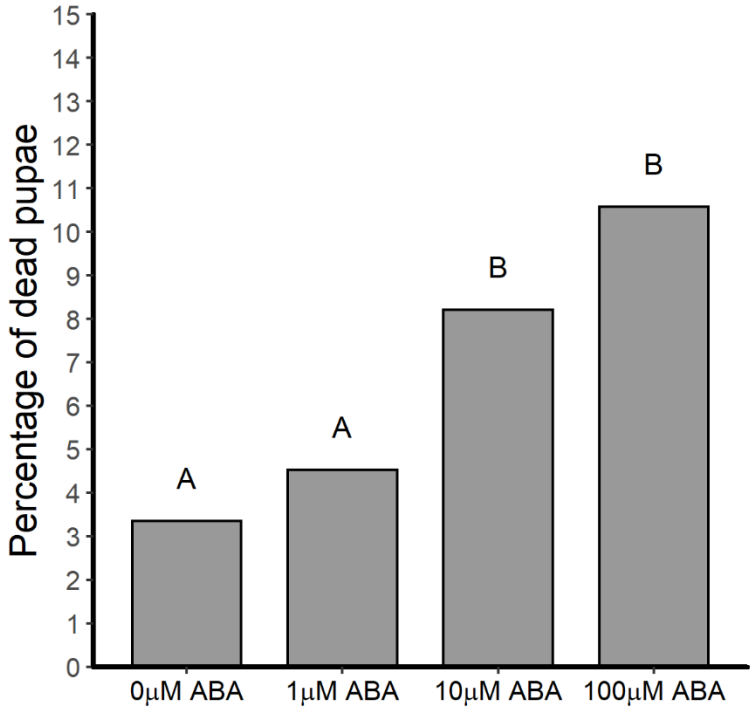

Figure 5. ABA treatment of larval *A. stephensi* increased the percentage of pupae dying prior to adult eclosion. While treatment of *A. stephensi* larvae with 1 μM ABA had no effect on pupae death relative to control (G-test $p = 0.249$), larvae that were exposed to 10 and 100μM ABA exhibited increased percentages of pupae death compared to control larvae (G-test $p < 0.001$ and $p < 0.001$, respectively). Data are represented as percentages of pupae out of the total collected that died prior to adult eclosion. Data from three biological replicates were combined for analysis. Different capitalized letters indicate significant differences among controls and treatments. Control = 776 larvae, 1 μM ABA = 772 larvae, 10 μM ABA = 767 larvae and 100 μM = 700 larvae.

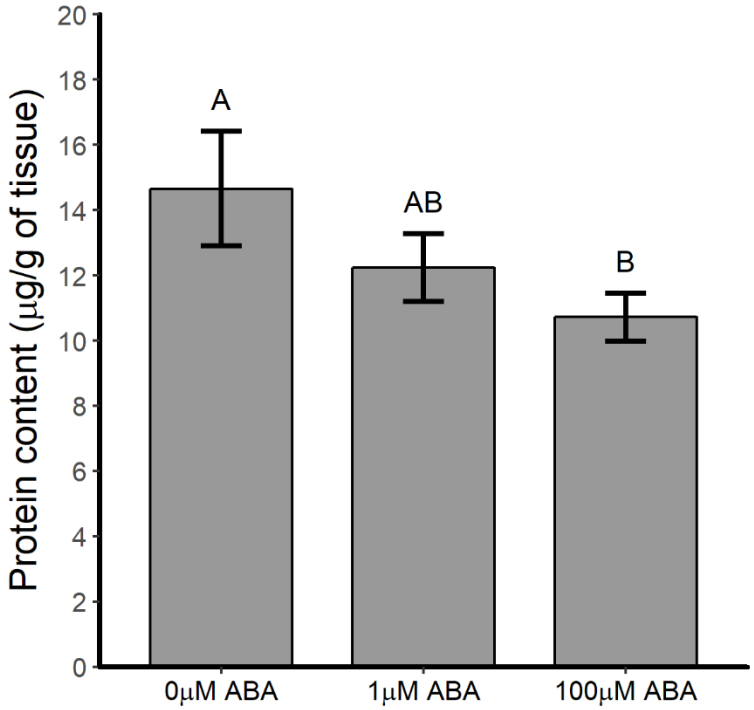

Figure 6. ABA treatment of larval *A. stephensi* reduced the protein content of newly eclosed adult females. Females emerged from larvae treated with 1 µM ABA exhibited a trend towards reduced protein content relative to controls (Tukey p = 0.213). Females emerged from larvae treated with 100 µM ABA exhibited reduced protein content relative to controls (Tukey p = 0.046), but were not significantly different from females emerged from larvae treated with 1 µM ABA (Tukey p = 0.489). Each biological sample was analyzed in triplicate to confirm assay reproducibility. Data from three biological replicates were combined for analysis and are shown as mean ± SEM. Different capitalized letters indicate significant differences among controls and treatments.

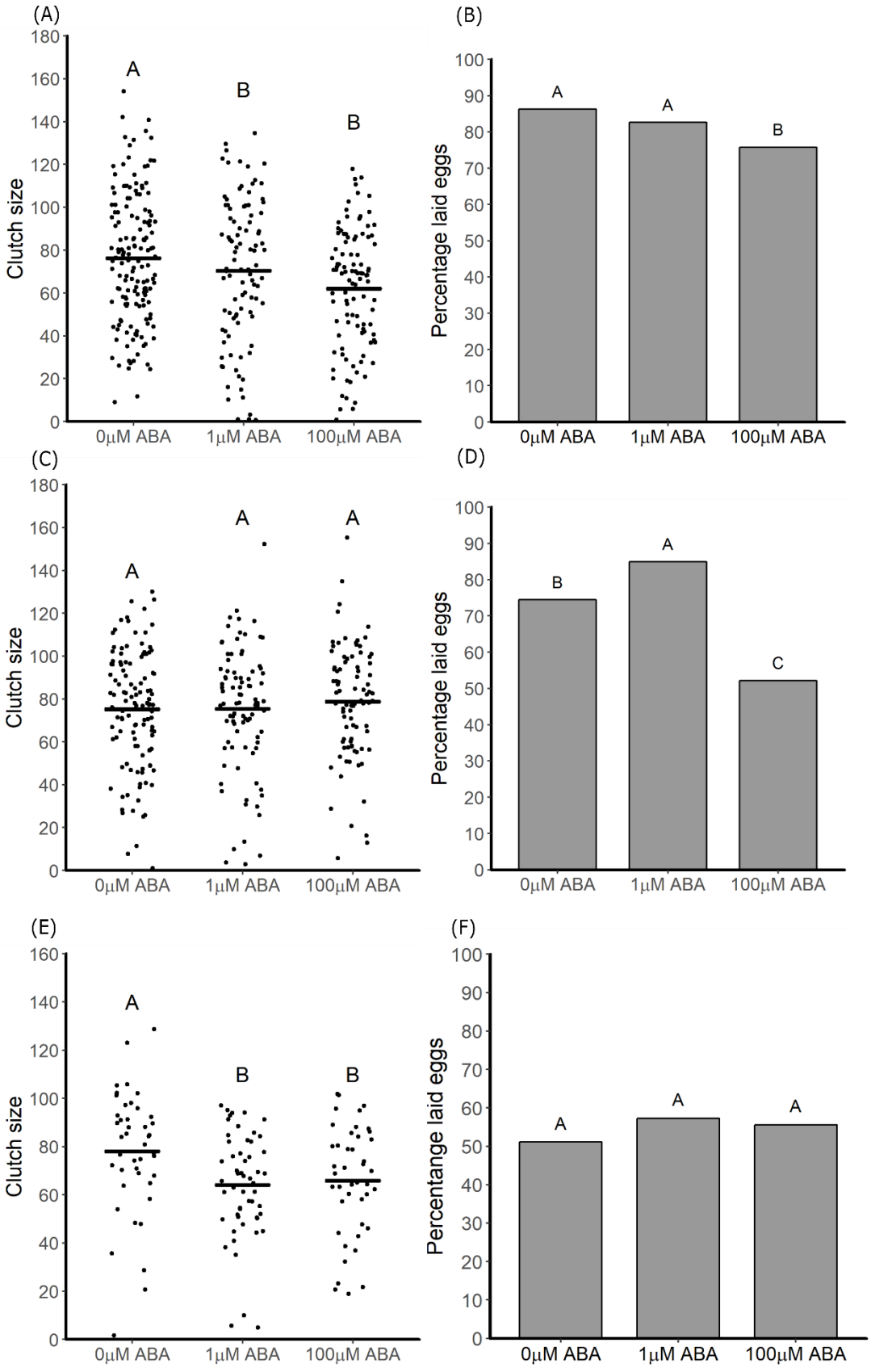
(A)
Clutch size
0
20
40
60
80
100
120
140
160
180
A
B
B
0μM ABA
1μM ABA
100μM ABA
(B)
Percentage laid eggs
0
10
20
30
40
50
60
70
80
90
100
A
A
B
0μM ABA
1μM ABA
100μM ABA
(C)
Clutch size
0
20
40
60
80
100
120
140
160
180
A
A
A
0μM ABA
1μM ABA
100μM ABA
(D)
Percentage laid eggs
0
10
20
30
40
50
60
70
80
90
100
B
A
C
0μM ABA
1μM ABA
100μM ABA
(E)
Clutch size
0
20
40
60
80
100
120
140
160
A
B
B
0μM ABA
1μM ABA
100μM ABA
(F)
Percentange laid eggs
0
10
20
30
40
50
60
70
80
90
100
A
A
A
0μM ABA
1μM ABA
100μM ABA

Figure 7. ABA treatment of larval A. stephensi variably reduced clutch sizes and percentages of ovipositing adult females across the first (**A, B**), second (**C, D**) and third (**E, F**) gonotrophic cycles. **(A)** Female mosquitoes derived from larvae treated with 1 and 100 µM ABA had reduced clutch sizes in the first gonotrophic cycle compared to controls (Tukey $p < 0.001$ and $p < 0.001$, respectively). Each dot represents the clutch size of a single female mosquito from four biological replicates; black bars represent means. **(B)** Treatment of larvae with 100 µM ABA reduced the percentage of ovipositing females relative to controls in the first gonotrophic cycle (Gtest $p = 0.013$). Data are shown as the means from three biological replicates, n = 389. **(C)** Larval treatment with ABA had no effect on clutch sizes of adult female A. stephensi (ANOVA $F = 0.009$, $p = 0.99$) in the second gonotrophic cycle. Each data point represents the clutch size of a single female mosquito from four biological replicates; black bars represents means. **(D)** Treatment of larvae with 1 µM ABA increased the percentage of ovipositing females mosquitoes compared to controls in the (GTest $p = 0.038$), while treatment of larvae with 100 µM ABA reduced the percentage of ovipositing females compared to controls (GTest $p < 0.001$). Data are shown as the means of three biological replicates, n = 298. **(E)** Larval treatment with ABA reduced clutch size in the third gonotrophic cycle (ANOVA $p = 0.012$; Tukey $p = 0.017$ for 1 µM ABA, Tukey $p = 0.034$ for 100 µM ABA). Each dot represents the clutch size of a single female mosquito from four biological replicates, black bars represent the mean of each treatment. **(F)** Percentage of fully engorged A. stephensi that laid eggs was not affected by larval treatment with ABA (GTest $p = 0.62$). Data are shown as the means of three biological replicates, n = 141.

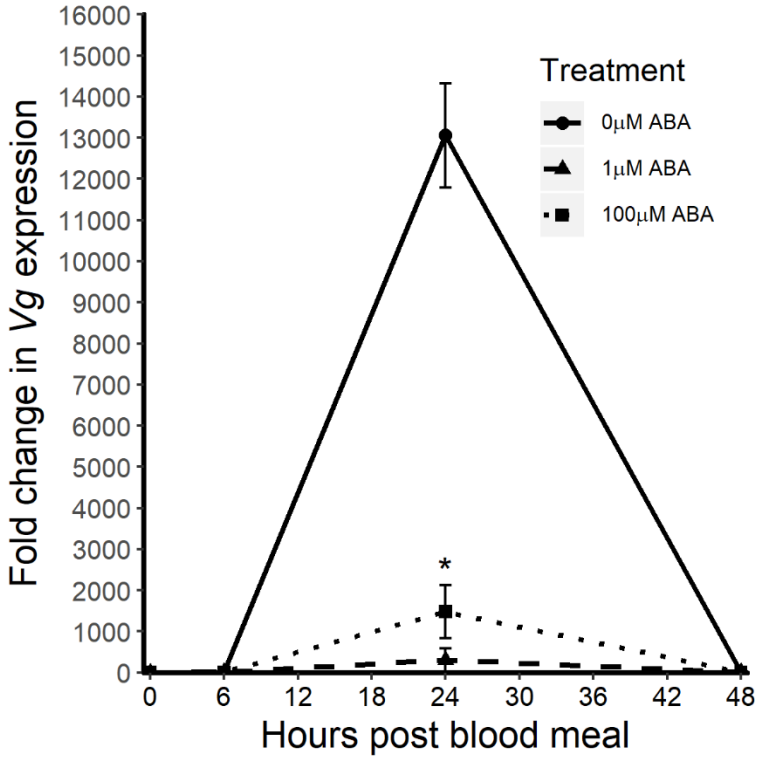

Figure 8. ABA treatment of larval A. stephensi reduced Vg transcript expression in adult female mosquitoes during the first 24 hr following a blood meal. At 6 hr post blood meal there was no difference in Vg expression in females derived from larvae treated with 1 µM or 100 µM ABA compared to controls (ANOVA $F = 0.722$, $p = 0.519$). At 24 hr post blood meal, however, Vg expression levels of females derived from larvae treated with 1 µM and 100 µM ABA were significantly reduced relative to controls (ANOVA $F = 56.374$, $p < 0.001$; Tukey $p < 0.001$ for 1 µM ABA, Tukey $p < 0.001$ for 100 µM ABA). Data are shown for three biological replicates as log fold change normalized within treatment group to Vg expression before the blood meal.

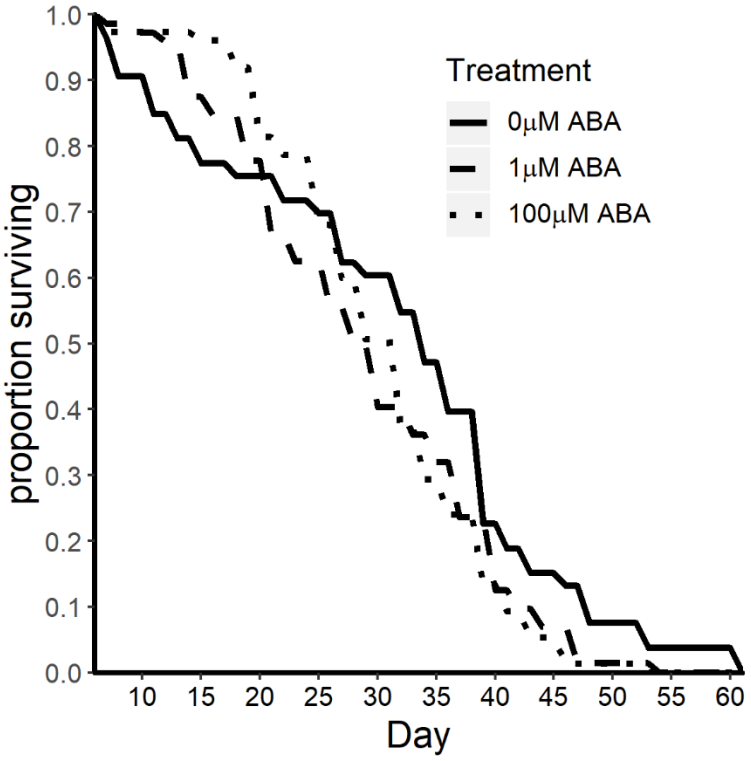

Figure 9. ABA treatment of larval A. stephensi reduced adult female survival. Survival curves of adult female mosquitoes derived from control untreated larvae and larvae treated with 1μM ABA (log-rank p = 0.028) and 100μM ABA (two-stage p = 0.025). Median survival was reduced from 34 ± 14.2 days in controls to 28 ± 10.78 days (1μM ABA) and to 30 ± 14.22 days (100 μM ABA). Data are representative of two biological replicates.

Supplementary Material

Table 1. Concentrations of ABA detected over time after submerging 2 g of Solanum lycopersicum (tomato) leaves, roots or leaves and roots in 200 mL of water. At 6, 24, and 48 hours, 1mL samples of water from each treatment were collected for analysis by LC-MS/MS. Samples were analyzed in triplicate, with an internal standard of deuterated 6-ABA. Data are shown as mean ± SEM.

Leaf	**ABA concentration (µM)**
6 hours	2.22 ± 0.46
24 hours	1.39 ± 0.12
48 hours	2.90 ± 0.66
Root	**ABA concentration (µM)**
6 hours	1.03 ± 0.56
24 hours	1.56 ± 0.30
48 hours	0.51 ± 0.29
Leaf + Root	**ABA concentration (µM)**
6 hours	0.65 ± 0.08
24 hours	0.89 ± 0.15
48 hours	1.48 ± 0.57

Table 2. Primer sequences for qRT-PCR assays

qRT-PCR primer sequences		
Gene	**Forward**	**Reverse**
Vg	5’-CAACATCATGTCCAAGTCGGAGGTGA-3’	5’-CTTGAAGCTTTCGTGCTCTTCCTCCG-3’
hmgcr	5’-GCGACACAACAGATCATCGGTAGC-3’	5’-AGATGATGGTGGATTCGTTTCACCAC-3’
jhamt	5’-TCAGCACACTGCGTTCTCCAAC-3’	5’-GGGCGAACGAGACAGTTGGTT-3’
ilp1	5’-GCTCGCTTCACTTGGTGTAACA-3’	5’-AAAGAAGCGAATGAAGTTTGATGA-3’
ilp2	5’-TAACCGCCGATCGCTTCT-3’	5’-GCAGTGTGGCTACCTCAACCTT-3’
ilp3	5’-GCGCGTCGATCTACAGTTTGA-3’	5’-GGTCGTGTCCGTCTTCATGA-3’
ilp4	5’-GAGTTGACATCACAGTGGTTTAGGA-3’	5’-TCGCATCCGTGCCTTAATG-3’
ilp5	5’-CAGTCGGCGGGACAAAAT-3’	5’-CGTAGGCCACTTCACGATCA-3’
rps7	5’-GATTGCTGGTTTCGTGACCCATTTGA-3’	5’-GGATCCACCTCAATGATGTCCTGC-3’
rps17	5’-GATTGCTGGTTTCGTGACCCATTTGA-3’	5’-GGATCCACCTCAATGATGTCCTGC-3’

Table 3. Mean wing size of adult female *A. stephensi* emerged from control untreated larvae or from larvae treated with 1, 10 or 100 µM ABA. In two of three replicates, larval treatment with 10 and 100 µM ABA was associated with increased wing size of emerged female mosquitoes relative to controls. There was no effect of larval treatment with 1 µM ABA in any replicate. Bold, underlined text indicates significance ($p < 0.05$) relative to control mosquitoes.

	Wing size (millimeters)				
Replicate	**Control**	**1 µM ABA**	**10 µM ABA**	**100 µM ABA**	**ANOVA p**
1	3.04 ± 0.14	3.03 ± 0.11	**3.12 ± 0.10**	**3.15 ± 0.12**	<0.0001
2	3.15 ± 0.07	3.17 ± 0.10	3.17 ± 0.10	3.16 ± 0.10	0.7375
3	3.12 ± 0.10	3.38 ± 0.17	**3.36 ± 0.12**	**3.24 ± 0.11**	0.0004

Table 4. Treatment of *A. stephensi* larvae with 1, 10, and 100 µM ABA reduced lifespan of adult female mosquitoes relative to untreated controls, regardless of additional adult supplementation with 100 nM ABA. In accord with previous observations (Glennon et al., 2017), lifespan of adult females derived from untreated larvae and supplemented with 100 nM ABA in a weekly blood meal was not significantly different from females derived from untreated larvae and not supplemented as adults (Log-Rank $p = 0.904$). In the presence and absence of adult ABA supplementation, lifespan of females derived from larvae treated with 1 µM ABA was significantly reduced relative to adult females derived from untreated larvae (Log-Rank $p = 0.006$); similarly, in the absence of adult supplementation, lifespan of females derived from larvae treated with 1 µM ABA was significantly reduced relative to adult females derived from untreated larvae (Log-rank $p = 0.028$). However, there was no difference in lifespan of adult females derived from larvae treated with 1 µM ABA and supplemented or not as adults with 100 nM ABA (Log-Rank $p = 0.958$). In the presence of adult supplementation, lifespan of adult females derived from larvae treated with 10 µM ABA was reduced relative to females derived from untreated larvae (Log-Rank $p = 0.005$);

similarly, in the absence of adult supplementation, lifespan of adult females derived from larvae treated with 10 µM ABA was reduced relative to females derived from untreated larvae (Log-Rank p = 0.025). However, there was no difference in lifespan of adult females derived from larvae treated with 10 µM ABA and supplemented or not as adults (Log-Rank p = 0.952). Finally, in the presence of adult supplementation, lifespan of adult females derived from larvae treated with 100 µM ABA was reduced relative to females derived from untreated larvae (Log-Rank p = 0.015); similarly, in the absence of adult supplementation, lifespan of adult females derived from larvae treated with 100 µM ABA was reduced relative to females derived from untreated larvae (Log-Rank p = 0.025). However, there was no difference in lifespan of adult females derived from larvae treated with 100 µM ABA and supplemented or not as adults (Log-Rank p = 0.116).

	Adult ABA = 0 µM Larval ABA = 0 µM	Adult ABA = 0 µM Larval ABA = 1 µM	Adult ABA = 0 µM Larval ABA = 10 µM	Adult ABA = 0 µM Larval ABA = 100 µM	Adult ABA = 100 nM Larval ABA = 0 µM	Adult ABA = 100 nM Larval ABA = 1 µM	Adult ABA = 100 nM Larval ABA = 10 µM
Adult ABA = 0 µM Larval ABA = 1 µM	**0.028**	-	-	-	-	-	-
Adult ABA = 0 µM Larval ABA = 10 µM	**0.025**	0.894	-	-	-	-	-
Adult ABA = 0 µM Larval ABA = 100 µM	**0.025**	**0.044**	0.063	-	-	-	-
Adult ABA = 100 nM Larval ABA = 0 µM	0.904	**0.007**	**0.004**	**0.024**	-	-	-
Adult ABA = 100 nM Larval ABA = 1 µM	**0.027**	0.958	0.725	**0.032**	**0.006**	-	-
Adult ABA = 100 nM Larval ABA = 10 µM	**0.024**	0.968	0.952	0.086	**0.005**	0.926	-
Adult ABA = 100 nM Larval ABA = 100 µM	**0.048**	0.531	0.654	0.116	**0.015**	0.524	0.530