Role of fatty acid synthase (FAS1) in blood-fed *Aedes aegypti* mosquitoes

Author: Amy L. Alabaster
Project Advisor: Roger L. Miesfeld

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Signatures below indicate approval of the scope and content of this senior thesis.

Project Advisor _____ Date _____

Undergraduate
Committee Member _____ Date _____

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Role of fatty acid synthase (FAS1) in blood-fed *Aedes aegypti* mosquitoes

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Abstract: The study of anti-mosquito targets specific to the unique mode in which female mosquitoes acquire their nutrients is a novel approach to the increasing global pandemic of vector borne diseases such as Malaria and Dengue Fever Virus. Mosquito proliferation, and thus disease proliferation, is rigidly dependent on the acquisition of the vertebrate blood meal since female mosquitoes require nutrients obtained from blood to synthesize egg yolk components and to replenish maternal reserves used to produce eggs. This project focuses on the lipid synthesis component of blood meal metabolism, and in particular on the expression and functional role of two fatty acid synthase genes, FAS1 and FAS2. Quantitative real-time reverse transcriptase polymerase chain reaction data suggests that FAS1 transcript levels in the midgut of blood fed mosquitoes are regulated by blood feeding, showing that *Aedes aegypti* mosquitoes synthesize lipids from blood meal proteins. A correlation between fatty acid synthesis and egg development was confirmed in three different ways from the phenotypic analysis of FAS1 dsRNA injected mosquitoes: by qualitative photographs, follicle measurements, and oviposited egg counts. It was concluded that there is a significant detrimental effect on mosquito reproduction when dsFAS1 RNA is injected. This is important to disease control because reduced mosquito populations could bring infection rates below a level needed to sustain disease endemism.

Introduction

Female mosquitoes often ingest or transmit pathogenic diseases in the process of consuming an equivalent of their own body weight in blood (1). *Aedes aegypti* mosquitoes, in particular, are responsible for the transmission of such deadly viruses as Yellow Fever Virus and Dengue Fever Virus. Mosquito proliferation, and thus disease proliferation, is rigidly dependent on the acquisition of the vertebrate blood meal since female *Aedes aegypti* mosquitoes require nutrients obtained from blood to synthesize egg yolk components and to replenish maternal reserves used to produce eggs (2). Mosquito borne diseases are on the rise, due in part to a lack of adequate mosquito control measures. Broad spectrum insecticides are under scrutiny because of their harmful effects on the environment and because insects are rapidly developing resistance to some of the most commonly used compounds. Vaccines against the disease organism similarly cannot keep up with selective resistance and often fail to be efficacious. A new approach then is to find anti-mosquito targets specific to the unique mode in which female mosquitoes acquire their nutrients.

Ideally, modern insecticides should inhibit metabolic processes vital for mosquito population survival without any negative repercussions to non-targeted organisms, including humans. The first step in designing mosquito selective inhibitors is to screen the vector's genome for potential targets. RNA interference can be used to test whether there is a significant detrimental effect on mosquito viability when a candidate gene is knocked out. If a bioinformatics search confirms that a candidate knockout is unlikely to be detrimental to the health of other organisms, small molecule inhibitors could be designed and tested as vector control agents.

In this way, a meticulous analysis of the metabolic and regulatory pathways involved in mosquito lipid utilization could uncover interesting mosquito control targets. This is because vector mosquitoes require a blood meal for reproduction, yet vertebrate blood is primarily composed of proteins and the mosquito egg contains large amounts of lipid (3). Besides the blood meal and larval stores, the only other source of carbon for lipid biosynthesis is carbohydrate contained in nectar meals, which does not provide sufficient carbon for lipid biosynthesis during *multiple* gonotrophic cycles . Therefore, degradation of proteins derived from the mosquito blood meal must be providing essential carbon skeletons for ongoing lipid synthesis, especially if nectar sources are limiting between available blood meals.

The project presented here focuses on the expression and functional role of two fatty acid synthase (FAS) genes in *Aedes aegypti*. The animal and fungal FAS is a very large multifunctional protein which carries out all enzymatic steps in the synthesis of palmitate from malonyl-CoA. This is in contrast to fatty acid synthesis carried out in prokaryotes and plants in which each step is catalyzed by several monofunctional enzymes. Even though structural organization diverges, the basic steps in fatty acid synthesis remain the same across all organisms. First, acetyl transferase (AT) transfers the acetyl moiety of acetyl coenzyme A to acyl carrier protein (ACP) which then shuttles it to the catalytic site of ketoacyl synthase (KS). After a malonyl moiety from malonyl coenzyme A is transferred to ACP by malonyl transferase (MT), KS condenses the acytyl and ACP-attached malonyl moiety. After condensation, the beta-ketoacyl-ACP product is reduced to a fully saturated carbon chain in three sequential reactions by three different enzymes, ketoreductase (KR), a dehydratase (DH), and enoyl reductase (ER). The chain continues to grow in two carbon units as further malonyl moieties are condensed with the growing chain and are subsequently reduced. Thioesterase (TE) releases the final product,

either a C16 palmitate or C18 stearate, as a free fatty acid. In animals the functions of AT and MT are carried out by one bi-functional domain, and in fungi the MT and TE domains are combined. The active animal FAS is a homodimer, with each polypeptide containing all seven functional domains. It is organized with a lower condensing part and an upper beta-modification part that come together to form two reaction compartments. The ACP and ET domains are loosely tethered at the terminal end of the enzyme (4).

Nothing is known yet about the tissue specific expression or metabolic role of FAS in *Aedes aegypti* mosquitoes. Therefore the goal of this senior project was to begin investigating this vital metabolic gene and to possibly learn more about exploitable regulatory processes involved in lipid synthesis.

Methods

Mosquitoes

Aedes aegypti (NIH-Rockefeller strain) were reared under standard conditions (5). Females were allowed to feed on 10% sucrose for up to five days after adult emergence and then fed porcine blood supplemented with ATP (5.0 mM final concentration). For dsRNA microinjection, *Aedes aegypti* female mosquitoes were used 1-2 days after emergence.

Expression Analysis

Quantitative real-time reverse transcriptase polymerase chain reaction (QRT-PCR) was carried out to quantify tissue specific mRNA expression patterns of FAS1 and FAS2 during blood meal digestion and oogenesis. cDNA was synthesized using 1.0μg of total RNA isolated from midgut

and fat body tissue samples as described by Scaraffia et al. (6). Tissues were collected from two separate cohorts of mosquitoes at 10 discreet time points (0, 3, 6, 12, 24, 36, 48, 72, 120 h post blood meal). The resulting cDNA was diluted 8-fold. QRT-PCR reactions were set up and amplified, and data analyzed according to the procedure described by Isoe et al.(7). Levels of FAS1 and FAS2 transcripts were normalized to ribosomal S7 protein gene transcripts. Sense and antisense primers used in these reactions are described in Table 1. The lowest transcript level (FAS2 in midgut of unfed mosquitoes) was chosen as the reference point for all other RNA samples (Figure 2).

RNA Interference

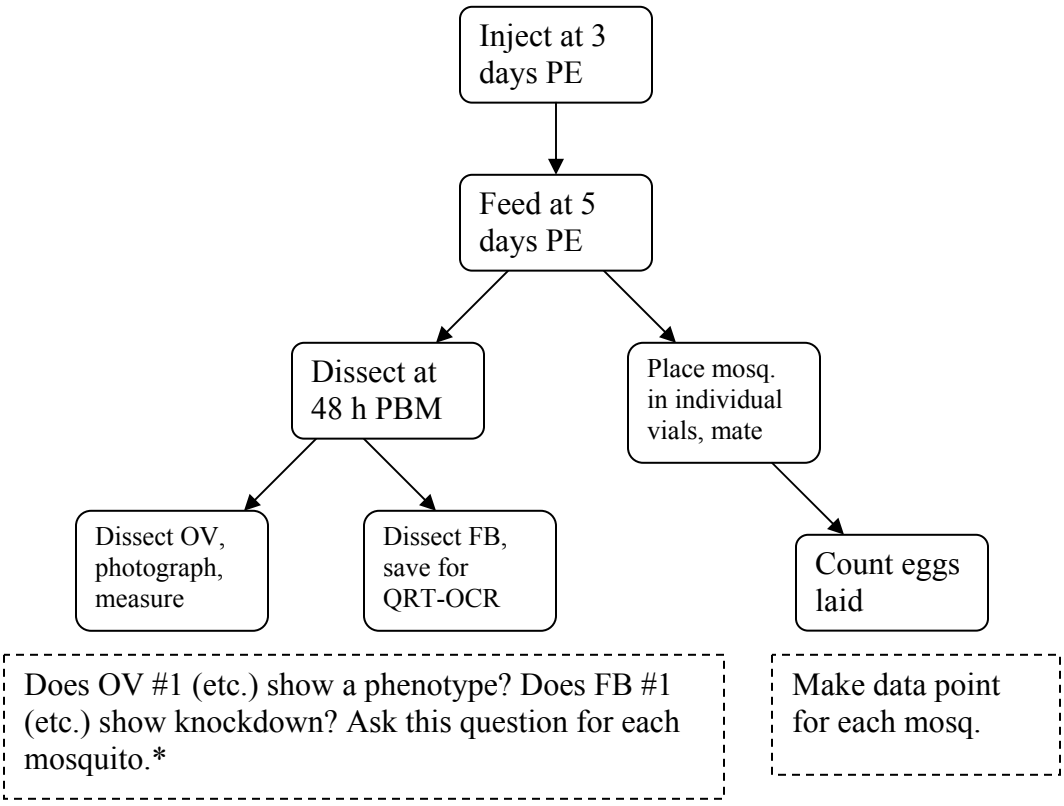
Double stranded FAS1 RNA (dsRNA) was synthesized using the same procedure described in Lu et al.(8). The FAS1 gene was cloned and sequenced as described by Scaraffia et al. (6). The cloned FAS1 was then used as a template to add T7 promoters to each strand. Control luciferase (FLUC) dsRNA was also constructed. Three day old adult mosquitoes were injected six times with 5.0 mg/ml FAS1 or FLUC dsRNA using a Drummond Nanoject injector (Drummond Scientific Company, Broomall, PA) set at 69 nl/injection. Approximately 2µg was injected into the thorax of each mosquito. Mosquitoes were maintained on 10% sucrose for two days before being fed a blood meal. Half of the FAS1 injected mosquitoes, and half of the FLUC injected mosquitoes, were dissected at 48 hours post blood feeding for phenotypic analysis. The remaining mosquitoes were individually put into cups to measure ovipositioning.

Phenotypic analysis

Figure 1 shows a schematic of the steps taken post-injection to track the reproductive phenotypes of FAS1-injected mosquitoes. At 48 hours post blood feeding, half of the FAS1 and FLUC injected mosquitoes were dissected. The fat body from each mosquito was placed in an individual tube with 40µl Trizol reagent (Invitrogen). Photos were taken of each pair of ovaries, keeping image magnification constant. Representative photos were chosen and are presented below. Six follicles chosen at random from each pair of ovaries were measured using a micrometer set in the microscope objective. The average follicle size from each mosquito (small-sized follicles, medium-sized follicles, large-sized follicles), was used to separate the fat body samples into separate pools for subsequent QRT-PCR analysis. Knockdown effect was determined from the QRT-PCR analysis and presented as RNA levels relative to FLUC injected fat body samples.

Mosquitoes that were saved for ovipositing were placed in individual cups along with 2-3 male mosquitoes. Mosquitoes deposited their eggs on papers placed in small beakers filled halfway with water. After all mosquitoes had deposited their eggs, the papers were removed, and the eggs were counted. Data was collected for individual mosquitoes and is presented in a scatter plot, with each point representing a single mosquito (Figure 5).

Figure 1. Schematic of RNAi phenotypic analysis experiments. PE: post eclosion. PBM: post blood meal. OV: ovary. FB: fat body. Dotted line boxes represents plans for future experiments. * Analysis done with pools rather than individual mosquitoes in present experiment.



Results

A human FAS protein coding sequence (2511aa) was identified using Entrez protein analysis software with an NCBI reference sequence of NP_004095.4. The protein sequence of human FAS was then used for a Basic Local Alignment Search Tool (BLAST) query on vectorbase.org, an online bioinformatics resource for common invertebrate disease vectors including *Aedes aegypti* mosquitoes (9,10). In agreement

with the results obtained by Nene et al. (10), six gene sequences match the human FAS sequence with E-values equal to zero. Sequence homology between *Aedes* FAS genes and the human FAS gene range from 33.07% to 49.47%. The sequences with the greatest homology to human FAS are AAEL001194-RA (2422aa, henceforth called FAS1) and AAEL008160-RA (2386aa, henceforth called FAS2). Using the VectorBase sequence database, it was found that FAS1 and FAS2 have 198 and 47 unique EST hits respectively, which were also identified using the vectorbase.org BLAST tool. By comparison, the other four *Aedes* FAS genes have EST hits ranging from 3 to 32. Therefore, the FAS1 and FAS2 genes were chosen for the experiment described here because the EST database suggests that they are likely to be the most highly expressed.

FAS1 gene expression is induced by feeding in mosquito midgut

As shown in figure 2A, we found that FAS2 expression in the midgut is not significantly altered after blood feeding. FAS2 in the midgut of unfed mosquitoes also has the lowest transcript levels out of the four gene/tissue combinations studied, which is why it was chosen to be the baseline reference for relative value comparisons. Both FAS1 and FAS2 in the fat body (Figure 2c, 2d) are similarly unchanged following a blood meal. On the other hand, FAS1 transcript levels in the midgut of blood fed mosquitoes do appear to be regulated, with peak expression levels at 6 hours and then decreasing (Figure 2a). This blood meal-induced expression of nearly 20-fold is consistent with earlier metabolic data showing that *Ae. aegypti* mosquitoes do in fact synthesize lipids from blood meal proteins, as was also deduced by Briegel et al (11). Moreover, at peak expression points in the midgut, FAS1 is expressed more than 400 times greater than FAS2 in blood fed

mosquitoes, and more than 50 times greater in the fat body. This parallels the finding on Vector Base that FAS1 has a higher number of EST hits. Note also that the levels of both FAS1 and FAS2 transcript levels appear to vary by 3-7 fold in the fat bodies of blood fed mosquitoes, however, these levels do not follow a predictable pattern and need to be confirmed using additional mosquito cohorts.

RNA interference leads to significant reproductive phenotypes

The correlation between fatty acid synthesis and egg development is confirmed in three different ways from the phenotypic analysis of FAS1 dsRNA injected mosquitoes. First, it is shown qualitatively by the characteristic photos in Figure 3 that mosquito follicles from FAS1 injected mosquitoes are underdeveloped at 48 hours post blood meal. Individual follicles appear smaller and more irregularly shaped. Second, it is shown quantitatively that follicles chosen at random are on average smaller in FAS1 injected mosquitoes than in FLUC injected mosquitoes (Figure 4). FAS1 injected mosquitoes that have follicles of the same or greater size than FLUC injected mosquitoes could represent inefficient knockdown by the dsRNA. Several of the mosquitoes do indeed have follicle sizes well below the natural variation seen in most of the FLUC injected mosquitoes.

Figure 2. QRT-PCR data for A) FAS2 and B) FAS1 in midgut and C) FAS2 and D) FAS1 in fat body measured at discreet times points post blood meal. The lowest unfed transcript level (FAS2 midgut) was chosen to be equal to 1 and all other data points are relative to this value. Presented in this way, FAS1 is clearly much more highly expressed in both midgut and fat body than FAS2. Gene regulation only appears to be significant for FAS1 expression in the *Aedes* midgut.

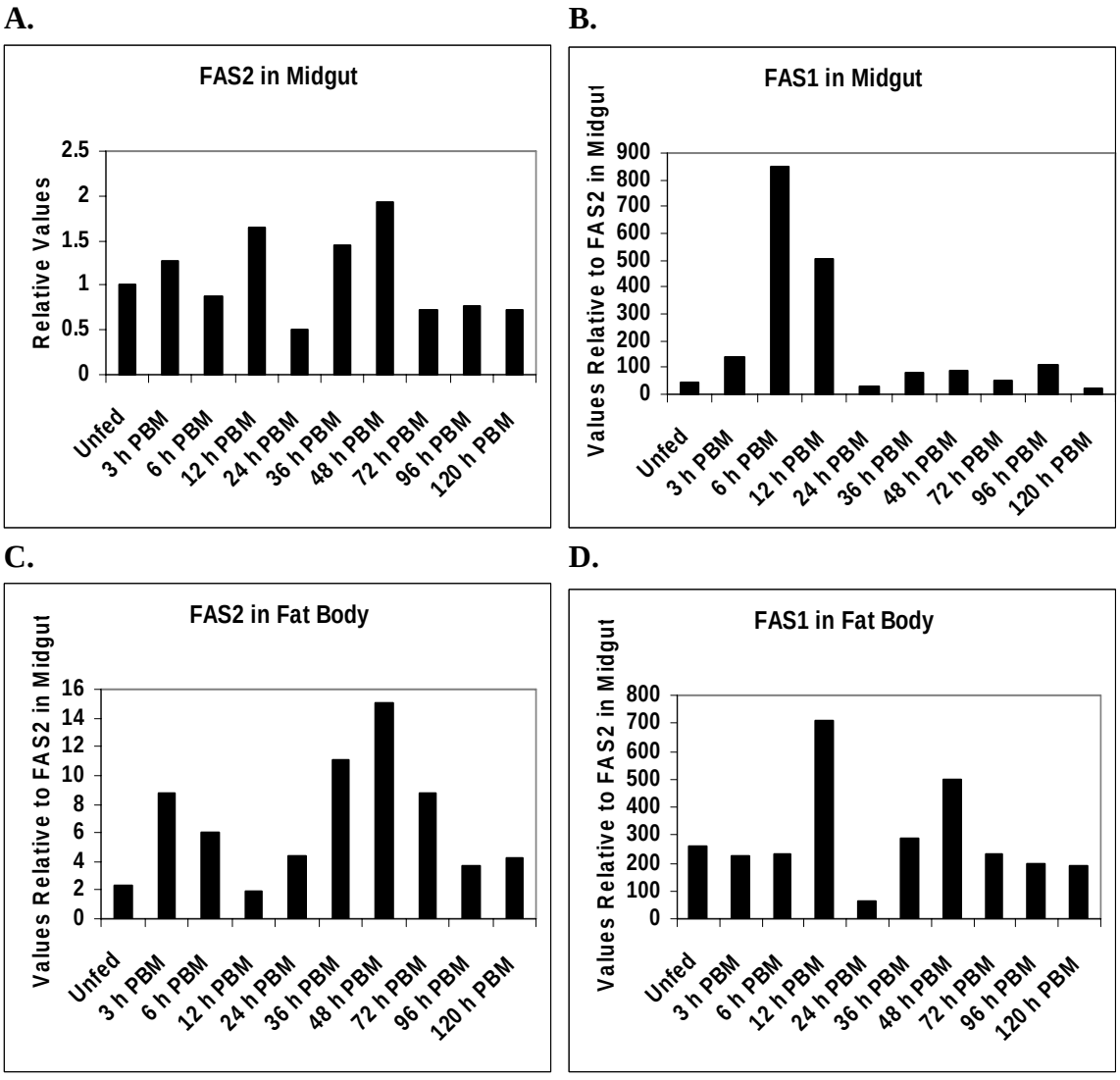


Figure 3. Representative photos of mosquito ovaries taken 48 hours post blood feeding. Photos taken using the same magnification. A) Representative photo of FLUC injected mosquito. B) Representative photo of FAS1 injected mosquito. Results suggest that interference of FAS1 transcription interferes with proper ovary development.

A. FLUC Injected **B. FAS1 Injected**

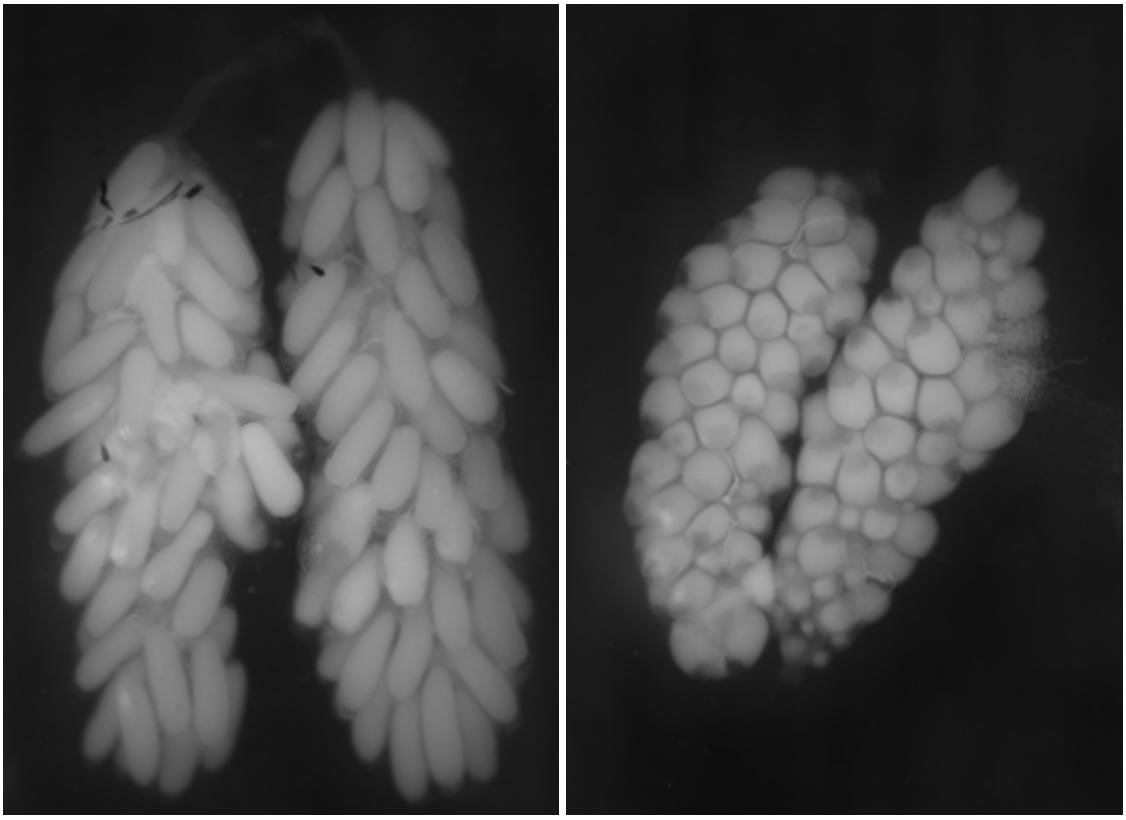
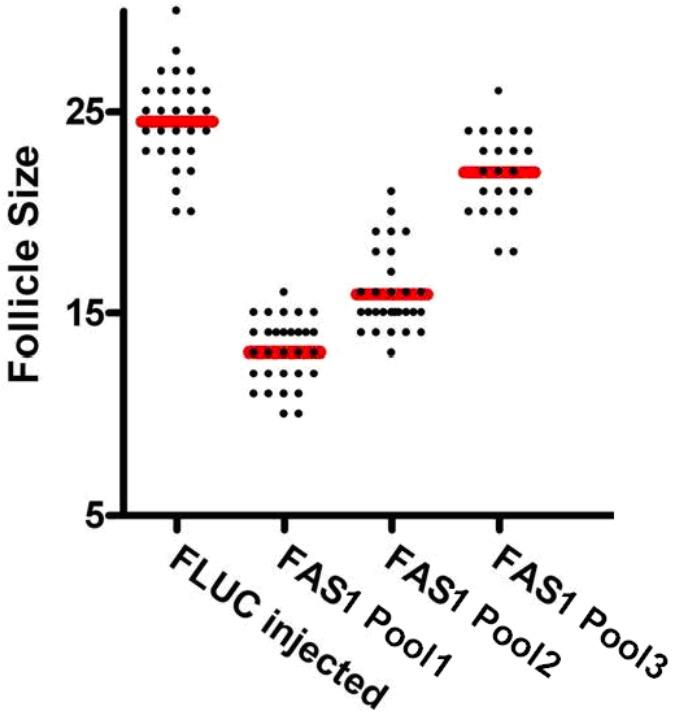
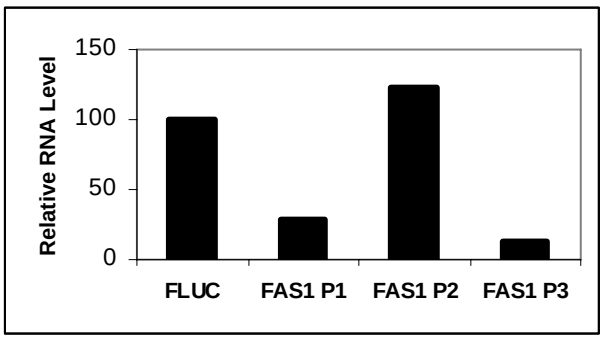


Figure 4. A. Mosquito ovaries dissected at 48 hours post blood feeding. Six follicles chosen at random and measured using an objective micrometer. Each data point represents the size of a single follicle measured in arbitrary units. Follicle sizes from FLUC injected mosquitoes show the natural variation in follicle sizes at this stage of development. Based on the follicle sizes of individual FAS1-injected mosquitoes (small-sized follicles, medium-sized follicles, large-sized follicles), the mosquito samples were assigned to one of three pools and plotted separately. B. Relative RNA levels represent the knockdown effect measured by QRT-PCR in each of the three pools shown in A.



A.

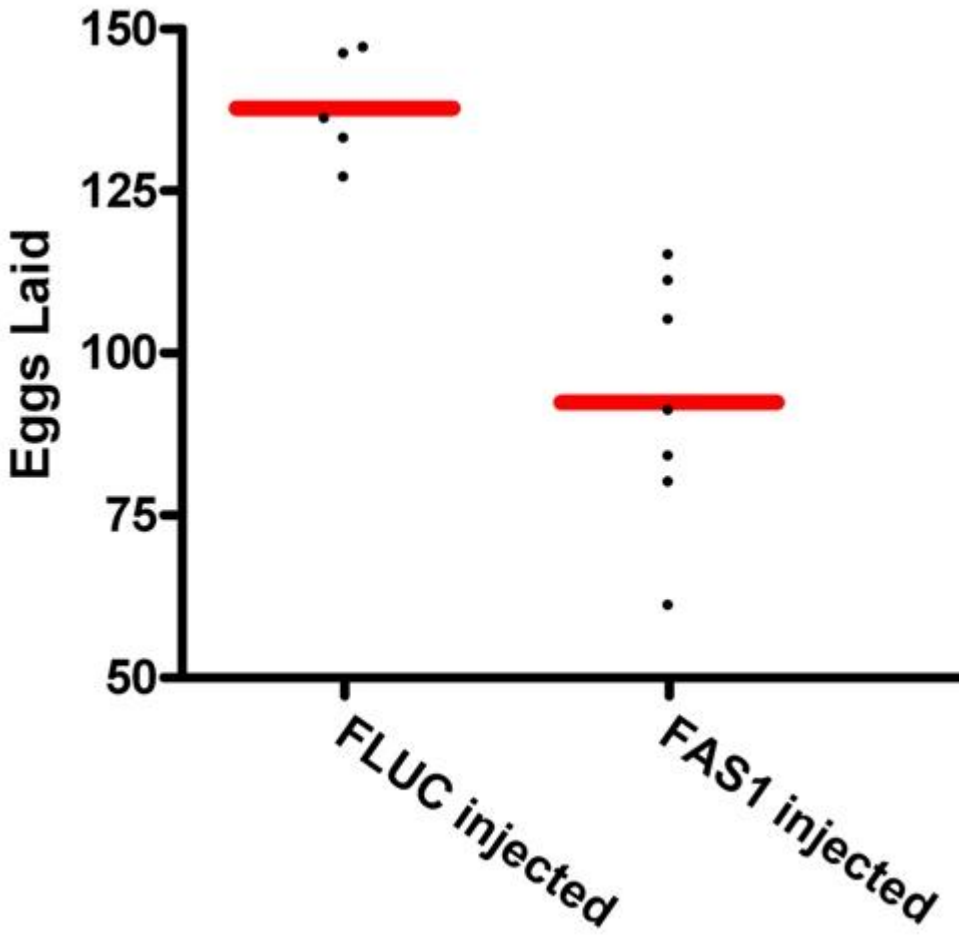


B.

Mosquitoes were separated into one of three pools based on follicle size (small, medium, large) and the data were plotted accordingly (figure 4A). This was done so that corresponding fat body RNA samples could be analyzed by QRT-PCR to see if there was a correlation between follicle size and extent of FAS1 knock-down. Note however, that it is possible that pool samples were mixed-up. Specifically, pool 1 and pool 3 had knockdown of 72% and 87% respectively, and pool 2 showed no knockdown. It is possible that actually pool 1 and pool 2 were knocked out. In this way, human error could account for the discrepancy between apparent phenotype and RNA transcript levels. New experiments are currently underway using single mosquitoes for both ovary phenotyping and QRT-PCR analysis.

The photographs and follicle measurements show a clear phenotype at 48 hours post blood meal but don't account for any biological events that may occur after this timepoint. Figure 5 shows that although mature eggs are deposited by both FLUC and FAS1 injected mosquitoes, number of eggs deposited is below natural variation in all FAS1 injected mosquitoes used for this experiment.

Figure 5. Number of eggs laid by individual mosquitoes. Mosquitoes were separated after blood feeding, and eggs from mated mosquitoes were collected on paper placed inside beakers filled partially with water. Results show a pronounced effect of FAS1 interference on mosquito fecundity.



Discussion

The RNAi knockdown results show that there is a significant detrimental effect on mosquito reproduction when dsFAS1 RNA is injected. However, not all mosquitoes showed a significant phenotype and some showed no phenotype at all. This could possibly be explained by ineffective knockdown by the FAS1 dsRNA, which still needs to be examined. The size and complexity of the FAS gene make designing RNAi primers more difficult than for smaller genes. Primers that target a more essential gene region or multiple gene regions may be needed. Also, the protocol for dsRNA synthesis and injection has hence been improved since these experiments were performed. Knockdown of 98% has been observed in nearly all injected mosquitoes with CD36 dsRNA[1], compared to 72% or 87% knockdown observed in two thirds FAS1 injected mosquitoes.

The protocol described here in which fat body samples were separated into pools based on phenotype strength is an improvement on a protocol in which fat bodies were analyzed in a single pool. This meant that tissues from mosquitoes that showed no knockdown were bulked with tissues from mosquitoes that did, skewing the actual knockdown result. An even better way to approach this problem is to collect RNA from individual mosquitoes to analyze the knockdown effect on individual mosquitoes. This last procedure is currently being implemented for other RNAi experiments in the lab[2].

Future phenotypic analysis will include photographs taken of dissected ovaries at more time points post blood meal. This could explain whether proper ovary development is actually impeded or just delayed. Also, mosquitoes will be kept in individual vials and monitored to see when ovipositing occurs, adding support to the idea that ovary

[1] Morton J., Isoe J., Unpublished data
[2] Isoe J., Unpublished data

development is delayed rather than obstructed. Eggs collected in individual vials will not only be counted, but they will also then be hatched to analyze whether fatty acid metabolism interference inhibits new larval viability. All of the above experiments will be repeated for more genes involved in fatty acid metabolism, including acetyl-CoA carboxylase, the first and rate-limiting step in fatty acid synthesis; fatty acid membrane binding proteins; intracellular fatty acid binding proteins; and diacylglycerol transferases, which are involved in lipid storage.

It should be noted that so far only significant detrimental RNAi effects have been incurred on mosquito reproduction and not on mosquito survival. A mosquito that imbibes on an infected blood meal can re-inject the disease even if it can no longer lay viable eggs. Future experiments done with improved dsRNA may show more detrimental effects to the female during blood meal digestion, which could affect survival. However, even if mosquito reproduction is the sole target, reduced mosquito populations could bring infection rates below a level needed to sustain disease endemism.

It should also be noted that FAS is an essential enzyme in all living organisms. The entire multifunctional enzyme or its parallel monofunctional homologs are highly conserved across all species. At first glance it would seem that this would make FAS a poor candidate as a mosquito selective target, but multiple studies on the therapeutic effects of FAS inhibition in humans are currently being carried out. For example, since FAS is highly expressed in most human cancer cells, it is being studied as a potential cancer screen and treatment. Studies have shown that FAS inhibition by such molecular inhibitors as cerulenin and C75 have selectively induced cancer cell death *in vitro* and *in vivo* (12). In studies on the inhibition of the bacterial FAS enzymes, over 20 molecular

inhibitors have been identified as potential broad-spectrum antibiotics. In particular, drugs that are efficacious against FabI, the prokaryotic enoyl-ACP reductase, in methicillin-resistant S. aureus (an antibiotic resistant infection most often incurred in health care settings, that can be fatal) have shown a great deal of promise (13). The compounds used as FAS inhibitors in these studies could be used *in vivo* in mosquitoes to see if they are effective anti-mosquito targets as well.

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