Open Access Insect Physiology

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Characterization of AMPK in female *Aedes aegypti* and *Georgecraigius atropalpus* mosquitoes in relation to low and adequate food intake

This article was published in the following Dove Press journal:
Open Access Insect Physiology
31 December 2015
Number of times this article has been viewed

Aparna Telang[1]
Amina Abdul Qayum[2]
Zeina E Jouni[3]

[1]Biology Program, University of South Florida Sarasota-Manatee, Sarasota, FL, [2]Department of Biology, University of Richmond, Richmond, VA, [3]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA

Abstract: AMPK is a sensor of cellular fuel and energy status in mammals and *Drosophila melanogaster*. When activated, AMPK switches off anabolic pathways that consume ATP and switches on catabolic processes that produce ATP. We investigated the role of AMPK in regulating energy metabolism in autogenous *Georgecraigius atropalpus* (formerly *Ochlerotatus atropalpus*) and anautogenous *Aedes aegypti* L. (*Stegomyia aegypti*), mosquitoes that differ to varying degrees on larval and adult-derived nutrients for reproduction. We fed chemical activators of AMPK to mosquitoes to signal metabolic stress, and then measured macronutrients utilized in response and whether this response affected mosquito egg development. Our results demonstrate a role for AMPK in mosquito energy metabolism and we report that catabolic pathways affected by AMPK activation differ according to female nutritional condition and breeding strategy. Glycogen was the first source of energy utilized by well-nourished females in response to chronic AMPK activation. In malnourished females, the catabolic pathway activated depended on female breeding strategy. Lastly, we observed delayed follicle development in female *Gc. atropalpus* in which AMPK was chronically activated. Our results indicate that AMPK signaling of metabolic stress leads to a decrease in macronutrients that may affect mosquito reproduction. The AMPK regulatory pathway offers an attractive target for disruption as an approach for mosquito vector population control.

Keywords: 5′-AMP-activated protein kinase, glycogen, lipid, energetics, nutrient utilization, metabolism, oogenesis

Introduction

Lipid and glycogen play vital roles in metabolically demanding processes such as reproduction and dispersal in insects. Adipokinetic hormone is a key player in the lipid and glycogen mobilization. This hormone helps cells communicate the need to mobilize lipid and glycogen reserves, however, the active players involved in this communication are still unknown. Recently, AMPK has been shown to be a key sensor of cellular fuel and energy status in mammals and recently in *Drosophila melanogaster*.[1,2] In mammalian systems, AMPK, upon stimulation, switches off anabolic pathways that consume ATP, such as protein, fatty acids (FA), and glycogen synthesis, and activates catabolic processes that produce ATP, such as FA oxidation and glycolysis.[3] AMPK regulates these pathways by phosphorylating metabolic enzymes, transcription factors, and co-activators that regulate gene expression.[3] As part of glycogen metabolism, AMPK activation via metabolic stressors or pharmacological agents leads to the inhibition of glycogen synthase and activation of PFK2 so that glycogen synthesis is reduced and glycogenolysis is increased, respectively. AMPK also regulates lipid metabolism by

Correspondence: Aparna Telang
Biology Program, University of South Florida Sarasota-Manatee, 8350 N Tamiami Trail, Sarasota, FL 34243, USA
Tel +1 941 359 4368
Fax +1 941 359 4489
Email atelang@usf.edu

phosphorylating ACC-1 and ACC-2, thereby switching off FA synthesis and switching on FA oxidation, respectively.[3] As is the case for most kinases, AMPK is itself activated by phosphorylation by upstream kinases. To date three mammalian kinases, LKB1, CaMKK, and Tak1 kinase, have been identified that phosphorylate the threonine residue (Thr-172) in the activation loop of the α-subunit catalytic domain of AMPK.[4] Analogous to the mammalian system, activation of AMPK in *D. melanogaster* via the phosphorylation of the α-subunit Thr-184 leads to downstream phosphorylation of the homologue DmACC involved in FA metabolism. So properties of AMPK activation and its downstream targets may be conserved between flies and mammals.[2]

We are investigating the activity of AMPK in mosquitoes as part of a long-term study on metabolic pathways involved in the reproductive physiology of this medically important insect. If mosquito AMPK exhibits the same regulatory effects on the downstream targets involved in energy metabolism, then prolonged activation of AMPK should switch on catabolic pathways leading to a decrease in macronutrients (lipid, glycogen, and protein) that may affect reproduction. In female mosquitoes, reproductive success depends on adequate nutrient acquisition during larval and adult stages. Using classical life history terms, "capital" breeders use stored nutrients or energy toward reproduction whereas "income" breeders use concurrent intake of nutrients for reproduction.[5] The downstream catabolic processes resulting from prolonged AMPK activation may differ according to female reproductive mode or to female nutritional condition regardless of her breeding strategy. To test these hypotheses, we utilized the capital breeder, rock pool mosquito *Georgecraigius atropalpus* (Coquillett) (formerly *Ochlerotatus atropalpus*) that is autogenous and provisions her first egg batch largely with larval-derived nutrient reserves,[6] thus providing an excellent system to examine if AMPK-activated catabolic processes are influenced by larval-derived nutritional conditions. In contrast, the income breeder, yellow fever mosquito *Aedes aegypti* L. (*Stegomyia aegypti*) is anautogenous and requires access to sugar and a blood meal to provision eggs, thus providing a system to examine if AMPK-activated catabolic processes are influenced by nutritional conditions derived at the adult stage, specifically sugar feeding as compared to water feeding (starvation).

In this study, prolonged activation of AMPK was carried out using two activators. The first activator was oligomycin, a highly stable macrolide antibiotic isolated from the fungus *Streptomyces diastochromogenes*. This chemical stressor inhibits mitochondrial ATP synthase and thus indirectly activates AMPK by elevating AMP levels in cells.[2,7,8] However, depletion of cellular ATP levels may have non specific side effects. The second activator we used was AICAR (5-aminoimidazole-4-carboxamide-ribonucleoside), an analog of adenosine that permeates cells via an adenosine transport system where it is phosphorylated by adenosine kinase to form 5-aminoimidazole-4-carboxamide-ribonucleotide (ZMP), a 5′-AMP analog. ZMP accumulates in cells and artificially stimulates AMPK directly without altering cellular levels of ATP, ADP, and AMP.[4,9] In addition to the biochemical studies, we profiled gene expression of the AMPK α-subunit in specific tissues of *Ae. aegypti* that would be involved in conveying and responding to information about energy status in this medically important mosquito.

For autogenous *Gc. atropalpus*, we hypothesized that AMPK activation would prompt use of glycogen over lipid or protein as the last two macromolecules are primarily obtained during the larval stage and used for yolk construction in the adult stage. For anautogenous *Ae. aegypti*, we hypothesized that AMPK activation would prompt use of protein and glycogen over lipid, as protein source for yolk construction is expected from a blood meal. Our results demonstrate an active role for AMPK in mosquito energy metabolism and we report here that downstream catabolic pathways affected by AMPK activation differ according to female nutritional condition and breeding strategy. Glycogen seems to be the first source of energy utilized by well-nourished autogenous and anautogenous females in response to chronic AMPK activation. In malnourished or starved females, the catabolic pathway activated depended on whether females reproduced autogenously or anautogenously.

Materials and methods

Gc. atropalpus colony

A laboratory colony of *Gc. atropalpus* (Coquillett) (Bass Rock strain) was maintained in an insectary at 28°C±1°C, 65% relative humidity with a daily photoperiod of 16 hours light and 8 hours dark.[6] The females are 100% autogenous for their first ovarian cycle, as measured by primary follicles advancing beyond Christopher's Stage II, similar to Clement's stage 2b without the need for a blood meal.[10,11] Food at the larval stage consisted of 10% solution of bovine liver powder (VWR). Larvae were raised in plastic pans (34×25×3.8 cm) containing 1.0 L of distilled water and at a larval density of 75–80 per pan. Larvae in each pan were provided 1.0 mL (100 mg) of food on days 2, 3, 5, and 7 (hatch day being day 1). Under these conditions, individuals of this strain

completed larval development in 7–8 days and were pupae on day 8–9. Pupae and remaining late fourth instars were collected on day 8, and the pupal stage lasted approximately 48 hours until adult eclosion. Adults were fed ad libitum on 3% sucrose for 2 days prior to autogenous egg laying.

Manipulation of larval nutrition and AMPK feeding strategy for *Gc. atropalpus*

We used *Gc. atropalpus* to examine the effect of larval-derived nutrient stores on AMPK-activated catabolic processes. Experimental larvae were reared under similar conditions as colony insects but raised at a density of 50 larvae per pan. The larval feeding regimen described for colony insects also served as the "high food quantity" treatment in experiments. For the "low food quantity" treatment, larvae were provided 1.0 mL (100 mg) of food only on days 2 and 8 (hatch day being day 1). Under this low food treatment, larvae completed development in 8–9 days and were pupae on days 9–10. Pupae and remaining late fourth instars were collected on day 9, and the pupal stage lasted approximately 48 hours until adult eclosion. The amount of food provided in the "low food quantity or nutritional stress" treatment was regularly depleted but still supported growth and development to pupation, whereas the "high food quantity" treatment provided nourishment in excess of that required for maximal growth. In our report, female *Gc. atropalpus* derived from larvae fed on high or low amounts of food are referred to as "high-reserve" or "low-reserve" females, respectively.

Immediately after emergence, small groups of high- and low-reserve female *Gc. atropalpus* were transferred to small (450 mL) cages in association with males from the high food quantity treatment. To examine the effect of only larval-derived nutrient stores on AMPK-activated catabolic processes, both high- and low-reserve females were fed AMPK activators along with sucrose rather than water. Providing AMPK activators with a sugar meal ensured that any reductions in nutrient stores observed in females were due to AMPK activation only and not to nutrient stress experienced at the adult stage. Females were fed using 6×50 mm glass tubes filled with either 3% sucrose (sugar control), 3% sucrose with 2 mM AICAR (Sigma-Aldrich Co, St Louis, MO, USA), or 3% sucrose with 5 µM oligomycin (Sigma-Aldrich) delivered through a cotton wick. After 36 hours of feeding, 5–8 mosquitoes that were visibly fed were frozen for analysis of metabolic reserves. This experiment was repeated with a separate cohort of females from a different egg hatch.

Ae. aegypti colony

Our laboratory colony of *Ae. aegypti* (L.) (Rockefeller strain) was maintained under similar insectary conditions with the following exceptions. Larval *Ae. aegypti* was fed a diet of ground tropical fish flakes (TetraMin/TetraFin Fish Flakes, PetsMart, Richmond, VA, USA), bovine liver powder (VWR), and Rodent diet (Teklad Global, Harlan Laboratories, Indianapolis, IN, USA) in a 10:1:10 mixture. Larvae were raised at a density of 100–120 per pan and provided the following amount of food: 0 mg for day 1 (hatch day), 32 mg for day 2, 130 mg for days 3 and 4, 161 mg for day 5, and 136 mg for day 6. Individuals of this strain completed larval development in 7 days and were pupae on day 7–8. Pupae and remaining late fourth instars were collected on day 7 and pupal stage approximately 48 hours until adult eclosion. Adult females were fed ad libitum 3% sucrose for 3–4 days prior to a blood meal. Using an artificial blood feeder, females 4–5 days old post-eclosion (PE) were fed porcine blood, supplemented with 100 mM ATP per 1.0 mL added to a Parafilm-lined glass vessel. Feeding stations were maintained at 37°C with the aid of a circulating water bath.

Manipulation of adult nutrition and AMPK treatments for *Ae. aegypti*

The nutritional condition of anautogenous *Ae. aegypti* is determined by a combination of larval-derived nutrient stores and nutrients acquired from sugar and blood feeding by adults. As it is important for female mosquitoes to take sugar after emergence, we used *Ae. aegypti* to examine the effect of sugar feeding or starvation (water only) on AMPK-activated catabolic processes. To remove effects related to nutrients acquired during the larval stage, female *Ae. aegypti* used in experiments were derived from well-nourished, experimental larvae reared under similar conditions as colony insects but raised at a density of 50 larvae per pan. Thus, we were able to focus our analysis on the effects of adult-acquired nutrients on AMPK-activated catabolic processes. Immediately after emergence, small groups of colony reared *Ae. aegypti* were transferred to small (450 mL) cages in association with males. One group of females were fed either 3% sucrose (sugar control), 3% sucrose with 2 mM AICAR, or 3% sucrose with 5 µM oligomycin. Another group of females were fed either water only (water control), water with 2 mM AICAR, or water with 5 µM oligomycin. This strategy of feeding AMPK activators with water served as the starvation treatment. After 36 hours of feeding, 5–8 mosquitoes that were visibly fed were frozen for analysis of metabolic reserves. This experiment was repeated with a separate cohort of females from a different egg hatch.

Quantification of macronutrients and measurement of follicle growth

Whole body homogenates of adult females were utilized for the extractions of glycogen, storage lipids (triacylglycerol), and soluble proteins using a procedure first described by Van Handel[12] and modified for *Ae. aegypti*.[13] Fractions of glycogen, lipid, and protein were frozen until quantification by colorimetric-based assays. Total lipid stores were determined by a modified Vanillin reagent assay,[14] total glycogen by a modified anthrone-based assay,[15] and soluble protein content by the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). All nutrients are reported on a microgram per mg dry mass basis. Each sample for biochemical analysis was assayed in duplicate and these duplicates were averaged for inclusion in our statistical model. Each experiment was repeated with a separate cohort of females from a different egg hatch for *Gc. atropalpus* (n=12, two food treatments × three AMPK treatments × two experimental replicates) and *Ae. aegypti* (n=12, two food treatments × three AMPK treatments × two experimental replicates).

For *Gc. atropalpus* only, we measured number and growth of primary vitellogenic follicles in females that had fed for 36 hours on 3% sucrose in the absence or presence of AMPK activators, AICAR, or oligomycin. Ovaries were dissected in saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 $CaCl_2$); primary follicles were counted, their developmental stage determined,[11] and their lengths were measured under a dissecting microscope using an ocular micrometer. This experiment was repeated with a separate cohort of females from a different egg hatch (n=36 females total).

RNA purification and cDNA synthesis

An additional goal of this study was the molecular characterization of AMPK in the medically important mosquito *Ae. aegypti*. Heads, guts (without blood), abdominal walls (including fat body), and ovaries were dissected from colony reared *Ae. aegypti* females (4–5 days PE) fed only water, or only sucrose, or at 4, 18, 24, and 48 hours post-blood meal (PBM). All tissues were dissected in saline solution, pooled in RNAlater (Sigma-Aldrich), incubated at 4°C overnight, and stored at −80°C until processed. Total RNA was extracted and treated with DNAase I using the RNeasy mini kit (Qiagen, Valencia, CA, USA), and RNA integrity was checked on a 1% agarose gel. RNA was quantified using the Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized from total RNA (0.3 µg for each body region sample/20 µL final volume) using iScript cDNA kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The integrity of cDNA obtained from every tissue sample was evaluated by polymerase chain reaction (PCR) amplification of *Ae. aegypti* ribosomal S7 products (GenBank accession AY380336), and the cDNA was not used if S7 products were not amplified.

Reverse transcription-PCR (RT-PCR)

RT-PCR was used to determine tissue distribution of AMPK transcripts in response to the aforementioned nutritional regimens. Primers were designed using PrimerSelect (Lasergene, DNASTAR) to the sequence for the catalytic subunit of AMPK, *AeagAMPK*-α (GenBank accession AY870330) and are as follows: forward primer (5′-CGGCATTCGGTCCCAGTCTAAAC-3′), reverse primer (5′-CATCCCGCCGAACCCAATC-3′). Specific transcript products were amplified from tissue-specific cDNA (1 µL of cDNA mixture equivalent to 15 ng total RNA) with gene-specific primers and GoTaq DNA polymerase (Promega Corporation, Fitchburg, WI, USA) by PCR (total volume 20 µL) under the following conditions: 94°C for 2 minutes; 35 or 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 72°C for 7 minutes. PCR mixtures (10 µL) were run on a 1% (w/v) agarose/TAE (40 mM Tris/acetate, 2 mM Ethylenediaminetetraacetic acid, pH 8.0) gel, and products were visualized by ethidium bromide stain and stored as digital images (Gel Logic 200, Kodak, Rochester, NY, USA). PCR products of the expected size were gel purified, cloned, and sequenced to confirm their expected sequence.[16] This experiment was repeated with independently isolated RNA samples from tissues from a second cohort of females.

Quantitative real-time PCR (qPCR) analysis of gene transcripts

We used qPCR to quantify changes in expression of AMPK transcripts in response to the aforementioned nutritional regimens. Primers were designed using PrimerSelect (Lasergene, DNASTAR) to the sequence of the catalytic subunit of AMPK, *AeagAMPK*-α (GenBank accession AY870330) and are as follows: forward primer (5′-AGCCGCTGGTAAAGATAGGTC-3′), reverse primer (5′-TGGCGGTTCAGGATTTTTAC-3′). The same two sets of tissue cDNA samples used for the RT-PCR study and gene specific primers (250 nM final concentration/10 µL reaction) were added to a master mix containing IQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and pure water. A non-template control was included, and three reactions per cDNA template and non-template control were performed in parallel to minimize pipetting differences. The

triplicate tissue cDNA reactions were averaged for inclusion in our statistical model. Reactions were conducted on a Chromo4 cycler (Bio-Rad Laboratories Inc.) using the program: 95°C for 3 minutes; 45 cycles of 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 20 seconds; followed by melt curve analysis. Melt curve analysis was used to confirm amplification of single products in each qPCR that contained cDNA template, and those samples that did not display a product specified by melt curve analysis were excluded from further analysis. Reaction products were collected and analyzed on a 1% (w/v) agarose/TAE gel to confirm product size. The threshold cycle (C_T), the cycle number at which each sample yields a detectable fluorescent signal, and PCR efficiency values for all samples were obtained from the Opticon Software (Bio-Rad Laboratories Inc.). We normalized transcript abundance in each sample relative to qPCR expression of ribosomal S7. We chose the 4–5 days old PE, water-fed females as the control sample (calibrator). Transcript expression values for the sucrose-fed and post-blood-fed groups were then reported as an increase or decrease relative to the calibrator for each tissue sample using the Pfaffl[17] method. The following formula was used to determine the expression ratio between sample and calibrator:

$$\text{Expression ratio} = (E_{\text{target}})^{\Delta CT,\ \text{target (calibrator-test)}} / (E_{\text{ref}})^{\Delta CT,\ \text{ref (calibrator-test)}} \quad (1)$$

where E is PCR efficiency. Real-time PCR results from two independent cohorts of females are summarized (n=48, four tissue samples × six nutrient categories × two cohorts).

Data analysis

For both species of mosquitoes, changes in percent dry mass protein, glycogen, and lipid in response to AMPK activators were analyzed by two-way analysis of variance (ANOVA), with diet, AMPK treatment, and an interaction term included. Differences between specific means were further analyzed using linear contrasts. For *Gc. atropalpus*, linear contrasts were set up to compare mean levels of macronutrients between high- and low-reserve control females (no AMPK activators given). Linear contrasts were then conducted to compare mean changes in the levels of macronutrients within high- and low-reserve females treated with AMPK activators (AICAR + oligomycin) to that of control females. For *Ae. aegypti*, linear contrasts were set up to compare mean levels of macronutrients between sucrose- and water-fed (starved) control females (no AMPK activators given). Linear contrasts were then conducted to compare mean changes in the levels of macronutrients within sucrose- and water-fed females treated with AMPK activators (AICAR + oligomycin) to those of control females. Next, females of both species that were well fed as larvae and given AMPK treatment with sucrose feeding were compared to examine species-specific differences in the effect of AMPK activators on macronutrient levels. Changes in percent dry mass protein, glycogen, and lipid in response to AMPK activators were analyzed by two-way ANOVA, with AMPK treatment, species, and an interaction term. For each species, linear contrasts were then conducted to compare mean changes in the levels of macronutrients in females treated with AMPK activators (AICAR + oligomycin) to those of control females. For *Gc. atropalpus* only, follicle number and development in response to AMPK activators were analyzed by two-way ANOVA, with diet, AMPK treatment, and an interaction term included followed by the Tukey-Kramer honestly significant difference (HSD) test to compare all means. qPCR results were analyzed by one-way ANOVA, and their means were compared by the Tukey-Kramer HSD test to compare all means. All data were statistically analyzed using JMP IN (version 4.0.3, SAS Institute Inc., Cary, NC, USA). Least square means (± standard errors of the mean) were obtained from statistical models and illustrated using GraphPad Prism 4.0 (2006; GraphPad Software, Inc., La Jolla, CA, USA).

Results

Glycogen is preferentially utilized in well-nourished, autogenous females in response to AMPK activators

Larval nourishment strongly influences the amount of macronutrients in newly emerged female *Gc. atropalpus*. Adult females derived from the high-food larvae and given access to sucrose for 36 hours had 112% higher lipid and 42% higher glycogen levels compared to the females derived from the low-food larvae (P=0.001 from linear contrast in both cases; Figure 1A and B). Larval dietary regimens had no significant effects on soluble protein levels (P=0.55 from linear contrast; Figure 1C).

Oral feeding of AMPK activators, AICAR, and oligomycin was associated with a 27%–33% reduction in glycogen levels in high-reserve *Gc. atropalpus* females (P=0.004 from linear contrast; Figure 1B) with no effects on lipid reserves (P=0.40 from linear contrast; Figure 1A). In contrast, feeding of AMPK activators was associated with a 52%–55% reduction in lipid levels in low-reserve females (P=0.001 from linear contrast; Figure 1A). The activators had no effect

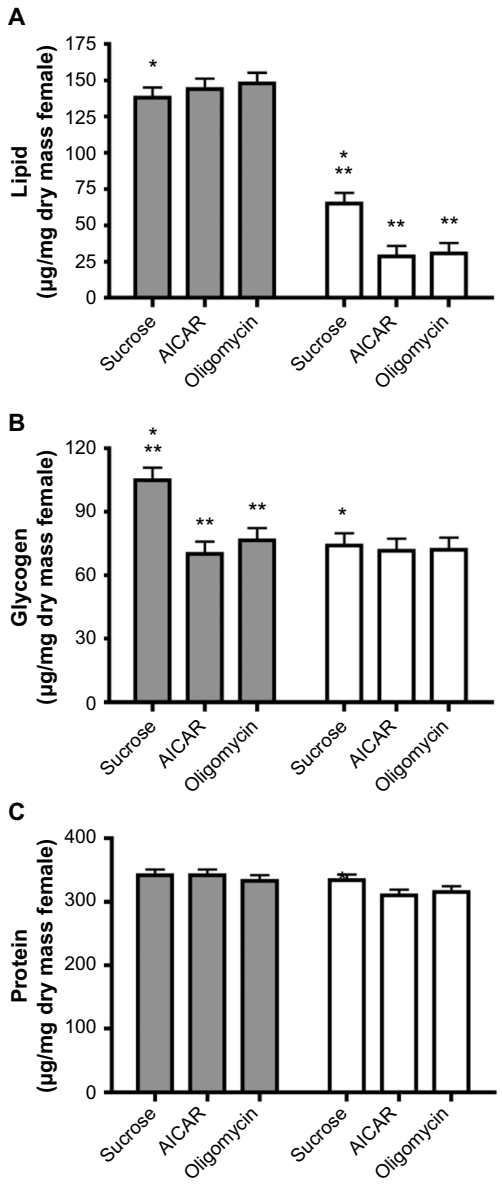

Figure 1 Effect of AMPK activators on nutrient reserves of adult female *Georgecraigius atropalpus*.
Notes: (**A**) Lipid amounts, (**B**) glycogen amounts, and (**C**) protein amounts in high-reserve females (dark gray bars) and in low-reserve females (white bars) given either 3% sucrose only, 3% sucrose with 2 mM AICAR, or 3% sucrose with 5 µM oligomycin for 36 hours. All nutrient values represent adjusted mean ± standard error of the mean; N=12. Within figure, columns representing control females with * are significantly different (from linear contrast test $P<0.001$). Columns representing females fed AMPK activators with ** are significantly different (from linear contrast test $P<0.001$).
Abbreviation: AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside.

on soluble protein levels in either group of females ($P=0.328$ from two-way ANOVA; Figure 1C).

Delayed follicle development in *Gc. atropalpus* treated with AMPK activators

Figure 2A and B displays the length and number of primary follicles of ovaries dissected from female *Gc. atropalpus* derived from variable larval feeding regimens that had fed

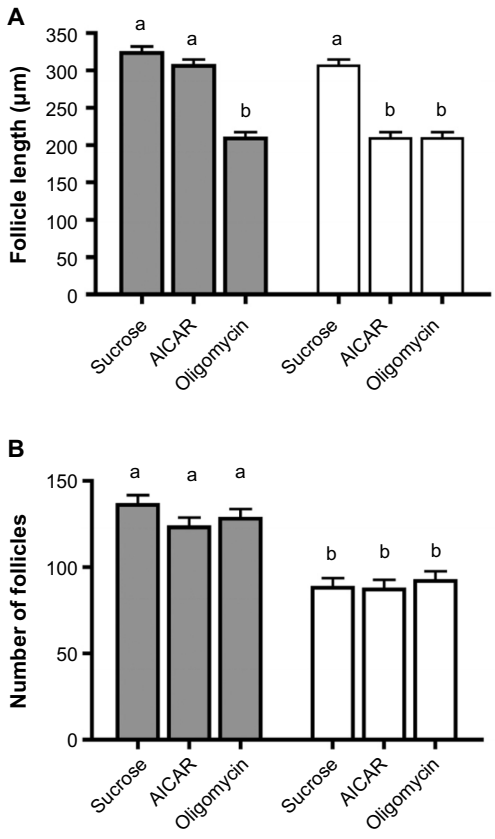

Figure 2 Effect of AMPK activators on follicle growth in adult *Georgecraigius atropalpus*.
Notes: (**A**) Growth of primary follicles and (**B**) number of primary follicles in ovaries of high-reserve (dark gray bars) and low-reserve (white bars) females given either 3% sucrose only, 3% sucrose with 2 mM AICAR, or 3% sucrose with 5 µM oligomycin for 36 hours. Within the figure, columns with different letters are significantly different (from a Tukey-Kramer HSD $P<0.05$).
Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside; HSD, honest significant difference.

as adults on sucrose only or in the presence of AICAR or oligomycin. While larval feeding regimen influenced the number of primary follicles produced ($P<0.0001$ from two-way ANOVA; Figure 2B), follicles developed to Christopher's Stage III regardless of larval nutrients ($P>0.05$ Tukey-Kramer HSD; Figure 2A). Prolonged AMPK activation by either AICAR or oligomycin delayed follicle development at Christopher's Stage II in low-reserve females compared to females given only sucrose (Figure 2A). Whereas, follicles were delayed at Christopher's Stage II in high-reserve females that ingested sucrose with oligomycin but not AICAR (Figure 2A) ($P\leq0.05$ Tukey-Kramer HSD).

Glycogen is preferentially utilized in sugar-fed, anautogenous females in response to AMPK activators

Protein, glycogen, and lipid levels were significantly different in control female *Ae. aegypti* fed either water or sucrose

only for 36 hours ($P<0.0001$ from linear contrast in all cases; Figure 3). Females given access to sucrose for 36 hours retained on average a larger body mass (4.2 mg/female) compared to females fed water only (2.7 mg/female) ($P<0.0001$). On a per mg dry mass basis, females given access to water for 36 hours presented greater levels of soluble protein compared to females fed on sucrose (Figure 3A). However, due to their heavier dry mass, sucrose-fed females contained greater total amounts (μg per individual) of all macronutrients, including protein.

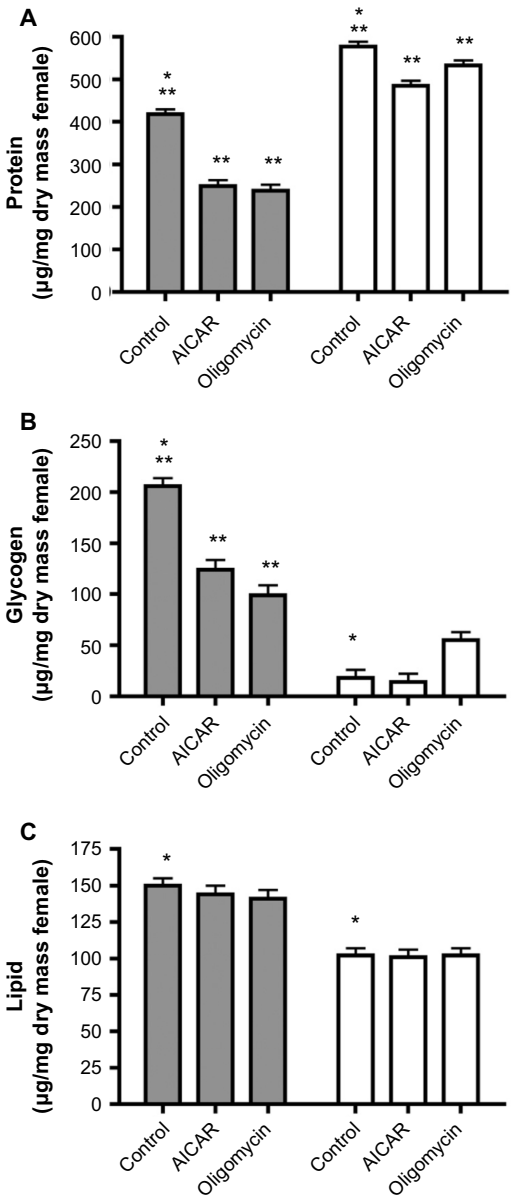

Figure 3 Effect of AMPK activators on nutrient reserves of adult female *Aedes aegypti*.
Notes: (**A**) Protein amounts, (**B**) glycogen amounts, and (**C**) lipid amounts in females given either 3% sucrose only, 3% sucrose with 2 mM AICAR, or 3% sucrose with 5 μM oligomycin (dark gray bars) or in females given either water only, water with 2 mM AICAR, or water with 5 μM oligomycin (white bars) for 36 hours. All nutrient values represent adjusted mean ± standard error of the mean; N=12. Within figure, columns representing control females with * are significantly different (from linear contrast test $P<0.001$). Columns representing females fed AMPK activators with ** are significantly different (from linear contrast test $P<0.001$).
Abbreviation: AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside.

Feeding AMPK activators with sucrose led to 40%–43% reductions in soluble protein and 40%–52% reductions in glycogen reserves ($P<0.0001$ from linear contrast in both cases) (Figure 3A and B). Ingestion of activators with water also led to noticeable reductions of 7%–16% in soluble protein ($P<0.0001$ from linear contrast) but not so in glycogen levels (Figure 3A and B). In contrast to soluble protein and glycogen levels, feeding of AMPK activators had no effect on lipid levels, regardless of whether activators were fed sucrose or water ($P=0.623$, two-way ANOVA) (Figure 3C).

In a separate statistical model, comparison was made between high-reserve *Gc. atropalpus* and standard colony reared *Ae. aegypti* when both species were fed AMPK activators with a sucrose meal. Feeding of AMPK activators led to reduced soluble protein and glycogen levels in *Ae. aegypti* ($P<0.0001$ from linear contrast in both cases) (Figure 4A and B). In *Gc. atropalpus* only glycogen was decreased in association with feeding of AMPK activators ($P=0.013$ from linear contrast) (Figure 4B). Finally, no changes in lipid levels were observed in association with AMPK activators in either species ($P=0.982$, two-way ANOVA) (Figure 4C).

AMPK transcripts are broadly distributed in *Ae. aegypti* in response to major food regimens

As shown by RT-PCR, *AaegAMPK*-α transcripts were observed in all tissues examined regardless of whether females were maintained on water, sucrose, or given a blood meal (data not shown). The integrity of all cDNA samples used in this study was verified by the presence of a ribosomal S7 PCR product, and it was consistently amplified in all tissues and adult food regimens (data not shown).

AMPK transcript levels vary in response to nutritional status of *Ae. aegypti* females

We used qPCR to perform a quantitative assessment of *AaegAMPK*-α transcript expression in specific tissues from non-blood-fed (given access to either water or sucrose only) and post-blood-fed female *Ae. aegypti*. Expression of *AaegAMPK*-α transcripts was low in head tissue from females given only water (the calibrator), and expression remained low in head tissue from sucrose-fed and recently blood-fed females but exhibited a nine fold increase in 48 hours PBM in females ($P\leq0.05$ Tukey-Kramer HSD, Figure 5). Relative to females given access to water only, the mid-gut exhibited

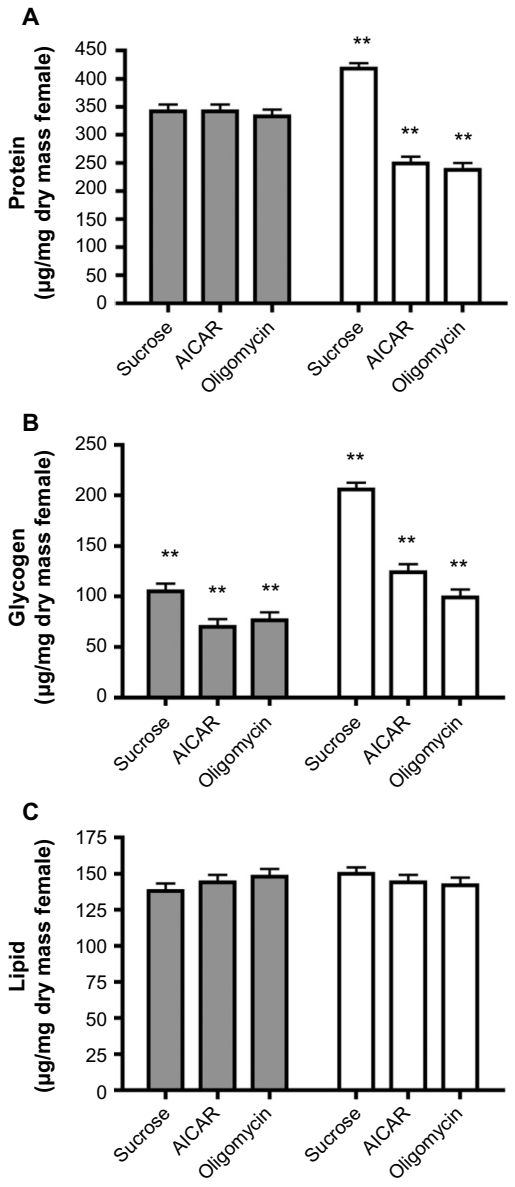

Figure 4 Comparison of the effect of AMPK activators on nutrient reserves of adult female *Georgecraigius atropalpus* and *Aedes aegypti.*

Notes: (**A**) Protein amounts, (**B**) glycogen amounts, and (**C**) lipid amounts in *Gc. atropalpus* (dark gray bars) and in *Ae. aegypti* (white bars) females given either 3% sucrose only, 3% sucrose with 2 mM AICAR, or 3% sucrose with 5 µM oligomycin for 36 hours. All nutrient values represent adjusted mean ± standard error of the mean; N=13. Within figure, columns representing females fed AMPK activators with ** are significantly different (from linear contrast test $P<0.01$).

Abbreviation: AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside.

a four fold increase of *AaegAMPK*-α transcripts at 18 and 24 hours PBM that may have begun a return to basal level at 48 hours PBM ($P\leq0.05$ Tukey-Kramer HSD, Figure 5). *AaegAMPK*-α transcripts in the fat body were also influenced by adult nutritional regimens, exhibiting a 3–4-fold increase by 18 hours PBM with an additional three fold increase at 48 hours PBM ($P\leq0.05$ Tukey-Kramer HSD, Figure 5). We do not currently know if transcripts returned to basal levels afterward. Compared to ovaries from females given only water, *AaegAMPK*-α expression increased only in ovaries from sucrose-fed females and remained at relatively low levels in ovaries from blood-fed females over the course of blood meal digestion ($P\leq0.05$ Tukey-Kramer HSD, Figure 5).

Discussion

AMPK has been extensively studied in mammalian systems and is considered a metabolic stress-sensing protein kinase under the influence of cellular AMP levels. Processes that deplete ATP subsequently lead to high AMP levels in cells. In response, AMPK switches off ATP-consuming anabolic pathways, such as protein, FA, and glycogen synthesis and activates ATP-producing catabolic processes such as FA oxidation and glycolysis.[4] In our study, chronic AMPK activation in vivo was used to signal metabolic stress and aid in determining which macronutrients would be utilized in response to metabolic stress and whether this response is associated with delayed egg development in mosquitoes.

Activation of AMPK in vivo exhibits the same effect on downstream ATP-producing catabolic processes, demonstrating a role for AMPK in regulating biochemical pathways in female mosquitoes. AMPK-activated catabolic pathways differed according to female nutritional condition, and glycogen seemed to be the first energy reserve utilized by well-nourished autogenous and anautogenous females. While glycogen levels only accounted for 18% of total macronutrients in high-reserve autogenous *Gc. atropalpus* females, these females utilized up to 33% of their glycogen in response to AMPK stimulation using AICAR or oligomycin. When given access to sugar, soluble protein and glycogen combined accounted for 81% of total macronutrients in female *Ae. aegypti* that then utilized 43% protein and 52% glycogen levels as a source of ATP in response to AMPK activation. So glycogenolysis may have resulted in well-nourished females of both species indicating that glycogen may be the first energy source to be utilized in response to metabolic stress signaling when sugar is available and presumably en route to replenish glycogen reserves.

Both *Gc. atropalpus* and *Ae. aegypti* accumulate similar levels of lipids when well fed as larvae, and accumulating high amounts of lipids during the larval stage plays an important role for both species.[18] Zhou et al[19] observed that approximately 35% lipid reserves and 60% glycogen and sugar reserves are utilized as sources of energy and egg provisions during a female's first gonotrophic cycle. In our current study, chronic activation of AMPK did not prompt

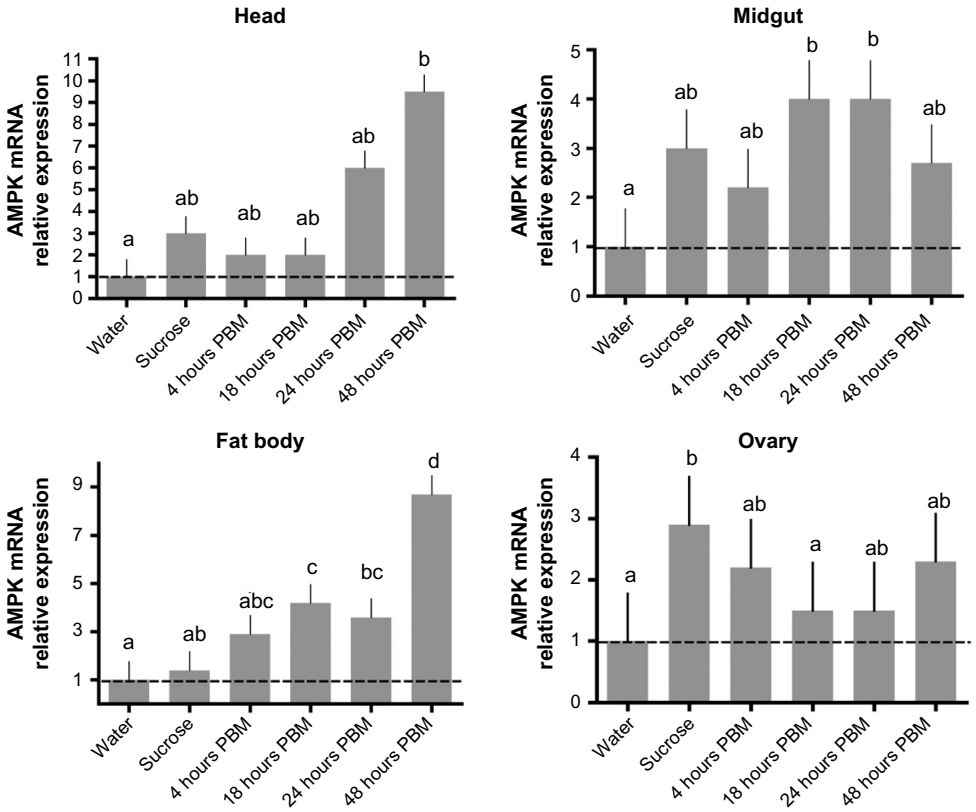

Figure 5 qPCR analysis of AMPK transcript abundance in heads, guts (without blood), abdominal walls (including fat body), and ovaries of *Aedes aegypti* females in response to feeding on water, sucrose, or blood meal.

Notes: Bars represent normalized levels of transcripts in each tissue with tissue from females given only water chosen as calibrator. Its ratio of 1.0 is indicated by dashed line. Within the figure, columns with different letters are significantly different (from a Tukey-Kramer HSD test $P<0.05$).

Abbreviations: qPCR, quantitative real-time polymerase chain reaction; HSD, honest significant difference; PBM, post-blood meal.

mobilization of lipid reserves in well-nourished females of either species, affirming the importance of lipid reserve for reproduction. In the case of female *Ae. aegypti* fed AMPK activators with water, these females were also derived from well-nourished larvae and perhaps they too had no need to mobilize lipids in response to AMPK signaling of metabolic stress. Our observations are supported by studies using the fruit fly *D. melanogaster* and the nematode *Caenorhabditis elegans* that show rapid turnover of lipid reserves in AMPK-impaired animals compared to AMPK-wild type expressing animals, indicating a central role for AMPK in directing maintenance of lipid reserves so that energy conservation is favored over expenditure during conditions of stress.[1,20]

In undernourished or sugar-starved females, macronutrients other than glycogen were reduced in response to chronic AMPK activation. Low-reserve *Gc. atropalpus* females mobilized lipids upon AMPK activation by AICAR or oligomycin. The decision by poorly nourished females to utilize lipids for survival was not surprising although females rely on a substantial amount of larval-derived lipids to provision eggs during their first egg cycle.[19] Under chronic activation of AMPK, starved *Ae. aegypti* females instead utilized soluble protein. Glycogen levels only made up 16% of total macronutrients in low-reserve *Gc. atropalpus* females given sugar and only 3% of total macronutrients in anautogenous *Ae. aegypti* females maintained on water (starved) for 36 hours. In both species, the low levels of glycogen may represent a threshold of sorts, at or under which glycogen cannot or will not be mobilized favoring use of other nutrient classes.

In mammals that are nutrient deprived or fasted, skeletal muscle switches from glucose to FA utilization and relies on glycogenolysis to contribute glucose to other tissues. Under nutrient stress, the mammalian liver can also undergo extensive glycogenolysis to support gluconeogenesis, the fuel for which comes from ATP provided by FA oxidation in adipose tissue. AMPK is activated by caloric restriction in these mammalian tissues and coordinates these metabolic pathways to restore cellular energy status.[21] Recent studies have also found that significant amounts of AMPK in normal-fed rat liver cells are actually bound to glycogen and may facilitate the ability of AMPK to act as a sensor of glycogen structure.[22] In our study, only well-provisioned female mosquitoes seemingly

relied on glycogenolysis in response to AMPK activation, indicating the importance of glycogen as a readily accessible energy store for various animals.

Female mosquitoes emerge with varying levels of larval-derived glycogen, lipids, and protein.[6,18,23–27] From those past studies, we know protein content is positively correlated with body size and levels of the other macronutrients; however, it is not clear how much larval-derived protein contributes directly to survival, flight, and reproduction. Interestingly, in response to AMPK activators that signal metabolic stress, soluble proteins were utilized to a large degree by anautogenous *Ae. aegypti*. Our results are supported by earlier studies that found *Ae. aegypti* females utilized soluble protein to varying degrees for survival in response to starvation (given water only).[24,28] By 36 hours post-emergence, *Ae. aegypti* females are likely close to initiating host-seeking behavior in order to secure a blood meal for their reproductive needs.[29] So it is perhaps not surprising that larval-derived protein was catabolized in anautogenous females that primarily utilize amino acids from blood to provision eggs.[30,31] Synthesis of yolk protein (vitellogenesis) by the fat body increases substantially in response to rising levels of amino acids PBM, this signal being transduced by the TOR pathway that increases protein synthesis.[32] AMPK inhibits protein synthesis at multiple points, including inhibition of the TOR pathway.[4] It is conceivable that chronic activation of AMPK exhibited similar inhibitory effects on TOR pathways, decreasing protein synthesis and favoring protein utilization.

Emerging evidence from mammalian systems points to an important role for AMPK in regulating whole-body energy metabolism by responding to adipocyte-derived hormones such as leptin and gut-derived hormones such as ghrelin.[33] These hormones act on AMPK in peripheral tissues such as muscle, liver, and skeletal tissue to alter metabolic pathways and in the hypothalamus to regulate food intake.[33] We examined expression of *AaegAMPK*-α transcripts in key tissues that would be expected to convey and respond to information about energy status. Transcripts were low in the mid-gut of non-blood-fed females and increased 18–24 hours PBM. When measuring protein digestion in blood-fed *Ae. aegypti* kept at 27°C, blood proteins were found to be completely digested within 36 hours.[34] In our study, an increase of AMPK transcripts in the mid-gut of females 18–24 hours PBM maintained at 28°C may be coinciding with a decrease in blood-meal protein substrate. This assessment agrees with the increased expression of AMPK transcripts in the mosquito head and fat body of 48 hours PBM females when blood-meal protein has been fully metabolized to provision eggs. While AMPK has been shown to be under post-translational regulation by phosphorylation,[3,35] our current study suggests α-subunit transcripts may be responsive to nutrient changes in key tissues of mosquitoes that would be involved in AMPK signal transduction. Future work will make use of an antibody specific for the phosphorylated Thr172 of *AaegAMPK*-α subunit to verify the pattern of AMPK activation in blood meal digestion.

At this stage in our research we do not know if effects of AMPK activation on nutrient utilization are restricted to the fat body, the source of fuel molecules, or whether other tissues are affected. We observed that follicle development was delayed in female *Gc. atropalpus* treated with AMPK activators, regardless of their reserve status. A future study will be undertaken to investigate whether AMPK signaling of metabolic stress leads to a temporary switch from using fuel molecules for egg development toward somatic maintenance. The role of AMPK in regulation of reproduction has only recently been studied in vertebrates. AMPK transcript and protein have been found in the ovaries of various animals, including rat, mouse, cow, pig, and chicken.[36] Activation of AMPK by either AICAR or metformin (another well-known activator of AMPK) decreases steroidogenesis in ovarian granulosa cells and increases the percentage of developmentally arrested oocytes in a number of those aforementioned species.[36]

In conclusion, the catabolic pathways affected by AMPK activation in female mosquitoes depended on their nutritional condition (starved or stocked with substrates) and reproductive mode (autogenous or anautogenous). Further, the expression of the fuel sensor AMPK in a number of tissues that are involved in nutrient signal transduction in mosquitoes suggests that AMPK may be a key signal regulating energy availability and reproduction. This important regulatory pathway can serve as a target for disruption as an approach for mosquito vector population control.

Acknowledgments

This paper has greatly benefitted from comments by anonymous reviewers. The authors would like to thank John Frey and Ben Peterson for mosquito rearing and the School of Arts and Science for a summer fellowship to AA Qayum. Portions of this research were funded by NIH/NIAID grant (K22AI070644) to A Telang as well as by University of South Florida Sarasota-Manatee Arts and Science Dean's funds to A Telang.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Johnson EC, Kazgan N, Bretz CA, et al. Altered metabolism and persistent starvation behaviors caused by reduced AMPK function in *Drosophila*. *PloS One*. 2010;5(9). pii: e12799.
2. Pan DA, Hardie DG. A homologue of AMP-activated protein kinase in *Drosophila melanogaster* is sensitive to AMP and is activated by ATP depletion. *Biochem J*. 2002;367(Pt 1):179–186.
3. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*. 2007;8(10):774–785.
4. Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol Rev*. 2009;89(3):1025–1078.
5. Stephens PA, Boyd IL, McNamara JM, Houston AI. Capital breeding and income breeding: their meaning, measurement, and worth. *Ecology*. 2009;90(8):2057–2067.
6. Telang A, Wells MA. The effect of larval and adult nutrition on successful autogenous egg production by a mosquito. *J Insect Physiol*. 2004;50(7):677–685.
7. Gleason CE, Lu D, Witters LA, Newgard CB, Birnbaum MJ. The role of AMPK and mTOR in nutrient sensing in pancreatic β-cells. *J Biol Chem*. 2007;282(14):10341–10351.
8. Hardie DG, Pan DA. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem Soc Trans*. 2002; 30(Pt 6):1064–1070.
9. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem*. 1995; 229(2):558–565.
10. Christophers SR. The development of the egg follicle in Anophelines. *Paludism*. 1911;2:73–87.
11. Clements AN. *The Biology of Mosquitoes, Volume 1*. London: Chapman & Hall; 1992.
12. Van Handel E. Microseparation of glycogen, sugars, and lipids. *Anal Biochem*. 1965;11(2):266–271.
13. Zhou G, Flowers M, Friedrich K, Horton J, Pennington J, Wells MA. Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. *J Insect Physiol*. 2004;50(4):337–349.
14. Van Handel E. Rapid determination of total lipids in mosquitoes. *J Am Mosq Control Assoc*. 1985;1(3):302–304.
15. Van Handel E. Rapid determination of glycogen and sugars in mosquitoes. *J Am Mosq Control Assoc*. 1985;1(3):299–301.
16. Telang A, Peterson B, Frame L, Baker E, Brown MR. Analysis of molecular markers for metamorphic competency and their response to starvation or feeding in the mosquito, *Aedes aegypti* (Diptera: Culicidae). *J Insect Physiol*. 2010;56(12):1925–1934.
17. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
18. Telang A, Li Y, Noriega FG, Brown MR. Effects of larval nutrition on the endocrinology of mosquito egg development. *J Exp Biol*. 2006;209(Pt 4): 645–655.
19. Zhou G, Pennington J, Wells MA. Utilization of pre-existing energy stores of female *Aedes aegypti* mosquitoes during the first gonotrophic cycle. *Insect Biochem Mol Biol*. 2004;34(9):919–925.
20. Narbonne P, Roy R. *Caenorhabditis elegans* dauers need LKB1/AMPK to ration lipid reserves and ensure long-term survival. *Nature*. 2009; 457(7226):210–214.
21. Fulco M, Sartorelli V. Comparing and contrasting the roles of AMPK and SIRT1 in metabolic tissues. *Cell Cycle*. 2008;7(23):3669–3679.
22. Bendayan M, Londono I, Kemp BE, Hardie DG, Ruderman N, Prentki M. Association of AMP-activated protein kinase subunits With glycogen particles as revealed in situ by immunoelectron microscopy. *J Histochem Cytochem*. 2009;57(10):963–971.
23. Briegel H. Fecundity, metabolism, and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria. *J Med Entomol*. 1990;27(5):839–850.
24. Briegel H. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *J Insect Physiol*. 1990;36(3):165–172.
25. Briegel H, Timmermann SE. *Aedes albopictus* (Diptera: Culicidae): physiological aspects of development and reproduction. *J Med Entomol*. 2001;38(4):566–571.
26. Foster WA. Mosquito sugar feeding and reproductive energetics. *Annu Rev Entomol*. 1995;40:443–474.
27. Timmermann SE, Briegel H. Larval growth and biosynthesis of reserves in mosquitoes. *J Insect Physiol*. 1999;45(5):461–470.
28. Klowden MJ, Chambers GM. Reproductive and metabolic differences between *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). *J Med Entomol*. 1992;29(3):467–471.
29. Clements AN. *The Biology of Mosquitoes, Volume 2*. New York: CABI Publishing; 1999.
30. Uchida K, Oda T, Matsuoka H, et al. Induction of oogenesis in mosquitoes (Diptera: Culicidae) by infusion of the hemocoel with amino acids. *J Med Entomol*. 2001;38(4):572–575.
31. Uchida K, Ohmori D, Yamakura F, Suzuki K. Changes in free amino acid concentration in the hemolymph of the female *Culex pipiens pallens* (Diptera: Culicidae), after a blood meal. *J Med Entomol*. 1990;27(3):302–308.
32. Hansen IA, Attardo G, Park J, Peng Q, Raikhel AS. Target of rapamysin-mediated amino acid signaling in mosquito anautogeny. *Proc Natl Acad Sci U S A*. 2004;101(29):10626–10631.
33. Andersson U, Filipsson K, Abbott CR, et al. AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem*. 2004; 279(13):12005–12008.
34. Briegel H, Lea AO. Relationship between protein and proteolytic activity in the midgut of mosquitoes. *J Insect Physiol*. 1975;21(9): 1597–1604.
35. Leff T. AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. *Biochem Soc Trans*. 2003; 31(Pt 1):224–227.
36. Dupont J, Chabrolle C, Ramé C, Tosca L, Coyral-Castel S. Role of the peroxisome proliferator-activated receptors, adenosine monophosphate-activated kinase, and adiponectin in the ovary. *PPAR Res*. 2008;2008:176275.