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Suppression of glycogen synthase expression reduces glycogen and lipid storage during mosquito overwintering diapause

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Abstract

During diapause in mosquitoes, efficient storage and utilization of energy are crucial for surviving prolonged periods of developmental arrest and for maximizing reproductive success once diapause is terminated and development recommences. In *Culex pipiens*, glycogen rapidly accumulates during early diapause (7-10 days after adult eclosion) and it is used to maintain energy homeostasis during the first month of diapause. In this study, a gene encoding glycogen synthase, which converts glucose residues into a polymeric chain for storage as glycogen, was characterized. After dsRNA directed against glycogen synthase was injected into mosquitoes programmed for diapause (reared under short day lengths), *Cx. pipiens* were fed 1% D-[¹³C₆]glucose, and the knockdown effects after 7-days were monitored by measuring ¹³C-labeled carbohydrate accumulation using solid-state NMR. The use of ¹³C cross-polarization magic-angle spinning spectrum showed a 46% reduction of ¹³C-labeled glycogen and a 6% reduction in lipid accumulation in glycogen synthase knockdown adult females. In addition, the suppression of glycogen synthase dramatically increased the mortality rate of diapausing *Cx. pipiens* by 88% at 30-days post injection. These findings indicated that glycogen synthase plays a critical role in regulating glycogen and lipid storages during overwintering diapause, and its function is essential for successful overwintering and survival of *Cx. pipiens*.

1. Introduction

The ability of an organism to adapt to the availability of food is an important factor for survival. Insects have evolved a variety of ecological and physiological adaptations to overcome either a continuous or transient lack of food. In insects, the main physiological driver for adaptation to overcome malnutrition is the regulation of energy metabolism through the regulation of hormones (Hahn and Denlinger, 2011; Saltiel and Kahn, 2001;

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Author contribution

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Chang et al., 2017). Physiological adaptation to extreme forms of malnutrition can be found in the diapause process of insects. In general, sugars, which are transported by the insect hemolymph after ingestion, are readily absorbed by the cells to produce energy through glycolysis. However, in diapausing insects, these sugars are converted to either glycogen or lipids and the excess is then stored in fat body cells or periphery cells. During the winter months, the stored glycogen and lipids are used in small increments as the sole energy source for survival.

It has been recently shown that the insulin/forkhead transcription factor (Foxo) plays a central role in controlling energy metabolism during diapause in mosquitoes (Sim and Denlinger 2008; 2013). In addition to diapause, this signal has been known as a common regulatory signal for growth and energy metabolism in other various insects (Lin and Smaghe 2018; Smykal, and Raikhel, 2015; Okamoto and Yamanaka, 2015). In the mosquito *Culex pipiens*, suppression of Foxo gene activity in early diapause of adult females resulted in halted nutrient storage accompanied by reduced lifespan (Sim and Denlinger 2008). The key downstream genes that are regulated by the Foxo transcription factor were recently elucidated using ChIP-sequencing (Sim and Denlinger 2015). One of the downstream genes identified is purported to be glycogen synthase which may be linked to a glycogen accumulation in the early diapause.

Glycogen synthase (GlyS) catalyzes the formation of glycogen, which is a branched polysaccharide of glucose that functions as glucose storage in many animals. GlyS is regulated by both allosteric effectors and phosphorylation at its N- and C-terminal domains (Bouskila et al., 2010). Phosphorylation of GlyS is carried out by several kinases, including glycogen synthase kinase 3 (GSK3) isoforms, which inactivate GlyS by reducing its affinity to its substrate uridine diphosphate glucose (UDP-glucose). In the presence of high levels of glucose-6-phosphate (G6P), GlyS is allosterically activated even when the enzyme is phosphorylated (Patel and Woodgett, 2017). GlyS plays a crucial role in long-term energy storage, utilizing glycogen accumulation as well as in lipid accumulation during this diapause period of insects (Hahn and Denlinger, 2011; Sim and Denlinger 2013). In most insect larvae, glycogen is stored primarily in body muscle but its distribution shifts to fat body cells and flight muscles in adults (Arrese and Soulages, 2010). Furthermore, glycogen accumulation also plays an important role during the developmental stage of the oocyte, where in fly oocytes high accumulation of glycogen is known to be associated with the conversion of the electron transport chain into a quiescent stage (Sieber et al. 2016).

In this study we evaluated the relationship between GlyS to glycogen and lipid accumulation in diapause. First, we used RNA interference (RNAi) to suppress GlyS in females during an early temporal window of diapause when this gene is up-regulated. To determine the *in situ* effects of GlyS suppression on glycogen and lipid accumulation, we used solid-state NMR to measure glycogen and lipid accumulations in intact diapause-destined females that were fed for 7 days on uniformly ^{13}C -labeled D- $^{13}\text{C}_6$ glucose following the RNAi injection. Solid-state NMR is a powerful tool well-suited for measuring glycogen and lipid accumulations in intact mosquitoes without sample extraction or purification. While the natural abundance ^{12}C -carbon is insensitive to NMR, ^{13}C isotope with nuclear magnetic moment of spin $\frac{1}{2}$ is NMR active. Thus, ^{13}C solid-state NMR was used to measure the

uptake of D-[$^{13}\text{C}_6$]glucose and accumulation of ^{13}C -labeled metabolites to provide insight into glucose utilization and storage in diapausing mosquitoes. We then evaluated the effects of GlyS suppression during the early diapause period in RNAi-injected *Cx. pipiens* using ^{13}C cross-polarization magic-angle spinning (CPMAS) NMR. We observed that the GlyS suppression during the early diapause period halted glycogen and lipid accumulation in *Cx. pipiens*. Furthermore, GlyS suppression markedly reduced the survival rate of the diapausing mosquitoes.

2. Materials and Methods

2.1. Insect Rearing

The stock colony of *Culex pipiens* was reared at 25 °C and 75% relative humidity under a 15 h light:9 h dark (L: D) photoperiod, according to previously described methods (Sim and Denlinger, 2008). When larvae reached the second instar, rearing containers were placed under diapause-inducing condition: mosquitoes were reared at 18 °C, 75% RH, and 9:15 L: D. To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of ovarian development was determined according to described methods (Christophers, 1911). In addition, fat body cells were examined by staining fixed tissues with BODIPY 493/503 (Invitrogen) that was diluted in PBS to a concentration of 1 mg/mL. Stained fat body was examined with Zeiss Axioskop fluorescent microscope.

2.2. Synthetic Dicer-substrate siRNA (dsi-RNA) Injection into Adult Female Mosquitoes.

We targeted glycogen synthase (CPIJ005086) using a method described previously (Sim and Denlinger 2009; 2011). We used the synthetic dicer-substrate siRNAs that correspond to target sequences in exon 2 in glycogen synthase silencing experiments. The dsi-RNAs were purchased from Integrated DNA Technology (IDT, Coralville, IA), confirmed through BLAST searches to have no significant homology to *Cx. pipiens* genes other than *glycogen synthase*, and were as follows: *dsi-glys*:

5'rArGrCrGrArCrUrCrCrArCrGrUrUrGrArArGrUrUrGrUrUrGrGr UrU 3' /

5'rCrCrArArCrArArCrUrUrCrArArCrGrUrGrGrArGrUrCrGCT3'. All phenotypes were confirmed following knockdown (KD) with *dsi-glys*. A scrambled negative control dsi-RNA, an siRNA duplex lacking significant sequence homology to any genes in the *Cx. pipiens* genome, was used for control experiments: *dsi-control*:

5'rGrArArGrArGrCrArCrUrGrArUrArGrArUrGr UrUrArGrCGT3' /

5'rArCrGrCrUrArArCrArUrCrUrArUrCrArGrUrGrCrUrCrUrUrCrCrG3. We didn't observe any significant changes in *dsi-control* injected mosquitoes compared to the wild type mosquitoes (no *dsi-RNA* injections).

2.3. RNAi Efficiency Evaluation Using qRT-PCR.

We carried out qRT-PCR of the dsi-RNA-injected mosquitoes as previously described (Sim et al 2009; 2015). Briefly, total RNA samples were extracted with TRIzol (Invitrogen) from three batches of 15 adult female mosquitoes on various days after dsi-RNA injection. To remove genomic DNA contamination, RNA samples were treated with DNase I following the manufacturer's instructions (50-375 units/μl; Invitrogen). For reverse transcription, 5 μg of total RNA was reverse-transcribed with SuperScript III RNase H-reverse transcriptase

(Invitrogen). All reactions were performed in triplicate in a total volume of 20 μ l containing 10 μ l SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and 300 nmol of each primer at the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The following qRT-PCR primers were used: q-glys, ACAACCGCAAATCTCTCCTG and CATGTGATGCAAGCGATCTT; q-rpl19, CGCTTTGTTTGATCGTGTGT and CCAATCCAGGAGTGCTTTTGT. The ribosomal protein large subunit 19 gene (rpl19) was used as a loading control. Geometric averages of transcript levels of the internal controls were used to normalize transcript levels of the *glycogen synthases* gene. Then, statistical significance of differences in transcript levels were determined using a Student's t-test between the relative transcript values of *dsi-glys* injected vs. control samples (*dsi-control* injected), using three biologically independent replicates for each gene. A P-value less than 0.05 was considered as a significant transcript-level change.

2.4. ^{13}C -isotope labeling of *Cx. pipiens*.

To monitor the carbon utilization in mosquitoes by solid-state NMR, we injected *dsi-glys* or *dsi-control* into the adult female mosquitoes. Then they were fed on sponges soaked with 10% glucose (^{13}C -natural abundance) mixed with 1% D- $^{13}\text{C}_6$ glucose for 7 days after *dsi-RNA* injections. Isotope-labeled D- $^{13}\text{C}_6$ glucose (uniformly ^{13}C labeled with 99% enrichment) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Following the 7-days post *dsi-RNA* injections, mosquitoes were frozen at -80°C then lyophilized. Lyophilized intact mosquitoes were weighed and packed into a 7-mm zirconia rotor for solid-state NMR analysis. The average dry weight of a mosquito was approximately 1.0 mg.

2.5. Solid-state NMR spectrometer.

The detailed setup for solid-state NMR is described elsewhere (Chang et al., 2016). Briefly, Carbon-13 CPMAS NMR measurements were performed on 7.1-T Bruker Avance III with a double resonance HX probe. Lyophilized mosquitoes were contained in a 7-mm outer diameter zirconia rotor with magic-angle spinning at 5 kHz. Spinning sidebands were suppressed using total-suppression of spinning sidebands sequence during echo in CPMAS. The proton-carbon matched cross polarization was set at 50 kHz with 2-ms contact time. The proton dipolar decoupling was achieved by applying two-pulse phase modulation on the ^1H channel during acquisition. The ^{13}C π -pulse length was 5 μ s with the recycle delay of 3 s. The line broadening for spectrum was 20 Hz. ^{13}C -CPMAS spectra were normalized to equal 175-ppm intensity which corresponded to the peptidyl-carbonyl carbons of proteins.

2.6. Total lipid and glycogen quantification and fat body staining.

After *dsi-glys*-injected and *dsi-control*-injected female *Cx. pipiens* after adult eclosion were fed 10% (10 g/100 mL) glucose for 7 days and frozen, then we measured total lipid and glycogen contents of individual mosquitoes using modified methods described by Van Handel (1985 and 1988). Briefly, a total of 7–8 mosquitoes were measured per each *dsi-RNA* treated group. For lipids, each sample had a single mosquito homogenized in 0.2 mL of chloroform:methanol (1:1) mixture. After evaporating the solvent, 0.2 mL of sulfuric acid was added, and samples were heated at 37°C for 10 min. After cooling, 2 mL of vanillin

reagent (0.12% vanillin in 68% phosphoric acid) was added. Samples were allowed to develop for 5 min, then the absorbance was measured at 525 nm. Sesame oil (Sigma, S3547) was used to generate a standard curve. For glycogen content, a single mosquito was homogenized in 0.2 mL of 2% sodium sulfate, followed by addition of methanol (1 mL) and centrifugation for 1 min. Supernatants were evaporated, and 2 mL of anthrone reagent (0.14% anthrone in 28% sulfuric acid) was added to each sample. Reactions were incubated at 37 °C for 15 min, then we measured the absorbance at 625 nm and generated the standard curve by using purified glycogen (Sigma, G0885). Comparisons of different groups were carried out using the two-tailed unpaired Student's t test. Differences were considered statistically significant at $p < 0.05$.

2.7. Survival following injection of *dsi-glys*

To evaluate the knock down effect of glycogen synthase on survival of diapausing mosquitoes, we intrathoracically injected either 0.5 µl *dsi-glys* or *dsi-control* (1.5 mg/ml) into 20 females per cohort. Mosquitoes were held at 18 °C, 75% R.H. and a 9:15 L:D cycle, with access to 10% glucose, and we assessed the survival of the treated mosquitoes on days 1, 15 and 30. Experiments were replicated five times.

3. Results

3.1. Increased fat body cells of diapausing *Cx. pipiens*.

The short-day-reared diapausing *Cx. pipiens* females exhibit the typical enlarged abdomen compared to the long-day-reared nondiapausing females (Fig. 1A). Diapausing female mosquitoes were fed a 10% glucose solution (10 g/100 mL), and the microscope images of BODIPY 493/503 stained fat body cells from the abdomen show an increase in the total amount of lipids and number of lipid droplets found in fat bodies compared to their control counterparts (Fig. 1B).

3.2. *Cx. pipiens* glycogen synthase (*glys*)

We identified *Cx. pipiens* glycogen synthase sequences (Accession No. [KP057863](#)) as one of the target genes of Foxo by using ChIP-sequencing (ChIP-seq) against the genome of *Cx. quinquefasciatus*, a sister species of *Cx. pipiens*. In addition, we found that mRNA level of the gene encoding glycogen synthase was approximately 4 times higher in diapausing females relative to nondiapausing females a week after eclosion from a previous study (Sim et. al. 2015). cDNA fragments of *Cx. pipiens* glycogen synthase (503 bp) share the highest identities with homologous genes from closely related mosquitoes, *Cx. quinquefasciatus* (99%, XM_001846844.1) and *Anopheles gambiae* (81%, XM_312352.5), respectively.

3.3. Dicer-substrate RNAi -glycogen synthase (*dsi-glys*) significantly reduce glycogen and lipid storage in diapausing females

We assessed RNAi efficiency by using qRT-PCR. In contrast to the relatively high induction of *glycogen synthase (glys)* in *dsi-control* injected mosquitoes, only 19% and 38% of *glys* mRNA was detected in *dsi-glys* injected, diapausing mosquitoes 3 and 7 days after injections, respectively. This result indicates that injection of *dsi-glys* successfully reduced induction of the *glycogen synthase* gene (Fig. 2A). The *rpl19* gene was used as an internal

control. qRT-PCR analysis of *rpl19* transcript levels 3 and 7 days after injections revealed no significant differences among the *dsi*-RNA treated groups.

3.4. Glucose metabolism in diapausing mosquitoes.

To investigate the changes in glucose metabolism in mosquitoes, we performed solid-state NMR measurements on lyophilized intact diapause-destined females that were fed 10% D-glucose in the presence or in the absence of ^{13}C -labeled glucose (1% D- $^{13}\text{C}_6$]glucose) for 7 days following the *dsi*-RNA-injection (Fig. 3A). Natural abundance ^{13}C -CPMAS spectrum of diapausing female *Cx. pipiens* which fed exclusively on 10% D-glucose is shown in Figure 3B. Spectral features include aliphatic carbons predominantly found in lipids visible at 10-40 ppm, O-alkyl carbons from carbohydrates at 60-110 ppm, aromatic carbons with contribution from nucleic acids at 130 ppm, and carbonyl carbons of proteins at 175 ppm.

^{13}C -CPMAS spectra of diapausing female *Cx. pipiens* fed for 7 days with 1% D- $^{13}\text{C}_6$]glucose following the *dsi*-RNA-injection are shown in Fig. 4 (bottom, black), and the ^{13}C -natural abundance spectrum of female *Cx. pipiens* fed for 7 days with D-glucose without RNA-injection is shown in red. The spectra are normalized to 175-ppm intensity of the peptidyl-carbonyl carbons. The ratio of 175-ppm to 130-ppm peak intensity is directly proportional to the total amounts of proteins to nucleic acids found in individual mosquitoes. This protein to nucleic acid ratio remains relatively constant in mosquitoes independent of feeding with either natural abundance glucose (Fig. 4A, bottom black) or in the presence of isotope labeled 1% D- $^{13}\text{C}_6$]glucose (Fig. 4A, bottom red). Therefore, the D- $^{13}\text{C}_6$]glucose provisioned during the diapause is not metabolized for the biosyntheses of proteins or nucleic acids. Instead, an intense 73-ppm peak in the CPMAS spectrum of 1% D- $^{13}\text{C}_6$]glucose fed mosquitoes, which is assigned to the O-alkyl carbons at C 2,3,5 positions of D- $^{13}\text{C}_6$]glucose, is consistent with the accumulation of ^{13}C -labeled glycogen during diapause. The ^{13}C -CPMAS spectrum of *dsi-glys* RNA-injected *Cx. pipiens* (Fig. 4B, bottom) shows a large reduction in 73-ppm peak intensity, compared to the control (Fig. 4A, bottom). This reduction indicated that GlyS suppression by *dsi-glys* RNA-injection prevented the biosynthesis of glycogen and its storage in diapausing mosquitoes.

To accurately quantify changes in glycogen storage of RNA-injected mosquitoes, the natural abundance contribution was removed from the CPMAS spectra of ^{13}C -labeled diapausing *Cx. pipiens* (Fig. 4, top). We carried out spectral subtraction of the natural abundance spectrum of diapausing female *Cx. pipiens* that fed for 7 days on 10% D-glucose (Fig. 4, bottom red line). The natural abundance labeled mosquitoes were not subjected to the *dsi-glys* injection. The resulting difference spectrum (Δ) for *dsi-control* and *dsi-glys*-injected *Cx. pipiens* are shown in Fig. 4A (top) and Fig. 4B (top), respectively. In the Δ spectra, only the ^{13}C from metabolized D- $^{13}\text{C}_6$]glucose are observed. The carbon resonances in the Δ spectra matches with the chemical shift assignments for glycogen which are: 61 ppm to C6; 73 ppm to C2, C3, and C5; 82 ppm to C4; and 93-104 ppm to anomeric C1 of glucose (figure inset). Anomeric C1 chemical shift is sensitive to the glycosidic linkages. In monosaccharide D-glucose, reducible C1 resonates at 93 ppm, whereas, C1 in linearly polymerized D-glucose with an $\alpha(1\rightarrow4)$ connection resonates at 104 ppm, and C1 in branched polymer with an $\alpha(1\rightarrow6)$ glycosidic bond resonates at 98 ppm (Cheatham et al.,

1998). The visible increase in 73-ppm intensity accompanied by increases in both 98 and 104 ppm resonances in the ^{13}C S spectra is consistent with glycogen accumulation in *dsi-control* injected *Cx. pipiens*. The uptake of D- $^{13}\text{C}_6$ glucose during the diapause is exclusively routed for the biosynthesis of glycogen but not for the biosynthesis of amino acids or chitin. This is evident by the absence of carbonyl carbons (proteins and chitin) at 175 ppm, and the C2 carbon of N-acetylglucosamine of chitin at 55 ppm, which is well resolved from the C2 of glucose resonating at 73 ppm in the ^{13}C S spectra. The ^{13}C S spectrum of *dsi-glys*-injected *Cx. pipiens* shows that the *dsi-glys* injection did not result in total inhibition of glycogen synthase but substantial knockdown that reduced glycogen accumulation in diapausing mosquitoes by 46% (Fig. 3B, top right). The reduced glycogen accumulation is in excellent agreement with the RNA interference efficiency of *dsi-glys*-injected *Cx. pipiens* measured by qRT-PCR at 7 days after the injection as shown in Figure 2A.

Earlier solid-state NMR study of mosquitoes (Chang et al., 2016) has shown that the feeding of diapausing *Cx. pipiens* with D- $^{13}\text{C}_6$ glucose increases the accumulation of both glycogen and lipids. However, the ^{13}C S spectra of *dsi*-RNA-injected *Cx. pipiens* (Fig. 3) show only glycogen accumulation, without visible increase in aliphatic carbons of lipids (15-40 ppm). The natural abundance spectrum of mosquitoes that were used to subtract from the ^{13}C -labeled mosquitoes spectrum to obtain the ^{13}C S were not subjected to *dsi*-RNA injection. Therefore, the ^{13}C S spectra not only highlights the accumulation of ^{13}C -labeled metabolites, as discussed above, it also demonstrates the effects of intrathoracic injection on insect metabolism. The absence of lipids in the ^{13}C S spectra of both *dsi-control* and *dsi-glys* injected *Cx. pipiens* may indicate that the intrathoracic injection is a traumatic event such that tissue repair and regeneration of the damaged tissue may have increased lipid utilization in fat body and thereby prevents significant lipid accumulation.

3.5. Differential carbohydrate utilization and overwinter survival.

We measured the total lipid and glycogen in individual diapausing females *Cx. pipiens* fed exclusively on 10% (10 g/100 mL) glucose for 7 days after adult eclosion using modified methods described by Van Handel (1985 and 1988) (Fig. 5B). We estimated the total quantity of lipids based on quantifying the reaction of phosphor-vanillin with unsaturated aliphatic chains primarily found in lipids by measuring the optical density (OD) at 525 nm. We also estimated total quantity of glycogen following acid hydrolysis of hexose by quantifying the hydroxy-methyl-furfural reaction through measuring the concentration of hydroxy-methyl-furfurylidene-anthrone with OD at 625 nm. Relative quantities of lipid (black) and glycogen (white) in non-diapausing mosquitoes fed 10% glucose were normalized to compare against amounts found in diapausing mosquitoes exclusively fed 10% glucose.

Before injection, females contained a mean $\pm$ SD of 44.1 ± 9.4 μg of glycogen per female, but 7 days post-injection the glycogen level increased approximately 4-fold in the a *dsi-control* (Fig. 5A). In response to an injection of *dsi-glys*, diapausing females accumulated less glycogen, a distinction that was already evident 3 days after injection. Although some glycogen continued to accumulate in the *dsi-glys*-injected females, the level attained was

approximately a quarter of what was observed in the control females by day 7 (Fig. 5A). In addition, lipid content revealed a slight reduction of fat storage and number of fat body cells when *dsi*-RNA was directed against the gene encoding glycogen synthase. Finally, compared to the control injection, the control diapausing females showed a higher level of total lipids than their knockdown counterparts (Fig. 5A).

Additionally, we compared the total lipid and glycogen quantifications using methods described by Van Handel with the solid-state NMR results. Figure 5B shows the scaled bar graph of scaled 70 and 33-ppm intensities from ^{13}C -CPMAS spectra of diapausing mosquitoes that were injected with *dsi-control* (left) or *dsi-glys* (right). The 70-ppm intensity is directly proportional to the total carbohydrates (glycogen), and the 33-ppm, to lipids in the mosquito. The raw 70-ppm intensity (glycogen) and 33-ppm intensity (lipid) in mosquitoes injected with either *dsi-control* or *dsi-glys* were scaled to normalize to the corresponding glycogen and lipids amounts shown in Figure 5A (dotted lines). Both results show a comparable trend of reduction in both glycogen and lipids in diapausing mosquitoes that were injected with *dsi-glys*, but the relative amount differs. This difference is due to solid-state NMR exclusively measuring ^{13}C -isotope distribution originating from the feeding of 1% D- $^{13}\text{C}_6$ glucose, whereas, the Van Handel assay measures the total lipids and glycogen of the entire organism.

3.6. Reduced survival of diapausing *Cx. pipiens* in response to *dsi-glys* injections

Expression levels of glycogen synthase in diapausing *Cx. pipiens* had a direct impact on the mortality rate. While >90% of the *dsi-control* injected diapausing mosquitoes survived at 15 days after the injection, only 62.5% of the *dsi-glys* injected females survived. The effect is more distinct at 30-days post injection where >80% of diapausing control mosquitoes survived, yet only 10.5% of the *dsi-glys* injected females survived at the 30-day mark (Fig. 6). This result suggests that, at least in early diapause, reduced expression levels of glycogen synthase significantly increased mortality of *Cx. pipiens*, possibly a consequence of reduced glycogen storage for overwintering survival.

4. Discussion

In diapausing insects, efficiency of glycogen utilization is crucial not only for energy homeostasis but also for cold tolerance during wintering periods (Sinclair and Marshall, 2018). Various cryoprotectants linked to the cold tolerance have been identified, including glycerol and small sugar derivatives as well as polyols (Toxopeus and Sinclair, 2018). In many insects, polyols provide a key antifreeze defense to maintain an organismal liquid state (Storey and Storey, 2013). The production of polyols is often catabolized from glycogen stores accumulated during the diapause preparatory period (Clark and Worland, 2008). In cold winter, glycogen and other carbohydrate derivatives are converted in response to changing temperature. It is also known that polyol synthesis is regulated by two enzymes in glycogen metabolic pathways: 1) glycogen phosphorylase kinase, and 2) protein phosphatase 1 in control of glycogen phosphorylase (Hayakawa 1985). Evidence suggests that the accumulation of small sugar derivatives and polyols are a common trait of cold

tolerant insects, which is closely linked to glycogen metabolism. Thus, the role of glycogen metabolic pathway proteins in insect cold response mechanisms needs further investigating.

In diapausing *Cx. pipiens* mosquitos, it has been reported that during the first week post-eclosion, the diapause-destined mosquito accumulates approximately twice the amount of carbohydrates compared to their nondiapause counterparts (Mitchell and Briegel, 1989). Interestingly, it has been demonstrated that glycogen, not lipid, is the primary energy source in the early diapause period (Zhou and Miesfeld, 2009). After the stored glycogen in diapausing females is depleted at the end of the first month, the stored lipid starts to be utilized for energy production. At this critical junction, the metabolic gene expression profile in *Cx. pipiens* changes by downregulating genes that are involved in fatty acid synthesis, while upregulating genes that are involved in beta-oxidation. This reflects a fundamental metabolic shift in how glycogen and lipids are utilized in early and late diapause periods (Sim and Denlinger, 2009). Our results imply that the energy metabolism shifts occur by a molecular mechanism that gauges glucose or glycogen levels during early diapause and stimulates the switch from glycogenolysis to lipolysis in late diapause period.

In various insects, high-glucose levels strongly activate the expression of lipogenic enzymes: ATP citrate lyase, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS), which convert citrate derived from the TCA cycle into lipids (Mattila et al., 2015; Mattila and Hietakangas, 2017). In diapausing *Cx. pipiens*, ACC and FAS genes are upregulated in the early diapause period (the first week after adult eclosion), and concomitantly the lipid levels increase at the fat body of diapausing females. Furthermore, RNA interference targeting the FAS gene resulted in reduced lipid levels and lowers survival rate in diapausing *Cx. pipiens* (Sim and Denlinger, 2009). By contrast, genes encoding lipid catabolic enzymes, such as triacyl-glycerol lipases and acyl-CoA dehydrogenase, are downregulated by high glucose levels. These genes were also suppressed when diapausing mosquitoes increase lipid and glycogen levels in the early diapause period (Sim and Denlinger, 2009). In *Drosophila*, a signaling complex containing Rack1, GSK-3B and AMPK assembles on glycogen particles to regulate the rate of synthesis through phosphorylating different amino acid residues of GlyS, with AMPK being responsible for targeting this complex to glycogen (Erdi et al. 2012). In addition, Atg8 interaction with GlyS implies a potential regulator of glycogen catabolism in flies. While the role of GlyS's activity in regulating diapause energetics is unclear, our study shows that genetic RNA interference of the GlyS's activity is detrimental for survival and GlyS plays a crucial physiological role in nutrient storage of diapausing mosquitoes on a sustained glucose diet.

In general, overwintering insects prefer lipids as an energy source, but some insect species primarily consume carbohydrates, even though lipid sources are abundant. For example, overwintering 2nd instar spruce budworm larvae use carbohydrates as a winter energy source (Han and Bause, 1993), and even *E. solidaginis* depends, at least in part, on carbohydrates (Storey and Storey, 1986). It appears that the timing and thresholds for the switching between glycogen and lipid stores are evolutionarily conserved in various diapausing insects. Diapause energetic strategies related to the timing and selection of energy stores are closely linked to the life-history trade-offs in individual species. Species that cannot replenish lipid reserves post-winter may conserve them for reproductive

investment, leading to egg deposits. By contrast, if carbohydrate stores are reserved to produce low-molecular-weight cryoprotectants such as glycerol to sorbitol (Storey, 1997), then the insect may prioritize lipid consumption to fuel overwinter energetics, making carbohydrates available for cryoprotectants. Finally, as described in the previous paragraph, lipid and carbohydrate metabolism appears to be co-regulated. The stored lipid can provide both the energy sources and precursors of cryoprotectants and beta oxidation that fuels the glucose levels in diapausing insects.

In summary, we suggest that the functional role of glycogen synthase responds to changes in carbohydrate intake and regulates diapause energetics; however, there is still a need for evaluating gene regulatory networks and signaling pathways, which act locally in fat body or other periphery tissues, as well as hormonal signals from brain and endocrine organs. The results from our study provide new directions for further study of the molecular mechanism of diapause energetics in the future.

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Abbreviations:

GlyS	glycogen synthase
<i>dsi-glys</i>	dicer-substrate RNA of <i>Cx. pipiens</i> glycogen synthase
<i>dsi-control</i>	dicer-substrate RNA of control gene
Foxo	forkhead transcription factor
qRT-PCR	quantitative real-time PCR
RNAi	RNA interference
ND	nondiapause
D	diapause
CPMAS	cross-polarization magic-angle spinning spectra

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Figure 1.

Morphological distinctions in abdomens and fat body cells between nondiapausing (ND) and diapausing (D) females of *Culex pipiens*. (A) Female *Cx. pipiens* fed with 10% (10 g/100 mL) glucose for 7 days after adult eclosion in non-diapausing (left) and diapausing (right) states. Diapausing female 7 days after adult eclosion with a visibly enlarged abdomen (right). (B) Fluorescent microscopy images of fat body cells. Fat body cells of diapausing female fed on 10% glucose shows fat hypertrophy. Lipids in adipocytes were stained using BIODIPY 493/503 (green). White bars represent 100 μ m.

Figure 2.

RNA interference efficiency targeting glycogen synthase (*glys*) in *Cx. pipiens*. (A) Transcript levels of the gene encoding glycogen synthase in females injected with dsi-*glys* (white bars) were compared with the dsi-control (black bars). Expression levels were measured by qRT-PCR at 3 and 7 days after dsi-RNA injection and normalized using a ribosomal protein large subunit 19 (*rpl19*) as a loading control. Bars (mean $\pm$ s.e., $n = 3$) with asterisks (*) indicate significant differences at $P < 0.05$, t-test.

Figure 3.

^{13}C -Natural abundance CPMAS spectrum of female *Cx. pipiens*. (A) Schematic sample preparation of dsi-glyc injected diapausing females for solid-state NMR analysis. (B) ^{13}C -Natural abundance cross-polarization magic-angle spinning (CPMAS) spectrum of lyophilized intact diapausing females that were fed for 7 days on 10% D-glucose. The carbonyl carbons of proteins are visible at 175 ppm, the aromatic carbons at 130 ppm, O-alkyl carbons from carbohydrates at 60-110 ppm, and aliphatic carbons that are predominantly found in lipids are visible at 10-40 ppm.

Figure 4.

^{13}C -CPMAS spectra of 1% $\text{D}-[^{13}\text{C}_6]\text{glucose}$ fed female *Cx. pipiens*. (A) ^{13}C -CPMAS spectrum of dsi-control-injected diapausing females fed for 7 days with 10% D -glucose in the presence of 1% $\text{D}-[^{13}\text{C}_6]\text{glucose}$ (bottom, black) is shown with ^{13}C -natural abundance spectrum (bottom, red) of Fig. 3B. Both CPMAS spectra are normalized to 175-ppm intensity of the peptidyl-carbonyl carbons. The difference spectrum (ΔS) is the spectral subtraction of the natural abundance spectrum (bottom, red) from the ^{13}C -CPMAS spectrum of mosquitoes fed in the presence of 1% $\text{D}-[^{13}\text{C}_6]\text{glucose}$ (bottom, black). In the ΔS spectrum (top), only the ^{13}C from metabolized $\text{D}-[^{13}\text{C}_6]\text{glucose}$ are observed. The carbon resonances in the ΔS matches with the chemical shift assignments of glycogen where 61 ppm is assigned to C6; 73 ppm to C2, C3, and C5; 82 ppm to C4; and 93-104 ppm to anomeric C1 of glucose (figure inset). (B) ^{13}C -CPMAS spectrum of dsi-glyS-injected diapausing females fed for 7 days with 10% D -glucose in the presence of 1% $\text{D}-[^{13}\text{C}_6]\text{glucose}$ (bottom, black) is shown with ^{13}C -natural abundance spectrum (bottom, red). The ΔS spectrum of dsi-glyS injected *Cx. pipiens* (top) shows 46% reduction in 73-ppm peak intensity, indicating that GlyS suppression by dsi-glyS injection led to reduced glycogen accumulation in diapausing mosquitoes.

Figure 5.

Lipid and glycogen quantifications of 10% glucose fed female *Cx. pipiens*. (A) Estimated total lipid and glycogen contents of individual diapausing females fed exclusively on 10% glucose for 7 days after adult eclosion as determined using the Van Handel method. Total amounts of lipid (black) and glycogen (white) per individual mosquitoes fed on 10% glucose were normalized. Error bars indicate standard error of mean. (B) Intensities of peaks at 70 and 33 ppm in ^{13}C -CPMAS spectra of diapausing mosquitoes fed exclusively on 10% D-[^{13}C 6]glucose (right) from Fig. 3. The 70-ppm intensity is proportional to the total carbohydrates (glycogen), and the 33-ppm intensity is proportional to the lipids in mosquitoes.

Figure 6.

The relative percentages (mean $\pm$ s.d., $n = 5$) of surviving mosquitoes after injection of dsi-RNAs targeting against the gene encoding glycogen synthase (dsi-glys) and control (dsi-control). (*) Indicate significant differences at $P = 0.05$, t-test. $N = 5$ groups of 20 individuals for each data point. White boxes = 1 day after adult dsi-RNA injection (1 d.p.i.), gray boxes = 15 days (15 d.p.i.), and black boxes = 30 days (30 d.p.i.).