Insect science

Insect Science (2022) 29, 691–703, DOI 10.1111/1744-7917.12972

ORIGINAL ARTICLE

Involvement of glucose transporter 4 in ovarian development and reproductive maturation of *Harmonia axyridis* (Coleoptera: Coccinellidae)

Yan Li[1,2], Sha-Sha Wang[2], Su Wang[3], Shi-Gui Wang[2], Bin Tang[2] and Fang Liu[1]

[1]*College of Horticulture and Plant Protection, Yangzhou University, Yangzhou, Jiangsu, 225009, China;* [2]*College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang, 310036, China and* [3]*Institute of Plant and Environment Protection, Beijing Academy of Agricultural and Forestry Sciences, Beijing, 100097, China*

Abstract Glucose is vital to embryogenesis, as are glucose transporters. Glucose transporter 4 (Glut4) is one of the glucose transporters, which is involved in rapid uptake of glucose by various cells and promotes glucose homeostasis. Although energy metabolism in insect reproduction is well known, the molecular mechanism of Glut4 in insect reproduction is poorly understood. We suspect that Glut4 is involved in maintaining glucose concentrations in the ovaries and affecting vitellogenesis, which is critical for subsequent oocyte maturation and insect fertility. *Harmonia axyridis* (Pallas) is a model organism for genetic research and a natural enemy of insect pests. We studied the influence of the Glut4 gene on the reproduction and development of *H. axyridis* using RNA interference technology. Reverse transcription quantitative polymerase chain reaction analysis revealed that HaGlut4 was most highly expressed in adults. Knockdown of the HaGlut4 gene reduced the transcript levels of HaGlut4, and the weight and number of eggs produced significantly decreased. In addition, the transcript levels of vitellogenin receptor and vitellogenin in the fat bodies and the ovaries of *H. axyridis* decreased after the interference of Glut4, and decreased the triglyceride, fatty acid, total amino acid and adenosine triphosphate content of *H. axyridis*. This resulted in severe blockage of ovary development and reduction of yolk formation; there was no development of ovarioles in the developing oocytes. These changes indicate that a lack of HaGlut4 can impair ovarian development and oocyte maturation and result in decreased fecundity.

Key words glucose transporter 4 (Glut4); *Harmonia axyridis*; RNAi; reproduction; vitellogenin

Introduction

The relationship between carbohydrate metabolism and oocyte development appears to be highly conserved in insects. The ovary has a high energy metabolism and requires an adequate energy supply to complete follicle development. Glucose is the main energy source and also the key source of fuel and metabolites for regulating hormone secretion, transcription, central nervous system function and enzyme function (Xiang *et al*., 2021). It is well known that glucose, glycogen, lipids and amino acids are key components of oocyte maturation and embryo development in insects. For example, a large number of studies have demonstrated that glucose regulation plays a key role in vitellogenesis and ovarian development in *Aedes aegypti*, *Tribolium castaneum* and *Drosophila melanogaster* (Yamazaki *et al*., 2003; Vital

Correspondence: Fang Liu, College of Horticulture and Plant Protection, Yangzhou University, Yangzhou, Jiangsu 225009, China. Email: liufang@yzu.edu.cn; Bin Tang, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang 310036, China. Email: tbzm611@163.com

et al., 2010; Fraga *et al*., 2013). Therefore, the mechanism by which glucose metabolism regulates insect reproduction is worthy of detailed examination.

Glucose is a hydrophilic molecule that cannot diffuse freely inside and outside cells; it requires transporters to cross the cell membrane. Glucose transporters (Gluts) are proteins involved in this function (Yang *et al*., 2018a; Wang *et al*., 2020). There are 14 known isoforms of glucose transporters, which have specific temporal and spatial distributions and show obvious transport kinetics, capacity and substrate selectivity (Deng *et al*., 2015; Stanirowski *et al*., 2017). Based on sequence similarity, *Gluts* can be divided into three subfamilies; class I sugar transport facilitators comprise the well-characterized isoforms *Glut1* to *4* and *Glut14* (Scheepers *et al*., 2004). *Glut1* is one of the earliest cloned membrane transporters, and it has been extensively studied and found to predominantly function in erythrocytes and the blood-brain barrier (Wang *et al*., 2020). *Glut2* is constitutively expressed in the intestine basolateral membrane in addition to glucose and can transport fructose (Scheepers *et al*., 2004; Bifano *et al*., 2010). *Glut3* is a high-affinity glucose transporter with predominant expression in tissues with a high glucose requirement (e.g., brain). *Glut4* is a high-affinity glucose transporter expressed in insulin-sensitive tissues (heart, skeletal muscle, adipose tissue) (Keyong *et al*., 2017). Class II facilitative glucose transporters include the fructose-specific transporter *Glut5* and *3* related proteins, as well as *Glut7*, *Glut9*, and *Glut11*. Class III comprises the transporter isoforms *Glut6*, *Glut8*, *Glut10*, and *Glut12* (Scheepers *et al*., 2004).

Among them, insulin-reactive *Glut4* was discovered in 1988 (Klip *et al*., 2019) and is responsible for insulin-dependent glucose transfer (Yuliya *et al*., 2018; Stanirowski *et al*., 2019). *Glut4* contains 12 transmembrane proteins with 509 amino acid residues, which are encoded by the *SLC2A4* gene and are mainly expressed in fat and muscle tissues (Chakraborty *et al*., 2013; Du & Zeng, 2016). It has been suggested that deletion of *Glut4* can lead to major metabolic defects (Wang *et al*., 2020). Most previous studies on *Glut4* focused on human diseases, such as diabetes or polycystic ovary syndrome (Yamashita *et al*., 2018; Fujimoto *et al*., 2019; Cabrera *et al*., 2019). In the related research on mammals, it is demonstrated that 80% of fetal energy is derived from glucose metabolism and *Glut4* is important for placental glucose exchange (Deng *et al*., 2015; Stanirowski *et al*., 2017). In addition, studies on *Glut4* in mouse endometrial epithelium showed that *Glut4* in the endometrial epithelium affects embryo development by altering glucose concentration in the uterine fluid. It can affect implantation by impairing endometrial receptivity due to dysfunction of *Glut4* (Long *et al*., 2021).

At present, *Glut4* in mammals has been extensively studied, but the research on *Glut4* in insect reproductive direction is very rare. Due to the metabolic differences between insects and mammals, *Glut4* applicability in physiological systems is unclear, and there is little information on the effect of glucose transporter 4 on animal reproduction, especially invertebrate reproduction. Therefore, in this study, taking *Harmonia axyridis* as the model, we investigated *Glut4* in *H. axyridis* regulatory effects of ovarian development and reproduction.

Predatory ladybird beetles (Coleoptera: Coccinellidae) are important natural enemies of insect pests. Among them, *H. axyridis* (Pallas) is a ladybird beetle native to Asia. It is a highly efficient predator of crop pests and widely used for the biological control of aphids, citrus pests, Lepidoptera larvae and mites. *H. axyridis* is also a well-known model organism for genetic studies (Li *et al*., 2019; Sun *et al*., 2019). However, the structural characteristics and biological functions of *H. axyridis Glut4* are largely unknown. Few studies have reported whether the metabolic regulation involved in *HaGlut4* signaling can affect insect reproduction. Therefore, in this study, we identified *HaGlut4* genes in *H. axyridis* by searching genome and transcriptome databases. The molecular mechanism of the *HaGlut4* gene in *H. axyridis* reproduction was analyzed by RNA interference (RNAi) and other techniques.

Our study provides the first analysis of the metabolism of the *H. axyridis* during vitellogenesis. *Glut4* appears to be essential for oogenesis and reproduction as judged by our functional analysis. The functional characterization of factors that drive successful reproductive biology of *H. axyridis* is important to answer fundamental biological questions and assist in the development of improved biocontrol.

Materials and methods

Experimental insects

H. axyridis was propagated in the Key Laboratory of Animal Adaptation and Evolution of Hangzhou Normal University. *H. axyridis* was fed with pea aphids *Acyrthosiphon pisum* (Hemiptera: Aphididae) reared on broad bean (*Vicia faba*, var. "Jinnong") seedlings. All insects were reared in an artificial climate chamber (23 ± 2 °C, 68% ± 5% relative humidity and 16 : 8 h L : D photoperiod) for the experiments.

Table 1 Primer sequences for double-stranded RNA synthesis and reverse transcription quantitative polymerase chain reaction.

Gene	Forward primers (5′–3′)	Reverse primers (5′–3′)
ds*GFP*	CCTGAAGTTCATCTGCACCA	ACAAGCAGAAGAACGGCATCA
ds*HaGlut4*	GCAGCATTACGTGACTTGGAG	TTGCCGGATTACTCGAAGGA
HaGlut4	ACTGTGTTCCTGTGGCCTTAT	ACTGCCGAATCTGATGCTTCT
Rp49	GCGATCGCTATGGAAAACTC	TACGATTTTGCATCAACAGT
HaVg	GCAACAGAGTCCGTGGTCTTT	GCTGCTTTCACCGTTCTTCAA
HaVgR	TGTAGGAGGCGAAGCAATGAT	TGGGATGTGACAGGGAAATAA
HaGS	CCCTTAGGATCGGATGTTCTC	CACCAGCCATCTCCCAGTT
HaTPS	GACCCTGACGAAGCCATACC	AAAGTTCCATTACACGCACCA
HaInR	CGATAACTCGGTTTCACT	GCTGTAGATTCCAGGTCC
T7	GGATCCTAATACGACTCACTATAGG	

Bioinformatics analysis

HaGlut4 sequences were searched against *H. axyridis* in the Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra) using the National Center for Biotechnology Information (NCBI) reference sequences. Nucleotide sequence data for *HaGlut4* has been deposited in the GenBank nucleotide sequence database, accession number MZ822109. Amino acid sequences were predicted using DNASTAR Lasergene EditSeq (https://www.dnastar.com/software/lasergene/), and amino acid sequence alignments were carried out using ClustalX (http://www.clustal.org/).

RNA extraction and reverse transcription quantitative polymerase chain reaction (qRT-PCR)

RNA extraction was carried out with TRIzol reagent according to manufacturer instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The final concentrations A260/280 ratio of the total RNAs were determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). All samples showed a ratio between 1.9 and 2.0. RNA integrity, including potential degradation products and DNA contamination, was evaluated by electrophoresis in a 1% agarose gel (FroggaBio Inc., Concord, ON, Canada). RNA was considered intact when the 18S ribosomal RNA subunit (18S rRNA) band was observed. The first-strand complementary DNA (cDNA) was synthesized using a reverse transcription kit (TaKaRa Co. Dalian, China).

A 2 mL sample of first-strand cDNA (diluted 10 times) was analyzed in each 20 μL reaction by qRT-PCR. All the tests were performed in three replicates. qRT-PCR was carried out on a CFX Connect^{TM} Real-Time System (Bio-Rad, Hercules, CA, USA) using SYBR Select Master Mix (TaKaRa Biotech, Osaka, Japan) under the following reaction program: qRT-PCR was performed in a 20 μL reaction volume containing 10 μL of SYBR Green PCR Master Mix, 2 μL of cDNA template (100 ng), and 1 μL each of forward and reverse primers. The qRT-PCR primers are listed in Table 1. Denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s and 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min with gene-specific primers. The relative transcript levels of each *HaGlut4* gene in double-stranded (ds) *HaGlut4* and ds*GFP* treatments were normalized (Bustin *et al*., 2009) using the ribosomal protein 49 (*Rp49*) threshold values (Ct). *Rp49* was used as a reference gene because of its relatively stable expression in different tissues of *H. axyridis* (Yang *et al*., 2018b). The qRT-PCR was determined for each gene using slope analysis with a linear regression model. Relative transcript levels in each sample were measured using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

dsRNA synthesis and RNAi

The open reading frame of *HaGlut4* around the 431-bp unique fragment was amplified and cloned into the pMD-19 T vector (TaKaRa), and dsRNA were synthesized *in vitro* using PCR-generated DNA templates using a T7 RiboMAXTM RNAi System (Promega, Madison, WI, USA). Newly emerged adult females were anesthetized with carbon dioxide and microinjected with approximately 250 ng of ds*Glut4* through the intersegmental membrane of abdominal segments, using a microinjection system (Eppendorf-Netheler-Hinz, Hamburg, Germany). *H. axyridis* microinjected with *GFP* dsRNA from *Aequorea victoria* were used as controls.

Sample collection for developmental stage expression analyses

To investigate the expression levels of the *HaGlut4* gene at different developmental stages, we collected *H. axyridis* on the first day of the 3rd instar larva, the first day of the 4th instar larva, the first day of the pupal stage, and the first day of adult emergence for detection.

Carbohydrate contents and substance contents in H. axyridis adults

Day 1 post-adult emergent (PAE) *H. axyridis* were deprived of *Glut4* for 48 h and subsequently used in carbohydrate metabolism assays. Fifteen females were pooled for each chemical assay, and a total of 45 females were used across three biological replicates. To measure the glucose level, ladybugs were homogenized in phosphate-buffered saline. Glucose levels were then measured with glucose oxidase reagent (Sigma-Aldrich) according to manufacturer instructions. To measure trehalose levels, 50% of the mixture was incubated with trehalase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C. To measure the glycogen level, the other half of the mixture was incubated with amyloglucosidase (Sigma-Aldrich) and protein concentrations were analyzed using a Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific), according to manufacturer instructions. The trehalose, glycogen and glucose contents were calculated based on three biological replicates.

Adult insect bodies were sampled from ds*Glut4* and ds*GFP*-injected females on the 3rd day after emergence. Following washing three times in 1 × phosphate-buffered saline, the insect bodies were homogenized in lysis buffer and kept at room temperature for 10 min. Then the homogenates were heated for 10 min and at 70 °C for 10 min, and centrifuged at 2000 × *g* for 5 min. The triglyceride content in the supernatant was determined from the manufacturer instructions (Applygen Technologies, Beijing, China). The fatty acid, total amino acid and adenosine triphosphate (ATP) contents were analyzed using the assay kit according to manufacturer instructions. Each set of experiments were performed using three biological replicates and three technical replicates. Protein concentrations were analyzed using a BCA Protein Assay Kit (Thermo Fisher Scientific).

Ovarian development of H. axyridis

Day 1 PAE *H. axyridis* were injected with ds*Glut4* or ds*GFP* and then collected for the paired mating assay. Each dsRNA-treated female ($n = 20$) was allowed to mate with two males in a glass tube. Ovary samples were collected from *H. axyridis* adults on each of the four dates, including those on d 1 PAE, d 3 PAE, d 4 PAE, and d 7 PAE. We performed vivisection of female *H. axyridis* in saline by cutting off the wings and head and attaching the body to an anatomical box (AGAR dish). Under a Leica EZ4HD stereoscopic microscope (Leica, Wetzlar, Germany), we cut along the middle abdomen of the larval body to the end of the abdomen and removed non-reproductive organs and tissues such as the digestive tract and the fat body. A total of 15 individuals were dissected from each group. We observed the ovarian development in each individual and collected photographic images.

Statistical analyses

Statistical analysis was performed using Student's *t*-test and analysis of variance. Data are presented as mean ± SD of three independent biological replicates. The significance values were determined using Student's *t*-test (*$P < 0.05$; **$P < 0.01$) with GraphPad Prism 8 software (San Diego, CA, USA).

Results

Bioinformatics analysis of Glut4 and assembly chaperone genes

Evolutionary relationships were inferred by the neighbor joining method with bootstrap branch support, which was performed 100 times. *Glut4* deduced amino acid sequences showed close phylogenetic relationships with their homologs in many insect species and humans (Fig. S1A). Bioinformatics analysis indicated that each *Glut4* contained a characteristic domain ubiquitin-regulatory X (UBX), including ZM, FH2, GARS, THEG, Cpn10, HintN, FYRC and HhH2, implying their functional diversity (Fig. S1B). The UBX domain is found in ubiquitin-regulatory proteins, which are members of the ubiquitination pathway, as well as a number of other proteins including FAF-1 (FAS-associated factor 1), the human Rep-8 reproduction protein and several hypothetical proteins from yeast. The function of the UBX domain is not known, although the fragment of avian FAF-1 containing the UBX domain causes apoptosis of transfected cells.

Effects of HaGlut4 on the development and reproduction of H. axyridis

The expression level of *HaGlut4* in adult females was significantly higher than in larvae (Fig. 2A). These results suggest that the *Glut4* gene plays a vital role in adult

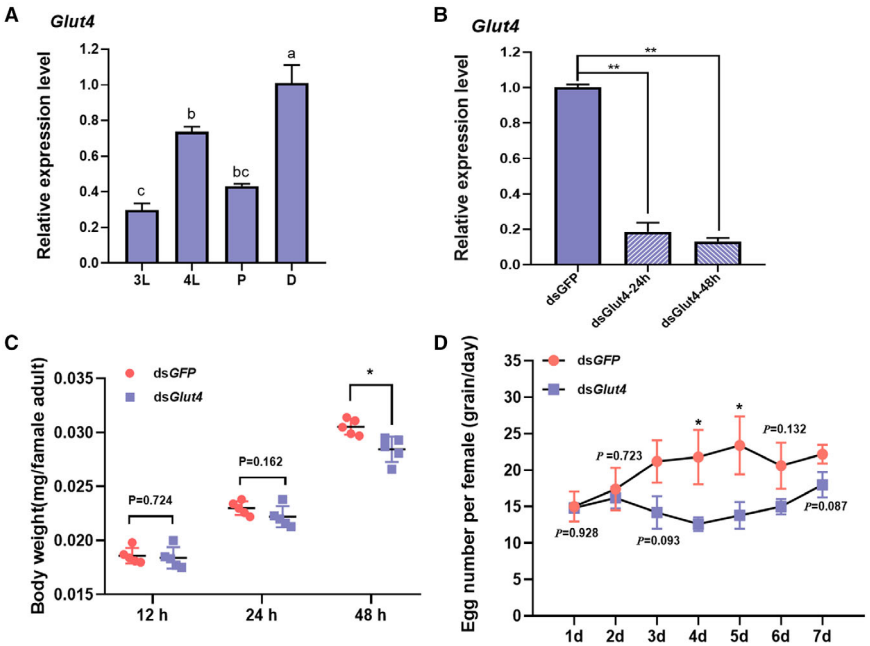

Fig. 1 Effects of silencing *HaGlut4* on the development and reproduction of *Harmonia axyridis*. A total of 250 ng of ds*Glut4* per adult was injected into an intersegmental region of the abdomen within 8 h after adult emergence. (A) Developmental stage expression of *HaGlut4*. Total RNAs were extracted from 3rd, 4th instar larvae, pupae and adults (n = 20–80 larvae, 20–80 pupae and 20 adults). 3L, 3rd instar larvae; 4L, 4th instar larvae; P, pupae; and D, adult *H. axyridis*. (B) RNA interference (RNAi) efficiency of *HaGlut4*. Total RNAs were extracted from *H. axyridis* at 24 h and 48 h after RNAi and transcript levels of each *HaGlut4* were analyzed by reverse transcription quantitative polymerase chain reaction. (C) Effect of *HaGlut4* RNAi on body weight. Adult female weights 12, 24, and 48 h after RNAi were measured. (D) Effect of *HaGlut4* RNAi on egg laying. Number of eggs laid by *H. axyridis* injected with ds*Glut4* from 1 to 7 d was counted. The relative transcript levels of each *HaGlut4* gene in ds*Glut4* and ds*GFP* treatments were normalized using the *H. axyridis* 49 ribosomal RNA cycle threshold (Ct) values. *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.

females and may have additional functions in ovarian tissues. Transcript levels of the *HaGlut4* gene were reduced at 24 h and 48 h in RNAi injected *H. axyridis* compared to the ds*GFP*-injected controls (Fig. 2B). Compared to the control group, the body weight of the *H. axyridis* decreased significantly after 48 h interference compared to the control (Fig. 2C). The oviposition of the interfering *HaGlut4* group began to decline on the third day compared with the control group and was significantly decreased at 4 d and 5 d. The number of eggs laid was upregulated in a time-dependent manner at 6 d (Fig. 2D). These results suggest that interference with *HaGlut4* may affect the eggs laid by impeding oocyte maturation in *H. axyridis*.

Effect of silencing HaGlut4 on ovarian morphology in H. axyridis

Adult females, at 1 d after injection with ds*Glut4*, showed normal ovaries with regular, banana-shaped mature oocytes. In contrast to ds*GFP*, on the 3rd, 5th and 7th d after injection of ds*Glut4*, the ds*Glut4* ovaries were obviously abnormal. The ds*GFP*-injected females had globose oocytes in the ovarioles, while the ds*Glut4*-injected females had segmented ovarioles with malformed, round-shaped oocytes. Females injected with ds*Glut4* had no obviously segmented ovarioles in the ovaries, but had abnormal-looking immature oocytes containing large lipid droplets, loosely distributed in the ovarioles. The lateral oviducts of the ds*Glut4* ovaries were milky white, compared with bright yellow oviducts in ds*GFP*-injected females (Fig. 2A). *HaGlut4* affects reproduction by modulating carbohydrates in *H. axyridis*.

To study the possible link between *Glut4* and insect reproduction, we measured the levels of glucose, glycogen and trehalose at 24 and 48 h after ds*Glut4* was injected into the ovary and hemolymph. Relative to *H. axyridis* injected with ds*GFP*, the injected ds*Glut4* into females of *H. axyridis* reduced glucose in ovaries and hemolymph (Figs. 3A,D), and markedly reduced

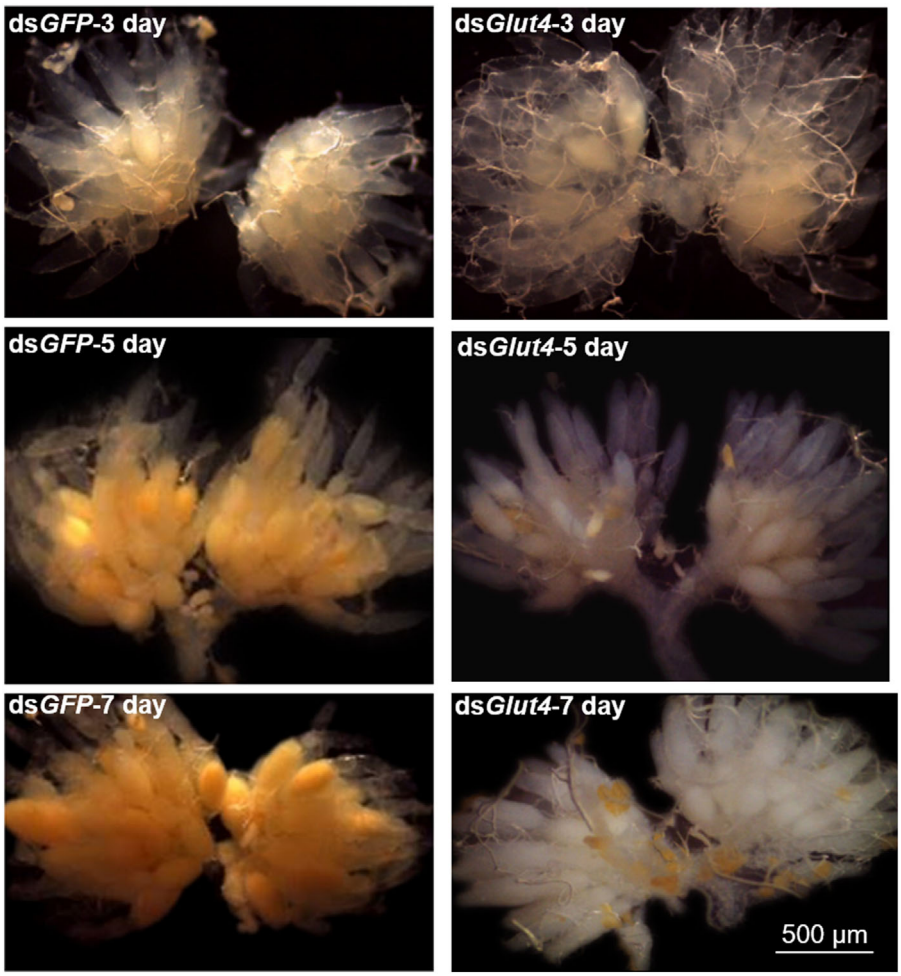

Fig. 2 Effect of silencing *HaGlut4* on ovarian morphology in *Harmonia axyridis*. Ovarian development and oocyte maturation. Ovaries were dissected from adult females on the 1st, 3rd and 5th d after RNA interference injection, and morphologies were observed under a stereomicroscope. Scale bars, 500 μm.

glycogen content in ovaries (Fig. 3B). In addition, the change trend of glycogen in the hemolymph was relatively consistent with that of trehalose content in the ovaries and hemolymph (Figs. 3C,E,F), which shows that it rises first and then falls.

Effects depleting specific HaGlut4 gene on key genes of sugar metabolism gene expression

We measured the levels of trehalose-6-phosphate synthase (*TPS*) and glycogen synthase (*GS*) in the hemolymph at 48 h after ds*Glut4* injection. Relative to *H. axyridis* injected with ds*GFP*, the ds*Glut4* injected adults had slightly reduced *HaGS* (Fig. 4A), but not *HaTPS* (Fig. 4B). These data indicate that *Glut4* affects insect reproduction by regulating carbohydrates.

HaGlut4 affects H. axyridis reproduction by regulating the content of nutrients

To study whether interference with *Glut4* effects key nutrients related to insect reproduction, we simultaneously analyzed ATP, fatty acids, triglycerides and total amino acid contents. In RNAi *HaGlut4* adults, ATP levels were reduced (Fig. 5A) and fatty acids levels were reduced (Fig. 5B); the ds*Glut4* lowered their triglyceride levels (Fig. 5C). In contrast to ds*GFP*, the total amino acid contents of all groups were reduced (Fig. 5D). Therefore, the nutritional status in *H. axyridis* may be regulated

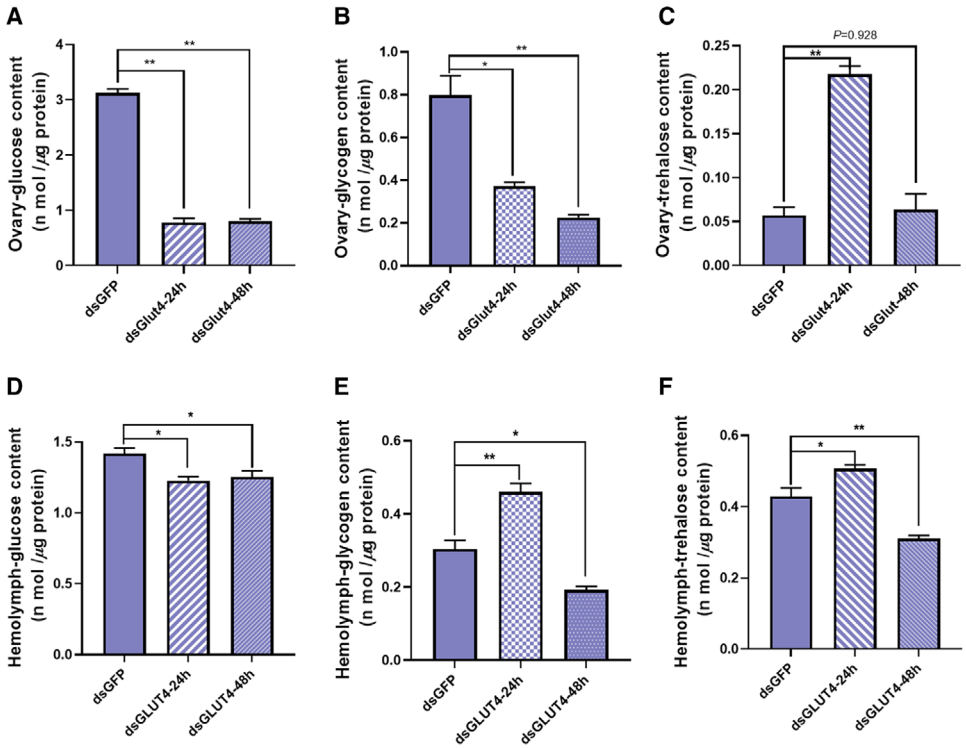

Fig. 3 *HaGlut4* influences reproduction by modulating carbohydrates in *Harmonia axyridis*. Circulating glucose (A), glycogen (B), and trehalose (C) levels in ovary were measured at 24 h and 48 h after ds*Glut4* injection. Circulating glucose (D), glycogen (E), and trehalose (F) levels in hemolymph were measured at 24 h and 48 h ds*Glut4* after injection. $n \geq 3$ each (≥ 7 adults per replicate). *$P <$ 0.05, significant difference; **$P < 0.01$, extremely significant difference.

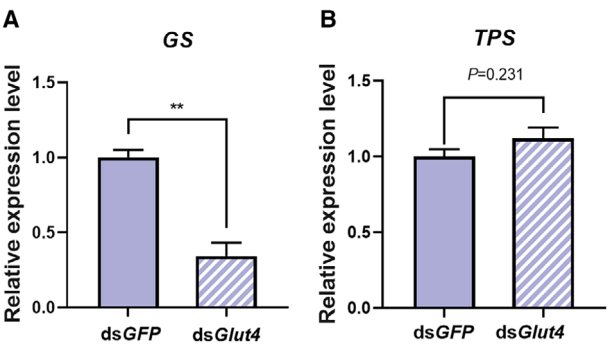

Fig. 4 RNA interference (RNAi) effects depleting specific *HaGlut4* gene on other gene expressions. (A) Glycogen synthase (*GS*) gene expression level. (B) Trehalose-6-phosphate synthase (*TPS*) gene expression level. Relative transcript levels of *HaTPS* and *HaGS* genes in each treatment were determined by reverse transcription quantitative polymerase chain reaction at 48 h after RNAi. ds*HaGFP*-injected samples were used as controls. Three independent biological replicates (mean ± SD; $n = 10$) were conducted. *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.

by *HaGlut4* expression at the messenger RNA (mRNA) level, and *Glut4* may modulate reproduction in response to carbohydrates and nutrients.

Effect of silencing HaGlut4 expression on HaVg, HaVgR and HaInR genes

Transcript levels of the *HaVg* gene in the fat body were reduced at 48 h in RNAi injected *H. axyridis* compared to the ds*GFP*-injected controls (Fig. 6A). To determine how *Glut4* regulates *Vg* deposition, the expression of *VgR*, which is responsible for the uptake of *Vg* into the ovary, was examined in the oocytes of the ds*Glut4* treated adults. qRT-PCR analysis showed that silencing of *HaGlut4* greatly decreased the *HaVgR* expression in the ovary of ds*HaGlut4*-treated adults compared to the controls (Fig. 6B). These results suggest that the suppression of *HaGlut4* in adults inhibits the deposition of *Vg* in the ovary and prevents oocyte maturation. The depletion of *HaGlut4* reduces *HaVg* accumulation, perturbs ovarian development, and arrests oogenesis. Insulin is a

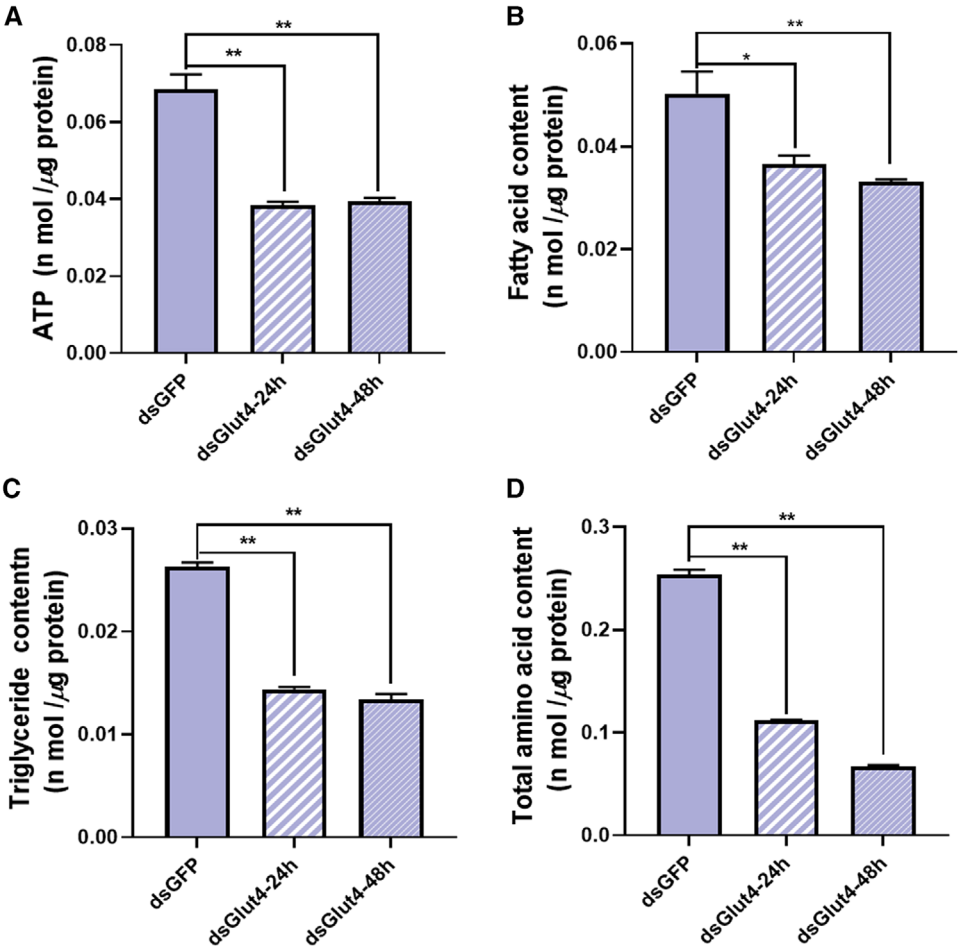

Fig. 5 *HaGlut4* influences insect reproduction by modulating nutrients. (A) Adenosine triphosphate, (B) fatty, (C) triglyceride and (D) total amino acid levels were measured at 24 h and 48 h after ds*Glut4* injection. $n \geq 3$ each (≥ 7 adults per replicate). *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.

key to insect reproduction, and to better understand the mechanism of *HaGlut4* regulation of *HaVg* and *HaVgR*, we determined the expression level of the insulin receptor (InR) gene after interfering with *HaGlut4*. The *HaInR* levels were visibly reduced at 48 h after ds*Glut4* injection (Fig. 6C). These data indicate that *Glut4* can affect insect reproduction by regulating insulin.

Discussion

In vertebrates and invertebrates, reproductive performance is an energy-demanding process, controlled by the complex interactions of diverse signaling pathways (Arrese & Soulages, 2010). Studies in *T. castaneum*, *C. chuxiongica* and *D. melanogaster* showed that a strong correlation of reproduction events and changes in glucose metabolism, and *Glut4* is the key to the glucose metabolism (Fraga *et al*., 2013; Almeida *et al*., 2021; Rong *et al*., 2021). Herein, to understand if *HaGlut4* participates in the regulation of insect reproduction and its mechanism of participation, we performed transcript knockdown assays using ds*Glut4* (Fig. 1). qRT-PCR analysis detected Ha*Glut4* expression throughout development, with the highest levels in females and the lowest levels in larvae, suggesting their functional importance in adult females. The changes in body weight and egg production of female *H. axyridis* after injection of ds*Glut4* further supported their physiological roles in female reproduction.

Based on these observations, we silenced *HaGlut4* expression in *H. axyridis* by RNAi. This resulted in the generation of *H. axyridis* with reduced egg yolk deposition and yolk that was milky white and distributed loosely

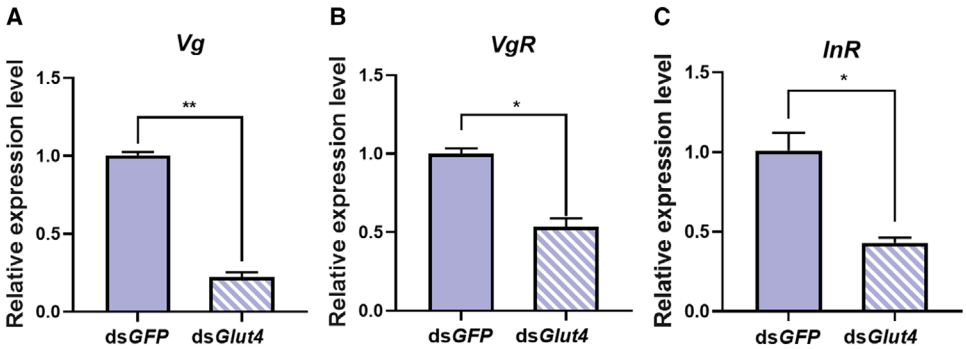

Fig. 6 Effects of RNA interference (RNAi) on *HaVg* and *HaVgR* gene expressions and on ovarian development. (A) Vitellogenin (*Vg*) gene expression level. (B) Vitellogenin receptor (*VgR*) gene expression level. (C) Insulin receptor (*InR*) gene expression level. Relative transcript levels of *HaVg*, *HaVgR* and *HaInR* genes in each treatment were determined by reverse transcription quantitative polymerase chain reaction at 48 h after RNAi. ds*GFP*-injected samples were used as controls. Three independent biological replicates (mean ± SD; $n = 10$) were conducted. *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.

in the ovary (Fig. 2). These results indicate that silencing of *HaGlut4* interfered with ovary development and affected ova development. Glucose metabolism in insects is closely correlated to embryonic stage development. Germ band retraction is a landmark regarding both glucose and glycogen metabolism (Santos *et al*., 2008). The time course of the accumulation of both glycogen and glucose follows the accumulation of yolk proteins (Arrese & Soulages, 2010). Our results showed that injection of ds*Glut4* into *H. axyridis* females markedly reduced glucose and glycogen content in the ovaries (Fig. 3). Under normal circumstances, the increase of glucose content during ovarian development can be explained by glycogenolysis. Glycogen is produced in the process of glucose decomposition and is consumed by other metabolic pathways. In addition, the change of glycogen in the hemolymph is relatively consistent with the level of trehalose, which rises first and then falls. This may be because when oocytes synthesize and accumulate glycogen, trehalose, the main sugar in insect hemolymph, is the main source of this synthesis (Li *et al*., 2019). Thus, *Glut4* could be involved in the transport of sugar into the oocytes during oogenesis, which leads to sugar metabolism disorder and affects ovary development.

Metabolic tracing studies revealed that *TPS* can limit glucose aggregation by interacting with glucose transport. *HaTPS* had no significant change in this study after being interfered by *HaGlut4*, which indicates that the utilization of *HaTPS* is reduced after the function of *HaGlut4* is blocked (Fig. 4). However, the expression level of *GS* decreased significantly, and *GS* is the key enzyme of glycogen synthesis (Li *et al*., 2019). Because glucose supply was not available after interference of *Glut4*, glycogen was the main form of carbohydrate storage in the ovary (Arrese *et al*., 2010). It is used to compensate for the deficiency of carbohydrate utilization in order to maintain energy metabolism. This indicates that *Glut4* is conservatively involved in glycogen metabolism during arthropod embryogenesis.

Energy metabolism is the process of generating energy (i.e., ATP) from nutrients. Cells require energy for growth and maintenance (Moriyama *et al*., 2016). During cell differentiation, *Glut4* is directly associated with regulation of glucose uptake (Li *et al*., 2016). The ATP of organisms is closely connected with glucose uptake. Therefore, according to the change of glucose metabolism after the above interference with *Glut4*, we detected the change of energy level, and the results showed that *HaGlut4* significantly affected the ATP level (Fig. 5A). Similarly, studies of *Spodoptera litura* and *Drosophila* confirmed that inhibition of ATP synthesis led to insufficient energy reserves and inhibition of fecundity and adult eclosion rate (Shi *et al*., 2018; Yu *et al*., 2019). *HaGlut4* was significantly inhibited, resulting in insufficient energy reserves and inhibition of oviposition and hatching ability of adults.

Lipids also are major sources of energy that support embryogenesis (Fruttero *et al*., 2017a; Gondim *et al*., 2018). Lipids are mostly triglycerides that are stored in lipid droplets in oocytes and fat body cells and serve as the main energy source for oocyte maturation and embryonic development (Yamasaki & Yasui, 2003; Jarc & Petan, 2020). We measured the triglyceride content of adult female *H. axyridis*. Knockdown of *HaGlut4* significantly reduced triglyceride levels in the adult females (Fig. 5B). These results indicated that lipid metabolism was adversely affected in the fat bodies, thereby disrupting the provision of triglycerides to the ovaries as well as the uptake and utilization of triglycerides in the oocytes.

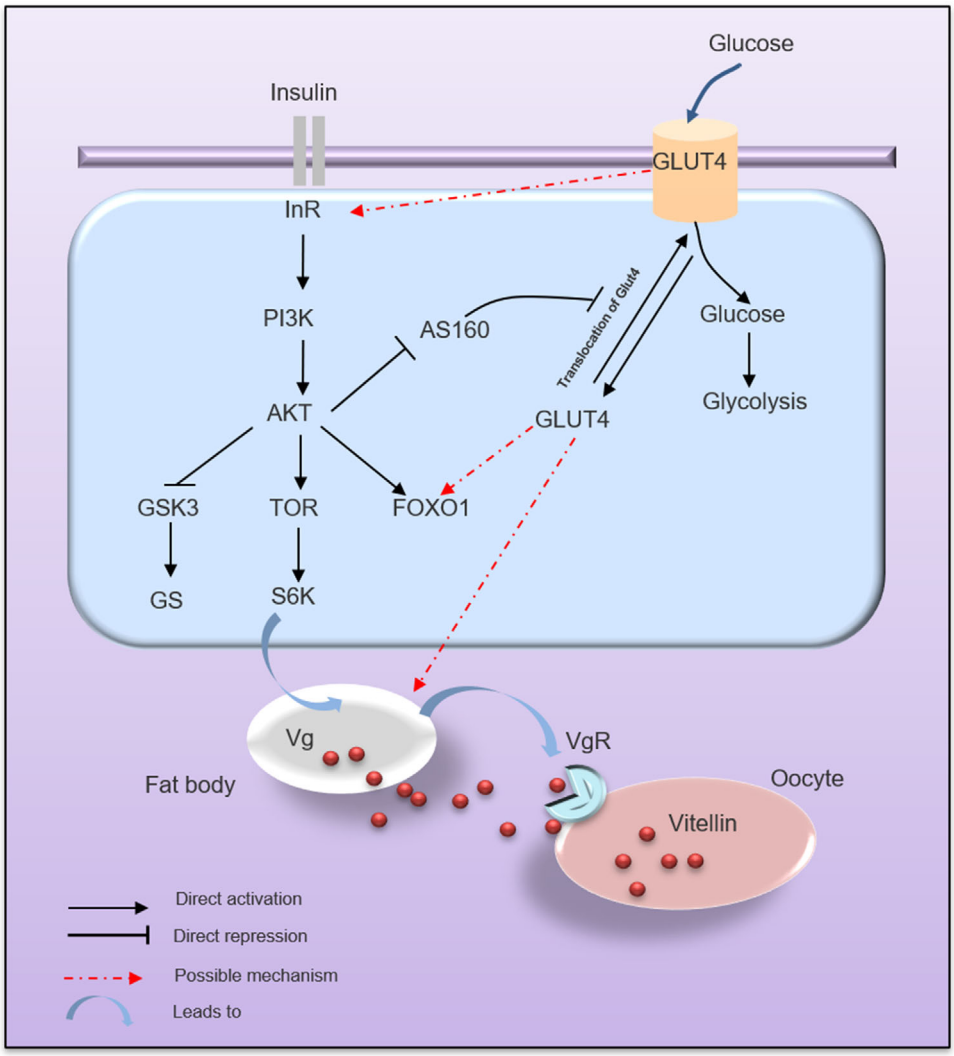

Fig. 7 Schematic diagram of *HaGlut4* affecting female reproduction in *Harmonia axyridis*. Knockdown of *HaGlut4* affects *HaVg* synthesis in the fat body and utilization in the developing oocytes and disrupts lipid metabolism in the fat body and oocytes. This leads to failure of oocyte maturation and development. Drawing idea reference (Zhu *et al*., 2020).

Adipose tissues function to take up fatty acids via the action of lipoprotein lipase, and they release fatty acids via hydrolysis (lipolysis) of stored intracellular triacylglycerol (Cerk *et al*., 2018). We also studied the reasons for triglyceride metabolic disorder by examining fatty acid contents in adult females following RNAi (Fig. 5C). Lipogenesis can occur in the presence of glucose, which acts as a carbon source for fatty acid synthesis and facilitates fatty acid esterification for lipid storage (Krycer *et al*., 2020). In this study, fatty acid content decreased significantly because the function of *HaGlut4* was blocked, resulting in insufficient supply of the carbon source. During oogenesis, lipids must be stored in developing oocytes because they are the main energy source to maintain embryo development (Fruttero *et al*., 2017b).

Nutrients from the hemolymph through the trophic cords transfer amino acids (AA), along with other components, to immature oocytes in order to maintain them in an optimal physiological condition (Calejman *et al*., 2020). For example, increasing the level of methionine can increase insect fecundity. And there was decrease in AA levels in this experiment after interference with *HaGlut4* (Fig. 5D). Methionine is the most limiting AA (Hoedjes *et al*., 2017), which might explain why fecundity is particularly sensitive to this specific AA.

Vg, along with carbohydrates and lipids, constitutes the main nutrient reserve to ensure the success of embryonic development (Yamasaki & Yasui, 2003; Ziegler & Antwerpen, 2006). Developing oocytes in oviparous insects accumulate large amounts of yolk to ensure embryo development (Tufail & Takeda, 2008). The uptake of *Vg* by developing oocytes during egg maturation is essential for successful female reproduction (Roy *et al*., 2018). *Vg* is involved in oocyte maturation and development and is a critical factor for insect reproduction (Zhang *et al*., 2017). Therefore, to understand the reasons for the abnormal ovarian dysplasia, we examined the changes in the levels of *HaVg* genes following *HaGlut4* knockdown and found that this significantly decreased the levels of *HaVg* in adult *H. axyridis* (Fig. 6A). Similarly, in a study of *Cimex lectularius*, *Vg* knockdown resulted in ovarian atrophy and reduced egg production (Moriyama *et al*., 2016). These data demonstrated that *Vg* expression was affected by knockdown of *HaGlut4*. The *Vg* gene is unique in oviparous animals and plays a significant role in yolk protein synthesis during embryo formation. *Vg* is also essential for insect reproduction. When the *Vg* content is reduced, the embryo fails to develop normally (Fruttero *et al*., 2017a).

To determine if knockdown of *HaGlut4* affects other ovary- or oocyte-specific gene expressions, we examined the transcript level variations of *HaVgR* gene by knockdown of individual *HaGlut4* expression in female ovaries. Knockdown of *HaGlut4* significantly reduced *HaVgR* transcript levels (Fig. 6B), indicating that knockdown of *Glut4* can downregulate *Vg* and *VgR* expression, which might impede the digestion and absorption of nutrients and energy in the developing oocytes, and thus inhibit oocyte development.

The regulation of glucose level in insects mainly depends on insulin. One of the key mechanisms of insulin is that it increases glucose uptake by fat and muscle cells by regulating the transport of glucose-carrying type 4 transport vesicles (Zhang *et al*., 2019). In this experiment, the *HaInR* gene decreased significantly after interfering with *HaGlut4*, and this indicated that *HaGlut4* participates in the insulin signaling pathway to regulate the reproduction of *H. axyridis* (Fig. 6C). In addition, the insulin signaling pathway reveals that phosphorylation events initiated by insulin receptors regulate the key *Glut4* transporter (Krycer *et al*., 2020). These findings indicate that *HaGlut4* is required for female reproductive processes in *H. axyridis*.

Further, we speculated that *HaGlut4* might regulate the expression of *HaVg* and *HaVgR*, probably through *HaInR*, metabolism-related enzymes and the transcription factors *FOXO1*, or might directly act on these genes (Fig. 7), to regulate the supply of nutrition, yolk protein up-taking and the metabolism of oocyte development.

Conclusions

Overall, the obtained results indicate that the lack of *HaGlut4* impairs ovarian development and oocyte maturation, resulting in decreased fecundity. These changes indicate that *HaGlut4* plays a key role in the ovarian development of *H. axyridis*. Furthermore, these findings have revealed a possible bridge connecting *HaGlut4* to the insulin signaling pathway. The present results contribute to a comprehensive insight into reproduction in *H. axyridis* and provide theoretical basis for the reproduction of natural enemy insects.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (Grant No. 2017YFD0201000) and the Hangzhou Science and Technology Development Program of China (Grant No. 20190101A01). We gratefully thank anonymous reviewers for their valuable comments on the manuscript.

Disclosure

The authors declare no competing or financial interests.

Author Contributions

YL conducted the molecular laboratory work, participated in data analysis, carried out sequence alignments, completed the statistical analyses and drafted the manuscript; BT, SW and FL participated in the design of the study and critically revised the manuscript; SSW and SGW carried out the molecular laboratory work. All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

References

Arrese, E.L. and Soulages, J.L. (2010) Insect fat body: Energy, metabolism, and regulation. *Annual Review of Entomology*, 55, 207–225.

Almeida-Oliveira, F., Tuthill, B.F., Gondim, K.C., Majerowicz, D. and Musselman, L.P. (2021) *Dhnf4* regulates lipid homeostasis and oogenesis in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, 133, 103569.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M. *et al*. (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, 611–622.

Bifano, T.D., Alegria, T. and Terra, W.R. (2010) Transporters involved in glucose and water absorption in the *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) anterior midgut. *Comparative Biochemistry & Physiology Part B Biochemistry & Molecular Biology*, 157, 1–9.

Cabrera-Cruz, H., Oróstica, L., Plaza-Parrochia, F., Torres-Pinto, I. and Vega, M. (2019) The insulin sensitizer mechanism of myo-inositol is associated to ampk activation and glut-4 expression in human endometrial cells exposed to a pcos environment. *AJP Endocrinology and Metabolism*, 318, 2.

Cerk, I.K., Wechselberger, L. and Oberer, M. (2018) Adipose triglyceride lipase regulation: An overview. *Current Protein and Peptide Science*, 19, 221–223.

Chakraborty, C., Bandyopadhyay, S., Maulik, U. and Agoramoorthy, G. (2013) Topology mapping of insulin–regulated glucose transporter glut4 using computational biology. *Cell Biochemistry & Biophysics*, 67, 1261–1274.

Calejman, C.M., Trefely, S., Entwisle, S.W., Luciano, A.K. and Guertin, D.A. (2020) mTORC2-AKT signaling to ATP-citrate lyase drives brown adipogenesis and de novo lipogenesis. *Nature Communications*, 11, 575.

Deng, D., Sun, P., Yan, C., Ke, M., Jiang, X., Xiong, L. *et al*. (2015) Molecular basis of ligand recognition and transport by glucose transporters. *Nature*, 526, 391–396.

Du, W. and Zeng, F. (2016) Identification of development-related genes in the ovaries of adult *Harmonia axyridis* (Pallas) lady beetles using a time-series analysis by RNA-seq. *Scientific Report*, 6, 39109.

Fraga, A., Ribeiro, L., Lobato, M., Santos, V., Silva, J.R. and Gomes, H. (2013) Glycogen and glucose metabolism are essential for early embryonic development of the red flour beetle *Tribolium castaneum*. *PLoS ONE*, 8, e65125.

Fruttero, L.L., Leyria, J. and Canavoso, L.E. (2017a) Lipids in insect oocytes: From the storage pathways to their multiple functions. *Results and Problems in Cell Differentiation*, 63,403–434.

Fruttero, L.L., Leyria, J., Ramos, F.O., Stariolo, R., Settembrini, B.P. and Canavoso, L.E. (2017b) The process of lipid storage in insect oocytes: The involvement of β-chain of atp synthase in lipophorin-mediated lipid transfer in the chagas' disease vector *Panstrongylus megistus* (Hemiptera: Reduviidae). *Journal of Insect Physiology*, 96, 82–92.

Fujimoto, T., Sugimoto, K., Takahashi, T., Yasunobe Xie, K., Tanaka, M., Ohnishi, Y.Y. *et al*. (2019) Overexpression of interleukin-15 exhibits improved glucose tolerance and promotes glut4 translocation via amp-activated protein kinase pathway in skeletal muscle. *Biochemical and Biophysical Research Communications*, 509, 994–1000.

Gondim, K.C., Atella, G.C., Pontes, E.G. and David, M. (2018) Lipid metabolism in insect disease vectors. *Lipid Metabolism in Insect Disease Vectors*, 101, 108–123.

Hoedjes, K.M., Rodrigues, M.A. and Flatt, T. (2017) Amino acid modulation of lifespan and reproduction in *Drosophila*. *Current Opinion in Insect Science*, 23, 118–122.

Jarc, E. and Petan, T. (2020) A twist of FATe: Lipid droplets and inflammatory lipid mediators. *Biochimie*, 169, 69–87.

Keyong, D., Murakami, S., Sun, Y., Kilpatrick, C.L. and Lusche, B. (2017) Dhhc7 palmitoylates glucose transporter 4 (glut4) and regulates glut4 membrane translocation. *Journal of Biological Chemistry*, 292, 2979–2991.

Klip, A., Mcgraw, T.E. and James, D.E. (2019) Thirty sweet years of glut4. *Journal of Biological Chemistry*, 294, 11369–11381.

Krycer, J.R., Quek, L.E., Francis, D., Zadoorian, A., Weiss, F.C. and Cooke, K.C. (2020) Insulin signaling requires glucose to promote lipid anabolism in adipocytes. *Journal of Biological Chemistry*, 295, 13250–13266.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods (San Diego, Calif.)*, 25, 402–408.

Li, Z., Frey, J.L., William, W.G., Marie-Claude, F., Wolfgang, M.J., Kim, J.K. *et al*. (2016) Glucose transporter-4 facilitates insulin-stimulated glucose uptake in osteoblasts. *Endocrinology*, 157, 4094–4103.

Li, Y., Chen, X., Wang, S.S., Pan, B.Y., Wang, S.G., Wang, S., et al. (2019) Evaluation of the expression and function of the TRE2-like and TRE2 genes in ecdysis of *Harmonia axyridis*. *Frontiers in Physiology*, 10, 1371.

Long, Y., Wang, Y.C., Yuan, D.Z., Dai, X.H. and Yue, L.M. (2021) *Glut4* in mouse endometrial epithelium: Roles in embryonic development and implantation. *Frontiers in Physiology*, 12, 674924.

Moriyama, M., Hosokawa, T., Tanahashi, M., Nikoh, N. and Fukatsu, T. (2016) Suppression of bedbug's reproduction by rna interference of vitellogenin. *PLoS ONE*, 11, e0153984.

Roy, S., Saha, T.T., Zou, Z. and Raikhel, A.S. (2018) Regulatory pathways controlling insect reproduction. *Annual Review of Entomology*, 63, 489–511.

Rong, X.A., Yhc, B., Jfx, A., Txz, C., Yhl, D. and Jyz, A. (2021) Metabolic dynamics across prolonged diapause development in larvae of the sawfly, *Cephalcia chuxiongica* (Hymenoptera: Pamphiliidae). *Journal of Asia-Pacific Entomology*, 24, 1–6.

Scheepers, A., Joost, H. and Schurmann, A. (2004) The glucose transporter families sglt and glut: Molecular basis of normal and aberrant function. *Journal of Parenteral and Enteral Nutrition*, 28, 364–371.

Santos, R., Mariano, A.C., Rosas-Oliveira, R., Pascarelli, B., Machado, E.A., Meyer-Fernandes, J.R. et al. (2008) Carbohydrate accumulation and utilization by oocytes of *Rhodnius prolixus*. *Archives of Insect Biochemistry and Physiology*, 67, 55–62.

Stanirowski, P.J., Szukiewicz, D., Pazura-Turowska, M., Sawicki, W. and Cendrowski, K. (2017) Expression of glucose transporter proteins in human diabetic placenta. *Canadian Journal of Diabetes*, 42, 209–217.

Shi, S., Zuo, H., Lu, G., Xin, Y. and Zhong, G. (2018) Silencing of rieske iron-sulfur protein impacts upon the development and reproduction of *Spodoptera exigua* by regulating ATP synthesis. *Frontiers in Physiology*, 9, 575.

Stanirowski, P.J., Szukiewicz, D., Pyzlak, M., Abdalla, N., Sawicki, W. and Cendrowski, K. (2019) Analysis of correlations between the placental expression of glucose transporters *Glut*-1, *Glut*-4 and *Glut*-9 and selected maternal and fetal parameters in pregnancies complicated by diabetes mellitus. *Journal of Maternal-Fetal & Neonatal Medicine*, 32, 650–659.

Sun, Y.X., Hao, Y.N., Liu, C.Z. and Wang, S.S. (2019) Investigating reproductive success of the ladybird beetle *Harmonia axyridis* from the perspective of micropyle variation. *Scientific Reports*, 9, 12742.

Tufail, M. and Takeda, M. (2008) Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology*, 54, 1447–1458.

Vital, W., Rezende, G.L., Abreu, L., Moraes, J., Lemos, F.J., Vaz, I.S. et al. (2010) Germ band retraction as a landmark in glucose metabolism during *Aedes aegypti* embryogenesis. *BMC Developmental Biology*, 25, 10–25.

Wang, T., Wang, J., Hu, X., Huang, X. and Chen, G.X. (2020) Current understanding of glucose transporter 4 expression and functional mechanisms. *World Journal of Biological Chemistry*, 11, 76–98.

Xiang, S., Xia, M.F., Song, J.Y., Liu, D.Q. and Lian, F. (2021) Effect of electro–acupuncture on expression of irs-1/pi3k/glut4 pathway in ovarian granulosa cells of infertile patients with polycystic ovary syndrome-insulin resistance of phlegm–dampness syndrome. *Chinese Journal of Integrative Medicine*, 27, 330–335.

Yamasaki, M. and Yasui, Y.H. (2003) Genetic basis of ovicidal response to whitebacked planthopper (*Sogatella furcifera* Horváth) in rice (O*ryza sativa* L.). *Molecular Breeding*, 12, 133–143.

Yuliya, S., Morgan, D., Claudia, C., Reilly, S.M., Chao-Wei, H., Xia, W. *et al*. (2018) Rala controls glucose homeostasis by regulating glucose uptake in brown fat. *Proceedings of the National Academy of Sciences USA*, 115, 7819–7824.

Yang, Y., Jiang, H., Xiao, L. and Yang, X. (2018a) MicroRNA-33b-5p is overexpressed and inhibits glut4 by targeting hmga2 in polycystic ovarian syndrome: An in vivo and in vitro study. *Oncology Reports*, 39, 3073–3085.

Yang, X., Pan, H., Yuan, L. and Zhou, X. (2018b) Reference gene selection for RT-qPCR analysis in *Harmonia axyridis*, a global invasive lady beetle. *Scientific Reports*, 8, 2689.

Yamashita, Y., Kishida, H., Nakagawa, K., Yoshioka, Y. and Ashida, H. (2018) Liquorice flavonoid oil suppresses hyperglycaemia accompanied by skeletal muscle myocellular Glut4 recruitment to the plasma membrane in kk–a y mice. *International Journal of Food Sciences and Nutrition*, 70, 1–9.

Yu, J., Chen, B., Zheng, B., Qiao, C., Chen, X. and Yan, Y. (2019) ATP synthase is required for male fertility and germ cell maturation in *Drosophila testes*. *Molecular Medicine Reports*, 19, 1561–1570.

Ziegler, R. and Antwerpen, R.V. (2006) Lipid uptake by insect oocytes. *Insect Biochemistry and Molecular Biology*, 36, 264–272.

Zhang, T., Zhang, G., Zeng, F., Mao, J., Liang, H. and Liu, F. (2017) Molecular cloning of the vitellogenin gene and the effects of vitellogenin protein expression on the physiology of *Harmonia axyridis* (Coleoptera: Coccinellidae). *Scientific Reports*, 7, 13926.

Zhang, N., Liu, X., Zhuang, L., Liu, X. and Fang, J. (2019) Berberine decreases insulin resistance in a pcos rats by improving glut4: Dual regulation of the PI3K/AKT and MAPK pathways. *Regulatory Toxicology and Pharmacology*, 110, 104544.

Zhu, S., Liu, F., Zeng, H., Li, N. and Li, S. (2020) Insulin/IGF signaling and TORC1 promote vitellogenesis via inducing juvenile hormone biosynthesis in the American cockroach. *Development (Cambridge, England)*, 147.

Manuscript received July 5, 2021
Final version received August 21, 2021
Accepted August 30, 2021

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Bioinformatics analysis of *Glut4* and assembly chaperone genes.