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

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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...
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; Staniorski 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; Staniorski 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; Staniorski 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.
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### Table 1

| Gene   | Forward primers (5′–3′)                        | Reverse primers (5′–3′)                        |
|--------|-----------------------------------------------|-----------------------------------------------|
| dsGFP  | CCTGAAGTTCATCTGCACCA                         | ACAAGCAGAAGAAGCGCATCA                         |
| dsHaGlut4 | GCAGCATTACGACTTGGAG                        | TTGCCGATTACTCGAAGGA                         |
| HaGlut4 | ACTGTGTTCCTGTGGCCTTAT                        | ACGTCGCTATCTCGAACCTTCT                       |
| Rp49   | GGGATGCCTATGGAACAAACTTCGT                    | TACGATTTTGCATCAACAGT                         |
| HaVg   | GCCACAGGTCCCTGGTTCTTT                        | GCTGCTTACAGGATTCTTCA                         |
| HaVgR  | TGTAGGGCCAGAAGCAGTGTCTTCT                    | TGGGATGACAGGGAATAAAA                        |
| HaGS   | CCTTTAGGATCGGTCTTCT                         | CACCACCCATCCACAGT                            |
| HaTPS  | GACCTCGAGGAAGCCATACC                        | AAAGTTCCATTACAGCCACCA                        |
| HaInR  | CGATAACTCGGTTTCACT                         | GCTGATACATCCAGGTCC                          |
| 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™ 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 dsGFP 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−ΔΔCt 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 dsGlut4 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 amyloligosidase (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 dsGlut4 and dsGFP-injected females on the 3rd day after emergence. Following washing three times in 1 X 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 dsGlut4 or dsGFP 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...
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Fig. 1. Effects of silencing HaGlut4 on the development and reproduction of Harmonia axyridis. A total of 250 ng of dsGlut4 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 dsGlut4 from 1 to 7 d was counted. The relative transcript levels of each HaGlut4 gene in dsGlut4 and dsGFP 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 dsGFP-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 up-regulated 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 dsGlut4, showed normal ovaries with regular, banana-shaped mature oocytes. In contrast to dsGFP, on the 3rd, 5th and 7th d after injection of Glut4, the dsGlut4 ovaries were obviously abnormal. The dsGFP-injected females had globose oocytes in the ovarioles, while the dsGlut4-injected females had segmented ovarioles with malformed, round-shaped oocytes. Females injected with dsGlut4 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 dsGlut4 ovaries were milky white, compared with bright yellow oviducts in dsGFP-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 dsGlut4 was injected into the ovary and hemolymph. Relative to H. axyridis injected with dsGFP, the injected dsGlut4 into females of H. axyridis reduced glucose in ovaries and hemolymph (Figs. 3A,D), and markedly reduced

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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 dsGlut4 injection. Relative to H. axyridis injected with dsGFP, the dsGlut4 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 dsGlut4 lowered their triglyceride levels (Fig. 5C). In contrast to dsGFP, the total amino acid contents of all groups were reduced (Fig. 5D). Therefore, the nutritional status in H. axyridis may be regulated
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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 dsGlut4 injection. Circulating glucose (D), glycogen (E), and trehalose (F) levels in hemolymph were measured at 24 h and 48 h dsGlut4 after injection. \( n \geq 3 \) each (\( \geq 7 \) adults per replicate). *\( P < 0.05 \), significant difference; **\( P < 0.01 \), extremely significant difference.

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

Transcript levels of the HaVg gene in the fat body were reduced at 48 h in RNAi injected H. axyridis compared to the dsGFP-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 dsGlut4 treated adults. qRT-PCR analysis showed that silencing of HaGlut4 greatly decreased the HaVgR expression in the ovary of dsHaGlut4-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. 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. dsHaGFP-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.
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 dsGlut4 injection. $n \geq 3$ each ($\geq 7$ adults per replicate). *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.

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 dsGlut4 (Fig. 1). qRT-PCR analysis detected HaGlut4 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 dsGlut4 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.
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 dsGlut4 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.
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**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 *HalnR* 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 *HalnR*, 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 uptake-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. 2017YFDF0201000) 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 anticipated in data analysis, carried out sequence alignments. All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

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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.