Adipokinetic hormone signaling mediates the fecundity of *Diaphorina citri* infected by '*Candidatus* Liberibacter asiaticus'

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Abstract

Diaphorina citri is the primary vector of the bacterium, '*Candidatus* Liberibacter asiaticus' (*C*Las) associated with the severe Asian form of huanglongbing. *C*Las-positive *D. citri* are more fecund than their *C*Las-negative counterparts and require extra energy expenditure. Therefore, understanding the molecular mechanisms linking metabolism and reproduction is of particular importance. In this present study, we found adipokinetic hormone (*DcAKH*) and its receptor (*DcAKHR*) were essential for increased lipid metabolism and fecundity in response to *C*Las infection in *D. citri.* Knockdown of *DcAKH* and *DcAKHR* not only resulted in triacylglycerol accumulation and a decline of glycogen, but also significantly decreased fecundity and *C*Las titer in ovaries. Combined *in vivo* and *in vitro* experiments showed that miR-34 suppresses *DcAKHR* expression by binding to its 3' untranslated region, whilst overexpression of miR-34 resulted in a decline of *DcAKHR* expression and *C*Las titer in ovaries and caused defects that mimicked *DcAKHR* knockdown phenotypes. In addition, knockdown of *DcAKH* and *DcAKHR* significantly reduced juvenile hormone (JH) titer and JH signaling pathway genes in fat bodies and ovaries, including the JH receptor, *methoprene-tolerant* (*DcMet*), and the transcription factor, *Krüppel homolog 1 (DcKr-h1)*, that acts downstream of it, as well as the egg development related genes *vitellogenin 1-like* (*DcVg-1-like*), *vitellogenin A1-like* (*DcVg-A1-like*) and the vitellogenin receptor (*DcVgR*). As a result, *C*Las hijacks AKH/AKHR-miR-34-JH signaling to improve *D. citri* lipid metabolism and fecundity, while simultaneously increasing the replication of *C*Las, suggesting a mutualistic interaction in *D. citri* ovaries with *C*Las.

Key words: huanglongbing; *Diaphorina citri*; *DcAKH*/*DcAKHR*; miR-34; mutualism

Introduction

The majority of devastating plant pathogens, including viruses and bacteria, are transmitted by insect vectors [1]. In recent decades, the interactions between vectors and pathogens have been widely studied [1-4]. However, studies on the mechanisms underlying these interactions have mainly focused on vector-virus or vector-fungus interactions; less research has been performed on vector-bacteria interactions. Knowledge of the relationships between vectors and pathogens is of great significance for understanding the epidemiology of plant pathogens, and this knowledge may help develop new strategies for controlling both vectors and pathogens. The severe Asian form of huanglongbing (HLB) is currently the most destructive citrus disease in Asia and the Americas where it is associated with the phloem-restricted, Gram-negative, α–Proteobacteria '*Candidatus* Liberibacter asiaticus' (*C*Las) and transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) [5-9]. However, since *C*Las has not been cultured *in vitro,* Koch's postulates have not been proven. Control of *D. citri* is the most effective way to prevent HLB epidemics [6,7] but experience in Asia [10] and the United States of America [11,12], and recent press reports from Brazil, indicate that it is impossible to suppress psyllid populations with insecticides to levels that prevent spread of *C*Las.

Previous studies have reported that infection with *C*Las significantly increases the fecundity of *D. citri* [13-15]. Reproduction is a costly process in terms of energy usage in the adult life of female insects [16], and there is an inevitable trade-off between lipid storage and use, because animals mobilize their lipid reserves during reproduction. How *D. citri* maintains a balance between lipid metabolism and increased fecundity after infection with *C*Las is not known.

In insects, energy mobilization is under control of adipokinetic hormone (AKH), which was the first insect neurohormone to be identified; it was shown to stimulate lipid mobilization for locomotor activity in locusts [17]. To date, more than 80 different forms of AKH have been identified or predicted in arthropod species due to similar structural characteristics of the enzyme [18]. The mechanisms of synthesis and biological activity of AKH have also been investigated. Insect store lipids in the form of triacylglycerides (TAG) and as carbohydrates in the form of glycogen in their fat bodies [19]. Under conditions of energy demand, AKHs are

67 synthesized in the corpora cardiaca, released into the hemolymph and bind to receptors
68 (AKHRs) located on the plasma membrane of fat body cells. AKHR binding and activation
69 triggers the release of energy-rich substrates after which various cellular signaling pathway
70 components come into play [20]. AKHRs are a class of G protein-coupled receptors and were
71 first identified in *Drosophila melanogaster* [21] and *Bombyx mori* [22]; subsequently, they were
72 identified in a large number of insect species [23-26].

73 Recent studies have shown that AKH/AKHR signaling plays a regulatory role during
74 female reproduction in insects. For example, depletion of AKHR in *Bactrocera dorsalis* resulted
75 in TAG accumulation, decreased sexual courtship activity, and fecundity [27]. AKHR
76 knockdown in *Nilaparvata lugens* interferes with trehalose homeostasis and vitellogenin
77 uptake by oocytes, which causes delayed oocyte development and reduced fecundity [28].
78 AKH/AKHR signaling also regulates vitellogenesis and egg development in *Locusta migratoria*
79 via triacylglycerol mobilization and trehalose homeostasis [29]. As outlined above, the
80 fecundity of *D. citri* is increased by *C*Las. Whether AKH/AKHR signaling participates in this
81 increase in fecundity due to infection via alterations in lipid metabolism is not currently known.

82 Although numerous studies have been conducted on the AKH/AKHR signaling pathway,
83 miRNA-mediated regulation of the AKH signaling pathway at the post-transcriptional level has
84 been little studied. MicroRNAs (miRNAs) are small, non-coding RNAs containing
85 approximately 22 nucleotides that act mostly on the 3' untranslated region (UTR) of target
86 mRNAs, resulting in translation repression or mRNA degradation [30]. miRNAs play important
87 roles in regulating cellular events at the post-transcriptional level during biological processes
88 [30,31,33]. For example, in term of host-virus interactions, miR-8 and miR-429 target *Broad*
89 *isoform Z2*, the gene involved in the hormonal regulation of single nucleopolyhedrovirus
90 (HaSNPV)-mediated climbing behavior in *Helicoverpa armigera* [33]. In relation to
91 reproduction, in *Aedes aegypti*, miR-275 is essential for egg development [34], miR-309 for
92 ovarian development [35] and miR-8 for other reproductive processes [36]. As for lipid
93 metabolism, knockdown of miR-277 in *A. aegypti* activates insulin/FOXO signaling by
94 increasing the expression of ilp7 and ilp8, which reduces TAG levels and inhibits ovarian
95 development [37]. Nevertheless, the functions of the miRNAs in insect-pathogen interactions
96 need further investigation. To date, there are no reports that miRNAs participate in *D.*

97 *citri*-*C*Las interactions or are hijacked by *C*Las to affect lipid metabolism and the fecundity of *D.*
98 *citri*. As a consequence, in this study, we used the *D. citri*-*C*Las system as a model to study the
99 molecular events associated with AKH/AKHR signaling that affect lipid metabolism and
100 increase fecundity of *D. citri* induced by *C*Las. Our study contributes to our understanding of
101 the interactions between vectors and pathogens and may also provide new ways for
102 controlling *D. citri* and HLB.
103

Results

Effects of *C*Las infection on the reproduction and metabolism of adult *D. citri*.

Total TAG and glycogen levels in fat bodies of *C*Las-negative and *C*Las-positive psyllids were determined. After infection with *C*Las, the TAG and glycogen levels of *C*Las-positive psyllids significantly increased compared with *C*Las-negative individuals (Figures 1A-B). Since TAG is mainly stored in fat bodies as lipid droplets, we next evaluated the changes in the lipid droplet size using Nile red staining. As shown in Figure 1C, the size of lipid droplets in the *C*Las-positive group was significantly larger than those of the *C*Las-negative group. In order to evaluate the effects of *C*Las on the reproduction of *D. citri*, the preoviposition period, oviposition period and fecundity of both *C*Las-negative and *C*Las-positive psyllids were examined. After infection with *C*Las, the preoviposition period was significantly shortened, the oviposition period was extended, and the fecundity was markedly increased (Figures 1D-E).

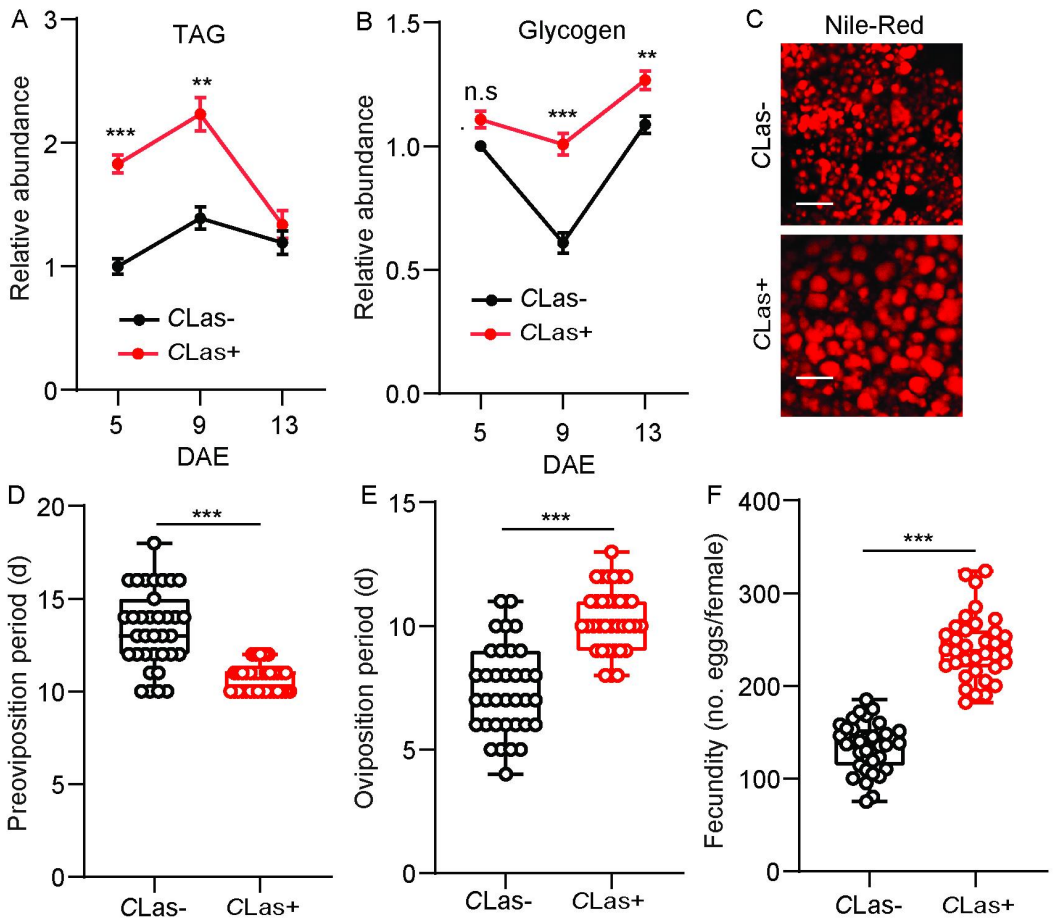

Figure 1. Effects of *C*Las on lipid metabolism and reproductive behavior of *D. citri*.

(A) Comparison of TAG levels in the fat bodies of *C*Las-positive (*C*Las+) and *C*Las-negative

119 (*C*Las-) females 5, 9, 13 days after emergence (DAE). (B) Comparison of glycogen levels in
120 the fat bodies of *C*Las+ and *C*Las- females 5, 9, 13 DAE. (C) Lipid droplets in fat bodies
121 dissected from *C*Las+ and *C*Las- females 9 DAE stained with Nile red. Scale bar = 40 μm.
122 (D-F) Comparison of the preoviposition period, oviposition period and the fecundity of *C*Las+
123 and *C*Las- adults. Data are shown as means ± SEM. The significant differences between
124 *C*Las-positive and *C*Las-negative psyllids were determined using Student's *t*-tests (**$P < 0.01$,
125 ***$P < 0.001$).
126
127 ***DcAKH* is involved in changes in metabolism and fecundity in *D. citri* mediated by *C*Las**
128 Using our transcriptome database, cDNA sequences of *DcAKH* were identified and cloned.
129 The cDNA sequence of *DcAKH* is 225 bp in length, encodes a deduced polypeptide of 74
130 amino acid residues and has the conserved structural characteristics of a neuropeptide. The
131 conserved structure was "signal peptide + mature peptide + related peptide", and the mature
132 peptide sequence of *Dc*AKH is "QVNFSPNW" (Figure S1A). A phylogenetic analysis was
133 conducted to evaluate the association of *Dc*AKH with other insect AKHs; *Dc*AKH was most
134 closely related to the AKHs of three hemipteran species, *Nilaparvata lugens*, *Sitobion avenae*
135 and *Acyrthosiphon pisum* (Figure S1B).
136 Tissue expression profiles showed that *Dc*AKH was expressed at the highest level in the
137 brain, significantly higher than in the ovaries, fat bodies, and midgut in *D. citri* (Figure S2). The
138 levels of *DcAKH* in ovaries of *C*Las-negative and -positive psyllids decreased during the
139 assessment period. However, levels in the ovaries of *C*Las-positive psyllids were significantly
140 higher than those that were *C*Las-negative at all assessment times (Figure 2A). To investigate
141 the role of *DcAKH* in metabolic and reproductive changes induced by *C*Las, we knocked down
142 *DcAKH* expression using RNAi. When fed with ds*DcAKH*, the mRNA level of *DcAKH* in
143 *C*Las-negative and *C*Las-positive ovaries significantly decreased by 89% and 66%,
144 respectively, compared with psyllids fed with ds*GFP* (Figure 2B). Knockdown of *DcAKH*
145 resulted in TAG accumulation and a significant decrease in glycogen (Figures 2C-D).
146 Correspondingly, depletion of *DcAKH* led to a significant increase the size of the lipid droplets
147 (Figure 2E). Compared with the controls, *DcAKH* RNAi slowed ovarian development (Figure
148 2F), extended the preoviposition period (from 13.3 to 14.2 days in *C*Las-negative psyllids and

149 from 10.7 to 13.4 days in *C*Las-positive psyllids), shortened the oviposition period (from 7.1 to
150 4.3 days in *C*Las-negative psyllids and from 10.1 to 5.7 days in *C*Las-positive psyllids), and
151 decreased fecundity (from 138.0 to 94.7 days in *C*Las-negative psyllids and from 230.0 to
152 118.1 days in *C*Las-positive psyllids) (Figures 2G-I). Furthermore, the *C*Las signal and relative
153 titer in the ovaries of *DcAKH* knockdown psyllids significantly decreased (Figures 2J-K). Taken
154 together, it can be concluded that *DcAKH* is involved in *C*Las-induced metabolic and
155 reproductive changes in *D.citri.*

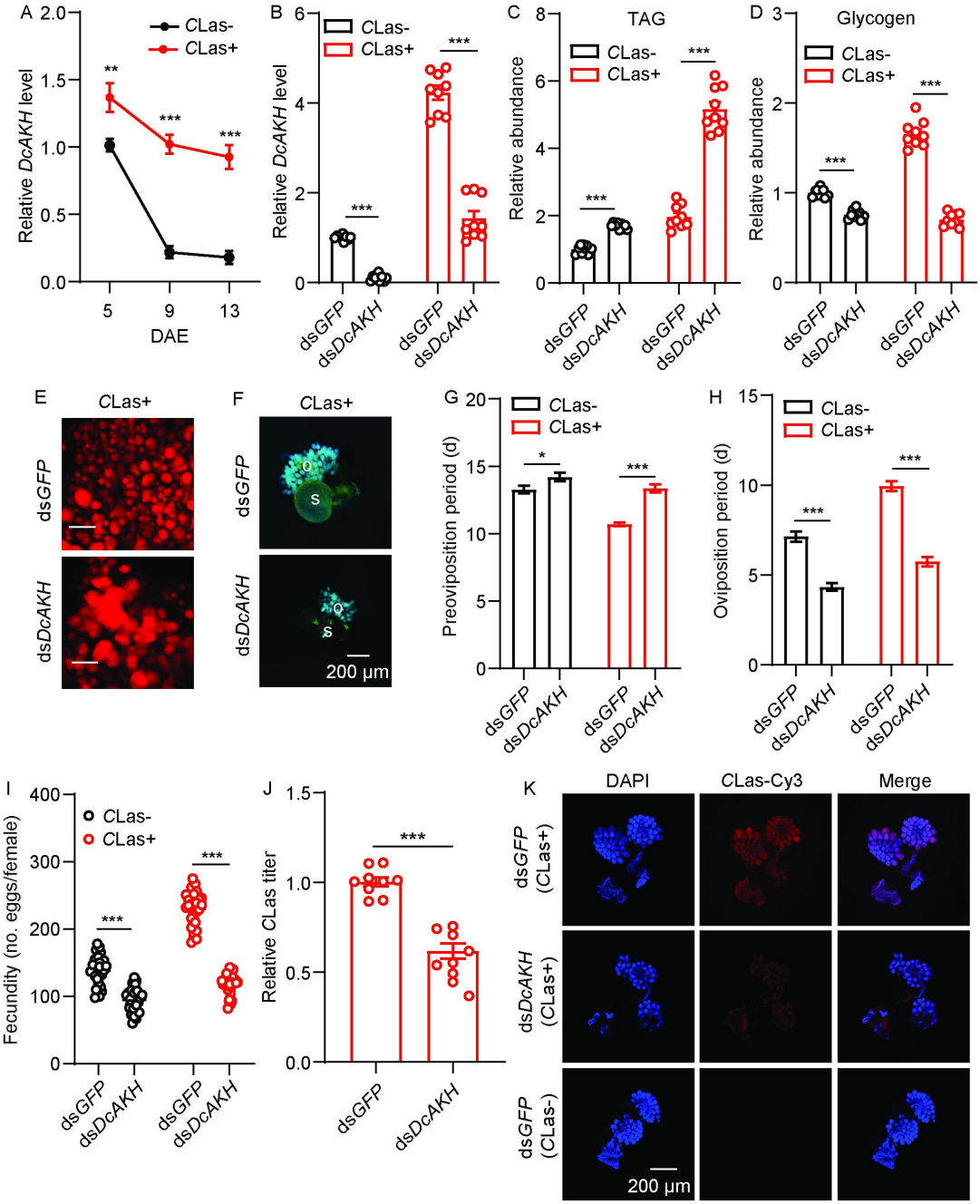

Figure 2. *DcAKH* is involved in the mutualistic relationship between *C*Las and *D. citri* resulting in increased fecundity. (A) Comparison of temporal expression patterns of *DcAKH* between the ovaries of *C*Las- and *C*Las+ psyllids. (B) Efficiency of RNAi of *DcAKH* in *C*Las- and *C*Las+ females treated with ds*DcAKH* for 48 h. (C) Comparison of TAG levels in fat bodies of *C*Las- and *C*Las+ females treated with ds*DcAKH* for 48 h. (D) Comparison of glycogen levels in fat bodies of *C*Las- and *C*Las+ females treated with ds*DcAKH* for 48 h. (E) Lipid droplets stained with Nile red in fat bodies dissected from *C*Las+ females treated with ds*DcAKH* for 48 h. Scale bar = 40 μm. (F) Ovary phenotypes of *C*Las+ females treated with

165 ds*DcAKH* for 48 h. Scale bar = 200 μm. o: ovary, s: spermathecae. (G-I) Comparison of the
166 preoviposition period, oviposition period and the fecundity between *C*Las- and *C*Las+ adults
167 treated with ds*DcAKH*. (J) The *C*Las titer in ovaries of *C*Las+ females treated with ds*DcAKH*
168 for 48 h. (K) Representative confocal images of *C*Las in the reproductive system of *C*Las+
169 females treated with ds*DcAKH* for 48 h. Scale bar = 200 μm. DAPI: the cell nuclei were stained
170 with DAPI and visualized in blue. *C*Las-Cy3: the *C*Las signal visualized in red by staining with
171 Cy3. Merge: merged imaging of co-localization of cell nuclei and *C*Las. Data are shown as
172 means ± SEM. The significant differences between treatment and controls are indicated by
173 asterisks (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).
174
175 ***DcAKHR* respond to *DcAKH* and participates in *D. citri*-CLas mutualism**
176 The protein tertiary structure of *DcAKHR* was predicted; *DcAKHR* has seven conserved
177 transmembrane (TM1-TM7) domains and is a typical G protein coupled receptor (Figure S3A).
178 A phylogenetic analysis was conducted to evaluate the association of *DcAKHR* with other
179 insect *AKHRs*; *DcAKHR* was most closely related to the *AKHR* of *Nilaparvata lugens* (Figure
180 S3B). To further confirm *Dc*AKH can activate *DcAKHR*, we performed a cell-based calcium
181 mobilization assay. As shown in Figure S3C, *DcAKHR* was strongly activated by *Dc*AKH in a
182 dose-dependent manner, with an EC_{50} value of 2.20×10^{-5} mM, but there were no responses
183 when challenged with an empty vector or other evolutionarily-related peptides. In addition,
184 after *DcAKH* knockdown, *DcAKHR* expression in the ovaries significantly decreased (Figure
185 S3D). *DcAKHR* was cloned, sequenced, designated and then used for further studies.
186 Sequence analysis showed that *DcAKHR* consisted of an open reading frame (ORF) of 1290
187 bp and a 185 bp 3' UTR. It encodes a putative protein consisting of 429 amino acids with a
188 predicted molecular weight of 48.32 kDa. Tissue expression patterns showed that *DcAKHR*
189 had the highest expression in the midgut, follow by the head, fat bodies, and ovaries (Figure
190 S4). In the ovaries of *C*Las-positive and -negative psyllids, the transcript and protein levels of
191 *DcAKHR* increased over the assessment period with levels of both being higher in
192 *C*Las-positive psyllids than in *C*Las-negative psyllids (Figure 3A). To investigate the role of
193 *DcAKHR* in metabolic and reproductive changes induced by *C*Las, we knocked down
194 *DcAKHR* expression by RNAi. When fed with ds*DcAKHR*, the mRNA levels of *DcAKHR* in the

195 ovaries of *C*Las-negative and *C*Las-positive were significantly decreased by 59.4 and 66.0%,
196 respectively, compared with psyllids fed with ds*GFP*; western blot analysis confirmed that
197 *Dc*AKHR protein levels were also significantly reduced (Figure 3B). Knockdown of *DcAKHR*
198 resulted in TAG accumulation and a significant decrease in glycogen (Figures 3C-D).
199 Correspondingly, depletion of *DcAKHR* led to a significant increase in the size of lipid droplets
200 (Figure 3E). Compared with the controls, *DcAKHR* RNAi interfered with ovarian development
201 (Figure 3F): the preoviposition period was extended (from 13.3 to 16.2 days in *C*Las-negative
202 psyllids and from 10.7 to 12.9 days in *C*Las-positive psyllids); oviposition period was shortened
203 (from 7.1 to 4.6 days in *C*Las-negative psyllids and from 10.1 to 5.3 days in *C*Las-positive
204 psyllids); and fecundity decreased (from 138.0 to 90.6 eggs per female in *C*Las-negative
205 psyllids and from 230.0 to 98.2 eggs per female in *C*Las-positive psyllids). These effects are
206 similar to those caused by *DcAKH* knockdown (Figures 3G-I). Furthermore, the *C*Las signal
207 and relative titer in the ovaries of *DcAKH* knockdown psyllids were significantly decreased
208 (Figures 3J-K). Taken together, *DcAKHR* responds to *DcAKH* and is involved in the
209 *C*Las-induced metabolic and reproductive changes in *D. citri.*
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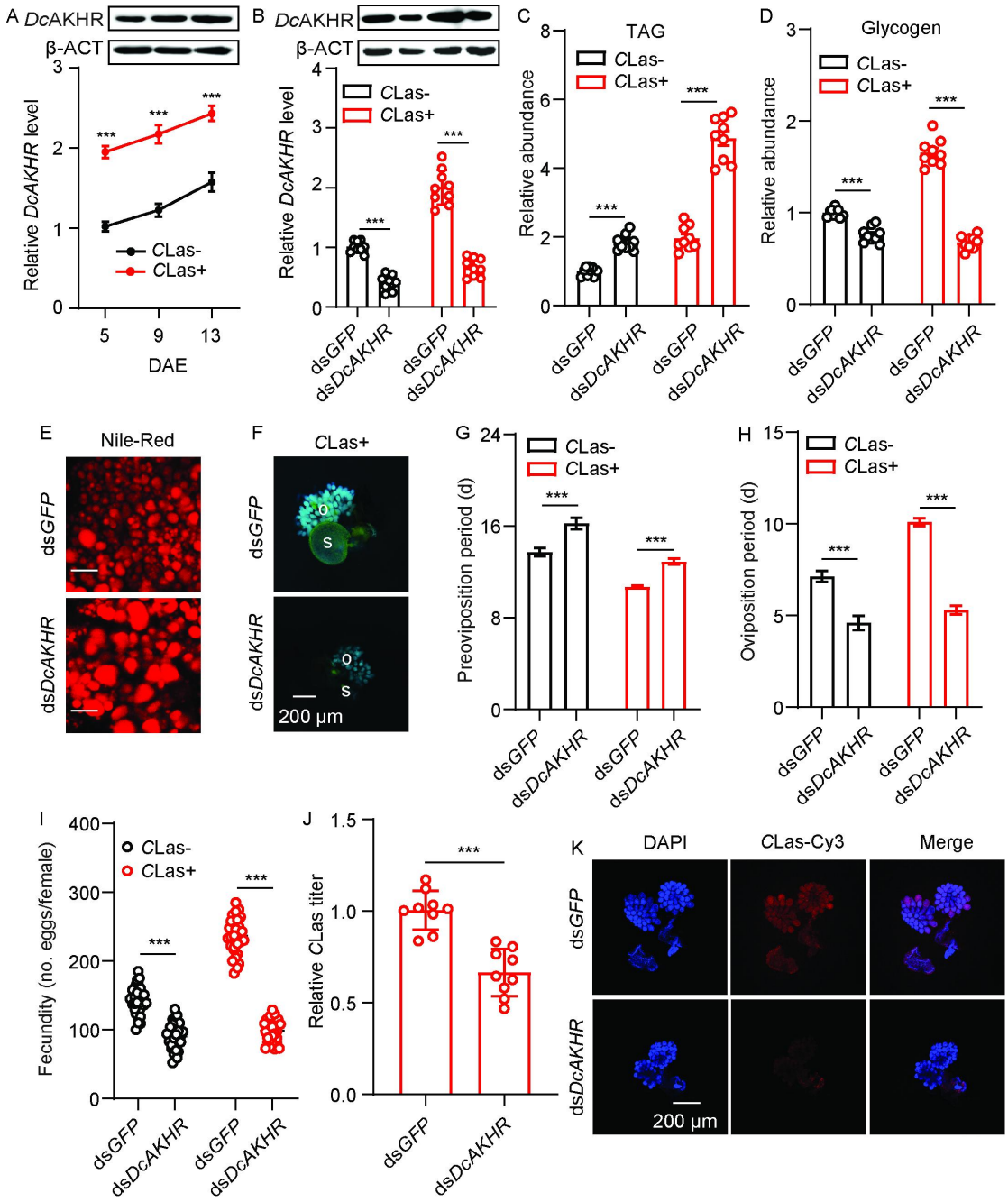
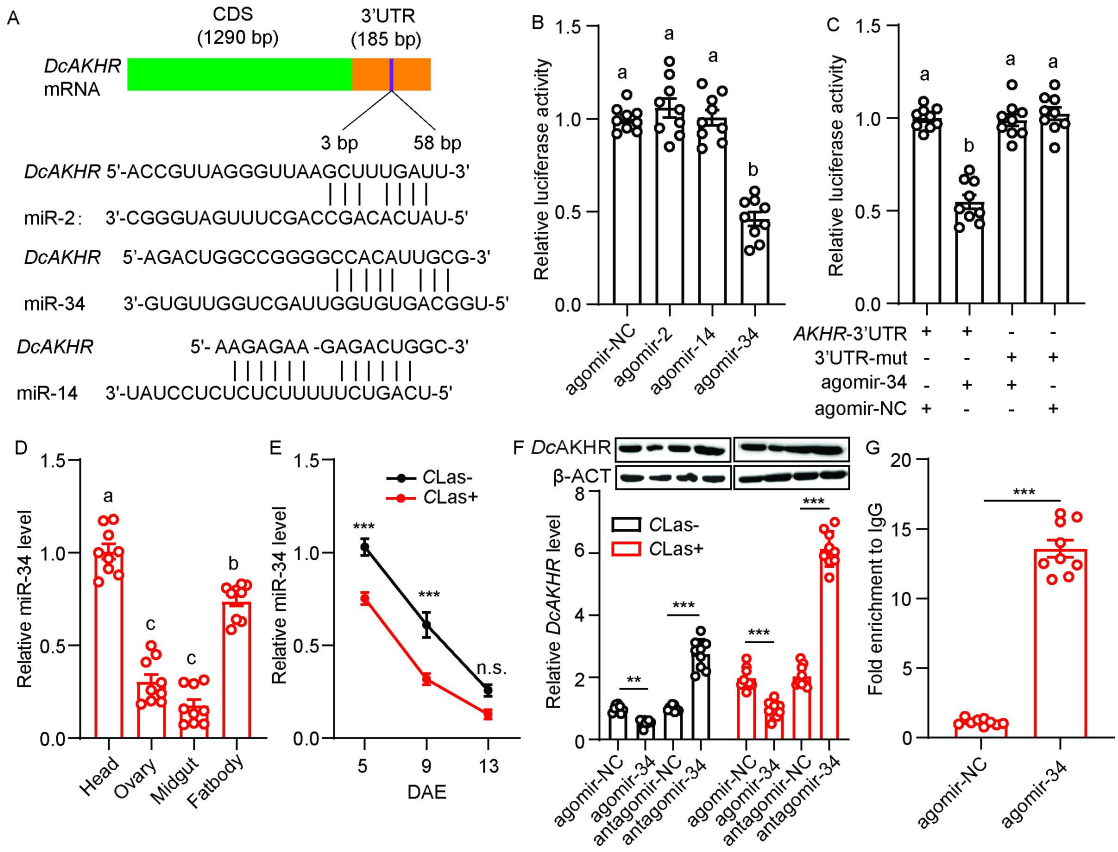


Figure 3. *DcAKHR* is involved in the mutualistic relationship between *C*Las and *D. citri* resulting in increased fecundity. (A) Comparison of temporal expression patterns of *DcAKHR* between the ovaries of *C*Las- and *C*Las+ psyllids. (B) The efficiency of RNAi of *DcAKHR* in *C*Las- and *C*Las+ psyllids treated with ds*DcAKHR* for 48 h. (C) Comparison of TAG levels in fat bodies of *C*Las- and *C*Las+ females treated with ds*DcAKHR* for 48 h. (D) Comparison of glycogen levels in fat bodies of *C*Las- and *C*Las+ females treated with ds*DcAKHR* for 48 h. (E) Lipid droplets stained with Nile red in fat bodies dissected from *C*Las-positive females treated with ds*DcAKHR* for 48 h. Scale bar = 40 μm. (F) Ovary

221 phenotypes of *C*Las+ females treated with ds*DcAKHR* for 48 h. Scale bar = 200 μm. o: ovary,
222 s: spermathecae. (G-I) Comparison of the preoviposition period, oviposition period and the
223 fecundity of *C*Las- and *C*Las+ adults treated with ds*DcAKHR*. (J) The *C*Las titer in the ovaries
224 of *C*Las+ females treated with ds*DcAKHR* for 48 h. (K) Representative confocal images of the
225 reproductive system of *C*Las+ females treated with ds*DcAKHR* for 48 h. Scale bar = 200 μm.
226 DAPI: the cell nuclei were stained with DAPI and visualized in blue. *C*Las-Cy3: the *C*Las signal
227 visualized in red by staining with Cy3. Merge: merged imaging of co-localization of cell nuclei
228 and *C*Las. Data are shown as means ± SEM. The significant differences between treatment
229 and controls are indicated by asterisks (Student's *t*-test, ***$P < 0.001$).
230
231 **miR-34 targeted *DcAKHR***
232 Based on the small RNA libraries of *D. citri*, we used miRanda and RNAhybrid software to
233 predict the potential miRNAs targeting *DcAKHR*; three putative miRNAs, namely miR-34,
234 miR-2 and miR-14 (Figure 4A), were identified. To confirm the binding activity of these miRNAs
235 with *DcAKHR*, several dual-luciferase reporter assays were conducted. Compared with the
236 controls, the luciferase activity was markedly reduced when miR-34 agomir was co-transfected
237 with the recombinant plasmid containing the full 3'UTR sequence of *DcAKHR*, while miR-2 and
238 miR-14 activities were not significantly changed (Figure 4B). In addition, the reporter activity
239 was recovered when the binding sites of miR-34 were mutated in the *DcAKHR* 3'-UTR (Figure
240 4C). To determine whether miR-34 specifically targets *DcAKHR*, the following experiments
241 were conducted. Firstly. tissue expression patterns showed that the miR-34 was highly
242 expressed in the head, followed by the fat bodies, ovaries, and midgut (Figure 4D). Secondly,
243 miR-34 has the opposite expression pattern to *DcAKHR* (Fig. 3A), and transcript levels
244 decreased over the assessment period with the levels of both being lower in *C*Las-positive
245 psyllids than in *C*Las-negative psyllids (Figure 4E). Thirdly, after feeding with agomir-34, the
246 relative levels of miR-34 mRNA in the ovaries of *C*Las-negative and *C*Las-positive psyllids
247 increased 1.9- and 2.3-fold, respectively (Figure S5). The mRNA and protein levels of
248 *DcAKHR* were correspondingly increased or decreased after separately feeding psyllids with
249 either antagomir-34 or agomir-34 (Figure 4F). Fourthly, an RNA immunoprecipitation (RIP)
250 assay was performed; *DcAKHR* mRNA was significantly enriched in the

251 anti-AGO-immunoprecipitated RNAs from ovaries of agomir-34-fed female psyllids relative to
252 the control samples (Figure 4G). Taken together, these results strongly suggest that *DcAKHR*
253 is a direct target of miR-34.



254

255 **Figure 4. Identification and validation of the target relationship between miR-34 and**
256 ***DcAKHR.*** (A) The putative binding sites of miRNAs in the *DcAKHR* 3'-UTR as predicted by
257 miRanda and RNAhybrid. (B) Dual-luciferase reporter assays using HEK293T cells
258 co-transfected with miRNA agomir and recombinant pmirGLO vectors containing the predicted
259 binding sites for miR-2, miR-14 and miR-34 in the CDS of *DcAKHR.* (C) Dual-luciferase
260 reporter assays using HEK293T cells co-transfected with miR-34 agomir plus recombinant
261 pmirGLO vectors containing *DcAKHR*-3'UTR or mutated *DcAKHR*-3'UTR. (D) Tissue
262 expression pattern of miR-34 in *C*Las+ female adults at 7 DAE in the head, ovary, fat body,
263 and midgut. (E) Comparison of temporal expression patterns of miR-34 in ovaries of *C*Las-
264 and *C*Las+ females. (F) Effect of miR-34 agomir and antagomir treatments on *Dc*AKHR mRNA
265 expression and protein level in ovaries of *C*Las- and *C*Las+ psyllids after 48 h. (G) Relative
266 expression of miR-34 targeted *DcAKHR in vivo* as demonstrated by an RNA
267 immunoprecipitation assay. Data are shown as mean ± SEM. For B-D, significant differences

268 among the different treatments are indicated by lowercase letters above the bars (one-way
269 ANOVA followed by Tukey's Honestly Significant Difference test at $P < 0.05$). The significant
270 differences between treatment and control are indicated by asterisks in E-G (Student's *t*-test,
271 $^{**}P < 0.01$, $^{***}P < 0.001$).
272

273 **miR34 participates in *D. citri*-*C*Las mutualism in ovaries**

274 To investigate the roles of miR-34 in the interaction between *D. citri* and *C*Las,
275 *C*Las-positive and –negative females were fed with agomir-negative control (NC) or with
276 miR-34 agomir. After feeding with miR-34 agomir, there was a significant accumulation of TAG
277 and a decrease in glycogen levels in the two populations (Figures 5A-B). The size of lipid
278 droplets in the fat bodies of *C*Las-positive psyllids were bigger than those of the control (Figure
279 5C). After treatment with miR-34 agomir, ovarian development of *C*Las-positive psyllids was
280 reduced (Figure 5D). In addition, when *C*Las-negative and *C*Las-positive psyllids were fed with
281 miR-34 agomir, the oviposition period of both psyllid types was shortened, preoviposition
282 period was markedly extended, and fecundity significantly decreased compared with the
283 relevant control groups (Figures 5E-G); these phenotypes mimic those expressed after
284 treatment with ds*DcAKHR*. Moreover, the *C*Las signals and relative titer in *C*Las-positive
285 ovaries were significantly reduced (Figures 5H-I). All the results indicate that miR-34
286 suppresses *DcAKHR* expression and is involved in the mutualistic interaction.

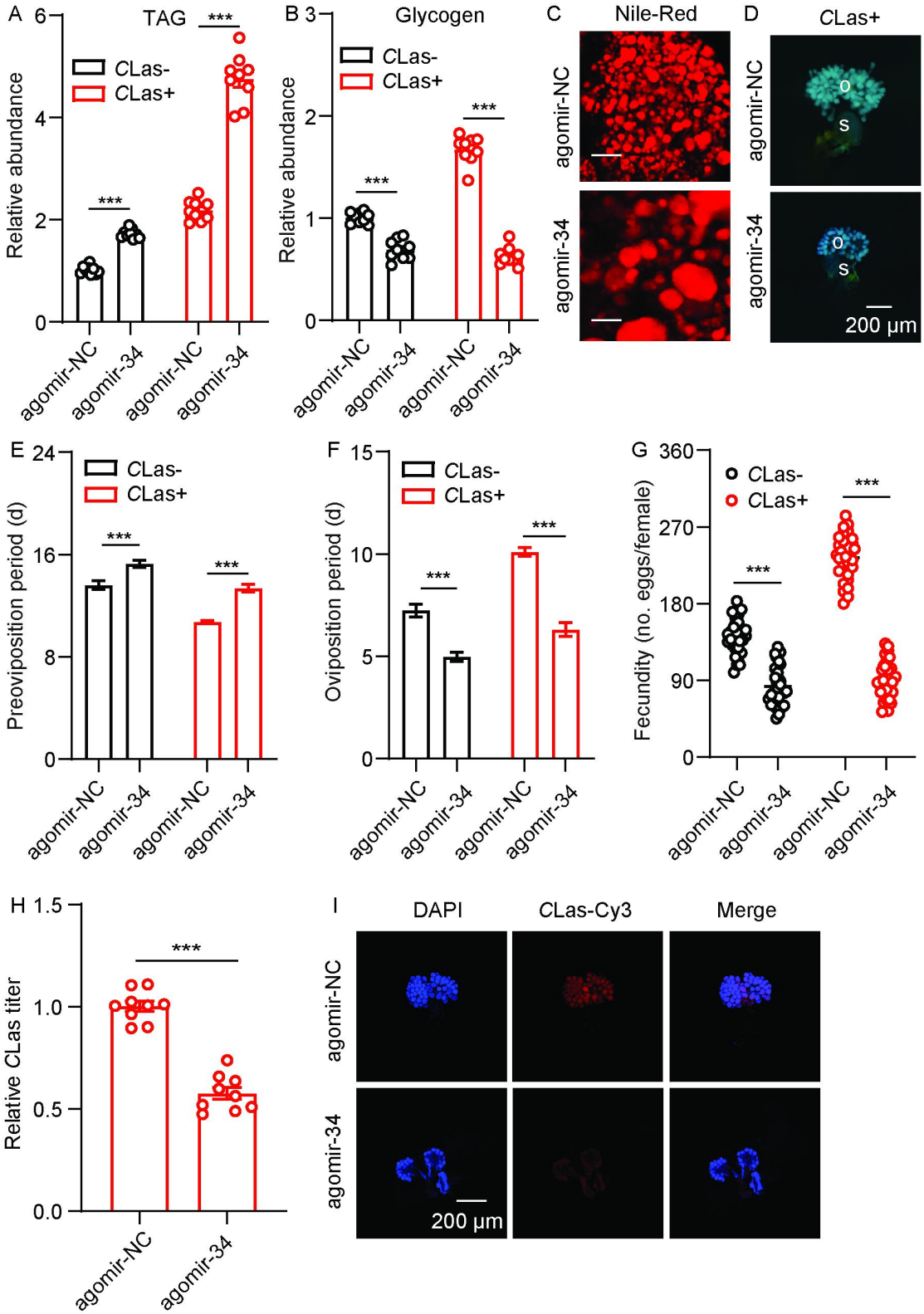


Figure 5. miR-34 participation in mutualistic interactions between *D. citri* and *C*Las. (A) Comparison of TAG levels in fat bodies of *C*Las- and *C*Las+ females treated with agomir-34 for 48 h. (B) Comparison of glycogen levels in the fat bodies of *C*Las- and *C*Las+ females treated with agomir-34 for 48 h. (C) Lipid droplets stained with Nile red in fat bodies

292 dissected from *C*Las+ females treated with agomir-34 for 48 h. Scale bar = 40 μm. (D) Ovary
293 phenotypes of *C*Las+ female treated with agomir-34 for 48 h. Scale bar = 200 μm. o: ovary, s:
294 spermathecae. (E-G) Comparison of the preoviposition period, oviposition period and the
295 fecundity between *C*Las- and *C*Las+ adults treated with agomir-34. (H) *C*Las titer in ovaries of
296 *C*Las+ females treated with agomir-34 for 48 h. (I) Representative confocal images of *C*Las in
297 the reproductive system of *C*Las+ females treated with agomir-34 for 48 h. Scale bar = 200 μm.
298 The signals of DAPI and *C*Las-Cy3 are same as described in Figure 2. Data are shown as
299 means ± SEM. The significant differences between treatment and controls are indicated by
300 asterisks (Student's *t*-test, ***$P < 0.001$).
301
302 **The JH signaling pathway is regulated by the AKH pathway and is involved in the**
303 **increase in fecundity induced by *C*Las**
304 To evaluate whether the JH signaling pathway is regulated by the AKH signaling pathway,
305 we assayed the relative JH titers and the expression levels of several JH pathway-related
306 genes in *DcAKH*-deficient, *C*Las-positive females. Compared to control females, the relative
307 JH titer in the abdomen decreased significantly after *dsDcAKH* treatment for 48 h (Figure 6A).
308 After feeding with *dsDcAKH* for 48 h, the expression levels of the JH receptor gene, *DcMet,*
309 and the downstream transcription factor, *DcKr-h1*, of the JH signaling pathway were
310 significantly reduced in the fat bodies and ovaries (Figures 6B-C). In addition, the expression
311 levels of *DcVg1-like*, *DcVgA1-like* and *DcV*gR, three important downstream genes of the JH
312 signaling pathway related to ovarian development were lower in *DcAKH*-deficient
313 *C*Las-positive females than in controls after dsRNA feeding for 48 h (Figures 6B-C). Similar
314 results were observed in the *DcAKHR*-deficient *C*Las-positive females (Figures 6D-F) as well
315 as *C*Las-positive females fed with agomir-34 (Figures 6G-I). These results suggest that JH
316 signaling pathway is regulated by the AKH-AKHR-miR-34 signaling pathway and is involved in
317 the increased fecundity of *D. citri* induced by *C*Las.

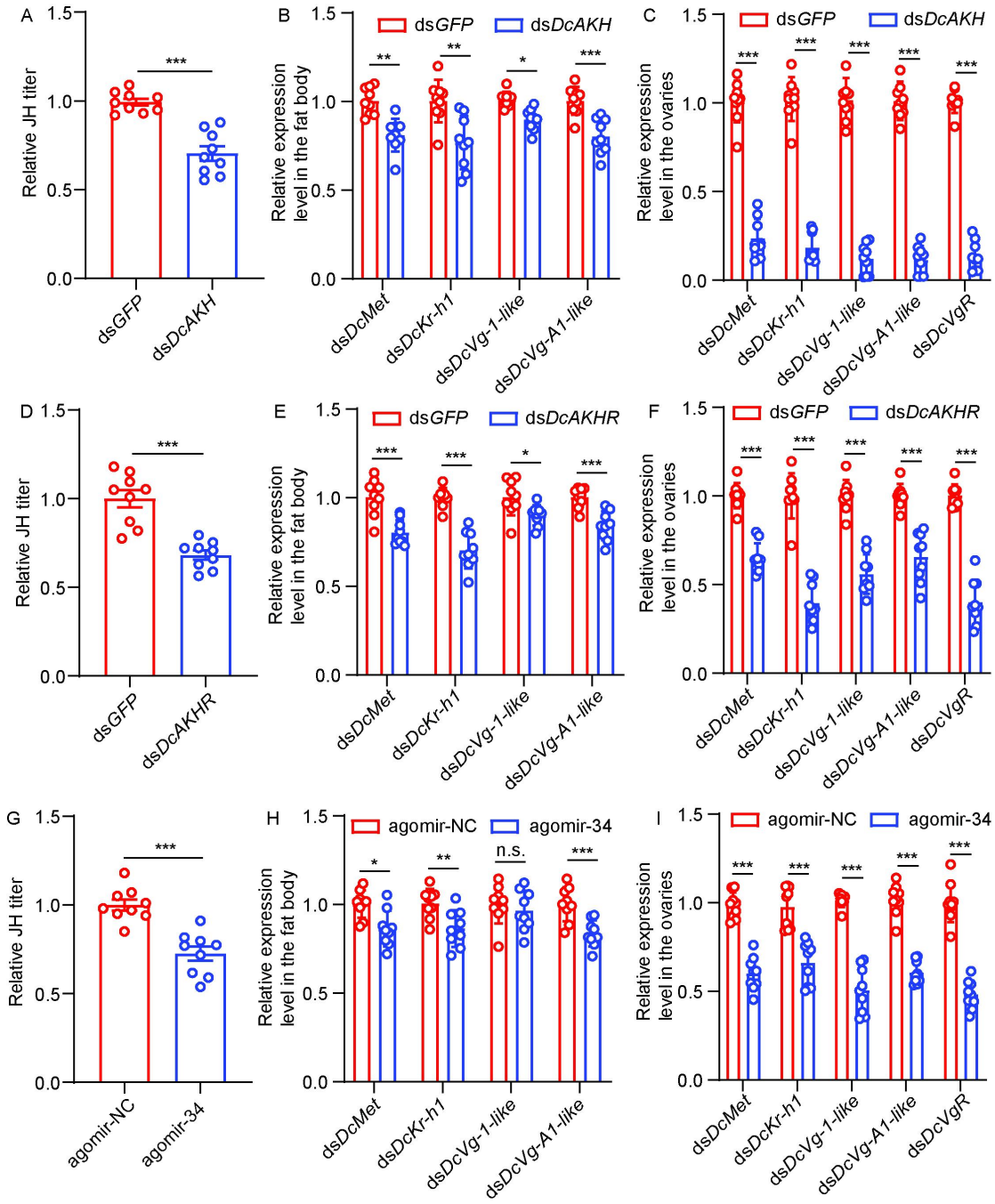


Figure 6. The JH signaling pathway is regulated by AKH signaling pathway and is involved in the increase in fecundity of *D. citri* induced by *C*Las. (A) JH titer in the abdomen of *C*Las+ females treated with ds*DcAKH* for 48 h. (B) Effects of ds*DcAKH* treatment on mRNA level of JH signaling pathway in fat bodies of *C*Las+ females. (C) Effects of ds*DcAKH* treatment on mRNA levels of components of the JH signaling pathway in the ovaries of *C*Las+ females. (D) JH titers in the abdomens of *C*las+ females treated with ds*DcAKHR* for 48 h. (E) Effects of ds*DcAKHR* treatment on mRNA level of JH signaling pathway in fat bodies of *C*Las+ females. (F) Effects of ds*DcAKHR* treatment on mRNA levels of components of the

327 JH signaling pathway in ovaries of *C*Las+ females. (G) JH titer in abdomen of *C*Las+ females
328 treated with agomir-34 for 48 h. (H) Effects of agomir-34 treatment on mRNA levels of
329 components of the JH signaling pathway in fat bodies of *C*Las+ females. (I) Effects of
330 agomir-34 treatment on mRNA levels of components of the JH signaling pathway in the
331 ovaries of *C*Las+ females.
332

333 ## Discussion

334 Increasing numbers of studies have focused on the effects of vector-virus interactions on
335 reproduction. For example, rice gall dwarf virus is vertically transmitted by hitchhiking on insect
336 sperm, thereby promoting long-term viral epidemics and persistence in nature [2]. Berasategui
337 et al. (2022) [3] found a mutualistic relationship between *Chelymorpha alternans* and *Fusarium*
338 *oxysporum*, where the pupae were protected in exchange for the dissemination and
339 propagation of the fungus. There are few studies on the mechanisms underlying
340 vector-bacteria interactions. However, Singh and Linksvayer (2020) [38] found that
341 *Wolbachia*-infected colonies of *Monomorium pharaonis* had increased colony-level growth,
342 accelerated colony reproduction, and shortened colony life cycles compared to those that were
343 uninfected.

344 The fat body is the major insect storage organ that produces the majority of
345 hemolymph-born vitellogenic proteins and critically contributes to insect ovary development
346 [16]. In this study, after infection with *C*Las, the preoviposition period of *D. citri* was shortened,
347 oviposition period was prolonged, and fecundity was significantly increased. These results
348 suggests that there is a mutualistic interaction in *D. citri* ovaries with *C*Las. *C*Las accelerates
349 ovarian development and prolongs the oviposition period thereby enhancing the fecundity of *D.*
350 *citri*; in turn, *D. citri* provides sites and nutrients for *C*Las replication during ovarian
351 development. *C*Las-positive *D. citri* require more energy to support this increased fecundity.
352 Hence, the TAG and glycogen levels of both *C*Las-positive and *C*Las-negative were assayed.
353 After infection with *C*Las, the TAG and glycogen levels significantly increased, and the average
354 size of lipid droplets in the fat bodies were distinctly larger, indicating that the increase in lipid
355 mobilization may prepare for an increase in fecundity.

356 The total amount of lipids present in fat bodies is a balance between lipogenesis and

357 lipolysis [39], and both processes are essential for lipid homeostasis. Disruption of either
358 lipogenesis or lipolysis could interfere with nutritional and physiological conditions in fat bodies
359 and affect vitellogenesis. AKH/AKHR mediated lipolytic systems are essential for lipid
360 provision and nutrient transfer during reproduction. In *Gryllus bimaculatus* [40,41] and
361 *Caenorhabditis elegans* [42], injections of AKH interfered with egg production by inhibiting the
362 production of vitellogenins and the accumulation of lipid stores. In *Bactrocera dorsalis*, AKHR
363 knockdown led to decreased lipolytic activity and delayed oocyte maturation [27] and these
364 authors suggested the decline in fecundity may be due to the inability to utilize the lipid stores
365 of fat bodies to promote oocyte maturation. In *Glossina morsitans*, the silencing of *AKHR* and
366 *brummer* led to the inability to mobilize lipid reserves in fat bodies during pregnancy, slowed
367 down the development of oocytes, and caused embryogenesis to fail, indicating that female
368 fertility depends on lipid metabolism regulated by AKHR [24]. These results suggest that lipid
369 homeostasis appears critical for insect fecundity, as its disruption substantially suppresses egg
370 production and negatively affects vitellogenesis.

371 In the present study, *DcAKH* and *DcAKHR* played important roles in the increased lipid
372 metabolism and fecundity of *D. citri* mediated by *C*Las. The cDNA sequences encoding AKH
373 and AKHR from *D. citri* were cloned and identified, and phylogenetic analysis showed that
374 *DcAKH* and *DcAKHR* were orthologous to other hemipteran AKHs and AKHRs. Functional
375 analysis showed that *DcAKHR* shares properties found with other AKHRs.
376 *DcAKHR*-transfected CHO cells could be activated by AKH peptide, while it did not response
377 to Crz peptide, despite the sequences and gene structures of AKH and Crz being closely
378 related [21]. *DcAKH* and *DcAKHR* were more highly expressed in the ovaries of *C*Las-positive
379 psyllids than those of *C*Las-negative individuals. Knockdown of *DcAKH* and *DcAKHR* resulted
380 in TAG accumulation and a significant decrease in glycogen levels in the fat bodies. Most
381 importantly, the *C*Las titer in the ovaries of *C*Las-positive psyllids and fecundity significantly
382 reduced when feeding with *dsDcAKH* and *dsDcAKHR*. Our results support the idea that
383 reproductive disruption from AKH/AKHR knockdown is due to the inability of female to mobilize
384 lipid reserves in the fat bodies required for oocyte maturation during vitellogenesis.

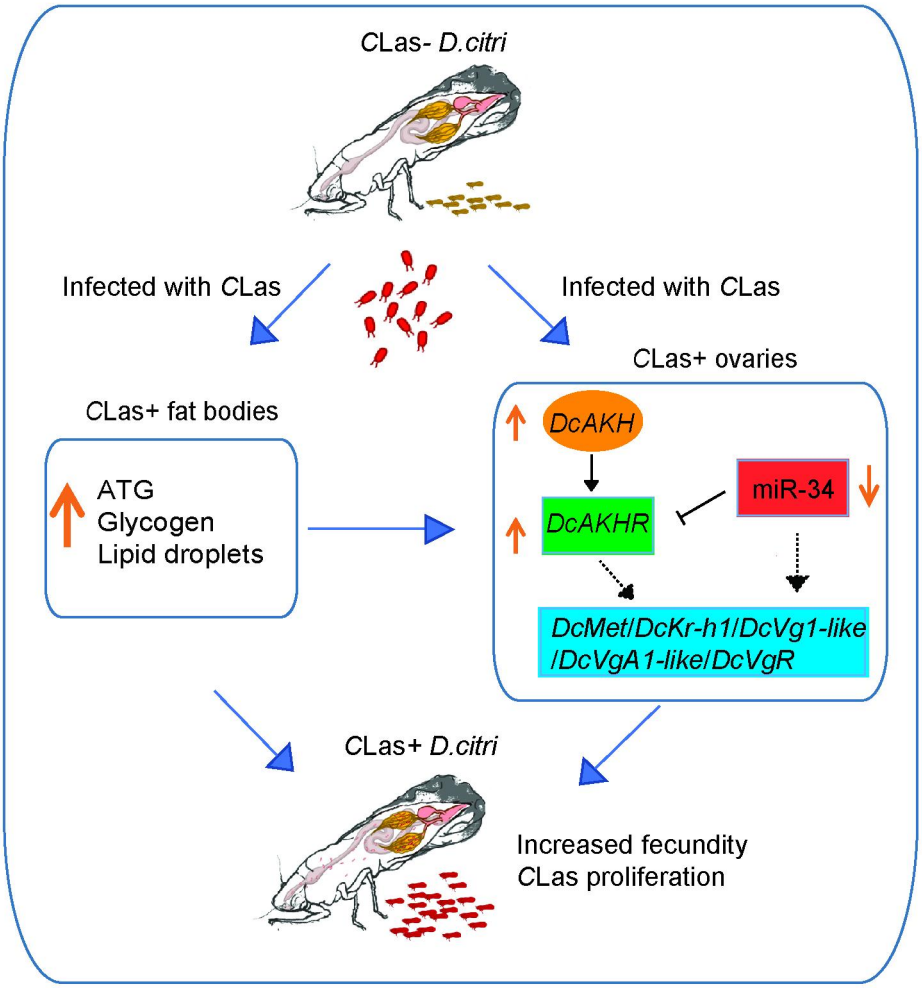
385 To date, there are no reports on the post-transcriptional regulation of AKHR. As important
386 post-transcriptional regulators, miRNAs generally suppress their target genes by triggering

387 mRNA degradation or translational repression [30]. More and more miRNAs have been
388 reported to be involved in the metabolic processes of insects including reproduction. In
389 addition to functions of miR-277 described in the Introduction, this miRNA targets ilp7 and ilp8
390 thereby controlling lipid metabolism and reproduction in *A. aegypti* [37]. miR-8 depletion in
391 mosquitoes resulted in an increase of its target gene, *secreted wingless-interacting molecule*,
392 and in lipid accumulation in developing oocytes [43]. Depletion of miR-8 in mosquitoes by
393 antagomir injection upregulates *secreted wingless-interacting molecule* and decreases the
394 level of lipid in ovaries to block vitellogenesis post blood meals [44]. A switch from catabolic to
395 anabolic amino acid metabolism in fat bodies mediated by miR-276 restricts mosquito
396 investment into oogenesis and benefits the development of *Plasmodium falciparum* [45]. In the
397 current study, based on *in vitro* and *in vivo* experiments, we found that host miR-34 targets
398 *DcAKHR*. miR-34 was observed to have an opposite expression trend to *DcAKHR*, and its
399 expression in the ovaries of *C*Las-positive psyllids was lower than in those of CLas-negative
400 individuals. Treatment of miR-34 agomir and antagomir, respectively, resulted in a significant
401 decrease and increase in *DcAKHR* mRNA and protein levels. In addition, miR-34
402 overexpression not only led to the accumulation of TAG and the reduction of glycogen levels in
403 fat bodies, but also decreased fecundity and *C*Las titers in the ovaries similar to the
404 phenotypes caused by silencing of *DcAKHR*. This indicates that *C*Las inhibited host miR-34 to
405 enhance *DcAKHR* expression and improve the lipid metabolism and fecundity. This study is
406 the first to report the manipulation of host miRNAs and target genes by a bacterium to affect
407 lipid metabolism and reproduction of their vector.

408 Vitellogenesis is strongly linked with insect JH production [46]. The fat body is an
409 important tissue that senses and integrates various nutritional and hormonal signals required
410 for the regulation of vitellogenesis [16]. Moreover, JH is also involved in the nutrient-dependent
411 regulation of insect reproduction, but the mechanism of action remains obscure. Lu et al.
412 (2016a, b) [47,48] have demonstrated that nutritional signaling during female reproduction
413 induces JH biosynthesis that in turn stimulates vitellogenin expression in fat bodies and egg
414 production in *N. lugens.* In order to evaluate the possible relationship between
415 AKH/AKHR-mediated lipolysis and JH-dependent vitellogenesis, the expressions of JH
416 signaling pathway-related genes in fat bodies and ovaries were determined after knockdown of

417 *DcAKH* and *DcAKHR*. We found that reducing *DcAKH* or *DcAKHR* by RNAi in *C*Las-positive *D.*
418 *citri* not only decreased JH titer but also resulted in a decrease in the expression of *DcMet* and
419 *DcKr-h1* in the fat bodies and ovaries, as well as the egg development related genes,
420 *DcVg-1-like*, *DcVg-A1-like* and *DcVgR*. Similar results were detected in the agomir-34
421 treatment. Our results suggest that AKH/AKHR-based lipolysis plays an important regulatory
422 role in the JH signaling pathway during *D. citri*-*C*Las interactions.

423 In conclusion, we proposed the scheme of events following *C*Las infection presented in
424 Figure 7. After infection with *C*Las, *D. citri* are more fecund than their uninfected counterparts.
425 The interaction between *C*Las and *D. citri* affecting reproduction is a win-win strategy; the
426 more offspring of *D. citri*, the more *C*Las in the field. *C*Las upregulates the AKH/AKHR
427 signaling and downregulates miR-34 to increase lipid metabolism and activate JH-dependent
428 vitellogenesis, thereby improving the fecundity of *C*Las-positive females. Overall, our results
429 not only broaden our understanding of the molecular mechanism underlying the *D. citri*-*C*Las
430 interaction, but also provide valuable information for further integrated management of *D.citri*
431 and HLB by targeting the *DcAKH/DcAKHR* signaling pathway.
432



433

434 **Figure 7. Mechanisms linking metabolism and reproduction of *D. citri* induced by CLas.**
435 After infection with CLas, the TAG and glycogen levels in fat bodies of CLas-positive psyllids
436 significantly increased as well as the size of lipid droplets. In ovaries, CLas upregulates the
437 AKH/AKHR signaling and downregulates miR-34 to increase lipid metabolism and activate
438 JH-dependent vitellogenesis, thereby improving the fecundity of CLas-positive females. *D. citri*
439 are more fecund than their uninfected counterparts. The interaction between CLas and *D. citri*
440 affecting reproduction is a win-win strategy; the more offspring of *D. citri*, the more CLas in the
441 field.
442

443 ## Materials and Methods

444 **Host plants and insect colonies**

445 The *C*Las-negative (healthy) and *C*Las-positive *D. citri* colonies were obtained from a
446 laboratory culture continuously reared on the healthy and *C*Las-infected lemon (*Citrus ×limon*
447 (L.) Osbeck) plants. The *C*Las-infected lemon plants and psyllids were monitored to detect
448 *C*Las infection monthly using the quantitative polymerase chain reaction (qPCR). The two
449 populations were raised in different incubators with same conditions (26 ± 1 ℃, 65 ± 5%
450 relative humidity (RH) and a 14-h light: 10-h dark cycle). *C*Las-negative and *C*Las-positive
451 lemon plants were grown in two separate glasshouses.

452

453 **Bioinformatics and phylogenetic analyses**

454 The physicochemical properties of *DcAKH* (GenBank accession number: MG550150.1)
455 and *DcAKHR* (GenBank accession number: OR259432) were analyzed using the online
456 bioinformatics ProtParam tool (http://web.expasy.org/protparam/). Homologous protein
457 sequences of AKH and AKHR were aligned with those of other insects using DNAman6.0.3
458 software. Phylogenetic trees were constructed with the neighbor-joining method in MEGA5.10
459 software, with 1000 bootstrap replicates; bootstrap values > 50% shown on the tree.

460

461 **Assays of reproductive parameters**

462 Tested females from two colonies were selected and paired with healthy males. One
463 female and one male were placed onto young vegetative shoots (flush) of healthy lemon plants
464 to promote oviposition; the flush and insects were sealed with tied white mesh bag (15 × 20
465 cm). These lemon plants were held in an incubator (25 ± 1 ℃, 65 ± 5% RH, 14L: 10D
466 photoperiod). After 24h, the preoviposition period and oviposition period were recorded, the
467 number of eggs laid per female were counted, and this pair of psyllids were transferred to new
468 flush to continue egg laying. Eggs were counted daily until all females died. All the experiments
469 were performed using three replications, and about 15 pairs of *D. citri* for each replication.

470

471 **Quantitative RT-PCR (qRT-PCR)**

472 For mRNA expression analysis, total RNA was extracted using TRIzol reagent (Invitrogen,

473 Carlsbad, CA, United States), and the synthesis of first-strand cDNA was performed using a
474 PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Beijing, China) in accordance with the
475 manufacturer's instructions. qRT-PCR was carried out by using the TB Green® Premix Ex
476 Taq™ II (Takara, Beijing, China) on an ABI PRISM® 7500 Real-Time System (Applied
477 Biosystems, Foster City, CA, USA). The beta-actin (Dcβ-ACT, GenBank XM_026823249.1)
478 gene was used as an internal control to normalize gene expression levels. For miRNA
479 expression analysis, miRNA was extracted using a miRcute miRNA Isolation Kit (TIANGEN,
480 Beijing, China), synthesized using a miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN,
481 Beijing, China), and quantified using the miRcute Plus miRNA qPCR Kit (SYBR Green)
482 (TIANGEN, Beijing, China). U6 snRNA was used as an internal control to normalize the
483 expression of miRNA. All the primers used are listed in Supplementary Table 1.
484
485 **Luciferase activity assay**
486 The 185 bp sequence of the 3'UTR containing the predicted target sites for miR-34 in
487 *DcAKHR* was cloned into the pmirGLO vector (Promega, Wisconsin, USA) downstream of the
488 luciferase gene to construct the recombinant plasmid, *DcAKHR*-3'UTR-pmirGLO, using the
489 pEASY®-Basic Seamless Cloning and Assembly Kit. The mutated 3'UTR sequence was
490 amplified and cloned into the pmirGLO vector to generate the *DcAKHR*-3'UTR
491 mutant-pmirGLO plasmid. The agomir and antagomir of miRNAs were chemically synthesized
492 and modified by GenePharma (Shanghai, China) with chemically modified RNA oligos of the
493 same sequence or anti-sense oligonucleotides of the miRNA. The negative control for agomir
494 and antagomir was provided by the manufacturer. According to the manufacturer's instructions,
495 the constructed vector (500 ng) and miRNA agomir (275 nM) were co-transferred into
496 HEK293T cells in a 24-well plate using a Calcium Phosphate Cell Transfection Kit (Beyotime,
497 Nanjing, China). After co-transfection for 24 h, the activities of firefly and Renilla luciferase
498 were detected using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA),
499 and the average luciferase activity was calculated.
500

501 **Fluorescence in situ hybridization (FISH)**

502 FISH using a *C*Las probe was carried out as previously described [49]. Under a dissecting
503 microscope, the ovaries of *C*Las-negative and *C*Las-postive *D. citri* were dissected in 1 ×
504 phosphate-buffered saline (PBS) (Beyotime Biotechnology, Shanghai, China). Firstly, the
505 isolated ovaries were fixed in Carnoy's fixative (glacial acetic acid-ethanol-chloroform, 1: 3: 6,
506 vol/vol) for 12 h at 25 ℃, washed four times (5 min each time) with 6% H_2O_2 in 80% ethanol
507 and then three times (10-15 min per time) with PBST (1 × PBS: TritonX-100, 99.7: 0.3,
508 (vol/vol)). Secondly, the samples were pre-incubated three times (10 min per time) in
509 hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl, 30% formamide (vol/vol), 0.01%
510 sodium dodecyl sulfate (wt/wol)) containing 10 pmol/mL of each probe for 24 h at 25 ℃, then
511 washed three times with PBST. Thirdly, nuclei were stained with 0.1 mg/mL of 40,
512 60-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) for 15 min, then
513 washed once with PBST. Finally, the stained ovaries were mounted in mounting medium. The
514 slides were scanned under a Leica TCS-SP8 (Leica Microsystems Exton, PA USA) confocal
515 microscope; excitation lasers emitting at 405 nm and 550 nm were used to detect the DAPI
516 and Cy3 signals, respectively, and sequential scanning was used to avoid the signal overlap of
517 probes. Image processing was completed using Leica LAS-AF software (v2.6.0). Specificity of
518 detection was carried out using without the probe and *C*Las-negative controls. Three FISH
519 tests were performed and at least 20 ovaries were viewed under the microscope to confirm the
520 repeatability. The probe sequences used in the current study are listed in Supplementary Table
521 1.

522

523 **Western blotting**

524 The total proteins from *D. citri* ovaries were extracted using RIPA protein lysis buffer (50
525 mM Tris pH 7.4, 1% Triton X-100, 150 mM NaCl, 1% sodium deoxycholate and 0.1% SDS)
526 with 1 mM PMSF. Proteins concentrations were quantified using a BCA protein assay kit
527 (Beyotime, Jiangsu, China). Aliquots of 60 μg protein were separated by 12% SDS-PAGE and
528 transferred to polyvinylidene fluoride membranes (Millipore). Subsequently, the membranes
529 were incubated with the primary antibody against *DcAKHR* (1: 1000 dilution, ABclonal
530 Technology Co., Ltd., Wuhan, China) for 12 h at 4 °C and then with the secondary antibody

531 (goat anti-rabbit IgG conjugated with HRP, 1: 10000 dilution) for 2 h at 25 °C. A mouse
532 monoclonal antibody against β-actin (TransGen Biotech, Beijing, China) was used as a control.
533 Immunoreactivity was imaged with the enhanced chemiluminescence with Azure C600
534 multifunctional molecular imaging system.
535
536 **Nile red staining**
537 The fat bodies were dissected from adult females 5 DAE, fixed in 4% paraformaldehyde
538 for 30 min at 25 ℃, and washed twice with 1 × PBS. Lipid droplets were incubated for 30 min
539 within the mixture of Nile red (0.1 μg/μL) and DAPI (0.05 μg/μL), and then washed twice with 1
540 × PBS. The samples were imaged using a laser scanning microscopy (TCS-SP8, Leica
541 Microsystems Exton, PA USA).
542
543 **Determination of TAG levels**
544 TAG levels were measured using a Triglycerides Colorimetric Assay (Cayman) in
545 accordance with the manufacturer's instructions. In short, thirty fat bodies were homogenized
546 in 100 μL of Diluent Assay Reagent. Then 10 μL of supernatant was incubated with Enzyme
547 Mixture solution, the TAG contents from the measurements were normalized to protein levels
548 in the supernatant of the samples determined using a BCA protein assay (Thermo Fisher
549 Scientific). Three independent biological replicates were analyzed for each treatment, and
550 each treatment included three technical replicates.
551
552 **Determination of glycogen levels**
553 Glycogen levels were assayed using a Glycogen Assay Kit (Cayman) in accordance with
554 the manufacturer's instructions. In brief, thirty fat bodies were homogenized in 100 μL of
555 Diluent Assay Reagent, and 10 μL of supernatant was incubated with Enzyme Mixture solution
556 and Developer Mixture. The glycogen levels from the measurements were normalized to
557 protein levels in the supernatant of the sample determined using a BCA protein assay (Thermo
558 Fisher Scientific). Three independent biological replicates were analyzed for each treatment,
559 and each treatment included three technical replicates.
560

561 **dsRNA synthesis and RNAi assay**

562 dsRNAs of *DcAKH* and *DcAKHR* were transcribed by a Transcript Aid T7 High Yield kit
563 (Thermo Scientific, Wilmington, DE, United states) and purified using the GeneJET RNA
564 Purification kit (Thermo Scientific). miRNA agomir, agomir negative control, miRNA antagomir,
565 and antagomir negative control were synthesized in the Shanghai GenePharma Co. Ltd.
566 (Shanghai, China). The RNAi and miRNA treatments were performed by feeding dsRNA or
567 miRNA antagomir/agomir through an artificial diet as described previously [50]. Briefly, twenty
568 females at 7 DAE were placed into a glass cylinder (25 × 75 mm) and sealed with two
569 stretched paramembranes. 200 μL of 20% (w:v) sucrose mixed with dsRNA was placed
570 between two paramembranes for feeding. The final ingestion concentrations of ds*DcAKH* and
571 ds*DcAKHR* were 200 ng/μL and 300 ng/μL, respectively. After feeding with dsRNA for 48 h, the
572 treated females were assayed as follows. The ovaries were dissected or qRT-PCR and
573 western blot analysis. Reproductive parameters including the pre-oviposition period,
574 oviposition period, and fecundity were recorded. *C*Las titers in the ovaries were assayed.
575 Ovary morphologies were observed using a Ultra-Depth Three-Dimensional Microscope
576 (VHX-500). Fat bodies were stained with Nile red. TAG levels and glycogen levels were
577 determined. Green fluorescent protein (GFP) was used as a reporter gene. Feeding ds*GFP*
578 was used as the control when psyllids were fed with dsRNA. All the experiments were
579 performed in three replications, and each replication included 15-20 pairs of *D. citri*.

580

581 **Heterologous expression and calcium mobilization assay**

582 The complete ORF of *DcAKHR* was cloned into the expression vector pcDNA3.1$^+$; cloning
583 was confirmed by sequencing (TSINGKE Bio). Endotoxin-free plasmid DNA of the vector was
584 extracted, and all tested peptides were synthesized by Sangon Biotech (Shanghai, China) at a
585 purity >95 % (see Fig. 1B for peptide structures). Chinese hamster ovary (CHO-WTA11) cells
586 supplemented with Gα16 subunit and aequorin were used [51] and transfected with
587 pcDNA3.1$^+$-*DcAKHR* or empty pcDNA3.1$^+$ (negative control) vectors using Lipofectamine
588 2000 (Thermo Fisher Scientifc, Waltham, MA USA). The calcium mobilization assay was
589 performed with slight modification as described by Gui et al. (2017) [52] and Shi et al. (2017)
590 [53]. In brief, the cells were incubated for 3 h in the dark with coelenterazine h (Promega,

591 Madison, WI, USA) 48 h after the transfection. Then, serial dilutions of peptide ligands (10^{-6} to
592 10^{1} μM) were loaded into opaque 96-well plates. Luminescence was measured over 15 s
593 using a SpectraMax i3x Multi Mode Microplate Reader (Molecular Devices). Medium alone
594 was used as a blank control and 100 μM ATP was served as a positive control. Each
595 experiment was repeated three times.
596
597 **JH titer detection**
598 JH titers were assessed using the Insect JH Ⅲ ELISA kit (Shanghai Enzyme-linked
599 Biotechnology Co., Ltd. Shanghai, China) following the manufacturer's protocol.
600
601 **Statistical analysis**
602 All statistical values are presented as means ± SEM. Statistical analyses were performed
603 by GraphPad Prism 8.0 software. Student's *t*-test was used to determine the pairwise
604 comparisons at the following significance levels (*P < 0.05, **P < 0.01, ***P < 0.001). For
605 multiple comparisons, one-way ANOVA with Tukey's Honest Significant Difference tests used
606 to separate means at P < 0.05.
607

608 ## Acknowledgments

609 This research was supported by the National Natural Science Foundation of China, grant
610 number 32102193, and the Open Competition Program of Ten Major Directions of Agricultural
611 Science and Technology Innovation for the 14th Five-Year Plan of Guangdong Province, grant
612 number 2022SDZG07.
613

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631 Writing-review & editing: Jiayun Li, Paul Holford, George Andrew Charles Beattie, Xiaoge Nian,
632 Yijing Cen.
633

634 ## Competing interests

635 The authors declare no conflict of interest.
636

637 ## Data Availability Statement

638 The published article includes all data generated or analyzed during this study. The full
639 sequences were submitted to NCBI, accession number: MG550150.1 for *DcAKH*, OR259432
640 for *DcAKHR*, OP251123 for *DcMet*, XM_026820026.1 for *DcKr-h1*, XM_008488883.3 for
641 *DcVg-1-like*, XM_026832896.1 for *DcVg-A1-like* and OP251122 for *DcVgR*.
642

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