Pesticide-induced resurgence in brown planthopper is mediated by action on a suite of genes that promote juvenile hormone biosynthesis and female fecundity

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Abstract

Pesticide-induced resurgence, increases in pest insect populations following pesticide application, is a serious threat to the sustainable control of many highly damaging crop pests. Resurgence can result from pesticide-enhanced pest reproduction, however, the molecular mechanisms mediating this process remain unresolved. Here we show that brown planthopper (BPH) resurgence following emamectin benzoate (EB) exposure results from the coordinated action of a diverse suite of actors that regulate juvenile hormone (JH) levels, resulting in increased JH titer in adult females and enhanced fecundity. Following reports of BPH resurgence in rice crops when this species is exposed to EB, we demonstrate that EB treatment results in profound changes in female BPH fitness including enhanced ovarian development and elevated egg production. This enhanced reproductive fitness results from the EB-mediated upregulation of key genes involved in the regulation of JH, including *JHAMT, Met and Kr-h1* and the downregulation of allatostatin (*AstA*) and allatostatin receptor (*AstAR*) expression. The remodulation of gene expression following EB exposure is dependent on the action of this insecticide on its molecular target the glutamate-gated chloride channel (GluCl) receptor. Collectively, these results provide mechanistic insights into the regulation of negative pesticide-induced responses in insects and reveal the key actors involved in the JH-signaling pathway that underpin pesticide resurgence.

Keywords: resurgence; reproduction; pesticide; brown planthopper

Significance

Pesticides remain a key means of controlling many of the world's insect pests, however, in some cases, pesticide applications can result in resurgence of pest populations due to pesticide-induced increases in fecundity. In the current study we show that pesticide resurgence in the brown planthopper (BPH) following exposure to the insecticide emamectin benzoate results from the transcriptional reprogramming of a diverse suite of positive and negative regulators of juvenile hormone (JH), a critical regulator of insect development and reproduction. This in turn leads to profound increases in female BPH reproductive fitness and enhanced fecundity. Our findings unravel the molecular mechanisms mediating pesticide-induced pest resurgence and inform the development of novel strategies to control highly damaging crop pests.

47 **Introduction**

48 Chemical pesticides remain the primary means of controlling many of the world's
49 most damaging arthropod crop pests (1, 2). However, pesticide applications can result
50 in pest resurgence, increases in pest insect populations that exceed natural, untreated
51 population sizes, following an initial reduction of the pest population (3-5). Two
52 mechanisms have been implicated in pest resurgence - the loss of beneficial insects
53 including natural enemies and pesticide-enhanced pest reproduction (3). In the case
54 of the latter, several pesticides, such as the insecticides triazophos, deltamethrin and
55 the fungicide jinggangmycin, have been reported to stimulate pest reproduction (6-8).
56 Pesticide-enhanced pest reproduction has been linked to changes in physiology and
57 biochemistry of pest organisms after exposure to pesticides (3, 4). However, the
58 molecular mechanisms underlying enhanced reproduction associated with pest
59 resurgence remain poorly resolved.

60 The brown planthopper (BPH), *Nilaparvata lugens* (Stål), is a notorious pest of
61 rice crops throughout Asia causing annual losses of ~300 million dollars across major
62 rice producing countries (2, 3). BPH inhibits the growth of rice plants by feeding, and
63 also transmits highly damaging plant viruses including rice grassy stunt virus and rice
64 ragged stunt virus (9). Currently, chemical insecticides play an indispensable role in
65 the control of BPH due to their efficiency, rapid effect, and low cost. However, due to
66 the widespread and intensive use of chemical insecticides, BPH has developed
67 resistance to the majority of compounds used for control (2, 10).

68 Emamectin benzoate (EB) and abamectin are avermectin pesticides, and act as

69 allosteric modulators of insect glutamate gated chloride channels (GluCls), inhibiting
70 muscle contractions that lead to the cessation of insect feeding and subsequent death
71 (11). These insecticides exhibit particularly strong activity against Lepidoptera such as
72 the rice leaffolder, *Cnaphalocrocis medinalis* Guénee, an important foliage-feeding
73 insect which attacks rice during the vegetative stage (12). Both BPH and the rice
74 leaffolder are migratory pests with overlapping migratory paths, however, their
75 occurrence period in the field differs by approximately one month, with leaffolders
76 appearing earlier than BPH. Therefore, the use of EB to control rice leaffolder has the
77 potential to impact BPH arriving later, via exposure to sublethal concentrations of this
78 compound. In this regard, we have observed that when farmers use EB and abamectin
79 to control leaffolders on rice crops in China, BPH outbreaks frequently occur in the
80 same field. While sublethal doses of certain pesticides have been shown to enhance
81 fecundity in BPH, including the insecticides triazophos and deltamethrin (6, 7, 13, 14)
82 and the fungicides carbendazim and jinggangmycin (8), whether avermectins trigger
83 resurgence in BPH via insecticide-enhanced reproduction remains unclear.
84 Reproduction in insects is influenced by external factors such as light (15),
85 temperature (16), humidity (17) and nutrition (18), and endogenous factors such as the
86 juvenile hormone (JH) (19), ecdysone (20), insulin (21) and TOR (22) pathways (23,
87 24). Of these, JH has been particularly implicated in insecticide-induced enhanced
88 fecundity, with triazophos and deltamethrin treatments leading to increased circulating
89 JH III titers in BPH females (3). JH is synthesized and secreted by the corpora allata
90 in insects (25), and can promote reproduction by regulating the synthesis and secretion

91 of vitellogenin (vg) in the female fat body, and stimulating the absorption of vg by the
92 developing oocyte (19). However, the regulation of JH is complex (19) and the key
93 actors involved in JH-mediated pesticide-enhanced reproduction remains an open
94 question. Previous finding reported that GluCl receptor is involved in JH biosynthesis
95 in cockroach (26-28). However, we know little whether insecticide which target on the
96 GluCl can stimulate reproduction in insect pest and how GluCl receptor regulates JH
97 production is still unknown.

98 In this study, we used a diverse range of approaches to investigate the impact of
99 sublethal doses of avermectins on BPH fecundity, and unravel the molecular
100 mechanisms mediating enhanced reproduction following exposure to this insecticide
101 class. We show that avermectin exposure results in profound changes in the
102 expression of a key suite of genes that in combination regulate JH, resulting in
103 increased JH titer in adult females, which promotes fecundity. Interestingly, we found
104 that GluCl channel modulators can inhibit Ast/AstAR expression and then promotes JH
105 increasing.

106

107 **RESULTS**

108 **GluCl allosteric modulators (emamectin benzoate and abamectin) stimulate**

109 **fecundity of female *N. lugens***

110 To investigate whether GluCl modulators affect fecundity in BPH, we first

111 determined the sub-lethal and median lethal doses of emamectin benzoate (EB) to

112 4th instar nymphs, newly emerged males and females of BPH (Table S1). For this we

113 employed two different bioassay methods, the rice seedling dip bioassay method and

114 topical application bioassay method (2, 29), in order to assess both the systemic and

115 contact toxicity of these insecticides (Table S1). We then systemically treated 4th

116 instar nymphs of BPH with the estimated LC_{15} and LC_{50} concentrations of EB and

117 examined the fecundity of BPH after these individuals mated with treated or

118 untreated individuals. We use the term “t” to represent individuals treated with EB

119 and “ck” to indicate control individuals that were treated with insecticide diluent minus

120 insecticide. After treatment with the LC_{15} and LC_{50} concentrations of EB, the number

121 of eggs laid per female of BPH in ♀t × ♂t crosses increased by 1.48 and 1.40 times

122 compared with control ♀ck × ♂ck crosses (Figure 1A); the number of eggs laid per

123 female of BPH in ♀t × ♂ck crosses increased by 1.53 and 2.07 times compared with

124 control crosses (Figure 1B); However, the number of eggs laid by per female of BPH

125 in ♀ck × ♂t crosses did not increase significantly compared to control ♀ck × ♂ck

126 crosses (Figure 1C).

127 **Table S1.** Determination of the toxicity of emamectin benzoate against BPH in systemic and topical application bioassays.

Treatment method	Developmental stages	Slope ± SE	LC_{15} (95%F.L.) (mg/L) or LD_{15} (95%F.L.) (pg/insect)	LC_{50} (95%F.L.) (mg/L) or LD_{50} (95%F.L.) (pg/insect)	χ^2(df)	*P value*
Systemic route	4th nymph	2.56 ± 0.487	0.87 (0.39-1.32)	2.21 (1.53-2.82)	2.68 (4)	0.61
	Male	2.42 ± 0.35	0.80 (0.40-1.20)	2.13 (1.48-2.78)	2.12 (4)	0.72
	Female	2.47 ± 0.41	2.33 (1.09-3.56)	6.12 (4.18-8.00)	3.56 (4)	0.50
Contact route	4th nymph	2.52 ± 0.35	15.54 (9.46-21.42)	39.90 (30.66-49.56)	2.60 (4)	0.63
	Male	2.77 ± 0.57	6.72 (2.52-10.92)	15.96 (9.66-21.84)	1.23 (3)	0.75

	Female	3.31 ± 0.76	18.48 (6.72-28.14)	37.80 (23.10-50.40)	0.79 (3)	0.85

131 Exposure of 4th instar nymphs with the LC_{15} and LC_{50} concentrations of EB in

132 contact bioassays also significantly stimulated fecundity. After treatment with the LC_{15}

133 and LC_{50} concentrations of EB, the number of eggs laid by per female of BPH in ♀t ×

134 ♂t crosses increased by 1.18 and 1.26 times compared with the control (♀ck × ♂ck)

135 (Figure 1D); The number of eggs laid per female of BPH in ♀t × ♂ck crosses

136 increased by 1.27 and 1.56 times compared with the control crosses (Figure 1E);

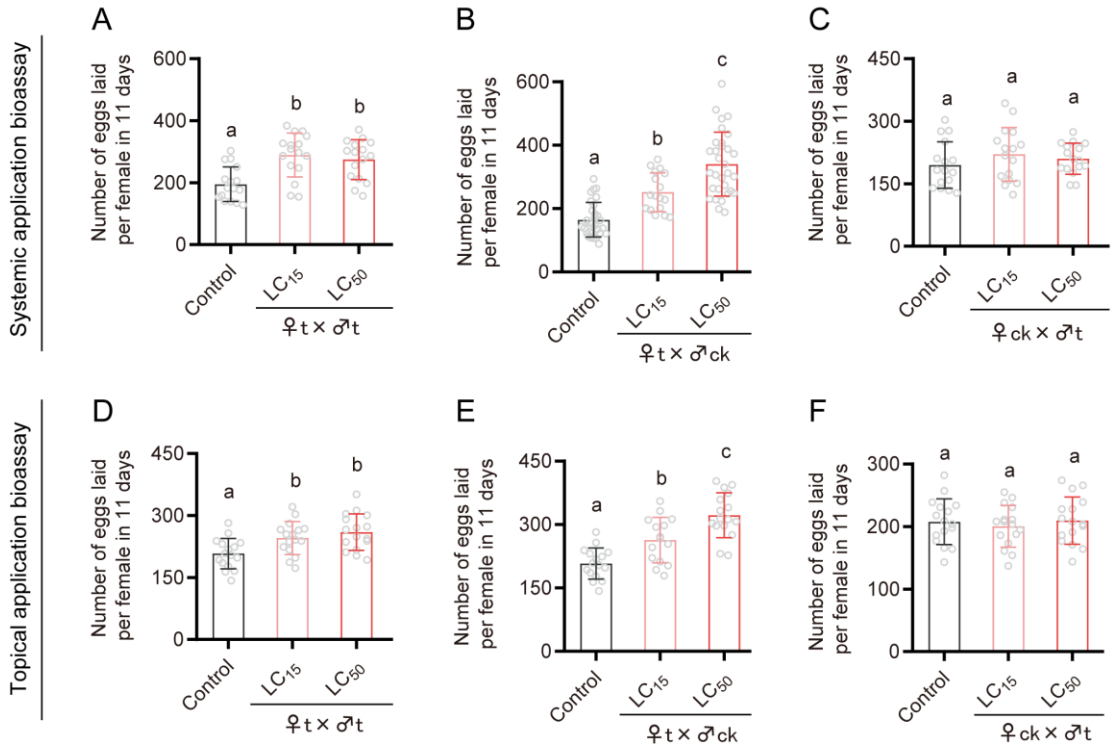
137 However, there was no significant difference in egg-laying number between ♀ck × ♂t

138 crosses and controls (♀ck × ♂ck) (Figure 1F). These results reveal that EB can

139 stimulate the fecundity of females following both the systemic and contact routes of

140 exposure.

141 **Figure 1**



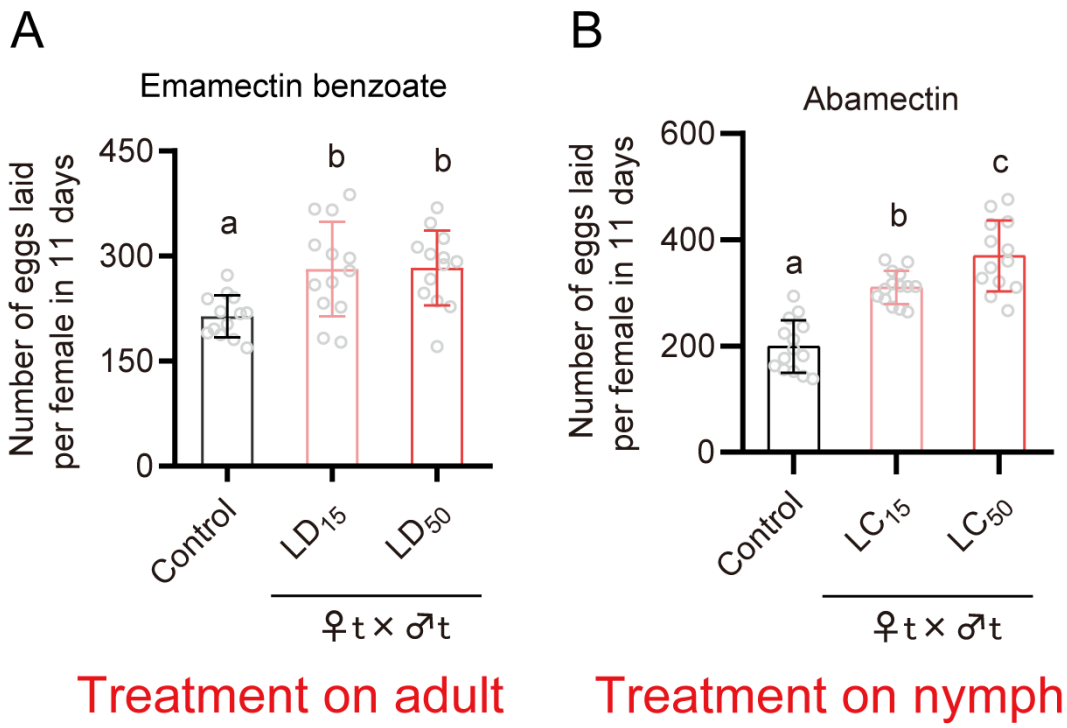
142

143 **Figure 1.** Fecundity of BPH following exposure to sub-lethal (LC_{15}) and median lethal (LC_{50})

144 concentrations of emamectin benzoate following system application bioassays (A: ♀t × ♂t;

145 B: ♀t × ♂ck; C: ♀ck × ♂t) and topical application bioassays (D: ♀t × ♂t; E: ♀t × ♂ck; F:

146 ♀ck × ♂t), respectively. The letter "t" represents treatment with insecticide, while "ck"
147 indicates controls that was not treated with insecticide. All data are presented as the mean ±
148 s.e.m. Different lower-case letters above the bars indicate significant differences (One-way
149 ANOVA with Tukey's Multiple Range Test, $p < 0.05$).
150
151 We next examined whether EB treatment of adult BPH also stimulates
152 reproduction. Indeed, treating newly emerged adults with the LC_{15} and LC_{50}
153 concentrations of EB significantly stimulated the number of eggs laid per female
154 (Figure 1-figure supplement 1A). Furthermore, sub-lethal exposure of 4th instar BPH
155 nymphs to another GluCl allosteric modulator, abamectin (LC_{15} and LC_{50}
156 concentrations) was also found to significantly enhance reproduction (Figure 1-figure
157 supplement 1B).
158 **Figure 1-figure supplement 1**

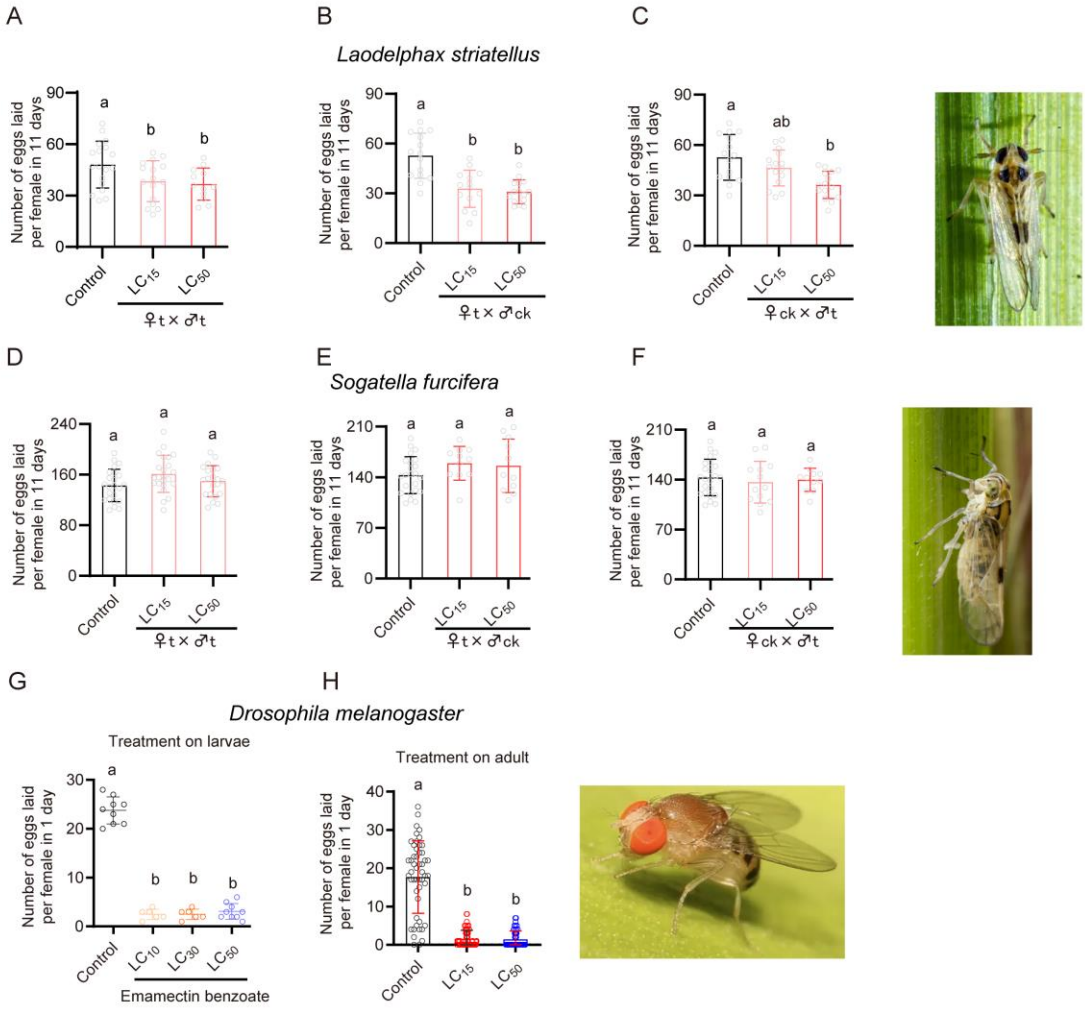


159
160 **Figure 1-figure supplement 1.** (A) Fecundity of BPH when newly emerged adults were
161 treated with sub-lethal (LD_{15}) and median lethal (LD_{50}) concentrations of emamectin benzoate

162 via topical application. (B) Fecundity of BPH when 4th instar nymphs were treated with sub-
163 lethal (LC_{15}) and median lethal (LC_{50}) concentrations of abamectin via systemic exposure. All
164 data are presented as the mean ± s.e.m. Different lower-case letters above the bars indicate
165 significant differences (One-way ANOVA with Tukey's Multiple Range Test, $p < 0.05$).

166 To examine if EB also stimulates egg-laying in other insect species we
167 conducted bioassays on the small brown planthopper, *Laodelphax striatellus*, the
168 white backed planthopper, *Sogatella furcifera* and fruit flies *Drosophila melanogaster*.
169 In contrast to our findings on BPH, we found that sub-lethal doses (LC_{15} and LC_{50}) of
170 EB inhibits fecundity of female *L. striatellus*, (Figure1-figure supplement 2A-C) and
171 has no impact on the fecundity of *S. furcifera*, (Figure1-figure supplement 2D-F). In
172 addition, we found that sublethal doses (LC_{15} or LC_{50}) of EB also inhibit fecundity in
173 *D. melanogaster* (Figure1-figure supplement 2G and H). These results indicate that
174 the stimulation of reproduction by EB in BPH is species-specific and does not extend
175 to even related insect species.

176 **Figure 1-figure supplement 2**



177

178 **Figure 1-figure supplement 2.** Fecundity of small brown planthopper, *Laodelphax striatellus*,

179 (A-C) white backed planthopper, *Sogatella furcifera* (D-F) and fruit fly, *Drosophila*

180 *melanogaster* (G and H) when larvae and newly emerged adults were treated with sub-lethal

181 concentrations of emamectin benzoate. All data are presented as the mean ± s.e.m. Different

182 lower-case letters above the bars indicate significant differences (One-way ANOVA with

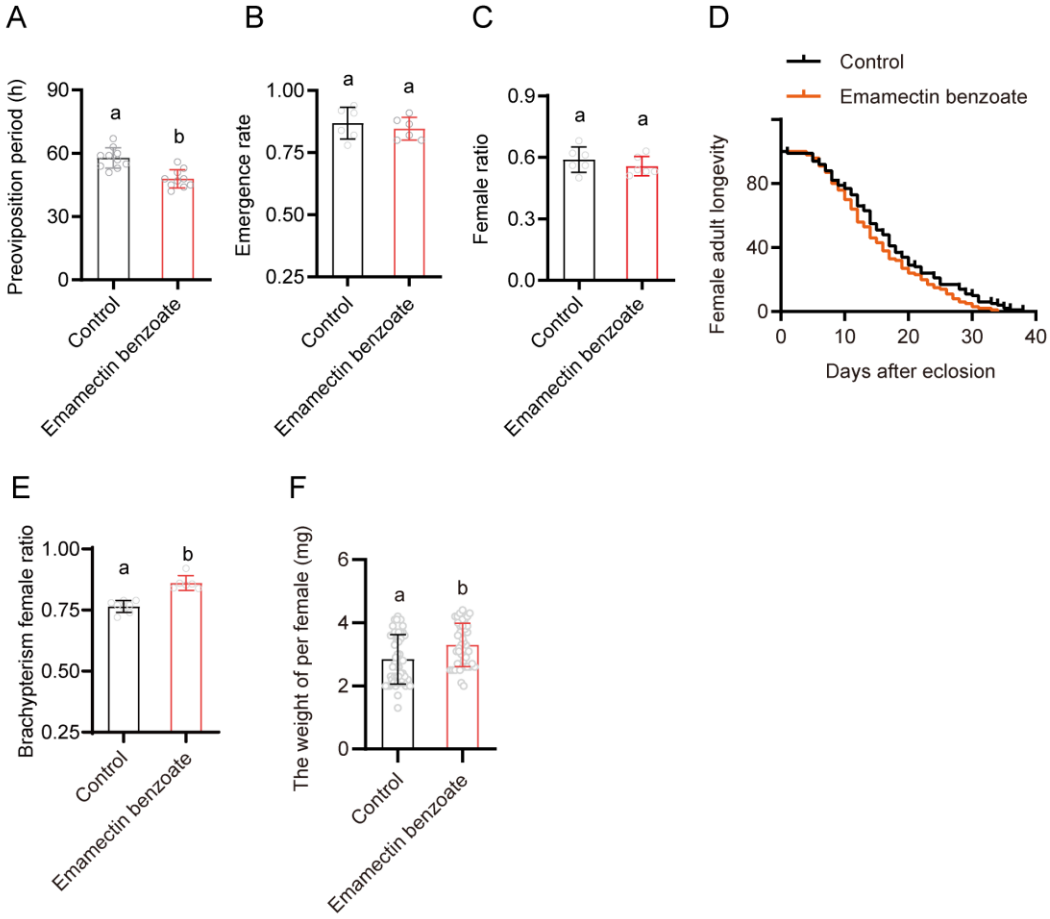
183 Tukey's Multiple Range Test, $p < 0.05$).

184

185 **The impact of EB treatment on BPH reproductive fitness**

186 To better understand the effects of EB on the reproductive fitness of BPH, the
187 preoviposition period, emergence rate, female ratio, female longevity and female
188 weight were evaluated following exposure using the systemic bioassay. The
189 preoviposition period of females treated with the LC_{50} of EB decreased significantly
190 compared with the control (Figure 1-figure supplement 3A). In contrast no significant
191 effects of EB on emergence rate and female ratio were observed (Figure 1-figure
192 supplement 3B and C). Female survival analysis showed that exposure of 4th instar
193 nymphs to the LC_{50} of EB has no impact on female longevity (Figure 1-figure
194 supplement 3D). Interestingly, brachypterism (long-wing) female ratio and female
195 weight were significantly increased after EB exposure (Figure 1-figure supplement 3E
196 and F).
197

198 **Figure 1-figure supplement 3**



199

200 **Figure 1-figure supplement 3.** The impact of emamectin benzoate on the reproductive

201 fitness of BPH. Fourth instar nymphs were treated with the LC_{50} concentration of emamectin

202 benzoate in systemic bioassays. All data are presented as the mean ± s.e.m. Different lower-

203 case letters above the bars indicate significant differences (Student's *t* test, $p < 0.05$).

204

205 **EB promotes ovarian development in BPH**

206 To investigate the cause of increased female weight following EB exposure we

207 examined if EB influences ovarian development in BPH. We dissected and compared

208 the ovaries of females treated with the LC_{50} of EB at 1, 3, 5 and 7 days after eclosion

209 (DAE) with control females. At 3, 5 and 7 DAE, the number of detained eggs of BPH

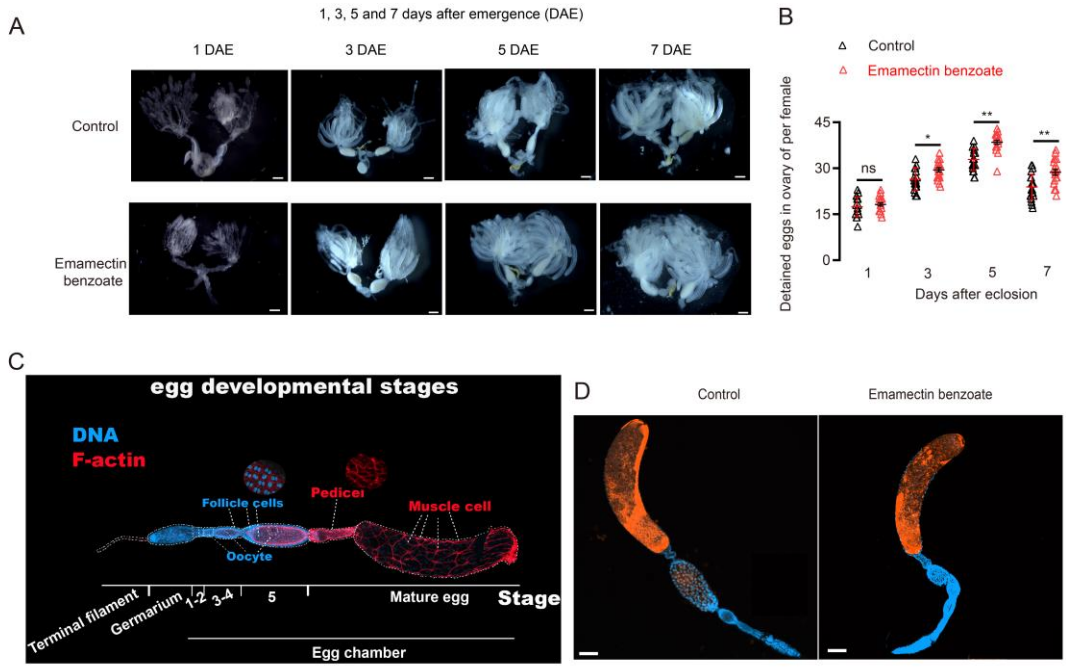
210 in the EB treated group were significantly higher than that of controls (Figure 2A and

211 B). We also explored whether EB treatment could enhance or impair oogenesis in

212 BPH. However, dissection of various developmental stages revealed that emamectin

213 benzoate treatment has no significant effects on oogenesis (Figure 2C and D).

214 **Figure 2**



215

216 **Figure 2.** The impact of emamectin benzoate on ovarian development in BPH. Fourth instar

217 nymphs were treated with the LC_{50} concentration of emamectin benzoate in systemic bioassays.

218 (A) Ovarian development in EB treated BPH at 1, 3, 5 and 7 days after eclosion (DAE)

219 compared to untreated controls. Scale bar = 1,000 μm. (B) Number of detained eggs in the

220 ovaries of EB treated BPH females measured at 1, 3, 5 and 7 DAE compared to controls. All

221 data are presented as the mean ± s.e.m. Asterisks indicate values significantly different from

222 the control using student *t* test (ns, no significant; $*p < 0.05$ and $**p < 0.01$). (C) Different

223 developmental stages of BPH eggs. (D) No impairment of emamectin benzoate on oogenesis

224 of BPH. Scale bar = 100 μm.

225

226 **EB exposure enhances the abundances of storage macromolecules and**

227 **circulating sugars in BPH**

228 Nutritional status is an important indicator of reproductive fitness. Thus, to

229 investigate whether EB affects intermediary metabolism and energy storage of BPH,

230 glycogen, triacylglyceride (TAG), total protein content, cholesterol and four circulating

231 carbohydrates were quantified in 4th instar BPH nymphs following exposure to the

232 LC_{50} of EB.

233 We found that EB exposure has no impact on glycogen levels (Figure 2-figure

234 supplement 1A). The amounts of TAG in EB-treated BPH were 27% higher ($p < 0.05$)

235 than those in controls, but only in BPH of the late fifth instar (5L) stage, with no

236 significant differences observed in subsequent developmental stages (Figure 2-figure

237 supplement 1B). The amount of total protein content in EB-treated BPH was higher

238 than the control groups in the case of all developmental stages from 5L nymph to

239 7DAE (Figure 2-figure supplement 1C). EB exposure also increased cholesterol

240 levels at 4 and 5 DAE (Figure 2-figure supplement 1D). Compared with the solvent

241 control, EB treatment caused significant increases ($p < 0.05$) in the levels of sucrose,

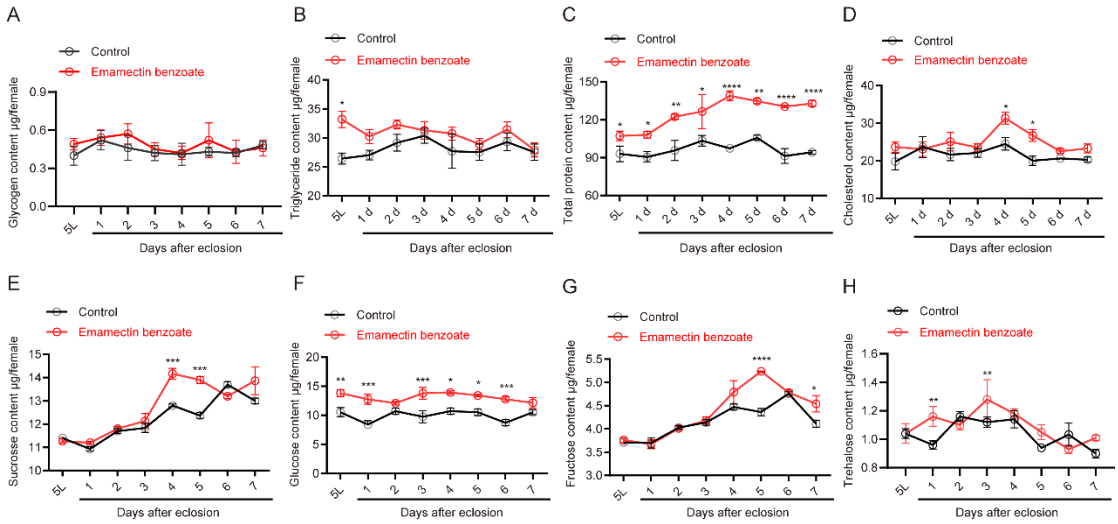
242 glucose, fructose, and trehalose (Figure 2-figure supplement 1E-H). Thus,

243 collectively, these data provide evidence that EB exposure leads to energy

244 mobilization and the metabolism of carbohydrates and lipids in BPH.

245

246 **Figure 2-figure supplement 1**



247

248 **Figure 2-figure supplement 1.** Amounts of Glycogen (A), TAG (B), total protein content (C),
249 cholesterol (D) and four circulating sugars including sucrose, glucose, fructose and trehalose
250 (E-H) after BPH exposure to EB. All data are presented as the mean ± s.e.m. The differences
251 between the EB-treated and solvent-treated BPH were analyzed using unpaired student *t*-test
252 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

253

254 **EB stimulates egg-laying that is mediated by the JH signaling pathway**

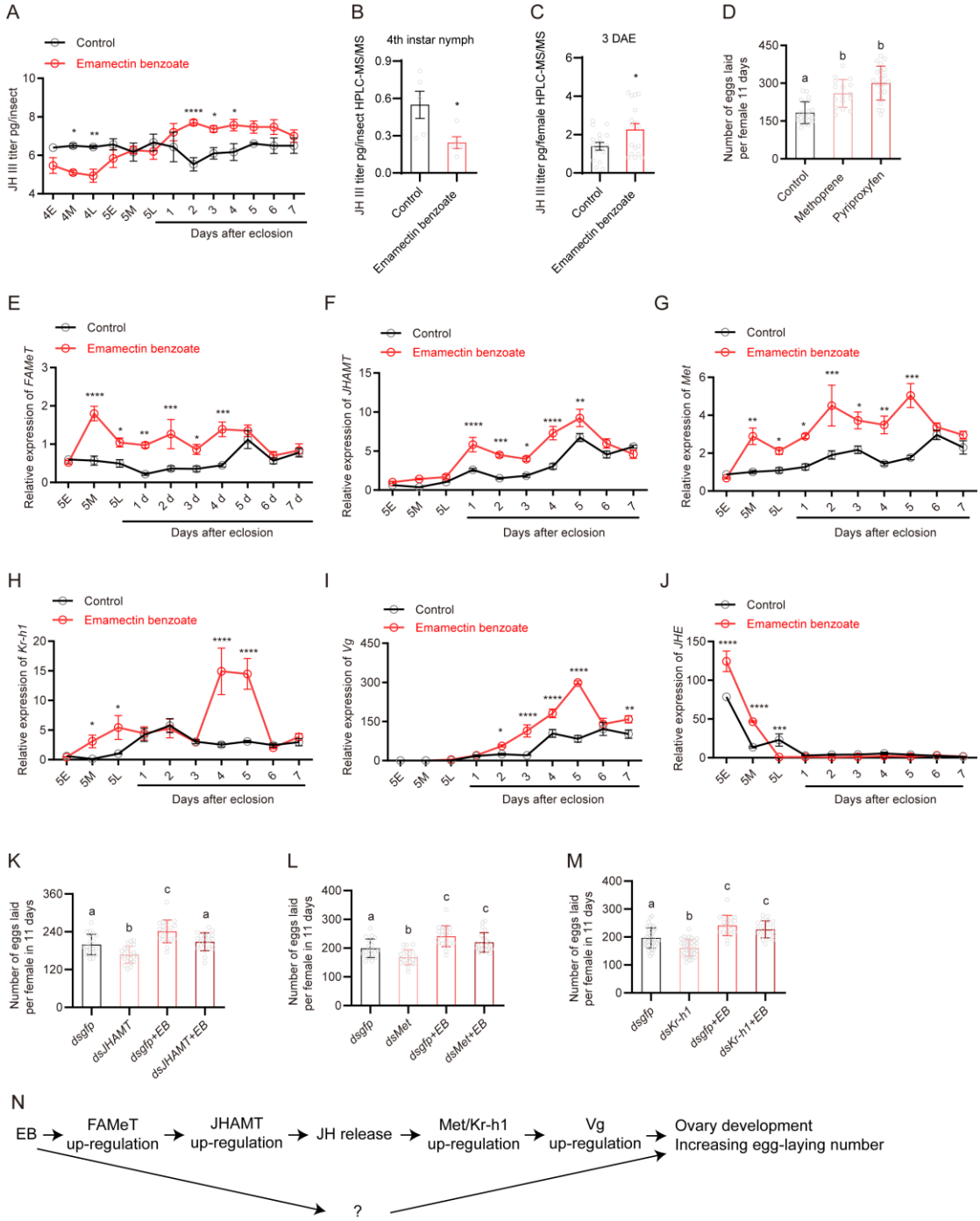
255 Given the important role of juvenile hormone (JH) in vitellogenesis and egg
256 development in insects (19, 25, 30-32), we asked whether EB-treatment could
257 influence the titer of JH in BPH. As measured by ELISA, the juvenile hormone titer of
258 BPH nymphs treated with the LC_{50} concentration of EB was significantly lower than
259 that of controls in systemic bioassays during the middle and late stages of the 4th
260 instar (Figure 3A). However, at 2, 3 and 4 DAE, the JH titer in the EB treated group
261 was significantly higher than that of the control (Figure 3A). Interestingly, the titer of
262 another important insect hormone, the steroid ecdysone, was not significantly
263 different between EB-treated BPH and solvent-treated controls (Figure 3-figure

264 supplement 1). To independently validate the results of ELISA assays, we employed
265 HPLC-MS/MS to measure JH titer in BPH following EB exposure (30, 33, 34). The
266 results showed that JH III titer significantly decreased after EB-treatment at the late
267 4th instar nymph stage (Figure 3A and B), but significantly increased at the third day
268 after eclosion (3 DAE) (Figure 3A and C). To further investigate the role of JH in EB-
269 enhanced fecundity in BPH, we treated BPH with methoprene and pyriproxyfen, JH
270 analogues or biologically active JH mimics respectively, to determine whether they
271 can stimulate fecundity in BPH. Both compounds significantly increased egg-laying in
272 BPH (Figure 3D). Takes together these results reveal that EB stimulates an increase
273 in JH titer that induces fecundity in BPH. Since we found that EB could induce JH
274 synthesis in the BPH, we asked whether EB could influence the expression of genes
275 that are involved in JH synthesis or degradation. For this we treated 4th instar
276 nymphs of BPH with the LC_{50} concentration of EB using systemic bioassays, and
277 then collected early (5E), middle (5M) and late (5L) stage of 5th instar nymph and 1-7
278 DAE female adults (DAE) for analysis. Quantitative PCR was then used to examine
279 the expression of key genes previously implicated in the regulation of JH (35, 36).
280 Farnesoic acid O-methyltransferase (FAMeT) (37), is known as an important
281 enzyme in the JH biosynthetic pathway, catalyzing methylation of farnesoic acid (FA)
282 to methyl farnesoate (MF) (36). We found that this gene was significantly upregulated
283 in 5M instar nymph to 4 DAE after EB- treatment (1.0-fold to 3.0-fold) (Figure 3E).
284 Another gene, juvenile hormone acid methyltransferase (JHAMT) (36, 38), which is
285 involved in the biosynthesis of JH, was also upregulated in EB-treated BPH at 1 to 5

286 DAE than controls (1.5-fold to 3.0-fold) (Figure 3F). Methoprene-tolerant (Met),
287 belongs the basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) family of transcription
288 factors and is the intracellular (nuclear) receptor of JH (39, 40). The levels of *met*
289 mRNA increased in EB-treated BPH at the 5M and 5L instar nymph and 1 to 5 DAE
290 stages compared to controls (1.7-fold to 2.9-fold) (Figure 3G). Krüppel homolog 1
291 (*Kr-h1*), a transcriptional target of JH signaling, is reported to be sensitive to levels of
292 JH and its expression levels are directly correlated with JH titers (35, 41, 42). We
293 found that *Kr-h1* was significantly upregulated in the adults of EB-treated BPH at the
294 5M, 5L nymph and 4 to 5 DAE stages (4.7-fold to 27.2-fold) (Figure 3H). Similarly, the
295 expression levels of vitellogenin (Vg), a key downstream component of JH signaling
296 triggering ovarian development in insects including BPH (43), was markedly
297 increased in females at 2–5 DAE by EB (1.7-fold to 5.5-fold) (Figure 3I). Juvenile
298 hormone esterase (JHE) is the primary JH-specific degradation enzyme that plays a
299 key role in regulating JH titers (44). Interestingly, we observed a significant
300 upregulation of JHE mRNA levels in the early and middle 5th instar nymph stage
301 followed by downregulation in 5L instar nymphs of EB-treated BPH (1.3-fold to 2.6-
302 fold) (Figure 3J). In combination these results reveal that EB has profound impacts
303 on the expression of key genes involved in the synthesis of JH or downstream
304 signaling pathway genes that might promote egg development.
305 To further understand whether these JH pathway-related genes were involved in
306 egg-laying behavior in BPH, we performed RNAi experiments to downregulate the
307 expression of *JHAMT, Met and Kr-h1* (Figure 3-figure supplement 2A-C). We found

308 that silencing of these three genes downregulated the titer of JH in BPH providing
309 functional evidence of their role in the regulation of JH (Figure 3-figure supplement
310 2D). Furthermore, silencing of *Kr-h1* inhibited *FAMeT* and *Vg* gene expression while
311 increasing *JHE* gene expression (Figure 3-figure supplement 2E-H). Importantly,
312 silencing of *JHAMT*, *Met* and *Kr-h1* gene in female BPH was also found to suppress
313 egg-laying (Figure 3K-M). However, this phenotype was rescued by EB treatment,
314 which restored egg-laying to normal levels in BPH injected with *JHAMT*, *Met* and *Kr-*
315 *h1* dsRNA (Figure 3K-M). Together these results provide a mechanistic
316 understanding of how EB enhances fecundity in BPH by modulating the expression
317 of key genes involved in JH synthesis (Figure 3N).
318

319 **Figure 3**

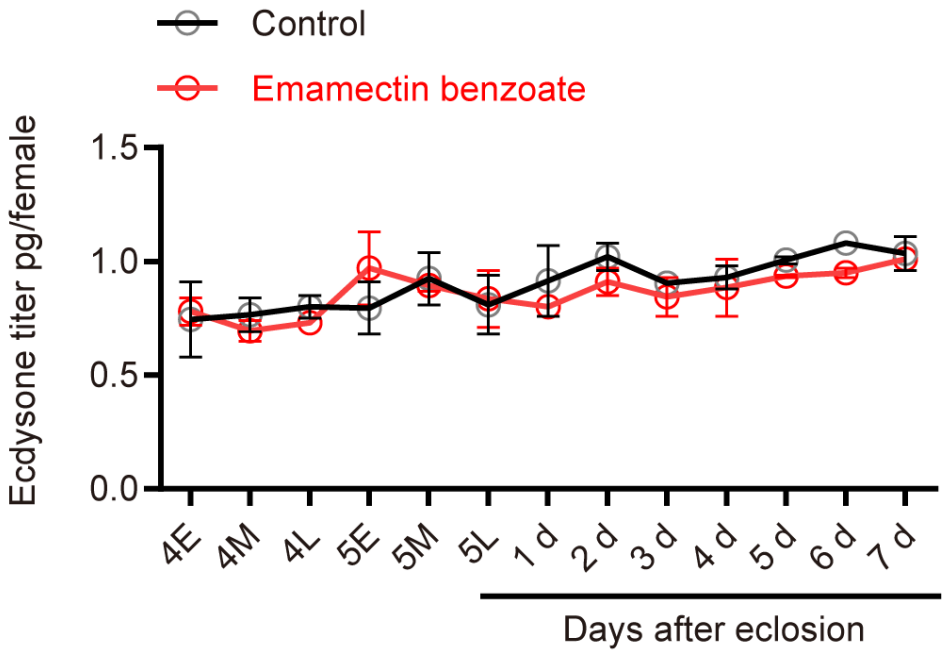


320

321 **Figure 3. EB induced reproduction in BPH is mediated by components of the JH**
322 **signaling pathway.** (A) The titer of JH III (as measured by ELISA assay) at different
323 developmental stages of BPH when 4th instar nymphs were treated with the median lethal
324 concentrations of EB. (B and C) The titer of JH III (as measured by HPLC-MS/MS) in BPH
325 females at 4L and 3 DAE when treated with median lethal concentrations of EB. (D) Oviposition
326 rate of BPH when 4th instar nymphs were treated with 4 ppm methoprene or 10 ppm

327 pyriproxyfen. (E-J) Expression of selected JH-related genes (*FAMeT*, *JHAMT*, *Met*, *Kr-h1*, *Vg*,
328 and *JHE*) in EB-treated BPH. (K) Egg production following silencing of *JHAMT* with or without
329 EB application. (L) Egg production following silencing of *met* with or without EB application. (M)
330 Egg production after silencing *Kr-h1* with or without EB application. (N) Schematic illustrating
331 the proposed impact of EB on the JH signaling pathway leading to enhanced reproduction. The
332 question mark indicates one or more potential additional signals. All data are presented as
333 means ± s.e.m. Student's *t* test was used to compare the two samples. One-way ANOVA with
334 Tukey's multiple comparisons test was used to compare more than two samples. ns, no
335 significant difference; Asterisks indicate values significantly different from the control (ns, no
336 significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). Different lower-case letters
337 above the bars indicate significant differences ($p < 0.05$).

338 **Figure 3-figure supplement 1**



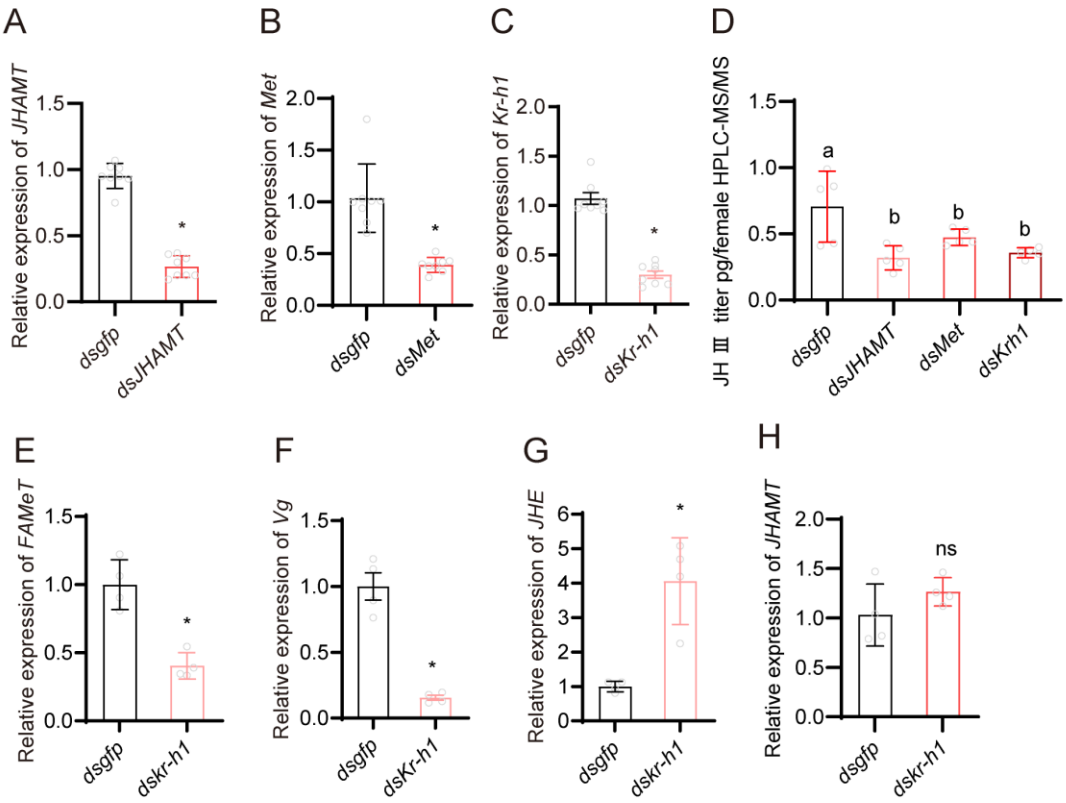
339

340 **Figure 3-figure supplement 1.** Ecdysone titer at different developmental stages of BPH when
341 4th instar nymphs were treated with median lethal concentrations of EB.

342

343

344 **Figure 3-figure supplement 2**



345

346 **Figure 3-figure supplement 2.** (A-C) Expression of *JHAMT* (A), *Met* (B) and *Kr-h1* (C)
347 following RNAi knockdown. (D) The titer of JHⅢ (as measured by HPLC-MS/MS) at 3 DAE
348 when female adults were injected with *dsJHAMT, dsMet,* and *dsKr-h1* at 1 DAE. (E-H)
349 Expression of *FAMeT*, *Vg*, *JHE* and *JHAMT* when *Kr-h1* was silenced in BPH. All data are
350 presented as means ± s.e.m. *$p < 0.05$; Mann–Whitney test.

351

352 **EB induces JH biosynthesis through the peptidergic AstA/AstAR signaling**
353 **pathway**

354 The timing and level of JH biosynthesis can be precisely regulated by the
355 neuropeptides, stimulatory allatotropins (ATs) and inhibitory allatostatins (Asts), in
356 many insects (45-52). Insects can, in a species-specific manner, produce one type of
357 AT and three types of Asts: FGL-amide Ast (AstA) (46, 53); myoinhibitory peptide
358 (MIP or AstB) and PISCF Ast (AstC) (50, 54). In some species, there also exists two
359 paralogue genes of AstCs which are named AstCC and AstCCC (55, 56).
360 Interestingly, the allatostatic activity of these three types of Ast peptides varies
361 between insect species so that in each species only one type of Ast (for example
362 AstA) controls JH production (50, 51, 54, 57).

363 Analysis of the BPH neural transcriptome sequence data has revealed the
364 presence of one AT, four types of Asts and four corresponding receptors, allatotropin
365 receptor (A16, ATR), AstA receptor (A2, AstAR), AstB (MIP) receptor (A10, AstBR or
366 MIPR) and AstC receptor (A1, AstCR) (58). We cloned the five neuropeptide genes
367 (AT, AstA, AstB/MIP, AstCC and AstCCC) and confirmed the sequence obtained from
368 transcriptome data (Figure 4-figure supplement 1) (58). Interestingly, we found that
369 AstC is missing in the genome of BPH and only AstCC and AstCCC are present
370 (Figure 4-figure supplement 1). Next, we also cloned their corresponding receptors
371 (55) including ATR (A16), AstAR (A2), AstBR (A10) and AstCR (A1) which might be
372 activated by AstCC and/or AstCCC (42, 55, 59). Sequence alignments and
373 phylogenetic analysis are shown in Figure 4-figure supplement 2.

374 Quantitative PCR was then used to examine if EB treatment could influence the
375 expression of the genes encoding these neuropeptides and their receptors. Treating
376 BPH with the LC_{50} concentration of EB significantly increased the expression of *AT*,
377 *ATR* and *AstCCC* while resulting in the downregulation of *AstA, AstB/mip, AstCC*,
378 AstAR and *AstBR/mipr* at the adult stage (Figure 4A and B, Figure 4-figure
379 supplement 3A-G). Among these, *AstA* and *AstAR* were the most downregulated
380 genes after EB treatment (Figure 4A and B, Figure 4-figure supplement 3H) and thus
381 the *AstA/AstAR* signaling system was selected for subsequent functional analysis.
382 Silencing of *AstAR* in female BPH using RNAi (Figure 4C), resulted in an increased
383 number of eggs laid per female compared with *dsgfp*-injected controls (Figure 4D).
384 Interestingly, silencing *AstAR* also resulted in the upregulation of *FAMeT, JHAMT,*
385 *Met* and *Kr-h1* which are involved in the JH biosynthesis/signaling (Figure 4E-H).
386 However, *JHE* was not influenced by AstAR silencing (Figure 4I). We therefore
387 investigated whether silencing the *AstAR* gene could influence JH titer in BPH. A
388 significantly increased JH titer was observed in *AstAR* silenced BPH compared with
389 controls (Figure 4J). Thus, our data strongly suggest that AstA is a key inhibitor of JH
390 production in BPH.
391 Finally, we investigated whether injection of mature Ast and AT peptides could
392 influence the number of eggs laid and JH titer in BPH. We synthesized one AT, six
393 AstAs (AstA1 to AstA6), one AstCC and one AstCCC peptide according to their
394 determined sequences (Figure 4-figure supplement 1). Indeed, we found that
395 injection of AstA1 and AstA6 reduced the number of eggs laid per female in 48 h

396 (Figure 4K). We also observed that AstA1 injection after 16h and 48h decreased the

397 JH titer significantly (Figure 4L), and AT injection increased the JH titer after 2h but

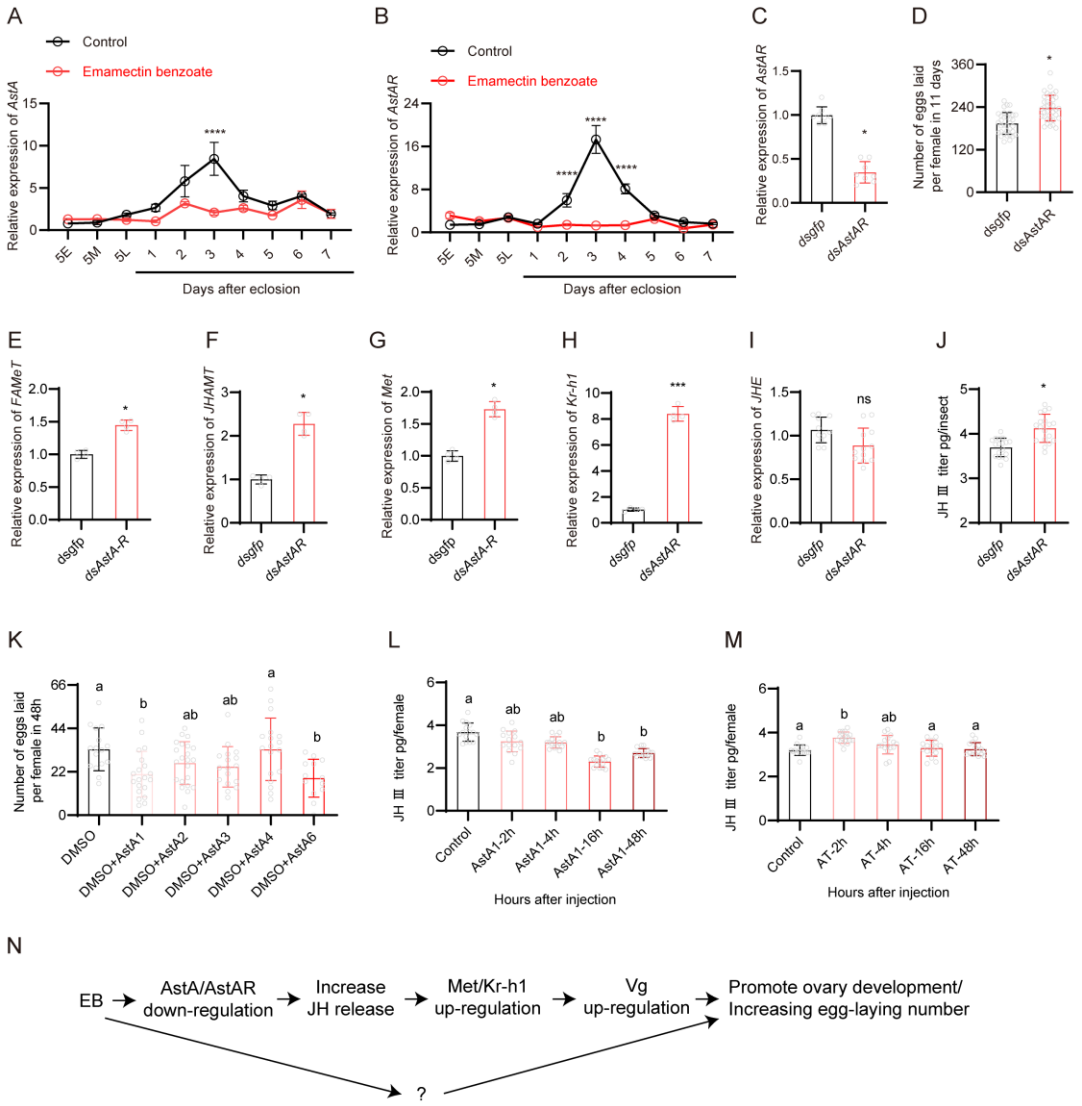
398 levels returned to normal 4h after injection (Figure 4M). Collectively, these results

399 provide compelling evidence that EB induces reproduction in BPH through the

400 AstA/AstAR and JH signaling pathways (Figure 4N) and further supports the role of

401 AstA and AT in regulation of JH titer.

402 **Figure 4**



403

404 **Figure 4. EB induced reproduction in BPH is mediated by the AstA/AstAR and JH**

405 **signaling pathway.**

406 (A and B) Expression of *AstA* and *AstAR* in different stages of BPH following EB treatment. (C)

407 Downregulation of *AstAR* using RNAi leads to a reduction in mRNA expression level. (D) Egg

408 production in female BPH following silencing of *AstAR* gene. (E-I) Expression of selected JH

409 signaling pathway related genes (*FAMeT*, *JHAMT*, *Met*, *Kr-h1* and *JHE*) in *AstAR* silenced BPH.

410 (J) JHIII titer of BPH females after *AstAR* gene silencing determined by HPLC-MS/MS. (K)

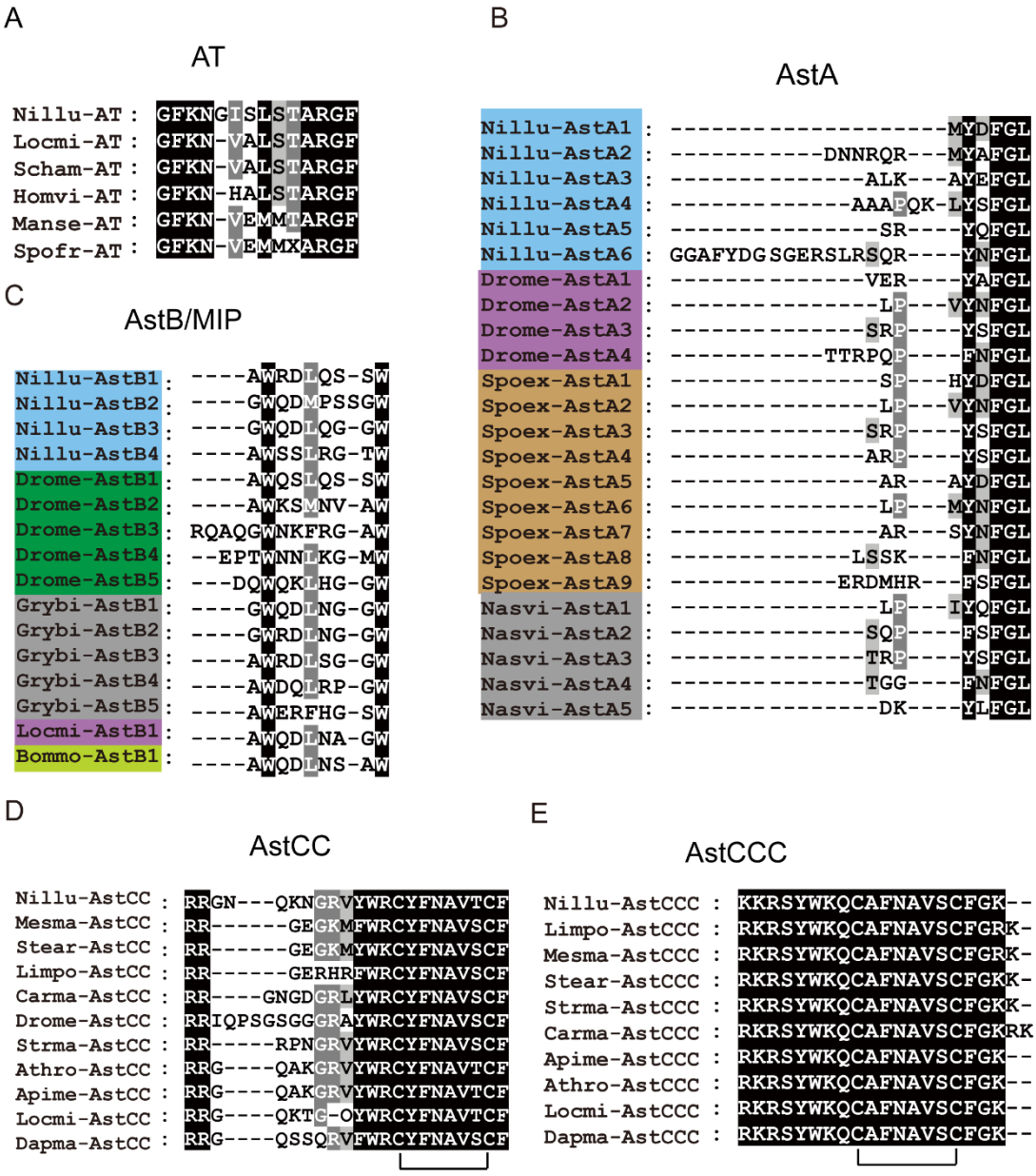
411 Number of eggs laid per female in 48h following injection of six AstA1-AstA6 mature peptides

412 and one AT mature peptide. Fifty nanoliter of PBS (as control) and seven peptides (20

413 pmol/insect) were injected into female BPH three days after eclosion. (L and M) The JH III titer

414 of BPH females at different time points following AstA or AT injection. (N) Schematic of the
415 proposed role of AstA/AstAR in the regulation of JH following EB exposure. The question
416 mark indicates one or more possible additional signals. All data are presented as means ± s.e.m.
417 Student's *t* test was used to compare the two samples. One-way ANOVA with Tukey's multiple
418 comparisons test was used to compare more than two samples. ns, no significant difference;
419 Asterisks indicate values significantly different from the control (ns, no significant; *$p < 0.05$, **p
420 < 0.01, ***$p < 0.001$ and ****$p < 0.0001$). Different lower-case letters above the bars indicate
421 significant differences ($p < 0.05$).
422

423 **Figure 4-figure supplement 1**

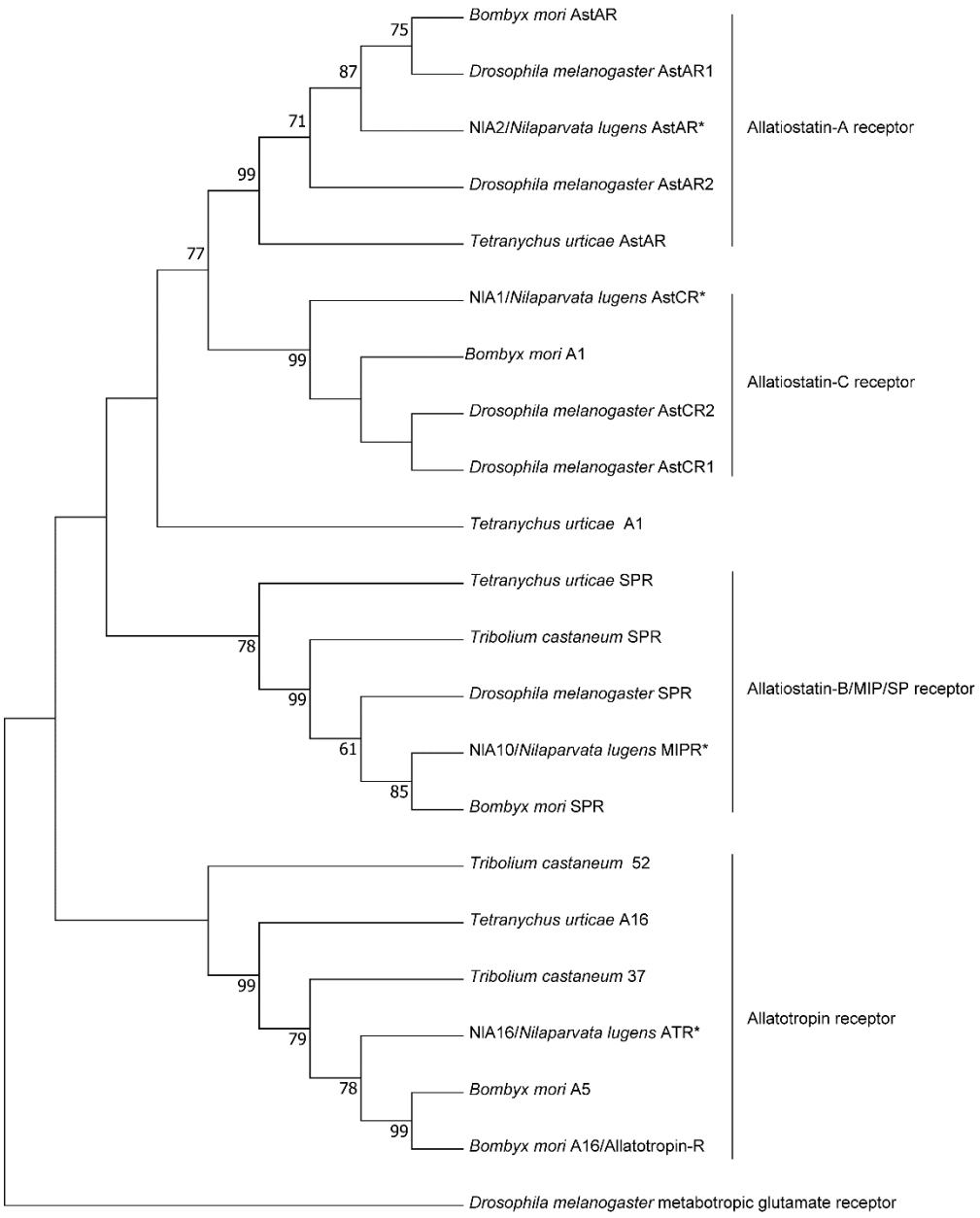


424

425 **Figure 4-figure supplement 1.** Alignments of the amino acid sequences of: (A) AT, (B) AstA,
426 (C) AstB/MIP, (D) AstCC and (E) AstCCC peptides from select species. AT, AstA, AstB/MIP and
427 AstCCC are predicted to have a C-terminal amide. The mature peptides belonging to the same
428 species have been highlighted with the same color. Species names are as follows: Nillu
429 (*Nilaparvata lugens*), Locmi (*Locusta migratoria*), Scham (*Schistocerca americana*), Homvi
430 (*Homalodisca vitripennis*), Manse (*Manduca sexta*), Spofr (*Spodoptera frugiperda*), Drome
431 (*Drosophila melanogaster*), Spoex (*Spodoptera exigua*), Nasvi (*Nasonia vitripennis*), Grybi
432 (*Gryllus bimaculatus*), Bommi (*Bombyx mori*); Mesma (*Mesobuthus martensii*), Stear

433 (*Stegodyphus araneomorph*), Limpo (*Limulus polyphemus*), Carma (*Carcinus maenas*), Strma
434 (*Strigamia maritima*), Athro (*Athalia rosae*), Apime (*Apis mellifera*), Dapma (*Daphnia magna*).
435 Black lines under the sequences indicate the locations of the disulfide bridges in the mature
436 peptides. The accession numbers of the sequences are listed in Figure 4-figure supplement 1
437 source data.
438

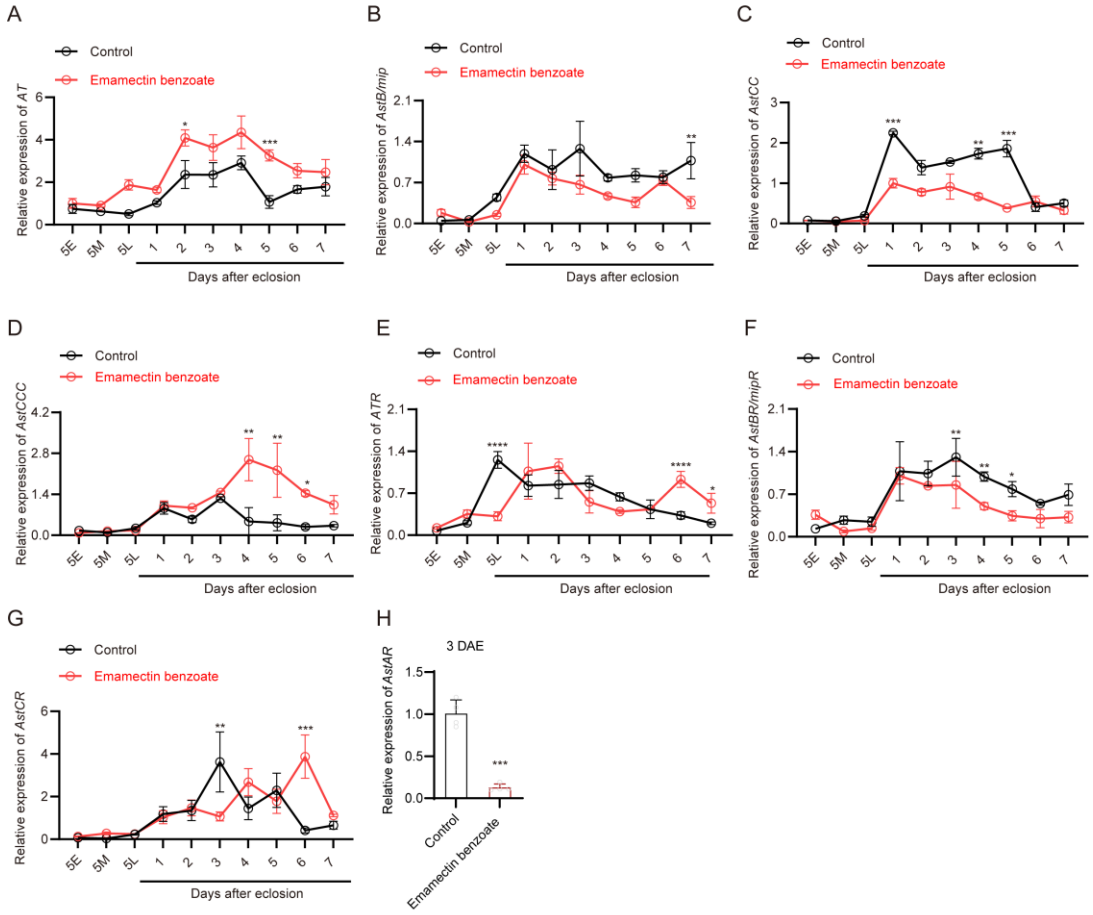
439 **Figure 4-figure supplement 2**



440

441 **Figure 4-figure supplement 2.** Phylogenetic tree of the predicted BPH (*) allatotropin receptor
442 (A16, ATR), allatostatins A receptor (A2, AstAR), AstB (MIP) receptor (A10, AstBR or MIPR)
443 and allatostatins C receptor (A1, AstCR) with other insect species. The tree was generated
444 using the maximum likelihood method. *Drosophila melanogaster* metabotropic glutamate
445 receptor was included as an outgroup. The accession numbers of the sequences used for this
446 phylogenetic tree are listed in Figure 4-figure supplement 2 source data.

447 **Figure 4-figure supplement 3**



448

449 **Figure 4-figure supplement 3. EB induced changes in the expression of *AT*, *AstB*, *AstCC*,**

450 ***AstCCC*, *ATR*, *AstBR* and *AstCR* in BPH.** All data are presented as means ± s.e.m. Student's

451 t test was used to compare the two samples. ns, no significant difference; Asterisks indicate

452 values significantly different from the control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

453

454 **EB-enhanced fecundity in BPH is dependent on its molecular target protein the**

455 **GluCl**

456 EB and abamectin, are allosteric modulators, which target glutamate-gated

457 chloride channels (GluCls) (60-62). Hence, we examined whether EB-stimulated

458 fecundity in BPH is influenced by its molecular target GluCl. The full length *GluCl*

459 coding sequence from BPH was cloned and sequenced (Figure 5-figure supplement

460 1) and the impact of EB on *GluCl* gene expression examined using quantitative PCR.

461 Treatment of BPH with the LC_{50} concentration of EB significantly downregulated

462 *GluCl* gene expression at the 5E and 5M nymph stages while upregulating *GluCl*

463 gene expression at 2 DAE and 5 DAE in the adult stage (Figure 5A). To examine the

464 role of *GluCl* gene in BPH fecundity, RNAi was used to knockdown expression of this

465 gene in female BPH (Figure 5B). A significant decrease in the number of eggs laid by

466 per female was observed in *dsGluCl*-injected insects compared with *dsgfp*-injected

467 insects (Figure 5C). However, treatment with EB was found to rescue the decreased

468 egg-laying phenotype induced by *dsGluCl* injection (Figure 5C). To investigate the

469 mechanism by which *GluCl* expression modulates fecundity we examined if silencing

470 *GluCl* influences JH titer and JH-related gene expression. Indeed, we observed that

471 RNAi knockdown of *GluCl* leads to a decrease in JH titer (Figure 5D) and down-

472 regulation of genes including *JHAMT* which is responsible for JH synthesis, and the

473 JH signaling downstream genes *Met and Kr-h1* (Figure 5E-G). In contrast,

474 expression of *FAMeT* and *JHE* were not changed in the *GluCl* silencing insects

475 (Figure 5H and I). We also examined whether silencing *GluCl* impacts the

476 *AstA*/*AstAR* signaling pathway. Silencing *GluCl* was found to have no impact on the

477 expression of *AT*, *AstA*, *AstB*, *AstCC*, *AstAR*, and *AstBR*. However, the expression of

478 *AstCCC* and *AstCR* was significantly upregulated in *dsGluCl*-injected insects (Figure

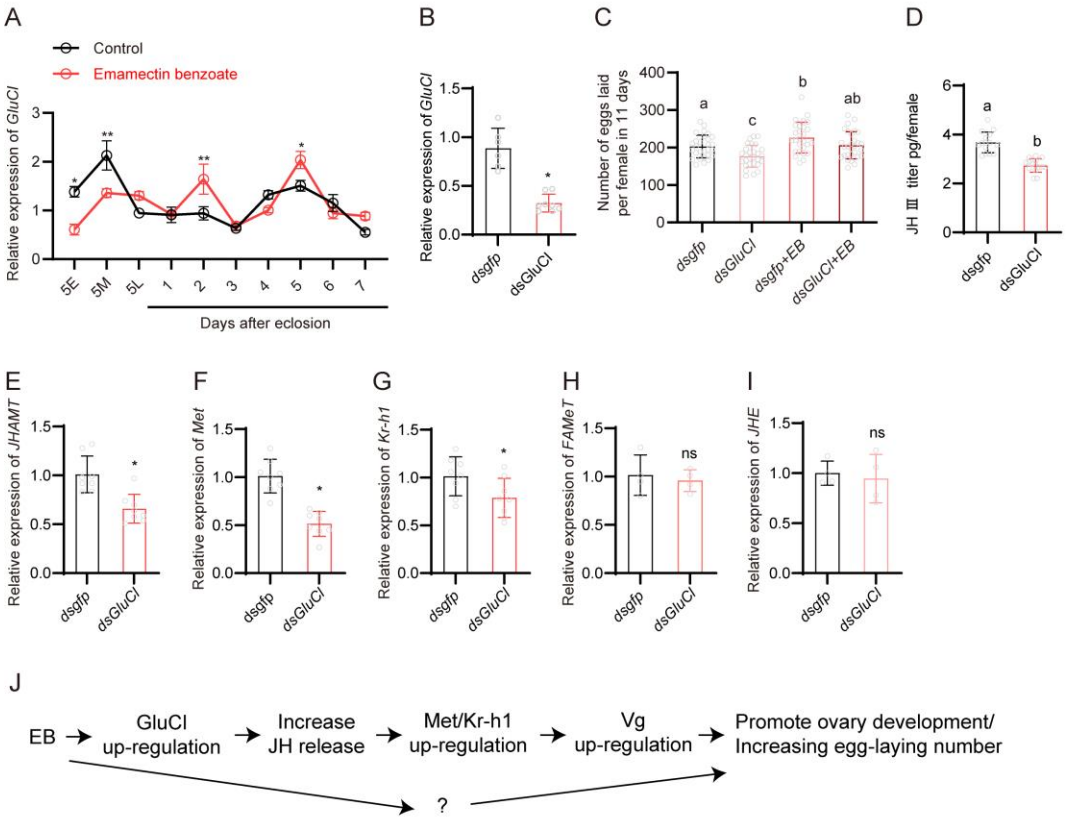
479 5-figure supplement 2A-H). These results suggest that EB activates *GluCl* which

480 induces JH biosynthesis and release, which in turn stimulates reproduction in BPH

481 (Figure 5J).

482

483 **Figure 5**



484

485 **Figure 5. EB induced reproduction in brown planthoppers through its molecular target**

486 **protein GluCl.**

487 (A) Expression of *GluCl* in EB-treated and untreated BPH. (B) Expression of *GluCl* following

488 injection of *dsGluCl* in BPH. (C) Egg production after *GluCl* gene knockdown in EB-treated and

489 untreated BPH. (D) The JH III titer of BPH females after *GluCl* gene silencing as quantified

490 using the ELISA method. (E-I) Expression patterns of selected JH-related genes (*JHAMT*, *Met*,

491 *Kr-h1*, *FAMeT* and *JHE*) in *GluCl* silenced BPH. (J) Schematic of the proposed role of GluCl as

492 a molecular target of EB and EB-enhanced reproduction in BPH. The question mark indicates

493 one or more possible additional signals. All data are presented as means ± s.e.m. Student's t

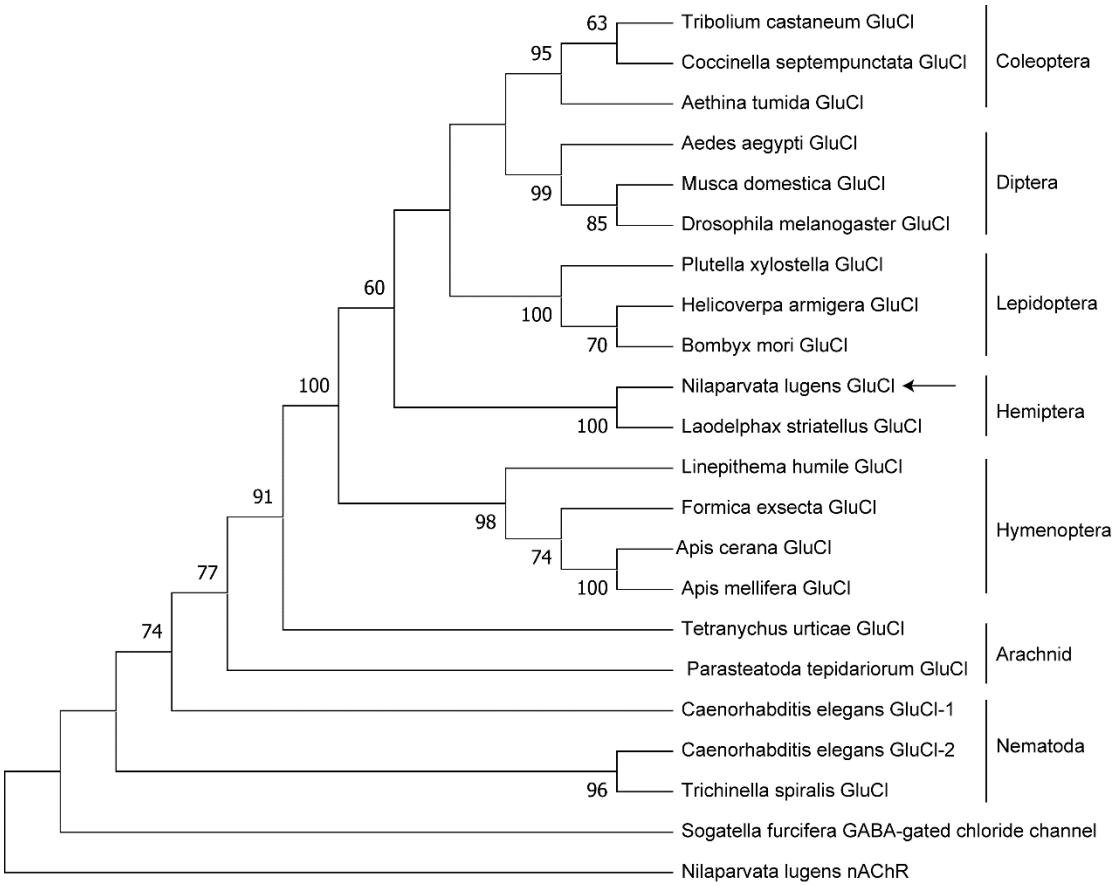
494 test was used to compare the two samples. One-way ANOVA with Tukey's multiple

495 comparisons test was used to compare more than two samples. ns, no significant difference;

496 Asterisks indicate values significantly different from the control (ns, no significant; *$p < 0.05$ and

497 **$p < 0.01$). Different lower-case letters above the bars indicate significant differences ($p < 0.05$).

498 **Figure 5-figure supplement 1**

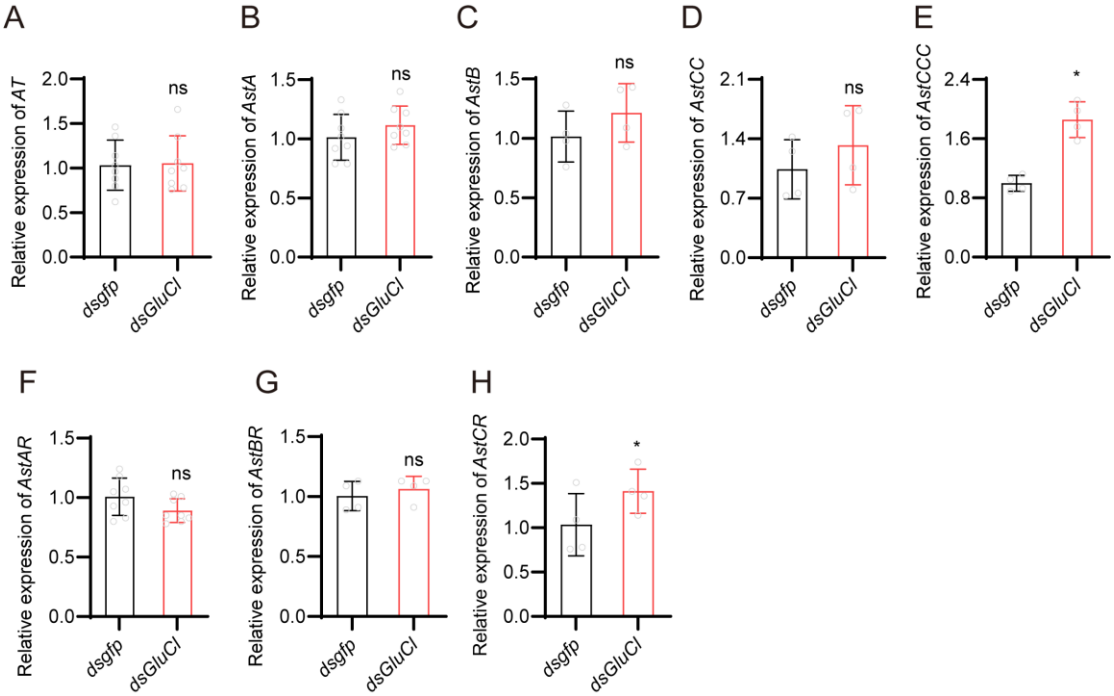


499

500 **Figure 5-figure supplement 1.** Phylogenetic analysis of glutamate-gated chloride channels in
501 different species. The numbers at the nodes of the branches represent the percentage
502 bootstrap support (1000 replications) for each branch. The *Sogatella furcifera* GABA-gated
503 chloride channel and *Nilaparvata lugens* nAchR were used as outgroup. Alignment was
504 performed with amino acid sequences from TM1-7. The receptor names are listed in the tree.
505 The accession numbers of the sequences used for this phylogenetic tree are listed in Figure 5-
506 figure supplement 1 source data.

507

508 **Figure 5-figure supplement 2**



509

510 **Figure 5-figure supplement 2.** The expression of *AT*, *AstA*, *AstB*, *AstCC*, *AstCCC*, *AstAR*,

511 *AstBR* and *AstCR* in BPH injected with *dsGluCl or dsgfp*. All data are presented as means ±

512 s.e.m. Student's *t* test was used to compare the two samples. ns, no significant. Different lower-

513 case letters above the bars indicate significant differences ($p < 0.05$).

514

515 **Discussion**

516 Pesticide-induced resurgence of pest insects is a serious problem in rice and
517 several other cropping systems (3). However, the mechanisms underpinning pesticide-
518 enhanced reproduction in insects remain poorly understood. Here we reveal that a
519 suite of molecular actors underlie this trait that, in combination, mediate profound
520 physiological changes in the reproductive fitness of female BPH. Our data provide
521 fundamental insights into the molecular mechanisms by which xenobiotics modify
522 insect reproduction and have applied implications for the control of a highly damaging
523 crop pest. We discuss these topics below.

524

525 **Sublethal doses of GluCl modulators, EB and abamectin, stimulates fecundity in**
526 **BPH**

527 We show that in both contact and systemic assays EB and abamectin stimulate
528 reproduction in BPH. Thus, insecticide-enhanced reproduction is likely a key factor in
529 the BPH resurgence observed when farmers use EB and abamectin to control
530 leaffolders in rice crops in China (29). Although this is the first report of sublethal doses
531 of avermectins enhancing insect fecundity, our findings are consistent with previous
532 studies which have shown that certain insecticides, herbicides and fungicides stimulate
533 BPH reproduction (3, 7, 14, 63-68). Intriguingly, we show that EB only induces
534 fecundity in female adults and is specific to BPH, with EB exposure failing to enhance
535 reproduction in two related species, the small brown planthopper, *L. striatellus* and the
536 white backed planthopper, *S. furcifera*, or the model insect *D. melanogaster*. Thus, the

537 mechanisms underpinning this trait appear to act exclusively on female BPH and may
538 be specific to this species.

539 Pesticides may stimulate insect reproduction through a variety of physiological and
540 molecular mechanisms. Our data reveal that exposure to sub-lethal concentrations of
541 EB results in profound changes to female BPH fitness, leading to increases in female
542 weight, total protein content, cholesterol content, sugar content and egg production
543 and decreases in duration of the preoviposition period. Some of these findings exhibit
544 parallels with previous studies, which demonstrated that treating third-instar BPH
545 nymphs with either deltamethrin, triazophos, or imidacloprid led to increased soluble
546 sugar levels in the corresponding adults (69). Such metabolites provide the energy that
547 drives BPH reproduction and resurgence (3). Thus, together with prior work, our results
548 suggest that pesticides associated with resurgence stimulate nutritional intake in BPH
549 to fuel enhanced energy-intensive reproduction.

550

551 **The JH signaling pathway plays an essential role in EB-induced fecundity in BPH**

552 JH is a pleiotropic hormone which plays important roles in development and
553 reproduction in insects (19, 70). Circulating JH titers are regulated by factors that
554 control JH production in the corpora allata including biosynthetic enzymes and
555 catabolic enzymes that regulate JH levels. Our results show that EB increases
556 circulating JH III titers in BPH females over 2–4 days after eclosion (DAE) and
557 promotes ovarian development. Previous studies have reported that triazophos and
558 deltamethrin treatments also lead to increased circulating JH III titers in BPH females

559 over 1–3 days post emergence. Similarly, jinggangmycin treatments were found to lead
560 to increased JH titers (by approximately 45–50%) in BPH females over two days post
561 emergence (71). Thus our findings, in combination with these previous studies,
562 demonstrate that insecticide treatments can have dramatic effects on the regulation of
563 key insect hormones involved in pest reproduction which can in turn drive pesticide
564 resurgence.

565 Although increased JH titers following pesticide exposure have been correlated
566 with reduced levels of active JH esterase during the first three days PE (6), the type
567 and number of mechanisms mediating the observed increase in hormone titer has
568 remained an open question. Our data reveal that elevated JH titer in EB-exposed BPH
569 is associated with the upregulation of genes that encode biosynthetic enzymes for JH
570 (*JHAMT*) and downstream signaling genes that can induce *vg* gene expression (*met*
571 and *kr-h1*). Using RNAi we provide functional evidence of the role of these genes in
572 the regulation of JH III and fecundity of female BPH, and demonstrate that EB can
573 restore the reduction in egg production resulting from the knockdown of *JHAMT*, *met*
574 and *kr-h1* expression.

575 JHMAT is an enzyme that catalyzes the conversion of inactive precursors of JH to
576 active JH in the final stages of JH biosynthesis (36, 38). Interestingly, while it has not
577 been previously implicated in pesticide resurgence, treatment of the stored product
578 pest *Sitotroga cerealella* with diallyl trisulfide, an insecticidal compound in garlic
579 essential oil, was found to increase JHAMT mRNA levels (72). Because JHMAT is the
580 key rate-limiting enzyme in regulation of JH titer our results suggest that its enhanced

581 expression is a key molecular mechanism of pesticide resurgence in BPH.

582 Met is a ligand-activated member of the basic helix–loop–helix Per/Arnt/Sim
583 (bHLH-PAS) transcription factors and is the intracellular receptor for JH (39, 40). Kr-h1
584 is a zinc finger protein that acts downstream of Met and is expressed in response to
585 JH signaling. Although the genes encoding these proteins have not been previously
586 linked to pesticide resurgence, our finding that they are upregulated following EB
587 exposure, and demonstration of their role of in promoting fecundity, is consistent with
588 previous studies. Specifically, treatment of BPH with JH III or the insecticidal analogues
589 methoprene or pyriproxifen was found to induce the expression of *Kr-h1* (73).
590 Furthermore, knockdown of *Met* and *Kr-h1* in BPH brachypterous females was found
591 to result in delayed ovariole development and this was significantly more pronounced
592 than the response observed in BPH treated separately with ds*NlMet* or ds*NlKr-h1* (74).
593 This finding provides evidence of a possible interaction between *Met* and *Kr-h1* and,
594 in combination with our data, suggests that *Met* and *Kr-h1* may act in concert to
595 mediate EB-enhanced fecundity.

596

597 **EB-induced fecundity in BPH is dependent on the allatostatin signaling pathway**

598 In addition to regulatory proteins that promote JH production, insects have
599 peptides that inhibit JH biosynthesis. These include the allatostatins: FGLamides
600 (FGLa; AstA), the W(X)6Wamides (AstB), and the PISCFs (AstC) (50, 51, 57, 58, 75,
601 76). Interestingly, our results showed that EB exposure results in the marked
602 downregulation of the expression of the genes encoding allatostatin *AstA* and its

603 receptor *AstAR*. We provide clear evidence of the functional impact of this on JH
604 synthesis and BPH fecundity by: i) demonstrating that RNAi knockdown of *AstAR*
605 expression results in increased JH titer and enhanced female egg production, and, ii)
606 showing that injection of female BPH with synthetic AStA peptide reduces JH titer and
607 decreases egg production. Thus, our data provide unequivocal evidence that AstA is a
608 key inhibitor of JH production in BPH. This finding is consistent with previous work
609 which has shown that FGLa/ASTs inhibit JH biosynthesis in cockroaches, and termites
610 (46, 77). To our knowledge, our study is the first report of insecticides inhibiting the
611 expression of the neuropeptide receptor, *AstAR*, and linking this to increases in JH titer
612 and enhanced reproduction in insects.

613 Interestingly knockdown of *AstAR* resulted in significant increases in the
614 expression of genes involved in JH synthesis/signaling including *FAMeT* and *JHAMT*
615 *Met* and *Kr-h1*. Related to this finding, previous work has shown that knockdown of the
616 AstA receptor gene, *Dar-2*, in *D. melanogaster* results in changes in the expression of
617 genes encoding Drosophila insulin-like peptides (DILPs) and adipokinetic hormone
618 (AKH) signaling proteins (78). Together with our findings, this demonstrates that AstA
619 receptors may modulate the expression of numerous downstream genes involves in
620 metabolism, energy store and reproduction. In the case of pesticide resurgence our
621 results imply significant cross-talk in the expression of genes that inhibit JH production
622 and those that promote it.

623

624 **The GluCl plays an essential role in EB-induced fecundity in BPH**

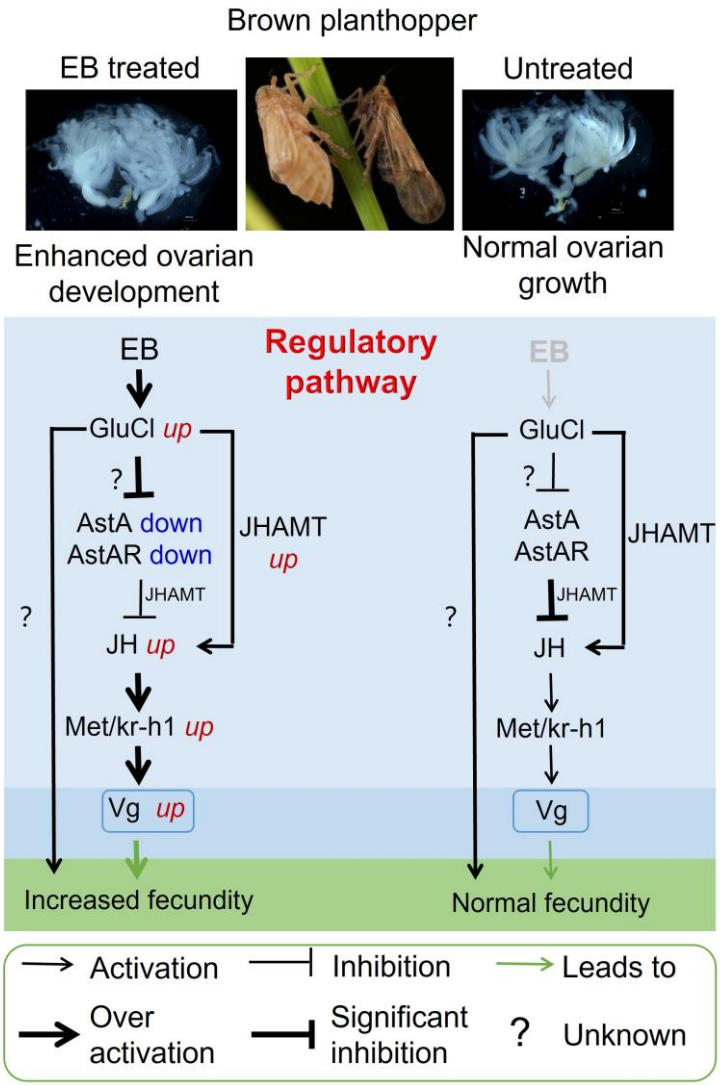
625 EB and abamectin are allosteric modulators of GluCls (60-62). Our data revealed
626 that EB exposure modifies expression of the GluCl in BPH, and knockdown of GluCl
627 expression resulted in a reduction in both JH levels and egg production. Interestingly,
628 the GluCl has been reported to inhibit the biosynthesis of JH in the cockroach,
629 *Diploptera punctata* (28). Recent work has also reported that modulation of
630 glutamatergic signals may contribute to the photoperiodic control of reproduction in
631 bean bug, *Riptortus pedestris* (79). Interestingly, work on *D. punctata* has revealed that
632 application of the GluCl channel agonist ivermectin, which like EB belongs to the
633 avermectin family, caused a decline in JH synthesis in corpus allatum glands (28).
634 While the inhibitory effect of ivermectin observed in this previous study differs from the
635 activating effect of EB we observed in our study, it is consistent with our finding of a
636 role for GluCl channel in the regulation of JH regulation. Interestingly, we found that
637 knockdown of *GluCl* gene expression results in the down-regulation of *JHAMT*, *Met*
638 *and Kr-h1*, further revealing significant convergent relationships between genes
639 underpinning pesticide resurgence.
640

641 **Conclusion**

642 Our study has revealed a diverse suite of genes that act in combination to enhance
643 JH titer and thus fecundity following BPH exposure to EB. A schematic of how these
644 factors promote ovarian development in the adult stage of *N. lugens* through the JH
645 signaling pathway is provided in Figure 6. Our findings provide the foundation for
646 further work to understand exactly how these genes interact and the mechanisms by

647 which their expression is activated or repressed by EB. Furthermore, our findings
648 provide fundamental insights into the molecular response in insects to xenobiotic
649 stress and illustrate that pesticides can have unexpected and negative impacts on pest
650 populations. In this regard our findings also have applied implications for the control of
651 a highly damaging crop pest. Previous studies have reported that avermectins such as
652 abamectin are toxic to the wolf spider *Pardosa pseudoannulata*, which is the main
653 predator of BPH in rice crops (80, 81). Thus, these insecticides both stimulate
654 reproduction in BPH while killing their natural enemies providing a 'perfect storm' for
655 damaging BPH outbreaks. Based on these findings, to avoid BPH resurgence, we
656 suggest that the GluCls, EB and abamectin, should not be (or rarely be) applied to rice
657 plants at growth stages when BPHs are present. On a more optimistic note, our
658 findings have identified numerous genes that play key roles in BPH reproduction and
659 thus represent promising targets for the development of novel controls against this
660 important pest.
661

662 **Figure 6**



663

664 **Figure 6. Schematic of the proposed regulatory pathway of EB-enhanced fecundity**
665 **in BPH.** Emamectin benzoate (EB) exposure results in the upregulation of genes that promote
666 JH production (*JHAMT, Met and Kr-h1*) and the downregulation of genes that inhibit it
667 (allatostatin, Ast*A* and allatostatin A receptor, *AstAR*). This transcriptome reprograming is
668 dependent on the action of EB on its molecular target the glutamate-gated chloride channel
669 (GluCl) receptor. The resulting increased JH titer promotes vg synthesis and increased
670 fecundity in EB exposed insects. We observe significant cross-talk in the expression of genes
671 that inhibit JH production and those that promote it, with *AstAR* inhibiting the expression of
672 *JHAMT, Met* and *Kr-h1* and *GluCl* activating the expression of *JHAMT* which is responsible for
673 JH synthesis, and the JH signalling downstream genes *Met and Kr-h1*.

674 ## Materials and methods

675 ### Insects

676 BPH was initially collected from Wanan, JiangXi Province in 2020, reared on
677 'Taichung Native 1' (TN1) rice seedlings in the laboratory without exposure to any
678 insecticides. The strain was maintained in a climatic chamber at 27 ± 1°C, with relative
679 humidity of 70 ± 10% and a light: dark = 16 h: 8 h photoperiod.

680 ### Chemicals

681 Emamectin benzoate (95.2%) was obtained from Hebei Weiyuan Co., Ltd. (Hebei,
682 China). Abamectin (96.8%) was obtained from Hebei Weiyuan Co., Ltd. (Hebei, China).
683 Pyriproxyfen (98%) was obtained from Shanghai Shengnong Co., Ltd. (Shanghai,
684 China). Methoprene (S)-(+) (mx7457-100mg) was purchased from Shanghai MaoKang
685 Biotechnology Co., Ltd., (Shanghai, China). Juvenile hormone standard sample
686 (J912305-10mg) was purchased from Shanghai Macklin Biotechnology Co., Ltd.,
687 (Shanghai, China).

688 ### Bioassay

689 Different life stages of insects were used to perform bioassay to investigate the
690 effects of insecticide on nymphs and adults. To test whether treatment of the nymph
691 stage of insects would promote reproduction in female, we used 4th instar nymphs of
692 BPH or 3rd instar nymph of *Laodelphax striatellus* and *Sogatella furcifera* to perform
693 bioassays. To test whether treatment of the adult stage of insects would promote
694 reproduction in female, we used newly emerged male and female BPH.

695 **Systemic route:** The rice-seeding dipping bioassay method was used to evaluate

696 the susceptibility of BPH, *L. striatellus* and *S. furcifera* to EB. Technical-grade
697 insecticides were dissolved in acetone as stock solution then diluted in a series of six
698 concentrations with water containing 0.1% Triton. Selected rice seedlings at the 6-8
699 cm growth stage were dipped in insecticide solutions for 30 s and then air-dried at
700 room temperature. The roots of the rice seedlings were wrapped with cotton strips and
701 placed seedlings placed in a plastic cup 5 cm in diameter. Fifteen insects were
702 introduced into each plastic cup for each replicate. The top of the cup was then selaed
703 with gauze to prevent escape. All experiments comprised at least three biological
704 replicates. Control rice seedings were treated with 0.1% Triton X-100 water solution
705 only. All treatments were maintained under standard conditions of 27 ± 1 °C and 70–
706 80% relative humidity with a 16 h light/8 h dark photoperiod. Mortality was assessed
707 after 4 d for *N. lugens* or 2 d for *L. striatellus* and *S. furcifera* after treatment with
708 insecticides. The insects were considered dead if they were unable to move after a
709 gentle prodding with a fine brush.

710 For *Drosophila* larvae bioassay, we adopted a method described previously in our
711 lab with minor modifications (82). Briefly, twenty third instar larvae were placed in fly
712 vials containing fly food (based on corn powder, brown sugar, yeast and agar)
713 supplemented with EB of different concentrations. Four concentrations (LC_{10}, LC_{30} and
714 LC_{50}) were tested together with a negative (no insecticide) control. For *Drosophila* adult
715 bioassays, we selected virgin females three days after eclosion. Several
716 concentrations were overlaid onto fly food in standard *Drosophila* vials and allowed to
717 dry overnight at room temperature. 15 adult flies (three days after eclosion) were then

718 added to each vial and mortality assessed after 2 d. Four replicates were carried out
719 for each concentration. Control mortality was assessed using vials containing food with
720 solvent minus insecticide.

721 **Contact route:** For topical bioassays working insecticide solutions were prepared
722 in acetone. 4th instar nymphs or newly emerged males/females were anesthetized with
723 carbon dioxide for 5 s, and then 0.04 μl/insect test solution applied topically to the
724 dorsal plates with a capillary micro-applicator (29). Insects were then placed in an
725 artificial climate incubator with a temperature of 27±1°C, a photoperiod of 16:8 h (L:D),
726 and a humidity of 70%±10%. Mortality was determined 2 d after treatment. Data with
727 over 20% mortality in the control treatment were discarded, and the assay was
728 repeated at least three times.

729 **Fecundity assays**

730 Fourth instar nymphs of BPH and 3rd instar nymph of *L. striatellus* and *S. furcifera*
731 were treated with the LC_{15} and LC_{50} of EB, and then transferred to fresh rice seedlings.
732 After eclosion, the adults were used in the following experiment. Newly emerged
733 treated adults and untreated adults were paired to produce four groups: untreated
734 males and untreated females (♂ck×♀ck; ck indicates untreated); untreated males and
735 treated females (♂ck×♀t; t indicates insecticide treated); treated males and untreated
736 females (♂t×♀ck); treated males and treated females (♂t×♀t). Each group comprised
737 at least 10 mating pairs. All pairs were transferred to glass cylinders (diameter 5 cm
738 and height 20 cm) containing rice plants (25 days old almost 20 cm high) as a food
739 source for eleven days. The number of eggs and nymphs in plants were counted by a

740 microscope.

741 For *Drosophila* egg-laying assay, we adopted our previous method (83). Briefly,
742 insecticide-treated virgin females were paired with untreated males for three days and
743 then the mated females transferred into the *Drosophila* ovipositing apparatus. The
744 number of eggs were counted after 16 hours.

745 **Fitness analysis**

746 The fitness of EB-treated BPH were analyzed using methods reported previously
747 (10). We selected two groups, (♂ck×♀ck) and (♂ck×♀t), to study the effects of the LC_{50}
748 concentration of EB on BPH fitness. In the case of systemic exposure, 4th instar
749 nymphs of BPH were treated with the LC_{50} of EB for 4 days and then transferred to
750 tubes containing untreated rice plants for individual rearing. The rice plants were
751 replaced every three days with untreated plants. The emergence ratio, female ratio,
752 preoviposition period, female longevity, brachypterism female ratio and female weight
753 were calculated.

754 **Examination of ovarian development**

755 Adult females from ♂ck×♀ck control and ♂ck×♀t group on 1, 3, 5, 7 DAE were
756 dissected for observe the ovarian development. The detained eggs in ovary were
757 photographed and recorded. Each group has at least fifteen replicates.

758 To examination whether EB treated impairs egg maturation, we dissected
759 untreated or EB-treated ovaries and fixed them in 4% paraformaldehyde (PFA) in
760 phosphate buffered saline (PBS) for 30 min at room temperature. After four washes of

761 10 min (4 × 10 min) in PAT3 (PBS with 0.5% Triton X-100 and 0.5% bovine serum
762 albumin), the ovaries were then incubated with DAPI (4',6-diamidino-2-phenylindole,
763 100 nM) and Actin-stain 670 Fluorescent Phalloidin (200 nM). Imaging was performed
764 using Zeiss LSM980 confocal laser microscope.

765 **Measurements of glycogen, triglyceride, total protein content, cholesterol and**
766 **four sugars**

767 The content of glycogen, triglyceride, cholesterol and total protein was
768 determined by spectrophotometry at 620 nm, 510 nm, 510 nm and 562 nm
769 respectively using the glycogen assay kit (A043-1-1), triglyceride reagent kit (A110-1-
770 1), cholesterol assay kit (A111-1-1) and total protein assay kit (A045-2-2) obtained
771 from Nanjing Jiancheng Bioengineering Institute following to the manufacturer's
772 instructions. The determined results were normalized to the protein content in the
773 sample, which was determined using BCA Protein Assay Reagent Kit (Thermo
774 Scientific, Waltham, USA). Each sample contained tissue extracts from five adult
775 female BPH, with three biological replicates per sample.

776 To assess the content of four sugars (sucrose, glucose, fructose and trehalose)
777 in the extract of BPH tissue, the same extraction method was used as above. Sugar
778 content was quantified using the colorimetric method by scanning at 290 nm, 505
779 nm, 285 nm and 620 nm respectively using the sucrose reagent kit (A099-1-1),
780 glucose reagent kit (F006-1-1), fructose reagent kit (A085-1-1) and trehalose reagent
781 kit (A150-1-1) obtained from Nanjing Jiancheng Bioengineering Institute based on the
782 manufacturer's instructions. Each sample contained tissue extracts from five adult

783 female *N. lugens*, with three biological replicates per sample.

784 **Determination of Juvenile hormone III and ecdysone titers of BPH by ELISA**

785 The titer of Juvenile hormone III in BPH was measured using the Juvenile
786 hormone ELISA Kit (Lot Number: HLE92086, Shanghai HaLing Biotechnology Co., Ltd.,
787 Shanghai, China) which employs the dual-antibody sandwich ELISA method. The titer
788 of ecdysone in BPH were measured using the ecdysone ELISA Kit (Lot Number:
789 ZK14705, Shanghai ZhenKe Biotechnology Co., Ltd., Shanghai, China). At least three
790 biological replicates were employed for each treatment.

791 **Determination of Juvenile hormone III titer in BPH using HPLC-MS/MS**

792 The whole bodies of 5 individuals BPH were mixed with 1 ml of n-hexane, followed
793 by centrifugation at 10,000×g for 10 min, the upper hexane layer was then dried with
794 nitrogen, dissolved in methanol and sonicated for 10 min, after centrifugation at
795 10000×g for 10 min, the supernatant was collected through the organic filter membrane
796 of 0.22 μm into 2 ml vials for JH Ⅲ determination. JH Ⅲ standard sample (J912305-
797 10 mg) purchased from (Shanghai McLean Biochemical Technology Co. Ltd),
798 dissolved in methyl alcohol as stock solution 10,000 mg/L was diluted in a series of six
799 concentration gradients to serve as a reference. Liquid chromatography-tandem mass
800 spectrometry (LC-MS/MS) was then carried out using UPLC Xevo TQ-S Micro (Waters
801 technology), quantitative method according to the external standard, the
802 chromatographic column was EC-C18 (4.6 mm×150 mm, 2.7 μm), column temperature
803 was 30°C，injection volume was 20 μl, elution flow rate was 0.3 ml/min, and the mobile

804 phase was acetonitrile:formic acid water (90:10), detection wavelength was 218 nm,
805 the peak height was used for quantification.

806 **Cloning, sequence and phylogenetic analysis**

807 The NCBI database and BLAST program were used to carry out sequence
808 alignment and analysis. Open Reading Frames (ORFs) were predicted with EditSeq.
809 Primers were designed using the primer design tool in NCBI. Total RNA Extraction was
810 extracted from 30 adults BPH using TRIzol reagent (Invitrogen, Carlsbad, CA, USA)
811 according to the manufacturer's instructions. cDNA was synthesized using the Biotech
812 M-MLV reverse transcription kit. Full-length gene sequences were amplified by PCR
813 using cDNA as template and Rapid Taq Master Mix (Vazyme Biotech, Cat# P222-02).
814 The PCR product was purified on a 1% agarose gel, cloned into pClone007 Simple
815 Vector Kit (Tsingke Biotech, Cat# TSV-007S), and then sequenced using the 3730 XL
816 DNA analyzer (Applied Biosystems, Carlsbad, CA, USA). Table S2 contains a list of
817 the primers used in this study.

818 The exon and intron architectures of AT, AstA, AstB, AstCC and AstACCC were
819 predicted based on the alignments of putative ORFs against their corresponding
820 genomic sequences. Sequence similarity/annotations and orthologous gene searches
821 were performed using BLAST programs available in NCBI. Multiple alignments of the
822 complete amino acid sequences were performed with Clustal Omega
823 (http://www.ebi.ac.uk/Tools/msa/clustalo). Phylogeny was conducted using the
824 maximum likelihood technique to create phylogenetic trees and these were

825 bootstrapped with 1000 replications were used using the MEGA 6 software (84).

826 **RNA interference**

827 Double-stranded RNA (dsRNA) of *gfp* (green fluorescent protein), *JHAMT*
828 (juvenile hormone acid O-methyltransferase,), *Met* (methoprene-tolerant), *Kr-h1*
829 (krüppel homolog 1), *AstAR* (allatostatin-A receptor) and *GluCl* (glutamate-gated
830 chloride channel) was prepared using Ambion's MEGAscript T7 kit instructions
831 following the manufacturer's instructions. The primer sequences for double-stranded
832 RNA synthesis are listed in Table S2. Newly emerged females were injected with 40
833 nl (5,000 ng/μl) of double-stranded RNA of *gfp* (*dsgfp*) or double-stranded RNA of the
834 target genes in the conjunctive part between prothorax and mesothorax of insects. In
835 the RNAi experiments, BPH were then treated with the LC_{50} of EB 24h after dsRNA
836 injection and the whole body sampled for qRT-PCR analysis.

837 **Quantitative RT-PCR**

838 Fourth instar nymphs of BPH were treated with EB after which total RNA was
839 extracted from 5th instar nymphs and 1-7 day post-eclosion females of *N. lugens* using
840 the methods detailed above. The HiScript® II RT SuperMix for qPCR (+gDNA wiper)
841 kit from Vazyme, Nanjing, China, was used to create first-strand cDNA. Primer3
842 (http://bioinfo.ut.ee/primer3/) was used to design real-time quantitative PCR (qPCR)
843 primers listed in Table S2. mRNA levels of candidate genes were detected by qPCR
844 using the UltraSYBR Mixture (with ROX) Kit (CWBIO, Beijing, China). Each reaction
845 contained 2 μL of cDNA template (500 ng), 1 μL each forward and reverse qPCR primer

846 (10 μM), 10 μL of UltraSYBR mixture buffer, and 6 μL of RNase-free water. Q-PCR
847 was run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA)
848 under the following conditions: 5 min at 95°C, followed by 40 cycles of 95°C for 10 s
849 and 60°C for 30 s. Three independent biological replicates and four technical replicates
850 were used in each qPCR experiment. The housekeeping genes 18S ribosomal RNA
851 of BPH were selected to normalize the expression of candidate genes. The $2^{-\Delta\Delta Ct}$
852 method (Ct represents the cycle threshold) was used to measure relative expression
853 levels (85). Three biological replicates were used for statistical comparison between
854 samples. Table S2 contains a list of the primers used in this study.

855 **Statistics**

856 PoloPlus v2.0 (LeOra Sofware 2008) was used to calculate the lethal
857 concentration (LC_{50}) and 95% fiducial limits (95% F.L.). GraphPad Prism 8.0 software
858 (GraphPad Software Inc., San Diego, USA) was used to generate graphs and perform
859 statistical analysis of data. Data presented in this study were first verified for normal
860 distribution using the D'Agostino–Pearson normality test. One-way analysis of
861 variance (ANOVA) with Duncan's multiple range test was used to test differences
862 among multiple groups of normally distributed groups of data. Student's t test was used
863 to test the differences between two groups. If not normally distributed, Mann–Whitney
864 test was used for pairwise comparisons, and Kruskal–Wallis test was used for
865 comparisons among multiple groups, followed by Dunn's multiple comparisons. All
866 data are presented as mean ± s.e.m. The sample sizes and statistical tests used for
867 each experiment are stated in the figures or figure legends.

868 **Table S2** Sequences of oligonucleotide primers used in this study.

Primers	Primer sequences (5' - 3')
For cDNA cloning	
Allatotropin：	
NlAT-F	TACGCGGCCAAACACACTTA
NlAT-R	AGGGAAAGAGGGCGAAATTCA
Allatostatins：	
NlAstA-F	TCGGCCGTCACAAGTCAAG
NlAstA-R	CCGAACCCGTACTTCATGCT
NlAstB-F	ACCGGGCTCACAGGAATTTT
NlAstB-R	TGTAGGCGCAGATCTTGAGG
NlAstCC-F	AACACAGCTCTACGAGGCAC
NlAstCC-R	CCAAGCAGGTGACTGCCATA
NlAstCCC-F	TTTGTGTGTGCTTGCAGGTG
NlAstCCC-R	GGATAGAAACGGTAGATTTGGTAGA
Allatostatins receptor：	
NlA16-F	CCTCATTGTGGAACCACCGA
NlA16-R	CGCAGCTGTAAGGTGGAAGA
NlA2-F	GAACGTAATGGGAGTCGGCA
NlA2-R	GTTTTTGTGAGCGCCGACTT
NlA10-F	ATGCAAAACACGGCCAGCCT
NlA10-R	TTAATCGTCTCTGCTCAACTCCAAAGGAAGGT
NlA1-F	CGACCAGACCACTCTACTGC
NlA1-R	ACGTGGACCTCACTATACCAAAAA
For Quantitative RT-PCR	
Q-Nl18S-F	CGCTACTACCGATTGAA
Q-Nl18S-R	GGAAACCTTGTTACGACTT
Q-vitellogenin-F	GTGGCTCGTTCAAGGTTATGG
Q-vitellogenin-R	GCAATCTCTGGGTGCTGTTG
Q-Vitellogenin receptor-F	AGGCAGCCACACAGATAACCGC
Q-Vitellogenin receptor-R	AGCCGCTCGCTCCAGAACATT
Q-JHE-F	GAGCCTCACATCCACAGC
Q-JHE-R	AATGGGAGCCCTACGC
Q-NlMet-F	GGTGGTAAACGGATTGGAAA
Q-NlMet-R	CATCGTCAGCCAACTCGATA
Q-NlFAMet-F	GCAAAGTCAGCAATCCGCAAGAAC
Q-NlFAMet-R	ACACCGTAGTGGGTGACAACGAATG
Q-JHAMT-F	GAACCTGCAGGCCAAACACA
Q-JHAMT-R	ACCACTCGGTTGGGCTGAAT
Q-NlKr-h1-F	TGATGAGGCACACGATGACT
Q-NlKr-h1-R	ATGGAAGGCCACATCAAGAG
Q-NlAT-F	CACGATACGTGGCTTCAAGA
Q-NlAT-R	ACGATCACTTTCGCCAATTC
Q-NlAstA-F	AGGACTTACTGGGCGAGGAT

Q-NlAstA-R	GGTGTCTCGTTTCCTGGTGT
Q-NlAstB-F	AGCGAGCTAGACGAGGACAA
Q-NlAstB-R	TCGTCTCTGCTCAACTCCAA
Q-NlAstCC-F	CTGCTCCCAGTGAAAAGGAG
Q-NlAstCC-R	GCTTCCAGTAACTGCGCTTC
Q-NlAstCCC-F	TGTGCTTGCAGGTGGTAGTC
Q-NlAstCCC-F	AGAAGCATGTGACTGCGTTG
Q-NlA2-F	TCCTGGTGCTGAAGAGTGTG
Q-NlA2-R	CTTTTCGGGCCCATTAATTT
Q-NLA1-F	ATATCGGCACCGAAGATGAG
Q-NLA1-R	GTCTGACCCGACAGGTTCTC
Q-NLA10-F	ACTGGGTGTCGACCAATCTC
Q-NLA10-R	TCGGTAGCGAGGAAGACAGT
Q-NLA16-F	TACCGTTCTGTGGGATGTCA
Q-NLA16-R	CCGGGATATCAAAGACGAGA
Q-NlGluCl-F	CACTGACTGAGGCCAACAGA
Q-NlGluCl-R	GCTGGCCATTCTTAGTGAGC

For double-stranded RNA synthesis

T7-EGFP-F	TAATACGACTCACTATAGGGCGTAAACGGCCACAAGTTCA
T7-EGFP-R	TAATACGACTCACTATAGGGGACTGGGTGCTCAGGTAGTG
T7-Kr-h1-F	TAATACGACTCACTATAGGGCGCCAGTGAAAGTGAGACCT
T7-Kr-h1-R	TAATACGACTCACTATAGGGGAGACCGCAAGTGGTTCTGA
T7-Met-F	TAATACGACTCACTATAGGGCCACCAACCAGCAGATGAACCTGA
T7-Met-R	TAATACGACTCACTATAGGGCCACGCAAAGCCTCGTACTCTTGG
T7-JHAMT-F	TAATACGACTCACTATAGGGCTCCAGGCCATTGTCCCTCA
T7-JHAMT-R	TAATACGACTCACTATAGGGTTGGCCTGCAGGTTCTTTGG
T7-AstA-R-F	TAATACGACTCACTATAGGGTACTGCCGTTCTGGCCTTTT
T7-AstA-R-R	TAATACGACTCACTATAGGGGTGATCTGGAAGAGCGGCTT
T7-Glucl-F	TAATACGACTCACTATAGGGACACATCACCTGCTCACCTG
T7-Glucl-R	TAATACGACTCACTATAGGGGTGTGTTTGCCTGCTGTCTG

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