Pesticide-induced resurgence of the brown planthopper is mediated by diverse actors that promote juvenile hormone biosynthesis and female fecundity

Yang Gao[a※], Shao-Cong Su[a※], Zhao-Yu Liu[a], Dick R. Nässel[b], Chris Bass[c], Cong-Fen Gao[a], Shun-Fan Wu[a,*]

[a] College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China.

[b] Department of Zoology, Stockholm University, Stockholm, Sweden

[c] College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, UK

※ These authors contributed equally to this work.

*Corresponding author. E-mail address: wusf@njau.edu.cn (S.-F. Wu)

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 ovary 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.

Introduction

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

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

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

allosteric modulators of insect glutamate gated chloride channels (GluCls), inhibiting muscle contractions that lead to the cessation of insect feeding and subsequent death [11]. These insecticides exhibit particularly strong activity against Lepidoptera such as the rice leaffolder, *Cnaphalocrocis medinalis* Guénee, an important foliage-feeding insect which attacks rice during the vegetative stage [12]. Both BPH and the rice leaffolder are migratory pests with overlapping migratory paths, however, their occurrence period in the field differs by approximately one month, with leaffolders appearing earlier than BPH. Therefore, the use of EB to control rice leaffolder has the potential to impact BPH arriving later, via exposure to sublethal concentrations of this compound. In this regard, we have observed that when farmers use EB and abamectin to control leaffolders on rice crops in China, BPH outbreaks frequently occur in the same field. While sublethal doses of certain pesticides have been shown to enhance fecundity in BPH, including the insecticides triazophos and deltamethrin [6, 7, 13, 14] and the fungicides carbendazim and jinggangmycin [8], whether avermectins trigger resurgence in BPH via insecticide-enhanced reproduction remains unclear.

Reproduction in insects is influenced by external factors such as light [15], temperature [16], humidity [17] and nutrition [18], and endogenous factors such as the juvenile hormone (JH) [19], ecdysone [20], insulin [21] and TOR [22] pathways [23, 24]. Of these, JH, has been particularly implicated in insecticide-induced enhanced fecundity, with triazophos and deltamethrin treatments leading to increased circulating JH III titers in BPH females [3]. JH is synthesized and secreted by the corpora allata in insects [25], and can promote reproduction by regulating the synthesis and secretion

of vitellogenin (vg) in the female fat body, and stimulating the absorption of vg by the developing oocyte [19]. However, the regulation of JH is complex [19] and the key actors involved in JH-mediated pesticide-enhanced reproduction remains an open question.

In this study, we used a diverse range of approaches to investigate the impact of sublethal doses of avermectins on BPH fecundity, and unravel the molecular mechanisms mediating enhanced reproduction following exposure to this insecticide class. We show that avermectin exposure results in profound changes in the expression of a key suite of genes that in combination regulate JH, resulting in increased JH titer in adult females, which promotes fecundity.

RESULTS

GluCl allosteric modulators (emamectin benzoate and abamectin) stimulate fecundity of female *N. lugens*

To investigate whether GluCl modulators affect fecundity in BPH, we first determined the sub-lethal and median lethal doses of emamectin benzoate (EB) to 4th instar nymphs, newly emerged males and females of BPH (Table S1). For this we employed two different bioassay methods, the rice seedling dip bioassay method and topical application bioassay method [2, 26], in order to assess both the systemic and contact toxicity of these insecticides (Table S1). We then systemically treated 4th instar nymphs of BPH with the estimated LC_{15} and LC_{50} concentrations of EB and examined the fecundity of BPH after these individuals mated with treated or untreated individuals. We use the term “t” to represent individuals treated with EB and “ck” to indicate control individuals that were treated with insecticide diluent minus insecticide. After treatment with the LC_{15} and LC_{50} concentrations of EB the number of eggs laid per female of BPH in ♀t × ♂t crosses increased by 1.48 and 1.40 times compared with control ♀ck × ♂ck crosses (Figure 1A); the number of eggs laid per female of BPH in ♀t × ♂ck crosses increased by 1.53 and 2.07 times compared with control crosses (Figure 1B); However, the number of eggs laid by per female of BPH in ♀ck × ♂t crosses did not increase significantly compared to control ♀ck × ♂ck crosses (Figure 1C).

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

Exposure of 4th instar nymphs with the LC_{15} and LC_{50} concentrations of EB in contact bioassays also significantly stimulated fecundity. After treatment with the LC_{15} and LC_{50} concentrations of EB, the number of eggs laid by per female of BPH in ♀t × ♂t crosses increased by 1.18 and 1.26 times compared with the control (♀ck × ♂ck) (Figure 1D); The number of eggs laid per female of BPH in ♀t × ♂ck crosses increased by 1.27 and 1.56 times compared with the control crosses (Figure 1E); However, there was no significant difference in egg-laying number between ♀ck × ♂t crosses and controls (♀ck × ♂ck) (Figure 1F). These results reveal that EB can stimulate the fecundity of females following both the systemic and contact routes of exposure.

Figure 1

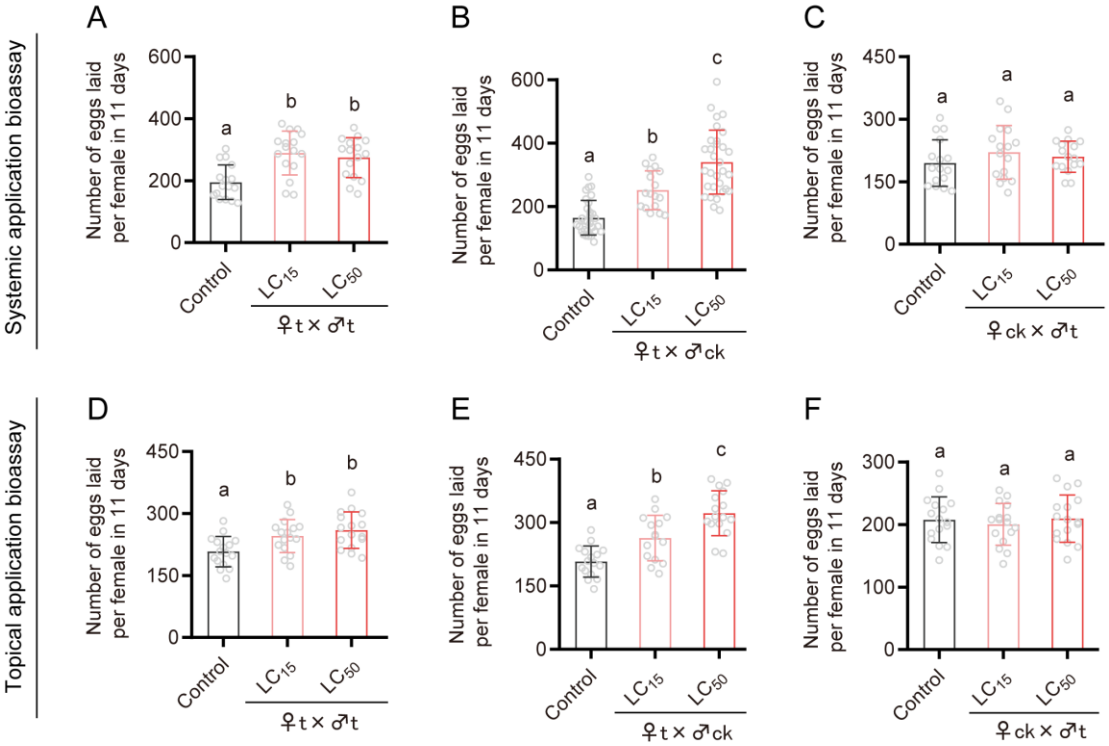


Figure 1. Fecundity of BPH following exposure to sub-lethal (LC_{15}) and median lethal (LC_{50}) concentrations of emamectin benzoate following system application bioassays (A: ♀t × ♂t; B: ♀t × ♂ck; C: ♀ck × ♂t) and topical application bioassays (D: ♀t × ♂t; E: ♀t × ♂ck; F:

♀ck × ♂t), respectively. The letter "t" represents treatment with insecticide, while "ck" indicates controls that was not treated with insecticide. All data are presented as the mean ± s.e.m. Different lower-case letters above the bars indicate significant differences (One-way ANOVA with Tukey's Multiple Range Test, $p < 0.05$).

We next examined whether EB treatment of adult BPH also stimulates reproduction. Indeed, treating newly emerged adults with the LC_{15} and LC_{50} concentrations of EB significantly stimulated the number of eggs laid per female (Figure 1-figure supplement 1A). Furthermore, sub-lethal exposure of 4th instar BPH nymphs to another GluCl allosteric modulator, abamectin (LC_{15} and LC_{50} concentrations) was also found to significantly enhance reproduction (Figure 1-figure supplement 1B).

Figure 1-figure supplement 1

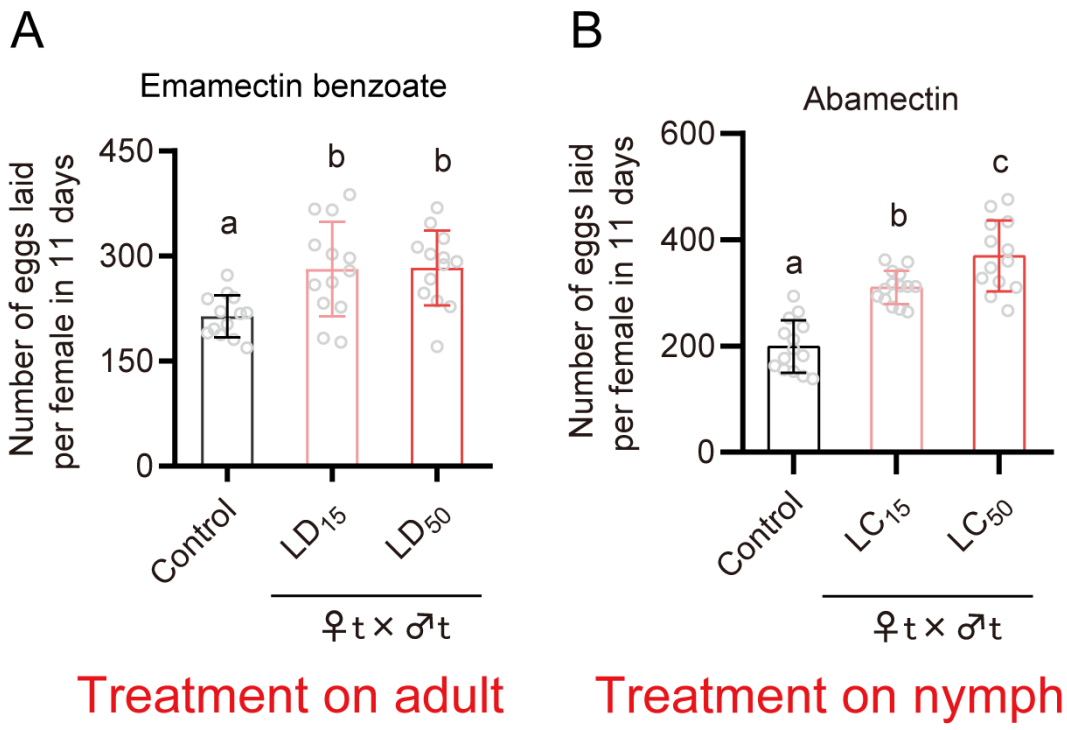
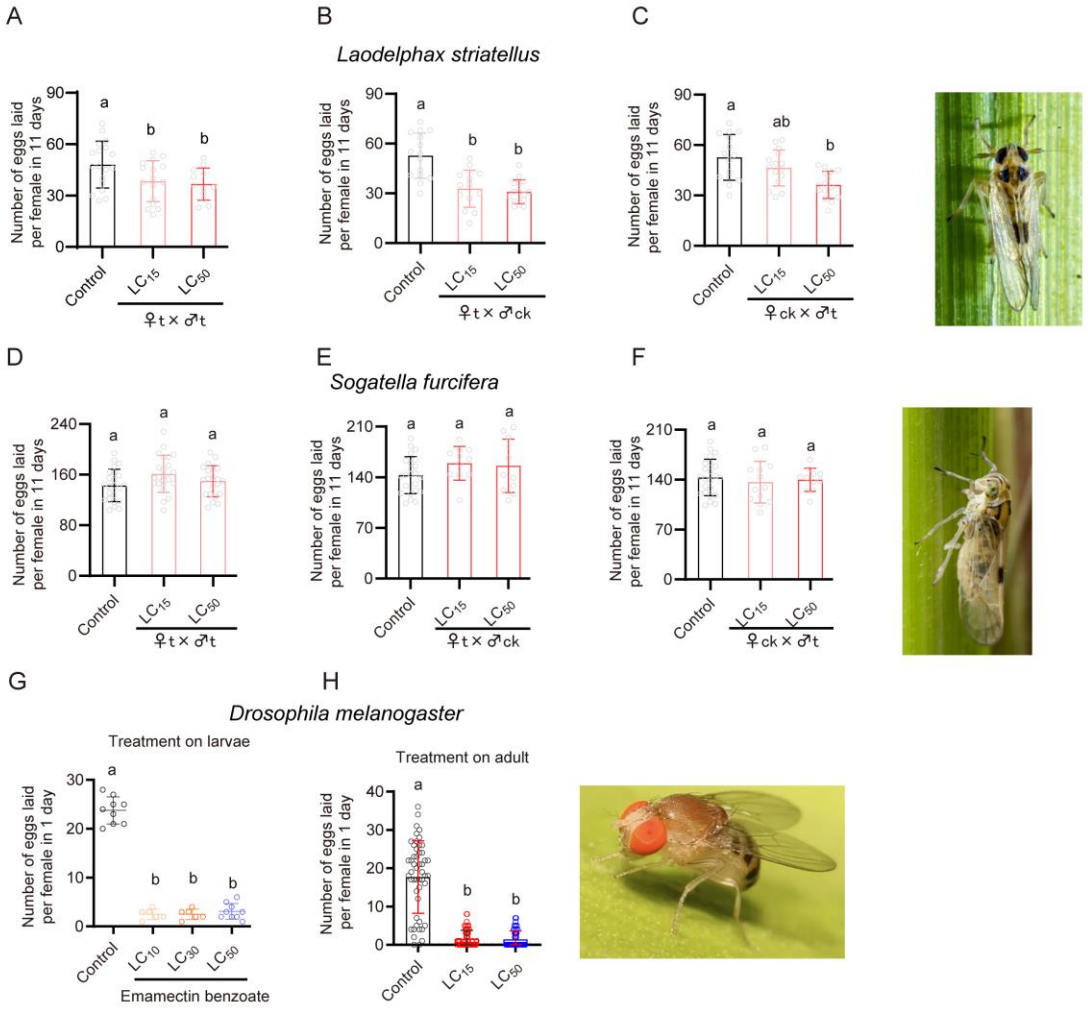


Figure 1-figure supplement 1. (A) Fecundity of BPH when newly emerged adults were treated with sub-lethal (LD_{15}) and median lethal (LD_{50}) concentrations of emamectin benzoate

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

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

158 **Figure 1-figure supplement 2**



159

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

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

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

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

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

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

166

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

168 To better understand the effects of EB on the reproductive fitness of BPH, the
169 preoviposition period, emergence rate, female ratio, female longevity and female
170 weight were evaluated following exposure using the systemic bioassay. The
171 preoviposition period of females treated with the LC_{50} of EB decreased significantly
172 compared with the control (Figure 1-figure supplement 3A). In contrast no significant
173 effects of EB on emergence rate and female ratio were observed (Figure 1-figure
174 supplement 3B and C). Female survival analysis showed that exposure of 4^{th} instar
175 nymphs to the LC_{50} of EB has no impact on female longevity (Figure 1-figure
176 supplement 3D). Interestingly, brachypterism (long-wing) female ratio and female
177 weight were significantly increased after EB exposure (Figure 1-figure supplement 3E
178 and F).
179

Figure 1-figure supplement 3

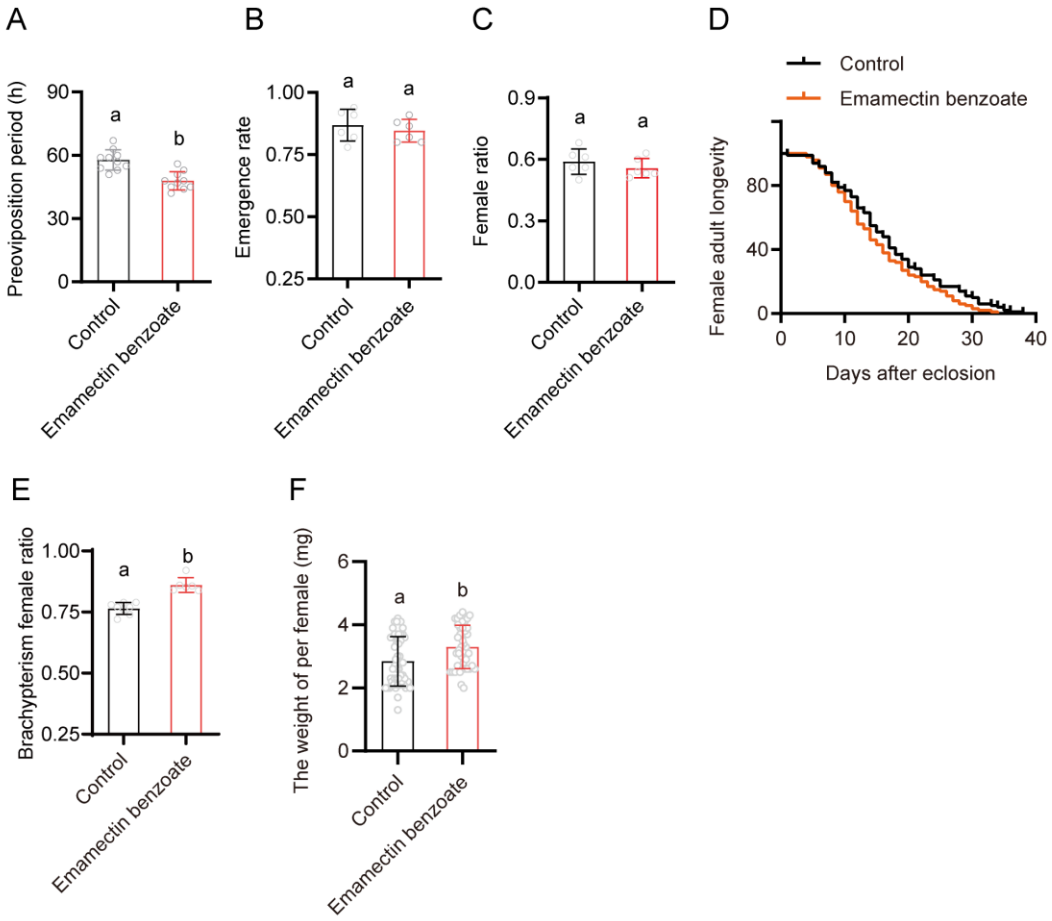


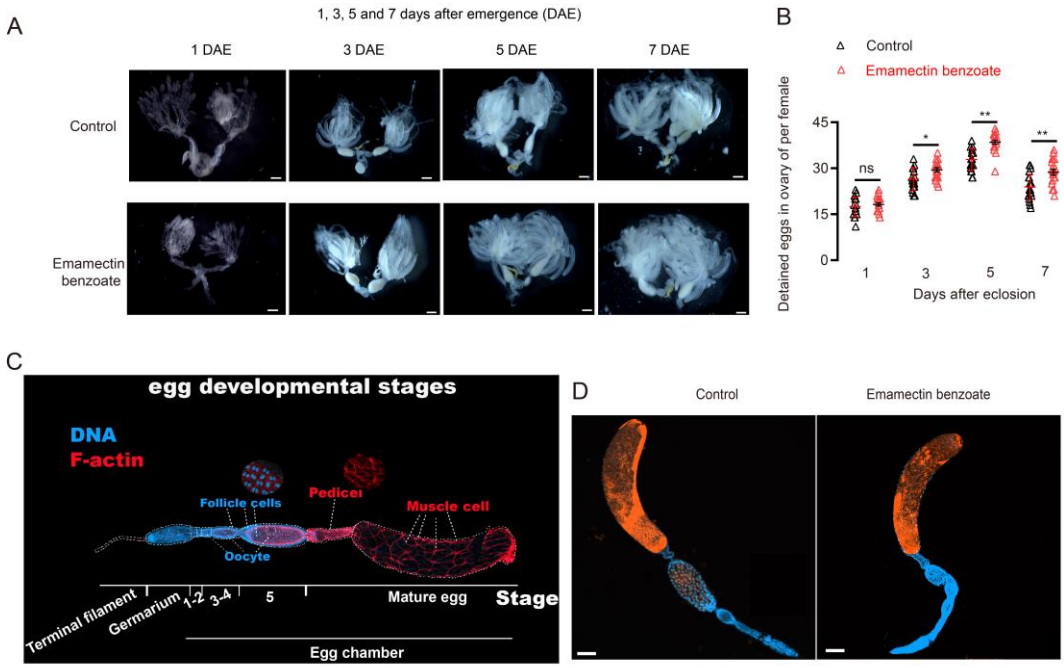
Figure 1-figure supplement 3. The impact of emamectin benzoate on the reproductive fitness of BPH. Fourth instar nymphs were treated with the LC_{50} concentration of emamectin benzoate in systemic bioassays. All data are presented as the mean ± s.e.m. Different lower-case letters above the bars indicate significant differences (Student's *t* test, $p < 0.05$).

EB promotes ovarian development in BPH

To investigate the cause of increased female weight following EB exposure we examined if EB influences ovary development in BPH. We dissected and compared the ovaries of females treated with the LC_{50} of EB at 1, 3, 5 and 7 days after eclosion (DAE) with control females. At 3, 5 and 7 DAE, the number of detained eggs of BPH

192 in the EB treated group were significantly higher than that of controls (Figure 2A and
193 B). We also explored whether EB treatment could enhance or impair oogenesis in
194 BPH. However, dissection of various developmental stages revealed that emamectin
195 benzoate treatment has no significant effects on oogenesis (Figure 2C and D).

196 **Figure 2**



197

198 **Figure 2.** The impact of emamectin benzoate on ovary development in BPH. Fourth instar
199 nymphs were treated with the LC_{50} concentration of emamectin benzoate in systemic bioassays.
200 (A) Ovary development in EB treated BPH at 1, 3, 5 and 7 days after eclosion (DAE) compared
201 to untreated controls. Scale bar = 1,000 μm. (B) Number of detained eggs in the ovaries of EB
202 treated BPH females measured at 1, 3, 5 and 7 DAE compared to controls. All data are
203 presented as the mean ± s.e.m. Asterisks indicate values significantly different from the control
204 using student *t* test (ns, no significant; *$p < 0.05$ and **$p < 0.01$). (C) Different developmental
205 stages of BPH eggs. (D) No impairment of emamectin benzoate on oogenesis of BPH. Scale
206 bar = 100 μm.

207

208 **EB exposure enhances the abundances of storage macromolecules and**
209 **circulating sugars in BPH**

210 Nutritional status is an important indicator of reproductive fitness. Thus, to
211 investigate whether EB affects intermediary metabolism and energy storage of BPH,
212 glycogen, triacylglyceride (TAG), total protein content, cholesterol and four circulating
213 carbohydrates were quantified in 4th instar BPH nymphs following exposure to the
214 LC_{50} of EB.

215 We found that EB exposure has no impact on glycogen levels (Figure 2-figure
216 supplement 1A). The amounts of TAG in EB-treated BPH were 27% higher ($p < 0.05$)
217 than those in controls, but only in BPH of the late fifth instar (5L) stage, with no
218 significant differences observed in subsequent developmental stages (Figure 2-figure
219 supplement 1B). The amount of total protein content in EB-treated BPH was higher
220 than the control groups in the case of all developmental stages from 5L nymph to
221 7DAE (Figure 2-figure supplement 1C). EB exposure also increased cholesterol
222 levels at 4 and 5 DAE (Figure 2-figure supplement 1D). Compared with the solvent
223 control, EB treatment caused significant increases ($p < 0.05$) in the levels of sucrose,
224 glucose, fructose, and trehalose (Figure 2-figure supplement 1E-H). Thus,
225 collectively, these data provide evidence that EB exposure leads to energy
226 mobilization and the metabolism of carbohydrates and lipids in BPH.
227

Figure 2-figure supplement 1

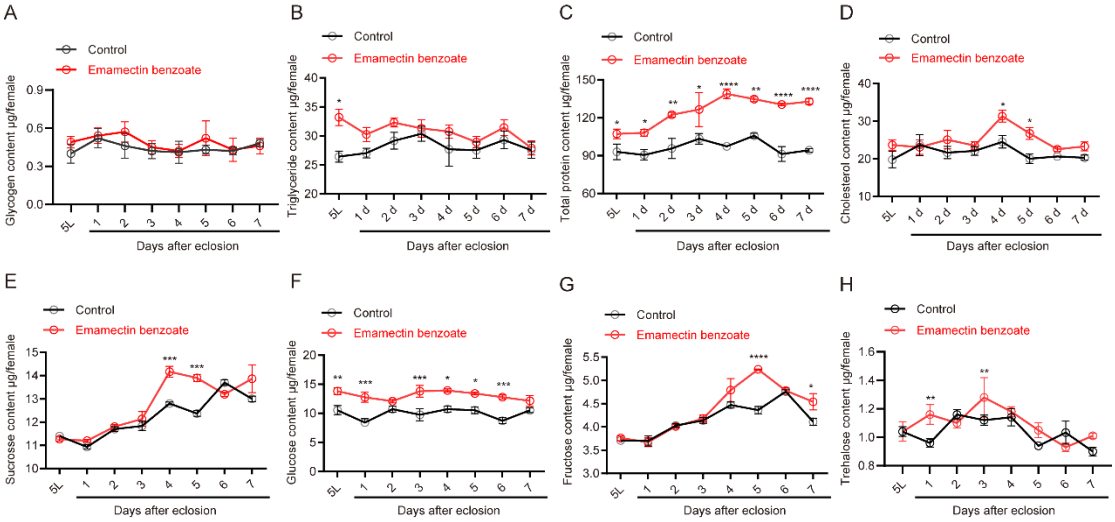


Figure 2-figure supplement 1. Amounts of Glycogen (A), TAG (B), total protein content (C), cholesterol (D) and four circulating sugars including sucrose, glucose, fructose and trehalose (E-H) after BPH exposure to EB. All data are presented as the mean ± s.e.m. The differences between the EB-treated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

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

Given the important role of juvenile hormone (JH) in vitellogenesis and egg development in insects [19, 25, 27-29], we asked whether EB-treatment could influence the titer of JH in BPH. As measured by ELISA, the juvenile hormone titer of BPH nymphs treated with the LC_{50} concentration of EB was significantly lower than that of controls in systemic bioassays during the middle and late stages of the 4th instar (Figure 3A). However, at 2, 3 and 4 DAE, the JH titer in the EB treated group was significantly higher than that of the control (Figure 3A). Interestingly, the titer of another important insect hormone, the steroid ecdysone, was not significantly different between EB-treated BPH and solvent-treated controls (Figure 3-figure

supplement 1). To independently validate the results of ELISA assays, we employed HPLC-MS/MS to measure JH titer in BPH following EB exposure [27, 30, 31]. The results showed that JH III titer significantly decreased after EB-treatment at the late 4th instar nymph stage (Figure 3A and B), but significantly increased at the third day after eclosion (3 DAE) (Figure 3A and C). To further investigate the role of JH in EB-enhanced fecundity in BPH, we treated BPH with methoprene and pyriproxyfen, JH analogues or biologically active JH mimics respectively, to determine whether they can stimulate fecundity in BPH. Both compounds significantly increased egg-laying in BPH (Figure 3D). Takes together these results reveal that EB stimulates an increased in JH titer that induces fecundity in BPH. Since we found that EB could induce JH synthesis in the BPH, we asked whether EB could influence the expression of genes that are involved in JH synthesis or degradation. For this we treated 4th instar nymphs of BPH with the LC_{50} concentration of EB using systemic bioassays, and then collected early (5E), middle (5M) and late (5L) stage of 5th instar nymph and 1-7 DAE female adults (DAE) for analysis. Quantitative PCR was then used to examine the expression of key genes previously implicated in the regulation of JH [32, 33].

Farnesoic acid O-methyltransferase (FAMeT) [34], is known as an important enzyme in the JH biosynthetic pathway, catalyzing methylation of farnesoic acid (FA) to methyl farnesoate (MF) [33]. We found that this gene was significantly upregulated in 5M instar nymph to 4 DAE after EB- treatment (1.0-fold to 3.0-fold) (Figure 3E). Another gene, juvenile hormone acid methyltransferase (JHAMT) [33, 35], which is

involved in the biosynthesis of JH, was also upregulated in EB-treated BPH at 1 to 5 DAE than controls (1.5-fold to 3.0-fold) (Figure 3F). Methoprene-tolerant (Met), belongs the basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) family of transcription factors and is the intracellular (nuclear) receptor of JH [36, 37]. The levels of *met* mRNA increased in EB-treated BPH at the 5M and 5L instar nymph and 1 to 5 DAE stages compared to controls (1.7-fold to 2.9-fold) (Figure 3G). Krüppel homolog 1 (*Kr-h1*), a transcriptional target of JH signaling, is reported to be sensitive to levels of JH and its expression levels are directly correlated with JH titers [32, 38, 39]. We found that *Kr-h1* was significantly upregulated in the adults of EB-treated BPH at the 5M, 5L nymph and 4 to 5 DAE stages (4.7-fold to 27.2-fold) (Figure 3H). Similarly, the expression levels of vitellogenin (Vg), a key downstream component of JH signaling triggering ovary development in insects including BPH [40], was markedly increased in females at 2–5 DAE by EB (1.7-fold to 5.5-fold) (Figure 3I). Juvenile hormone esterase (JHE) is the primary JH-specific degradation enzyme that plays a key role in regulating JH titers [41]. Interestingly, we observed a significant upregulation of JHE mRNA levels in the early and middle 5th instar nymph stage followed by downregulation in 5L instar nymphs of EB-treated BPH (1.3-fold to 2.6-fold) (Figure 3J). In combination these results reveal that EB has profound impacts on the expression of key genes involved in the synthesis of JH or downstream signaling pathway genes that might promote egg development.

To further understand whether these JH pathway-related genes were involved in egg-laying behavior in BPH, we performed RNAi experiments to downregulate the

290 expression of *JHAMT, Met and Kr-h1* (Figure 3-figure supplement 2A-C). We found
291 that silencing of these three genes downregulated the titer of JH in BPH providing
292 functional evidence of their role in the regulation of JH (Figure 3-figure supplement
293 2D). Furthermore, silencing of *Kr-h1* inhibited *FAMeT* and *Vg* gene expression while
294 increasing *JHE* gene expression (Figure 3-figure supplement 2E-H). Importantly,
295 silencing of *JHAMT*, *Met* and *Kr-h1* gene in female BPH was also found to suppress
296 egg-laying (Figure 3K-M). However, this phenotype was rescued by EB treatment,
297 which restored egg-laying to normal levels in BPH injected with *JHAMT*, *Met* and *Kr-*
298 *h1* dsRNA (Figure 3K-M). Together these results provide a mechanistic
299 understanding of how EB enhances fecundity in BPH by modulating the expression
300 of key genes involved in JH synthesis (Figure 3N).
301

Figure 3

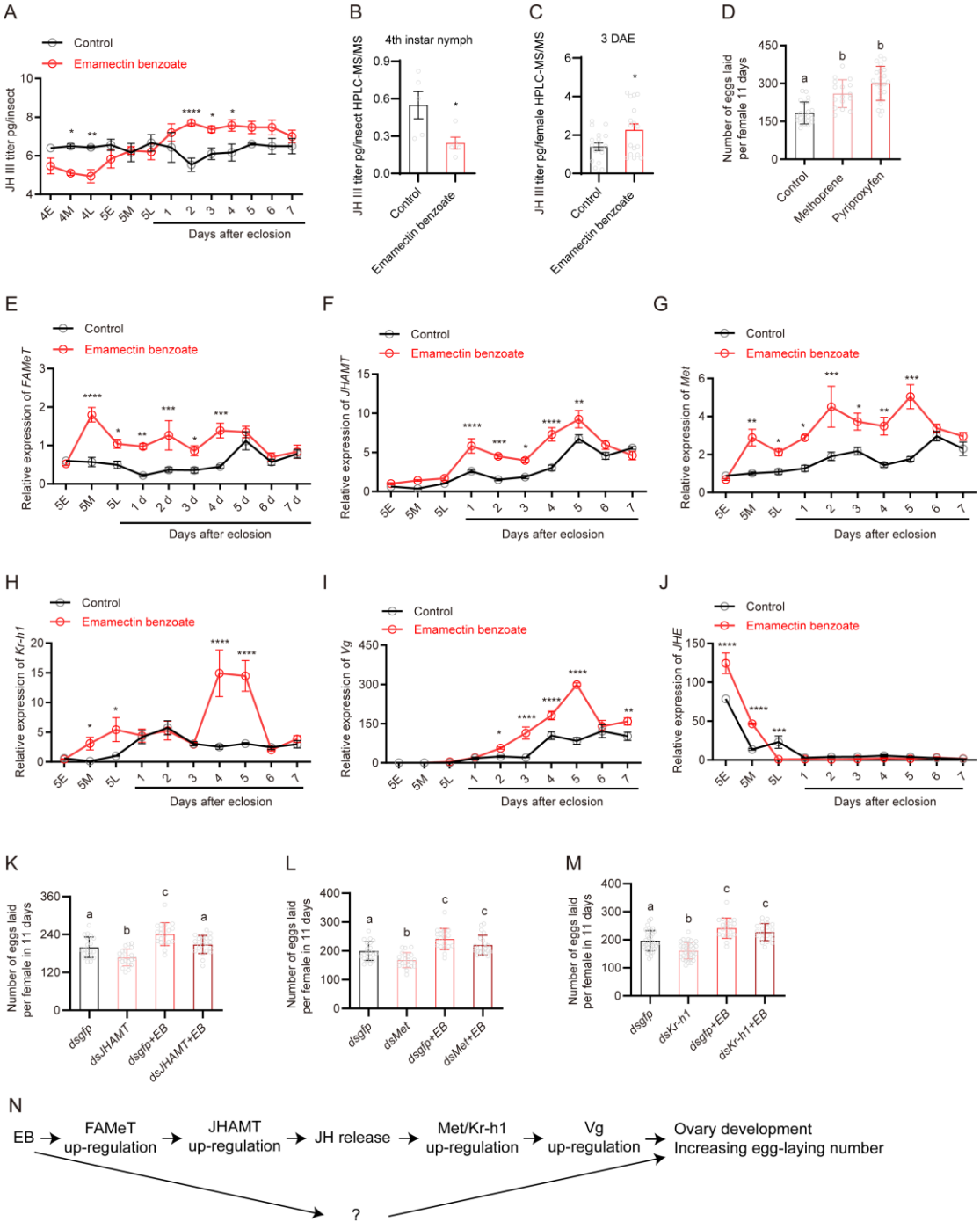


Figure 3. EB induced reproduction in BPH is mediated by components of the JH signaling pathway. (A) The titer of JH III (as measured by ELISA assay) at different developmental stages of BPH when 4th instar nymphs were treated with the median lethal concentrations of EB. (B and C) The titer of JH III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

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

Figure 3-figure supplement 1

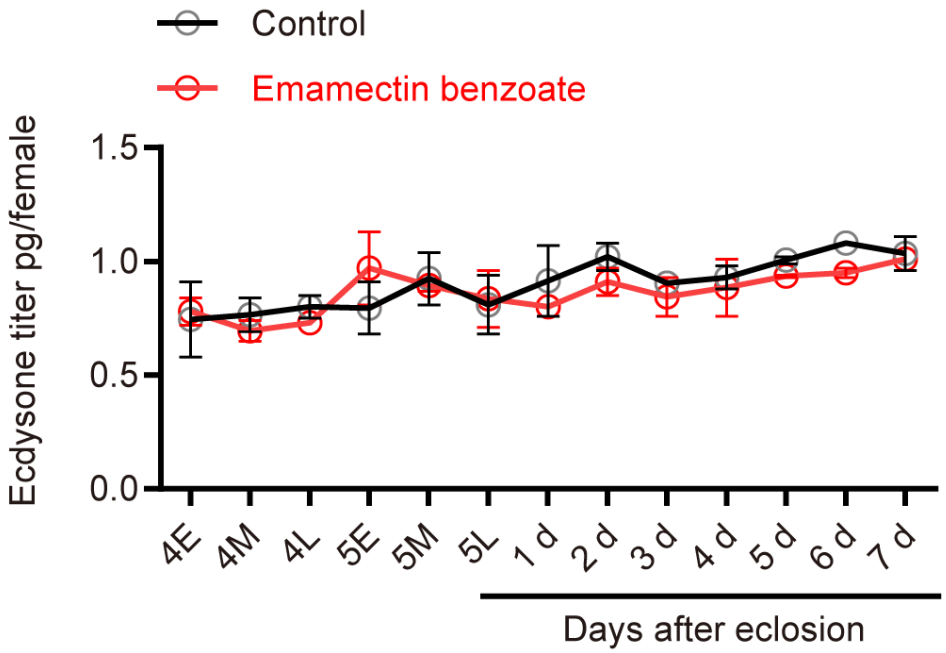


Figure 3-figure supplement 1. Ecdysone titer at different developmental stages of BPH when 4th instar nymphs were treated with median lethal concentrations of EB.

Figure 3-figure supplement 2

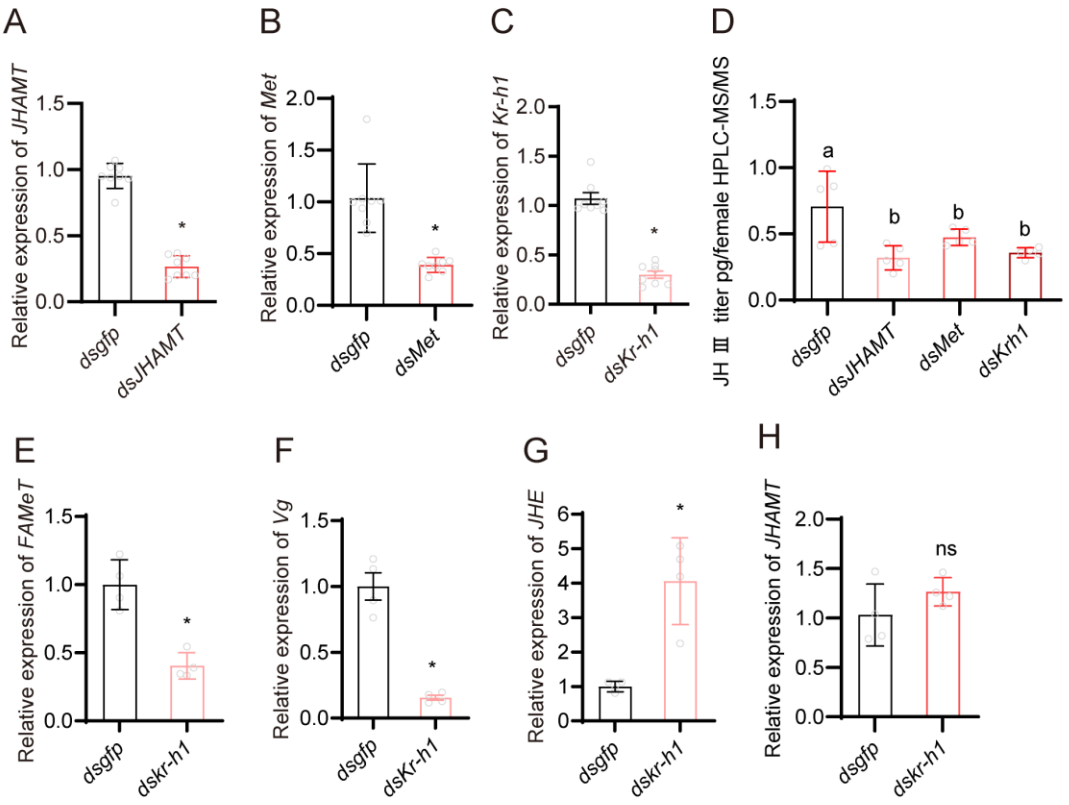


Figure 3-figure supplement 2. (A-C) Expression of *JHAMT* (A), *Met* (B) and *Kr-h1* (C) following RNAi knockdown. (D) The titer of JHIII (as measured by HPLC-MS/MS) at 3 DAE when female adults were injected with *dsJHAMT, dsMet,* and *dsKr-h1* at 1 DAE. (E-H) Expression of *FAMeT*, *Vg*, *JHE* and *JHAMT*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

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

The timing and level of JH biosynthesis can be precisely regulated by the neuropeptides, stimulatory allatotropins (ATs) and inhibitory allatostatins (Asts), in many insects [42-49]. Insects can, in a species-specific manner, produce one type of AT and three types of Asts: FGL-amide Ast (AstA) [43, 50]; myoinhibitory peptide (MIP or AstB) and PISCF Ast (AstC) [47, 51]. In some species, there also exists two paralogue genes of AstCs which are named AstCC and AstCCC [52, 53]. Interestingly, the allatostatic activity of these three types of Ast peptides varies between insect species so that in each species only one type of Ast (for example AstA) controls JH production [47, 48, 51, 54].

Analysis of the BPH neural transcriptome sequence data has revealed the presence of one AT, four types of Asts and four corresponding receptors, allatotropin receptor (A16, ATR), AstA receptor (A2, AstAR), AstB (MIP) receptor (A10, AstBR or MIPR) and AstC receptor (A1, AstCR) [55]. We cloned the five neuropeptide genes (AT, AstA, AstB/MIP, AstCC and AstCCC) and confirmed the sequence obtained from transcriptome data (Figure 4-figure supplement 1) [55]. Interestingly, we found that AstC is missing in the genome of BPH and only AstCC and AstCCC are present (Figure 4-figure supplement 1). Next, we also cloned their corresponding receptors [52] including ATR (A16), AstAR (A2), AstBR (A10) and AstCR (A1) which might be activated by AstCC and/or AstCCC [39, 52, 56]. Sequence alignments and phylogenetic analysis are shown in Figure 4-figure supplement 2.

357 Quantitative PCR was then used to examine if EB treatment could influence the
358 expression of the genes encoding these neuropeptides and their receptors. Treating
359 BPH with the LC_{50} concentration of EB significantly increased the expression of *AT*,
360 *ATR* and *AstCCC* while resulting in the downregulation of *AstA, AstB/mip, AstCC*,
361 AstAR and *AstBR/mipr* at the adult stage (Figure 4A and B, Figure 4-figure
362 supplement 3A-G). Among these, *AstA* and *AstAR* were the most downregulated
363 genes after EB treatment (Figure 4A and B, Figure 4-figure supplement 3H) and thus
364 the *AstA/AstAR* signaling system was selected for subsequent functional analysis.
365 Silencing of *AstAR* in female BPH using RNAi (Figure 4C), resulted in an increased
366 number of eggs laid per female compared with *dsgfp*-injected controls (Figure 4D).
367 Interestingly, silencing *AstAR* also resulted in the upregulation of *FAMeT, JHAMT,*
368 *Met* and *Kr-h1* which are involved in the JH biosynthesis/signaling (Figure 4E-H).
369 However, *JHE* was not influenced by AstAR silencing (Figure 4I). We therefore
370 investigated whether silencing the *AstAR* gene could influence JH titer in BPH. A
371 significantly increased JH titer was observed in *AstAR* silenced BPH compared with
372 controls (Figure 4J). Thus, our data strongly suggest that AstA is a key inhibitor of JH
373 production in BPH.

374 Finally, we investigated whether injection of mature Ast and AT peptides could
375 influence the number of eggs laid and JH titer in BPH. We synthesized one AT, six
376 AstAs (AstA1 to AstA6), one AstCC and one AstCCC peptide according to their
377 determined sequences (Figure 4-figure supplement 1). Indeed, we found that
378 injection of AstA1 and AstA6 reduced the number of eggs laid per female in 48 h

(Figure 4K). We also observed that AstA1 injection after 16h and 48h decreased the JH titer significantly (Figure 4L), and AT injection increased the JH titer after 2h but levels returned to normal after 4h injection (Figure 4M). Collectively, these results provide compelling evidence that EB induces reproduction in BPH through the AstA/AstAR and JH signaling pathways (Figure 4N) and further supports the role of AstA and AT in regulation of JH titer.

Figure 4

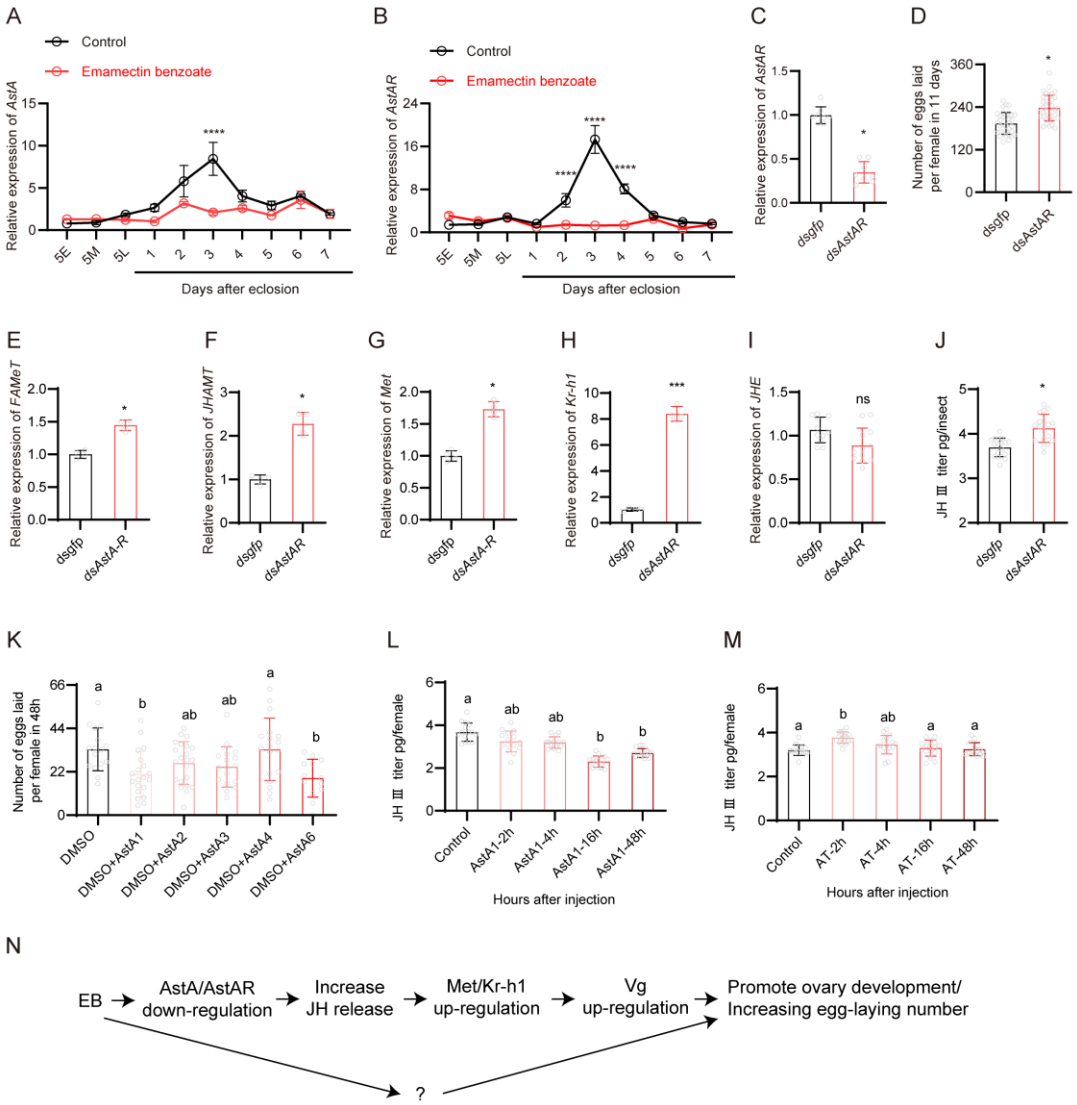
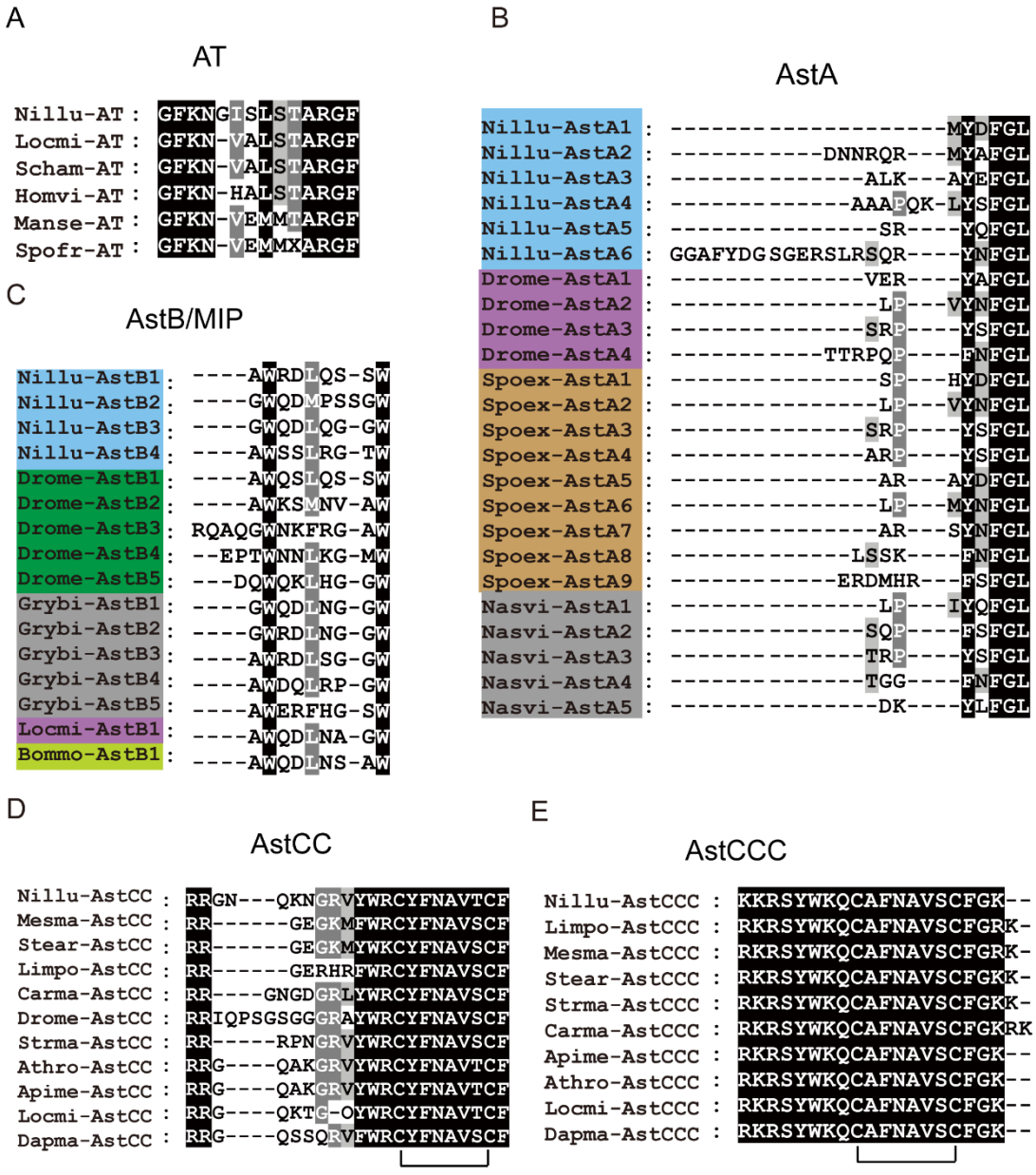


Figure 4. EB induced reproduction in BPH is mediated by the AstA/AstAR and JH signaling pathway.

(A and B) Expression of *AstA* and *AstAR* in different stages of BPH following EB treatment. (C) Downregulation of *AstAR* using RNAi leads to a reduction in mRNA expression level. (D) Egg production in female BPH following silencing of *AstAR* gene. (E-I) Expression of selected JH signaling pathway related genes (*FAMeT*, *JHAMT*, *Met*, *Kr-h1* and *JHE*) in *AstAR* silenced BPH. (J) JHIII titer of BPH females after *AstAR* gene silencing determined by HPLC-MS/MS. (K) Number of eggs laid per female in 48h following injection of six AstA1-AstA6 mature peptides and one AT mature peptide. Fifty nanoliter of PBS (as control) and seven peptides (20 pmol/insect) were injected into female BPH three days after eclosion. (L and M) The JH III titer

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

406 **Figure 4-figure supplement 1**



407

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

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

Figure 4-figure supplement 2

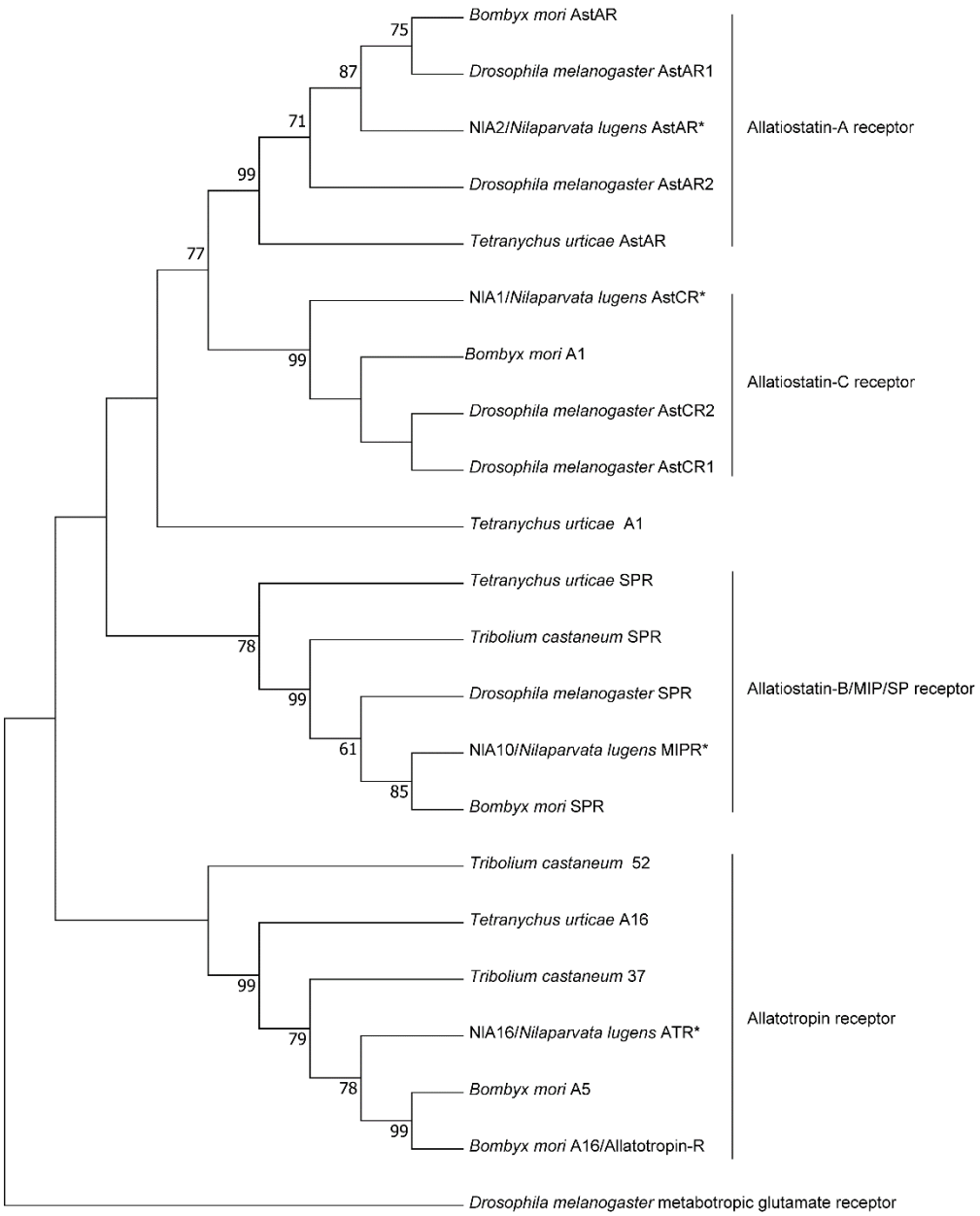


Figure 4-figure supplement 2. Phylogenetic tree of the predicted BPH (*) allatotropin receptor (A16, ATR), allatostatins A receptor (A2, AstAR), AstB (MIP) receptor (A10, AstBR or MIPR) and allatostatins C receptor (A1, AstCR) with other insect species. The tree was generated using the maximum likelihood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

Figure 4-figure supplement 3

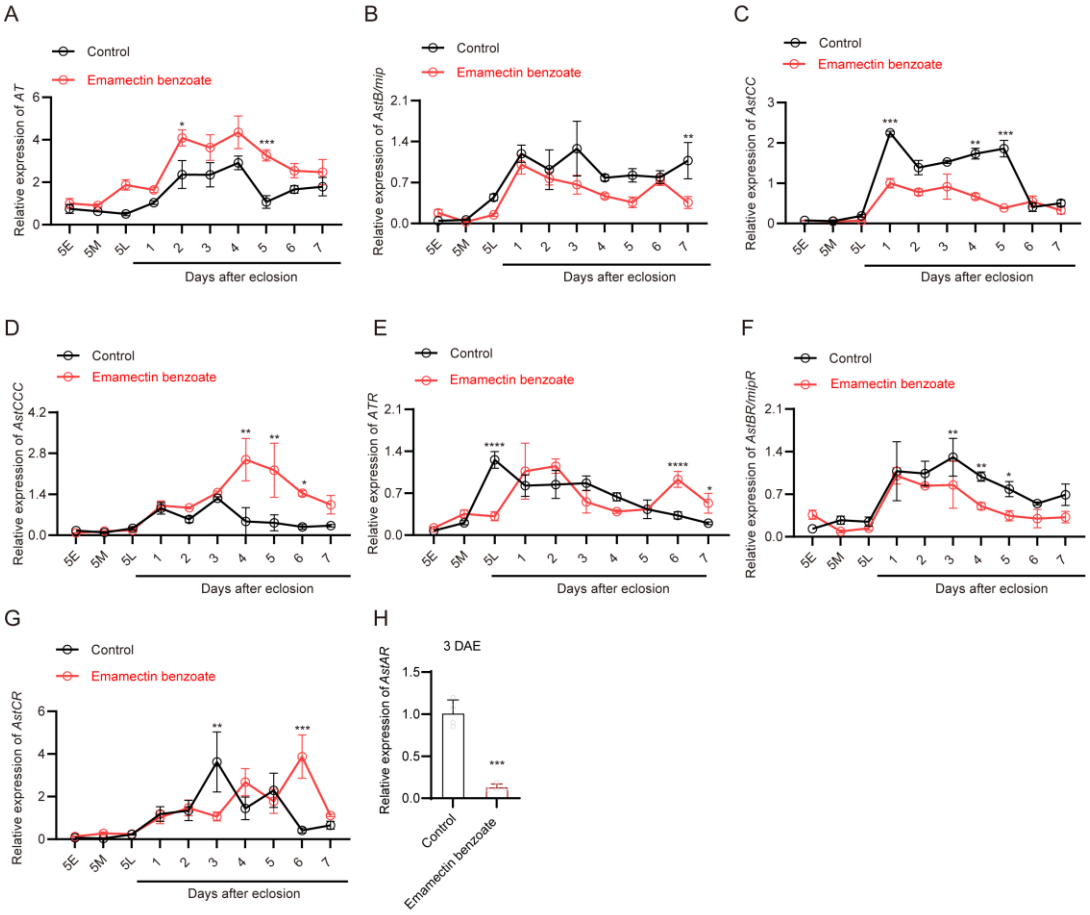


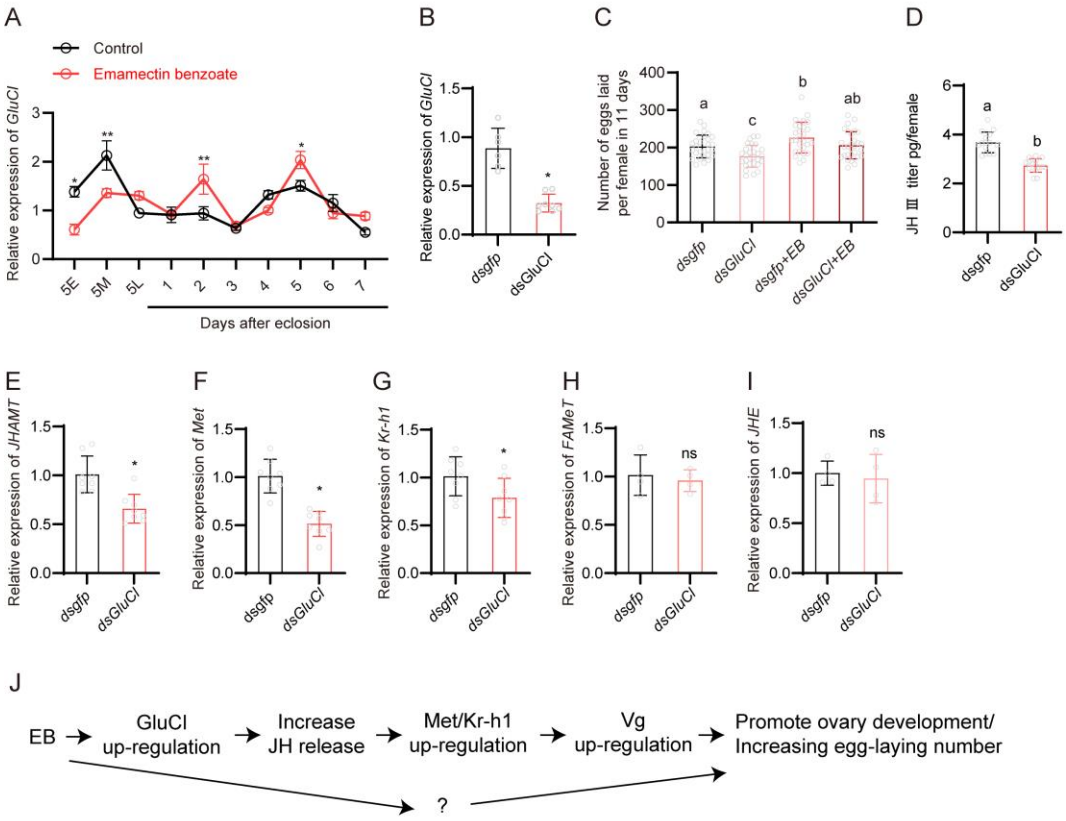
Figure 4-figure supplement 3. EB induced changes in the expression of *AT*, *AstB*, *AstCC*, *AstCCC*, *ATR*, *AstBR* and *AstCR* in BPH. All data are presented as means ± s.e.m. Student's t test was used to compare the two samples. ns, no significant difference; Asterisks indicate values significantly different from the control (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

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

EB and abamectin, are allosteric modulators, which target glutamate-gated chloride channels (GluCls) [57-59]. Hence, we examined whether EB-stimulated fecundity in BPH is influenced by its molecular target GluCl. The full length *GluCl* coding sequence from BPH was cloned and sequenced (Figure 5-figure supplement 1) and the impact of EB on *GluCl* gene expression examined using quantitative PCR. Treatment of BPH with the LC_{50} concentration of EB significantly downregulated *GluCl* gene expression at the 5E and 5M nymph stages while upregulating *GluCl* gene expression at 2 DAE and 5 DAE in the adult stage (Figure 5A). To examine the role of *GluCl* gene in BPH fecundity, RNAi was used to knockdown expression of this gene in female BPH (Figure 5B). A significant decrease in the number of eggs laid by per female was observed in *dsGluCl*-injected insects compared with *dsgfp*-injected insects (Figure 5C). However, treatment with EB was found to rescue the decreased egg-laying phenotype induced by *dsGluCl* injection (Figure 5C). To investigate the mechanism by which *GluCl* expression modulates fecundity we examined if silencing *GluCl* influences JH titer and JH-related gene expression. Indeed, we observed that RNAi knockdown of *GluCl* leads to a decrease in JH titer (Figure 5D) and down-regulation of genes including *JHAMT* which is responsible for JH synthesis, and the JH signaling downstream genes *Met and Kr-h1* (Figure 5E-G). In contrast, expression of *FAMeT* and *JHE* were not changed in the *GluCl* silencing insects (Figure 5H and I). We also examined whether silencing *GluCl* impacts the

459 *AstA*/*AstAR* signaling pathway. Silencing *GluCl* was found to have no impact on the
460 expression of *AT*, *AstA*, *AstB*, *AstCC*, *AstAR*, and *AstBR*. However, the expression of
461 *AstCCC* and *AstCR* was significantly upregulated in *dsGluCl*-injected insects (Figure
462 5-figure supplement 2A-H). These results suggest that EB activates *GluCl* which
463 induces JH biosynthesis and release, which in turn stimulates reproduction in BPH
464 (Figure 5J).
465

466 **Figure 5**



467

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

469 **protein GluCl.**

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

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

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

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

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

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

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

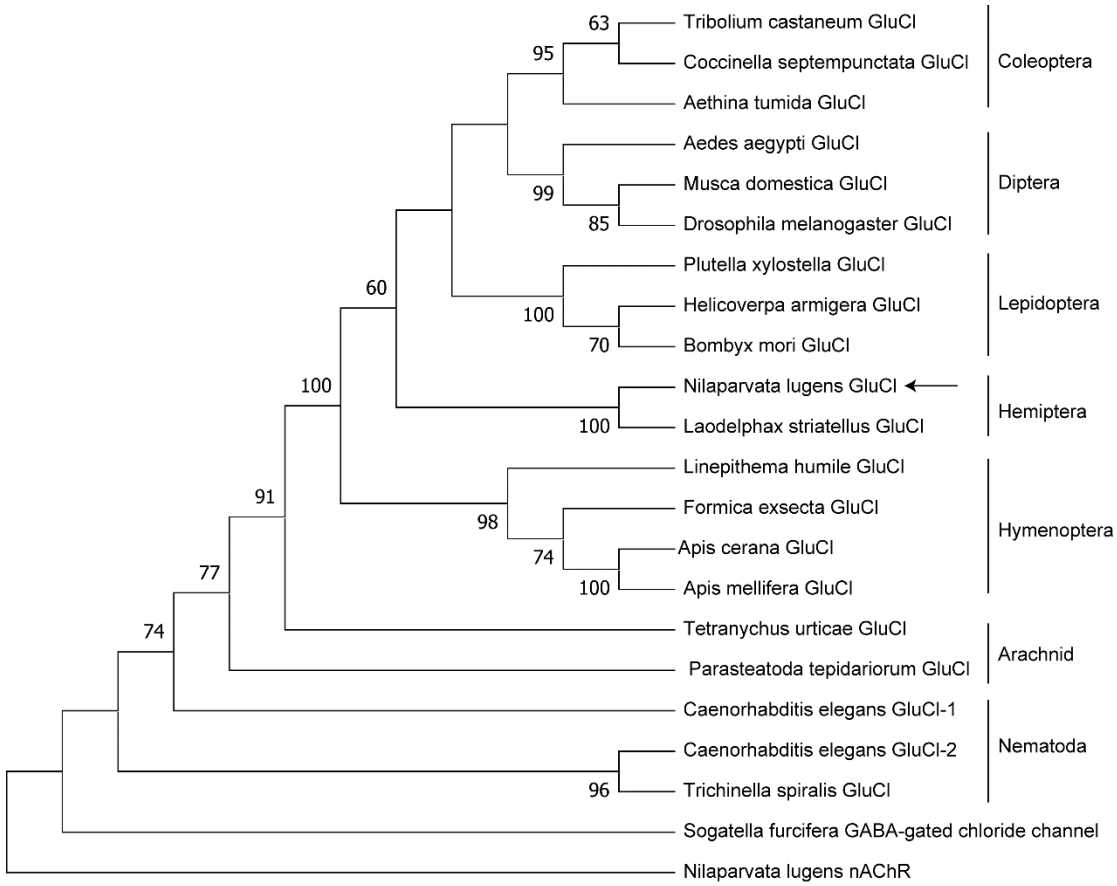
477 test was used to compare the two samples. One-way ANOVA with Tukey's multiple

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

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

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

481 **Figure 5-figure supplement 1**

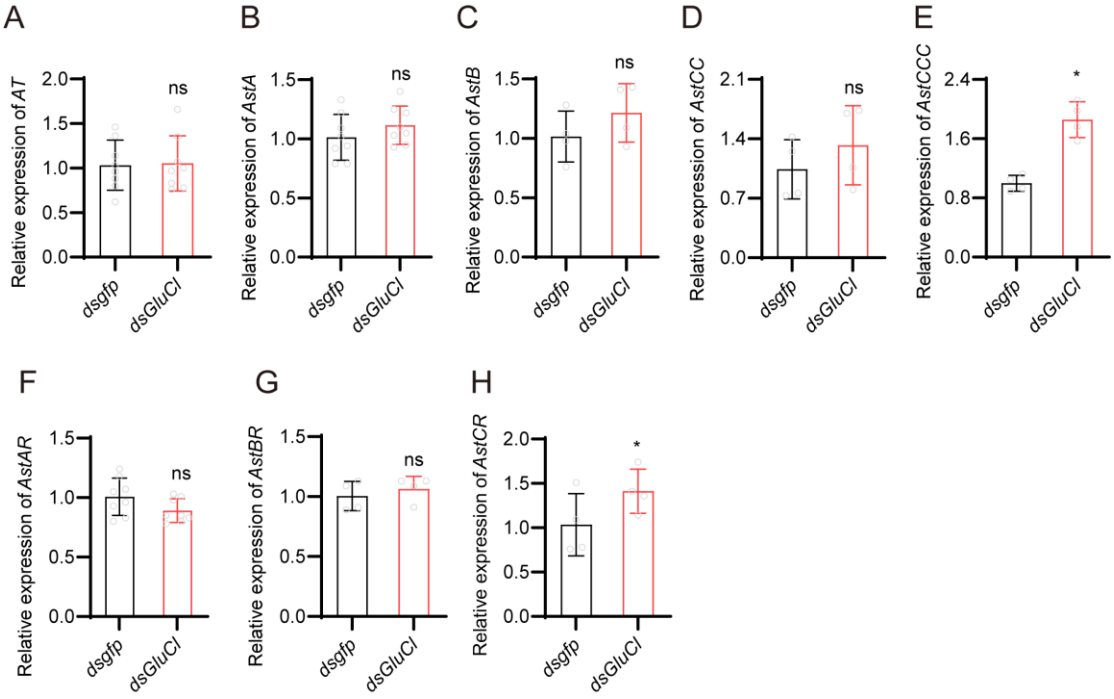


482

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

490

491 **Figure 5-figure supplement 2**



492

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

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

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

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

497

Discussion

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

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

We show that in both contact and systemic assays EB and abamectin stimulate reproduction in BPH. Thus, insecticide-enhanced reproduction is likely a key factor in the BPH resurgence observed when farmers use EB and abamectin to control leaffolders in rice crops in China [26]. Although this is the first report of sublethal doses of avermectins enhancing insect fecundity, our findings are consistent with previous studies which have shown that certain insecticides, herbicides and fungicides stimulate BPH reproduction [3, 7, 14, 60-65]. Intriguingly, we show that EB only induces fecundity in female adults and is specific to BPH, with EB exposure failing to enhance reproduction in two related species, the small brown planthopper, *L. striatellus* and the white backed planthopper, *S. furcifera*, or the model insect *D. melanogaster*. Thus, the

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

Pesticides may stimulate insect reproduction through a variety of physiological and molecular mechanisms. Our data reveal that exposure to sub-lethal concentrations of EB results in profound changes to female BPH fitness, leading to increases in female weight, total protein content, cholesterol content, sugar content and egg production and decreases in duration of the preoviposition period. Some of these findings exhibit parallels with previous studies, which demonstrated that treating third-instar BPH nymphs with either deltamethrin, triazophos, or imidacloprid led to increased soluble sugar levels in the corresponding adults [66]. Such metabolites provide the energy that drives BPH reproduction and resurgence [3]. Thus, together with prior work, our results suggest that pesticides associated with resurgence stimulate nutritional intake in BPH to fuel enhanced energy-intensive reproduction.

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

JH is a pleiotropic hormone which plays important roles in development and reproduction in insects [19, 67]. Circulating JH titers are regulated by factors that control JH production in the corpora allata including biosynthetic enzymes and catabolic enzymes that regulate JH levels. Our results show that EB increases circulating JH III titers in BPH females over 2–4 days after eclosion (DAE) and promotes ovary development. Previous studies have reported that triazophos and deltamethrin treatments also lead to increased circulating JH III titers in BPH females

542 over 1–3 days post emergence. Similarly, jinggangmycin treatments were found to lead
543 to increased JH titers (by approximately 45–50%) in BPH females over two days post
544 emergence [68]. Thus our findings, in combination with these previous studies,
545 demonstrate that insecticide treatments can have dramatic effects on the regulation of
546 key insect hormones involved in pest reproduction which can in turn drive pesticide
547 resurgence.

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

558 JHMAT is an enzyme that catalyzes the conversion of inactive precursors of JH to
559 active JH in the final stages of JH biosynthesis [33, 35]. Interestingly, while it has not
560 been previously implicated in pesticide resurgence, treatment of the stored product
561 pest *Sitotroga cerealella* with diallyl trisulfide, an insecticidal compound in garlic
562 essential oil, was found to increase JHAMT mRNA levels [69]. Because JHMAT is the
563 key rate-limiting enzyme in regulation of JH titer our results suggest that its enhanced

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

565 Met is a ligand-activated member of the basic helix–loop–helix Per/Arnt/Sim
566 (bHLH-PAS) transcription factors and is the intracellular receptor for JH [36, 37]. Kr-h1
567 is a zinc finger protein that acts downstream of Met and is expressed in response to
568 JH signaling. Although the genes encoding these proteins have not been previously
569 linked to pesticide resurgence, our finding that they are upregulated following EB
570 exposure, and demonstration of their role of in promoting fecundity, is consistent with
571 previous studies. Specifically, treatment of BPH with JH III or the insecticidal analogues
572 methoprene or pyriproxifen was found to induce the expression of *Kr-h1* [70].
573 Furthermore, knockdown of *Met* and *Kr-h1* in BPH brachypterous females was found
574 to result in delayed ovariole development and this was significantly more pronounced
575 than the response observed in BPH treated separately with ds*NlMet* or ds*NlKr-h1* [71].
576 This finding provides evidence of a possible interaction between *Met* and *Kr-h1* and,
577 in combination with our data, suggests that *Met* and *Kr-h1* may act in concert to
578 mediate EB-enhanced fecundity.

579

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

581 In addition to regulatory proteins that promote JH production, insects have
582 peptides that inhibit JH biosynthesis. These include the allatostatins: FGLamides
583 (FGLa; AstA), the W(X)6Wamides (AstB), and the PISCFs (AstC) [47, 48, 54, 55, 72,
584 73]. Interestingly, our results showed that EB exposure results in the marked
585 downregulation of the expression of the genes encoding allatostatin *AstA* and its

receptor *AstAR*. We provide clear evidence of the functional impact of this on JH synthesis and BPH fecundity by: i) demonstrating that RNAi knockdown of *AstAR* expression results in increased JH titer and enhanced female egg production, and, ii) showing that injection of female BPH with synthetic AStA peptide reduces JH titer and decreases egg production. Thus, our data provide unequivocal evidence that AstA is a key inhibitor of JH production in BPH. This finding is consistent with previous work which has shown that FGLa/ASTs inhibit JH biosynthesis in cockroaches, and termites [43, 74]. To our knowledge, our study is the first report of insecticides inhibiting the expression of the neuropeptide receptor, *AstAR*, and linking this to increases in JH titer and enhanced reproduction in insects.

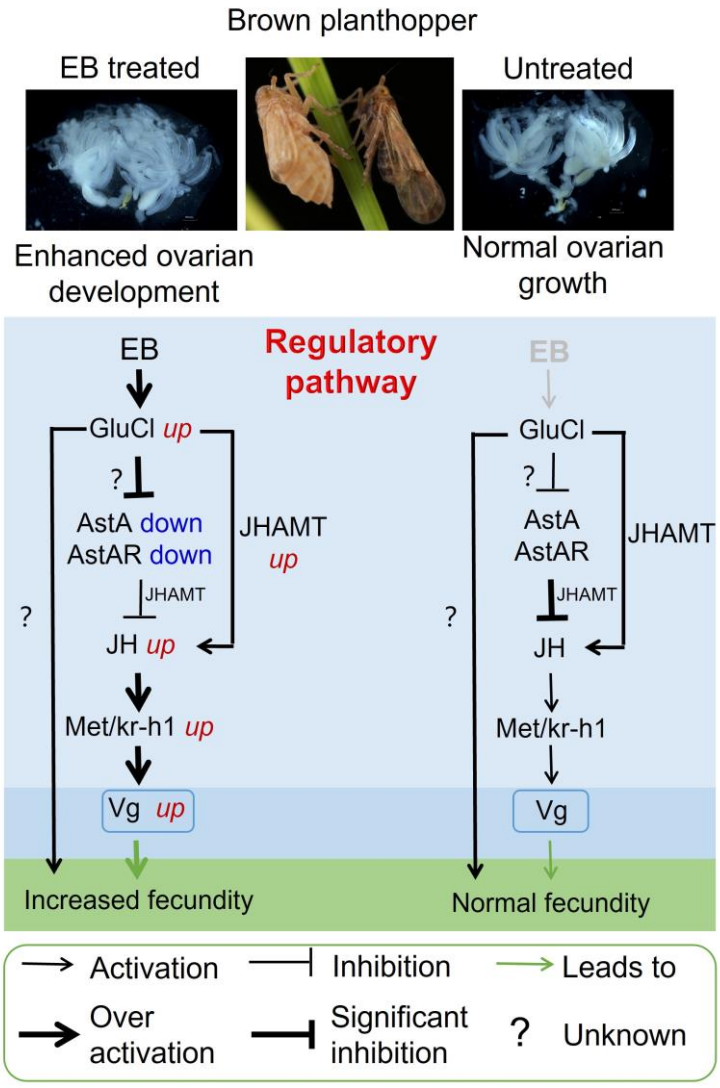
Interestingly knockdown of *AstAR* resulted in significant increases in the expression of genes involved in JH synthesis/signaling including *FAMeT* and *JHAMT Met* and *Kr-h1*. Related to this finding, previous work has shown that knockdown of the AstA receptor gene, *Dar-2*, in *D. melanogaster* results in changes in the expression of genes encoding Drosophila insulin-like peptides (DILPs) and adipokinetic hormone (AKH) signaling proteins [75]. Together with our findings, this demonstrates that AstA receptors may modulate the expression of numerous downstream genes involves in metabolism, energy store and reproduction. In the case of pesticide resurgence our results imply significant cross-talk in the expression of genes that inhibit JH production and those that promote it.

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

608 EB and abamectin are allosteric modulators of GluCls [57-59]. Our data revealed
609 that EB exposure modifies expression of the GluCl in BPH, and knockdown of GluCl
610 expression resulted in a reduction in both JH levels and egg production. Interestingly,
611 the GluCl has been reported to inhibit the biosynthesis of JH in the cockroach,
612 *Diploptera punctata* [76]. Recent work has also reported that modulation of
613 glutamatergic signals may contribute to the photoperiodic control of reproduction in
614 bean bug, *Riptortus pedestris* [77]. Interestingly, work on *D. punctata* has revealed that
615 application of the GluCl channel agonist ivermectin, which like EB belongs to the
616 avermectin family, caused a decline in JH synthesis in corpus allatum glands [76].
617 While the inhibitory effect of ivermectin observed in this previous study differs from the
618 activating effect of EB we observed in our study, it is consistent with our finding of a
619 role for GluCl channel in the regulation of JH regulation. Interestingly, we found that
620 knockdown of *GluCl* gene expression results in the down-regulation of *JHAMT*, *Met*
621 *and Kr-h1*, further revealing significant convergent relationships between genes
622 underpinning pesticide resurgence.
623
624 **Conclusion**
625 Our study has revealed a diverse suite of genes that act in combination to enhance
626 JH titer and thus fecundity following BPH exposure to EB. A schematic of how these
627 factors promote ovary development in the adult stage of *N. lugens* through the JH
628 signaling pathway is provided in Figure 6. Our findings provide the foundation for
629 further work to understand exactly how these genes interact and the mechanisms by

630 which their expression is activated or repressed by EB. Furthermore, our findings
631 provide fundamental insights into the molecular response in insects to xenobiotic
632 stress and illustrate that pesticides can have unexpected and negative impacts on pest
633 populations. In this regard our findings also have applied implications for the control of
634 a highly damaging crop pest. Previous studies have reported that avermectins such as
635 abamectin are toxic to the wolf spider *Pardosa pseudoannulata*, which is the main
636 predator of BPH in rice crops [78, 79]. Thus, these insecticides both stimulate
637 reproduction in BPH while killing their natural enemies providing a 'perfect storm' for
638 damaging BPH outbreaks. Based on these findings, to avoid BPH resurgence, we
639 suggest that the GluCls, EB and abamectin, should not be (or rarely be) applied to rice
640 plants at growth stages when BPHs are present. On a more optimistic note, our
641 findings have identified numerous genes that play key roles in BPH reproduction and
642 thus represent promising targets for the development of novel controls against this
643 important pest.
644

645 **Figure 6**



646

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

Materials and methods

Insects

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

Chemicals

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

Bioassay

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

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

679 the susceptibility of BPH, *L. striatellus* and *S. furcifera* to EB. Technical-grade
680 insecticides were dissolved in acetone as stock solution then diluted in a series of six
681 concentrations with water containing 0.1% Triton. Selected rice seedlings at the 6-8
682 cm growth stage were dipped in insecticide solutions for 30 s and then air-dried at
683 room temperature. The roots of the rice seedlings were wrapped with cotton strips and
684 placed seedlings placed in a plastic cup 5 cm in diameter. Fifteen insects were
685 introduced into each plastic cup for each replicate. The top of the cup was then selaed
686 with gauze to prevent escape. All experiments comprised at least three biological
687 replicates. Control rice seedings were treated with 0.1% Triton X-100 water solution
688 only. All treatments were maintained under standard conditions of 27 ± 1 °C and 70–
689 80% relative humidity with a 16 h light/8 h dark photoperiod. Mortality was assessed
690 after 4 d for *N. lugens* or 2 d for *L. striatellus* and *S. furcifera* after treatment with
691 insecticides. The insects were considered dead if they were unable to move after a
692 gentle prodding with a fine brush.

693 For *Drosophila* larvae bioassay, we adopted a method described previously in our
694 lab with minor modifications [80]. Briefly, twenty third instar larvae were placed in fly
695 vials containing fly food (based on corn powder, brown sugar, yeast and agar)
696 supplemented with EB of different concentrations. Four concentrations (LC_{10}, LC_{30} and
697 LC_{50}) were tested together with a negative (no insecticide) control. For *Drosophila* adult
698 bioassays, we selected virgin females three days after eclosion. Several
699 concentrations were overlaid onto fly food in standard *Drosophila* vials and allowed to
700 dry overnight at room temperature. 15 adult flies (three days after eclosion) were then

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

Contact route: For topical bioassays working insecticide solutions were prepared in acetone. 4th instar nymphs or newly emerged males/females were anesthetized with carbon dioxide for 5 s, and then 0.04 μl/insect test solution applied topically to the dorsal plates with a capillary micro-applicator [26]. Insects were then placed in an artificial climate incubator with a temperature of 27±1°C, a photoperiod of 16:8 h (L:D), and a humidity of 70%±10%. Mortality was determined 2 d after treatment. Data with over 20% mortality in the control treatment were discarded, and the assay was repeated at least three times.

Fecundity assays

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

723 microscope.

724 For *Drosophila* egg-laying assay, we adopted our previous method [81]. Briefly,
725 insecticide-treated virgin females were paired with untreated males for three days and
726 then the mated females transferred into the *Drosophila* ovipositing apparatus. The
727 number of eggs were counted after 16 hours.

728 **Fitness analysis**

729 The fitness of EB-treated BPH were analyzed using methods reported previously
730 [10]. We selected two groups, (♂ck×♀ck) and (♂ck×♀t), to study the effects of the LC_{50}
731 concentration of EB on BPH fitness. In the case of systemic exposure, 4^{th} instar
732 nymphs of BPH were treated with the LC_{50} of EB for 4 days and then transferred to
733 tubes containing untreated rice plants for individual rearing. The rice plants were
734 replaced every three days with untreated plants. The emergence ratio, female ratio,
735 preoviposition period, female longevity, brachypterism female ratio and female weight
736 were calculated.

737 **Examination of ovary development**

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

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

744 10 min (4 × 10 min) in PAT3 (PBS with 0.5% Triton X-100 and 0.5% bovine serum
745 albumin), the ovaries were then incubated with DAPI (4',6-diamidino-2-phenylindole,
746 100 nM) and Actin-stain 670 Fluorescent Phalloidin (200 nM). Imaging was performed
747 using Zeiss LSM980 confocal laser microscope.

748 **Measurements of glycogen, triglyceride, total protein content, cholesterol and**
749 **four sugars**

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

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

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

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

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

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

775 The whole bodies of 5 individuals BPH were mixed with 1 ml of n-hexane, followed
776 by centrifugation at 10,000×g for 10 min, the upper hexane layer was then dried with
777 nitrogen, dissolved in methanol and sonicated for 10 min, after centrifugation at
778 10000×g for 10 min, the supernatant was collected through the organic filter membrane
779 of 0.22 μm into 2 ml vials for JH Ⅲ determination. JH Ⅲ standard sample (J912305-
780 10 mg) purchased from (Shanghai McLean Biochemical Technology Co. Ltd),
781 dissolved in methyl alcohol as stock solution 10,000 mg/L was diluted in a series of six
782 concentration gradients to serve as a reference. Liquid chromatography-tandem mass
783 spectrometry (LC-MS/MS) was then carried out using UPLC Xevo TQ-S Micro (Waters
784 technology), quantitative method according to the external standard, the
785 chromatographic column was EC-C18 (4.6 mm×150 mm, 2.7 μm), column temperature
786 was 30°C，injection volume was 20 μl, elution flow rate was 0.3 ml/min, and the mobile

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

Cloning, sequence and phylogenetic analysis

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

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

bootstrapped with 1000 replications were used using the MEGA 6 software [82].

RNA interference

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

Quantitative RT-PCR

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

829 (10 μM), 10 μL of UltraSYBR mixture buffer, and 6 μL of RNase-free water. Q-PCR
830 was run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA)
831 under the following conditions: 5 min at 95°C, followed by 40 cycles of 95°C for 10 s
832 and 60°C for 30 s. Three independent biological replicates and four technical replicates
833 were used in each qPCR experiment. The housekeeping genes 18S ribosomal RNA
834 of BPH were selected to normalize the expression of candidate genes. The $2^{-\Delta\Delta Ct}$
835 method (Ct represents the cycle threshold) was used to measure relative expression
836 levels [83]. Three biological replicates were used for statistical comparison between
837 samples. Table S2 contains a list of the primers used in this study.

838 **Statistics**

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

851 **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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