1   **E93 expression and links to the juvenile hormone in hemipteran mealybugs with insights on**

2   **female neoteny**

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10 **Abstract**

11 Insect metamorphosis generates reproductive adults and is commonly accompanied by the direct or

12 indirect development of wings. In some winged insects, the imago is altered by life history changes.

13 For instance, in scale insects and mealybugs, reproductive females retain juvenile features and are

14 wingless. The transcription factor *E93* triggers metamorphosis and plays in concert with the

15 juvenile hormone pathway to guarantee the successful transition from juvenile to adult. We

16 previously provided evidence of an atypical down-regulation of the juvenile hormone pathway

17 during female adult development in the Japanese mealybug. Here, we further investigate how *E93*

18 is involved in the production of neotenic wingless females, by identifying its isoforms, assessing

19 their expression patterns and evaluating the effect of exogenous juvenile hormone mimic treatment

20 on *E93*. This study identifies three *E93* isoforms on the 5' end based on Japanese mealybug cDNA

21 and shows that female development occurs with the near absence of *E93* transcripts, as opposed to

22 male metamorphosis. Additionally, while male development is typically affected by exogenous

23 juvenile hormone mimic treatments, females seem to remain insensitive to the treatment, and up-

24 regulation of the juvenile hormone signaling is not observed. Furthermore, juvenile hormone mimic

25 treatment on female nymphs did not have obvious effect on *E93* transcription, while treatment on

26 male prepupae resulted in decreased *E93* transcripts. In this study, we emphasize the importance of

27 examining cases of atypical metamorphosis as complementary systems to provide a better

28 understanding on the molecular mechanisms underlying insect metamorphosis. For instance, the

29 factors regulating the expression of *E93* are largely unclear. Investigating the regulatory mechanism

30 of *E93* transcription could provide clues towards identifying the factors that induce or suppress *E93*

31 transcription, in turn triggering male adult development or female neoteny.

32

33 **Graphical abstract**

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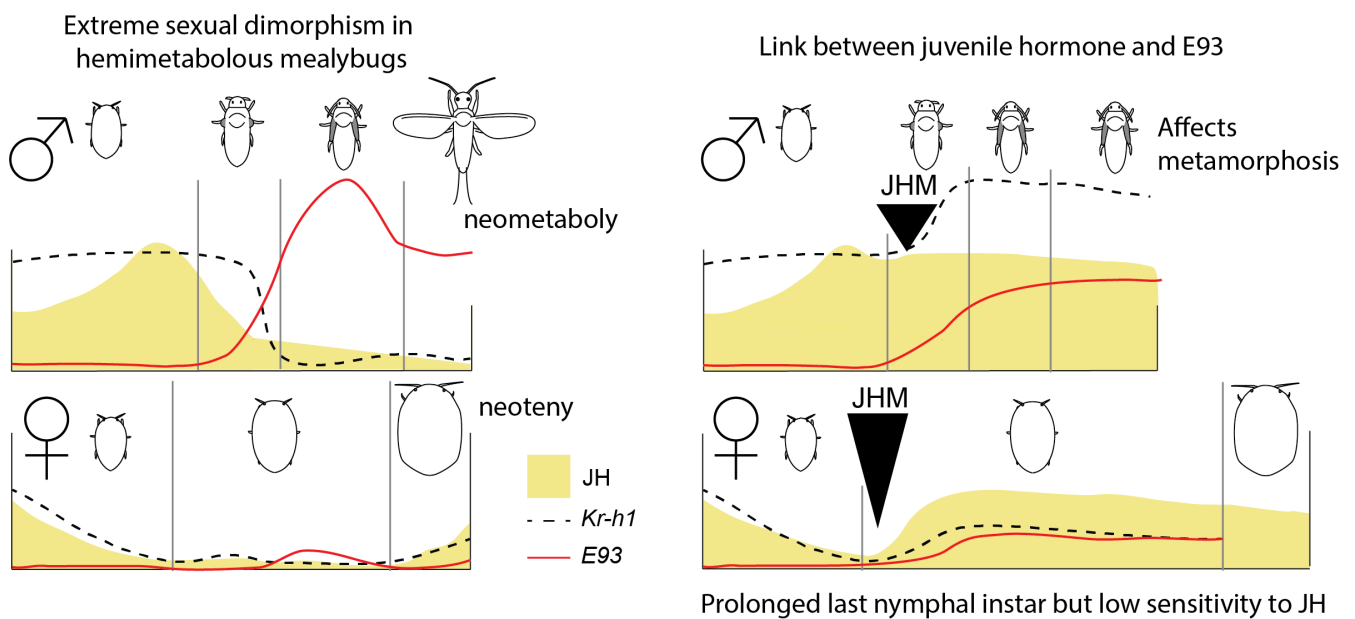
35 **Highlights**

36 - Neotenic female *Planococcus kraunhiae* (Japanese mealybug) develops with low *E93*

37   expression.

38 - *E93* expression pattern during male development is typical to other insects.

39 - Juvenile hormone mimic treatment on male prepupae results in decreased *E93* transcripts.

40 - Juvenile hormone mimic treatment on female nymphs does not have obvious effects on *E93*

41   transcription.

42 - Female mealybugs have low sensitivity to juvenile hormone mimic treatments compared to

43   males and other insects.

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45 **Keywords: E93, insect metamorphosis, neoteny, hemimetaboly, juvenile hormone, mealybugs**

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1. Introduction

48   The evolution of metamorphosis undeniably contributed to the diversity of insect forms, life

49   histories and increasing opportunities for ecological niche exploitation (Grimaldi and Engel, 2005;

50   Truman and Riddiford, 1999)**.** While two types of metamorphosis -- holometaboly and

51   hemimetaboly – predominate in insects, in a few lineages, life cycles deviate to form unusual

52   developmental instances, nonetheless leading to important adaptations. For example,

53   hypermetamorphosis, manifested by two types of larvae, arose from holometaboly. Found in

54   Strepsiptera, Meloidae and Rhipiphoridae beetles, neuropteran Mantispidae and many Hymenoptera

55   and some Diptera, hypermetamorphosis is often associated to parasitic life (Truman and Riddiford,

56   2002) or predatory habits (Belles, 2011). Neometaboly is another metamorphosis found in plant

57   sap-feeding hemimetabolous Paraneoptera (Aleyrodoidea, Thysanoptera, and male Coccomorpha),

58   in which the formation of quiescent stages (prepupae and pupae) is reminiscent to holometaboly

59   (Belles, 2011).

60   Two hormones orchestrate insect metamorphosis: ecdysone and the juvenile hormone (JH).

61   JH, in particular, dictates the identity of subsequent stages and is therefore of special interest for

62   understanding how peculiar life cycles arise. An essential player, the transcription factor *E93*,

63   triggers adult metamorphosis and is universally up-regulated at the end of insect juvenile

64   development (Ureña et al., 2014). *E93* involvement was first reported in *Drosophila melanogaster*

65   cell death process in the prepupa (Baehrecke and Thummel, 1995; Buszczak et al., 2000; Lee et al.,

66   2000) and acts as a developmental switch to control the responsiveness of target genes during

67   metamorphosis (Mou et al., 2012). Later, functional studies on *E93* orthologs in both

68   hemimetabolous and holometabolous species confirmed *E93* is a universal adult specifier in insect

69   metamorphosis (Ureña et al., 2014). Finally, a communication between *E93* and JH signaling

70   pathway exists. In fact, *Krüppel homolog 1* (*Kr-h1*), an early response gene of JH signaling, acts as

71   a repressor of *E93* until the onset of adult metamorphosis: knocking down *Kr-h1* at the penultimate

3

72   juvenile instar of hemimetabolous *Blattella germanica* results in the early increase of *E93* (Belles

73   and Santos, 2014). Thereafter, functional studies in hemimetabolous *Cimex lectularius* (Gujar and

74   Palli, 2016) and *Tribolium castaneum* pupal stage (Ureña et al., 2016) confirmed the interaction of

75   JH signaling and *E93* in other insect lineages. Finally, the direct transcriptional repressor role of *Kr-*

76   *h1* on *E93* promoter region was confirmed in *Bombyx mori* (Kayukawa et al., 2017).

77   Some unusual life cycle alterations can also result in reproductive forms retaining juvenile

78   features: neoteny. Considered merely as curiosities, neotenic forms originated multiple times in

79   various insects and can be associated to particular adaptive traits. Examples include parasitism in

80   Strepsiptera (Kathirithamby, 2009), Isoptera sociality (Higashi and Abe, 1997; Roisin, 2000), and

81   bioluminescence-generating Elateriformia (fireflies, jewel beetles, click beetles etc…) (Bocakova et

82   al., 2007; South et al., 2011). Nevertheless, few studies have addressed the underlying molecular

83   mechanisms of neoteny. The emergence of diverse ways to metamorphose could be associated to

84   the change in maturation timing (heterochrony), which implies that variations in controlling

85   hormones may be an essential factor in establishing these forms (Gould, 1977). As such, female-

86   specific neotenic forms should be tightly linked to the reproductive function of JH. So far, the main

87   hypothesis for the creation of juvenile-like reproductive females resides in excessive levels of JH,

88   simultaneously affecting female developmental progress and the timing of activating reproductive

89   function (Matsuda, 1976).

90   Excessive JH titers are indeed observed in termite neotenics of *Reticulitermes speratus*. Here,

91   the female reproductive neotenic caste shows significantly higher JH titers than those of the nymphs

92   or worker castes. Additionally, knocking down JH receptor (*RsMet*) depletes *vitellogenin* transcript

93   levels. However, it is still unclear whether the phenotypic features attributed to neotenics are

94   affected (Saiki et al., 2015). The only other molecular studies on insect neotenic forms were

95   undertaken on holometabolous insects, where the role of ecdysone was investigated as the

96   responsible factor. Strepsiptera (twisted-wing insects) display sex-specific neotenic forms, whereby

97   females in extreme groups are larviform and endoparasitic. The expression patterns of the pupal

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98 specifier *broad* in *Xenos vesparum* was also examined. In holometabolous insects, *broad* is up-

99 regulated during the last larval instar, at the onset of metamorphosis with an ecdysone titer increase

100 (Kiss et al., 1988; Konopova and Jindra, 2008; Parthasarathy et al., 2008; Uhlirova et al., 2003). In

101 *X. vesparum*, only males that undergo metamorphosis showed the increase of *broad* BTB domain

102 expression, while it was not observed in last larval instar females (Erezyilmaz et al., 2014). Finally,

103 Cecidomyiidae (gall midges) possess a facultative paedogenetic life cycle where ovaries then

104 differentiate and grow precociously in the larval stage. In this instance, a shift in timing of *ecdysone*

105 *receptor* and *ultraspiracle* expression kick-starts the facultative life cycle and creates larval

106 reproductive females (Hodin and Riddiford, 2000).

107 Scale insects and mealybugs (Coccomorpha) belong to Hemiptera, an insect order that mostly

108 develop through hemimetaboly. However, Coccomorpha species have departed from the traditional

109 nymphal instars with progressive wing growth. Males undergo two quiescent stages reminiscent to

110 complete metamorphosis (neometaboly, as mentioned above). In striking contrast, reproductive

111 females retain juvenile features, as they develop through successive molts without wing growth,

112 reduction of nymphal stages and features linked to mobility in many species (Gullan and Kosztarab,

113 1997). This life history trait not only gives rise to extremely sexually dimorphic organisms, but

114 offers a successful strategy, as plant-sap feeding insects, to allocate energy to reproduction,

115 sedentarize and adapt to their host-plant habitats. Female scale insects and mealybugs are often

116 described as neotenic (Danzig, 1980; Koteja, 1990). However, which type of neoteny, or the

117 mechanisms by which adult females keep juvenile features remains unknown. This prevents us

118 from understanding the link between the development and evolution of neoteny. More importantly,

119 this lineage includes some of the most damaging agricultural pests in human activities, likely a

120 consequence of the evolution of neotenic females and their adaptive life history to host plants.

121 We previously presented a study on the variation of JH in the Japanese mealybug

122 *Planococcus kraunhiae* (Pseudococcidae) to examine variations between male and female

123 development as a possible mechanism leading to extreme sexual dimorphism in scale insects. In

5

124   addition to significant differences observed in JH early-response gene *Kr-h1* when male and female

125   mealybugs start to differentiate, we reported that JH signaling remained unusually low throughout

126   female adult development, suggesting a different JH regulation of female neoteny in this case (Vea

127   et al., 2016). To further examine the involvement of *E93* in female neoteny, in relation to the JH

128   signaling, we compared the expression pattern of *E93*, in *P. kraunhiae* male and female

129   postembryonic development and performed hormonal assays using a JH mimic, pyriproxyfen. We

130   hypothesize that in scale insects, females fail to express *E93*, resulting in the maintenance of

131   juvenile features in their external morphology even after reproductive maturation. Additionally, we

132   test whether increasing levels of JH during the last nymphal instars in females readjusts the

133   expression of *Kr-h1* at similar levels as seen in males, which in turn could allow to initiate *E93*

134   expression. It turned out that females are insensitive to exogenous JHM treatment in the context of

135   the effects on *Kr-h1* and *E93* expression as well as on adult development.

136

2. Materials and methods

2.1. Mealybug rearing and sampling strategy

Mealybug culture and sampling strategy for gene expression profile are described in a previous study on JH variations in the *P. kraunhiae* (Vea et al., 2016). In this study, we carried out an independent sampling to ensure reproducibility of the previous study. As such, we collected samples every 24 hours after oviposition. Eggs oviposited during the first day were used for male-biased samples and eggs oviposited during the fifth day for female-biased samples (see Vea et al., 2016 for sex-biased sample strategy). All stages are abbreviated as follows: E = embryonic stage after oviposition, N1= first-instar nymph, N2= second-instar nymph, N2f= female second-instar nymph, N2m= male second-instar nymph, N3= female third-instar nymph, pre=male prepupa, pu= male pupa, m=male adult, f=female adult.

2.2. cDNA cloning and identification of sequences

The total RNA of pooled individuals from different stages was extracted with TRIzol (as described in Vea et al., 2016) and Oligo-dT-primed reverse transcription was performed with the PrimeScript II 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). The conserved region of *E93* sequence in *B. germanica* was blasted against a transcriptome of *P. kraunhiae* [accession number DRA004114; (Sugahara et al., 2015)], and primers for RT-PCR were designed to amplify a partial region of the gene. Primers for RACE PCR were designed based on this partial sequence, and 5' and 3' RACE was conducted with SMARTer RACE cDNA Amplification Kit (Takara Bio USA, Inc., Mountain View, CA) in order to retrieve the full-length cDNA sequences. All PCR products were cloned in a pGEM-T Easy Vector (Promega, Madison, WI) and sequenced. DNA sequence data were deposited in the DDBJ/EMBL-Bank/GenBank International Nucleotide Sequence Database with the following accession numbers: *PkE93* isoform 1 (LC374380), *PkE93* isoform 2 (LC374381) and *PkE93* isoform 3 (LC374382). The primer sequences are listed in Table S1.

2.3. RNA extraction and quantitative RT-PCR

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163   Total RNA was extracted from all samples using the sex-biased sampling as described in Vea et al.

164   (2016). Each sample consisted of 0.5 to 2 mg of pooled individuals homogenized in TRIzol reagent,

165   total RNA was extracted using nuclease-free glycogen (Thermo Fisher Scientific) as a carrier, and

166   reverse transcribed using the PrimeScript RT reagent Kit with the gDNA Eraser (Takara Bio).

167   Expression profiles for the post-oviposition development of males and females were established by

168   quantifying the levels of transcripts for targeted fragments using absolute quantitative RT-PCR

169   (qRT-PCR), performed on a Thermal Cycler Dice Real Time System (model TP800, Takara Bio) as

170   described previously (Vea et al., 2016). Six serial dilutions of a plasmid containing a fragment of

171   each gene was used as the standard. Primer sequences for each *PkE93* isoform used for qRT-PCR

172   are listed in Table S1. The values obtained by the second derivative maximum (SDM) method were

173   normalised with the *ribosomal protein L32* (*rpL32*) transcript levels. Primers for *PkrpL32,* our

174   reference gene, were from our previous study (Vea et al., 2016).

175   *2.4. JH mimic assays on male prepupae and female juvenile instars*

176   For JH mimic (JHM) treatments, we applied 2 µL of pyriproxyfen (5 mM dissolved in methanol) to

177   batches of 3 to 5 male prepupae on a filter paper, 24-48 hours after molting. Excess chemical

178   solution was immediately absorbed by the filter paper. After treatment, we waited for the solvent to

179   evaporate completely before transferring them in 1.5 mL microcentrifuge tubes (Ina Optica, Osaka,

180   Japan), bearing a paper disc (8 mm in diameter, 1.5 mm in thickness; Toyo Roshi, Japan) with 10

181   µL of distilled water to control humidity. Female N3D0 (0-24 h after molting to the N3 stage) were

182   treated in the same manner except that 0.5 µL of pyriproxyfen (20 mM dissolved in methanol) was

183   applied on the tergite of individual N3D0. After the N3D0 started to move again, it was transferred

184   into a glass dish containing a sprouted broad bean on top of a filter paper. The glass dishes were

185   sealed with parafilm. Treated samples were left to incubate at 23ºC for various numbers of days

186   after treatment before being homogenized in TRIzol and stored at -80 ºC for RNA extraction. RNA

187   extraction, reverse transcription and qRT-PCR analyses were performed as described in 2.3.

188

189   *2.5. Graphs and statistical analyses*

190   Graphs from qRT-PCR SDM values were generated using the R package ggplot2 and the statistical

191   significance of JHM effects were calculated by comparing the overall effect of the mimic over the

192   days using a linear model. We also tested the effect of JHM on individual days 4 and 8 after

193   treatment in females using the Student's *t*-test. All analyses using qRT-PCR SDM values are

194   detailed and publically available in GitHub and Zenodo

195   (https://zenodo.org/badge/latestdoi/116843862).

196

197   **3. Results and Discussion**

198   *3.1. Structure of E93 in P. kraunhiae*

199   The *PkE93* sequence identified from RT-PCR includes a Pipsqueak DNA binding domain

200   characteristic of *E93* (Siegmund and Lehmann, 2002) and highly conserved (**Fig. 1A**). Using 5' and

201   3' RACE PCR combined to designed primers from the conserved region, we cloned and sequenced

202   three complete transcripts of 5008, 5056 and 5233 bp long. All of them resulted from either usage

203   of different transcription initiation site and/or alternative splicing on 5'end only (**Fig. 1B**). Each

204   isoform was arbitrarily designated as *PkE93-1*, *PkE93-2* and *PkE93-3*. The region common to all

205   transcripts counts 4676 bp, *PkE93-1* and *PkE93-3* predicted protein sequence is of 1050 aa, while

206   *PkE93-2* has a predicted protein sequence of 1090 aa. In summary, *PkE93-1* and *PkE93-3* differ in

207   the 5' untranslated region, while *PkE93-2* has a longer coding region.

208

209   *3.2. Sex-specific expression profiles of E93*

210   We first examined the expression profile of each *PkE93* isoform during the post-

211   oviposition development in male and female mealybugs, using absolute qRT-PCR (**Fig. 2A**).

212   *PkE93-1* isoform has the highest expression compared to the two other isoforms. Generally, *PkE93-*

213   *1* stays at low levels in the embryo, N1 and N2 in both males and females (**Fig. 2A; top**). In males

214   only, *PkE93-1* suddenly increases at the beginning of prepupa and reaches a peak of expression

9

215 before adult metamorphosis. In females, however, *PkE93-1* does not show such dramatic

216 expression, although slight increases at the end of N2 and N3 are observed (**Fig. 2B**). *PkE93-2*

217 isoform followed a similar expression pattern although around 20-fold lower (**Fig. 2A; middle**),

218 which suggests that *PkE93-2* may be a minor isoform. Finally, *PkE93-3*, despite its also lower

219 expression, shows a distinct pattern during the embryonic stage (**Fig. 2A; bottom**), with peaks of

220 expression in both males and females, similar to that of *PkKr-h1* (**Fig. S1**). At the end of N2,

221 *PkE93-3* expression also increases in males but decreases during the pupal stage, while its

222 expression reaches near-zero levels after N2 in females. *PkE93* expression differs between males

223 and females more strikingly than the early JH-response gene *PkKr-h1*. This is especially true

224 towards the end of post-embryonic development, where a peak of expression is observed in males,

225 but not in females. At this point, males enter quiescent stages (prepupa and pupa) where wings

226 develop. In contrast, females molt only once then become reproductively mature but retain juvenile

227 features. Along with previous results on *PkKr-h1* and *Pkbr* (Vea et al., 2016), we suggest that sex-

228 specific expression patterns of *PkE93* may contribute to the development of sexual dimorphism in

229 mealybugs.

230

231   *3.3. Expression pattern of male mealybugs consistent with JH signaling of other insects*

232   The origin of holometaboly has been debated for decades. Although the consensus is that

233 endocrinological changes are responsible for the transition between hemimetaboly and

234 holometaboly, two hypotheses were advanced regarding the details of stage homologies [see details

235 in (Truman and Riddiford, 1999)]. More recently, based on study of *E93* expression in *B.*

236 *germanica*, it was proposed that the last nymphal stage in hemimetabolous insects is

237 ontogenetically homologous to the pupal stage of holometabolous insects because of the similar

238 *E93* expression pattern during these stages (Belles and Santos, 2014). Furthermore, the propupal

239 and pupal stages in neometabolous Thysanoptera, could be together homologous to the

240 holometabolous pupal stage (Minakuchi et al., 2011). In scale insects, a similar neometabolous

241 development occurs in males (stages traditionally coined as "prepupa" and "pupa") and our

242 expression profile pattern in male *P. kraunhiae* (**Fig. 2A**) shows that *PkE93* expression starts at the

243 beginning of the prepupa and peaks at the beginning of the pupal stage. Finally, hormonal treatment

244 using JHM at the beginning of the prepupal stage, when *Kr-h1* expression drops suddenly, prevents

245 adult metamorphosis by creating a supernumerary pupal stage (Vea et al., 2016). In addition to

246 maintaining high levels of *PkKr-h1* for at least six days, JHM treatment results in the significant

247 decrease of *PkE93-1* and *PkE93-2,* while *PkE93-3* is not significantly affected (**Fig. 3A**). Although

248 functional analyses are still necessary to confirm this in mealybugs, the effects of JHM on *E93*

249 expression in neometabolous males are congruent with previous results found in other

250 holometabolous and hemimetabolous insects, and *Kr-h1* and *E93* must be acting as reciprocal

251 inhibitors.

252

253 *3.4. Female atypical regulation of JH signaling and E93*

254 Based on our expression profile of *PkE93* in females, all isoforms are very low throughout

255 the successive molting events, contrasting with male neometabolous development. However, if we

256 compare *PkE93* and *PkKr-h1* expression only during the last instar nymphs, we see that even at

257 very low levels, a slight decrease of *PkKr-h1* is accompanied with a small peak of expression of

258 *PkE93-1* (**Fig. 2B**). Although the female expression is 20-fold lower than in males, this small peak

259 could explain the sexual maturation of females after N3, while somatic differentiation does not

260 occur in the imago. Additionally, this small peak likely indicates tissue-specific expression, like

261 ovaries and related reproductive organs. As *PkKr-h1* expression begins to decrease progressively at

262 the penultimate nymphal stage in females (**Figs. 2B and S1**; Vea et al. 2016), we decided to

263 increase the expression of *Kr-h1* with JHM treatment at the early last nymphal stage (N3D0). By

264 doing so, we assessed whether *Kr-h1* can artificially reach a threshold necessary for the switch to

265 adult fate controlled by *E93*. Twenty mM of pyriproxyfen were applied on N3D0, concentration

266 four times higher than male treatments. Following this treatment, 7 out of 19 (37 %) treated

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267   individuals died before the last molt (**Table 1**). The 12 survivors (63 %), showed a tendency to

268   prolong the N3 stage. While the control samples molted 9.6±1.5 days (N=14) after treatment, 12 out

269   of 19 JHM-treated individuals molted to adult stage 13±2.5 days after treatment. We also followed

270   the effect of JHM treatment on *PkKr-h1* and *PkE93* expression (**Fig. 3B**), one to eight days after

271   treatment (last days before the female adult molt). At 20 mM, pyriproxyfen induced the up-

272   regulation of *PkKr-h1* after four days, which then lasted several days. However, it is worth

273   mentioning that *PkKr-h1* levels were significantly lower than the response observed in males, even

274   with a concentration of pyriproxyfen four times lower. We suggest that female mealybugs may have

275   mechanisms preventing them to respond to JH as sensitively or efficiently as in males. Previously,

276   we showed that *PkMet* and *PkTai*, forming the JH receptor complex, were highly expressed at the

277   end of male development, while in females the expression remained low (Vea et al., 2016). After

278   JHM treatment on females at N3D0, although *PkKr-h1* starts to be affected only four days after

279   treatment by an upregulation, the expression of all *PkE93* isoforms does not change significantly

280   over time (interaction between treatment and time not significant; **Fig. 3B**). However, when

281   removing the interaction, the treatment alone leads to an overall significant effect for two isoforms;

282   this is probably explained by the increase in expression at Day 2, Day 4 and Day 8 after treatment

283   for *PkE93-1* and *PkE93-2*. We therefore conclude that applying JHM on early ultimate juvenile

284   instars in females does not significantly change *PkE93* expression overtime, but a small effect

285   might be observed at Day 8 after treatment by an increase of *PkE93-1* and *PkE93-2*.

286   Several studies have demonstrated that at the onset of metamorphosis in holometabolous

287   insects, juvenile instars need to reach a critical weight for adult metamorphosis (Davidowitz et al.,

288   2003; Nijhout, 2003; Nijhout et al., 2014). In mealybugs, males and females are phenotypically

289   identical before their development starts to diverge. If we consider that critical weight is a factor in

290   at least male metamorphosis, the threshold should be reached at the start of prepupa, where we see

291   the sudden decrease of *PkKr-h1*. By contrast, females should attain their critical weight for

292   metamorphosis in N3 stage. In females, *PkKr-h1* down-regulation takes place in a progressive

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293 decrease from the middle of N2 rather than the sudden decline of transcripts. Moreover, *Pkjhamt*

294 (*juvenile hormone acid O-methyltransferase*), involved in the last steps of JH biosynthesis, starts

295 decreasing even at the beginning of N2, while in males, the transcripts are still expressed until

296 prepupa (Vea et al., 2016). This indicates an early arrest in JH synthesis in female development.

297 Another possibility is that a sudden decrease combined with attaining a threshold of *Kr-h1*

298 expression is necessary to induce *E93* expression. In this case, maintaining low levels of *PkKr-h1*

299 during female development may explain why *PkE93* expression never peaks.

300

301 **4. Concluding remarks**

302 JH anti-metamorphic role in insect metamorphosis suggests that high levels of this hormone delays

303 metamorphosis. Female neoteny has therefore been believed to be the result of disrupted JH down-

304 regulation leading to constantly high JH titers. Although this hypothesis was first proposed based

305 only on the differential size of scale insect *corpora allata* (Matsuda, 1976), we recently reported

306 contradictory evidence that the last juvenile stages in female Japanese mealybug develop under

307 surprisingly lower JH titers compared with males (Vea et al., 2016). In this study, we provide

308 additional data to support an atypical hormonal regulation in mealybugs, and show the first example

309 of possible failure in *E93* induction, linked to neotenic reproductive females in an hemimetabolous

310 insect. The response of female development to JH modulations is intriguing and suggests that

311 mealybug neotenic forms are insensitive to JH signaling as opposed to males, has consequences on

312 *E93* expression, and leads to extreme sexual dimorphism. So far, gene expression manipulation on

313 mealybug juvenile stages by dsRNA injection has been ineffective. Alternatively, identifying

314 suppressors of *E93* promoter in neotenic females, coupled with functional studies through genome

315 editing should provide novel insights on the function and interaction between *E93* and JH signaling

316 pathway in neotenic female scale insects.

317

318

319   **Acknowledgements**

324

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428 **Figures**

A

Homo sapiens LCoR-2	RKKRGRYRQYNSEILEEAISVVMSGKMSVSKAQSIYGIPHSTLEYKVKERLGTL
Caenorhabditis elegans MBR1	RPKRGQYRKYDKNALDEAVRSVRRGEMTVHRAGSFFGVPHSTLEYKVKERNLMR
Blattella germanica E93	RPKRGKYRNYDRDSLIEAVRAVQRGEMSVHRAGSHFGVPHSTLEYKVKERHLMR
Planococcus kraunhiae E93	RPKRGKYRNYDRDSLVEAVRAVQRGEMSVHRAGSYYGVPHSTLEYKVKERHLMR
Tribolium castaneum E93	RPKRGKYRNYDRDSLVEAVRAVQRGEMSVHRAGSYYGVPHSTLEYKVKERHLMR
Drosophila melanogaster E93	RPKRGKYRNYDRDSLVEAVKAVQRGEMSVHRAGSYYGVPHSTLEYKVKERHLMR
Bombyx mori E93	RPKRGKYRNYDRDSLVEAVKAVQRGEMSVHRAGSYYGVPHSTLEYKVKERHLMR

B

PkE93-1 (5233 bp, 1050 aa)

PkE93-2 (5008 bp, 1090 aa)

PkE93-3 (5056 bp, 1050 aa)

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430 **Figure 1:** Identification of PkE93 sequence and the structure of its isoforms. A. Amino acid alignment of the Pipsqueak
431 DNA-binding domain with other insects' E93, *Homo sapiens* LCoR-2 and *Caenorharbidtis elegans* MBR1 [sequences
432 from (Ureña et al., 2014)]. B. General structure of cDNA sequences obtained from 5' and 3' RACE PCR. The
433 sequences are identical on the 3' end, while the 5' end differ among the three isoforms, the common region is of 4676
434 bp starting from 3' end. Grey: open reading frame. Arrows: primers designed for qRT-PCR. Scale bar: 500 bp.
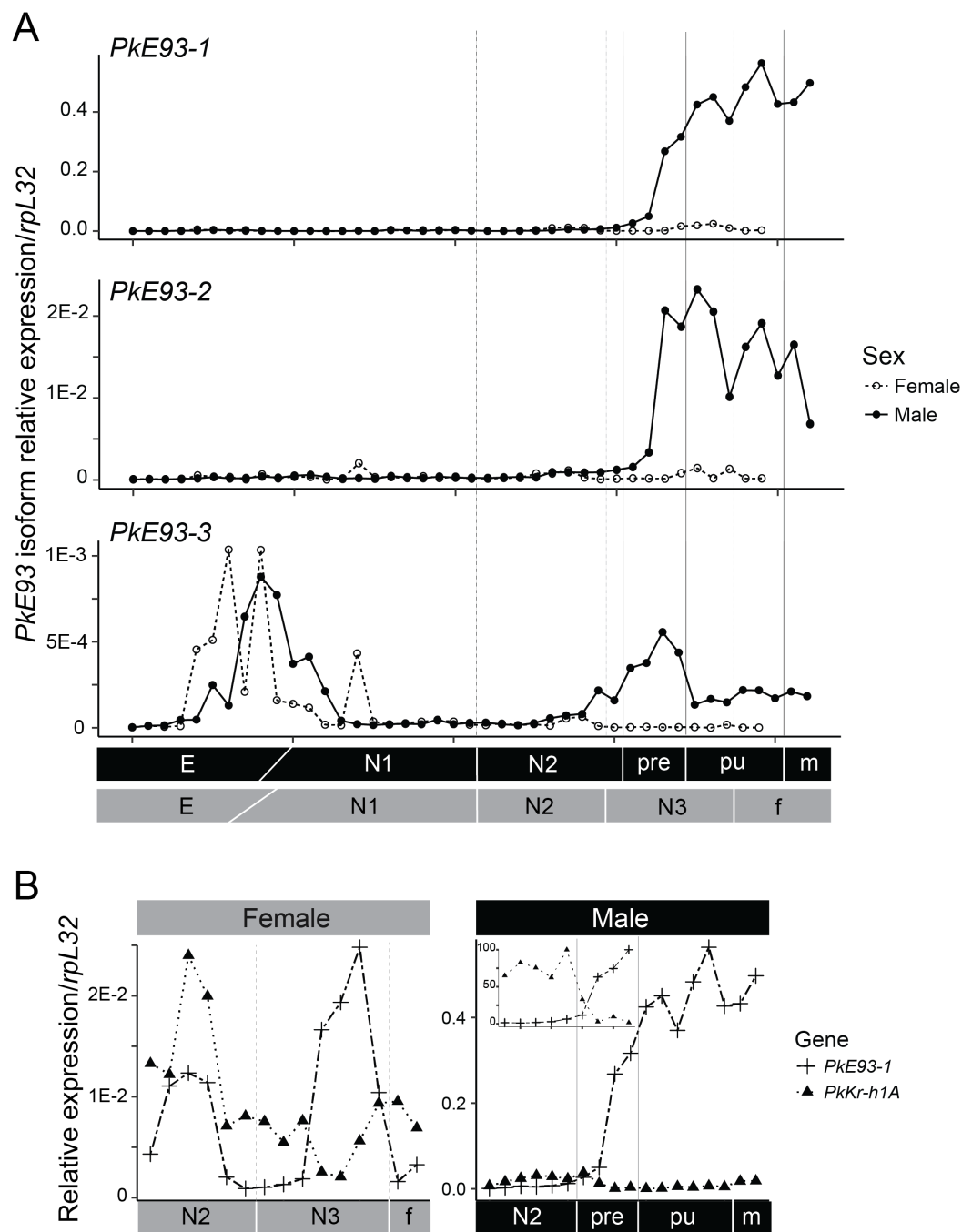
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Figure 2: Expression profiles of *PkE93* isoforms throughout post-oviposition in males and females, and comparison with those of *PkKr-h1* at the end of development. A. *PkE93-1*, *PkE93-2* and *PkE93-3* expression profiles from qRT-PCR of samples collected every 24 hours from oviposition. The primers used for qRT-PCR are shown in Fig. 1 and their sequences are listed in Table S1. B. Comparison of *PkE93-1* and *PkKr-h1A* expression from the second-instar nymph, when female and male can be differentiated, to the adult stage. The small graph on top left part of male expression graph shows the relative expression in percentage when the switch of expression occurs between the two genes.
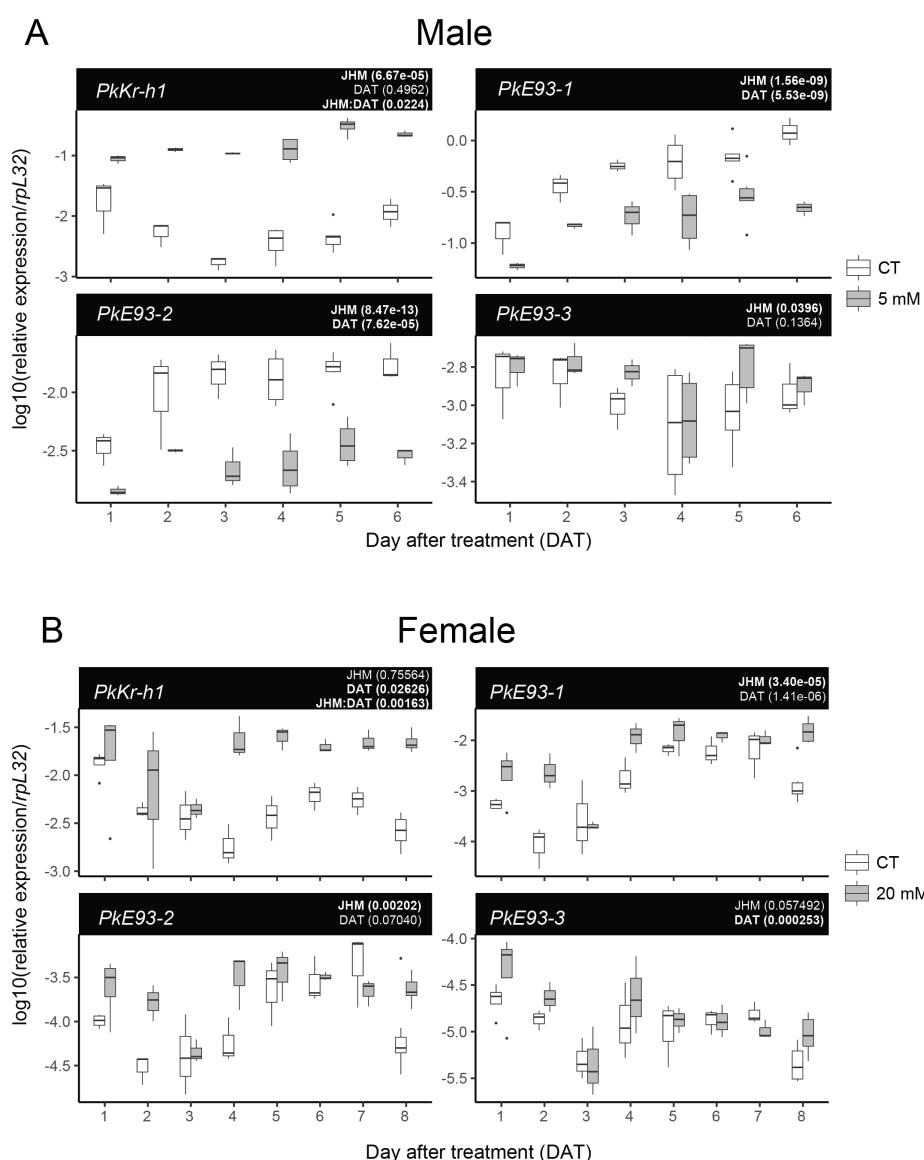
Figure 3: Effect of juvenile hormone mimic treatment on *Kr-h1* and *E93* expression at the end of male and female development. The transcript levels were analyzed with qRT-PCR, and normalized with the *rpL32* levels. A. Expression of *PkKr-h1, PkE93-1, PkE93-2* and *PkE93-3*, 1 to 6 days after male 1 day old prepupae (PreD1) were treated with 5 mM pyriproxyfen or methanol. B. Expression of *PkKr-h1, PkE93-1, PkE93-2* and *PkE93-3*, 1 to 8 days after 0-24 h old third instar females (N3D0) were treated with 20 mM pyriproxyfen or methanol. Statistical significance: we used a linear model in R on log10 transformed data, to test the significance of pyriproxyfen treatments and also considered day after treatment as a factor (see GitHub link in Materials and Methods for details on the analysis). P-values for each factor are indicated on top right side of each graph. We first considered an interaction between the two tested variables in our linear model. If the interaction is significant, the p-values of treatment (JHM), day after treatment (DAT) and interaction between them (JHM:DAT) are indicated (as it is the case for *PkKr-h1*). If no interaction was found, we used the linear model excluding the interaction and indicated p-values for treatment and day after treatment only (for all three *PkE93* isoforms). We considered an effect significant when p-value < 0.01.

List of supplementary material

Table S1: List of primers used for cloning, 5' and 3' RACE PCR and quantitative RT-PCR.

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460   Figure S1: Expression profile of *PkKr-h1* during male and female Japanese mealybug development
461   after oviposition
462
463   GitHub/Zenodo repository for data analysis: https://zenodo.org/badge/latestdoi/116843862
464   Protocol repository: protocols for sex biased collecting strategy in mealybugs
465   (dx.doi.org/10.17504/protocols.io.mh9c396) and pyriproxyfen treatments on mealybugs
466   (dx.doi.org/10.17504/protocols.io.miac4ae)

467   Table 1: Effect of pyriproxyfen treatment on last nymphal instar females on adult metamorphosis

	N	Number of dead individuals during N3 (%)	Number of individuals alive that molted to adult (%)									(days after treatment)
			8	9	10	11	12	13	14	15	16	
Methanol	14	0 (0)	3 (21)	6 (43)	1 (7.1)	3 (21)	0 (0)	1(7.1)	0 (0)	0 (0)	0 (0)	
20 mM pyriproxyfen	19	7 (37)	0 (0)	2 (11)	1 (5.3)	1 (5.3)	0 (0)	5 (26)	0 (0)	0 (0)	3 (16)	

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