

1 BmCncC/keap1-pathway is involved in high-temperature induced metamorphosis
2 regulation of silkworm, *Bombyx mori*

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19

20 **Abstract**

21

22 The global warming has affected the growth, development and reproduction of insects. However,
23 the molecular mechanism of high temperature stress-mediated metamorphosis regulation of
24 lepidopteran insect has not been elucidated. In this study, the relationship between the insect
25 developmental process and endogenous hormone level was investigated under high temperature
26 (36 ° C) stress in *Bombyx mori* (*B. mori*). The results showed that the duration of 5th instar larvae
27 were shortened by 28 ± 2 h, and the content of 20E was up-regulated significantly after 72 h of
28 high temperature treatment, while the transcription levels of 20E response genes *E93*, *Br-C*, *USP*,
29 *E75* were up-regulated 1.35, 1.25, 1.28, and 1.27-fold, respectively. The high temperature
30 treatment promoted the phosphorylation level of Akt and the downstream BmCncC/keap1
31 pathway was activated, the transcription levels of 20E synthesis-related genes *cyp302a1*,
32 *cyp306a1*, *cyp314a1* and *cyp315a1* were up-regulated by 1.12, 1.51, 2.17 and 1.23-fold,
33 respectively. After treatment with double stranded RNA of BmCncC (dsBmCncC) in BmN cells,
34 the transcription levels of *cyp302a1* and *cyp306a1* were significantly decreased, whereas
35 up-regulated by 2.15 and 1.31-fold, respectively, after treatment with CncC activator Curcumin.
36 These results suggested that BmCncC/keap1-mediated P450 genes (*cyp302a1*, *cyp306a1*)
37 expression resulted in the changes of endogenous hormone level, which played an important role
38 in the regulation of metamorphosis under high temperature stress. Studies provide novel clues for
39 understanding the CncC/keap1 pathway-mediated metamorphosis regulation mechanism in
40 insects.

41

42 **Author Summary**

43

44 Mammalian nuclear transcription factor Nrf2 (NF-E2-related factor 2) plays an important role in
45 the stress response of cells. CncC is a homolog of mammalian Nrf2 in insect, regulating the genes
46 expression of insect antioxidant enzymes and cytochrome P450 detoxification enzyme. Evidence
47 suggests that the CncC/Keap1 pathway also plays an important role in regulating insect
48 development. Here, we investigated the regulatory mechanism between the CncC/Keap1 pathway
49 and metabolism of silkworm hormones in Lepidoptera. We found that high temperature induction
50 accelerated the development of silkworm, the ecdysone content and related metabolic genes in
51 hemolymph were significantly up-regulated, the CncC/Keap1 pathway was activated, and the
52 expression of *BmCncC* was significantly increased, indicating that the CncC/Keap1 pathway plays
53 an important role in this process. The expression of *cyp302a1* and *cyp306a1* was significantly
54 decreased by RNA interference with *BmCncC*, which indicated that CncC in silkworm had a
55 regulatory relationship with downstream 20E synthetic gene. In summary, the results indicate that
56 the CncC/Keap1 pathway plays an important role in regulating hormone metabolism in silkworm,
57 providing a basis for further study of the relationship between CncC/Keap1 pathway and
58 development in insects.

59

60

61 **Introduction**

62

63 The global warming resulted from greenhouse-effect adversely affects the ecosystems and

64 survival of organisms, changing the living habitats and causing deterioration of the ecological
65 environment [1, 2]. Insects are the most abundant species on earth and play an important role in
66 the balance of ecosystems and the human agroforestry economy [3]. Insects are
67 temperature-variable animals that are extremely sensitive to environmental changes. The high
68 temperature environment directly affects the growth and development of insects, changing the
69 biological characteristics, reproductive ability and life span [4,5,6]. Silkworm (*Bombyx mori*)
70 belongs to the Lepidoptera and is an important economic insect [7, 8] which is susceptible to high
71 temperature environment during rearing, resulting in reduction of survival rate, cocoon rate, and
72 pupa yield [9,10]. The molecular mechanism of high temperature-mediated interaction between
73 metamorphosis processes and endogenous hormone metabolism has not been elucidated.

74

75 The mammalian Nrf2 (nuclear factor erythroid 2 related factor 2)/Keap1 (Kelch-like
76 ECH-associated protein 1) pathway regulates intracellular redox potential, metabolic
77 detoxification, enhances cell resistance and delays aging [11,12]. Under stress conditions, Nrf2
78 induces the expression of antioxidant and detoxification enzyme genes by selectively binding to
79 the antioxidant regulatory element (ARE) element in the promoter region to protect the cells
80 against external stress [13, 14]. CncC (cap 'n' collar isoform-C) is the homolog of Nrf2 in insects,
81 which plays an important role in metabolic detoxification and antioxidant enzyme genes
82 expression [15]. The cytochrome P450 family genes in insects are mainly involved in
83 detoxification of plant toxins and exogenous chemicals, which are related to insecticides
84 resistance, and also play important role in biosynthesis of insect hormones such as 20E and
85 pheromone et. [16]. Inhibition of CncC gene expression can change the developmental process of
86 *Leptinotarsa decemlineata* [17], suggesting that CncC plays an important role in the insect
87 metamorphosis. The P450 family genes in silkworm are divided into four subfamilies according to
88 their homology: CYP2, CYP3, CYP4 and mitochondrial P450 [18], and the Hollween family
89 genes *cyp302a1*, *cyp306a1*, *cyp314a1* and *cyp315a1* are mainly involved in 20E synthesis [19]. In
90 silkworm, whether the P450 family Hollween genes are regulated by the BmCncC/keap1 pathway
91 under high temperature stress have not been reported.

92

93 **Results**

94

95 **Effects of HT stress on the development and vitality of silkworm**

96

97 The results indicate that high temperature treatment resulted in smaller body size of larvae and
98 transparent epidermis (Fig 1A), the characteristics and behavior of mature silkworm larvae were
99 observed either. In addition, the larvae maturity time was advanced 28 ± 2 h in average and mostly
100 were concentrated around 144 h to 168 h (Fig 1C), indicating that high temperature treatment
101 shortens the developmental duration of larvae. Furthermore, the vitality and pupation rate in high
102 temperature group was reduced by 0.85 and 0.76-fold of the control group, respectively (Fig 1B
103 and D), indicating that high temperature treatment reduced the vitality of the silkworm.

104

105 **The effect of HT stress on endogenous hormone and its metabolism-related genes expression**

106

107 The content of JH in hemolymph was reduced by 0.63-fold at 72 h after high temperature

108 treatment (Fig 2A), and the mRNA levels of the JH metabolism genes *Met*, *JH3* and *Kr-h1* were
109 down-regulated by 0.75, 0.77, and 0.54-fold, respectively (Fig 2C). In addition, the content of 20E
110 was increased by 1.86-fold after 72 h treatment, and the expression of 20E response genes *USP*,
111 *Br-C*, *E75* and *E93* was up-regulated by 1.76, 1.35, 2.94 and 1.79-fold, respectively (Fig 2D).
112 These results indicated that high temperature treatment changed the level of endogenous hormone
113 in hemolymph.

114

115 **High temperature stress activated Akt/BmCncC/Keap1 pathway and induced expression of 116 20E-synthetic genes**

117

118 The mRNA levels of BmCncC and Maf were up-regulated by 1.89 and 1.32-fold, and keap1
119 was down-regulated by 0.85-fold. The transcription levels of upstream genes PI3K and Akt was
120 up-regulated significantly by 1.53 and 2.59-fold, respectively. Western blot analysis showed that
121 the protein levels of Akt, p-Akt and BmCncC were up-regulated by 1.45, 1.56 and 1.32-fold,
122 while the protein level of Keap1 was down-regulated by 0.76-fold, respectively. Furthermore, the
123 mRNA levels of 20E-biosynthesis genes *cyp302a1*, *cyp306a1*, *cyp314a1*, and *cyp315a1* were
124 significantly up-regulated, which was 1.12, 1.51, 2.17, and 1.23-fold, respectively (Fig 3A). The
125 results indicated that high temperature treatment activated the Akt/BmCncC/keap1 pathway and
126 induced the expression of downstream 20E biosynthesis genes.

127

128 **Regulation of downstream P450 genes by BmCncC**

129

130 The mRNA levels of *cyp302a1* and *cyp306a1* in BmN cells were significantly down-regulated
131 by 0.46 and 0.78-fold after treatment with dsBmCncC for 24 h. In contrast, after treatment with
132 CncC activator Curcumin for 24 h, the mRNA levels of *cyp302a1* and *cyp306a1* were
133 up-regulated by 2.15 and 1.31-fold, respectively (Fig 4). The transcriptional levels of *cyp314a1*
134 and *cyp315a1* were non-significantly changed after treatment (Fig 4). These results indicated that
135 BmCncC plays an important role in regulation of *cyp302a1* and *cyp306a1* genes.

136

137 **Discussion**

138

139 The high temperature environment caused by climate warming resulted in the deterioration of
140 the ecological environment and affects the growth and development of animals and plants [20].
141 The elevate in temperature can increase the annual generations of insects in the growing season,
142 affecting the growth and development of insects [21]. Studies have shown that high temperature
143 stress can reduce the vitality, body weight, and cocoon shell rate of *B. mori*, and promote the
144 expression of stress-related genes and CSP (chemosensory protein) genes [10]. In this study, the
145 larva epidermis became transparent and showed the characteristics of mature larva after high
146 temperature induction (Fig 1A), the survival and pupation rate of silkworm were significantly
147 decreased (Fig 1D), which was in consistent with previous studies [10]. BmCncC/keap1 pathway
148 plays an important role in response to oxidative stress and regulation of antioxidative and
149 detoxification genes expression [22]. Whether this pathway involved in metamorphosis processes
150 and regulates ecdysone-synthesis P450 genes in silkworm have not been reported. We found that
151 the duration of 5th instar larva was shortened and the maturity time was advanced and relatively

152 concentrated after high temperature induction (Fig 1C), indicating that the high temperature
153 promoting the development of silkworm. We also investigated the relationship between the 20E
154 synthesis-related P450 genes and BmCncC/keap1 pathway by alternating the endogenous
155 hormone levels under high temperature induction.

156 The steroid hormone ecdysone and JH coordinated to regulate the development of insects in an
157 opposite way. JH treatment can prolong the duration of larva and enlarge the body size [23]. In the
158 study, the content of JH and the transcriptional levels of response genes *Met*, *JH3*, and *Kr-h1* were
159 significantly down-regulated after high temperature treatment (Fig 2A and Fig 2C). The *JH3* and
160 *Met* genes play an important role in the JH signaling cascade [24]. *Kr-h1* can maintain *B. mori*
161 larval state by controlling 20E content mediated by inhibition of steroidogenic enzymes genes
162 transcription [25]. The 20E content was increased significantly after high temperature treatment,
163 and the transcriptional levels of 20E metabolism-related genes *E93*, *Br-C*, *USP* and *E75* were
164 significantly up-regulated (Fig 2D). Previous studies shown that treatment silkworm with 20E at
165 5th instar larva can accelerate the development and promote larvae maturation [26]. *E93* gene
166 promotes the development of silkworm and induces the expression of downstream 20E response
167 genes, including *Br-C*, *USP* and *E75*, that plays important role in 20E signal cascade [27]. The
168 results suggest that high temperature stress alternated the normal level of endogenous hormone,
169 and the significant up-regulation of 20E content and response-genes under high temperature
170 induction maybe one of the reasons for the accelerated development of silkworm.

171
172 Hollween genes belongs to the insect P450 genes family play an important role in the synthesis
173 of 20E [28]. In this study, the transcription levels of 20E-synthesis genes (*cyp302a1*, *cyp306a1*,
174 *cyp314a1*, *cyp315a1*) were significantly up-regulated after high temperature induction (Fig 3A).
175 Studies shown that treatment silkworm with exogenous substances (TiO₂ NPs) promoted the
176 expression of Hollween genes and increased the content of 20E [29], our results demonstrate that
177 high temperature treatment promotes the anabolic level of 20E as well. In this study, high
178 temperature treatment activated PI3K, resulting in phosphorylation of Akt and increased the
179 expression of downstream BmCncC (Fig 3C), suggesting that the PI3K/Akt/BmCncC axis plays
180 an important role in the up-regulation of Hollween genes expression under high temperature.
181 Evidence suggests that CncC regulates the expression of the downstream P450 family genes [30].
182 Studies shown that CncC constitute a homodimer or a heterodimer with Maf to regulate the
183 expression of the P450 genes [31]. The CncC and Maf complex regulates the expression of P450
184 genes by regulating the promoter activities of *CYP389B1* and *CYP392A28* genes in *Boisduval*
185 (*Tetranychus cinnabarinus*) [32]. Inhibition of the CncC/keap1 pathway altered the
186 metamorphosis process of the Colorado potato beetle (*Leptinotarsa decemlineata*), suggesting that
187 the CncC/keap1 pathway plays an important role in developmental regulation [33]. Our study
188 investigated whether BmCncC regulated the downstream 20E-synthesis P450 genes, found that
189 the mRNA level of *cyp302a1* and *cyp306a1* was significantly down-regulated after inhibition of
190 *BmCncC* (Fig 4), whereas significantly up-regulated after treatment with Curcumin, indicating
191 that a regulatory relationship exists between *BmCncC* and downstream P450 (*cyp302a1*
192 *cyp306a1*) genes. Furthermore, the mRNA levels of *cyp314a1* and *cyp315a1* were not changed
193 significantly after activation or inhibition of *BmCncC*, implying that there is an unknown pathway
194 of regulation.

195

196 In summary, our study demonstrated that high temperature induction can accelerate the
197 development of silkworm, elevate the content of 20E and promote related-genes expression,
198 indicating a regulatory relationship between *BmCncC* and downstream 20E-biosynthetic genes,
199 *cyp302a1* and *cyp306a1* in silkworm. Our results provided new clues for further studying of the
200 high temperature impact on insect hormone metabolism and the regulatory relationship between
201 *CncC* and P450 family genes.

202

203 **Methods**

204

205 **Insects and treatment.**

206 The larvae of *B. mori* (Jingsong × Haoyue strain) maintained in our laboratory were reared on
207 mulberry leaves under 12 h light/ 12 h dark conditions at $25 \pm 1^\circ\text{C}$ with 75-85% humidity. After
208 three days of normal rearing, the 5th instar silkworms were maintained at constant high
209 temperature of 36°C and $75 \pm 5\%$ humidity, and feed with fresh mulberry leaves.

210

211 **Cell culture and treatment.**

212 The BmN cells were maintained at 27°C in Grace insect medium, supplemented with 10% fetal
213 bovine serum and 1% antibiotics. DsRNA of *BmCncC* was purchased from Shanghai
214 GenePharma Co., Ltd, China. BmN cells were cultured in 12-well plates for 6 h and the medium
215 was replaced with serum-free medium without antibiotics. Cells were transfected using 1 μl (20
216 $\mu\text{M}/\mu\text{L}$) siRNA mixed with LipoHigh Liposome efficient transfection reagent (Sangon, Shanghai,
217 China) and incubated for 8 h. Cells were cultured with dsRNA for another 24 h after replacement
218 of medium. For Curcumin treatment (final concentration 10 $\mu\text{M}/\text{ml}$), the cells were analyzed after
219 24 h treatment with Curcumin. All data are expressed as mean of 3 replicates.

220

221 **Elisa analysis.**

222 Ecdysone and juvenile hormone (JH) content were determined using a kit (MEIMIAN,
223 Shanghai, China), refer to the instructions. The absorbance value was measured at wavelength of
224 450 nm to calculate the sample content. All data are expressed as mean of 3 replicates.

225

226 **Real time-quantitative PCR (qRT-PCR) analysis.**

227 Total RNA was extracted from silkworm fat body using RNA lysate (Takara, China). Primer
228 sequences are shown in Table 1. Use *Actin3* as the internal reference gene. QRT-PCR was
229 performed on a ViiA 7 System (ABI, Foster City, CA, USA). The reaction was in 20 μL volume.
230 Amplification conditions were as follows: denaturation at 95°C for 1 min, followed by 45 cycles
231 of 95°C for 5 s, 55°C for 10 s, and 72°C for 10 s. Data are expressed as the mean of three
232 independent experiments \pm SE (standard error).

233

234 **Western blotting analysis.**

235 The samples of fat body of the control and treated groups were homogenized in lysis buffer
236 supplemented with 1 mM PMSF. The samples were centrifuged at 12000 g for 10 min, and the
237 supernatant was collected for analysis. The procedure was carried out according to the method
238 before [26]. The Akt, p-Akt antibodies (CST, USA, 1:2000), polyclonal antibodies for *BmCncC*,
239 *Bmkeap1* (GenScript, Shanghai; 1:1500) were used as the primary antibody, and the

240 HRP-conjugated goat anti-rabbit IgG (CST, USA, 1:2000) was used as the secondary antibody.

241

242 **Statistical analysis.**

243 All data are expressed as mean of 3 replicates. The differences in means between multiple sets
244 of data were compared by one-way ANOVA. Dunnett's test was performed when compared with
245 the control. $P < 0.05$ was considered significant difference.

246

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248

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256

257 **References**

258

- 259 1. Richard Harrington, Richard A. Fleming, Ian P. Woiwod (2001) Climate change impacts on
260 insect management and conservation in temperate regions: can they be predicted? *Agricultural and
261 Forest Entomology* 3(4):233-240.
- 262 2. PC Tobin, S Nagarkatti, G Loeb, MC Saunders (2008) Historical and projected interactions
263 between climate change and insect voltinism in a multivoltine species. *Global Change Biology*
264 14:951–957.
- 266 3. Brown, Clarke, Whittaker, Hollier (1998) Direct and indirect effects of climate change on insect
267 herbivores: Auchenorrhyncha (Homoptera). *Ecological Entomology* 23 (1) :45–52.
- 269 4. M Van Asch, P Tienderen, L Holleman, M Visser (2007) Predicting adaptation of phenology in
270 response to climate change, an insect herbivore example. *Global Change Biology*
271 13(8):1596-1604.
- 273 5. EH Delucia, PD Naby, JA Zavala, MR Berenbaum (2012) Climate change: resetting
275 plant-insect interactions. *Plant Physiology* 160(4):1677.
- 276 6. N Sigrid, S Axel (2010) Potential effects of climate change on insect herbivores in European
277 forests—General aspects and the pine processionary moth as specific example. *Forest Ecology &
279 Management* 259(4):831-838.
- 280 7. Gong Y, Li L, Gong D, Yin H, Zhang J (2016) Biomolecular evidence of silk from 8,500
282 years ago. *PLoS One*. 11. e0168042.

283

284 8. Gu Z, Sun S, Wang Y, Wang B, Xie Y, et al. (2013) Transcriptional characteristics of gene
285 expression in the midgut of domestic silkworms (*Bombyx mori*) exposed to phoxim. Pestic
286 Biochem Physiol. 105:36-43.

287

288 9. Li JX, Xue B, Cheng XY, Hu JS, Tian JH, et al. (2018) TiO₂ NPs Alleviates High-Temperature
289 Induced Oxidative Stress in Silkworms. Journal of Economic Entomology 111(2): 879–884.

290

291 10. AH MIR, AQAMAR (2012) Effects of Starvation and Thermal Stress on the Thermal
292 Tolerance of Silkworm, *Bombyx mori*: Existence of Trade-offs and Cross-Tolerances. Neotrop
293 Entomol 13744-017-0559-2.

294

295 11. Motohashi H, Yamamoto M (2004) Nrf2-Keap1 defines a physiologically important stress
296 response mechanism. Trends Mol. Med 10(11):549-57.

297

298 12. Rushmore TH, Morton MR, Pickett CB. (1991) The antioxidant responsive element.
299 Activation by oxidative stress and identification of the DNA consensus sequence required for
300 functional activity. J Biol Chem 266(18): 11632-11639.

301

302 13. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, et al. (2004) Oxidative Stress
303 Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase to Regulate Proteasomal
304 Degradation of Nrf2. Mol Cell Biol 24(16): 7130-7138.

305

306 14. Chowdhry S, Zhang Y, Mcmahon M, Sutherland C, Cuadrado A, et al. (2013) Nrf2 is
307 controlled by two distinct β -TrCP recognition motifs in its Neh6 domain, one of which can be
308 modulated by GSK-3 activity. Oncogene 32(32): 3765-3781.

309

310 15. Xue F, Huang JW, Ding PY, Zang HG, Kou ZJ, et al. (2016) Nrf2/antioxidant defense
311 pathway is involved in the neuroprotective effects of Sirt1 against focal cerebral ischemia in rats
312 after hyperbaric oxygen preconditioning. Behav Brain Res. 309-1.

313

314 16. Bergé JB, Feyereisen R, Amichot M (1998) Cytochrome P450 monooxygenases and
315 insecticide resistance in insects. Philos Trans R Soc Lond B Biol Sci 353:1701-5.

316

317 17. Megha K, Subba RP. (2017) Transcription factor cap n collar C regulates multiple
318 cytochrome P450 genes conferring adaptation to potato plant allelochemicals and resistance to
319 imidacloprid in *Leptinotarsa decemlineata* (Say). Insect Biochemistry and Molecular Biology 83:
320 1e12.

321

322 18. Li FC, Ni M, Zhang H, Wang BB, Xu KZ, et al. (2015) Expression profile analysis of
323 silkworm P450 family genes after phoxim induction. Pesticide Biochemistry and Physiology 122:
324 103-109.

325

326 19. Ai JW, Zhu Y, Duan J, Yu QY, Zhang GJ, et al. (2011) Genome-wide analysis of cytochrome
327 P450 monooxygenase genes in the silkworm, *Bombyx mori*. Gene 480: 42–50.

328

329 20. Jaramillo J, Chabiolaye A, Kamonjo C, Jaramillo A, Vega FE. (2009) Thermal Tolerance
330 of the Coffee Berry Borer *Hypothenemus hampei*: Predictions of Climate Change Impact on a
331 Tropical Insect Pest. Plos One 4(8): e6487.

332

333 21. Bale JS, Masters GJ, Hodkinson ID, Awmack C, Bezemer TM (2002) Herbivory in global
334 climate change research: direct effects of rising temperature on insect herbivores. Global Change
335 Biology 8(1):1–16.

336

337 22. Hu JS, Chen J, Wang H, Mao TT, Li JX, et al. (2018) Cloning and Functional Analysis of
338 CncC and Keap1 Genes in Silkworm. J Agric Food Chem. 66: 2630–2636.

339

340 23. Saito K, Su ZH, Emi A, Mita K, Takeda M. (2010). Cloning and expression analysis of
341 takeout/JHBP family genes of silkworm, *Bombyx mori*. Insect Molecular Biology. 15
342 (3) :245-251.

343

344 24. Li ZQ, Cheng DJ, Wei L, Zhao P, Shu X, et al. (2010) The silkworm homolog of
345 Methoprene-tolerant (Met) gene reveals sequence conservation but function divergence. Insect
346 Science 17 (4) :313-324.

347

348 25. Hu Q, Zhu Z, Li C, Feng Q, Deng H. (2016) Gene Cloning and Expression Analysis of
349 Transcription Factor BmKr-h1 in Juvenile Hormone Signaling Pathway of Silkworm (*Bombyx*
350 *mori*). Science of Sericulture.

351

352 26. Muramatsu D, Kinjoh T, Shinoda T, Hiruma K. (2008) The role of 20-hydroxyecdysone and
353 juvenile hormone in pupal commitment of the epidermis of the silkworm, *Bombyx mori*.
354 Mechanisms of Development. 125 (5-6):411-420.

355

356 27. Li K, Tian L, Guo Z, Guo S, Zhang J, et al. (2016) 20-Hydroxyecdysone (20E) Primary
357 Response Gene E75 Isoforms Mediate Steroidogenesis Autoregulation and Regulate
358 Developmental Timing in Bombyx. Journal of Biological Chemistry. 291 (35) :18163.

359

360 28. Alexandra C, Stephane F, Gaëlle LG, Philippe F, Yves A, et al. (2008) Role of
361 CYTOCHROME p450 in the perception of caffeine in *Drosophila melanogaster*. PLOS
362 ONE 236 (1) :261-272.

363

364 29. Balarabe R, Mohammed C, Wilding P, Collier J, Yusu Y. Deeni. (2014) Bioinformatic
365 analysis of regulatory elements within the promoter region of the cytochrome P450 Gene,
366 CYP6M2 in *Anopheles gambiae*. European Journal of Biotechnology and Bioscience 2 (1): 24-31.

367

368 30. Peng T, Pan Y, Gao X, Xi J, Zhang L. (2016) Cytochrome P450 CYP6DA2 regulated by cap
369 'n'collar isoform C (CncC) is associated with gossypol tolerance in *Aphis gossypii* Glover. Insect
370 Molecular Biology 25(4):450-459.

371

372 31. Li S, Wang MY, Zhang YC, Shen GM, Di HS, et al. (2017) The expression of P450 genes
373 mediating fenpropathrin resistance is regulated by CncC and Maf in *Tetranychus cinnabarinus*
374 (Boisduval). Comparative Biochemistry and Physiology, Part C 198: 28–36.

375

376 32. Sharath C, Gaddelapati M, Kalsi A, Subba R. (2018) Cap 'n' collar C regulates genes
377 responsible for imidacloprid resistance in the Colorado potato beetle, *Leptinotarsa decemlineata*.
378 Insect Biochemistry and Molecular Biology 99: 54-62.

379

380 33. Megha K, Subba R. (2015) Transcription factors, CncC and Maf, regulate expression of
381 CYP6BQ genes responsible for deltamethrin resistance in *Tribolium castaneum*. Insect
382 Biochemistry and Molecular Biology 65: 47e56.

383

384 **Table 1. Primer sequences used in qRT-PCR**

Gene name	Primer sequence (5'-3')
<i>Actin3</i>	F: CGGCTACTCGTTCACTACC R: CCGTCGGGAAGTCGTAAG
<i>Met</i>	F: ACTCGTCGCCAGCCAGTAC R: CGTGTTCGTTACGCAGTT
<i>JH-3</i>	F: ACGCTTTACTGATTAGATAGG R: ACATTAACAAACAGCACCACAA
<i>Kr-h1</i>	F: GTGGCTCATTGACTATGTAATCTAA R: ATGTATCCAATTCCACCTCCT
<i>USP</i>	F: ATGGCTAACATAGAGTCAG R: TCAAGGGATGGTTAGGG
<i>Br-C</i>	F: GATCGCTGCACGGATGACA R: GGGCGGAATGAATGGTGAG
<i>E75</i>	F: AGTCGTCCCGAGGTATCTTT R: GCCAAGTCTCGTTACTCTTT
<i>E93</i>	F: ACATTACCTGATTACGCACCT R: GCTGATTCCCTCCACTACGG
<i>PI3K</i>	F: GACACTCGTGATGGAACCTTT R: ACTTATTGCAACCACCTTTC
<i>Akt</i>	F: ACTTCGGACTATGCAAGGTGAA R: GCGGGACCATAATCGGAGTC
<i>CncC</i>	F: CATGGACGAGTTCAACGAGAG R: GCGAGCGAGGTTATCTGGT
<i>Keap1</i>	F: ATGACCTGCCCTCCGATTAGT R: TCCAACCTCCAACACGACATC
<i>Maf</i>	F: TAGTCACGGTGGAGCAAGG R: GCTGTGGCATCTCGGATT
<i>Cyp302a1</i>	F: CGATACAACAGCCTACACGAC R: AACGTCAGCGGTTATCTCATC

<i>Cyp306a1</i>	F: ATCAACCAGGGCTACGCT
	R: CTCCACGATCCTCGATAAG
<i>Cyp314a1</i>	F: TTTGGGACCTTATTGCTCG
	R: TCTTTCGTTGATCGTTCCCTGTC
<i>Cyp315a1</i>	F: GCCCTCAGCCTCTTCCCTT
	R: GGCGTCGTCTCCATGAACACT

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A

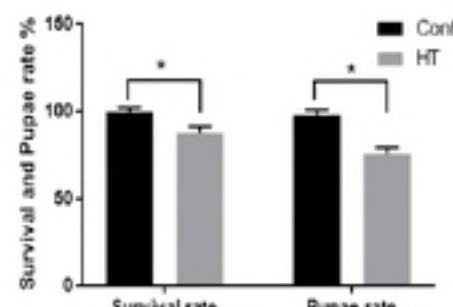
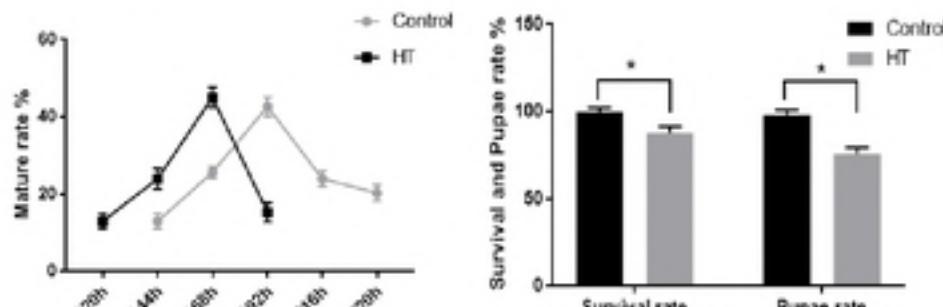
B

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C

D



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6 **Fig 1. Effect of HT on the development and vitality of *B. mori*.** A. Effect of HT on the larvae of
7 silkworm. B. Effect of HT on the development of pupa. C. Effect of HT on silkworm duration of
8 developmental stage. D. Effect of HT on silkworm vitality and percentage of pupation. HT: High
9 Temperature. The experiment was repeated three times independently. The results were shown to
10 be the mean values, and the significant differences were expressed by * ($P \leq 0.05$).
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