Imidacloprid disrupts larval molting regulation and nutrient energy metabolism, causing developmental delay in honey bee *Apis mellifera*

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Abstract

Imidacloprid is a global health threat that severely poisons the economically and ecologically important honeybee pollinator, *Apis mellifera*. However, its effects on developing bee larvae remain largely unexplored. Our pilot study showed that imidacloprid causes developmental delay in bee larvae, but the underlying toxicological mechanisms remain incompletely understood. In this study, we exposed bee larvae to imidacloprid at environmentally relevant concentrations of 0.7, 1.2, 3.1, and 377 ppb. There was a marked dose-dependent delay in larval development, characterized by reductions in body mass, width, and growth index. However, imidacloprid did not affect on larval survival and food consumption. The primary toxicological effects induced by elevated concentrations of imidacloprid (377 ppb) included inhibition of neural transmission gene expression, induction of oxidative stress, gut structural damage, and apoptosis, inhibition of developmental regulatory hormones and genes, suppression of

gene expression levels involved in proteolysis, amino acid transport, protein synthesis, carbohydrate catabolism, oxidative phosphorylation, and glycolysis energy production. In addition, we found that the larvae may use antioxidant defenses and P450 detoxification mechanisms to mitigate the effects of imidacloprid. Ultimately, this study provides the first evidence that environmentally exposed imidacloprid can affect the growth and development of bee larvae by disrupting molting regulation and limiting the metabolism and utilization of dietary nutrients and energy. These findings have broader implications for studies assessing pesticide hazards in other juvenile animals.

Keywords: Imidacloprid; *Apis mellifera*; Toxicity effects; Developmental retardation; Juvenile animal;

1. Introduction

Hazardous pesticides are used extensively around the world to protect crop production, particularly in agriculture-dependent developing countries. However, exposure to these chemicals has become a global health concern. Imidacloprid, introduced in 1991, is known for its high animal toxicity and has received international attention (Jeschke et al., 2011). The World Health Organization and the U.S. Environmental Protection Agency have listed it as an acute oral and dermal toxicant for animals. The excessive use of imidacloprid can lead to environmental contamination, endangering human and animal health through food chains, poisoning non-target pollinators, and disrupting ecological balances (Tudi et al., 2021).

Imidacloprid is also known for its adverse effects on human health. Adults exposed to high doses of imidacloprid (9.6%) experience dizziness, apathy, movement disorders, labored breathing, and temporary growth retardation (Wu et al., 2001). Imidacloprid also causes adverse congenital, hematological, hepatic, and renal effects, along with degenerative changes in various organs (Khan et al., 2010; Shaw et al., 2014; Yang et al.,

2014). Imidacloprid even at low doses causes severe liver, thyroid, and body weight problems, reproductive toxicity, developmental retardation, and neurobehavioral deficits in rats and rabbits (Anatra-Cordone M, 2005). Additionally, imidacloprid affects the singing ability of birds, making it difficult for them to attract mates and reproduce. It is a significant contributor to the decline in terrestrial insect populations since 1991, averaging 9% per decade (Cardoso et al., 2020; Sánchez-Bayo and Wyckhuys, 2019). Honey bees (*Apis mellifera* L.) are valuable model organisms in insects with significant economic and ecological importance as crop pollinators and in maintaining ecological balance (Zheng and Fu-Liang, 2009). However, the widespread use of pesticides, including imidacloprid, has resulted in colony collapse disorder (CCD), causing severe impacts on the beekeeping industry and ecological balance (Mahé et al., 2021). Studies have shown that imidacloprid is applied as a seed coating (Simon-Delso et al., 2015) and accumulates in the plant (Dively and Kamel, 2012; Rocha et al., 2022), exposing non-target bees to imidacloprid residues during pollen and nectar collection (Tong et al., 2018). Even low levels of systemic imidacloprid cause sublethal effects in adult bees, including neurotoxicity (Belzunces et al., 2012), behavioural changes (Karahan et al., 2015; Medrzycki et al., 2003), reproductive impairment (Chaimanee et al., 2016), reduced immunity (Pettis et al., 2013), apoptosis and autophagy (Carneiro et al., 2022), and shortened lifespan (Anderson and Harmon-Threatt, 2021). A recent finding shows that imidacloprid induces oxidative stress in honey bees (Balieira et al., 2018). This oxidative stress is a result of the long-term synergistic effect between imidacloprid and its target receptors (nAChRs) (Makoto et al., 2020) in bees, which leads to an excessive influx of Ca^{2+} into the bee brain (Tahirare, 2013), disrupting Ca^{2+} homeostasis and ultimately causing mitochondrial dysfunction and increased mitochondrial ROS generation, exacerbating oxidative stress.

The development and health status of juvenile animals play a critical role in population structure and future growth (Gill et al., 2012). However, they are more susceptible to the toxic effects of pesticides than adults. Despite this, there is limited

information on the toxic effects of imidacloprid on juvenile animals. Available reports mainly focus on the epistatic effects of toxicity, with limited investigation into the involved biochemical mechanisms. Imidacloprid is known to pose a potential health risk to children through dietary exposure (Anderson and Harmon-Threatt, 2021), and may cause lead to developmental abnormalities in amphibians (Samojeden et al., 2022) and fish (Islam et al., 2019). Furthermore, negative effects on pupal development in insects have been observed (Jia and Li, 2023). For instance, cat fleas (*Ctenocephalides felis*) larvae treated with imidacloprid become immobile, and their intestines show pulsatile movements for up to one hour, eventually dying after two hours (Mehlhorn et al., 1999). Additionally, imidacloprid exposure can affect the average weight gain per day in *Harmonia axyridis* (Coleoptera: Coccinellidae) larvae (Charles et al., 2000). It has also been found that imidacloprid exposure affects various cellular aspects, such as mitochondria, energy, lipids, and the transcriptome in Drosophila (Felipe et al., 2020), and prolongs the larval pupation duration and affects forewing development in the butterfly *Pieris brassicae* (Whitehorn et al., 2018).

In bees, larvae play a crucial role in the growth and development of colonies. However, the broader toxic effects of imidacloprid on bee larvae remain to be investigated. Limited reports suggest that developing bee larvae are exposed to imidacloprid through contact with contaminated hives or ingestion of contaminated food (Bhme et al., 2018). However, their survival, developmental rate, and body weight are not affected (Dai et al., 2019). Nevertheless, other studies have shown that imidacloprid can lead to impaired olfactory learning behavior (Peng and Yang, 2016), and other behavioral abnormalities (Wu et al., 2017). The current understanding is that honey bee larvae are more tolerant to imidacloprid than adults, as they can survive treatment and develop into adults (Dai et al., 2017). However, they may experience developmental delays, such as delayed plumage (Woyciechowski and Moroń, 2009; Wu et al., 2011), and reduced rates of capping, pupation, and eclosion (Yang et al., 2012). A recent study using next-generation sequencing has indicated that sublethal imidacloprid

110 treatment during the larval stage causes changes in gene expression in larvae, pupae,
111 and adults, suggesting a persistent sublethal impact on honey bee development (Chen
112 et al., 2021). Furthermore, a latest study has demonstrated that imidacloprid induces
113 differential gene expression in bumble bee larvae, resulting in unique expression
114 patterns related to various biological processes such as starvation response, cuticle
115 genes, neural development, and cell growth (Martín-Blázquez et al., 2023).

116 Although progress has been made in understanding the toxic effects of imidacloprid
117 on honey bee larvae, the molecular basis of this toxicity remains poorly understood.
118 Further research is needed to uncover the mechanisms and pathways involved in the
119 developmental toxicity of imidacloprid in honey bee larvae. In our pilot study, we
120 observed a significant developmental delay in bee larvae exposed to imidacloprid;
121 However, the molecular and biochemical mechanisms underlying this delay have not
122 been systematically investigated. Therefore, this study aims to comprehensively
123 evaluate the toxicity of imidacloprid on honey bee larvae at multiple levels, including
124 histology, neurotoxicity, molting regulation, oxidative stress, detoxification, nutrition,
125 and energy metabolism. Our study provides first-hand evidence of the developmental
126 delay caused by imidacloprid in bee larvae. Because these mechanisms involved are
127 relatively conserved in animals, our findings have broader implications for
128 understanding and assessing the potential risk of continuous exposure to imidacloprid
129 during animal development.

130

131

132 ## Results

133 ### Imidacloprid causes bee larval developmental retardation

134 The 96-hour observation period showed no significant difference in larval survival
135 between the four groups exposed to imidacloprid and the control group. However, as
136 the exposure concentration and duration increased, here was a significant decrease in
137 larval developmental progress, weight, and width (Fig. 1A-1E). Further analysis was

138 conducted on the most adverse exposure level of 377 ppb, which is the highest reported
139 environmental residue concentration detected in bee products, such as beeswax
140 (Kapoor et al., 2014). It is important to note that while the tested doses of the 377 ppb
141 was found in bee products, it is likely that the bees themselves were exposed to even
142 higher doses. Out result demonstrate that exposure to 377 ppb imidacloprid did not
143 have a significant impact on survival, feeding (Fig. 1C, 1F, and 1G), and the time required
144 for larvae to enter the pupal stage successfully (Fig. 1H). However, it did lead to slow
145 developmental progress, a lower initial rate of successful pupation, and lower growth
146 index. Additionally, imidacloprid consistently resulted in lower larval weight (Fig. 1I) and
147 width (Fig. 1J) up to the pupal stage (Fig. 1B).

148 **Imdacloprid neurotoxicity in bee larvae**

149 Under imidacloprid environmental stress at 377 ppb, AChE activity, which is closely
150 related to the disruption of neurotransmission, was significantly inhibited in larvae (Fig.
151 2A). Neither acetylcholine receptor (nAChR) alpha 1 (*Alph1*) nor acetylcholinesterase 2
152 (*Ace2*) gene expression was significantly different from the control. In contrast,
153 acetylcholine receptor alpha 2 (*Alph2*) and acetylcholinesterase 1 (*Ace1*) gene
154 expression was significantly higher than that in the control group (Fig. 2B).

155 **Imidacloprid disrupts the homeostasis of developmental regulation in larvae**

156 In imidacloprid-exposed larvae, the developmental regulatory gene juvenile hormone
157 acid methyl transferase (*JHAMT*) and the transcription factor broad complex (*Br-c*) were
158 downregulated, while vitellogenin (*Vg*) gene expression was upregulated by seven-fold
159 in comparison to control groups (Fig. 3A). In parallel, imidacloprid exposure decreased
160 the major regulator of insect metamorphosis 20-hydroxyecdysone (20E) titers but had
161 no effect on preventing premature metamorphosis juvenile hormone (JH-3) titers (Fig.
162 3B).

163 **Imidacloprid induces detoxification in larvae**

164 According to the qRT-PCR analysis, the expression levels of cytochrome P450
165 monooxygenase family members such as CYPq1, CYPq2, CYP450, CYP6AS14, CYP4G11,
166 and CYP306A1, except for CYPq3, were observed to be significantly upregulated by
167 several fold in imidacloprid-induced developmental retardation larvae. Among them,
168 CYPq1 showed an abnormal 94-fold increase (Fig. 4).

169 **Imidacloprid toxicity causes oxidative stress and induces antioxidant defense in larvae**

170 Larvae exposed to imidacloprid exhibited a significant increase in reactive oxygen
171 species (ROS) levels (Fig. 5A) and upregulation of the antioxidant genes GPX and Trx
172 expression (Fig. 5C), as well as increased activities of the antioxidants CAT, SOD and GSH
173 (Fig. 5B), resulting in a significant increase in total antioxidant capacity (T-AOC) (Fig. 5B)
174 compared to the control group. Under these conditions, MDA levels, used as a marker of
175 lipid peroxidation, were observed to increase, while protein carbonylation damage
176 indicator PCO levels remained unchanged (Fig. 5A).

177 **Imidacloprid induces gut apoptosis and tissue damage in larvae**

178 Hematoxylin-eosin staining revealed a significant reduction in the number of cells in
179 the muscular layer of the larval gut exposed to imidacloprid with clear apoptotic
180 characteristics compared to the standard feeding control groups (Fig. 5D). This was
181 evidenced by increased nuclear staining and compacted chromatin, as well as disrupted
182 cell arrangement in the basal layer of the gut, increased nuclear staining of basal lamina
183 cells, and apoptotic signals. The integrity of the peritrophic membrane structure of the
184 gut was better in the control group, while imidacloprid-exposed larvae were still in the
185 formation stage, resulting in the accumulation of undigested food residues in their guts
186 (Fig. 5D).

187 **Imidacloprid impairs larval digestion and breakdown of dietary nutrients**

188 Exposure to imidacloprid did not significantly affect the daily food intake of
189 developmentally delayed larvae compared to controls (Fig. 1F and 1G). However,

190 imidacloprid toxicity suppressed the expression of nutrient catabolism genes, including
191 alpha-amylase (*α-Am*) and alpha-glucosidase (*α-Glu*), which are involved in
192 carbohydrate catabolism (Fig. 6A), carboxypeptidase (*CPs*) and aminopeptidase (*APs*)
193 involved in proteolysis (Fig. 6B), and glycine tRNA ligase (*Aats-Gly*) and tyrosine tRNA
194 ligase (*Aats-Tyr*) involved in amino acid transport (Fig. 6C).

195 **Imidacloprid causes restriction of larval protein synthesis and energy metabolic**
196 **dysfunction**

197 RT-qPCR analysis revealed that imidacloprid exposure reduced the expression of
198 critical genes involved in energy metabolism, including cytochrome c oxidase copper
199 chaperone (*COX17*) and NADH dehydrogenase [ubiquinone] 1β subcomplex subunit 7
200 (*NDUFB7*), in developmentally delayed larvae (Fig. 6D). Imidacloprid exposure also
201 inhibited the expression of genes related to glycolysis and energy production, such as
202 glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and glucosamine-6-phosphate
203 isomerase (*Oscillin*) (Fig. 6E). In addition, the expression of transcription initiation
204 factors related to protein synthesis, including translation initiation factor 3 subunit M
205 (*Tango7*), translation initiation factor 3 subunit B (*eIF3-S9*), and translation initiation
206 factor 3 subunit A (*eIF3-S10*), was significantly suppressed by imidacloprid toxicity (Fig.
207 6F). Finally, the total levels of ATP, glycogen and protein, which are closely related to
208 energy production and reserves, were significantly lower in developmentally delayed
209 larvae than in controls (Fig. 6G-6I).

210 **Correlation between developmental phenotypic changes and molecular features**
211 **caused by imidacloprid toxicity**

212 To better understand the mechanism of imidacloprid-induced larval developmental
213 retardation, we performed a comprehensive correlation analysis between epigenetic
214 growth traits and molecular characteristics (Fig. 7). The results showed that in larvae
215 with imidacloprid-induced developmental retardation, body weight, width, growth
216 index, and developmental rate were positively correlated with energy metabolism and

217 reserves, nutrient catabolism, protein synthesis, and developmental regulation.
218 Conversely, negative correlations were observed between these growth traits and
219 parameters associated with detoxification and antioxidant defense processes that
220 require additional energy expenditure. Moreover, oxidative stress and damage,
221 detoxification, and antioxidant defense all exhibited significant positive correlations (Fig.
222 7).

223

224 ## Discussion

225 **Toxic effects and the molecular basis of environmental concentrations of imidacloprid**
226 **on bee larvae**

227 Previous research has shown that bee larvae are more susceptible to the adverse
228 effects of pesticides than adult bees (Tomé et al., 2020). In this study, larvae were
229 exposed to environmental concentrations of imidacloprid, resulting in sublethal effects
230 such as decreased body weight, width, developmental rate, and growth index (Fig. 1).
231 Our results support the notion that pesticides can affect larval development (Dai et al.,
232 2018; Zhu et al., 2014). However, current research has primarily focused on
233 characterizing the effects of imidacloprid on larval phenotype and behavior, and the
234 molecular mechanisms underlying its ecotoxicological effects remain poorly understood.

235 To obtain molecular evidence of imidacloprid toxicity to bee larvae, we first examined
236 the expression of acetylcholine receptors and AChE activity. The results showed that
237 imidacloprid exposure increased the expression of the *Alph2* gene and inhibited AChE
238 activity (Fig. 2). Normally, acetylcholine stimulates nerve impulses by binding to the Alph
239 receptor, and AChE degrades acetylcholine to terminate nerve conduction. However,
240 imidacloprid competitively binds to AChE receptors, inhibits AChE activity, and causes
241 continuous excitation of nerve conduction, ultimately leading to paralysis and death
242 (Katić et al., 2021; Shan et al., 2020). Therefore, our results strongly suggest that
243 imidacloprid is neurotoxic to bee larvae.

244 The growth and development of juvenile animals depends on the digestion,
245 absorption and utilization of food nutrients. Therefore, we next investigated whether
246 imidacloprid affects the digestion and utilization of dietary by larvae. The results
247 indicate that exposure to imidacloprid suppressed the hydrolysis genes *CPs* and *APs* and
248 the amino acid transport pathway genes *Aats-Gly* and *Aats-Tyr* in larvae (Fig. 6B and 6C).
249 Since these genes are responsible for cleaving dietary proteins into absorbable peptides
250 and amino acids and transporting them for metabolism (2019), inhibiting the expression
251 of these genes would severely affect larval digestion, resulting in protein deficiency and
252 impaired metabolic activity. In addition, imidacloprid also affected the expression of *α-*
253 *Am* and *α-Glu* genes (Fig. 6A), which are involved in carbohydrate digestion, indicating
254 that imidacloprid also affects the digestion of dietary carbohydrates. Indeed, histological
255 observations also confirmed that a large amount of undigested food remained in the
256 intestine of larvae exposed to imidacloprid (Fig. 5D). In conclusion, imidacloprid inhibits
257 the digestion and utilization of dietary proteins and carbohydrates by larvae, which is
258 extremely detrimental to their growth and development.

259 In addition to nutrition, energy metabolism is critical for the maintenance of all life
260 activities. In this study, imidacloprid exposure inhibited the expression of genes related
261 to mitochondrial oxidative phosphorylation (*COX17*, *NDUFB7*) and its alternative
262 glycolytic pathway (*Gapdh*, *Oscillin*). Mitochondrial oxidative phosphorylation is the
263 central pathway for ATP generation during energy metabolism (Tian et al., 2020a), while
264 glycolysis is an alternative mechanism for ATP synthesis (Jiang et al., 2020). Since both
265 biochemical processes are essential for energy production, their inhibition resulted in
266 reduced ATP levels in bee larvae (Fig. 6G). These results strongly suggest that
267 imidacloprid induces energy metabolism dysfunction leading to insufficient energy
268 production in larvae. Since larval energy production is insufficient, this is likely to lead to
269 a depletion of energy reserves. Indeed, further analysis revealed a significant decrease
270 in glycogen and protein energy reserves in larvae exposed to imidacloprid (Fig. 6H and
271 6I). These findings are consistent with previous reports that exposure to pesticides such

272 as endosulfan, chlorpyrifos, insecticides, and fipronil can lead to a reduction in energy
273 reserves in organisms (Bouayad et al., 2012a; Dutra et al., 2009; Radwan et al., 2008;
274 Rambabu and Rao, 1994; Ribeiro et al., 2001). In conclusion, our results suggest that
275 imidacloprid induces energy metabolism dysfunction and affects energy production and
276 reserves in bee larvae.

277 Insects rely primarily on cytochrome P450 detoxification to resist toxic compounds (Li
278 et al., 2007), and the level of CYP450 expression indicates the severity of pesticide
279 toxicity. While adult worker bees have shown a positive response to imidacloprid (Mao
280 et al., 2009), it is uncertain whether bee larvae can respond to pesticide toxicity. In this
281 study, we found that seven cytochrome P450 family transcripts, including CYPq1, CYPq2,
282 CYP450, CYP6AS14, CYP4G11, and CYP306A1, were significantly upregulated in bee
283 larvae, with CYP9q1 being 94-fold higher than the control group (Fig. 4). These results
284 are consistent with previous findings that the CYP450 family genes CYP6AS3, CYP6AS4,
285 and CYP9S1 were significantly upregulated in adult worker bees exposed to quercetin,
286 coumaphos, and fluvalinate (Mao et al., 2011) and that CYP9Q1, CYP9Q2, and CYP9Q3
287 help bees detoxify fluvalinate and coumaphos (Gregorc et al., 2018). This suggests that
288 the P450 detoxification system is already established in bee larvae and they respond
289 positively by activating the detoxification function of CYP450 upon exposure to
290 imidacloprid.

291 Oxidative stress is the excessive production of ROS in aerobic organisms (Felton, 1995;
292 Ozcan and Ogun, 2015). Pesticides, including coumaphos (Gregorc et al., 2018), fipronil
293 (Paris et al., 2017), paraquat (Li-Byarlay et al., 2016), organophosphorus, pyrethroid,
294 organochlorinated (Chakrabarti et al., 2015), and chlorpyrifos (Shafiq-ur-Rehman et al.,
295 2012), have been shown to cause oxidative stress in bees. In this study, exposure to
296 imidacloprid resulted in increased levels of ROS and MDA in larvae (Fig. 5A), indicating
297 severe oxidative stress and lipid damage. Organisms have evolved antioxidant defense
298 mechanisms to scavenge excessive ROS and resist oxidative stress, including increasing

299 levels of antioxidants such as CAT, SOD, GSH, TrxR, and GSH (Dong et al., 2013; Tian et
300 al., 2020b). In this regard, we have recently confirmed this in worker bees (Li et al.,
301 2022), which is also well reflected in the present study. We observed a significant
302 increase in the activities of antioxidant enzymes SOD and CAT, gene expression of Trx
303 and Gpx, and GSH levels in larvae after exposure to imidacloprid (Fig. 5B and 5C). These
304 results show that the antioxidant system in developing larvae is established and used to
305 resist oxidative stress caused by imidacloprid, similar to that in adult worker bees.
306 Notably, compared to our recent study in adult bees (Li et al., 2022), the larvae in this
307 study exhibited more robust antioxidant activity when exposed to higher concentrations
308 of imidacloprid toxicity than worker bees. However, the underlying mechanism requires
309 further investigation. The underlying mechanisms involved require further study.

310 Notably, in addition to routine antioxidants, imidacloprid induces a 7-fold increase in
311 Vg expression (Fig. 3A). Vg is known to provide nutrition to developing embryos
312 (Jorgensen et al., 2009) and to help animals cope with pollution-induced stress (Matozzo
313 et al., 2008). In bees, Vg plays an important antioxidant role by protecting other
314 hemolymph molecules from ROS and increasing cellular oxidative stress tolerance
315 (Corona et al., 2007). Previous studies have shown a correlation between high Vg
316 expression in adult worker bees and resistance to oxidative stress resulting from
317 exposure to the pesticide paraquat (Seehuus et al., 2006). In this study, our results
318 support this observation and suggest that larvae may also resist oxidative stress caused
319 by imidacloprid through increased Vg production.
320

321 **Mechanisms of imidacloprid-induced developmental retardation in bee larvae**

322 Impaired juvenile development can have a significant impact on the growth and
323 sustainability of entire populations. Deviations in juvenile development due to
324 environmental stress have recently received considerable attention. *Drosophila*
325 *melanogaster* exposed to heat stress exhibit developmental delays due to the failure of

326 early embryos to fully inhibit the synthesis of non-heat shock proteins (Bergh and Arking,
327 1984). Similarly, exposure to nutrient and heavy metal stress can cause delayed or
328 arrested development in *Caenorhabditis elegans* due to altered gene expression and
329 disruption of developmental signaling pathways (Carranza-García and Navarro, 2020;
330 Rashid et al., 2021). In the present study, we also observed typical developmental delays
331 in honeybee larvae exposed to imidacloprid, but the underlying mechanisms remain
332 unclear.

333 Insect development is regulated by the hormones JH and 20E, which control
334 morphological remodeling events during molting. 20E promotes pupation (Yuan et al.,
335 2020), while JH maintains juvenile characteristics and prevents metamorphosis by
336 antagonizing the effects of 20E (Luo et al., 2021). To elucidate the mechanism
337 underlying imidacloprid-induced larval developmental delay, we first focused on the
338 hormonal regulation of molting development and found that imidacloprid inhibits the
339 expression of JHAMT (Fig. 3A), a positive regulator of JH synthesis (Liu et al., 2018), but
340 did not affect JH titer levels (Fig. 3B). This suggests that the antagonistic effect of JH still
341 restricts the developmental process, thereby maintaining larval morphology. In parallel,
342 imidacloprid toxicity also resulted in reduced ecdysteroid 20E titer and Br-c expression
343 (Fig. 3). The role of 20E is to induce larval molting and pupation by triggering
344 downstream response factors, such as Br-c (Deng et al., 2012). Therefore, our results
345 suggest that larval developmental delay may be caused by imidacloprid reducing 20E
346 titer and inhibiting *Br-c* expression, thereby blocking molting. Notably, we also observed
347 that a decrease in 20E levels was accompanied by a decrease in AChE activity (Fig. 2A),
348 which is known to cause delayed pupation (He et al., 2012) and developmental arrest
349 (Desneux et al., 2007) in insect larvae. Therefore, our results suggest that imidacloprid
350 neurotoxicity may cause developmental delay in bee larvae by inhibiting the molting
351 hormone 20E. This conclusion is supported by a correlation analysis showing that
352 decreases in 20E titer, *Br-c* expression and AChE activity were positively correlated with
353 decreased developmental rates and growth index, weight and width growth (Fig. 7). In

354 conclusion, imidacloprid neurotoxicity inhibits the 20E and *Br-c* genes, thereby blocking
355 molting, which may be an important cause of delayed larval development in honey bees.

356 Animals employ selective foraging as a survival strategy to avoid ingesting toxic foods
357 that may cause adverse physiological effects (Berenbaum and Johnson, 2015). Although
358 bees cannot actively reject toxins innately, postingestive malaise allows them to learn to
359 avoid ingesting toxic-containing foods with adverse physiological effects (Hurst et al.,
360 2014). Given this, we speculate that it is likely that the honey bee larvae in this study
361 reduced their intake of toxic imidacloprid-containing foods, making it difficult to meet
362 their nutritional and energy needs, which may have resulted in delayed development.
363 However, monitoring of daily food intake did not show a significant difference between
364 larvae exposed to imidacloprid and the control group (Fig. 1F and 1G), indicating that
365 larval food intake was not reduced and larvae did not initiate avoidance behavior
366 toward toxic food. Therefore, food intake was not a critical factor in the delayed larval
367 development caused by imidacloprid.

368 Nutrition and energy are critical for the growth and development of juvenile animals.
369 Exposure to heavy metals and neonicotinoid pesticides has been shown to affect
370 digestion, energy reserves, production, and utilization in animals (Jiang et al., 2020; Li et
371 al., 2021). Given this, since the lack of the necessary correlation between delayed larval
372 development and food intake in this study prompts us to consider potential problems in
373 larval food and energy utilization that led to delayed development. Consequently, our
374 focus shifted to the investigation of larval nutrition and energy metabolism.
375 Interestingly, we found severe pathological changes in the gut tissues of
376 developmentally delayed larvae exposed to imidacloprid (Fig. 5D). Imidacloprid caused a
377 significant reduction in the tightness of the cell arrangement in the basal layer of the gut,
378 and a drastic decrease in the number of cells. The peritrophic membrane was
379 incompletely formed, and undigested food residues were present in the gut. qRT-PCR
380 analysis revealed a significant decrease in larval digestion and catabolism of dietary

381 proteins and carbohydrates, amino acid transport, oxidative phosphorylation, and
382 glycolysis (Fig. 6D-6F). Biochemical assays showed that imidacloprid stress also
383 decreased ATP levels and total glycogen and protein content, resulting in a lack of
384 energy reserves and increased energy expenditure (Fig. 6G-6I). Correlation analysis
385 indicated a positive relationship between impaired catabolism and dietary nutrient
386 utilization caused by imidacloprid and larval developmental delay (Fig. 7). These
387 evidence strongly suggest that imidacloprid exposure affects the digestion and
388 utilization of nutrients and energy in larvae, resulting in insufficient nutrients and energy
389 for growth and development, which may be another important factor contributing to
390 imidacloprid-induced larval developmental delay.

391 The Dynamic Energy Budget (DEB) theory suggests that environmental stress can
392 significantly affect the energy balance of organisms, leading to increased energy
393 expenditure (Kooijman, 2009). To cope with stress, animals may deplete their energy
394 reserves, such as glycogen and protein (Bouayad et al., 2012b; Matsukura et al., 2008).
395 In this study, we found that larvae with delayed development caused by imidacloprid
396 had lower energy reserves (ATP, total protein, and total glycogen), and these energy
397 reserves were negatively correlated with larval antioxidant and detoxification (Fig. 7),
398 suggesting that the reduction in energy reserves caused by imidacloprid is related to the
399 energy-consuming activities of CYP450 detoxification and antioxidant defense. To meet
400 the high energy cost demands of environmental stress, animals often have to allocate
401 most of their food intake to defense (Guedes et al., 2006), and this redistribution of
402 energy resources will inevitably lead to an insufficient energy supply for growth and
403 development (Beyers et al., 1999). Therefore, the P450 detoxification and antioxidant
404 defense used by the larvae in this study to resist imidacloprid toxicity will inevitably
405 force them to consume more food resources that should have been used for growth and
406 development, resulting in a severe lack of energy needed for development. This may
407 also be another important reason why imidacloprid causes delayed development in bee
408 larvae.

409 It is worth noting that our investigation into the causes of imidacloprid-induced
410 developmental delay in honey bee larvae has provided limited answers. Future
411 comparative analyses of different tissues and durations of exposure, using genetic
412 manipulation and histochemical methods, would improve our understanding of the
413 effects of sublethal doses of imidacloprid on larval development, growth and survival.

414

415 ## Conclusion

416 Imidacloprid toxicity caused growth and developmental delay in *A. mellifera* larvae.
417 The toxic mechanism may include imidacloprid disrupting the regulatory balance of molt
418 development, resulting in restricted development. In addition, the gut damage caused
419 by imidacloprid restricts the metabolism and utilization of food nutrients and energy in
420 the larvae. Third, the additional energy consumed by larval P450 detoxification and
421 antioxidant defenses further reduces the supply of nutrients and energy for growth and
422 development (Fig. 8). This study is the first multi-level investigation of the toxic effects
423 of imidacloprid on *A. mellifera* larvae. These findings have broader implications for
424 understanding and assessing the threat of harmful pesticides to animal larvae.

425

426 **Materials and Methods**

427 **Larval rearing**

428 The Animal Bioethics Committee at Chongqing Normal University, China approved all
429 experimental protocols. The chosen colonies were healthy and not exposed to
430 pathogens or pesticides. Two-day-old larvae from the same frames of the same hive
431 were individually transferred to sterile 24-well cell culture plates. The plates contained
432 standard food, which included royal jelly, glucose, fructose, water, and yeast extract
433 (Aupinel et al., 2005). The larvae were cultured in dark conditions at 35°C and 96% RH,
434 with daily feeding volumes adjusted according to their age (20 μL for three-day-old

435 larvae, 30 μL for four-day-old larvae, 40 μL for five-day-old larvae, and 50 μL for six-day-
436 old larvae). Any remaining food was recorded and removed.
437
438 **Experimental design**
439 This study employed four different concentrations of imidacloprid, which were
440 residual concentrations found in honey (0.7 ppb) (Chauzat et al., 2009), bees (1.2 ppb)
441 (Chauzat et al., 2011), pollen (3.1 ppb) (Mullin et al., 2010), and beeswax (377 ppb)
442 (Pareja et al., 2011). The experiment was conducted with three treatment groups:
443 control group (CK), solvent control group (CKac), and imidacloprid-treated group (IMI),
444 each with three replicates. Each replicate contained 32 bees that were distributed in
445 standard 24-well cell culture plates (1 bee/well). The CK group consisted of larval bees
446 fed acetone-royal jelly glucose-fructose-yeast extract-distilled water (0.00377%-50%-
447 6%-6%-1%-36.99623%, v/w/w/w/w/v). The IMI group consisted of larvae-fed control
448 solution containing imidacloprid (99% active ingredient, Hubei Norna Technology Co. Ltd,
449 Hubei, China) at 0.7 ppb, 1.2 ppb, 3.1 ppb, and 377 ppb. The CKac group served as a
450 solvent control and contained only acetone at a concentration of 0.00377%. A total of
451 312 larvae were used for survival statistics (96 bees), body weight and width (96 bees),
452 and gene expression, enzyme activity, histochemical content, and histopathologic
453 analysis. Food was administered daily according to age (20 μL for three-day-old larvae,
454 30 μL for four-day-old larvae, 40 μL for five-day-old larvae, and 50 μL for six-day-old
455 larvae). Dead individuals and food remain were recorded daily, and incubation
456 conditions were 35°C and 96% RH. Surviving larvae were collected 72 h after
457 consumption of the test solution and stored at −80°C for further analysis, including
458 evaluations of gene expression, enzyme activity, and chemical content evaluations.
459 Furthermore, HE-stained sections were used to evaluate any imidacloprid-induced
460 tissue damage and apoptosis.
461
462 **Developmental phenotypic traits**

463 Larval individuals were randomly selected daily from the CK and IMI groups to
464 measure body weight and width. Food residues, larval mortality, developmental
465 progression, and the amount of time spent in the larval developmental stage (from the
466 first imidacloprid exposure to before pupa) were recorded daily to calculate food
467 consumption, survival rates, developmental rates, and development times. The growth
468 index (GI) was calculated for larvae exposed to imidacloprid from the three-day-old to
469 the six-day-old using the equations described by Zhang (Zhang et al., 1993). The
470 developmental rate was measured as the number of larvae successfully reaching the
471 pupal stage as a percentage of the total initial sample size.
472

473 **RNA extraction, cDNA synthesis, and real-time quantitative PCR analysis**

474 Total RNA was extracted from larvae using TRIzol reagent (Thermo Fisher Scientific)
475 and RNAex Pro Reagent (Accurate Biology, China) as instructed by the manufacturer.
476 The RNA quality was evaluated by gel electrophoresis and absorbance determination
477 using a NanoDrop 2000 spectrophotometer (Thermo Scientific, New York, USA).
478 Genomic DNA was removed, and cDNA was synthesized using a reverse transcription kit
479 (Evo M-MLV RT Mix Kit with gDNA Clean for qPCR, Accurate Biology, China) according to
480 the manufacturer's instructions. Briefly, 1 μg of RNA reacted with the 5X gDNA Clean
481 Reaction Mix provided in the kit at 42°C for 5 min to remove residual genomic DNA to
482 ensure the accuracy of the quantitative results. After the reaction, 4 μL of 5X Evo M-
483 MLV RT Reaction Mix and 4 μL of RNase-free water were added to the reaction tube
484 for 20 μL. The mixture was incubated at 37°C for 15 min and 85°C for 5 sec to generate
485 cDNA. Then, quantitative RT–PCR was performed with a Bio-Rad CFX96™ Real-time PCR
486 Detection System (Bio-Rad, Hercules, CA, USA) according to standard protocols and
487 cycling conditions. RP49 (ribosomal protein 49, Accession no. AF441189) (Lourenco et al.,
488 2008) was used as the endogenous control. The primers for target genes and RP49 are
489 listed in Table S1. qPCR system (20 μL): EvaGreen Express 2X qPCR MasterMix (10 μL),
490 each forward and reverse primer (0.4 μL, 0.2 μmol/L), cDNA (1 μL, 100 ng/μL), ddH_2O

491 (8.2 μL). The thermal cycling program was as follows: 95°C for 30 sec, followed by 40
492 cycles of 95°C for 5 s and 60°C for 30 s. The qPCR products were sequenced by Shanghai
493 Biological Engineering Technology Services Co., Ltd. to confirm whether the amplified
494 fragments were the target sequences. Relative mRNA expression levels of target genes
495 were calculated using the $2^{-\Delta\Delta Ct}$ method.
496
497 **Histochemical measurement**
498 Juvenile hormone 3 (JH-III), 20-hydroxyecdysone (20E) titers, and reactive oxygen
499 species (ROS) levels in larvae were determined using ELISA enzyme-linked
500 immunosorbent assay kits (Jining, Shanghai, China) according to the manufacturer's
501 instructions. Briefly, the larva was homogenized on ice in 5 volumes (M: V) of
502 phosphate-buffered saline (PBS) buffer, and the supernatant was collected after
503 centrifugation at 12,000 x g for 20 min. Subsequently, 50 μL of reference standard
504 solution or 10 μL of sample supernatant plus 40 μL of dilution were added to the
505 microtiter wells and incubated at 37°C for 60 min with 100 μL of HRP-labeled detection
506 antibody. Each well was washed five times with 400 μL of PBS buffer containing 0.05%
507 Tween 20, pat dried, and left to stand at room temperature for 5 min. TMB chromogen
508 solution A (50 μL) and solution B (50 μL) were added to each well, gently mixed, and
509 incubated at 37°C for 15 min in the dark. Then, 50 μL of H_2SO_4 (4 mol/L) was added to
510 each well to terminate the reaction. Finally, the OD value was measured at 450 nm with
511 a NanoDrop 2000 spectrophotometer (Thermo Scientific, New York, USA), and the
512 content of the detected substance was calculated from a calibration curve constructed
513 by reference standards.
514 To prepare samples for analysis of protein carbonylation (PCO), acetylcholinesterase
515 (AchE), catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T-AOC),
516 superoxide dismutase (SOD), and glutathione (GSH), larvae was weighed and
517 homogenized on ice in 5 volumes (M: V) of PBS buffer, then centrifuged at 12,000 x g for
518 15 min to collect the supernatant. Subsequently, PCO content was measured using the

519 2,4-dinitrophenyl-hydrazine (DNPH) method with slight modifications. The above-
520 prepared supernatant was mixed with streptomycin sulfate at a 9:1 volume (V: M), kept
521 at room temperature for 10 min, and then centrifuged at 12,000 x g for 15 min. Then, 80
522 µL of supernatant was mixed with 160 µL of 10 mM DNPH in 2 M HCl and reacted at
523 37°C for 30 min. Sample controls were also prepared by adding an equal volume (500 µL)
524 of 2 M HCl. Then, 200 µL of 20% TCA was added, mixed thoroughly, and centrifuged at
525 4°C for 10 min at 12,000 x g, and the precipitate was collected. Add 400 µL of ethanol:
526 ethyl acetate mixture (1:1), vortex and mix, centrifuge at 4°C 12,000 x g for 10 min, and
527 collect the precipitate to remove unbound DNPH. This DNPH removal step was repeated
528 once. The pellet was mixed with 400 µL of 6 M guanidine hydrochloride, kept at room
529 temperature for 15 min, and then centrifuged at 4°C 12,000 x g for 10 min to collect the
530 supernatant. The carbonyl and protein contents were determined by reading the
531 absorbance at λ = 370 nm and 280 nm with a SpectraMax 190 spectrophotometer
532 (Molecular Devices, California, USA) for each sample against an appropriate blank.
533 Finally, the carbonyl content per unit of protein was calculated based on the protein
534 content.

535 AChE activity was measured by the Ellman method as described by Orhan et al.
536 (Orhan et al., 2007) with slight modifications. Briefly, 20 µL of the prepared sample
537 supernatant was mixed with 160 µL of 0.1 mM PBS buffer (pH 8.0) and 10 µL of an AChE
538 (0.28 U mL^{-1}) and incubated for 15 min at room temperature. Then, 10 µL of ATChI
539 (acetylthiocholine iodide, 4 mg mL^{-1}) was added together with 20 µL of DTNB (5,5-
540 dithiobis-2-nitrobenzoic acid, 1.2 mg mL^{-1}) and immediately mixed, and the absorbance
541 was read at 412 nm. The results were expressed as activity percentages relative to the
542 sample solvent negative control.

543 The CAT activity was determined using a kit (Beyotime, Shanghai, China) according to
544 the manufacturer's instructions with slight modifications. Briefly, 10 µL of the prepared
545 sample supernatant was mixed with 30 µL of Tris-HCl buffer (pH 7.0, 50 mmol L^{-1}), 10 µL
546 of 250 mM H_2O_2 was added, and the mixture was immediately mixed and kept at 25°C

547 for 5 min. Then, 450 µL of 1.8 mol L-1 H_2SO_4 was added, and the mixture was vortexed
548 to terminate the reaction. Subsequently, 10 µL of this termination reaction solution was
549 mixed with 40 µL of Tris-HCl buffer (pH 7.0, 50 mmol L^{-1}). Then, 10 µL of this mixture
550 was added to a 96-well plate mixed with 200 µL of color development working solution
551 containing peroxidase and incubated for 15 min at 25°C. Finally, the absorbance was
552 measured at 520 nm, and the CAT activity was calculated using a calibration curve of 5
553 mM H_2O_2.

554 T-AOC was measured using the total antioxidant capacity assay kit with the ABTS
555 (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method based on a previous report
556 (Chen et al., 2019). Add 10 µL of the prepared sample supernatant to 200 µL of ABTS
557 working solution. After 6 min of reaction, the absorbance was measured at 734 nm.
558 Finally, the antioxidant activity was displayed by Trolox equivalents antioxidant capacity
559 as mmol Trolox equivalents/g extract. The ABTS working solution was prepared as
560 follows: 400 µL of potassium persulfate was added to 400 µL of ABTS to generate an
561 ABTS stock solution, which was then incubated for 16 h at room temperature in the dark.
562 Before use, the stock solution was diluted with phosphate buffer, and the absorbance at
563 734 nm was adjusted to 0.70 ± 0.05.

564 Additionally, MDA, SOD, and GSH levels were detected using ELISA enzyme-linked
565 immunosorbent assay kits (Beyotime, Shanghai, China) according to our recent study (Li
566 et al., 2022).

567 **Histopathological analysis**

568 Microsectioning and hematoxylin-eosin (HE) staining was performed according to
569 standard histological protocols (Khan et al., 2010) with some modifications. Briefly,
570 entire fresh larvae were fixed in 10% neutral-buffered formalin solution for 12 h at 4°C
571 after 72 h of imidacloprid exposure. After isopropyl alcohol dehydration, samples were
572 embedded in paraffin and sectioned. Three-micron-thick sections were stained with a
573 Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China), observed with an
574 Olympus BX51 microscope, and photographed with an Olympus digital camera.

575

576 **Statistical analysis**

577 Data were obtained from three independent experiments performed in triplicate and
578 are presented as the mean ± SEM. SPSS statistical package was used for statistical
579 analysis using Student's t-test and one-way ANOVA followed by Tukey's multiple
580 comparison test (*P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically
581 significant). GraphPad software was used for data normalization, Pearson correlation
582 coefficient calculation was analyzed using SPSS, and a heat map was generated.

583

584 ## Acknowledgments

585 The authors would like to thank the other colleagues in our laboratory who
586 contributed to this study. This study received financial support from the grants from the
587 earmarked fund for China Agriculture Research System (No. CARS-44); the Science and
588 Technology Project of the Chongqing Municipal Education Commission of China (No.
589 KJZD-K202100502); the Natural Science Foundation Project of Chongqing of China (No.
590 cstc2021jcyj-msxmX0422); and the Natural Science Foundation Project of the State Key
591 Laboratory of Silkworm Genome Biology of China (No. SKLSGB-ORP202103).

592 ## Additional information

593 ## Author contributions

594 **Zhi Li:** Supervision, Designed research, Conceptualization, Methodology, Funding
595 acquisition, Writing-original draft, Writing-review & editing. **Yuedi wang:** Investigation,
596 Methodology, Validation, Formal analysis, Project administration. **Lanchun Chen:**
597 Investigation, Data curation. **Qiqian Qing:** Investigation, Data curation. **Xiaoqun Dang:**
598 Data curation. **Zhengang Ma:** Data curation. **Zeyang Zhou:** Conceived the research,
599 Research guidance. All the authors read, corrected, and approved the manuscript.

600

601 ## Ethics

Animal experimentation: This study was performed in strict accordance with the guidelines of the Animal Bioethics Committee at Chongqing Normal University, China approved all experimental protocols.

Data Availability

All the data needed to evaluate the conclusions in this paper are present in the paper and/or Supplementary Materials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional files

Supplementary files：Table S1. Primers used in real-time quantitative PCR.

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843

844 ## Figure legends

845 **Figure 1.** Imidacloprid causes larval developmental retardation in *Apis mellifera*. Larvae
846 were orally administered imidacloprid (IMI) or artificial food (CK) beginning at three-
847 day-old, followed by developmental monitoring for 96 hours. (A) Larval developmental
848 phenotypes at 0.7, 1.2, 3.1, and 377 ppb environmental concentrations of imidacloprid
849 exposure; (B) Larval developmental phenotype at 377 ppb imidacloprid exposure; (C)
850 Effect of 0.7, 1.2, 3.1, and 377 ppb imidacloprid on larval survival; (D) and (E) body width
851 and weight of larvae exposed to 0.7, 1.2, 3.1, and 377 ppb imidacloprid for 96 hours; (F)
852 and (G) statistics for daily (F) and three-day cumulative feeding (G) for larvae exposed to
853 377 ppb imidacloprid; (H) larval development time from three-day-old to pupal stage; (I)
854 The number of larvae that successfully reached the pupal stage as a percentage of the
855 total initial sample size; (J) The growth index of larvae. Statistical significance was set at
856 $*P < 0.05$ and $**P < 0.01$.
857
858 **Figure 2.** Imidacloprid neurotoxicity in *Apis mellifera* larvae. (A) AChE activity and (B)
859 nerve conduction-related gene expression analysis in larvae exposed to 377 ppb
860 imidacloprid for 72 hours or a control group. IMI is the larvae exposed to imidacloprid
861 for 72 hours. CK is the artificial food control group. Statistical significance was set at $*P <$
862 0.05 and $**P < 0.01$.
863
864 **Figure 3.** Effects of imidacloprid on the homeostasis of developmental regulation in *Apis*
865 *mellifera* larvae. (A) Relative gene expression and (B) hormone levels of developmental
866 regulatory-related genes in larvae exposed to 377 ppb imidacloprid for 72 h or a control
867 group. Statistical significance was set at $*P < 0.05$ and $**P < 0.01$.
868
869 **Figure 4.** Imidacloprid induced a detoxification response in *Apis mellifera* larvae. Relative
870 expression levels of cytochrome P450 monooxygenase family genes in larvae exposed to
871 imidacloprid for 72 hours. IMI is the larvae exposed to imidacloprid for 72 hours. CK is

872 the artificial food control group. Statistical significance was set at *$P < 0.05$, **$P < 0.01$,
873 and ***$P < 0.001$.
874
875 **Figure 5.** Imidacloprid exposure causes oxidative stress, gut apoptosis, and tissue
876 structural damage while inducing antioxidant defenses in *Apis mellifera* larvae. (a)
877 Analysis of oxidative stress and damage in larvae exposed to imidacloprid for 72 hours.
878 ROS levels indicate the degree of oxidative stress. MDA is a marker for lipid peroxidation.
879 PCO levels reflect the extent of protein carbonylation damage; (b) Activity of
880 antioxidants in larvae exposed to imidacloprid for 72 hours; (c) The relative expression
881 levels of antioxidant genes in larvae exposed to imidacloprid for 72 hours; (d)
882 Histological sections of the larval gut stained with H&E. The circled inserts in the figure
883 show magnified views of the muscle layer. The blue arrow indicates the cell nucleus of
884 the muscle layer. The boxed inset in the figure is a magnified view of the basal layer
885 (black arrow), peritrophic membrane (red arrow), and food residues (green arrow). IMI is
886 the larvae exposed to imidacloprid for 72 hours. CK is the artificial food control group.
887 Statistical significance was set at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
888
889 **Figure 6.** Imidacloprid toxicity inhibited the expression of genes involved in nutrient
890 catabolism, protein synthesis, and energy metabolism and reserves. (A) Carbohydrate
891 catabolism; (B) Proteolysis; (C) Amino acid transport; (D) Mitochondrial oxidative
892 phosphorylation; (E) Glycolysis; (F) Protein synthesis. (G) The total contents of ATP,
893 glycogen (H), and protein (I). IMI is the larvae exposed to imidacloprid for 72 hours. CK is
894 the artificial food control group. Statistical significance was set at *$P < 0.05$, **$P < 0.01$,
895 and ***$P < 0.001$.
896
897 **Figure 7.** Correlation between phenotypes and molecular characteristics in imidacloprid-
898 exposed larvae with developmental retardation. Data were normalized using GraphPad
899 software, followed by a Pearson correlation coefficient calculation using SPSS, and a
900 heatmap was generated.
901

902 **Figure 8.** A model interpreting the ecotoxicological effects of imidacloprid on *Apis*
903 *mellifera* larvae and the mechanisms of imidacloprid-induced developmental retardation.
904 Imidacloprid toxicity caused growth and developmental delay in *A. mellifera* larvae. It
905 disrupts the regulatory balance of molt development, resulting in restricted
906 development. In addition, the damage caused by imidacloprid restricts the metabolism
907 and utilization of food nutrients and energy in larvae. The additional energy consumed
908 by larval P450 detoxification and antioxidant defenses further reduces the supply of
909 nutrients and energy supply for growth and development. The arrows indicate induction
910 or promotion, the straight lines indicate inhibition, and the green dotted lines indicate
911 speculative inhibition.
912
913
914
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917
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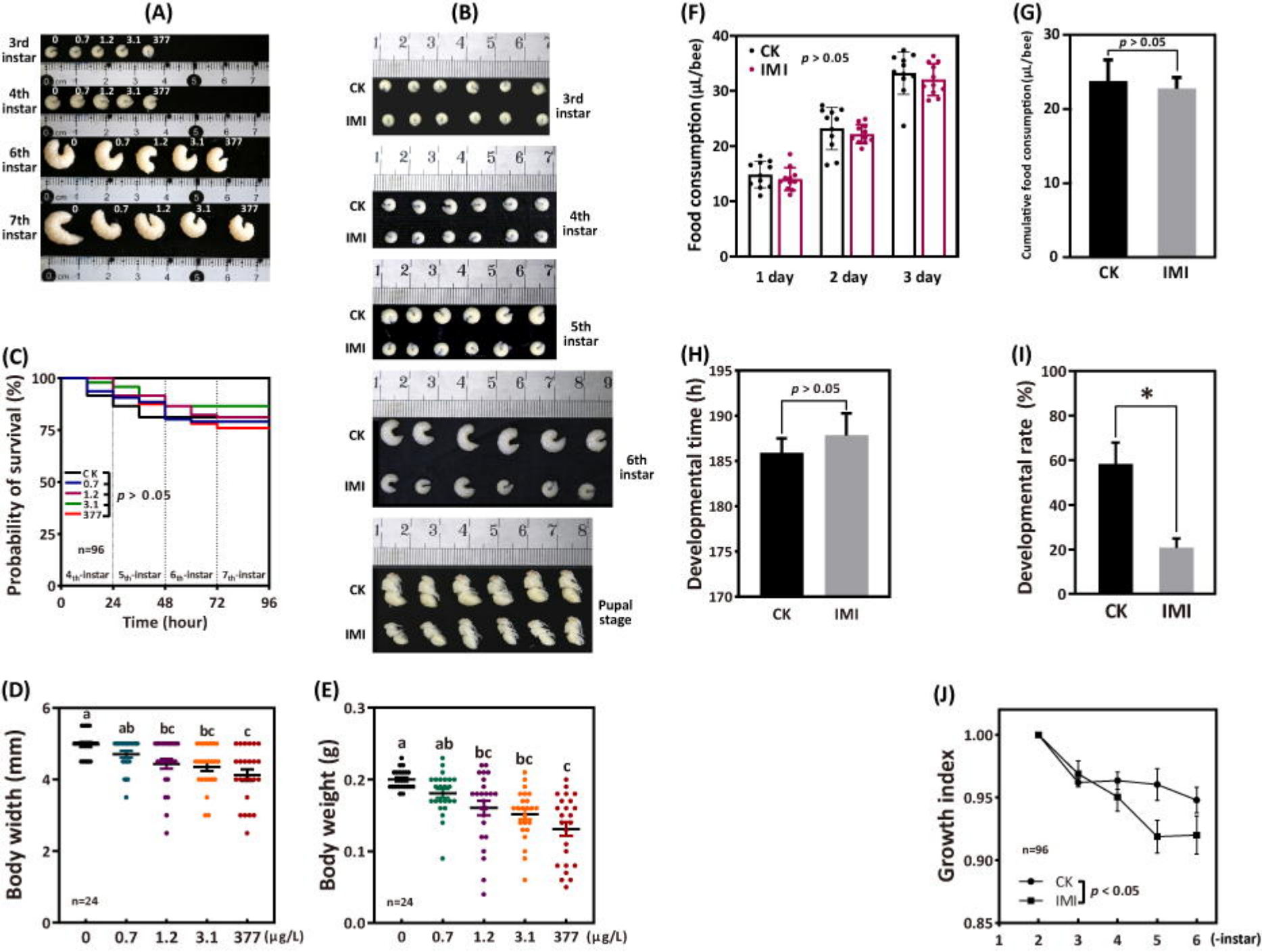
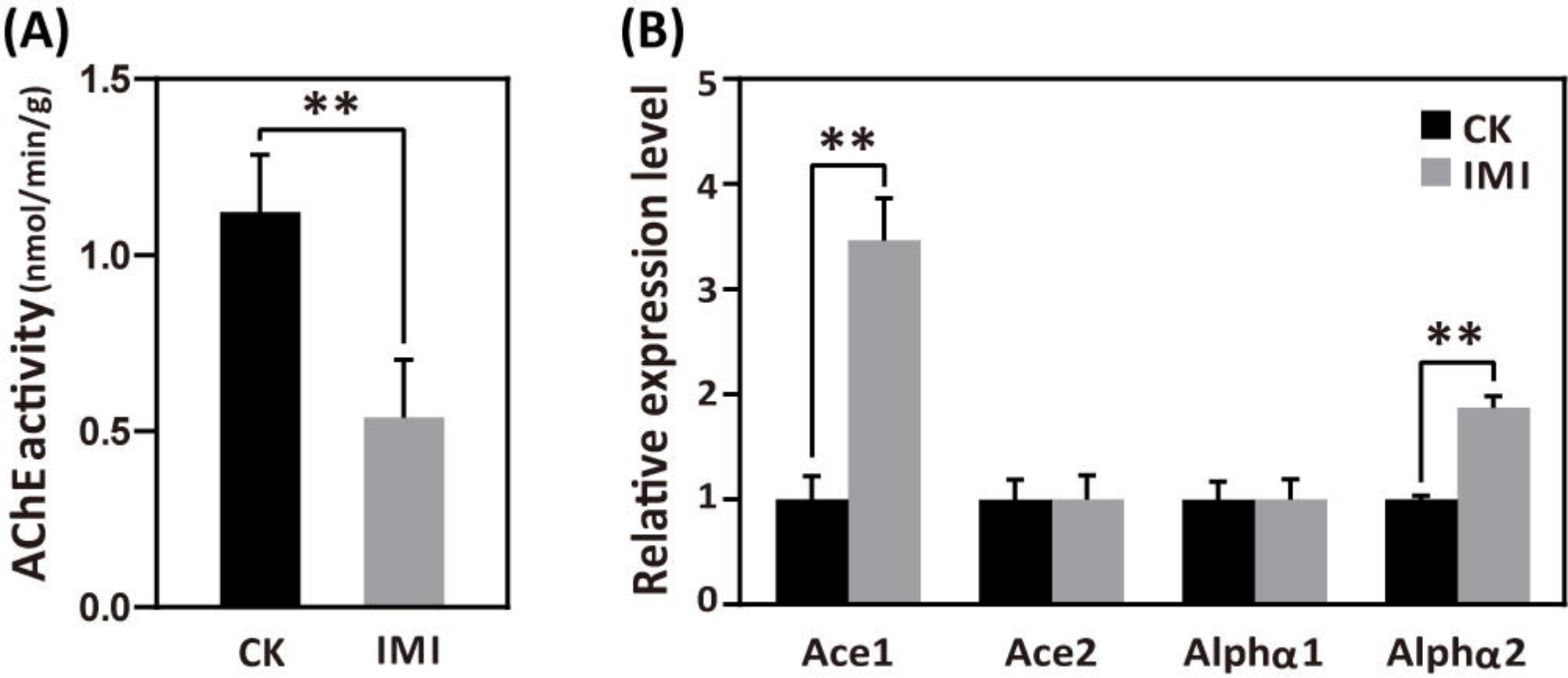
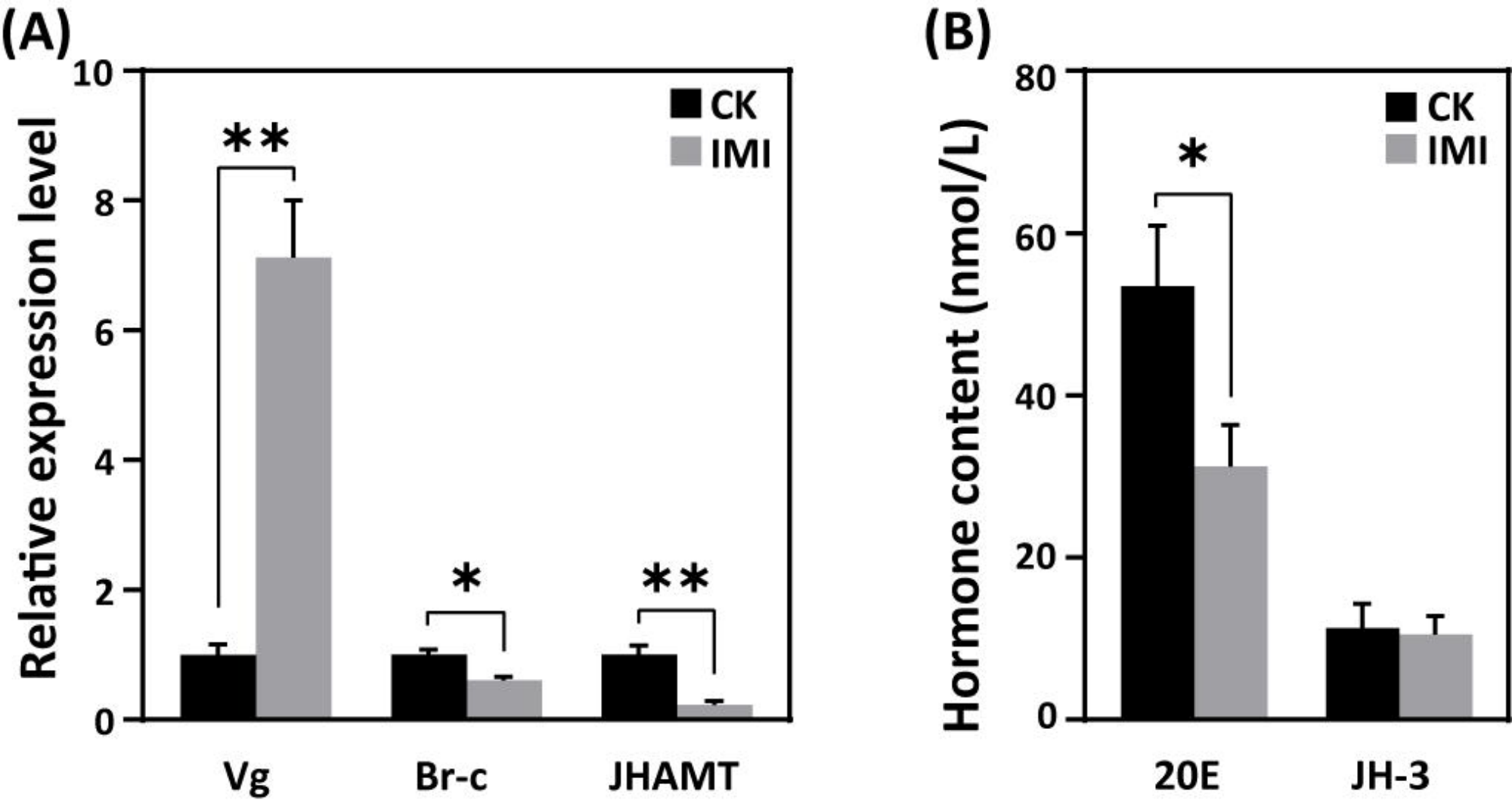
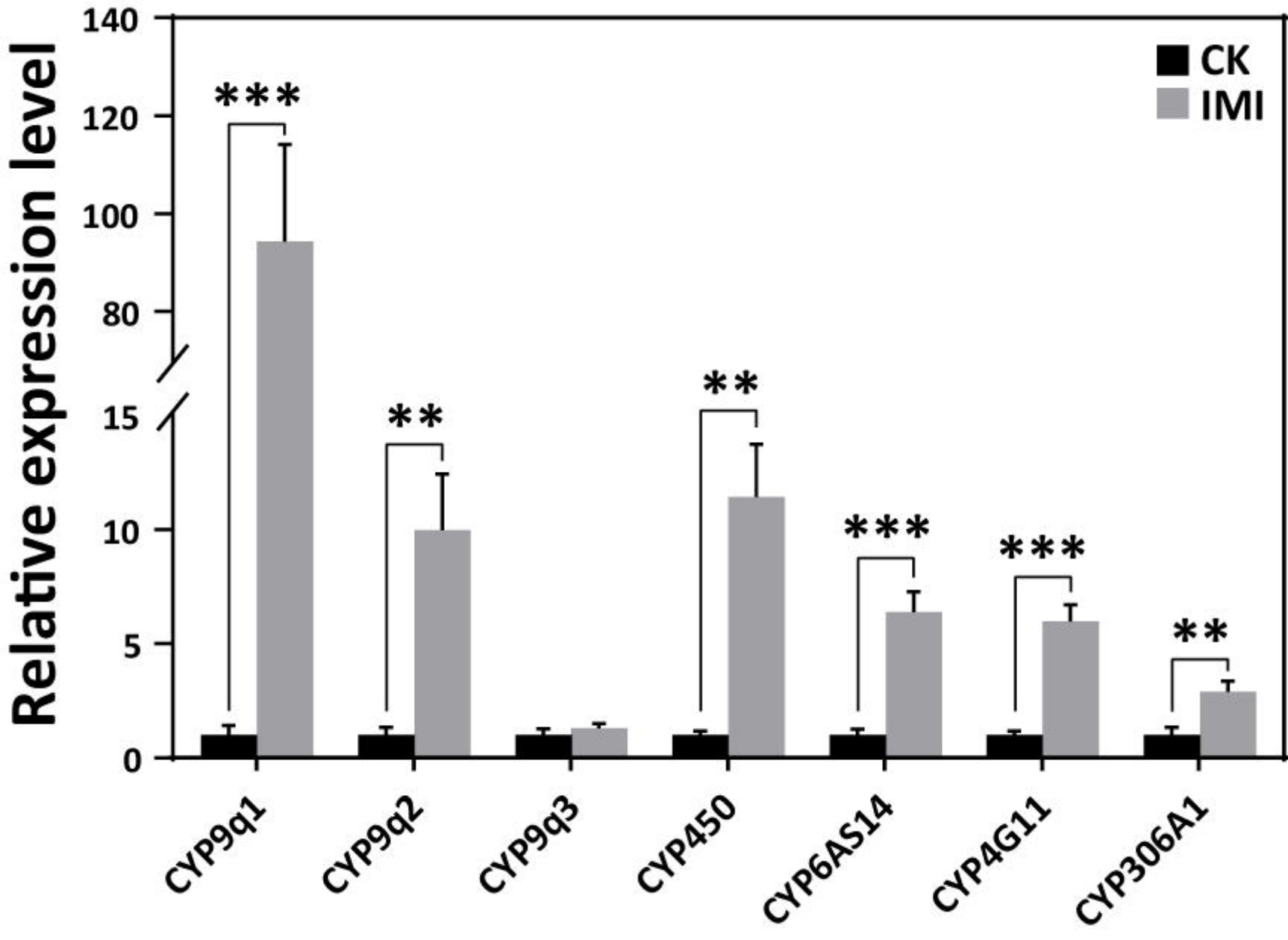
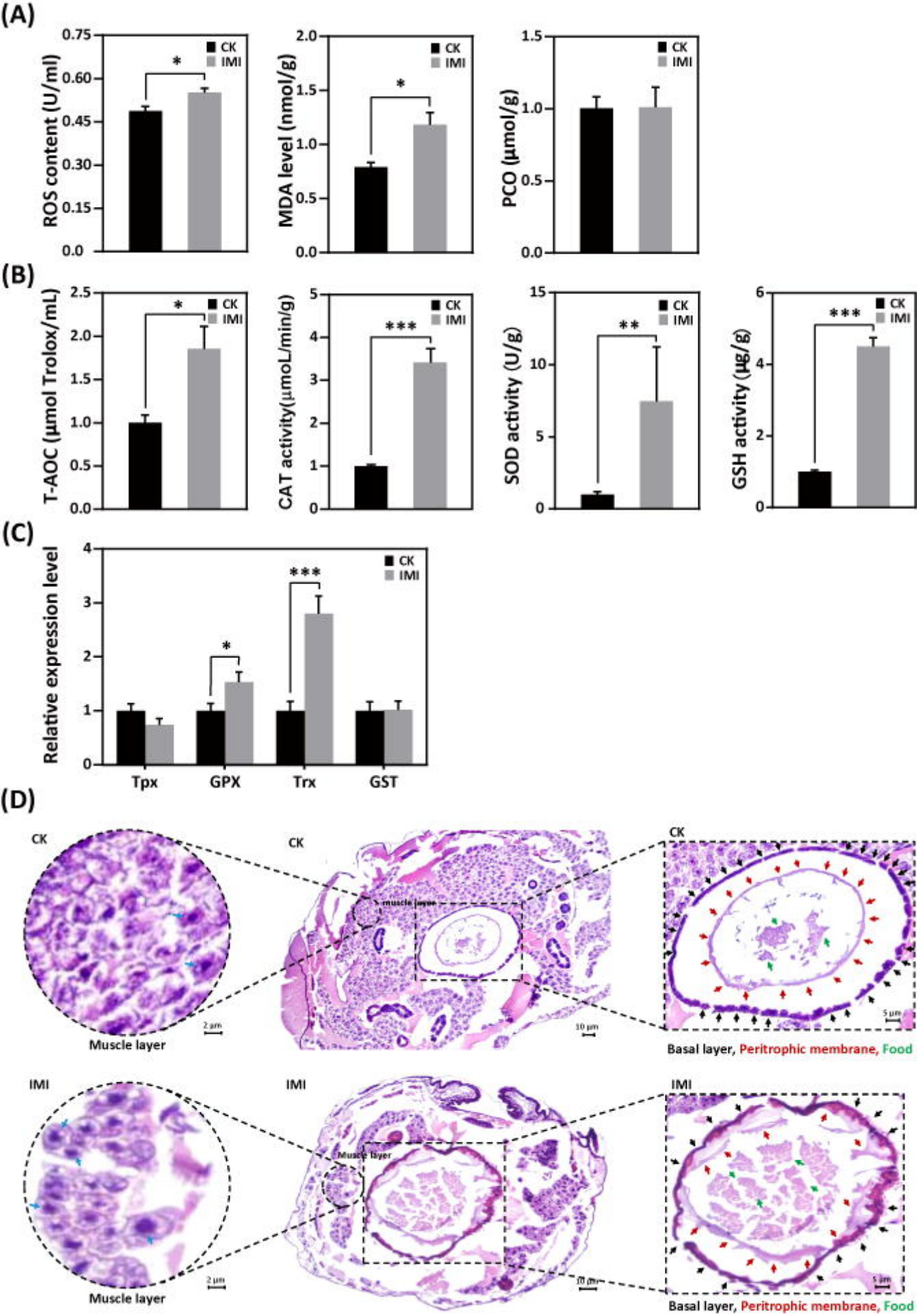
919
920

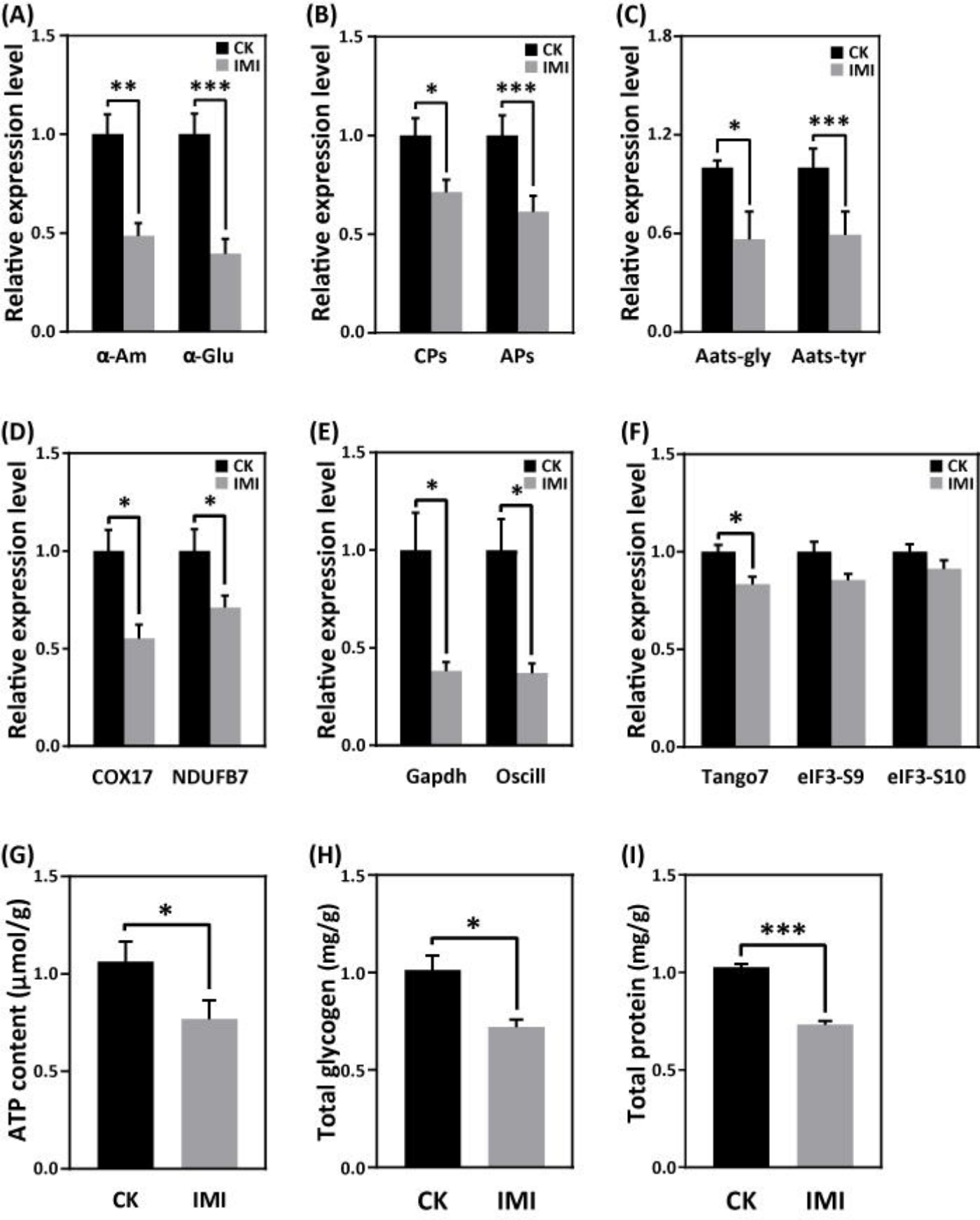

(A)
3rd instar
4th instar
6th instar
7th instar
0
0.7
1.2
3.1
377
(B)
CK
IMI
3rd instar
4th instar
5th instar
6th instar
Pupal stage
(C)
Probability of survival (%)
Time (hour)
CK
0.7
1.2
3.1
377
p > 0.05
n=96
4th-instar
5th-instar
6th-instar
7th-instar
(D)
Body width (mm)
a
ab
bc
bc
c
n=24
0
0.7
1.2
3.1
377 (μg/L)
(E)
Body weight (g)
a
ab
bc
bc
c
n=24
0
0.7
1.2
3.1
377 (μg/L)
(F)
Food consumption (μL/bee)
CK
IMI
p > 0.05
1 day
2 day
3 day
(G)
Cumulative food consumption (μL/bee)
p > 0.05
CK
IMI
(H)
Developmental time (h)
p > 0.05
CK
IMI
(I)
Developmental rate (%)
*
CK
IMI
(J)
Growth index
n=96
CK
IMI
p < 0.05
(-instar)

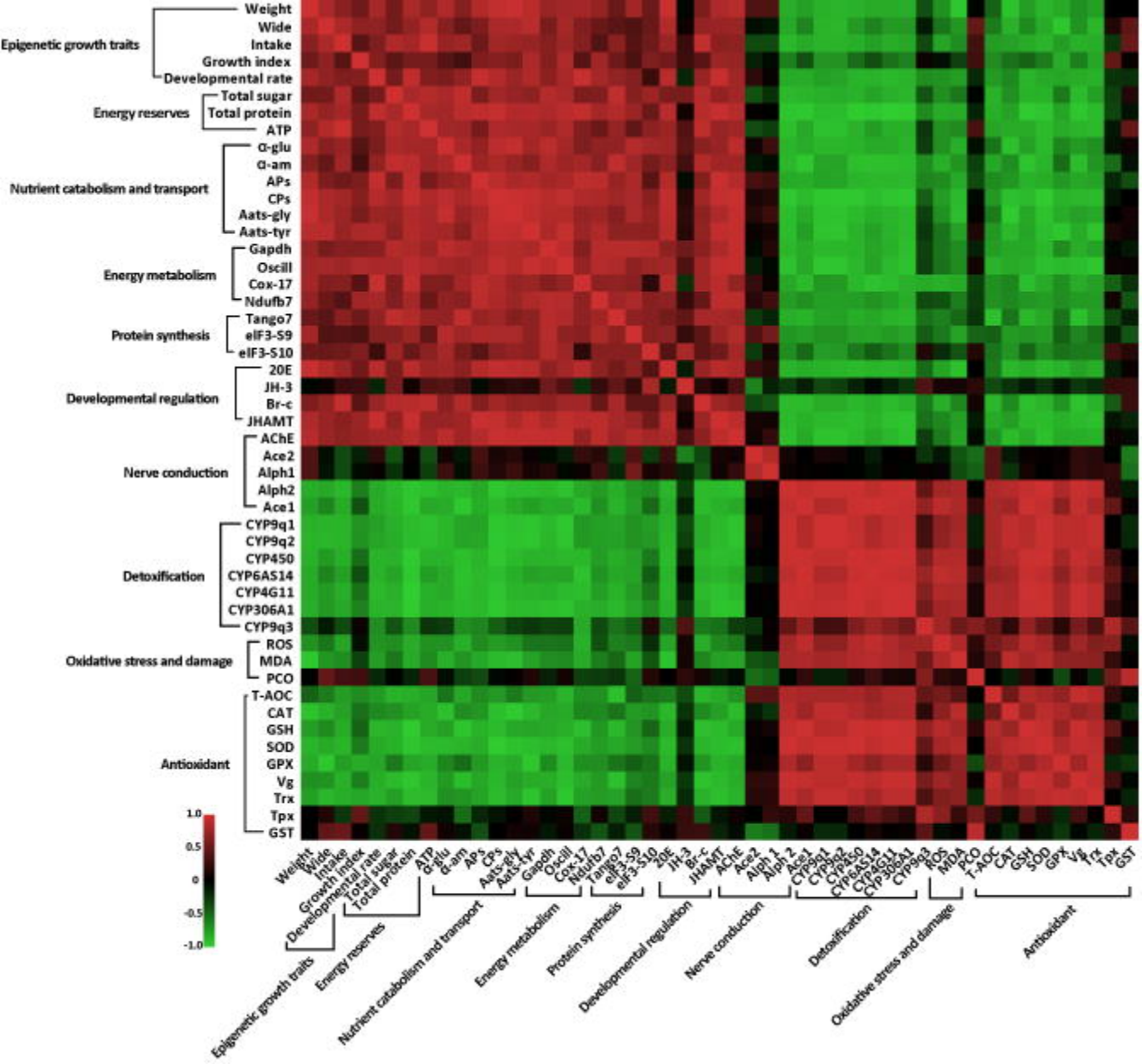

(A)
AChE activity (nmol/min/g)
1.5
1.0
0.5
0.0
**
CK
IMI
(B)
Relative expression level
5
4
3
2
1
0
**
**
CK
IMI
Ace1
Ace2
Alphα1
Alphα2

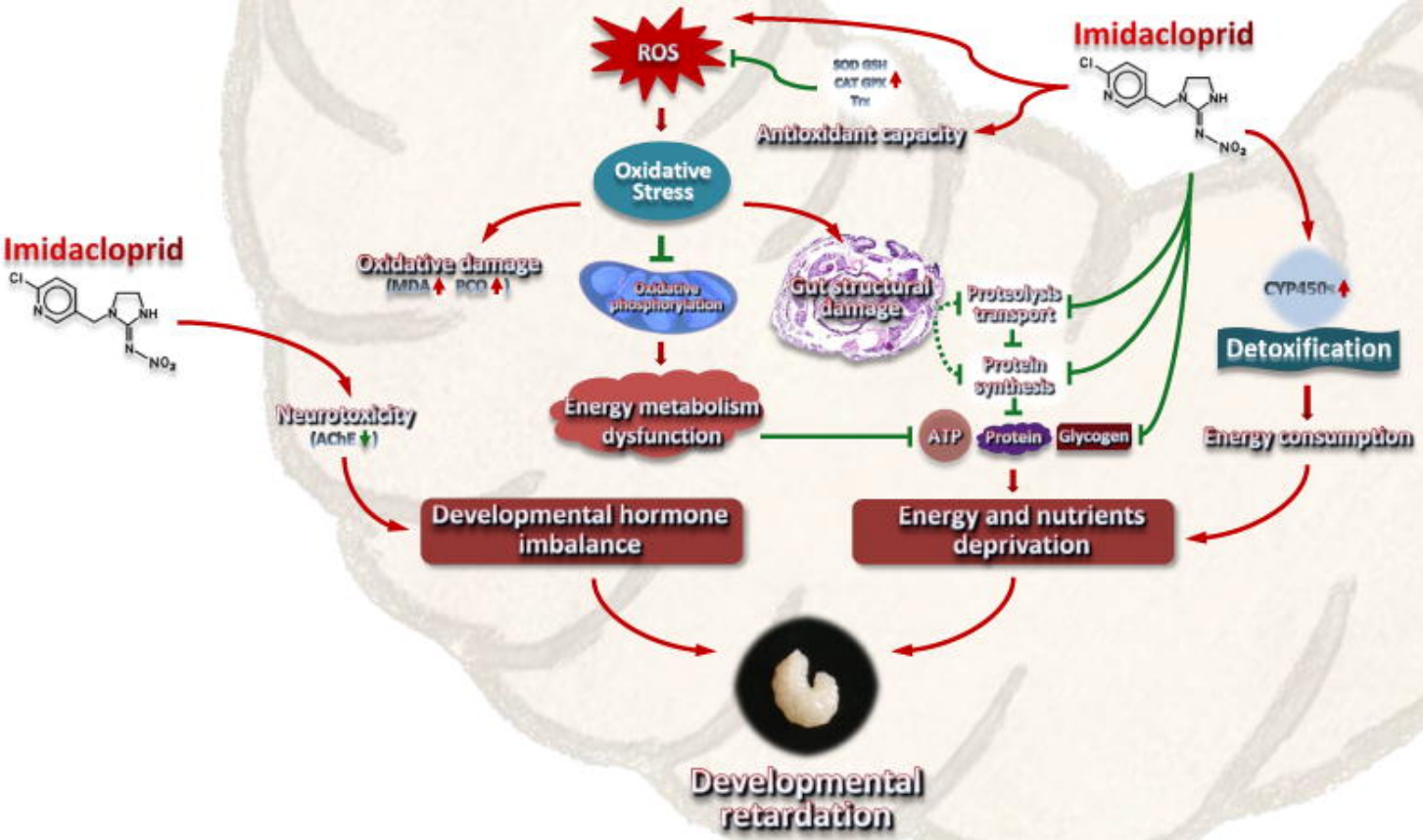

(A)
Relative expression level
10
8
6
4
2
0
CK
IMI
**
*
**
Vg
Br-c
JHAMT
(B)
Hormone content (nmol/L)
80
60
40
20
0
CK
IMI
*
20E
JH-3


Relative expression level
140
120
100
80
15
10
5
0
CK
IMI
***
**
**
***
***
**
CYP9q1
CYP9q2
CYP9q3
CYP450
CYP6AS14
CYP4G11
CYP306A1


(A)
ROS content (U/ml)
MDA level (nmol/g)
PCO (μmol/g)
CK
IMI
(B)
T-AOC (μmol Trolox/mL)
CAT activity(μmoL/min/g)
SOD activity (U/g)
GSH activity (μg/g)
(C)
Relative expression level
Tpx
GPX
Trx
GST
(D)
CK
IMI
Muscle layer
Basal layer, Peritrophic membrane, Food

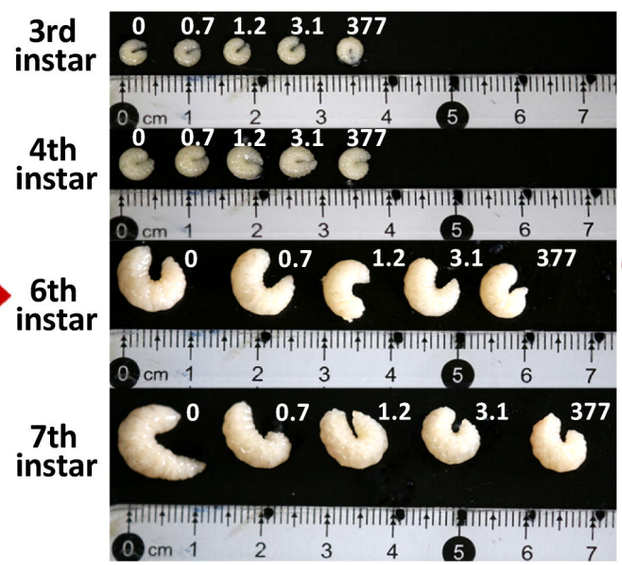

(A)
Relative expression level
CK
IMI
**
***
α-Am
α-Glu
(B)
Relative expression level
CK
IMI
*
***
CPs
APs
(C)
Relative expression level
CK
IMI
*
***
Aats-gly
Aats-tyr
(D)
Relative expression level
CK
IMI
*
*
COX17
NDUFB7
(E)
Relative expression level
CK
IMI
*
*
Gapdh
Oscill
(F)
Relative expression level
CK
IMI
*
Tango7
eIF3-S9
eIF3-S10
(G)
ATP content (μmol/g)
*
CK
IMI
(H)
Total glycogen (mg/g)
*
CK
IMI
(I)
Total protein (mg/g)
***
CK
IMI


Epigenetic growth traits
Weight
Wide
Intake
Growth index
Developmental rate
Energy reserves
Total sugar
Total protein
ATP
Nutrient catabolism and transport
α-glu
α-am
APs
CPs
Aats-gly
Aats-tyr
Energy metabolism
Gapdh
Oscill
Cox-17
Ndufb7
Protein synthesis
Tango7
eIF3-S9
eIF3-S10
Developmental regulation
20E
JH-3
Br-c
JHAMT
Nerve conduction
AChE
Ace2
Alph1
Alph2
Ace1
Detoxification
CYP9q1
CYP9q2
CYP450
CYP6AS14
CYP4G11
CYP306A1
CYP9q3
Oxidative stress and damage
ROS
MDA
PCO
Antioxidant
T-AOC
CAT
GSH
SOD
GPX
Vg
Trx
Tpx
GST
1.0
0.5
0
-0.5
-1.0

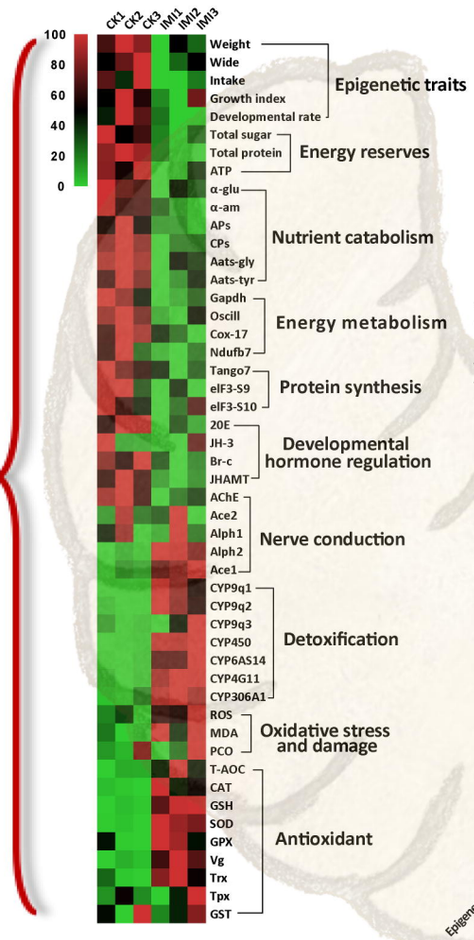

ROS
SOD GSH
CAT GPX
Trx
Antioxidant capacity
Imidacloprid
Oxidative Stress
Oxidative damage
(MDA PCO)
Oxidative phosphorylation
Gut structural damage
Proteolysis transport
Protein synthesis
CYP450
Detoxification
Energy consumption
Imidacloprid
Neurotoxicity
(AChE)
Energy metabolism dysfunction
ATP
Protein
Glycogen
Developmental hormone imbalance
Energy and nutrients deprivation
Developmental retardation

Imidacloprid toxicity and the underlying mechanism causing larval development delay

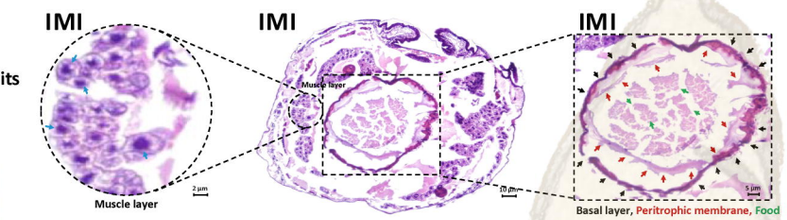
72 hours Exposure



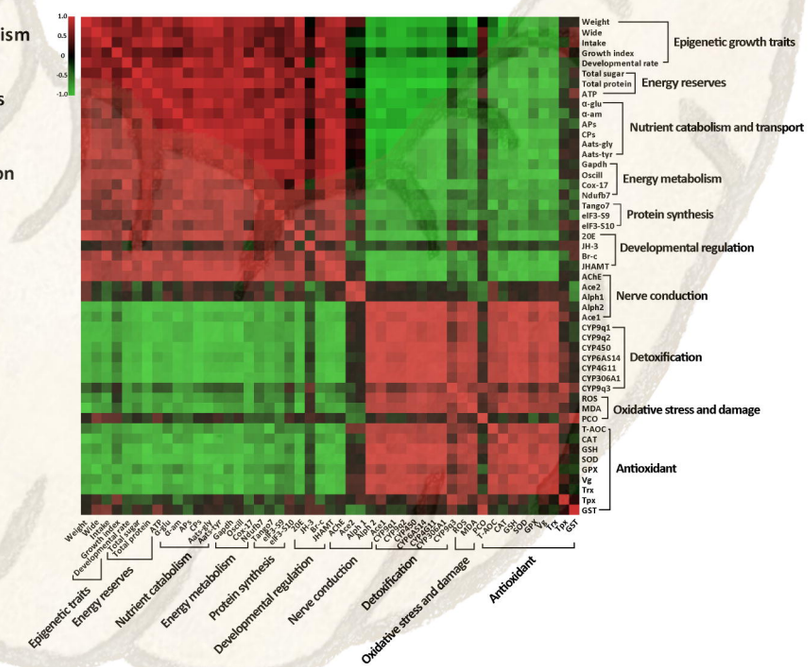
Developmental retardation



Toxic effects



Gut tissue damage



Correlation Analysis