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

53 degenerative changes in various organs (Khan et al., 2010; Shaw et al., 2014; Yang et al.,
54 2014). Imidacloprid even at low doses causes severe liver, thyroid, and body weight
55 problems, reproductive toxicity, developmental retardation, and neurobehavioral
56 deficits in rats and rabbits (Anatra-Cordone M, 2005). Additionally, imidacloprid affects
57 the singing ability of birds, making it difficult for them to attract mates and reproduce. It
58 is a significant contributor to the decline in terrestrial insect populations since 1991,
59 averaging 9% per decade (Cardoso et al., 2020; Sánchez-Bayo and Wyckhuys, 2019).
60 Honey bees (*Apis mellifera* L.) are valuable model organisms in insects with significant
61 economic and ecological importance as crop pollinators and in maintaining ecological
62 balance (Zheng and Fu-Liang, 2009). However, the widespread use of pesticides,
63 including imidacloprid, has resulted in colony collapse disorder (CCD), causing severe
64 impacts on the beekeeping industry and ecological balance (Mahé et al., 2021). Studies
65 have shown that imidacloprid is applied as a seed coating (Simon-Delso et al., 2015) and
66 accumulates in the plant (Dively and Kamel, 2012; Rocha et al., 2022), exposing non-
67 target bees to imidacloprid residues during pollen and nectar collection (Tong et al.,
68 2018). Even low levels of systemic imidacloprid cause sublethal effects in adult bees,
69 including neurotoxicity (Belzunces et al., 2012), behavioural changes (Karahan et al.,
70 2015; Medrzycki et al., 2003), reproductive impairment (Chaimanee et al., 2016),
71 reduced immunity (Pettis et al., 2013), apoptosis and autophagy (Carneiro et al., 2022),
72 and shortened lifespan (Anderson and Harmon-Threatt, 2021). A recent finding shows
73 that imidacloprid induces oxidative stress in honey bees (Balieira et al., 2018). This
74 oxidative stress is a result of the long-term synergistic effect between imidacloprid and
75 its target receptors (nAChRs) (Makoto et al., 2020) in bees, which leads to an excessive
76 influx of Ca^{2+} into the bee brain (Tahirare, 2013), disrupting Ca^{2+} homeostasis and
77 ultimately causing mitochondrial dysfunction and increased mitochondrial ROS
78 generation, exacerbating oxidative stress.

79 The development and health status of juvenile animals play a critical role in
80 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 (Vincent 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

109 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 (Figure 1A-E). 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 (Figure 1C and Figure 1 – Source Data 1), feeding
144 (Figure 1F and Figure 1– Source Data 4, Figure 1G and Figure 1– Source Data 5) and the
145 time required for larvae to enter the pupal stage successfully (Figure 1H and Figure 1–
146 Source Data 6). However, it did lead to slow developmental progress, a lower initial rate
147 of successful pupation (Figure 1I and Figure 1– Source Data 7), and lower growth index
148 (Figure 1J and Figure 1– Source Data 8). Larval growth index gradual decrease from the
149 4th instar, and then stabilized by the 6th instar. At the 4th, 5th and 6th instars, the
150 growth index of the imidacloprid-treated groups was significantly lower than that of the
151 control group by an average of 1.35%, 4.49%, and 2.76%, respectively ($p<0.05$) (Figure 1,
152 source data 8). In addition, imidacloprid consistently resulted in reduced larval width
153 (Figure 1D and Figure 1– Source Data 2) and weight (Figure 1E and Figure 1– Source
154 Data 3) up to the pupal stage (Figure 1B). Comparing the 377 ppb imidacloprid-exposed
155 larvae to the control, the mean body weight and body width were significantly lower by
156 34.38% and 17.15%, respectively ($p<0.05$).

157 **Imdacloprid neurotoxicity in bee larvae**

158 Under imidacloprid environmental stress at 377 ppb, AChE activity, which is closely
159 related to the disruption of neurotransmission, was significantly inhibited in larvae
160 (Figure 2A and Figure 2– Source Data 9) and showed a significant average reduction of
161 48% ($p<0.05$) compared to the control group. Neither acetylcholine receptor (nAChR)
162 alpha 1 (*Alph1*) nor acetylcholinesterase 2 (*Ace2*) gene expression was significantly
163 different from the control. In contrast, acetylcholine receptor alpha 2 (*Alph2*) and

164 acetylcholinesterase 1 (*Ace1*) gene expression was significantly higher than that in the
165 control group, with an average of 1.87 and 3.46 times higher (Figure 2B and Figure 2–
166 Source Data 10), respectively ($p<0.05$).

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

168 In imidacloprid-exposed larvae, the developmental regulatory gene juvenile hormone
169 acid methyl transferase (*JHAMT*) and the transcription factor broad complex (*Br-c*) were
170 downregulated, while vitellogenin (*Vg*) gene expression was upregulated by seven-fold
171 in comparison to control groups ($p<0.05$)(Figure 3A and Figure 3– Source Data 11). In
172 parallel, imidacloprid exposure decreased the major regulator of insect metamorphosis
173 20-hydroxyecdysone (20E) titers but had no effect on preventing premature
174 metamorphosis juvenile hormone (JH-3) titers (Figure 3B and Figure 3– Source Data 12).

175 **Imidacloprid induces detoxification in larvae**

176 According to the qRT-PCR analysis, the expression levels of cytochrome P450
177 monooxygenase family members such as CYPq1, CYPq2, CYP450, CYP6AS14, CYP4G11,
178 and CYP306A1, except for CYPq3, were observed to be significantly upregulated by
179 several fold in imidacloprid-induced developmental retardation larvae. Among them,
180 CYPq1 showed an abnormal 94-fold increase ($p<0.05$)(Figure 4 and Figure 4– Source
181 Data 13).

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

183 Larvae exposed to imidacloprid exhibited a significant increase in reactive oxygen
184 species (ROS) levels (Figure 5A and Figure 5– Source Data 14) and the activities of the
185 antioxidants CAT, SOD and GSH (Figure 5B and Figure 5– Source Data 15), as well as
186 upregulation of the antioxidant genes GPX and Trx expression (Figure 5C and Figure 5–
187 Source Data 16), resulting in a significant increase in total antioxidant capacity (T-AOC)
188 (Figure 5B and Figure 5– Source Data 15) compared to the control group. Under these

189 conditions, MDA levels, used as a marker of lipid peroxidation, were observed to
190 significant increase 58% ($p<0.05$), while protein carbonylation damage indicator PCO
191 levels remained unchanged (Figure 5A and Figure 5– Source Data 14).

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

193 Hematoxylin-eosin staining revealed a significant reduction in the number of cells in
194 the muscular layer of the larval gut exposed to imidacloprid with clear apoptotic
195 characteristics compared to the standard feeding control groups (Figure 5D and Figure 5
196 – Source Data 17 and 18). This was evidenced by increased nuclear staining and
197 compacted chromatin, as well as disrupted cell arrangement in the basal layer of the gut,
198 increased nuclear staining of basal lamina cells, and apoptotic signals. The integrity of
199 the peritrophic membrane structure of the gut was better in the control group, while
200 imidacloprid-exposed larvae were still in the formation stage, resulting in the
201 accumulation of undigested food residues in their guts (Figure 5D and Figure 5– Source
202 Data 18).

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

204 Exposure to imidacloprid did not significantly affect the daily food intake of
205 developmentally delayed larvae compared to controls (Figure 1F and 1G, Figure 1–
206 Source Data 4 and 5). However, imidacloprid toxicity suppressed the expression of
207 nutrient catabolism genes, including alpha-amylase (*α-Am*) and alpha-glucosidase (*α-*
208 *Glu*), which are involved in carbohydrate catabolism (Figure 6A and Figure 6– Source
209 Data 19), carboxypeptidase (*CPs*) and aminopeptidase (*APs*) involved in proteolysis
210 (Figure 6B and Figure 6– Source Data 20), and glycine tRNA ligase (*Aats-Gly*) and
211 tyrosine tRNA ligase (*Aats-Tyr*) involved in amino acid transport (Figure 6C and Figure 6–
212 Source Data 21). Carbohydrate catabolism and amino acid transport were significantly
213 suppressed by over 50% ($p<0.05$).

214 **Imidacloprid causes restriction of larval protein synthesis and energy metabolic**
215 **dysfunction**

216 RT-qPCR analysis revealed that imidacloprid exposure reduced the expression of
217 critical genes involved in energy metabolism, including cytochrome c oxidase copper
218 chaperone (*COX17*) and NADH dehydrogenase [ubiquinone] 1β subcomplex subunit 7
219 (*NDUFB7*), in developmentally delayed larvae (Figure 6D and Figure 6– Source Data 22).
220 Imidacloprid exposure also inhibited the expression of genes related to glycolysis and
221 energy production, such as glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and
222 glucosamine-6-phosphate isomerase (*Oscillin*) (Figure 6E and Figure 6– Source Data 23).
223 Both had expression levels below 50% of the control group ($p<0.05$). In addition, the
224 expression of transcription initiation factors related to protein synthesis, including
225 translation initiation factor 3 subunit M (*Tango7*), translation initiation factor 3 subunit
226 B (*eIF3-S9*), and translation initiation factor 3 subunit A (*eIF3-S10*), was significantly
227 suppressed by imidacloprid toxicity (Figure 6F and Figure 6– Source Data 24). Finally, the
228 total levels of ATP, glycogen and protein, which are closely related to energy production
229 and reserves, were significantly lower in developmentally delayed larvae than in
230 controls ($p<0.05$)(Figure 6G-I and Figure 6– Source Data 25-27).

231 **Correlation between developmental phenotypic changes and molecular features**
232 **caused by imidacloprid toxicity**

233 To better understand the mechanism of imidacloprid-induced larval developmental
234 retardation, we performed a comprehensive correlation analysis between epigenetic
235 growth traits and molecular characteristics (Figure 7 and Figure 7– Source Data 28). The
236 results showed that in larvae with imidacloprid-induced developmental retardation,
237 body weight, width, growth index, and developmental rate were positively correlated
238 with energy metabolism and reserves, nutrient catabolism, protein synthesis, and
239 developmental regulation. Conversely, negative correlations were observed between
240 these growth traits and parameters associated with detoxification and antioxidant

241 defense processes that require additional energy expenditure. Moreover, oxidative
242 stress and damage, detoxification, and antioxidant defense all exhibited significant
243 positive correlations (Figure 7 and Figure 7– Source Data 28).

244

245 Discussion

246 Toxic effects and the molecular basis of environmental concentrations of imidacloprid 247 on bee larvae

248 Previous research has shown that bee larvae are more susceptible to the adverse
249 effects of pesticides than adult bees (Tomé et al., 2020). In this study, larvae were
250 exposed to environmental concentrations of imidacloprid, resulting in sublethal effects
251 such as decreased body weight, width, developmental rate, and growth index (Figure 1).
252 Our results support the notion that pesticides can affect larval development (Dai et al.,
253 2018; Zhu et al., 2014). However, current research has primarily focused on
254 characterizing the effects of imidacloprid on larval phenotype and behavior, and the
255 molecular mechanisms underlying its ecotoxicological effects remain poorly understood.

256 To obtain molecular evidence of imidacloprid toxicity to bee larvae, we first examined
257 the expression of acetylcholine receptors and AChE activity. The results showed that
258 imidacloprid exposure increased the expression of the *Alph2* gene and inhibited AChE
259 activity (Figure 2 and Figure 2– Source Data 10). Normally, acetylcholine stimulates
260 nerve impulses by binding to the Alph receptor, and AChE degrades acetylcholine to
261 terminate nerve conduction. However, imidacloprid competitively binds to AChE
262 receptors, inhibits AChE activity, and causes continuous excitation of nerve conduction,
263 ultimately leading to paralysis and death (Katić et al., 2021; Shan et al., 2020). Therefore,
264 our results strongly suggest that imidacloprid is neurotoxic to bee larvae.

265 The growth and development of juvenile animals depends on the digestion,
266 absorption and utilization of food nutrients. Therefore, we next investigated whether

267 imidacloprid affects the digestion and utilization of dietary by larvae. The results
268 indicate that exposure to imidacloprid suppressed the hydrolysis genes *CPs* and *APs* and
269 the amino acid transport pathway genes *Aats-Gly* and *Aats-Tyr* in larvae (Figure 6B-C
270 and Figure 6– Source Data 20-21). Since these genes are responsible for cleaving dietary
271 proteins into absorbable peptides and amino acids and transporting them for
272 metabolism (2019), inhibiting the expression of these genes would severely affect larval
273 digestion, resulting in protein deficiency and impaired metabolic activity. In addition,
274 imidacloprid also affected the expression of *α-Am* and *α-Glu* genes (Figure 6A and Figure
275 6– Source Data 19), which are involved in carbohydrate digestion, indicating that
276 imidacloprid also affects the digestion of dietary carbohydrates. Indeed, histological
277 observations also confirmed that a large amount of undigested food remained in the
278 intestine of larvae exposed to imidacloprid (Figure 5D and Figure 5– Source Data 18). In
279 conclusion, imidacloprid inhibits the digestion and utilization of dietary proteins and
280 carbohydrates by larvae, which is extremely detrimental to their growth and
281 development.

282 In addition to nutrition, energy metabolism is critical for the maintenance of all life
283 activities. In this study, imidacloprid exposure inhibited the expression of genes related
284 to mitochondrial oxidative phosphorylation (*COX17*, *NDUFB7*) and its alternative
285 glycolytic pathway (*Gapdh*, *Oscillin*). Mitochondrial oxidative phosphorylation is the
286 central pathway for ATP generation during energy metabolism (Tian et al., 2020a), while
287 glycolysis is an alternative mechanism for ATP synthesis (Jiang et al., 2020). Since both
288 biochemical processes are essential for energy production, their inhibition resulted in
289 reduced ATP levels in bee larvae (Figure 6G and Figure 6– Source Data 25). These results
290 strongly suggest that imidacloprid induces energy metabolism dysfunction leading to
291 insufficient energy production in larvae. Since larval energy production is insufficient,
292 this is likely to lead to a depletion of energy reserves. Indeed, further analysis revealed a
293 significant decrease in glycogen and protein energy reserves in larvae exposed to
294 imidacloprid (Figure 6H-I and Figure 6– Source Data 26-27). These findings are

295 consistent with previous reports that exposure to pesticides such as endosulfan,
296 chlorpyrifos, insecticides, and fipronil can lead to a reduction in energy reserves in
297 organisms (Bouayad et al., 2012a; Dutra et al., 2009; Radwan et al., 2008; Rambabu and
298 Rao, 1994; Ribeiro et al., 2001). In conclusion, our results suggest that imidacloprid
299 induces energy metabolism dysfunction and affects energy production and reserves in
300 bee larvae.

301 Insects rely primarily on cytochrome P450 detoxification to resist toxic compounds (Li
302 et al., 2007), and the level of CYP450 expression indicates the severity of pesticide
303 toxicity. While adult worker bees have shown a positive response to imidacloprid (Mao
304 et al., 2009), it is uncertain whether bee larvae can respond to pesticide toxicity. In this
305 study, we found that seven cytochrome P450 family transcripts, including CYPq1, CYPq2,
306 CYP450, CYP6AS14, CYP4G11, and CYP306A1, were significantly upregulated in bee
307 larvae, with CYP9q1 being 94-fold higher than the control group (Figure 4 and Figure 4–
308 Source Data 13). These results are consistent with previous findings that the CYP450
309 family genes CYP6AS3, CYP6AS4, and CYP9S1 were significantly upregulated in adult
310 worker bees exposed to quercetin, coumaphos, and fluvalinate (Mao et al., 2011) and
311 that CYP9Q1, CYP9Q2, and CYP9Q3 help bees detoxify fluvalinate and coumaphos
312 (Gregorc et al., 2018). This suggests that the P450 detoxification system is already
313 established in bee larvae and they respond positively by activating the detoxification
314 function of CYP450 upon exposure to imidacloprid.

315 Oxidative stress is the excessive production of ROS in aerobic organisms (Felton, 1995;
316 Ozcan and Ogun, 2015). Pesticides, including coumaphos (Gregorc et al., 2018), fipronil
317 (Paris et al., 2017), organophosphorus, pyrethroid, organochlorinated (Chakrabarti et al.,
318 2015), and chlorpyrifos (Shafiq-ur-Rehman et al., 2012), as well as paraquats such as
319 paraquat (Li-Byarlay et al., 2016), have been shown to cause oxidative stress in bees. In
320 this study, exposure to imidacloprid resulted in increased levels of ROS and MDA in
321 larvae (Figure 5A and Figure 5– Source Data 14), indicating severe oxidative stress and

322 lipid damage. Organisms have evolved antioxidant defense mechanisms to scavenge
323 excessive ROS and resist oxidative stress, including increasing levels of antioxidants such
324 as CAT, SOD, GSH, TrxR, and GSH (Dong et al., 2013; Tian et al., 2020b). In this regard,
325 we have recently confirmed this in worker bees (Li et al., 2022), which is also well
326 reflected in the present study. We observed a significant increase in the activities of
327 antioxidant enzymes SOD and CAT, gene expression of Trx and Gpx, and GSH levels in
328 larvae after exposure to imidacloprid (Figure 5B-C and Figure 5– Source Data 15-16).
329 These results show that the antioxidant system in developing larvae is established and
330 used to resist oxidative stress caused by imidacloprid, similar to that in adult worker
331 bees. Notably, compared to our recent study in adult bees (Li et al., 2022), the larvae in
332 this study exhibited more robust antioxidant activity when exposed to higher
333 concentrations of imidacloprid toxicity than worker bees. However, the underlying
334 mechanism requires further investigation. The underlying mechanisms involved require
335 further study.

336 Notably, in addition to routine antioxidants, imidacloprid induces a 7-fold increase in
337 Vg expression (Figure 3A and Figure 3– Source Data 11). Vg is known to provide nutrition
338 to developing embryos (Jorgensen et al., 2009) and to help animals cope with pollution-
339 induced stress (Matozzo et al., 2008). In bees, Vg plays an important antioxidant role by
340 protecting other hemolymph molecules from ROS and increasing cellular oxidative
341 stress tolerance (Corona et al., 2007). Previous studies have shown a correlation
342 between high Vg expression in adult worker bees and resistance to oxidative stress
343 resulting from exposure to the pesticide paraquat (Seehuus et al., 2006). In this study,
344 our results support this observation and suggest that larvae may also resist oxidative
345 stress caused by imidacloprid through increased Vg production.
346

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

348 Impaired juvenile development can have a significant impact on the growth and
349 sustainability of entire populations. Deviations in juvenile development due to
350 environmental stress have recently received considerable attention. *Drosophila*
351 *melanogaster* exposed to heat stress exhibit developmental delays due to the failure of
352 early embryos to fully inhibit the synthesis of non-heat shock proteins (Bergh and Arking,
353 1984). Similarly, exposure to nutrient and heavy metal stress can cause delayed or
354 arrested development in *Caenorhabditis elegans* due to altered gene expression and
355 disruption of developmental signaling pathways (Carranza-García and Navarro, 2020;
356 Rashid et al., 2021). In the present study, we also observed typical developmental delays
357 in honeybee larvae exposed to imidacloprid, but the underlying mechanisms remain
358 unclear.

359 Several mechanisms may be responsible for the delayed development of honey bee
360 larvae, including endocrine disruption, altered gene expression of metabolic pathways,
361 and increased energy expenditure due to detoxification mechanisms. Nonetheless, no
362 evidence has been reported in detail to date. Insect development is regulated by the
363 hormones JH and 20E, which control morphological remodeling events during molting.
364 20E promotes pupation (Yuan et al., 2020), while JH maintains juvenile characteristics
365 and prevents metamorphosis by antagonizing the effects of 20E (Luo et al., 2021).
366 Multiple studies show that neonicotinoid insecticides disrupt honey bee endocrine
367 systems (Christen et al., 2018; Cook 2019). This suggests that imidacloprid may affect
368 molt-related endocrine hormones. Nevertheless, this phenomenon has not been
369 reported in honeybees yet. Therefore, we first focused on the hormonal regulation of
370 molting development and found that imidacloprid inhibits the expression of JHAMT
371 (Figure 3A and Figure 3– Source Data 11), a positive regulator of JH synthesis (Liu et al.,
372 2018), but did not affect JH titer levels (Figure 3B and Figure 3– Source Data 12). This
373 suggests that the antagonistic effect of JH still restricts the developmental process,
374 thereby maintaining larval morphology. In parallel, imidacloprid toxicity also resulted in
375 reduced ecdysteroid 20E titer and Br-c expression (Figure 3 and Figure 3– Source Data

376 11-12). The role of 20E is to induce larval molting and pupation by triggering
377 downstream response factors, such as Br-c (Deng et al., 2012). Therefore, our results
378 suggest that larval developmental delay may be caused by imidacloprid reducing 20E
379 titer and inhibiting *Br-c* expression, thereby blocking molting. Notably, we also observed
380 that a decrease in 20E levels was accompanied by a decrease in AChE activity (Figure 2A
381 and Figure 2– Source Data 9), which is known to cause delayed pupation (He et al., 2012)
382 and developmental arrest (Desneux et al., 2007) in insect larvae. Therefore, our results
383 suggest that imidacloprid neurotoxicity may cause developmental delay in bee larvae by
384 inhibiting the molting hormone 20E. This conclusion is supported by a correlation
385 analysis showing that decreases in 20E titer, *Br-c* expression and AChE activity were
386 positively correlated with decreased developmental rates and growth index, weight and
387 width growth (Figure 7 and Figure 7– Source Data 28). In conclusion, imidacloprid
388 neurotoxicity inhibits the 20E and *Br-c* genes, thereby blocking molting, which may be
389 an important cause of delayed larval development in bee larvae. However, we cannot
390 rule out the possibility that the decline in the growth index may be due to other factors,
391 such as oxidative stress impairing mitochondria, dysregulated neuro-endocrine axis
392 caused by imidacloprid targeting neurons, poor nutrient absorption, impaired
393 movement, etc, as animal growth and development are collectively regulated by
394 numerous physiological, biochemical, and genetic factors.

395 Animals employ selective foraging as a survival strategy to avoid ingesting toxic foods
396 that may cause adverse physiological effects (Berenbaum and Johnson, 2015). Although
397 bees cannot actively reject toxins innately, postingestive malaise allows them to learn to
398 avoid ingesting toxic-containing foods with adverse physiological effects (Hurst et al.,
399 2014). Given this, we speculate that it is likely that the bee larvae in this study reduced
400 their intake of toxic imidacloprid-containing foods, making it difficult to meet their
401 nutritional and energy needs, which may have resulted in delayed development.
402 However, monitoring of daily food intake did not show a significant difference between
403 larvae exposed to imidacloprid and the control group (Figure 1F and G, Figure 1– Source

404 Data 4 and 5), indicating that larval food intake was not reduced and larvae did not
405 initiate avoidance behavior toward toxic food. Therefore, food intake was not a critical
406 factor in the delayed larval development caused by imidacloprid.

407 Nutrition and energy are critical for the growth and development of juvenile animals.
408 Exposure to heavy metals and neonicotinoid pesticides has been shown to affect
409 digestion, energy reserves, production, and utilization in animals (Jiang et al., 2020; Li et
410 al., 2021). Given this, since the lack of the necessary correlation between delayed larval
411 development and food intake in this study prompts us to consider potential problems in
412 larval food and energy utilization that led to delayed development. Interestingly, we
413 found severe pathological changes in the gut tissues of developmentally delayed larvae
414 exposed to imidacloprid (Figure 5D and Figure 5– Source Data 17). Imidacloprid caused a
415 significant reduction in the tightness of the cell arrangement in the basal layer of the gut,
416 and a drastic decrease in the number of cells. This inevitably impacts the larval digestion
417 and absorption of food nutrients. Indeed, the peritrophic membrane was incompletely
418 formed, and undigested food residues were found in the gut of imidacloprid-exposed
419 larvae compared to controls. In addition, qRT-PCR evidence showed a significant
420 decrease in digestion and catabolism of dietary proteins and carbohydrates, amino acid
421 transport, oxidative phosphorylation, and glycolysis (Figure 6D-F and Figure 6– Source
422 Data 22-24). Biochemical assays showed that imidacloprid stress also decreased ATP
423 levels and total glycogen and protein content, resulting in inadequate energy reserves
424 and increased energy expenditure (Figure 6G-I and Figure 6– Source Data 25-27).
425 Furthermore, correlation analysis provided some support for a positive relationship
426 between imidacloprid-induced impaired catabolism and dietary nutrient utilization and
427 larval developmental delay (Figure 7 and Figure 7– Source Data 28). In conclusion, these
428 findings clearly suggest that imidacloprid exposure affects the digestion and utilization
429 of nutrients and energy in larvae, resulting in insufficient nutrients and energy for
430 growth and development, which may be another important factor contributing to
431 imidacloprid-induced larval developmental delay.

432 In addition, mitochondria are the main producers of ATP for cellular metabolism,
433 accounting for approximately 90% of the total. However, mitochondria are also involved
434 in the generation of reactive oxygen species (ROS). Excessive accumulation of ROS in
435 mitochondria leads to oxidative stress, which in turn damages mitochondria and further
436 increases ROS levels, creating a vicious cycle (Boovarahan and Kurian, 2018). In the
437 present study, it was found that imidacloprid exposure led to increased ROS and MDA
438 levels in larvae (Figure 5A and Figure 5-source data 14), indicating that imidacloprid
439 induced severe oxidative stress and lipid damage, which may damage mitochondria and
440 in turn affect mitochondrial ATP production, resulting in insufficient energy supply for
441 larval development. This factor may also be an important explanation for the larval
442 developmental delay caused by imidacloprid.

443 The Dynamic Energy Budget (DEB) theory proposes that environmental stress can
444 significantly affect the energy balance of organisms, leading to increased energy
445 expenditure (Kooijman, 2009). To cope with stress, animals may deplete their energy
446 reserves, such as glycogen and protein (Bouayad et al., 2012b; Matsukura et al., 2008).
447 In this study, we found that larvae with delayed development caused by imidacloprid
448 had lower energy reserves (ATP, total protein, and total glycogen). Furthermore, this
449 decrease in energy reserves was negatively correlated with larval antioxidant and
450 detoxification (Figure 7 and Figure 7– Source Data 28). It is commonly acknowledged
451 that antioxidant defense and detoxification for stress resistance is an energy-consuming
452 process. Therefore, our results indirectly imply that imidacloprid-induced reduction in
453 energy reserves is likely associated with the energy-consuming activities of CYP450
454 detoxification and antioxidant defense. In nature, to meet the high energy cost
455 demands of environmental stress, animals often have to allocate most of their food
456 intake to defense (Guedes et al., 2006), and this redistribution of energy resources will
457 inevitably lead to an insufficient energy supply for growth and development (Beyers et
458 al., 1999). Consequently, the P450 detoxification and antioxidant defense used by the
459 larvae in this study to resist imidacloprid toxicity most likely force them to consume

460 more food resources that should have been used for growth and development,
461 consequently resulting in a severe lack of energy needed for development. This may be
462 another important factor contributing to delayed development in bee larvae caused by
463 imidacloprid. However, on this point, it remains to be supported in the future by
464 overexpression of P450 and antioxidant enzymes, combined with transcriptomic
465 analysis of the overall expression of other P450s, antioxidant genes to obtain direct
466 evidence that depletion of larval antioxidants and detoxification leads to a decrease in
467 the growth index.

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

473

474 ## Conclusion

475 Imidacloprid toxicity caused growth and developmental delay in *A. mellifera* larvae.
476 The toxic mechanism may include imidacloprid disrupting the regulatory balance of molt
477 development, resulting in restricted development. In addition, the gut damage caused
478 by imidacloprid restricts the metabolism and utilization of food nutrients and energy in
479 the larvae. Third, the additional energy consumed by larval P450 detoxification and
480 antioxidant defenses further reduces the supply of nutrients and energy for growth and
481 development (Figure 8). This study is the first multi-level investigation of the toxic
482 effects of imidacloprid on *A. mellifera* larvae. These findings have broader implications
483 for understanding and assessing the threat of harmful pesticides to animal larvae.

484

485 **Materials and Methods**

486 **Larval rearing**

487 The protocol of the current research was approved by the Animal Bioethics
488 Committee of the Chongqing Normal University, China (Protocol Number: 201001). The
489 chosen colonies were healthy and not exposed to pathogens or pesticides. Two-day-old
490 larvae from the same frames of the same hive were individually transferred to sterile
491 24-well cell culture plates. The plates contained standard food, which included royal
492 jelly, glucose, fructose, water, and yeast extract (Aupinel et al., 2005). The larvae were
493 cultured in dark conditions at 35°C and 96% RH, with daily feeding volumes adjusted
494 according to their age (20 μL for three-day-old larvae, 30 μL for four-day-old larvae, 40
495 μL for five-day-old larvae, and 50 μL for six-day-old larvae). Any remaining food was
496 recorded and removed.

497

498 **Experimental design**

499 This study employed four different concentrations of imidacloprid, which were
500 residual concentrations found in honey (0.7 ppb) (Chauzat et al., 2009), bees (1.2 ppb)
501 (Chauzat et al., 2011), pollen (3.1 ppb) (Mullin et al., 2010), and beeswax (377 ppb)
502 (Pareja et al., 2011). The experiment was conducted with three treatment groups:
503 control group (CK), solvent control group (CKac), and imidacloprid-treated group (IMI),
504 each with three replicates. Each replicate contained 32 larvaes that were distributed in
505 standard 24-well cell culture plates (1 bee/well). The CK group consisted of larvaes fed
506 acetone-royal jelly glucose-fructose-yeast extract-distilled water (0.00377%-50%-6%-
507 6%-1%-36.99623%, v/w/w/w/w/v). The IMI group consisted of the two-day-old larvae
508 that were orally exposed to a control solution containing imidacloprid (99% active
509 ingredient, Hubei Norna Technology Co. Ltd, Hubei, China) at 0.7 ppb, 1.2 ppb, 3.1 ppb,
510 and 377 ppb, respectively, after which the larvae were fed a standard food from three-
511 day-old. The CKac group served as a solvent control and contained only acetone at a
512 concentration of 0.00377%. A total of 312 larvae were used for survival statistics (96
513 bees), body weight and width (96 bees), and gene expression, enzyme activity,

514 histochemical content, and histopathologic analysis. Food was administered daily
515 according to age (20 µL for three-day-old larvae, 30 µL for four-day-old larvae, 40 µL for
516 five-day-old larvae, and 50 µL for six-day-old larvae). Dead individuals and food remain
517 were recorded daily, and incubation conditions were 35°C and 96% RH. Surviving larvae
518 were collected 72 h after consumption of the test solution and stored at −80°C for
519 further analysis, including evaluations of gene expression, enzyme activity, and chemical
520 content evaluations. Furthermore, HE-stained sections were used to evaluate any
521 imidacloprid-induced tissue damage and apoptosis.
522
523 **Developmental phenotypic traits**
524 Larval individuals were randomly selected daily from the CK and IMI groups to
525 measure body weight and width. Food residues, larval mortality, developmental
526 progression, and the amount of time spent in the larval developmental stage (from the
527 first imidacloprid exposure to before pupa) were recorded daily to calculate food
528 consumption, survival rates, developmental rates, and development times. The growth
529 index (GI) was calculated for larvae exposed to imidacloprid from the three-day-old to
530 the six-day-old using the equations described by Zhang (Zhang et al., 1993). The
531 experiment was conducted in three biological replicates, each containing 24 randomly
532 selected larvae (24-well cell culture plate, 1 bee/well). The two-day-old larvae were
533 administered imidacloprid orally (after which they were maintained on standard chow),
534 and then the developmental progress of the larvae was monitored daily for 12 days
535 starting at four-day-old, and the developmental time and developmental rate were
536 counted. Sample data that died abnormally or failed to enter the pupal stage during this
537 period were excluded. Developmental time was calculated as the length of time from
538 four-day-old until the larvae entered the pupal stage after oral administration of
539 imidacloprid to two-day-old larvae. Developmental rate was defined as the percentage
540 of the number of larvae that developed into pupae out of 24 two-day-old larvae that
541 received oral doses of imidacloprid.

542

543 The developmental rate was measured as the number of larvae successfully
544 reaching the pupal stage as a percentage of the total initial sample size.

545

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

547 Total RNA was extracted from larvae using TRIzol reagent (Thermo Fisher Scientific) a
548 nd RNAex Pro Reagent (Accurate Biology, China) as instructed by the manufacturer. The
549 RNA quality was evaluated by gel electrophoresis and absorbance determination using a
550 NanoDrop 2000 spectrophotometer (Thermo Scientific, New York, USA). Genomic DNA
551 was removed, and cDNA was synthesized using a reverse transcription kit (Evo M-MLV R
552 T Mix Kit with gDNA Clean for qPCR, Accurate Biology, China) according to the manufact
553 urer's instructions. Briefly, 1 μg of RNA reacted with the 5X gDNA Clean Reaction Mix pr
554 ovided in the kit at 42°C for 5 min to remove residual genomic DNA to ensure the accura
555 cy of the quantitative results. After the reaction, 4 μL of 5X Evo M-MLV RT Reaction Mix
556 and 4 μL of RNase-free water were added to the reaction tube for 20 μL. The mixture wa
557 s incubated at 37°C for 15 min and 85°C for 5 sec to generate cDNA. Then, quantitative R
558 T–PCR was performed with a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad,
559 Hercules, CA, USA) according to standard protocols and cycling conditions. RP49 (riboso
560 mal protein 49, Accession no. AF441189) (Lourenco et al., 2008) was used as the endoge
561 nous control. The primers for target genes and RP49 are listed in Supplementary File 1.
562 qPCR system (20 μL): EvaGreen Express 2X qPCR MasterMix (10 μL), each forward and re
563 verse primer (0.4 μL, 0.2 μmol/L), cDNA (1 μL, 100 ng/μL), ddH_2O (8.2 μL). The thermal c
564 ycling program was as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 s and
565 60°C for 30 s. The qPCR products were sequenced by Shanghai Biological Engineering Te
566 chnology Services Co., Ltd. to confirm whether the amplified fragments were the target
567 sequences. Relative mRNA expression levels of target genes were calculated using the 2^{-}
568 $^{\Delta\Delta Ct}$ method.

569

570 **Histochemical measurement**

571 Juvenile hormone 3 (JH-III), 20-hydroxyecdysone (20E) titers, and reactive oxygen
572 species (ROS) levels in larvae were determined using ELISA enzyme-linked
573 immunosorbent assay kits (Jining, Shanghai, China) according to the manufacturer's
574 instructions. Briefly, the larva was homogenized on ice in 5 volumes (M: V) of
575 phosphate-buffered saline (PBS) buffer, and the supernatant was collected after
576 centrifugation at 12,000 x g for 20 min. Subsequently, 50 μL of reference standard
577 solution or 10 μL of sample supernatant plus 40 μL of dilution were added to the
578 microtiter wells and incubated at 37°C for 60 min with 100 μL of HRP-labeled detection
579 antibody. Each well was washed five times with 400 μL of PBS buffer containing 0.05%
580 Tween 20, pat dried, and left to stand at room temperature for 5 min. TMB chromogen
581 solution A (50 μL) and solution B (50 μL) were added to each well, gently mixed, and
582 incubated at 37°C for 15 min in the dark. Then, 50 μL of H_2SO_4 (4 mol/L) was added to
583 each well to terminate the reaction. Finally, the OD value was measured at 450 nm with
584 a NanoDrop 2000 spectrophotometer (Thermo Scientific, New York, USA), and the
585 content of the detected substance was calculated from a calibration curve constructed
586 by reference standards.

587 To prepare samples for analysis of protein carbonylation (PCO), acetylcholinesterase
588 (AchE), catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T-AOC),
589 superoxide dismutase (SOD), and glutathione (GSH), larvae was weighed and
590 homogenized on ice in 5 volumes (M: V) of PBS buffer, then centrifuged at 12,000 x g for
591 15 min to collect the supernatant. Subsequently, PCO content was measured using the
592 2,4-dinitrophenyl-hydrazine (DNPH) method with slight modifications. The above-
593 prepared supernatant was mixed with streptomycin sulfate at a 9:1 volume (V: M), kept
594 at room temperature for 10 min, and then centrifuged at 12,000 x g for 15 min. Then, 80
595 μL of supernatant was mixed with 160 μL of 10 mM DNPH in 2 M HCl and reacted at
596 37°C for 30 min. Sample controls were also prepared by adding an equal volume
597 (500 μL) of 2 M HCl. Then, 200 μL of 20% TCA was added, mixed thoroughly, and

598 centrifuged at 4°C for 10 min at 12,000 x g, and the precipitate was collected. Add 400
599 µL of ethanol: ethyl acetate mixture (1:1), vortex and mix, centrifuge at 4°C 12,000 x g
600 for 10 min, and collect the precipitate to remove unbound DNPH. This DNPH removal
601 step was repeated once. The pellet was mixed with 400 µL of 6 M guanidine
602 hydrochloride, kept at room temperature for 15 min, and then centrifuged at 4°C 12,000
603 x g for 10 min to collect the supernatant. The carbonyl and protein contents were
604 determined by reading the absorbance at λ = 370 nm and 280 nm with a SpectraMax
605 190 spectrophotometer (Molecular Devices, California, USA) for each sample against an
606 appropriate blank. Finally, the carbonyl content per unit of protein was calculated based
607 on the protein content.

608 AChE activity was measured by the Ellman method as described by Orhan et al.
609 (Orhan et al., 2007) with slight modifications. Briefly, 20 µL of the prepared sample
610 supernatant was mixed with 160 µL of 0.1 mM PBS buffer (pH 8.0) and 10 µL of an AChE
611 (0.28 U mL^{-1}) and incubated for 15 min at room temperature. Then, 10 µL of ATChI
612 (acetylthiocholine iodide, 4 mg mL^{-1}) was added together with 20 µL of DTNB (5,5-
613 dithiobis-2-nitrobenzoic acid, 1.2 mg mL^{-1}) and immediately mixed, and the absorbance
614 was read at 412 nm. The results were expressed as activity percentages relative to the
615 sample solvent negative control.

616 The CAT activity was determined using a kit (Beyotime, Shanghai, China) according to
617 the manufacturer's instructions with slight modifications. Briefly, 10 µL of the prepared
618 sample supernatant was mixed with 30 µL of Tris-HCl buffer (pH 7.0, 50 mmol L^{-1}), 10 µL
619 of 250 mM H_2O_2 was added, and the mixture was immediately mixed and kept at 25°C
620 for 5 min. Then, 450 µL of 1.8 mol L-1 H_2SO_4 was added, and the mixture was vortexed
621 to terminate the reaction. Subsequently, 10 µL of this termination reaction solution was
622 mixed with 40 µL of Tris-HCl buffer (pH 7.0, 50 mmol L^{-1}). Then, 10 µL of this mixture
623 was added to a 96-well plate mixed with 200 µL of color development working solution
624 containing peroxidase and incubated for 15 min at 25°C. Finally, the absorbance was

625 measured at 520 nm, and the CAT activity was calculated using a calibration curve of 5
626 mM H_2O_2.
627 T-AOC was measured using the total antioxidant capacity assay kit with the ABTS
628 (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method based on a previous report
629 (Chen et al., 2019). Add 10 µL of the prepared sample supernatant to 200 µL of ABTS
630 working solution. After 6 min of reaction, the absorbance was measured at 734 nm.
631 Finally, the antioxidant activity was displayed by Trolox equivalents antioxidant capacity
632 as mmol Trolox equivalents/g extract. The ABTS working solution was prepared as
633 follows: 400 µL of potassium persulfate was added to 400 µL of ABTS to generate an
634 ABTS stock solution, which was then incubated for 16 h at room temperature in the dark.
635 Before use, the stock solution was diluted with phosphate buffer, and the absorbance at
636 734 nm was adjusted to 0.70 ± 0.05.
637 Additionally, MDA, SOD, and GSH levels were detected using ELISA enzyme-linked
638 immunosorbent assay kits (Beyotime, Shanghai, China) according to our recent study (Li
639 et al., 2022).
640 **Histopathological analysis**
641 Microsectioning and hematoxylin-eosin (HE) staining was performed according to
642 standard histological protocols (Khan et al., 2010) with some modifications. Briefly,
643 entire fresh larvae were fixed in 10% neutral-buffered formalin solution for 12 h at 4°C
644 after 72 h of imidacloprid exposure. After isopropyl alcohol dehydration, samples were
645 embedded in paraffin and sectioned. Three-micron-thick sections were stained with a
646 Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China), observed with an
647 Olympus BX51 microscope, and photographed with an Olympus digital camera.
648
649 **Statistical analysis**
650 Data were obtained from three independent experiments performed in triplicate and
651 are presented as the mean ± SEM. SPSS statistical package was used for statistical
652 analysis using Student's t-test and one-way ANOVA followed by Tukey's multiple

comparison test (*P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant). GraphPad software was used for data normalization, Pearson correlation coefficient calculation was analyzed using SPSS, and a heat map was generated.

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Additional information

Ethics

The protocol of the current research was approved by the Animal Bioethics Committee of the Chongqing Normal University, China (Protocol Number: 201001).

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

680 **Supplementary files：Supplementary File 1. Primers used in real-time quantitative**

681 **PCR.**

682

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915

916 ## Figure legends

917 **Figure 1.** Imidacloprid causes larval developmental retardation in *Apis mellifera*. Larvae
918 were orally administered imidacloprid (IMI) or artificial food (CK) beginning at three-
919 day-old, followed by developmental monitoring for 96 hours. (A) Larval developmental
920 phenotypes at 0.7, 1.2, 3.1, and 377 ppb environmental concentrations of imidacloprid
921 exposure; (B) Larval developmental phenotype at 377 ppb imidacloprid exposure; (C)
922 Effect of 0.7, 1.2, 3.1, and 377 ppb imidacloprid on larval survival; (D) and (E) body width
923 and weight of larvae exposed to 0.7, 1.2, 3.1, and 377 ppb imidacloprid for 96 hours; (F)
924 and (G) statistics for daily (F) and three-day cumulative feeding (G) for larvae exposed to
925 377 ppb imidacloprid; (H) larval development time from three-day-old to pupal stage; (I)
926 The number of larvae that successfully reached the pupal stage as a percentage of the
927 total initial sample size; (J) The growth index of larvae. Statistical significance was set at
928 $*P < 0.05$ and $**P < 0.01$.
929
930 **Figure 2.** Imidacloprid neurotoxicity in *Apis mellifera* larvae. (A) AChE activity and (B)
931 nerve conduction-related gene expression analysis in larvae exposed to 377 ppb
932 imidacloprid for 72 hours or a control group. IMI is the larvae exposed to imidacloprid
933 for 72 hours. CK is the artificial food control group. Statistical significance was set at $*P <$
934 0.05 and $**P < 0.01$.
935
936 **Figure 3.** Effects of imidacloprid on the homeostasis of developmental regulation in *Apis*
937 *mellifera* larvae. (A) Relative gene expression and (B) hormone levels of developmental
938 regulatory-related genes in larvae exposed to 377 ppb imidacloprid for 72 h or a control
939 group. Statistical significance was set at $*P < 0.05$ and $**P < 0.01$.
940
941 **Figure 4.** Imidacloprid induced a detoxification response in *Apis mellifera* larvae. Relative
942 expression levels of cytochrome P450 monooxygenase family genes in larvae exposed to
943 imidacloprid for 72 hours. IMI is the larvae exposed to imidacloprid for 72 hours. CK is

944 the artificial food control group. Statistical significance was set at *$P < 0.05$, **$P < 0.01$,
945 and ***$P < 0.001$.
946
947 **Figure 5.** Imidacloprid exposure causes oxidative stress, gut apoptosis, and tissue
948 structural damage while inducing antioxidant defenses in *Apis mellifera* larvae. (a)
949 Analysis of oxidative stress and damage in larvae exposed to imidacloprid for 72 hours.
950 ROS levels indicate the degree of oxidative stress. MDA is a marker for lipid peroxidation.
951 PCO levels reflect the extent of protein carbonylation damage; (b) Activity of
952 antioxidants in larvae exposed to imidacloprid for 72 hours; (c) The relative expression
953 levels of antioxidant genes in larvae exposed to imidacloprid for 72 hours; (d)
954 Histological sections of the larval gut stained with H&E. The circled inserts in the figure
955 show magnified views of the muscle layer. The blue arrow indicates the cell nucleus of
956 the muscle layer. The boxed inset in the figure is a magnified view of the basal layer
957 (black arrow), peritrophic membrane (red arrow), and food residues (green arrow). IMI is
958 the larvae exposed to imidacloprid for 72 hours. CK is the artificial food control group.
959 Statistical significance was set at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
960
961 **Figure 6.** Imidacloprid toxicity inhibited the expression of genes involved in nutrient
962 catabolism, protein synthesis, and energy metabolism and reserves. (A) Carbohydrate
963 catabolism; (B) Proteolysis; (C) Amino acid transport; (D) Mitochondrial oxidative
964 phosphorylation; (E) Glycolysis; (F) Protein synthesis. (G) The total contents of ATP,
965 glycogen (H), and protein (I). IMI is the larvae exposed to imidacloprid for 72 hours. CK is
966 the artificial food control group. Statistical significance was set at *$P < 0.05$, **$P < 0.01$,
967 and ***$P < 0.001$.
968
969 **Figure 7.** Correlation between phenotypes and molecular characteristics in imidacloprid-
970 exposed larvae with developmental retardation. Data were normalized using GraphPad
971 software, followed by a Pearson correlation coefficient calculation using SPSS, and a
972 heatmap was generated.
973

974 **Figure 8.** A model interpreting the ecotoxicological effects of imidacloprid on *Apis*
975 *mellifera* larvae and the mechanisms of imidacloprid-induced developmental
976 retardation. Imidacloprid toxicity caused growth and developmental delay in *A. mellifera*
977 larvae. It disrupts the regulatory balance of molt development, resulting in restricted
978 development. In addition, the damage caused by imidacloprid restricts the metabolism
979 and utilization of food nutrients and energy in larvae. The additional energy consumed
980 by larval P450 detoxification and antioxidant defenses further reduces the supply of
981 nutrients and energy supply for growth and development. The arrows indicate induction
982 or promotion, the straight lines indicate inhibition, and the green dotted lines indicate
983 speculative inhibition.
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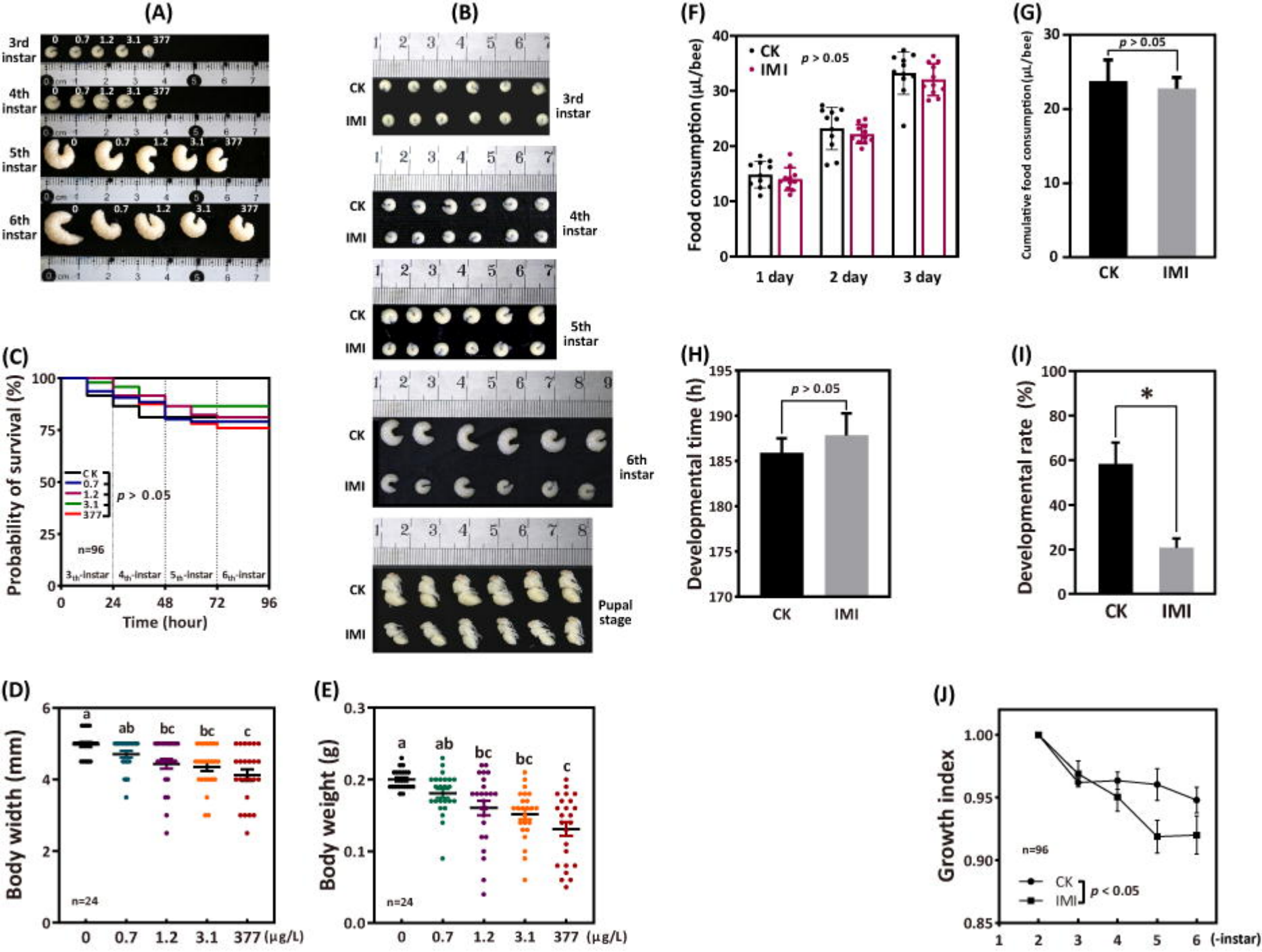
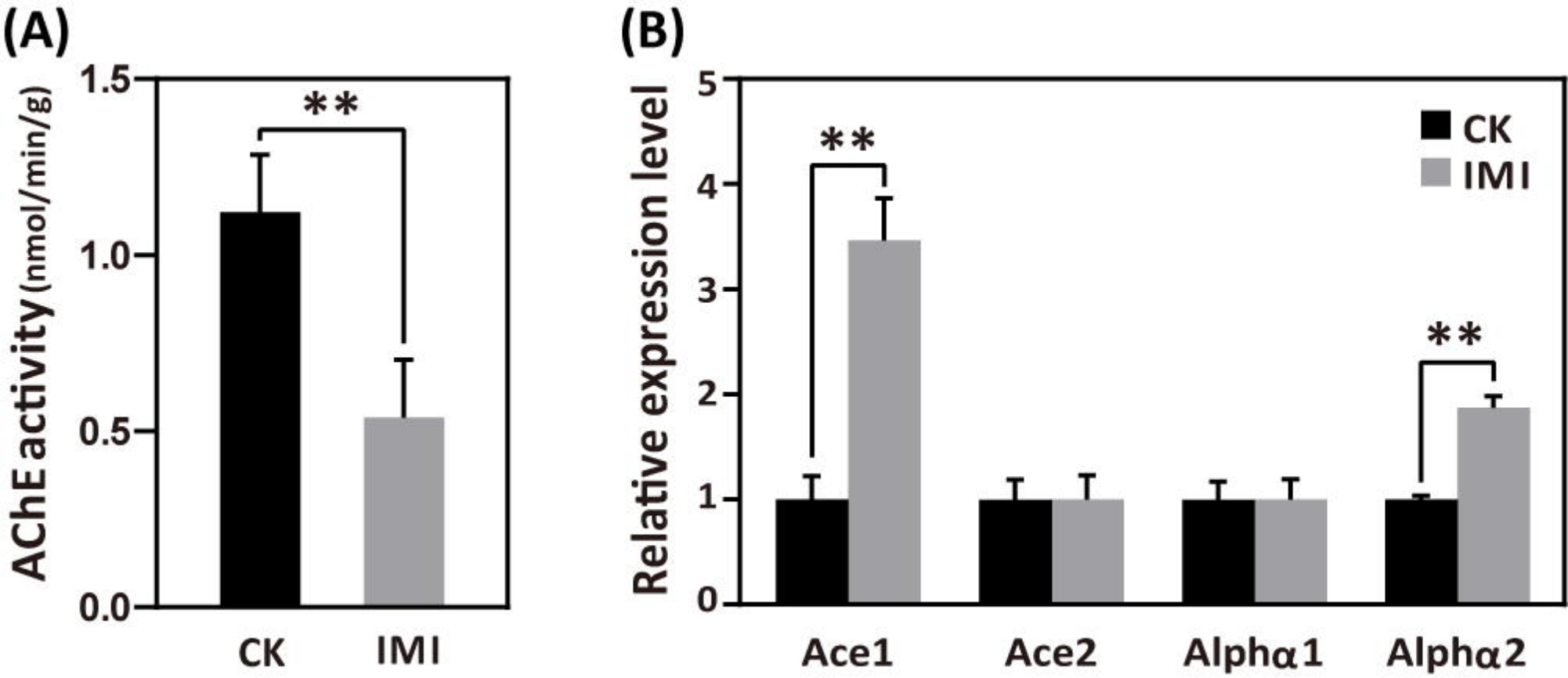
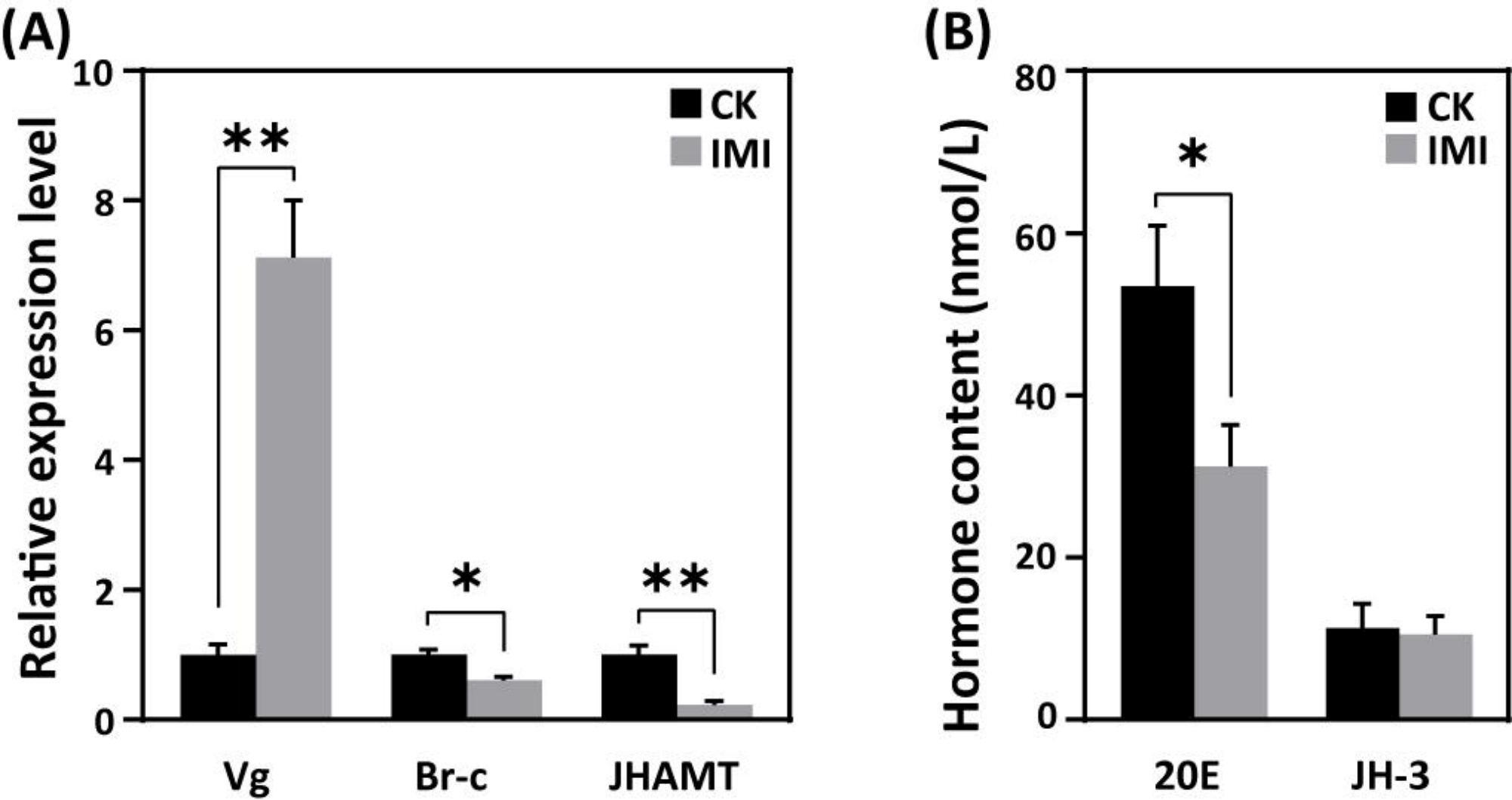
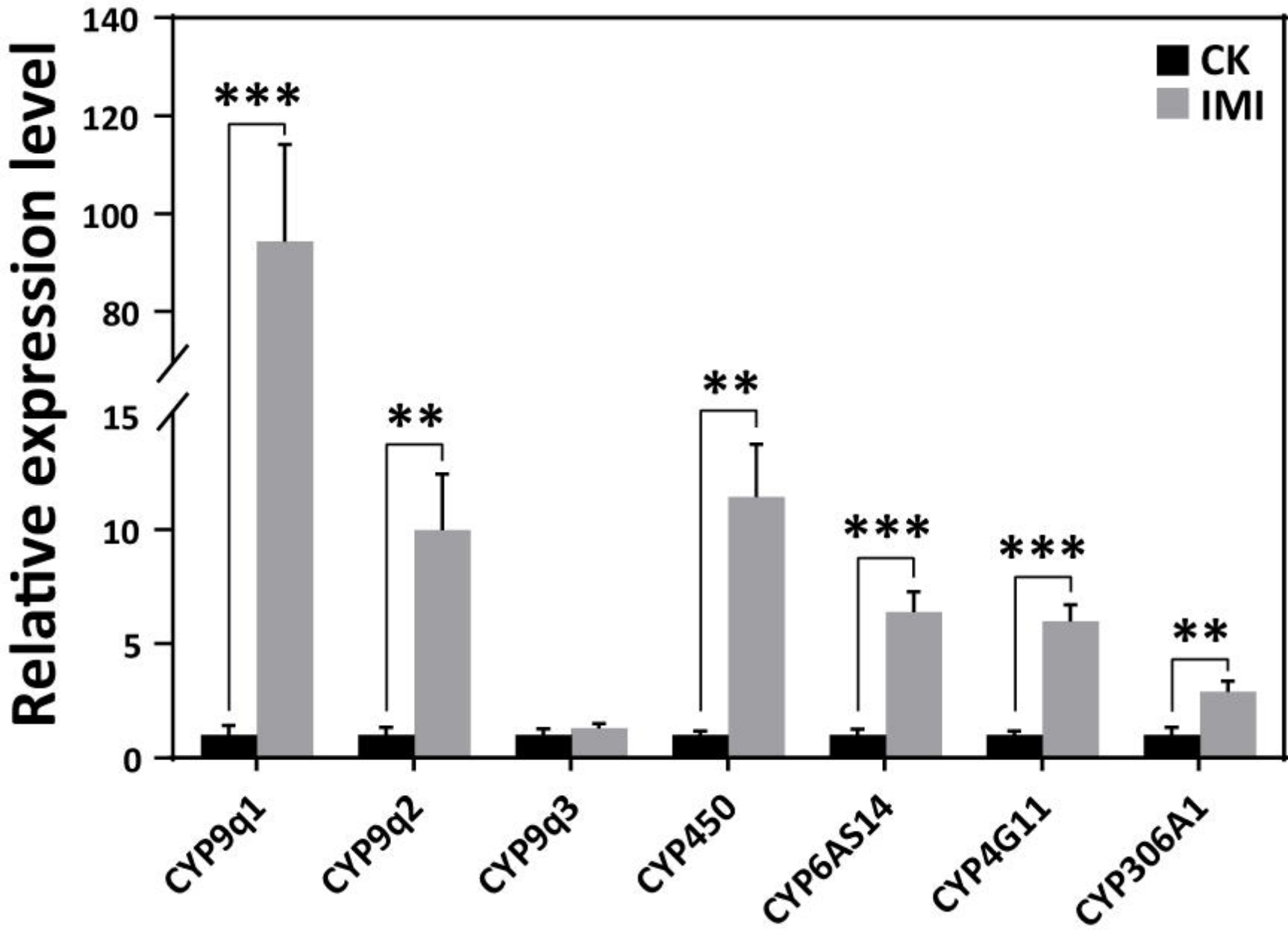
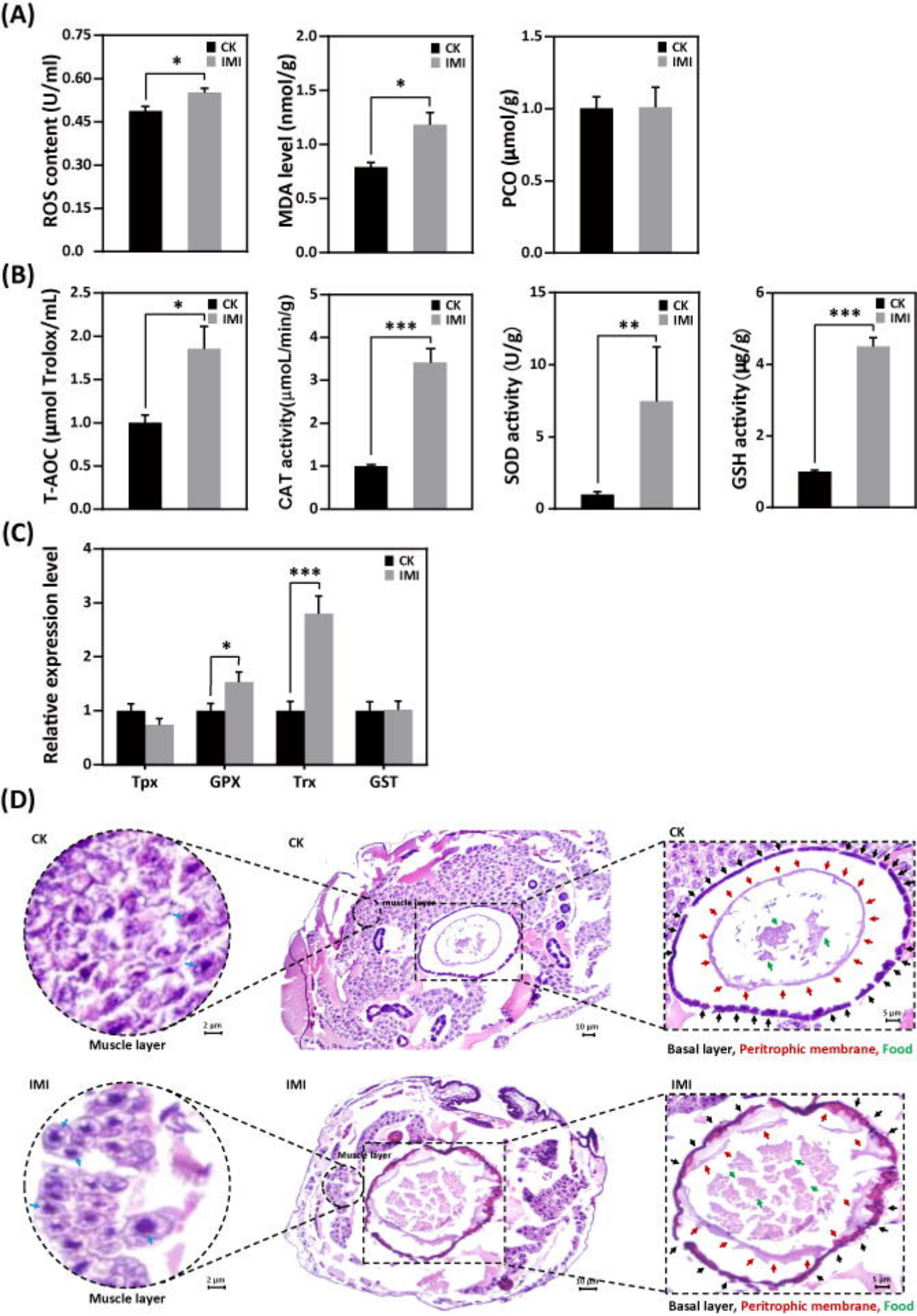
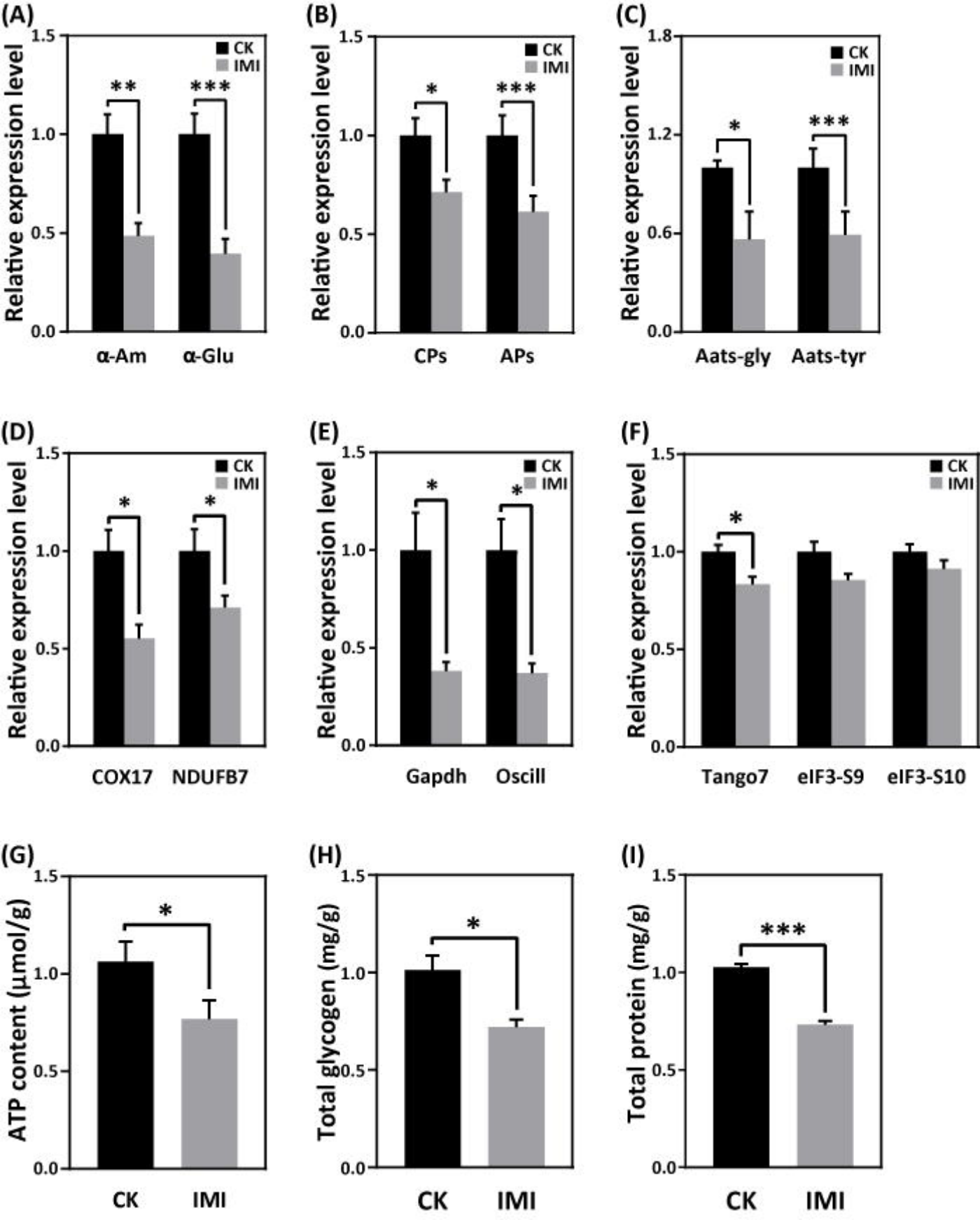

(A)
3rd instar
4th instar
5th instar
6th instar
0
0.7
1.2
3.1
377
(B)
CK
IMI
3rd instar
4th instar
5th instar
6th instar
Pupal stage
(C)
Probability of survival (%)
Time (hour)
CK
0.7
1.2
3.1
377
p > 0.05
n=96
3th-instar
4th-instar
5th-instar
6th-instar
(D)
Body width (mm)
a
ab
bc
bc
c
n=24
0
0.7
1.2
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)

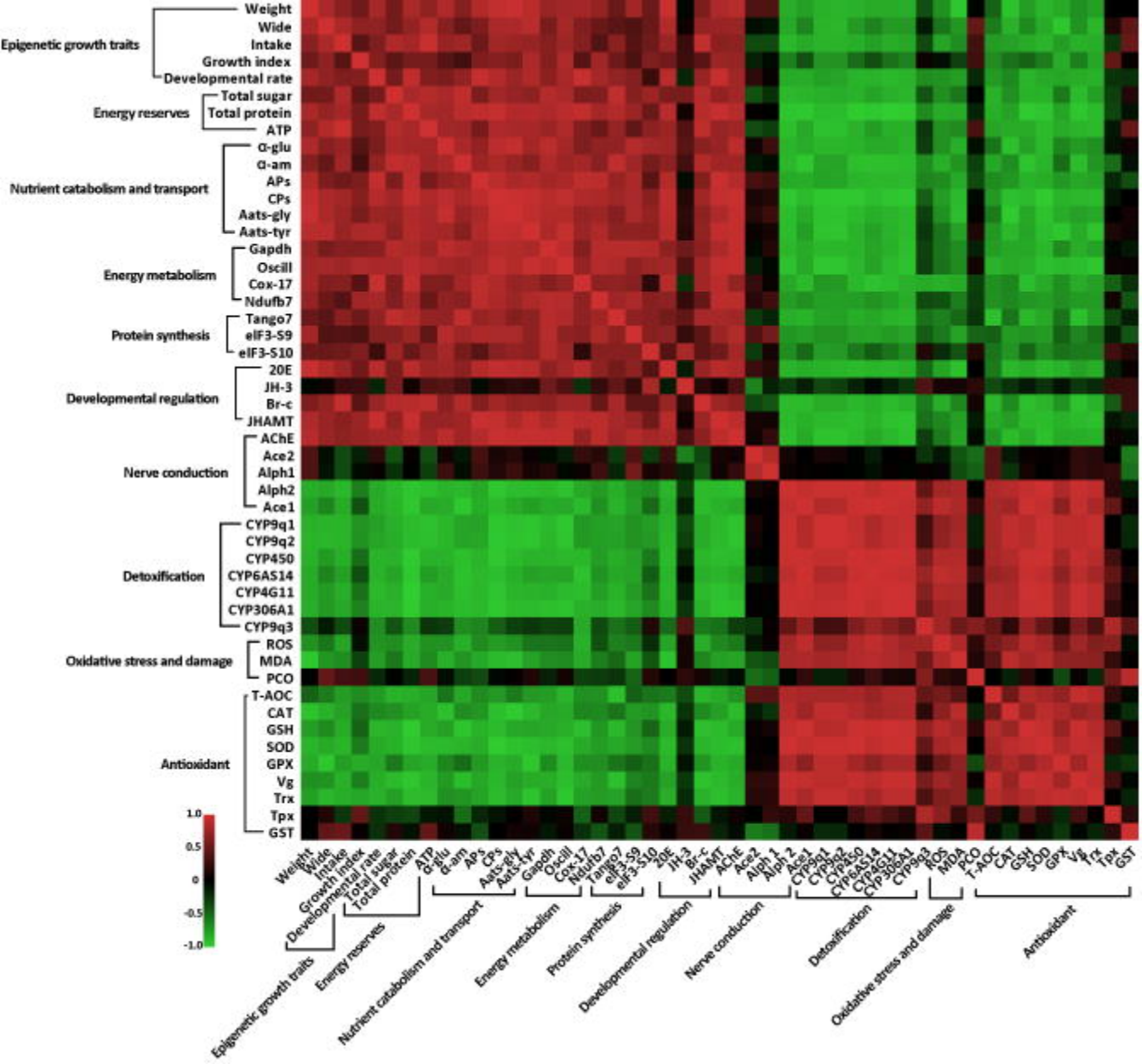

(A)
AChE activity (nmol/min/g)
1.5
1.0
0.5
0.0
**
CK
IMI
(B)
Relative expression level
5
4
3
2
1
0
**
**
CK
IMI
Ace1
Ace2
Alphα1
Alphα2

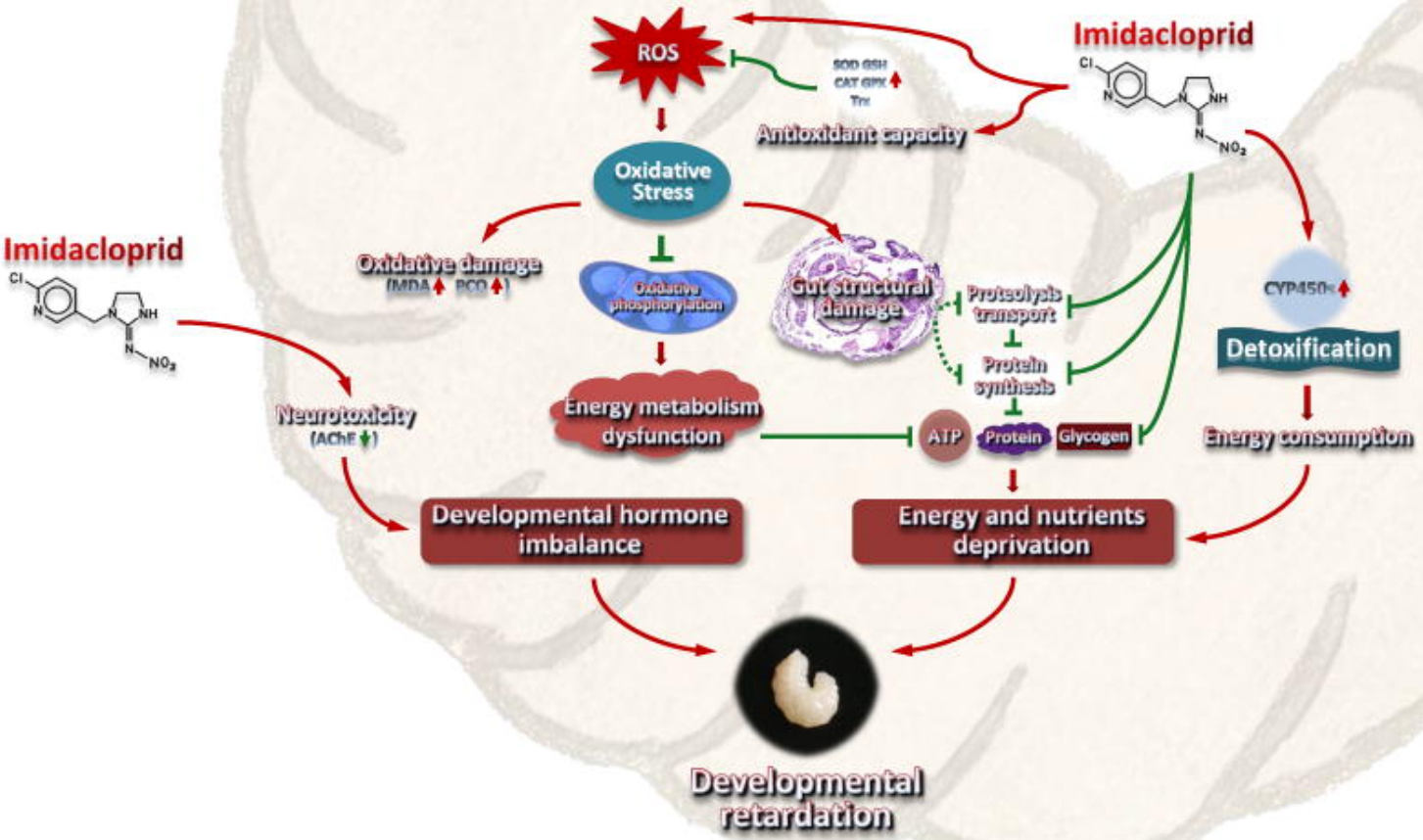

(A)
Relative expression level
10
8
6
4
2
0
CK
IMI
**
*
**
Vg
Br-c
JHAMT
(B)
Hormone content (nmol/L)
80
60
40
20
0
CK
IMI
*
20E
JH-3


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

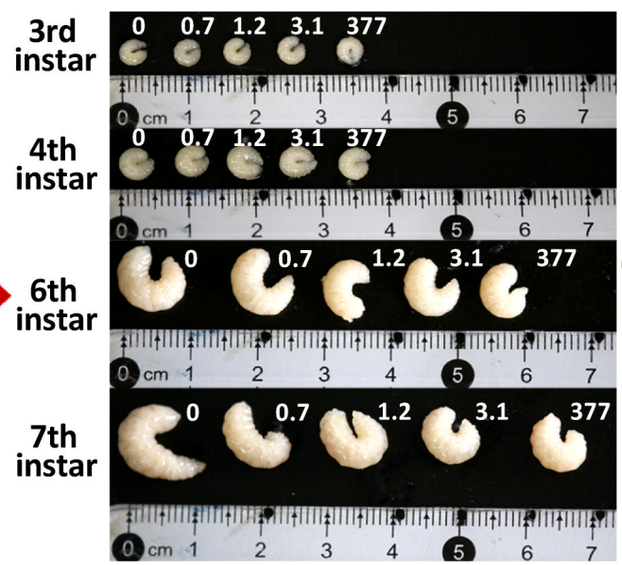

(A)
Relative expression level
CK
IMI
**
***
α-Am
α-Glu
(B)
Relative expression level
CK
IMI
*
***
CPs
APs
(C)
Relative expression level
CK
IMI
*
***
Aats-gly
Aats-tyr
(D)
Relative expression level
CK
IMI
*
*
COX17
NDUFB7
(E)
Relative expression level
CK
IMI
*
*
Gapdh
Oscill
(F)
Relative expression level
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

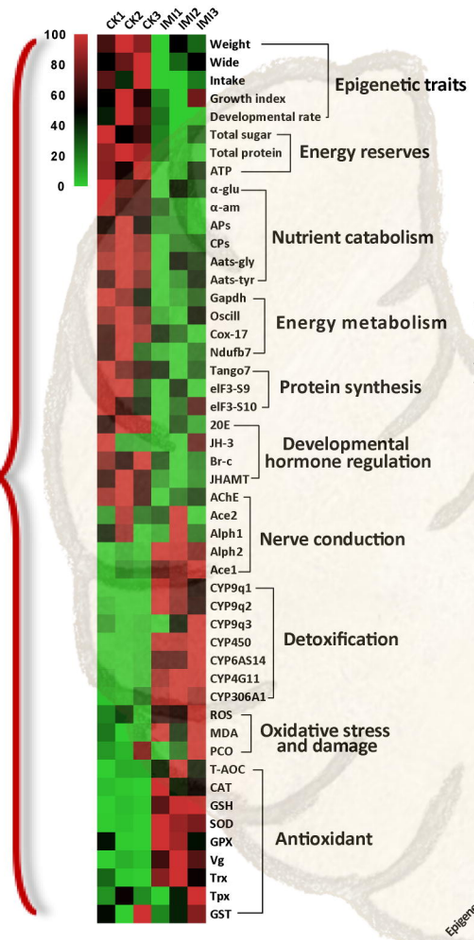

ROS
SOD GSH
CAT GPX
Trx
Antioxidant capacity
Imidacloprid
Oxidative Stress
Oxidative damage
(MDA PCO)
Oxidative phosphorylation
Gut structural damage
Proteolysis transport
Protein synthesis
CYP450
Detoxification
Energy consumption
Imidacloprid
Neurotoxicity
(AChE)
Energy metabolism dysfunction
ATP
Protein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

Imidacloprid toxicity and the underlying mechanism causing larval development delay

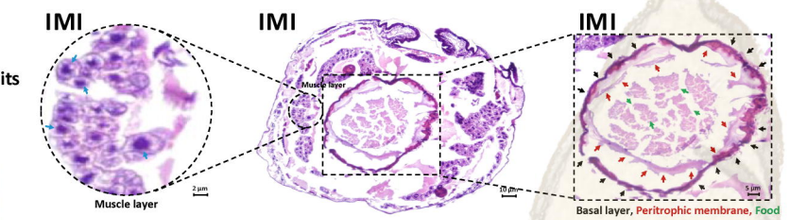
72 hours Exposure



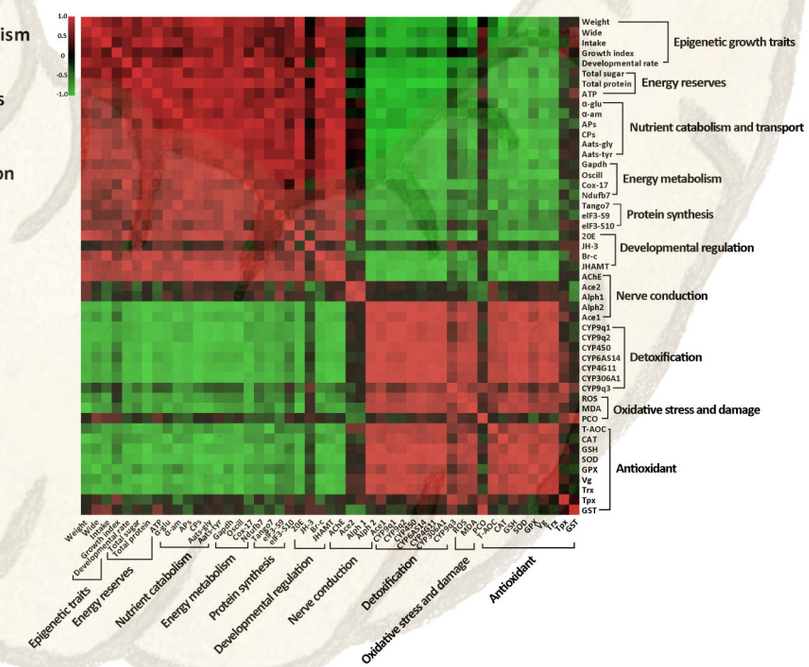
Developmental retardation



Toxic effects



Gut tissue damage



Correlation Analysis