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Absence of juvenile hormone signalling regulates the dynamic expression profiles of nutritional metabolism genes during diapause preparation in the cabbage beetle *Colaphellus bowringi*

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Abstract

Temperate insects have evolved diapause, a period of programmed developmental arrest during specific life stages, to survive unfavourable conditions. During the diapause preparation phase (DPP), diapause-destined individuals generally store large amounts of fat by regulating nutrition distribution for the energy requirement during diapause maintenance and postdiapause development. Although nutritional patterns during the DPP have been investigated at physiological and biochemical levels in many insects, it remains largely unknown how nutritional metabolism is regulated during the DPP at molecular levels. We used RNA sequencing to compare gene expression profiles of adult female cabbage beetles *Colaphellus bowringi* during the preoviposition phase (POP) and the DPP. Most differentially expressed genes were involved in specific metabolic pathways during the DPP. Genes related to lipid and carbohydrate metabolic pathways were clearly highly expressed during the DPP, whereas genes related to protein metabolic pathways were highly expressed during the POP. Hormone challenge and RNA interference experiments revealed that juvenile hormone via its nuclear receptor *methoprene-tolerant* mediated the expression of genes associated with nutritional metabolism during the DPP. This work not only sheds light on the mechanisms of diapause preparation, but also provides new insights into the molecular basis of environmental plasticity in insects.

Keywords: *Colaphellus bowringi*, RNA sequencing, diapause preparation phase, nutritional metabolism, juvenile hormone, methoprene-tolerant.

Introduction

Seasonal polyphenism and phenotypic plasticity are strategies that allow animals to survive environmental change (Scheiner, 1993; Simpson *et al.*, 2011). For example, many animals have evolved polyphenism in wing structure, migration, and diapause (a programmed type of developmental arrest that occurs in specific life stages) to survive adverse climatic conditions (Tauber *et al.*, 1986; Lucas *et al.*, 2001). Diapause is relatively common in nematodes, crustaceans, fish and insects (Hand *et al.*, 2016). It is initiated by environmental cues, such as temperature and photoperiod, which in diapause-capable species and individuals trigger a series of physiological changes (Denlinger, 2002). Ecological and physiological studies in many insects have revealed that diapause is a dynamic process comprised of successive phases, including the induction, preparation, initiation, maintenance, termination and postdiapause development phases (Kostal, 2006). Although mechanisms that regulate diapause have been identified in several insect species (Bajgar *et al.*, 2013; Denlinger & Armbruster, 2014), the specific molecular events that take place during the different phases of diapause remain unclear. Recent advances in high-throughput gene expression profiling based on RNA sequencing and proteomics that allow this technique to be used to investigate diapause in insects, particularly nonmodel species (Zhang *et al.*, 2012; Poelchau *et al.*, 2013a; Tu *et al.*, 2015; Zhao *et al.*, 2015), could help resolve this question.

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The prediapause phase is divided into two subphases; the induction phase and the preparation phase (Kostal, 2006). Physiological changes in diapause-destined (DD) individuals are initiated during the diapause preparation phase (DPP) (Denlinger, 2002). Energy accumulation is one of the most important aspects of the DPP. As insects typically cannot feed during diapause, accumulating sufficient energy during the DPP is vital to their ability to sustain diapause, undergo postdiapause development and thereby survive adverse conditions (Hahn & Denlinger, 2011). Many studies have demonstrated the nutritional differences between reproduction and diapause preparations at physiological and biochemical levels, but the molecular events remain unclear. The molecular events that take place during the DPP have been investigated in larvae of the mosquito *Aedes albopictus* (Poelchau *et al*., 2013a) and pupae of the cotton bollworm *Helicoverpa armigera* (Zhang *et al*., 2012, 2013), but little is known of these processes in adult insects. In addition, although environmentally regulated hormone signalling is considered the primary mechanism regulating diapause (Denlinger *et al*., 2012), little is known about how hormones regulate diapause preparation, especially with regard to nutritional metabolism.

The cabbage beetle pest *Colaphellus bowringi* is in many respects an ideal animal model for research on reproductive diapause preparation. At 25 °C, short day lengths induce adult females of this species to become reproductive, whereas long day lengths induce them to enter diapause (Xue *et al*., 2002). *Co. bowringi* perceives diapause-inducing photoperiod information during the larval stage, completes diapause preparation within 4 days of eclosion and then digs into the soil to begin diapause (Xue *et al*., 2002; Tan *et al*., 2016). Changes in photoperiod during the adult stage do not affect an individual cabbage beetle's decision to enter diapause (Xue *et al*., 2002). The DPP can therefore be readily distinguished from the diapause induction and initiation phases. Another attribute of the cabbage beetle is that the DPP of DD individuals, and the preoviposition phase (POP) of reproductive (nondiapause-destined, NDD) individuals, both take place during the 4-day posteclosion period (PEP) (Tan *et al*., 2016), a feature that greatly facilitates comparative research. During the POP, yolk proteins are accumulated in the ovaries of NDD female adults, whereas during the DPP, large amounts of fat are stored in the fat body of DD female adults. The carbohydrate content of DD female adults also gradually increases and their ovarian development stops (Tan *et al*., 2016). These clear differences in nutrient accumulation and distribution during the POP and DPP make *Co. bowringi* an ideal animal model for investigating the metabolic regulation of energy reserves during the DPP.

In this study, we used RNA sequencing to compare dynamic gene expression profiles in DD and NDD female adult *Co. bowringi*. We also analysed the basic molecular regulation of nutritional metabolism during the DPP and conducted additional experiments using hormone challenge and RNA interference to examine the role of juvenile hormone (JH) in the regulation of diapause preparation. We argue that our findings not only provide new insights on the mechanisms of diapause preparation, but also shed light on the molecular basis of ecological adaptation in animals.

Results and discussion

RNA sequencing reveals the gene expression differences during the POP and DPP

To investigate the dynamic gene expression profiles involved in the DPP, we used RNA sequencing to compare gene expression on days 0, 2 and 4 of the PEP in DD and NDD females (Fig. 1A, Tables S1, S2). There were relatively few differentially expressed genes (DEGs) associated with diapause early in diapause preparation (NDD0 vs. DD0), but then the number of DEGs went up during diapause preparation progressing to 2 and 4 days (Fig. 1B), which suggests that the dynamic molecular events involved in both the POP and DPP begin in both DD and NDD females on day 2 of the PEP. Similar nutritional and developmental differentiation in POP and DPP female *Co. bowringi* was also apparent on day 2 of the PEP in a previous study (Tan *et al*., 2016). Moreover, consistent with the suppression of gene expression in other DD insects (Zhang *et al*., 2012; Poelchau *et al*., 2013a), most of the more than 2800 DEGs we identified were downregulated in DD females on days 2 and 4 of the PEP (Fig. 1B). These results suggest the convergent evolution of diapause regulation in which gene expression is generally downregulated during the DPP in different types of diapause, such as larval (Poelchau *et al*., 2013a) and pupal (Zhang *et al*., 2012) diapause. However, at least 900 genes, presumably including some that promote diapause, were upregulated during the DPP (Fig. 1B). Taken together, these results suggest that the last 2 days of the DPP are critical and that future research should focus on the activity of DEGs during this period.

Nutritional metabolic pathways were significantly enriched during the DPP

Based on the DEGs identified in NDD and DD female adults on days 2 and 4 of the PEP, we investigated the molecular events of the DPP by pathway enrichment using Kyoto Encyclopedia of Genes and Genomes (KEGG). Compared to NDD females, pathways such as the cell cycle (Table 1, Fig. S1D), metabolism (Fig. S1F), DNA replication and hormone biosynthesis (Table

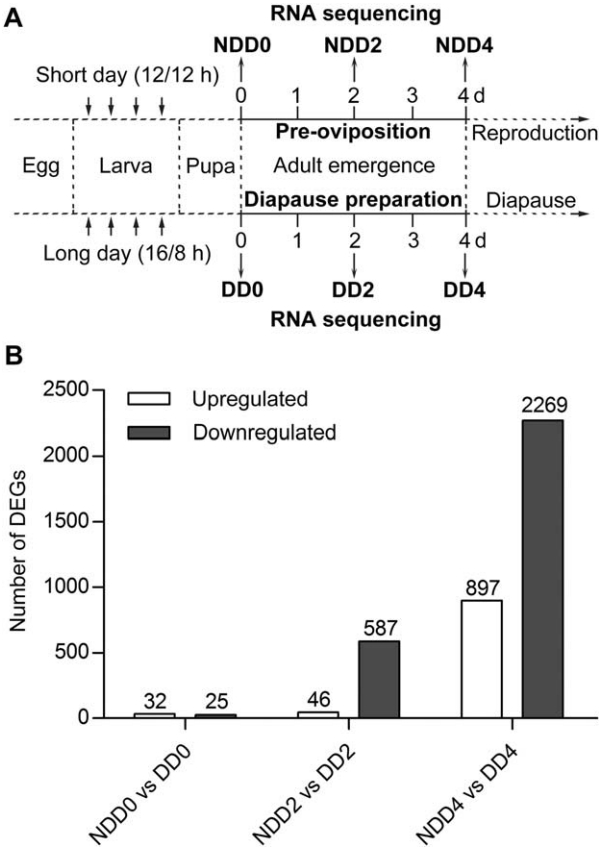

Figure 1. Design and results of experiments designed to identify differentially expressed genes (DEGs). (A) Schematic of RNA sequencing of diapause-destined (DD) and nondiapause-destined (NDD) female adult *Colaphellus bowringi*. The 4-day posteclosion period (PEP) is the preoviposition phase in NDD female adults, but is the diapause preparation phase in DD female adults at 25 °C. RNA was collected for sequencing from DD and NDD females on days 0, 2 and 4 of the PEP, identified as DD0, DD2 and DD4 for DD females, and NDD0, NDD2 and NDD4 for NDD females, respectively. (B) Relative frequency of DEGs. Comparisons between NDD and DD, such as NDD0 vs. DD0, mean that genes were up- or downregulated in DD0. DEGs were identified by RNA sequencing from three biological replicates.

1, Fig. S1D, F) were relatively inactive in DD females. Interestingly, these pathways have also been found to be suppressed in other types of diapause, such as embryonic diapause in the cricket *Allonemobius socius* (Reynolds & Hand, 2009) and annual killifish *Nematolebias whitei* (Thompson & Orti, 2016), larval diapause in *Ae. albopictus* (Poelchau *et al*., 2013a) and the drosophilid fly *Chymomyza costata* (Kostal *et al*., 2009), and pupal diapause in the flesh fly *Sarcophaga crassipalpis* (Tammariello & Denlinger, 1998) and *H. armigera* (Bao & Xu, 2011). Therefore, suppression of these pathways in DD individuals suggests the convergent evolution of diapause regulation.

We found that the galactose metabolism, glycerolipid metabolism and ribosome pathways were significantly enriched ($P < 0.05$) during DPP on day 4 of the PEP

Table 1. KEGG pathway enrichment of differentially expressed genes (DEGs) on days 2 and 4 of the 4-day posteclosion period.

Enriched KEGG pathway	Gene number	*P*-value
NDD2 vs. DD2		
Cell cycle	33	2.811896e-11
p53 signalling pathway	17	3.214353e-09
DNA replication	17	6.263617e-09
Wnt signalling pathway	14	0.0004143309
Oocyte meiosis	12	0.0008798592
NDD4 vs. DD4		
Metabolic pathways	378	5.854522e-16
Lysosome	118	2.002408e-27
Galactose metabolism	44	1.502144e-07
Glycerolipid metabolism	44	5.022731e-06
Ribosome	42	7.973873e-07
Amino sugar and nucleotide sugar metabolism	42	6.55171e-09
Glutathione metabolism	33	3.716735e-07

The pathways of DEGs in the diapause-destined (DD) and nondiapause-destined (NDD) female adults were enriched by Kyoto Encyclopedia of Genes and Genomes (KEGG); enrichment values of $P < 0.05$ are considered significant. p53, the tumor proteins which were originally named because of their 53-kilodalton molecular mass; Wnt, wingless and integration 1.

(Table 1). This suggests that regulation of nutritional metabolism may be the main molecular event that takes place in the DPP in *Co. bowringi*. Similarly, more than 60% of DEGs identified in the larval brain of *H. armigera* during the DPP were related to metabolic pathways (Zhang *et al*., 2012), and many DEGs associated with metabolism were also found during the DPP in *Ae. albopictus* (Poelchau *et al*., 2013a).

Analysis of the metabolic gene expression associated with three major nutrients (carbohydrates, lipids and proteins) using the k-means clustering method revealed that the abundance of carbohydrate metabolic genes increased on day 2, but decreased on day 4, of the PEP in DD females compared to NDD females (Fig. 2A, Table S3). Lipid metabolic genes were more highly expressed in DD females than in NDD females at both days 2 and 4 of the PEP (Fig. 2B, Table S3). Conversely, protein metabolic genes had lower expression in DD females compared to NDD females (Fig. 2C, Table S3). These data suggest that the carbohydrate and lipid metabolic pathways are activated during the DPP, whereas the protein metabolic pathway is activated during the POP. These findings are consistent with the actual distribution of nutrients during the DPP and POP in *Co. bowringi*; DD females have higher carbohydrate and lipid content, but lower protein content, than NDD females (Tan *et al*., 2016). Upregulation of the lipid metabolic pathway and suppression of the protein metabolic pathway is consistent with the suppression of ovarian development, and consequently yolk protein synthesis, in females entering reproductive diapause (Kawakami *et al*., 2009). DD individuals need to store large fat reserves to survive unfavourable conditions, such as periods of food shortage, cold, or heat stress (Hahn & Denlinger, 2011). In

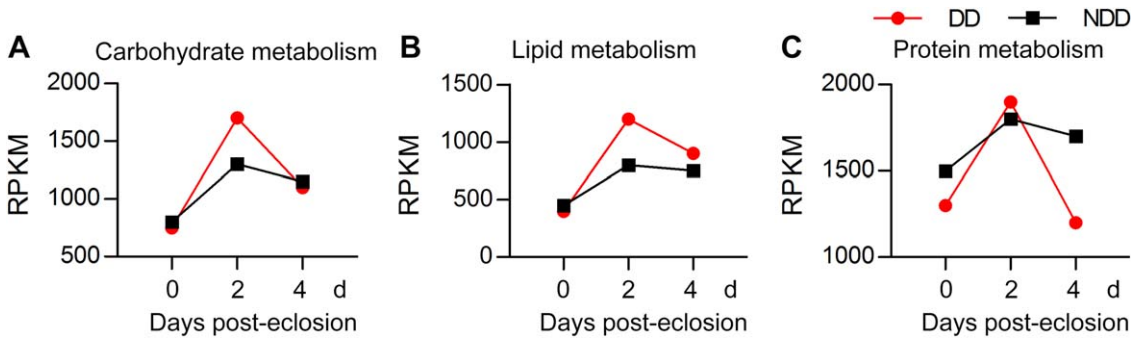

Figure 2. Molecular patterns of differentially expressed genes (DEGs) associated with nutritional metabolism during the preoviposition phase and diapause preparation phase. Expression patterns of DEGs in carbohydrate (A), lipid (B) and protein (C) metabolic pathways in adult, female *Colaphellus bowringi* were analysed using the k-means clustering method. DD, diapause destined; NDD, nondiapause destined; RPKM, reads per kb per million reads. [Colour figure can be viewed at wileyonlinelibrary.com]

addition, upregulation of the carbohydrate metabolic pathway in DD females may contribute to lipid storage because triacylglycerol, the main type of stored fat in insects during diapause, can be produced from carbohydrates via the tricarboxylic acid cycle (Canavoso *et al.*, 2001; Hahn & Denlinger, 2007).

To more accurately determine the mechanisms regulating nutritional metabolism in DD and NDD females we extended our analysis to carbohydrate, lipid and protein metabolism subpathways. In carbohydrate metabolism, genes related to the glycolysis and pentose phosphate pathways (PPP) were highly expressed in DD females compared to NDD females (Fig. 3A, B, and Table S3). Expression of genes associated with the galactose metabolism and citrate cycle [also known as the tricarboxylic acid cycle (TCA)] pathways was upregulated on day 2, and downregulated on day 4, of the PEP in DD females but not in NDD females (Fig. 3C, D, and Table S3). We think that the upregulation of the carbohydrate metabolism-related genes may contribute to lipid accumulation in DD female adults. Glycolysis can promote triacylglycerol synthesis by producing glycerol-3-phosphate, one of the two primary precursors required for triacylglycerol synthesis (Coleman & Lee, 2004). The upregulation of an essential glycolysis enzyme (fructose 1, 6-bisphosphate aldolase) in the pupal brain of DD *H. armigera* (Zhang *et al.*, 2012) supports the hypothesis that the glycolysis pathway plays the same role in diapause regulation in different insect species. The PPP also plays a role in lipid storage by providing nicotinamide adenine dinucleotide phosphate (NADPH), a reductant in fatty acid synthesis. NADPH also enhances the ability of cancer cells to survive stress (Patra & Hay, 2014), suggesting that the PPP may promote lipid storage and stress tolerance. The galactose metabolism pathway potentially also plays a role in triacylglycerol synthesis because this pathway generates glucose 6 phosphate, which can be used in both glycolysis and the PPP, and also contributes to lipid storage. The TCA functions as a hub integrating carbohydrate, lipid and protein metabolic pathways (Akram, 2014), so we think the upregulation of TCA genes in DD female adults on day 2 of the PEP may be related to nutrient absorption from food and the transformation and synthesis of fat. However, as has been observed in *H. armigera* (Xu *et al.*, 2012), DD females lowered their metabolism by suppressing TCA activity when they entered diapause. Therefore, the downregulation of the TCA in DD females on day 4 of the PEP, the late stage of diapause preparation, may be necessary for diapause entry and maintenance in *Co. bowringi*. Suppression of the TCA also happens in the larval diapause of the nematode *Caenorhabditis elegans* (McElwee *et al.*, 2006) and in diapausing embryos of the brine shrimp *Artemia franciscana* (Podrabsky & Hand, 2015).

In the lipid metabolism pathway, expression of genes involved in fatty acid biosynthesis and glycerolipid metabolism was upregulated in DD females relative to NDD females (Fig. 3E, F, and Table S3). In both DD and NDD females, expression of genes associated with fatty acid elongation was upregulated on day 2 compared to day 0 of the PEP (Fig. 3G and Table S3). However, expression of these genes was downregulated on day 4 of the PEP in DD females compared to NDD females. In addition, expression of genes associated with fat digestion and absorption decreased in DD females compared to NDD females (Fig. 3H and Table S3). The fatty acid biosynthesis and glycerolipid metabolism pathways can directly promote fat synthesis by providing fatty acids and glycerol (Reynolds *et al.*, 2012), so we think that upregulation of the expression of the genes involved in these two pathways is probably essential for lipid synthesis in DD female adults. Triacylglycerol is mainly produced from long-chain fatty acids and glycerol (Sul & Wang, 1998), so upregulation of the expression of the genes in the fatty acid elongation pathway on day 2 of the PEP, the peak stage of feeding, would also be expected in DD females. Low expression of genes related to this pathway on day 4 of

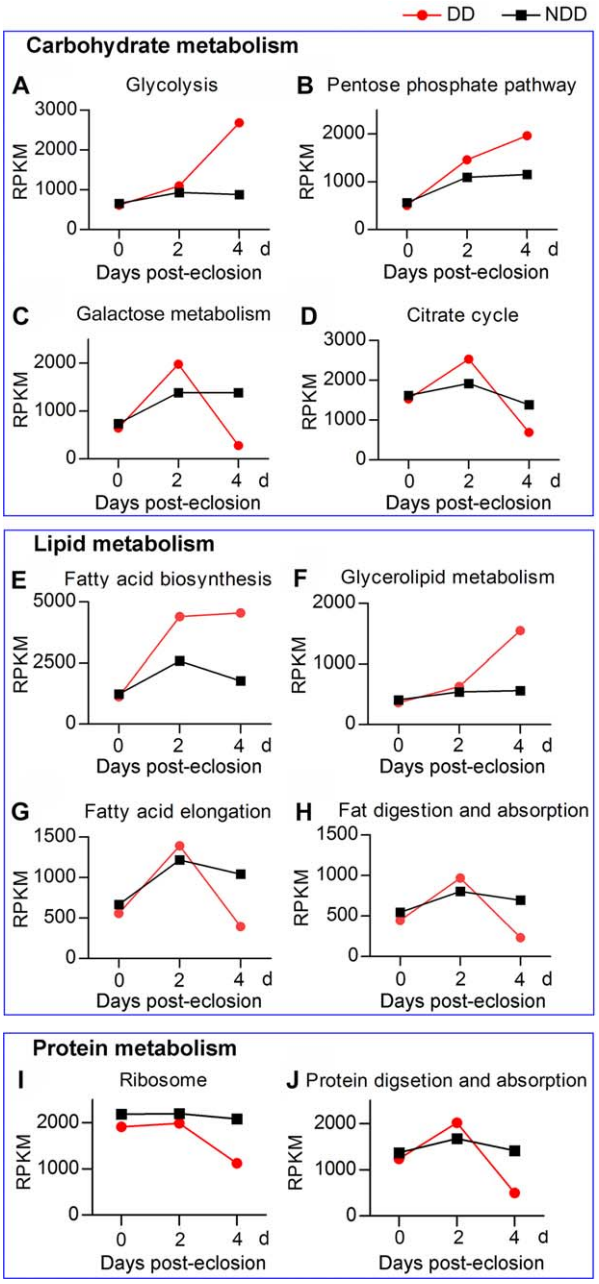

Figure 3. Molecular patterns of differentially expressed genes (DEGs) in different metabolic subcategories during the preoviposition phase and diapause preparation phase. Based on the analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, the expression patterns of DEGs in the corresponding metabolic pathways in female adult *Colaphellus bowringi* were analysed with the k-means clustering method. DD, diapause destined; NDD, nondiapause destined; RPKM, reads per kb per million reads. [Colour figure can be viewed at wileyonlinelibrary.com]

the PEP is consistent with fatty acid elongation being no longer necessary once DD females have entered diapause. Similarly, downregulation of the expression of the genes involved in the fat digestion and absorption pathways in DD females is consistent with the potential for these pathways to restrict lipid accumulation. Taken together, the temporal patterns of lipid metabolic pathway activity are consistent with actual patterns of lipid storage in DD female *Co. bowringi* (Tan *et al.*, 2016).

With regard to protein metabolism, expression of genes involved in the ribosome, protein digestion and absorption pathways was upregulated in NDD females compared to DD females (Fig. 3I, J, and Table S3). The ribosome pathway synthesizes peptides by using mRNA templates, and then regulates protein biosynthesis (Nierhaus & Wilson, 2004). Upregulation of the expression of the genes in this pathway is therefore not unexpected because NDD females need to synthesize a large number of yolk proteins as part of their reproductive development. We think that the high expression of the genes associated with protein digestion and absorption may help NDD females obtain nutrients from food, and also promote the absorption of vitellogenin by the ovaries. Protein metabolic pathways would therefore be expected to be more active in NDD females than in DD females. However, we suspect that some low-level expression of these pathways may also be required for the biosynthesis of diapause-related regulatory proteins in DD females.

Quantitative real-time PCR (qRT-PCR) analyses verify the quantitative accuracy of DEGs and reveal the candidates regulating nutritional metabolism during the DPP

To validate the DEGs identified by RNA sequencing we selected nine metabolic genes (Fig. S2) and examined their expression patterns during the POP and DPP. Based on our previously published *Co. bowringi* transcriptome (Tan *et al.*, 2015), we cloned the glycolysis pathway genes, including *alcohol dehydrogenase* (*ADH*, KX447599), *pyruvate dehydrogenase E2 component* (*PDHE2*, KX447600), *transketolase 2* (*TKT2*, KX447601, the key enzyme of the PPP) and *isocitrate dehydrogenase* (*IDH*, KX447602, the rate-limiting enzyme of the TCA). In the lipid metabolic pathway, we cloned the fatty acid biosynthesis related gene *aldehyde dehydrogenase 1* (*ALDH1*, KX447603), the glycerolipid metabolism pathway gene *triacylglycerol lipase 1* (*TGL1*, KX447604) and the fatty acid elongation pathway gene *elongation of very long chain fatty acids protein 4* (*ELOVL4*, KX447605). These genes generally regulate nutritional metabolism and may play important roles in the fat storage during the DPP. In the protein metabolic pathway, two ribosome pathway genes were cloned; the *large subunit ribosomal protein* (*LP0*, KX447597) and the *small subunit ribosomal protein* (*S2e*, KX447598).

The results of qRT-PCR show that, consistent with the corresponding pathway patterns determined by RNA sequencing (Fig. S3A–C), *ADH*, *PDHE2* and *TKT2* were upregulated in DD females (Fig. 4A–C). Glycolysis-

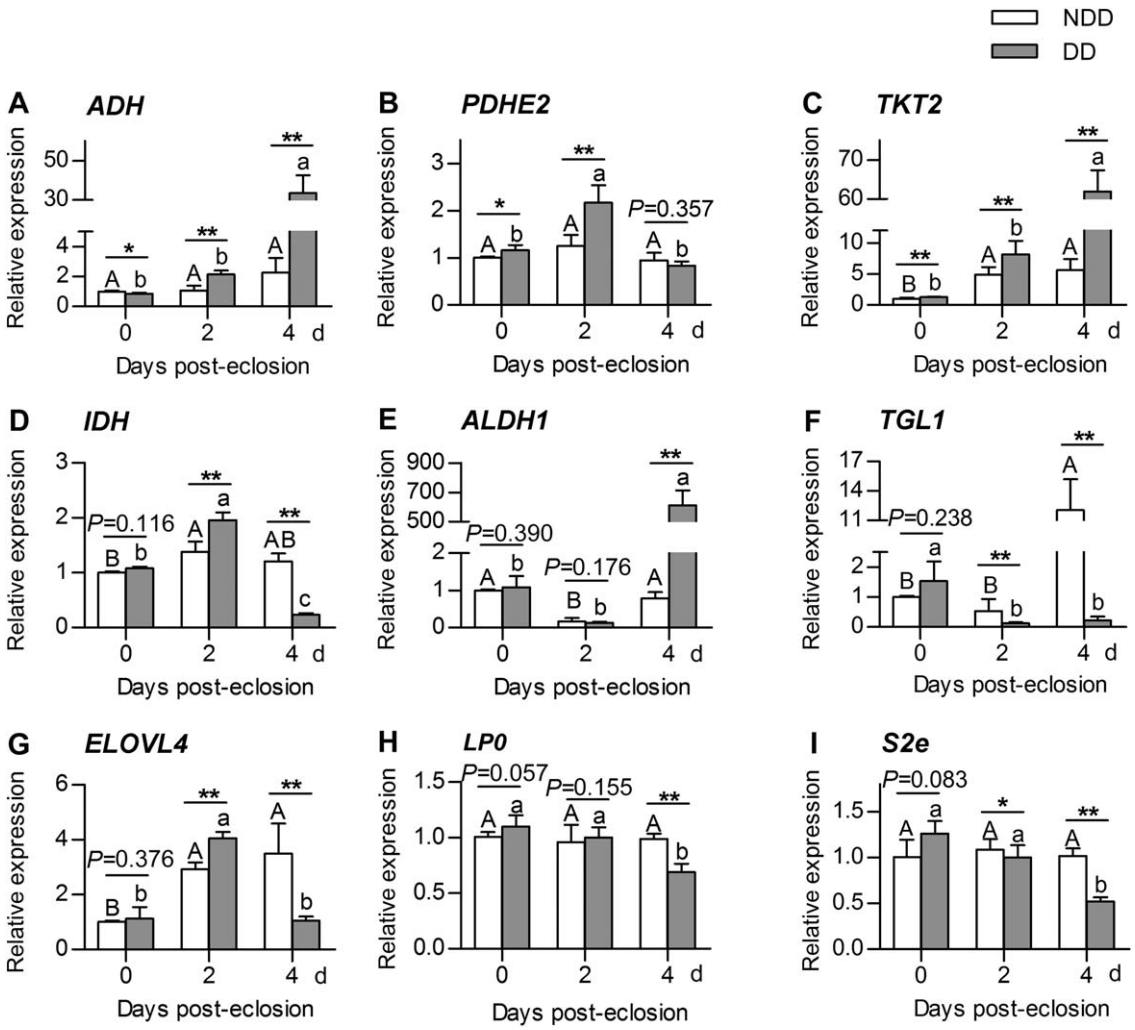

Figure 4. Quantitative real-time PCR (qRT-PCR) validation of gene expression profiles of differentially expressed genes identified by RNA sequencing. The mRNA profiles of three biological replicates of the carbohydrate metabolic pathway genes *alcohol dehydrogenase* (*ADH*; A), *pyruvate dehydrogenase E2 component* (*PDHE2*; B), *transketolase 2* (*TKT2*; C), *isocitrate dehydrogenase* (*IDH*; D); lipid metabolic pathway genes *aldehyde dehydrogenase 1* (*ALDH1*; E), *triacylglycerol lipase 1* (*TGL1*; F), *elongation of very long chain fatty acids protein 4* (*ELOVL4*; G); protein metabolic pathway genes *large subunit ribosomal protein* (*LP0*; H) and *small subunit ribosomal protein* (*S2e*; I) in nondiapause-destined (NDD) and diapause destined (DD) female adult *Colaphellus bowringi* were determined by qRT-PCR on days 0, 2 and 4 after eclosion. Relative expression values are means + SD. In all panels, the relative abundance of genes in NDD female adults on day 0 was regarded as 1 and those in other treatments expressed relative to this standard. Capital and lower case letters indicate multiple comparisons in NDD and DD female adults, respectively. Different letters indicate significant differences as determined by a one-way analysis of variance followed by Tukey's honestly significant difference (HSD) test ($P < 0.05$). Asterisks indicate the statistical significance of pairwise comparisons as determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

produced pyruvate can be converted into acetyl coenzyme A (acetyl-CoA) by a series of enzymatic reactions involving ADH and PDHE2. As acetyl-CoA is the basic unit of fatty acid synthesis, we think it likely that upregulation of *ADH* and *PDHE2* may promote lipid synthesis in DD females. In diapausing adults of the mosquito *Culex pipiens* (Kang *et al*., 2016), brine shrimp *A. franciscana* (Qiu *et al*., 2007) and *Ca. elegans* (McElwee *et al*., 2006), *ADH* was always highly expressed relative to NDD larvae. Interestingly, *PDH* and its subunits often decreased in diapausing *A. franciscana* (Patil *et al*., 2013; Podrabsky & Hand, 2015) and *Ca. elegans* larvae (Wang & Kim, 2003; McElwee *et al*., 2006). In contrast, we found high expression of *PDHE2* during the DPP in *Co. bowringi*. However, consistent with results on other species, *PDHE2* expression may be downregulated after female *Co. bowringi* enter diapause (Figs 4B and S3B).

In the non-oxidative phase of the PPP, TKT2 links the PPP and the glycolysis pathway, which suggests that the increase of *TKT2* in DD females may induce lipid storage. In the migratory locust, *Locusta migratoria*, several transketolases were upregulated in prediapause eggs (Tu *et al*., 2015). Hence TKT upregulation may be

a common feature of the DPP in different insects and diapause types. IDH catalyses irreversible reactions involving the TCA, and is therefore regarded as a rate-limiting enzyme for the TCA (Hartong *et al*., 2008). We found that the pattern of *IDH* expression was similar to that of the TCA in that it was upregulated on day 2 of the PEP and downregulated on day 4 of the PEP in DD females (Figs 4D and S3D). In view of the hub function of the TCA in the carbohydrate, lipid and protein metabolism pathways (Akram, 2014), we suspect that fluctuation of *IDH* expression may regulate the TCA to promote nutrient absorption from food in DD females on day 2 of the PEP (the peak of feeding), but act to suppress energy consumption after these enter diapause. The downregulation of IDH during diapause in the female ladybird *Coccinella septempunctata* supports this conjecture (Ren *et al*., 2016).

In the lipid metabolic pathways, ALDH1, also known as retinal dehydrogenase 1, plays a critical role in fatty acid synthesis. ALDH1 is highly expressed in the white adipose tissue of mice and a deficiency of this gene inhibits obesity, suggesting that ALDH1 promotes lipid synthesis in mice (Kiefer *et al*., 2012). We found that *ALDH1* expression was higher in DD females compared to NDD females (Figs 4E and S3E), which suggests that ALDH1 is also involved in the fat hypertrophy of DD insects. In studies of other types of insect diapause, such as embryonic diapause in *L. migratoria* (Tu *et al*., 2015), larval diapause in *Nasonia vitripennis* (Wolschin & Gadau, 2009) and pupal diapause in *H. armigera* (Lu & Xu, 2010), ALDH mRNA or protein was generally upregulated in DD individuals during the prediapause and early diapause stages. Expression of this gene also increased in DD embryos of the shrimp *A. franciscana* (Qiu *et al*., 2007), suggesting that its role in regulating animal diapause is highly conserved.

ELOVL4, a gene responsible for long chain fatty acid elongation, was upregulated in DD females on day 2 of the PEP, but it was more abundant under NDD conditions relative to DD conditions on day 4 of the PEP (Figs 4G and S3G). By promoting fatty acid elongation *ELOVL4* may directly contribute to lipid synthesis for diapause preparation in the early part of the DPP. High expression of this gene in the copepod *Calanus sinicus* during diapause supports this view (Ning *et al*., 2013). However, the high expression of *ELOVL4* on day 4 of the PEP in NDD females suggests that this gene may regulate reproduction. In addition to lipid synthesis, we speculate that lipolysis may regulate fat storage during DPP. It is known that TGL facilitates lipolysis by catalysing triacylglycerol into diacylglycerol and a carboxylate (Watt & Steinberg, 2008). We therefore monitored *TGL1* expression and found that this gene was downregulated in DD females (Figs 4F and S3F). We think that downregulation of *TGL1* may help DD females limit lipolysis. However, *TGL1* may be upregulated to metabolize lipid reserves as an energy source after DD individuals enter diapause (Poelchau *et al*., 2013b). Collectively, we think it likely that the large amounts of fat stored in DD insects are because of an increase in lipid synthesis and a decrease in lipolysis.

In the protein metabolic pathway, both *LP0* and *S2e*, the genes involved in protein synthesis, were more highly expressed in NDD females than DD females (Figs 4H, I, and S3H, I). This is consistent with the demand for yolk protein biosynthesis in reproductive female adults. However, the similar expression profiles of these two genes in DD and NDD females on day 2 of the PEP suggest that some basic protein synthesis is also necessary for DD females.

Absence of JH signalling promotes diapause preparation by regulating metabolic gene expression

Suppression of JH biosynthesis is believed to be the primary mechanism allowing reproductive diapause in adult insects (Denlinger *et al*., 2012). Consequently, we speculated that the JH titre may be reduced during the DPP in *Co. bowringi*. Compared to NDD females, expression of JH biosynthesis genes decreased on day 4 of the PEP in DD females, indicating that JH production is blocked in DD individuals (Fig. 5A). Furthermore, the abundance of JH degradation genes in DD females increased on day 2, but decreased on day 4, of the PEP, relative to NDD females (Fig. 5B). We think that the early upregulation of JH degradation genes may promote the quick degradation of JH, whereas the late downregulation of these genes reflects the low JH titre. Hence, we infer that the JH titre of DD females is very low. This conjecture is supported by the low expression of *Krüppel homologue 1* (*Kr-h1*) in DD females (Fig. 5C); *Kr-h1* is directly induced by JH at the transcriptional level in insects and is therefore commonly used as a marker for JH signalling (Kayukawa *et al*., 2012; Song *et al*., 2014).

JH can prevent reproductive diapause phenotypes, such as lipid accumulation (Sim & Denlinger, 2013; Liu *et al*., 2016), and that the JH receptor methoprene-tolerant (Met) is involved in this process (Liu *et al*., 2016). However, it remains largely unknown how JH-Met signalling regulates lipid accumulation. We therefore tested the effect of JH-Met signalling on nutritional metabolic genes to try to address this question. The expression of six highly expressed genes, *ADH*, *PDHE2*, *TKT2*, *IDH*, *ALDH1* and *ELOVL4*, was decreased by JH analogue (JHA) treatment in DD females (Fig. 5D–H, J). Conversely, the expression of the genes (*TGL1, LP0 and S2e*) that were highly expressed in NDD females, increased after JHA treatment (Fig. 5I, K, L). This suggests that JH signalling suppresses

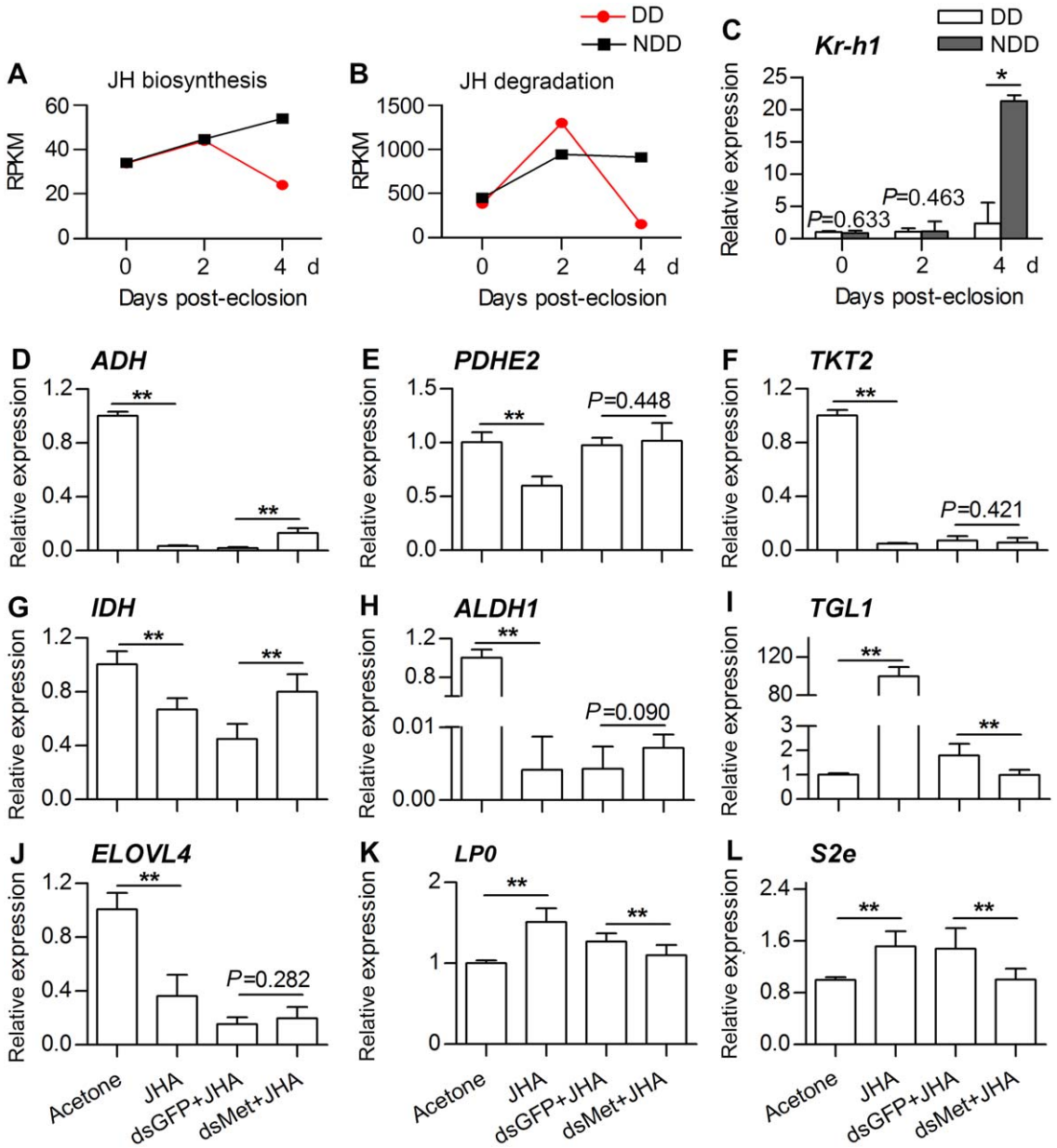

Figure 5. Effects of juvenile hormone (JH) signalling on the expression of metabolic genes. The expression patterns of genes related to JH biosynthesis (A) and degradation (B) in nondiapause-destined (NDD) and diapause-destined (DD) female adult *Colaphellus bowringi* were analysed with the k-means clustering method. (C) Quantitative real-time PCR shows the expression of the JH responsive gene *Krüppel homologue 1* (*Kr-h1*). The relative abundance of *Kr-h1* in DD female adults on day 0 after eclosion (within 12 h of eclosion but before feeding) was regarded as 1 and those in other treatments expressed relative to this standard. Expression levels of *alcohol dehydrogenase* (*ADH*; D), *pyruvate dehydrogenase E2 component* (*PDHE2*; E), *transketolase 2* (*TKT2*; F), *isocitrate dehydrogenase* (*IDH*; G), *aldehyde dehydrogenase 1* (*ALDH1*; H), *triacylglycerol lipase 1* (*TGL1*; I), *elongation of very long chain fatty acids protein 4* (*ELOVL4*; J), *large subunit ribosomal protein* (*LP0*; K) and *small subunit ribosomal protein* (*S2e*; L) were measured in the fat body of DD females 24 h after injection with the JH analogue (JHA) methoprene, or 24 h after injection with double-stranded RNA (dsRNA) against *Met* (dsMet) and JHA. Acetone and dsRNA against *green fluorescent protein* (dsGFP) were injected as controls for JHA and dsMet, respectively. Relative expression values are means + SD based on three biological replicates. The relative expression of genes in the acetone treatment was regarded as 1 and those in other treatments expressed relative to this standard. Asterisks indicate the statistical significance of pairwise comparisons as determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$). RPKM, reads per kb per million reads. [Colour figure can be viewed at wileyonlinelibrary.com]

diapause-related gene transcription but promotes the expression of genes related to reproduction.

To investigate the function of Met we injected DD females with both double-stranded RNA (dsRNA) against *Met* (dsMet) and JHA (Fig. S4A). Compared to the dsRNA against *green fluorescent protein* (dsGFP) control, *Met* depletion reversed the JH-induced suppression of *ADH* and *IDH* (Fig. 5D, G), and the upregulation of *TGL1*, *LP0*

and *S2e* (Fig. 5I, K, L). To confirm these results, we silenced *Met* in NDD females (Fig. S4B) and found that these metabolic genes could also be partly regulated by *Met* depletion (Fig. S5). Therefore, we argue that the JH-Met pathway is the main upstream signalling pathway regulating metabolic gene expression during the DPP in adult female *Co. bowringi*. In fact, JH signalling appears to inhibit diapause phenotypes (eg lipid accumulation) in several insects, such as the mosquito *Cu. pipiens* (Sim & Denlinger, 2013) and the linden bug *Pyrrhocoris apterus* (Bajgar *et al*., 2013). In addition, the JH-Met pathway is the primary signalling pathway regulating metabolic gene expression during reproduction in the mosquito *Aedes aegypti* (Zou *et al*., 2013; Hou *et al*., 2015). In the larval integument of the silkworm *Bombyx mori* JHA also modulates the expression of carbohydrate metabolism genes (Cheng *et al*., 2014). Collectively, these findings suggest that JH-Met signalling-mediated regulation of the nutritional metabolism pathways is a common feature of different physiological processes in different insect species. In particular, JH-Met-mediated regulation of metabolic gene expression in DD individuals may be important for fat storage during the DPP.

In fact, Met-mediated signalling may not be the only downstream mode by which JH regulates metabolism. For example, our results show that JH-inhibited expression of *ALDH1* was independent of *Met*. Many studies have also found evidence of other JH receptors in addition to Met. For example, JH was found to modulate *Tribolium castaneum* cellular immunity independently of Met (Hepat & Kim, 2014). JH also inhibits the transcription of *forkhead box O* (*FOXO*) independently of Met in *Co. bowringi* (Liu *et al*., 2016). Identifying any such alternative JH receptors involved in JH-regulated nutritional metabolism pathways should be a goal of future research.

Conclusions

Diapause is an adaptive strategy that allows many insects to survive seasonally adverse conditions, such as food shortage, dehydration and cold stress. During the DPP, DD insects will store a large quantity of fat to serve as an energy reserve during diapause and post-diapause development (Kostal, 2006; Hahn & Denlinger, 2011). Although nutritional patterns during the DPP have been investigated at physiological and biochemical levels, exactly how the nutritional metabolism pathway is regulated, and how lipid is accumulated at molecular levels, during the DPP remains unclear. In addition, inactivation of JH signalling is considered essential for reproductive diapause, but whether JH signalling regulates the nutritional metabolism pathway during the DPP required verification.

We addressed these questions in this study using the cabbage beetle *Co. bowringi* as a model because of its easily recognizable DPP. Our findings on the metabolic regulation of diapause preparation and preoviposition in *Co. bowringi* can be summarized in a model (Fig. 6). At 25 °C, short day lengths induce an increase in JH titre during the POP thereby stimulating the protein metabolic pathway and the consequent synthesis and absorption of yolk protein in female adults, while at the same time blocking the lipid and carbohydrate pathways involved in lipid storage. Conversely, long day lengths lead to lower JH titres during the DPP and the consequent activation of the lipid and carbohydrate pathways. Lipid is accumulated in female adults, enabling them to enter reproductive diapause. Combined with the results of previous studies on diapausing *Cu. pipiens* (Sim & Denlinger, 2008, 2013; Sim *et al*., 2015) and *Co. bowringi* (Liu *et al*., 2016), we believe that FOXO may be the key factor linking JH signalling to lipid storage during diapause, although this needs to be verified in more insects. However, we do not rule out the existence of additional factors connecting JH signalling to downstream lipid accumulation. Considering the different endocrine regulatory mechanisms involved in different types of diapause, the types of hormone signalling pathways that mediate diapause preparation are necessarily diverse. In addition to shedding light on the mechanisms that regulate diapause, the results of this study also provide new insights on the molecular basis of ecological adaptions in animals.

Experimental procedures

Experimental animals

Co. bowringi larvae were obtained from a wild population in Xiushui County (29°10′N, 114°40′E), Jiangxi Province, China, transported to our laboratory and raised on radish *Raphanus sativus* L. var. *longipinnatus* (Brassicales: Brassicaceae) leaves in plastic containers (7.5 × 7.5 × 6 cm). The offspring of these beetles were used in this study. When larvae were kept at 25 °C under a 12:12 h light : dark photoperiod, they developed into reproductive NDD female adults with developing ovaries, but when larvae were kept at the same temperature under a 16:8 h light : dark photoperiod they became DD female adults, which dug into soil to begin diapause. NDD and DD females could therefore be readily distinguished on the basis of ovarian development and digging behaviour (Xue *et al*., 2002; Tan *et al*., 2016).

RNA sequencing and pathway enrichment

The design of our RNA sequencing experiment is shown in Fig. 1A. Briefly, NDD female adults absorb nutrients from food for ovarian development during the POP, which occurs during the 4-day PEP. However, during the same 4-day period DD female adults transform these nutrients into fat in the fat body to provide energy reserves for diapause. Therefore, day 0 (within 12 h of eclosion and before feeding), day 2 and day 4, of the 4-day PEP correspond to the early, peak and late stages

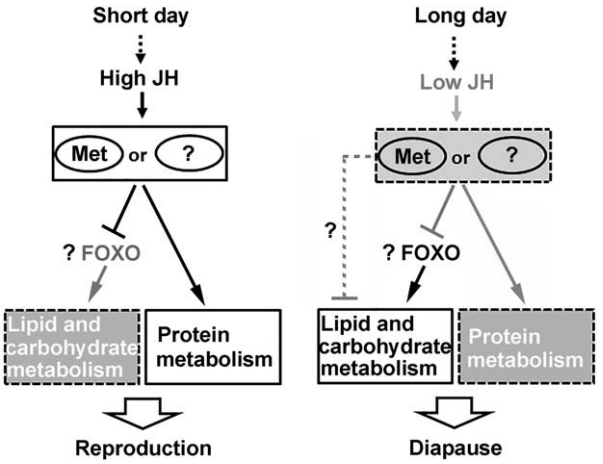

Figure 6. A model of the mechanism regulating nutrient accumulation during the preoviposition phase or diapause preparation phase in *Colaphellus bowringi*. At 25 °C, short day lengths induce juvenile hormone (JH) biosynthesis, thereby activating JH receptor methoprene-tolerant (Met)-mediated signalling. Forkhead box O (FOXO) is then suppressed, leading to the inhibition of lipid and carbohydrate synthesis and storage. At the same time, the protein metabolism pathway is upregulated and yolk protein is accumulated in the ovaries for reproduction. Under long day lengths, JH biosynthesis is suppressed, thereby preventing JH signalling. The suppression of FOXO is thus removed, inducing the upregulation of lipid and carbohydrate synthesis and storage and the consequent accumulation of fat in the fat body of diapausing individuals (Sim & Denlinger, 2008, 2013; Sim *et al.*, 2015; Liu *et al.*, 2016). In addition to Met, there may be other unknown JH receptors that regulate nutritional metabolism. We also speculate that FOXO may be not the only factor that links JH signalling to lipid storage.

of the POP phase in NDD females, or the DPP phase in DD females. Whole bodies of NDD and DD female adults were collected on days 0, 2 and 4 of the PEP for RNA extraction. Each treatment contained three biological replicates, each of which was comprised of 10 individuals. After mRNA purification, cDNA synthesis, PCR amplification and ligation of sequencing adaptors, samples were sequenced with an Illumina HiSeq™ 2000 at the Beijing Genomics Institute (Shenzhen, China). The clean data are accessible in NCBI under the BioProject PRJNA338895. Gene annotation and quantification were performed by referencing our previously reported *Co. bowringi* transcriptome, which contains 39 390 unigenes assembled from 57 000 000 sequence reads (Tan *et al.*, 2015). In the current work, over 85% of reads were mapped to the reference transcriptome. Over 60% of genes have a gene's coverage more than 50%. The expression level for each gene is determined by the numbers of reads uniquely mapped to the specific gene and the total number of uniquely mapped reads in the sample. Gene abundance was quantified by calculating the reads per kb per million reads (RPKM) values (Mortazavi *et al.*, 2008). We determined DEGs using a Poisson distribution model (Audic & Claverie, 1997), and screened groups of DEGs using the NOIseq method (probability $\geq$ 0.8 and |$\log_2$Ratio|$\geq$1) (Tarazona *et al.*, 2011). Gene function annotation and pathway analysis were performed by blasting the Gene Ontology (GO; Ashburner *et al.*, 2000) and KEGG (Kanehisa & Goto, 2000) databases, respectively. GO and KEGG pathway enrichment was performed using a hypergeometric distribution model and significant differences (*P*-values) were calculated by Functional Specification based on a previously described method (Robinson *et al.*, 2002; Rivals *et al.*, 2007). Details of gene annotation, expression, function annotation and pathway analysis are described in Tables S1 and S2.

Abundance of DEGs in metabolic pathways

To investigate metabolic processes during the POP and DPP, the abundance of DEGs in different metabolic pathways was quantified by 1gene (Hangzhou, China). Metabolic genes were selected and classified based on their annotation in the KEGG pathway. The expression patterns of DEGs in one pathway at each sampling time point were determined using the k-means clustering method (Eisen *et al.*, 1998; Birnbaum *et al.*, 2003). In detail, K points (RPKM values) were randomly selected and used as initial group centroids. By searching all the points, each point was assigned to the closest centroid, and the average of each clustering was used as the new centroid. After an iterative process, the centroid no longer moved. The stable centroids are the values (RPKM) presented in the k-means results.

cDNA cloning and sequence analysis

Based on the transcriptome database of *Co. bowringi*, we cloned nine DEGs associated with metabolism, and one JH responsive gene *Kr-h1* (Table S4). Protein sequences were deduced with the ExPASy Translate tool (http://web.expasy.org/translate/), and MEGA 4.1 software (Sudhir Kumar, Tempe, USA) was used to construct rooted neighbour-joining phylogenetic trees (Fig. S2).

qRT-PCR for the detection of mRNA abundance

qRT-PCR was performed following the Minimum Information for publication of Quantitative real time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2010) and according to previously published methods (Liu *et al.*, 2016). Based on reference gene evaluation in *Co. bowringi* (Tan *et al.*, 2015), two reference genes, *ribosomal protein L19* and *Actin1*, were used to normalize gene expression. See Table S5 for the PCR primers used. qRT-PCR data were collected from three biological replicates and three technical replicates, and analysed with the $2^{-\Delta\Delta CT}$ Cycle threshold (CT) method (Schmittgen & Livak, 2008). Values are presented as means + SD, and the statistical significance of differences between means was assessed using Student's *t*-test, or a one-way analysis of variance followed by Tukey's honestly significant difference (HSD) test, as appropriate ($\alpha = 0.05$).

JH treatment of DD female adults

Fifteen μg of JHA methoprene (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 200 nl solvent (acetone) and injected into DD female adults within 12 h of eclosion with a microinjector (World Precision Instruments, Sarasota, Florida, USA). An equal amount of acetone was injected into other DD females as a control. Total RNA of the fat body was extracted and subjected to cDNA synthesis and qRT-PCR 24 h after injection with JHA or the control.

RNA interference of the JH receptor Met

Knockdown of *Met* followed our previous protocol (Liu *et al*., 2016). Briefly, dsMet was synthesized with a T7 transcription kit (Fermentas, Vilnius, Lithuania) and the corresponding primers (Table S5). The control was dsGFP. 1 μg dsRNA (200 nl) was injected into newly emerged NDD females, the fat bodies of which were collected for RNA extraction and qRT-PCR 96 h later. Dual-injection with dsRNA and JHA was performed by first injecting 1 μg dsRNA into newly emerged DD female adults and injecting them with JHA 12 h later. Total RNA of the fat body was extracted after an additional 24 h and used in qRT-PCR.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Gene annotation, expression, function annotation and pathway analysis for the preoviposition phase of *Colaphellus bowringi*.

Table S2. Gene annotation, expression, function annotation and pathway analysis for the diapause preparation phase of *Colaphellus bowringi*.

Table S3. List of the genes included in the k-means cluster analysis presented in Figs 2 and 3.

Table S4. BLASTP analyses of putative *Colaphellus bowringi* genes.

Table S5. Primers for quantitative real-time PCR and RNA interference.

Figure S1. Top 20 statistics of pathway enrichment determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) in female adult *Colaphellus bowringi*. 'Upregulation' and 'Downregulation' indicate increases and decreases in pathways in diapause-destined females, respectively.

Figure S2. Neighbour-joining, rooted, phylogenetic trees of genes involved in regulating the nutritional metabolism of *Colaphellus bowringi*. All the trees, (A) Krüppel homologue 1 (Kr-h1), (B) alcohol dehydrogenase (ADH), (C) pyruvate dehydrogenase E2 component (PDHE2), (D) transketolase 2 (TKT2), (E) isocitrate dehydrogenase (IDH), (F) aldehyde dehydrogenase 1 (ALDH1), (G) triacylglycerol lipase 1 (TGL1), (H) elongation of very long chain fatty acids protein 4 (ELOVL4), (I) large subunit ribosomal protein (LP0) and (J) small subunit ribosomal protein (S2e) were constructed with MEGA 4.1 software. The Kr-h2 of *Drosophila melanogaster* was used as the outgroup for Kr-h1 and the corresponding genes of *Mus musculus* served as the outgroups for the other metabolic genes. Bootstrap values are marked on the nodes.

Figure S3. Expression profiles of the nine genes validated by quantitative real-time PCR were analysed based on the reads per kb per million reads (RPKM) values from RNA sequencing. The mRNA profiles of three biological replicates of *alcohol dehydrogenase* (*ADH*; A), *pyruvate dehydrogenase E2 component* (*PDHE2*; B), *transketolase 2* (*TKT2*; C), *isocitrate dehydrogenase* (*IDH*; D), *aldehyde dehydrogenase 1* (*ALDH1*; E), *triacylglycerol lipase 1* (*TGL1*; F), *elongation of very long chain fatty acids protein 4* (*ELOVL4*; G), *large subunit ribosomal protein* (*LP0*; H) and *small subunit ribosomal protein* (*S2e*; I) in nondiapause-destined and diapause-destined female adult *Colaphellus bowringi* were determined based on the RPKM values on days 0, 2 and 4 after eclosion. RPKM values are means + SD. 'Sig.' indicates a significant difference, which is filtered by probability ≥ 0.8 and $|\log_2\text{Ratio}| \geq 1$ (NOISeq method).

Figure S4. The effect of the juvenile hormone (JH) receptor *methoprene-tolerant* (*Met*) knockdown in the diapause-destined (DD) and nondiapause-destined (NDD) female adult *Colaphellus bowringi*. (A) *Met* knockdown in DD female adults. One μg double-stranded RNA (dsRNA) against *Met* (dsMet) was microinjected into newly emerged DD females. After 12 h, 15 μg JH analogue (JHA) was also injected and total RNA was extracted for quantitative real-time PCR after an additional 24 h. dsRNA against *green fluorescent protein* (dsGFP) and JHA were injected as controls. The relative abundance of *Met* in the control dsGFP+JHA was regarded as 1 and the abundance of dsMet+JHA expressed relative to this standard. (B) *Met* knockdown in NDD female adults. One μg dsMet was microinjected into newly emerged NDD female adults and their fat bodies collected for analysis after 96 h. The control was injection with 1 μg dsGFP. The relative abundance of *Met* in the control dsGFP group was regarded as 1 and that in the dsMet group expressed relative to this standard. Values are means + SD based on the three biological replicates. Asterisks indicate the statistical significance of pairwise comparisons as determined by Student's *t*-test ($^{**}P < 0.01$).

Figure S5. The effects of the juvenile hormone (JH) receptor *methoprene-tolerant* (*Met*) knockdown on metabolic gene expression in nondiapause-destined female adult *Colaphellus bowringi*. The fat body was collected from females 96 h after *Met* knockdown or injection with double-stranded RNA (dsRNA) against *green fluorescent protein* (dsGFP; the control), and the expression of *alcohol dehydrogenase* (*ADH*; A), *pyruvate dehydrogenase E2 component* (*PDHE2*; B), *transketolase 2* (*TKT2*; C), *isocitrate dehydrogenase* (*IDH*; D), *aldehyde dehydrogenase 1* (*ALDH1*; E), *triacylglycerol lipase 1* (*TGL1*; F), *elongation of very long chain fatty acids protein 4* (*ELOVL4*; G), *large subunit ribosomal protein* (*LP0*, H) and *small subunit ribosomal protein* (*S2e*; I) measured. Relative expression values are means + SD based on three biological replicates. The relative abundance of genes in the dsGFP control was regarded as 1, and that in the dsRNA against *Met* (dsMet) treatment expressed relative to this standard. Significant differences were determined by Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$).