Analysis of molecular mechanism for acceleration of polyembryony using gene functional annotation pipeline in Copidosoma floridanum

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Abstract

**Background:** Polyembryony is defined as the formation of several embryos from a single egg. This phenomenon can occur in humans, armadillo, and some endoparasitoid insects. However, the mechanism underlying polyembryogenesis in animals remains to be elucidated. The polyembryonic parasitoid wasp Copidosoma floridanum oviposits its egg into an egg of the host insect; eventually, over 2000 individuals will arise from one egg. Previously, we reported that polyembryogenesis is enhanced when the juvenile hormone (JH) added to the culture medium in the embryo culture. Hence, in the present study, we performed RNA sequencing (RNA-Seq) analysis to investigate the molecular mechanisms controlling polyembryogenesis of C. floridanum. Functional annotation of genes is not fully available for C. floridanum; however, whole genome assembly has been archived. Hence, we constructed a pipeline for gene functional annotation in C. floridanum and performed molecular network analysis.

**Results:** We obtained 11,117 transcripts in the JH treatment group and identified 217 differentially expressed genes compared with the control group. As a result, 76% of C. floridanum transcripts were assigned to human genes. Gene enrichment analysis revealed genes associated with platelet degranulation, fatty acid biosynthesis, cell morphogenesis in the differentiation and integrin signaling pathways were fluctuated following JH treatment. Furthermore, Cytoscape analysis revealed a molecular interaction that was possibly associated with polyembryogenesis.

**Conclusions:** We have constructed a pipeline for gene functional annotation of C. floridanum, and identified transcripts with high similarity to human genes during early embryo developmental. Additionally, this study reveals new molecular interactions associated with polyembryogenesis; these interactions could indicate the molecular mechanisms underlying polyembryony. Our results highlight the potential utility of molecular interaction analysis in human twins.

**Keywords:** Polyembryony, Xanthine dehydrogenase/oxidase, Endoparasitoid, Polyembryogenesis, Capidosoma floridanum
Background

The development of a single-cell egg into a multicellular organism begins with cell cleavage; however, polyembryogenesis—in which many embryos are produced from one egg—occurs in some species. Although identical twins are representative examples of human polyembryogenesis, the incidence of this situation is as low as 0.3% [1]. Armadillos (Dasypus) are the only mammals to exhibit obligatory polyembryony, developing from one egg to four individuals through embryonic shield separation [2, 3]. Owing to the ethical limitations of experiments with animal, the phenomenon of polyembryogenesis remains poorly studied.

Furthermore, the occurrence of polyembryogenesis has been reported for insects such as the endoparasitic wasps Braconidae, Dryinidae, Platygasteridae, and Encyrtidae, and research on developmental patterns has progressed rapidly in recent years [4]. The identification of molecules associated with polyembryogenesis could further our understanding of the mechanisms underlying the regulation of this process in animals.

The endoparasitic wasp Copidosoma floridanum (Hymenoptera: Encyrtidae) is an egg-larval parasitoid of the plusiine moth Thysanoplusia intermixta. The egg developmental stage of T. intermixta lasts 4 days, the larval developmental stage around 20 days, and pupal developmental lasts around 8 days under experimental conditions [5] (Fig. 1). In standard experimental conditions, C. floridanum parasitizes the 2-day old egg of T. intermixta, ultimately producing nearly 2000 cloned embryos from either a fertilized egg (which develops into females) or an unfertilized egg (which develops into males) (Fig. 1). Although almost all insects exhibit egg segmentation due to superficial cleavage, C. floridanum

![Fig. 1 The life cycle of Copidosoma floridanum.](https://example.com/copidosoma_life_cycle.png)

- **a**: C. floridanum oviposited its egg into the host egg.
- **b**, **c**: The C. floridanum egg starts developing from the two-cell stage to morula and invaginates into host embryo.
- **d**: The morula embryo clonally divided, and polyembryos are formed around 60 h after parasitism.
- **e**, **f**: A part of polyembryos starts segmentation and, then, develops into soldier larvae via soldier embryo.
- **g**: Each embryo achieves morphogenesis when the host insect develops at the end of the fifth instar larva.
- **h**: Finally, reproductive larvae emerge when the host insect achieves on the second day of sixth instar larvae, and the adult emerges from the mummy.
- **i**: T. intermixta life cycle.
- **j**: T. intermixta egg indicates black arrows.
- **k**: j-m: T. intermixta life cycle.
egg segments undergo holoblastic cleavage. In C. floridanum, cell cleavage begins when the egg is laid into a host egg (Fig. 1a). The C. floridanum egg then starts to develop from the two-cell stage to a morula after the C. floridanum embryo invades the host embryo (Fig. 1b). The egg of C. floridanum comprises an embryonic cell and an anterior cell from a polar body [6]. The anterior cell develops into an extraembryonic syncytium, which forms the outer part of the morula. The extraembryonic syncytium from the anterior cell wraps around the blastomeres after the chorion is removed and morula form (Fig. 1c). The motility of early morulae enable them to invade the host embryo and then develop into polyembryos when morulae movement ceases after entry into the host embryo [7] (Fig. 1d). A subset of polyembryos starts to develop into soldier larvae from embryos without primary germ cells (Fig. 1e and f). Each embryo undergoes morphogenesis when the host insect reaches the end of the final instar of larval development (Fig. 1g). Finally, reproductive larvae emerge when the host insect reaches the second day of the final instar of larval development (Fig. 1h), and adults finally emerge (Fig. 1i) [8, 9]. Although the process by which C. floridanum polyembryony progresses in host embryos is known, the molecular mechanisms remain obscure.

Previously, we reported polyembryogenesis to be accelerated by exposure to juvenile hormone (JH) or the JH analog methoprene, under culture conditions [10]. Although the molecular function of JH in the embryogenesis of C. floridanum remains unclear, we anticipated that either JH or methoprene would promote the progress of polyembryogenesis from the two-cell to polymorula stage. Functional annotation of genes is not fully available for C. floridanum; however, whole-genome sequence data is available. Thus, we were unable to analyze the molecular networks in C. floridanum as has been done for Drosophila melanogaster, a model insect. Accordingly, we constructed a pipeline for gene functional annotation of C. floridanum and performed molecular network analysis focusing on gene expression following polyembryony development in response to JH treatment in the two-cell developmental stage. We also performed RNA sequencing (RNA-Seq) analysis of C. floridanum as a model animal for polyembryogenesis to elucidate the molecular mechanisms underlying this process.

Results
Juvenile hormone accelerates polyembryogenesis
We collected the two-cell-stage embryos and cultured them for 5 days; then, we counted the cultured embryo and evaluated whether these embryos were polymorulae or not (Fig. 2). We found the JH-treated group to exhibit an increased rate of polymorulae compared with the control group. Additionally, polymorulae were identified in the JH-treated group from 2 days after the start of

![Fig. 2](image-url) Polymorula was accelerated by the juvenile hormone (JH) treatment in the culture condition. The early embryo of the two-cell stage was cultured with or without JH. The number of polymorula was counted and plotted on the graph. Notes: Black line, JH-treated group; blue line, control group; vertical axis, rate of polymorula (%); horizontal axis, culture period (days). Error bars indicate standard deviation (SD).
culturing, whereas these were only identified in the control group after 4 days after culture. Hence, the development of polymorulae was accelerated by JH treatment (Fig. 2; Table 1).

Identification of differentially expressed genes and assignment of human homolog

We constructed a pipeline for functional gene annotation of *C. floridanum* (Fig. 3). In the present study, we identified 13,160 human homologs of 17,308 total predicted protein dataset in *C. floridanum* using the current gene functional annotation pipeline. As a result, *C. floridanum* exhibits 76% gene similarity with humans. Following the results of polymorulae analysis, we extracted total RNA for RNA-Seq analysis after 48 h of cell culture. We performed RNA-Seq analysis on three control samples (SRA accession numbers: DRR138914, DRR138915, and DRR138916) and two JH-treated samples (DRR138917 and DRR138918). The RNA-Seq data were mapped with HISAT2 and StringTie. We obtained 11,117 transcripts from these RNA-Seq data and found that 10,908 of these were commonly expressed in the control and JH-treated groups.

We identified 217 differentially expressed genes (DEGs; false discovery rate [FDR] < 0.05; Fig. 4a). While the expression of 123 of these was increased, 94 were downregulated in the JH-treated group (Fig. 4a, orange-colored dots indicate differentially expressed transcripts). Next, we assessed the number of *C. floridanum* genes by comparing our transcript dataset with the *C. floridanum* RefSeq datasets. Of the 11,117 transcripts in our *C. floridanum* RNA-Seq datasets, 9417 were present in the *C. floridanum* RefSeq datasets (84.7% coverage). We identified 6098 human homologs out of the 11,117 total transcripts in *C. floridanum* using tblastx with a cutoff E-value of 1e−10. Of the 217 DEGs that we identified, 88 corresponded to human genes (Additional file 1: Table S1).

Gene enrichment analysis of differentially expressed genes

For the gene enrichment analysis using Metascape, we needed to assign human gene IDs to *C. floridanum* genes IDs. Thus, we chose *C. floridanum* 88 genes, which have sequence similarity to human genes. Of 88 genes that were chosen to compare expression between the two groups, the expression of 42 genes was increased in the JH-treated group, whereas 46 were downregulated following JH treatment. We imported the list of DEGs and their expression levels into Metascape and converted to their human homologs for gene enrichment analysis. Metascape generated 12 genetic function groups for Gene Ontology (GO), indicating that upregulated genes in the JH-treated group were assigned to platelet degranulation (GO:0002576) and fatty acid biosynthetic process (GO:0006633) (Fig. 4b). Furthermore, Metascape indicated that downregulated genes in the JH-treated group were assigned to cell morphogenesis was involved in differentiation (GO: 0000904) and ST Integrin Signaling Pathway (M3270) (Fig. 4c).

Screening for related molecules using the molecular network analysis by Cytoscape

We focused on the GO terms platelet degranulation, fatty acid biosynthetic process, and integrin signaling pathway to identify the molecular network that regulates polyembryogenesis. Using the public protein interaction database and Cytoscape, we further explored correlations among the genes involved in these processes. Hence, we identified molecular interactions including filamin-A (*FLNA*), xanthine dehydrogenase/oxidase (*XDH*), exportin-1 (*XPO1*), protein phosphatase slingshot homolog 2 (*SSH2*), and integrin alpha-4 (*ITGA4*) as genes that fluctuated in the JH-treated group (Fig. 5a). The Transcripts Per Kilobase Million (TPM) values of these molecules were plotted (Fig. 5b). We found that the mRNA expression of *FLNA* and *XDH* increased, while that of *SSH2* and *ITGA4* decreased following JH treatment (Fig. 5b). However, the mRNA expression of *XPO1* was not by JH treatment (Fig. 5b).

**Table 1** Impact of polyembryogenesis by the juvenile hormone treatment to the early embryo of *Cepidosoma floridanum*

| Treatment groups | Day of polymorula | Rate of polymorula (%) |
|------------------|-------------------|------------------------|
| Control          | 4.88 ± 0.13       | 25.0                   |
| JH               | 3.16 ± 0.23       | 60.3                   |

Control group: experimental 1, n = 10; experimental 2, n = 25; experimental 3, n = 21. Juvenile-hormone-treated group (experimental 1, n = 10; experimental 2, n = 24; experimental 3, n = 23). Polymorula was observed in experimental 1 to 3 groups and the day of polymorula is shown as mean ± standard deviation. Abbreviations: JH juvenile hormone.

**Discussion**

We investigated gene expression during polyembryogenesis of embryos of *C. floridanum* treated with JH in the two-cell stage. Additionally, we constructed a pipeline for functional gene annotation of *C. floridanum* and performed molecular network analysis.

Our previous study revealed that JH and methoprene accelerate polyembryogenesis; a phenomenon that was also observed when JH I or JH II were added to the culture medium [10]. Methoprene had the strongest effect on promotion of polyembryogenesis, while farnesol, farnesyl acetate, and methyl caproate did not promote polyembryogenesis [10]. Moreover, polyembryogenesis was not promoted by ecdysone (Additional file 2: Figure S1). Hence, only JH or methoprene promote polyembryogenesis in *C. floridanum*.
The insect hormone JH is unique in its structure; it has \( \alpha \)-, and \( \beta \)-unsaturated methyl ester groups and epoxy groups at both ends of the terpenoid backbone [11]. The importance of JH in processes such as regulation of molting, pheromone biosynthesis, maturation of gonads, egg development, homeostasis, maintenance of population, and body color change has been reported [12]. Thus, JH is a critical element of insect physiology.

Krüppel homolog 1 (Kr-h1) is a JH-responsive gene [13]. Kr-h1 was not affected by JH in this study (Additional file 3: Figure S2). Retinoid X receptor (RXR), a type of nuclear receptor that binds to 9-cis retinoic acid [14], may be able to bind to several chemicals that share the structure of retinoic acid [15]. Reportedly, the JH analog methoprene and methoprene acid can bind to RXR [15], which also functions as a JH receptor in *D. melanogaster* [16]. In this study, the expression of *C. floridanum* RXR-alpha-B tended to be increased following JH treatment (Additional file 3: Figure S2). Retinoic acid is involved in embryonic development and cell differentiation through its interaction with RXR [17]. This suggests that JH could act in a similar manner to retinoic acid to affect embryonic development and participate in the retinoic acid signaling pathway in our model of polyembryony.

Additionally, *C. floridanum* RefSeq datasets were constructed by analyzing male and female adult *C. floridanum* transcriptome. A reference genome sequence of *C. floridanum* from male has been published (https://www.ncbi.nlm.nih.gov/assembly/GCF_000648655.2/). Overall, 17,038 of the estimated proteins have been registered in the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome/12734?genome_assembly_id=358239).
Previously, we identified human homologs of Bombyx mori and D. melanogaster through systematic BLAST analysis. We found B. mori and D. melanogaster to contain 58 and 63% of human homologs, respectively [18]. In the present study, we found, C. floridanum exhibits 76% gene similarity with humans. The large number of human homologs that we observed in C. floridanum is therefore comparable with these model insects. In the present study, 88 C. floridanum genes that showed differential expression when comparing JH-treated and control groups corresponded to human genes, and we input these genes to gene enrichment and molecular network analyses.

Gene enrichment analysis revealed the expression of lipid metabolism-related (GO: 0006633) and platelet degranulation (GO: 0002576) related genes were upregulated in the JH-treated group. The characteristics of genes containing these GO terms have been shown to be involved with vesicle-associated V-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) in cellular membrane adhesion [19]. Additionally, genes encoding synaptosomal-associated protein (SNAP)25 and 29, (SNAPC)3 and 4, SNARE-associated protein Snapin (SNAPIN), and syntaxin-binding protein (STXBP) were shown to be commonly expressed in both groups of the present study. Reportedly, these genes are involved in the membrane fusion of neurosecretory cells [20]. Notably, STXBP interacts with these proteins to inactivate membrane fusion [20]. Thus the decreased mRNA expression of STXBP that we observed following JH treatment may lead to activation of cellular membrane fusion. As the molura promotes fusion with the extraembryonic syncytium, it is possible that JH treatment could accelerate molura to polyploidy.

The genes SNAP25 and SNAP29, SNAPC3 and SNAPC4, SNAPIN, and STXBP, which we observed to be expressed in C. floridanum molura embryos, also play a role for mediates membrane fusion during exocytosis in the neurosecretory cells of humans [21]. Therefore, C. floridanum might employ similar molecular mechanisms for cellular membrane adhesion as human neurosecretory cells.

The molura comprises the outer extraembryonic syncytium and the inner embryonic cell; the extraembryonic syncytium separates by dividing the embryonic cell [22]. Embryonic cells adhere to the extraembryonic syncytium via integrin in the molura. When integrin expression is

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**Fig. 4** The extraction of fluctuated transcripts in the juvenile hormone (JH) treatment and gene enrichment analysis. Fluctuated transcripts were extracted and plotted on the graph. Orange dots, transcripts that fluctuated more than two times compared with the control in the JH treatment (a). The gene enrichment analysis of fluctuated transcripts in the morula using Metascape. A heatmap of enriched terms across the input transcripts lists; different colored bars, P values. (b) JH upregulated genes; (c) JH downregulated genes.
decreased following exposure to JH, this adhesion may be loose, causing the extraembryonic syncytium to invaginate into the cell gap of embryonic cells. These cells might then divide, resulting in polyembryony. The actin filament crosslinking protein, FLNA, which is present in non-muscle cells [23] might be involved in the division of embryos interacting with SSH2 and ITGA4. Reportedly, SSH2 mediates actin dynamics [24], while ITGA4 belongs to the integrin family and plays a role in cell adhesion [25]. These molecular interactions might contribute to the polyembryony.

Xanthine dehydrogenase/oxidase is the rate-limiting enzyme of purine metabolism, and a key component in uric acid synthesis. During egg development of B. mori the amount of uric acid gradually declines until blastokinesis, after which it increases until egg pigmentation [26]. Remarkably, XHD knockdown has been shown to enhance cell mobility and invasion of HepG2 cells, although no effect on cell proliferation was observed [27]. Furthermore, XDH converts retinoic acid to 9-cis retinal [28], and activity of the enzyme is essential in order for JH to induce bristle formation and cuticle production on the abdominal epidermis during pupal and adult development [29].

The chemical structures of JH III and retinoic acid are similar, and it is possible that JH III binds to XDH. Then, XDH-bound JH could enter the nucleus via XPO1, and released JH might bind to RXR to control subsequent transcription; this molecular correlation could be a novel mechanism triggered by JH. Accordingly, the following model could be proposed for the progression of forming molura to polymolura: To start, embryos are wrapped with extraembryonic syncytium (Fig. 6a). During the formation of polymolurae, the degradation of actin is avoided by reducing SSH2 expression followed by suppression of ITGA4 expression. As the adhesion of embryonic cells is loose (Fig. 6b), the syncytial membrane facilitates fractionation (Fig. 6c and d) and promotes polyembryony (Fig. 6e). Hence, XDH plays a key role in embryogenesis via JH, as well as in uric acid synthesis. Overall, the present study reveals novel molecular interactions involved in polyembryogenesis and

**Fig. 5** Screening of the molecular interaction using Cytoscape. The molecular interaction of (a) Cytoscape automatically generates the corresponding molecules as a node on a molecular interaction in polyembryogenesis-related genes. A red colored node shows up-regulated genes, blue colored genes shows down-regulated genes, and a gray colored gene is not fluctuation gene. (b) Polyembryogenesis-related genes in juvenile hormone (JH) treatment of molura. The y-axis indicates the ratio of the average Transcripts Per Kilobase Million (TPM) values for molura between the control and JH treatment groups.
demonstrates the connections that are required for the progression of cell separation in polyembryogenesis.

**Conclusions**
In this study, we constructed a pipeline for gene functional annotation of *C. floridanum*. We identified *C. floridanum* transcripts with high similarity with several human genes during early embryogenesis, and found that *C. floridanum* has many more human homologs than *D. melanogaster*. Additionally, we identified new molecular interactions associated with polyembryogenesis, which may enable the elucidation of the molecular mechanisms underlying polyembryony. Our results highlight the potential utility of molecular interaction analysis for the investigation of polyembryogenesis of humans. In future studies, we intend to investigate the function of these molecules using RNAi in *C. floridanum*.

**Methods**

**Insect**
We obtained *C. floridanum* from parasitized *T. integrina* larvae from burdock fields in Tokyo, Chiba, and Ibaraki Prefectures. Larvae were maintained with an artificial diet [30] at 25 °C with a 16–h light/8–h dark cycle. *T. integrina* adults were fed a 10% sugar solution absorbed with cotton. *C. floridanum* adults were fed a 50% honey solution absorbed with cotton. We used *T. integrina* eggs within 17 h post-oviposition for parasitism by *C. floridanum*. The parasitized hosts were kept in the same conditions as non-parasitized hosts.

**Embryo culture**
We dissected the two-cell stage of the embryo from *T. integrina* eggs within 2-h post-oviposition and, then, cultured it with the modified MGM medium [31]. We dissolved JH III ([J2000; Sigma-Aldrich Co. Ltd., Munich, Germany]) in ethanol to prepare a stock solution (10 mg/mL); then, 1 μL of this stock solution was added to the modified MGM medium as the final concentration was 1 μg/mL. In the control group, an equivalent volume of ethanol alone was added to the culture medium. We used two-cell stage embryos produced from *T. integrina* eggs within 2 h of oviposition for the control group (experimental 1, *n* = 10; experimental 2, *n* = 25; experimental 3, *n* = 21) and the JH group (experimental 1, *n* = 10; experimental 2, *n* = 24; experimental 3, *n* = 23).

**RNA-Seq analysis**
We isolated total RNA from morulae cultured for 2 days in control (experimental 1, *n* = 24; experimental 2, *n* = 16; experimental 3, *n* = 11), and JH conditions (experimental 1, *n* = 17; experimental 2, *n* = 28; experimental 3, *n* = 23) using a combination of TRIzol® LS Reagent and the Pure-Link® RNA Extraction Kit (Thermo Fisher Scientific Inc., Valencia, CA) per the manufacturer’s instructions. Then, we used an Agilent TapeStation 2200 (Agilent Technologies, Santa Clara, CA) to assess the RNA quality. Additionally, single-end sequencing cDNA libraries were constructed with 100 ng of total RNA from these samples (control group: *n* = 3; JH group: *n* = 3) with a TruSeq® Stranded mRNA Sample Preparation Kit (Illumina Inc., San Diego, CA) per the manufacturer’s instructions. Next, libraries were sequenced (75 bp, single-end) on the Illumina NextSeq500 platform, and FASTQ files were assessed by Trim Galore! v0.4.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequencing of the JH group of experimental 3 was not possible as the creation of a library for RNA-Seq failed. Thus, we omitted the data of this group. Finally, we analyzed gene expression using three biological replicates in the control group and two biological replicates in the JH group. Notably, the *C. floridanum* genome (GCF_000648655.2) sequence is

**Fig. 6** The model for the molecular mechanism of polyembryogenesis in *Copidosoma floridanum*. a The embryonic cell and the extraembryonic syncytium are attached in the morula. b The juvenile hormone (JH) loosens the cell attachment between the embryonic cells and the extraembryonic syncytium. c The extraembryonic syncytium invaginates into the morula. d After the inside of the extraembryonic syncytium has fused, e each cell is separated and polymorula are formed.
available in the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Copidosoma_floridanum/101/). The obtained FASTQ sequence files were aligned to the genomic reference sequence by HISAT2 v2.1.0 with default parameters [32]. Next, SAM files were converted to BAM files by Samtools v1.8 [33]. Using StringTie v1.3.4, we estimated the transcript abundance, and the count data were extracted by Subread v1.6.0 [34, 35]. All statistical analyses were performed using R software version 3.4.3 (https://www.r-project.org). To normalize the data and compare the control and JH-treated groups, we used the TCC and DEseq2 packages [36]. We generated a scatter plot using TIBCO Spotfire Desktop v7.6.0 with the “Better World” program license (TIBCO Spot re, Inc., Palo Alto, CA; http://spotfire.tibco.com/better-world-donation-program/). Furthermore, the sequence data (FASTQ files) were deposited to the DDBJ Sequence Read Archive (accession numbers DRR138914-DRR138918).

C. floridanum gene functional annotation pipeline for molecular network analysis
The transcripts were extracted from the C. floridanum genome using gffread software (https://github.com/gper tea/gffread). To annotate C. floridanum gene, we identified genes homologous to those of human by conducting a systematic BLAST search (tblastx) with a cutoff E-value of significant similarity at 1e−10 (query: C. floridanum cDNA sequence; database: whole human cDNA sequence set from Ensembl database). Using the generated assignment table, we reconstructed conserved pathways common to C. floridanum and humans by projecting C. floridanum genes onto the human pathway.

Gene enrichment analysis and pathway analysis
We performed the gene enrichment analysis using Metascape (http://metascape.org/); a gene list for Metascape analysis was generated from the TCC output. Then, we performed the molecular network analysis using IntAct Molecular Interaction Database (https://www.ebi.ac.uk/intact/) and Cytoscape v3.6.1 (http://www.cytoscape.org). We converted the gene IDs from the C. floridanum RNA-Seq data to human Ensembl IDs, using the assignment table described above and, then, input the list of genes obtained from the RNA-Seq data into Cytoscape to obtain the significant molecular interactions with corresponding E values.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-6559-3.

Additional file 1: Table S1. C. floridanum genes assign to the human gene. Up-regulated genes and down-regulated genes were assigned to the human gene. These genes were used for gene set enrichment analysis.

Additional file 2: Figure S1. Polymorula was accelerated by the juvenile hormone (JH) treatment in the culture condition. The early embryo of the two-cell stage was cultured with or without JH or Eclysone. The number of polymorula was counted and plotted on the graph. Solid line, JH treatment; dashed line, treatment without JH; wide dashed line, treatment Eclyson, vertical axis, a rate of polymorula (%); horizontal axis, the culture period (day).

Additional file 3: Figure S2. Expression of Krueppel homolog 1-like genes, and retinoic acid receptor RXR-alpha-B in juvenile hormone (JH) treatment of molura. The y-axis indicates the ratio of the average Transcripts Per Kilobase Million (TPM) values for molura between the control and JH treatment groups.

Abbreviations
DEG: Differentially expressed genes; GO: Gene ontology; JH: Juvenile hormone; RQ: Relative quantification; RXR: Retinoid X receptor; SNARE: Sensitive factor attachment protein receptor; TPM: Transcripts per kilobase million

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Authors’ contributions
Conceived and designed the experiments: TS, HT, and KI. Performed the experiments: TS and MN. Contributed reagents/materials/analysis tools: JY, KI, and HT. Analyzed the data: TS, HB, TN and HT. Contributed to the writing of the paper under draft version: TS and HT. All authors discussed the data and helped with manuscript preparation. KI and HT supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials
The RNA-seq reads supporting the conclusions of this article are available in the Sequence Read Archive (SRA) with accession IDs: DRR138914, DRR138915, and DRR138916; control samples, DRR138917 and DRR138918; JH-treated samples.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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