Transcriptome Analysis Reveals the Endocrine Regulation on the Expression of IAG in *Litopenaeus vannamei*

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Abstract: The insulin-like androgenic gland hormone (IAG) plays a key role in male sexual differentiation and spermatogenesis in crustaceans. The expression of IAG is usually negatively regulated by neuropeptide hormones through the "eyestalk-AG" endocrine axis. However, the underlying mechanism is still largely unknown. In the present study, we performed a comparative transcriptome analysis on the androgenic gland (AG), the main secretory organ of IAG, of *L. vannamei* before and after unilateral eyestalk ablation. A total of 67 differentially expressed genes (DEGs) were identified, including some putative genes involved in sexual development. Interestingly, several genes related to molting and endocrine processes were found differentially expressed between the two treatments. Further investigation on the expression profiles of these genes and *Lv-IAG* showed that their expression patterns were closely correlated throughout the molting cycle. In addition, injection of 20-hydroxyecdysone (20E) could inhibit the expression of *Lv-IAG* at different molting stages in a dosage-dependent manner. The data indicated that ecdysteroids played important roles in regulating the *Lv-IAG* expression and might bridge the endocrine axis between eyestalk and AG. The present study provided new insights into understanding the regulation of sexual development in male crustacean.

Keywords: insulin-like androgenic gland hormone; sexual development; endocrine axis; ecdysteroids; crustacean

1. Introduction

Sexual dimorphism is a common phenomenon in decapod crustaceans [1]. *Litopenaeus vannamei* is one of the most successful commercial crustaceans in the world due to its high growth rate, resistance to diseases, and environmental stress, and adaptability to low salinity cultivation [2]. The female *L. vannamei* shows obvious superiority in growth rate comparing to male shrimp after sexual maturity [3]. Therefore, clarifying the molecular mechanism of shrimp sex determination and sex differentiation is essential for the development of its monosex culture techniques.

In crustacean, the androgenic gland (AG) is an essential player in male sex differentiation processes. The insulin-like androgenic gland hormone (IAG), which is secreted by AG, is regarded as the sexual "IAG-switch" during sex differentiation of crustaceans [4]. The silencing of *IAG* could induce full functional sex reversal in *Macrobrachium rosenbergii* [5]. Except for its function in sex differentiation at the early developmental stage, IAG also plays important roles in the maintenance of male characters and spermatogenesis at the

adult stage of male crustaceans [6–9]. The implantation of AG or injection of an active extract of AG induces masculinization and inhibits the development of female secondary sex characteristics in crustaceans [10,11].

Although several homologs in the sex determination pathway, including *Dsx*, *Sxl*, *Dmrt11E*, and Sox9, have been identified to participate in IAG expression in some crustaceans [12–14], it seems that the expression of IAG is mainly regulated by the "eyestalk-AG-testis" endocrine system [15]. Eyestalk ablation could cause hypertrophy and alter the cell types of AG and enhance RNA synthesis in AG cells of *Pandalus platyceros*, *L. vannamei*, *Portunus pelagicus*, *Macrobrachium rosenbergii*, and *Cherax quadricarinatus* [16–19]. Additionally, eyestalk ablation could also greatly promote the expression of IAG in *Callinectes sapidus* and *Portunus trituberculatus* [17,20].

The X-organ/sinus-gland (XO-SG) complex, located in the eyestalk of crustaceans, works as the center of the neuroendocrine system to regulate various physiological processes, including metabolism, molting cycle, sexual differentiation, and reproductive development [4,21,22]. Recently, several neuro-hormones secreted by the XO-SG complex, such as the crustacean hyperglycemic hormones (CHHs) [23,24], crustacean female sex hormone (CFSH) [25,26], gonad-inhibiting hormone (GIH), and molt-inhibiting hormone (MIH) [27], have been proved to be negative regulatory factors of IAG expression. As the XO-SG complex locates in the eyestalk, while the AG attaches to the distal vas deferens and terminal ampoule, there should be an efficient regulatory strategy to achieve an accurate regulation. However, the underlying molecular mechanism is still largely unknown.

In order to learn how the factors inside the eyestalk affect the expression of IAG, we performed a comparative transcriptome analysis on AG of *L. vannamei* after unilateral eyestalk ablation. We found that some genes related to molting and endocrine processes showed differential expressions in AG of shrimp after eyestalk ablation, as well as several putative genes involved in male sexual development. Further study showed that IAG expression in AG could be regulated by ecdysteroids. The data indicated the important roles of ecdysteroids in regulating *Lv-IAG* expression. These results might bridge the endocrine axis between eyestalk and AG, which will provide new insights into understanding the regulation of sex differentiation in male crustaceans.

2. Results

2.1. Transcriptome Data Mapping and Analysis

Six samples, including eAG1, eAG2, eAG3, cAG1, cAG2, and cAG3, were sequenced on the Illumina Hiseq 2500 sequencing platform. A total of 114,939,060 and 126,419,470 raw reads from a unilateral eyestalk ablation (ESA) group and a control group were obtained by RNA-sequencing, respectively. After filtering low-quality reads and adaptors, 113,214,646 and 123,993,288 clean reads were obtained in each group. About 87.85% and 87.76% of the total clean reads from two groups were mapped to the reference genome (Genebank accession: PRJNA438564). The sequencing and mapping output of all samples are summarized in Table 1.

Table 1. Summary of the sequencing and mapping output of all samples.

Sample	Raw Reads	Clean Reads	Q20 (%)	Q30 (%)	Total Mapped	GC Content (%)
eAG1	39,144,350	38,623,336	97.64	93.61	32,983,645 (88.06%)	48.65
eAG2	38,000,328	37,384,452	97.48	93.28	31,424,401 (87.06%)	47.65
eAG3	37,794,382	37,206,858	97.53	93.47	32,130,046 (88.44%)	48.77
cAG1	42,634,574	41,927,468	97.50	93.34	36,357,115 (88.87%)	49.85
cAG2	38,989,158	38,455,262	97.74	93.84	31,867,585 (86.36%)	49.13
cAG3	44,795,738	43,610,558	97.53	93.63	37,288,555 (88.04%)	50.29

2.2. Identification of Differentially Expressed Genes (DEGs)

As shown in Figure 1A, 67 genes were identified with a significant difference ($p < 0.05$) between the two groups, including 45 up-regulated genes and 22 down-regulated genes,

respectively. The gene accession number, annotation, and differential expression changes of all the identified DEGs are summarized in Table S1. Among these DEGs, 61 genes could be annotated with known functions. As expected, the expression level of *Lv-IAG* in the ESA group was significantly higher (Table S1) than that in the control group, which was consistent with the expression trends of *Lv-IAG* in the two groups detected by qPCR analysis (Figure 1B). The data showed that the identified DEGs were reliable.
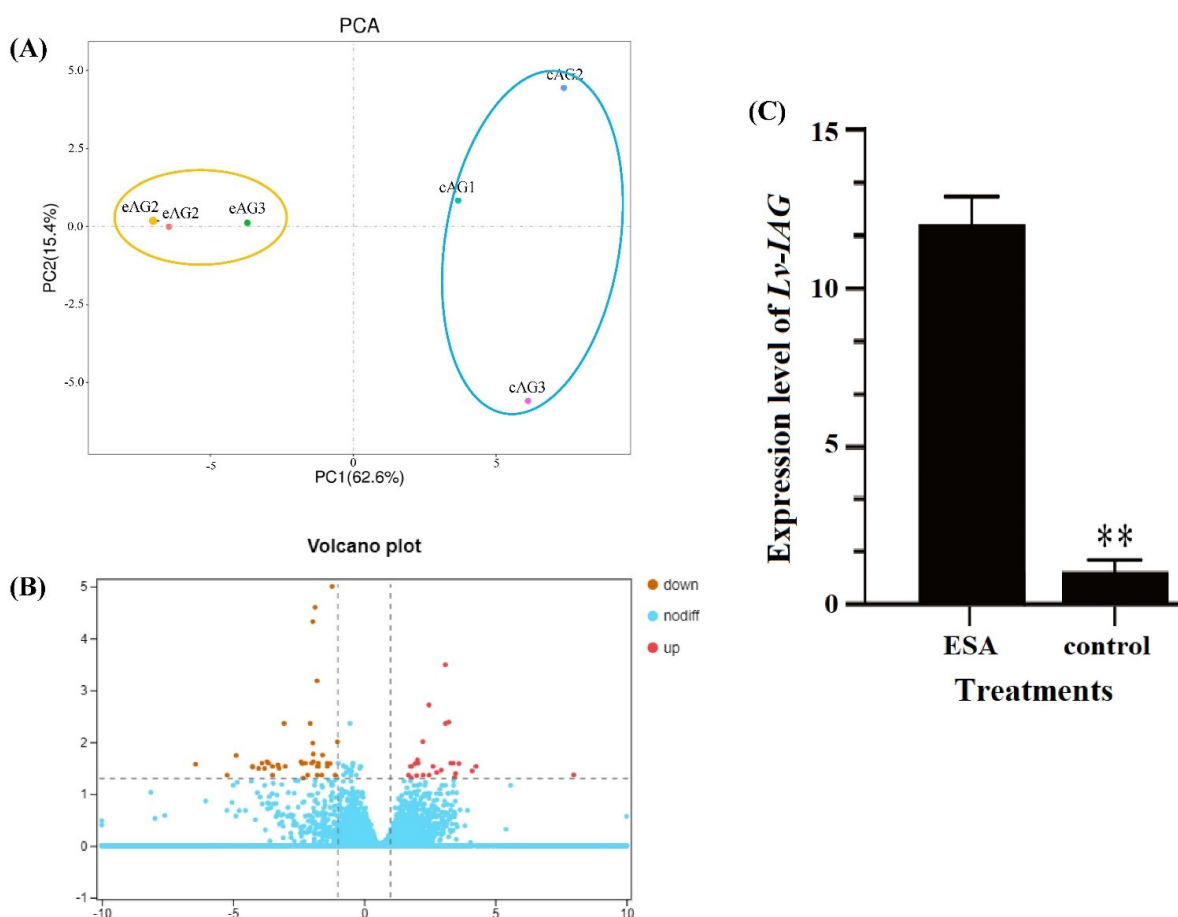
Figure 1. Principal component analysis (PCA) of the transcriptome, identification of differentially expressed genes (DEGs), and confirmation of *Lv-IAG* expression by RT-qPCR. (**A**) shows the PCA result of the ESA group (eAG1, eAG2, and eAG3) and the control group (cAG1, cAG2, and cAG3). The first two axes account for 15.4% (PC1) and 62.6% (PC2) of the variation. (**B**) shows the expression volcano plot of DEGs between the unilateral eyestalk ablation group and the control group. The abscissa represents the logarithm of the expression fold change of DEGs relative to control. The ordinate represents the negative logarithm of the statistical significance of the expression difference. The red dots represent up-regulated DEGs, the yellow dots represent down-regulated DEGs, and the blue dots represent non-DEGs. (**C**) shows the *Lv-IAG* expression profiles validated by RT-qPCR. Data are represented as means of fold change and standard deviations (*n* = 3). Statistical difference was determined using Student's *t*-test (** *p* < 0.01).

2.3. Identification of DEGs Related to Male Differentiation

Based on the functional annotation and published literature, 20 DGEs with annotation were identified as putative target genes related to sexual development and endocrine system (Table 2). Among them, most DEGs related to sexual development were up-regulated after the unilateral eyestalk ablation, like the expression trend of *Lv-IAG*. Only two DEGs in this category, zinc finger and SCAN domain-containing protein 31 and zinc finger/CW type with PWWP domain 1, were down-regulated after unilateral eyestalk ablation. Eleven DEGs related to endocrinology were identified, including the molting-related hormone receptor 4 (*Lv-Hr4*), several dopamine-metabolism-related genes, and

one co-receptor of the androgen receptor proliferation-associated protein 2G4. *Lv-Hr4* was down-regulated, whereas all dopamine-metabolism-related genes were up-regulated.

Table 2. Identified DEGs related to sexual development and endocrinology.

Categories	Gene Symbol	Accession Number	Annotation	Log2 (Fold Change ESA/Control)
Sexual development	*Lv-IAG*	MSTRG.32535	insulin-like androgenic gland hormone	2.41
	Lv-Mrr1	ROT78373.1	male reproductive-related protein A	3.09
	Lv-Mrr2	MSTRG.11644	male reproductive-related protein A	3.3
	Lv-GGCX	ROT82257.1	Vitamin K-dependent gamma-carboxylase	3.3
	Lv-Calu	ROT83784.1	Calumenin	2.76
	Lv-Hsp90-1	ROT62469.1	heat shock protein 90	2.76
	Lv-Hsp70	ROT68844.1	heat shock protein 70	3.1
	Lv-Hsp90-2	ROT76137.1	heat shock protein 90	2.63
	Lv-ZSCAN31	ROT76330.1	zinc finger and SCAN domain-containing protein 31	−4.18
	Lv-ZCWPW1	MSTRG.12712	zinc finger/CW type with PWWP domain 1	−1.89
Endocrine system	*Lv-Hr4*	ROT82234.1	hormone receptor 4	−1.75
	Lv-SPI	ROT62953.1	serine proteinase inhibitor	2.47
	Lv-SPI3	ROT75925.1	serine proteinase inhibitor-3	−6.43
	Lv-DBH1	ROT77266.1	dopamine beta-hydroxylase	2.35
	Lv-DBH2	ROT77268.1	dopamine beta-hydroxylase	2.75
	Lv-DBH3	ROT77267.1	dopamine beta-hydroxylase	2.54
	Lv-GRWD1	ROT82204.1	glutamate-rich WD repeat-containing protein 1	2.47
	Lv-LRP2	ROT73017.1	lipoprotein receptor-related protein 2	−4.25
	Lv-PA2G4	ROT64438.1	proliferation-associated protein 2G4	1.79
	Lv-ANK3	ROT77030.1	ankyrin-3-like isoform X1	−3.05

2.4. Expression Profiles of Selected Genes at Different Molt Stages

As the transcriptome data showed that some genes related to endocrine, especially the molting process, were identified as DEGs, we firstly detected the expression pattern of *Lv-IAG* in AG of the shrimp throughout the molting cycle. As shown in Figure 2, the expression of *Lv-IAG* showed the highest level at the late pre-molt stage (D_{3-4}) and rapidly declined at the post-molt stage (P_{1-2}) and inter-molt stage (C), whereas it started to increase at the early and middle pre-molt stage (D_{0-2}).

In order to know the expression correlation between *Lv-IAG* and endocrine-related DEGs, we detected the expression profiles of *Lv-Hr4* and three dopamine beta-hydroxylase genes *(Lv-DBH1, Lv-DBH2, Lv-DBH3)* throughout the molting cycle. As shown in Figure 2, the expression pattern of *Lv-Hr4* showed almost an opposite trend. It showed the highest expression level at the inter-molt stage, declined to the lowest level at the late pre-molt stage, and started to increase at the post-molt stage. The expression of three *Lv-DBH* genes showed similar expression profiles as that of *Lv-IAG*.

2.5. Lv-IAG Expression Profile in Molting Cycle with 20E Treatment

As the expression trends of endocrine-related genes were closely related to the expression pattern of *Lv-IAG* throughout the molting cycle, we further detected whether a regulation relation existed between the molting and male development. The expression level of the internal gene 18S rRNA among all groups was shown in Figure S1. The results showed that a low dosage (1 μg) of 20E injection could inhibit the expression of *Lv-IAG* at pre-molt and post-molt stages, and high dosage (5 μg) of 20E injection could significantly decrease the expression levels of *Lv-IAG* throughout the whole molting cycle (Figure 3A).

As the ecdysteroids activating gene, *Lv-Hr4* was positively regulated by 20E injection at different molting stages (Figure 3B).
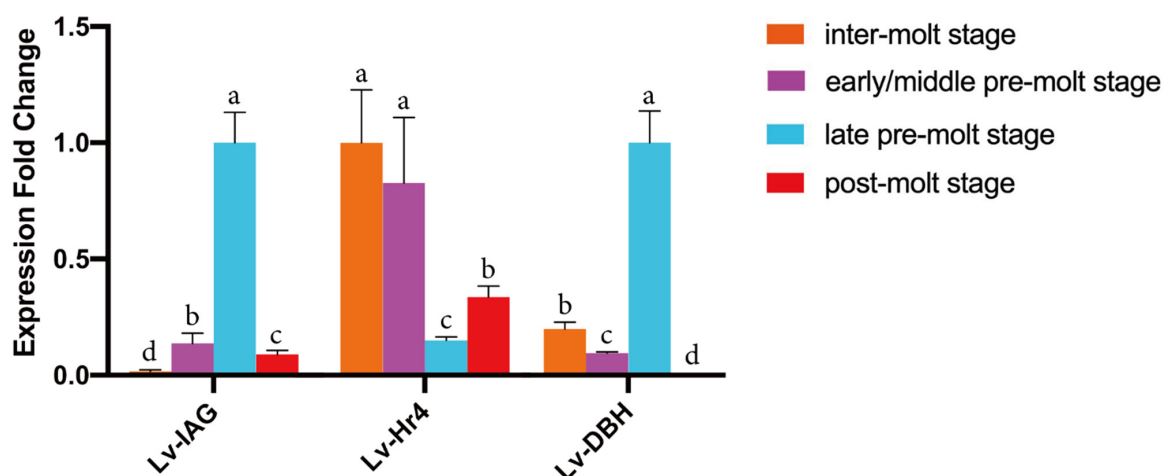
Figure 2. Expression profiles of *Lv-IAG*, *Lv-Hr4*, and three *Lv-DBH* genes in AG at different molting stages. Data are represented as means and standard deviations. Statistical differences were determined using Student's *t*-test. The different letters indicate significant differences in gene expression level at the same molt stage ($n = 3$).
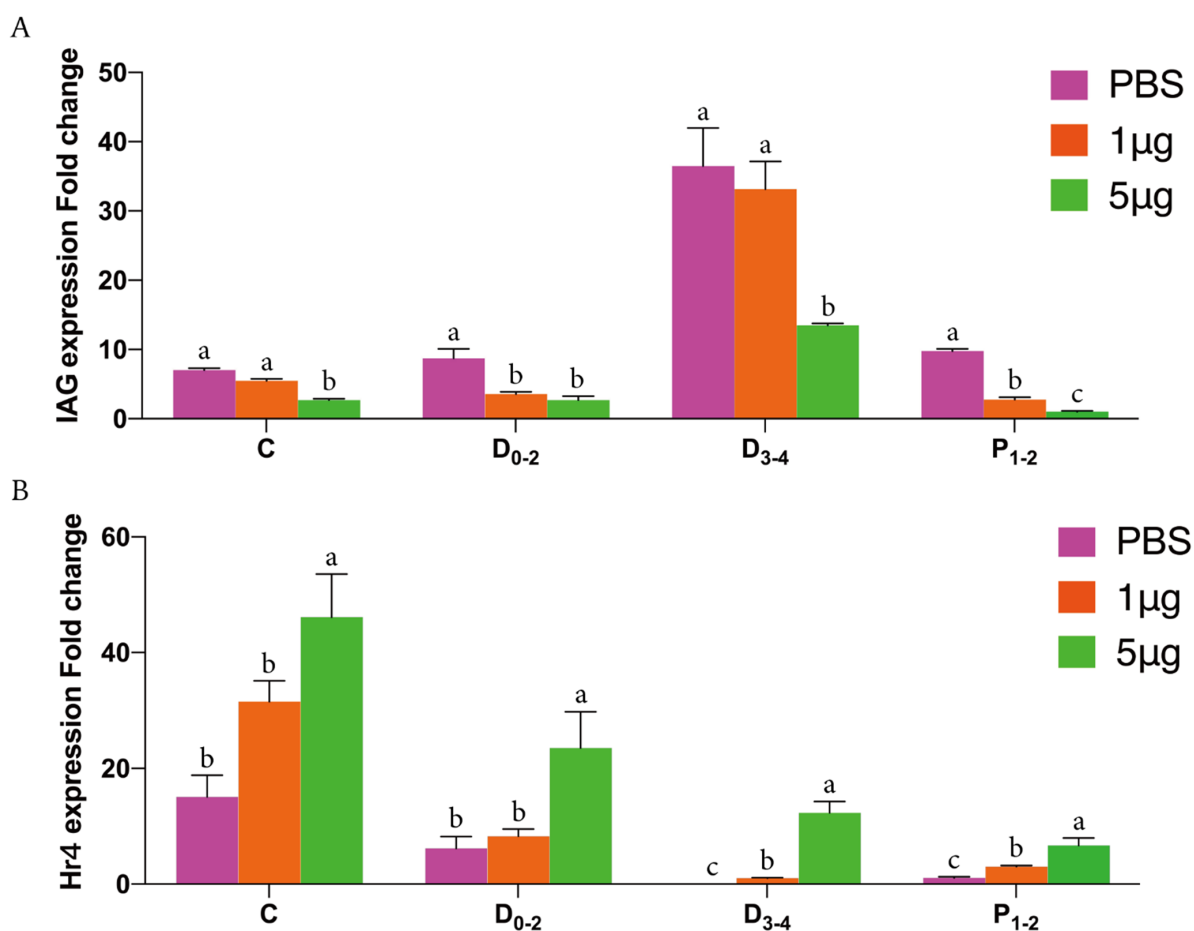
Figure 3. The 20E injection regulated the expression of *Lv-IAG* (**A**) and *Lv-Hr4* (**B**) throughout the molting cycle. Two dosages of 20E (1 μg and 5 μg per individual, respectively) were used, and an equal volume of PBS was injected as a negative control. Data are represented as means and standard deviations. Statistical differences were determined using Student's *t*-test. The different letters indicate significant differences in the gene expression level at the same molt stage ($n = 3$).

3. Discussion

More and more evidence indicates that the "eyestalk-AG-testis" endocrine axis in crustaceans plays an important role in regulating male sexual development by secreting peptide hormones from the X-organ/sinus-gland (XO-SG) complex of eyestalk [15]. However, it is not clear whether these neurohormones directly function on AG. In order to know how the eyestalk affects *Lv-IAG* expression, we performed a comparative transcriptome analysis between AG tissues in shrimp before and after unilateral eyestalk ablation to identify genes related to *Lv-IAG* expression inside the AG tissue.

Some identified DEGs have been reported to be involved in sex development in crustaceans or other animals. Besides *Lv-IAG*, two genes encoding male reproductive-related (*Mrr*) protein A were also up-regulated after unilateral eyestalk ablation. *Mar-Mrr* was identified as a sex-specific gene that was first detected in the epithelial cells of the sperm duct and terminal ampulla of *M. rosenbergii* [28] and later found to be more likely involved in acrosomal activation during fertilization in *P. pelagicus* [29]. Three heat shock proteins were also up-regulated upon unilateral eyestalk ablation, which have been reported to be involved in sexual differentiation of medaka since they could induce cortisol levels by elevating the expression of the corticotropin-releasing hormone b in the hypothalamus [30].

Two genes related to vitamin K metabolism, vitamin K-dependent gamma-carboxylase, and calumenin were also positively regulated by unilateral eyestalk ablation. Vitamin K-dependent gamma-carboxylase is a kind of enzyme catalyzing γ-carboxylation with vitamin K as a co-factor, which is evolutionary conservative and widely found in both vertebrates and invertebrates. In the lobster *Sagmariasus verreauxi*, Vitamin K-dependent gamma-carboxylase showed an AG-biased expression profile [31]. In fish, it is exclusively expressed in pituitary and gonad tissues, indicating its function in reproduction [32,33]. In mice, it is essential for epididymal sperm maturation [34]. The data showed that all the above-mentioned genes exhibited up-regulation trends like *Lv-IAG* after the inhibitory effect of eyestalk decreased by unilateral eyestalk ablation. These results indicated that these genes, like *Lv-IAG*, might be regulatory targets of the "eyestalk-AG" endocrine axis.

According to the endocrine axis hypothesis, some genes related to the transduction of extracellular signals from eyestalk should have existed in the androgenic gland. Interestingly, several genes related to endocrinology were differentially expressed after unilateral eyestalk ablation. *Lv-Hr4* encodes a downstream nuclear receptor of the ecdysteroid signaling transition [35,36], and its expression could be induced by ecdysteroids. Ecdysteroids are a kind of steroid hormone and play important roles in many biological processes including molting and reproduction in arthropods. In crustaceans, ecdysteroids are secreted by the Y-organ, and the process is mainly under the control of neuropeptide hormones, including MIH and CHH, which are produced in the XO-SG complex [37–40]. The coordination of reproduction and molt exists both in the female decapods [41] and in male ones. In the spider crab *Libinia emarginat*, ecdysteroids were mainly found in the testis of non-reproductive unabraded males rather than that in the actively mating abraded ones, indicating the role of ecdysteroids in gonad maturation in unabraded animals [42]. In *L. vannamei*, the reproductive cycle of male individuals is strictly associated with their molting cycle [43]. These data suggest that a coordination mechanism probably exists in shrimps to regulate the relationship between the molt cycle and male sexual development process, which is also supported by the expression profile of *Lv-IAG* and the inhibitory effect of 20E on *Lv-IAG* expression throughout the whole molting cycle.

In crustaceans, the ecdysteroids level changes periodically along with the molting process. Starting from basal levels at the post-molt stage, the level of ecdysteroids increases rapidly at the early pre-molt stage, followed by a sharp decline back to basal levels prior to ecdysis [44,45]. In the present study, *Lv-IAG* was mainly detected at the late pre-molt stage, which is also the time when ecdysteroids decline. The hemolymph titer of MIH, the inhibitory neuropeptide for both ecdysteroids synthesis and *IAG* expression, was decreased to the lowest level at the early pre-molt stage but kept at high levels at other molting stages in *Procambarus clarkii* [45]. Accordingly, the decrease in neuropeptide hormones secreted

by the X-organ/sinus gland complex activates synthesis of ecdysteroids in the Y-organ, while the decline in ecdysteroids levels leads to the high expression of *IAG* in the AG tissue (Figure 4A). Therefore, we deduce that ecdysteroids might mediate the inhibitory regulation of neuropeptide hormones on the expression of *Lv-IAG*. In other words, an "X-organ/sinus gland–Y-organ–AG" endocrine axis (Figure 4B) should exist in shrimp to regulate the *IAG* expression and the subsequent male sexual differentiation. Further investigations are necessary to provide more evidence on the hypothesis.
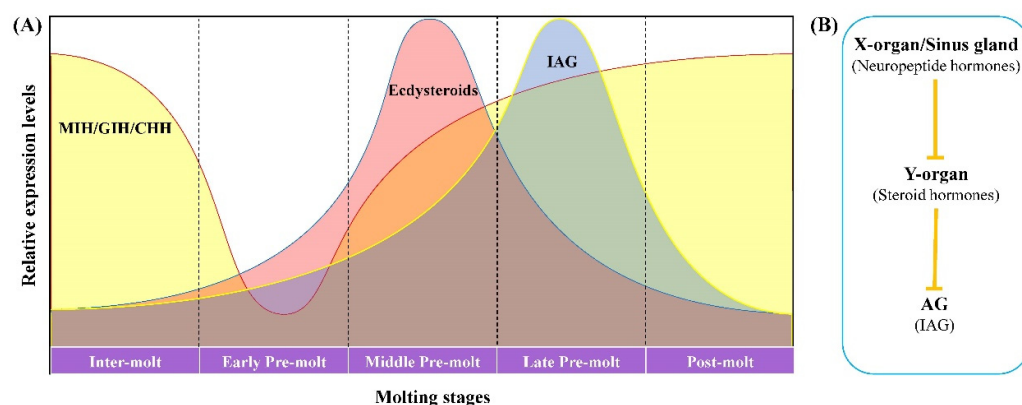
Figure 4. The hypothesis of the regulatory mechanism between the secretion of neuropeptide hormones and the *Lv-IAG* expression level via the mediating role of ecdysteroids. (**A**) shows the expression profile of the inhibitory neuropeptides, ecdysteroids, and *Lv-IAG* throughout the molting cycle of male *L. vannamei.* (**B**) shows a diagram illustrating the hypothetical "X-organ/sinus gland–Y-organ–AG" endocrine axis. The biosynthesis of ecdysteroids was triggered by the decreased secretion of neuropeptide hormones from X-organ/sinus gland complex at the early pre-molt stage. When the concentration of ecdysteroids decreased dramatically at the late pre-molt stage, the suppression effect of ecdysteroids on *Lv-IAG* expression was eliminated.

4. Materials and Methods

4.1. Experimental Animals

Healthy adult male shrimps were bought from an aquaculture farm nearby Qingdao city and kept in the aerated aquarium of our laboratory at a temperature of 25 °C for at least one week before experiments. Different sizes of shrimp were used since different experiments were not performed at the same time. Male individuals with a bodyweight of 10.5 ± 1.5 g were used for a unilateral eyestalk ablation experiment, male individuals with a bodyweight of 7.0 ± 1.0 g were used for collecting AG tissues at different molting stages, and male shrimps with a bodyweight of 9.0 ± 0.8 g were used in the 20-hydroxyecdysone (20E) injection experiment.

4.2. Unilateral Eyestalk Ablation

Eighty male shrimp individuals at the intermolt stage were used and separated into two groups: the unilateral eyestalk ablation (ESA) group and the control group. In the ESA group, the left eyestalk of shrimp was ablated. The AG tissue of shrimp in two groups was collected individually at 7 days after the ablation treatment.

4.3. Total RNA Preparation and cDNA Synthesis

The total RNA from each AG sample was extracted with RNAisol reagent (Takara, Japan) using the recommended manufacturer's instruction. The quality of RNA samples was identified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis on 1% agarose gel. One µg total RNA from each sample was used for cDNA synthesis. The cDNA was synthesized following the manufacturer's protocol of the PrimeScripxt RT reagent Kit with a gDNA Eraser kit (Takara, Japan).

4.4. RNA-Sequencing

Before performing the RNA-sequence, the expression levels of *Lv-IAG* in all AG samples were detected via RT-qPCR and calculated according to the $2^{-\Delta\Delta Ct}$ method [46]. After analyzing the relative expression of *Lv-IAG* in all AG samples, nine samples with high *Lv-IAG* expression levels in the ESA group and nine samples with low *Lv-IAG* expression levels in the control group were selected, respectively. Then the equal amount of total RNA from three samples in each group was mixed, and three biological replicates were prepared for each group. Three replicates from the ESA group were named eAG1, eAG2, and eAG3, and three replicates from the control group were named cAG1, cAG2, and cAG3.

The quantity and integrity of RNA samples were evaluated by Qubit2.0 Fluorometer and Agilent 2100 Bioanalyzer. Then, mRNA was enriched using magnetic beads with Oligo(dT) and broken into short fragments by ultrasound. The first-strand cDNA was synthesized in an M-MuLV reverse transcriptase system with random oligonucleotides as primers. Then the second cDNA strand was synthesized in the DNA polymerase I system after the RNA strands were eliminated by RNaseH. After purification, end repairment Poly(A) tail was added and connected to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced on the Illumina Hiseq 2500 by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China).

4.5. Bioinformatic Analyses and DEGs Identification

Clean reads were obtained after removing the adaptor and filtering raw reads with more than 10% of unknown nucleotides and low-quality raw reads (Q-value $\leq$ 20) by fastp (version 0.18.0). Ribosome RNA (rRNA) was mapped to the ribosome RNA (rRNA) database and removed by using the short reads alignment tool Bowtie2 (version 2.2.8). Then paired-end clean reads were mapped to the reference genome (Accession number: PRJNA438564) using HISAT2. Gene expression levels were obtained by calculating the fragments per kilobase of exon model per million mapped reads (FPKM) value. The differentially expressed genes (DEGs) were analyzed by DESeq2 [47] software. Genes/transcripts with the parameter of false discovery rate (FDR) less than 0.05 and absolute log2 (fold change) $\geq$1 were considered differentially expressed genes/transcripts. The method for adjusting *p*-values was according to Benjamini and Hochberg [48].

4.6. AG Collection at Different Molting Stages

In order to detect the expression profiles of selected genes in AG of shrimp at different molting stages, AG tissues of five males were collected from each of the four different molting stages separately, including inter-molt stage (C), early/middle pre-molt stage (D_{0-2}), late pre-molt stage (D_{3-4}), and post-molt stage (P_{1-2}) according to Gao et al. [49]. Extraction of the total RNA and synthesis of cDNA was performed on each sample according to 2.3.

4.7. 20-Hydroxyecdysone (20E) Injection

The pure powder of 20-hydroxyecdysone was bought from Aladdin Industrial Corporation (Shanghai, China) and dissolved in DMSO as a stock solution of 5 µg/µL. Before injection, the stock solution was dissolved with 1 $\times$ PBS into a suitable dosage. Animals at different molt stages were divided into four groups with 24 individuals in each group at one molting stage: inter-molt stage (C), early/middle pre-molt stage (D_{0-2}), late pre-molt stage (D_{3-4}), and post-molt stage (P_{1-2}). In each group, eight individuals were injected with 1 µg 20E, 5 µg 20E, or equal volume PBS, respectively. The doses of injected 20E were used according to previous studies [50]. At 48 h post-injection, AG tissue from six individuals was collected from each group and used as three biological replicates. The samples were used for total RNA extraction and cDNA synthesis according to Section 4.3.

4.8. RT-qPCR Analysis

RT-qPCR was performed to detect the expression levels of *Lv-IAG*, hormone receptor 4 (*Lv-Hr4*), and three dopamine beta-hydroxylase genes (*Lv-DBH1*, *Lv-DBH2*, *Lv-DBH3*).

The qPCR was performed on Eppendorf Mastercycler® ep realplex (Eppendorf, Germany) by using SuperReal PreMix Plus (SYBR Green, Toyobo, Japan) under the steps described below: denaturation at 94 °C for 1 min; 40 cycles of 94 °C for 20 s, annealing temperature for 20 s, and 72 °C for 20 s. As the internal reference gene, 18S rRNA was used. Primers were designed using Primer Premier 5 and are listed in Table 3. The specificity and accuracy of primers were determined by PCR assay and single peak in the melting curve of RT-qPCR assay.

Table 3. All primers used in this study.

Gene Symbol	Primer Name	Nucleotide Sequences (5'-3')	Annealing Temperature (°C)
Lv-IAG	Lv-IAG-qF Lv-IAG-qR	AGTGTCAAGGTCAGCCGATAC CGAGATTCCACGTTGGATTCAG	54.5 °C
Lv-18S	18S-F 18S-R	TATACGCTAGTGGAGCTGGAA GGGGAGGTAGTGACGAAAAAT	54.9 °C
Lv-Hr4	Lv-Hr4-F Lv-Hr4-R	AGACTCCCCAGCAGCTATGA GTTGCGCTGTGCCTTTGTAA	55.0 °C
Lv-DBH1	Lv-DBH1-qF Lv-DBH1-qR	TCGAAGCCGAAGACAGAACC AGGCGCTCACTTCTCTCAAC	56.0 °C
Lv-DBH2	Lv-DBH2-qF Lv-DBH2-qR	CAGCACCGATTTCAGCGATG ATCCTCTTCCCCTGGAGTCC	55.0 °C
Lv-DBH3	Lv-DBH3-qF Lv-DBH3-qR	TATGGTGCCAACATGCCCAT GGGACGATTTGGATTGGGGT	54.0 °C

4.9. Statistical Analysis

All real-time quantitative PCR experiments were performed with three replicates and normalized by using geometric averaging of the host gene 18S rRNA. The comparative Ct method was used with the formula $2^{-\triangle\triangle Ct}$ to calculate the relative expression level of the candidate genes [46]. Data of real-time quantitative PCR were statistically tested by one-way ANOVA, and p-value < 0.05 denoted a statistically significant difference.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse9060677/s1, Figure S1: Expression levels (Ct values) of the internal gene 18S rRNA in all groups after 20E injection. Table S1: Differentially expressed genes identified from the transcriptome analysis.

Author Contributions: Conceptualization, S.L. and F.L.; methodology, validation, formal analysis and data curation, K.C. and S.L.; writing—original draft preparation, K.C. and S.L.; writing—review and editing, S.L., J.X., A.S. and F.L.; funding acquisition, S.L. and F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Joint NSFC-ISF Research Program (31861143047), the National Key R&D Program of China (2018YFD0900202), and the China Agriculture Research System of MOF and MARA.

Institutional Review Board Statement: This study used shrimp and experimental animals, which is not endangered invertebrates. In addition, there is no genetically modified organism used in the study. According to the national regulation (Fisheries Law of the People's Republic of China), no permission is required to collect the animals and no formal ethics approval is required for this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest.

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