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Heart Failure-Like Reaction Is Likely Involved in the Feeding Behaviour of Blood-Sucking Leeches

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Abstract

Medicinal leeches have been utilized in therapy for thousands of years. However, the adaptation physiology between leeches and hosts is not fully understand. To disclose the molecular mechanisms of adaptation between leech and host, the body transcriptomes of hunger and fed blood-sucking *Poecilobdella javanica*, *Haemadipsa cavatuses*, and *Hirudo nipponia* leeches were obtained by RNA sequencing, after comparison, a stratified unigenes group was obtained, which closely correlated to body distension. In the group, Rfamide receptor decreased significantly ($P < 0.05$) while serotonin receptor increased significantly ($P < 0.05$). Moreover, four KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, including cardiac muscle contraction, complement and coagulation cascades, renin-angiotensin system, and hypertrophic cardiomyopathy were significantly enriched. The unigenes annotation, neuroregulators correlation analysis and induced function of the KEGG pathways, were consistently supported the same result as: vasoconstriction and systole reaction enhance in hunger leeches and vice versa vasodilation and diastole increase in fed leeches, meanwhile, Interspecific comparison and correlative analyses of physiological function showed that the strongest reaction of induced heart failure from four KEGG occur in strongest reaction of systole in hungry *P. javanica* and in strongest reaction of diastole in fed *H. nipponia.* Overall, heart failure is likely a physiological function involved in feeding behaviour.

Keywords

Blood-Sucking Leeches, Transcriptomic Analysis, Heart Failure, Feeding Behaviour

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1. Introduction

Leeches have been widely used for the treatment of diseases in cardiology, gynecology, urology, surgery, stomatology, and ophthalmology both in Europe and the United States [1], and *Hirudo medicinalis* is even officially approved by the Food and Drug Administration [2]. In traditional Chinese medicine, *Whitmania pigra*, *W. acranulata*, and *Hirudo nipponia* have been used for treating cardiovascular diseases and cerebral thrombosis over 1000 years [3]. *Poecilobdella javanica*, which belongs to Hirudinidae, is used in Indian blood-letting therapy [4]. *Haemadipsa cavatuses* is exclusively feeding on bats in caves, found near the border between China and Burma [5], which have a strong blood sucking ability, no medical reports used in therapy. Among the physiological functions of leeches used in medical treatment, blood sucking related functions, especially anticoagulant functions are mostly studied, but little is known about other adaptation physiology.

The anticoagulation system of blood-sucking leeches is the product of the co-evolution between leeches and their hosts. The saliva of leeches is known to contain anticoagulants. However, other sets of anticoagulants adapted to allow long-term blood storage should exist, in the leech body, and therefore the body wall should be examined. The tubular body wall of the adult leech is composed of four discrete layers: an outer epidermal layer, circumferential (circular) muscles, oblique muscles, and an inner layer of longitudinal muscles [6]. During periods of starvation, the gut of a locomoting leech can be empty and the animal can function as a muscular hydrostat in which the obliquely striated body wall musculature has mechanical properties similar to that of smooth muscles [7]. In addition, the leech body can distend tenfold from hunger to satiated with blood, and then shrink nearly to prototype after completely digesting the stored blood [8] [9]. Hungry leeches swim and bite more frequently during the blood-sucking phase of feeding; they extend their jaws, are less responsive to sensory input, produce mucus, and relax the body wall in a process that can last 30 - 45 min [10]. The blood sucked into the gut is desalted and then dehydrated, which concentrates the consumed blood; this concentrated blood does not coagulate, and some portions of red blood cells remain intact for several months [11]. When satiated, feeding-mediated distension inhibits swimming in medicinal leeches [12], indicating that the body wall has an autonomic regulation system, when removing central nervous system (CNS), residual amount of muscle activation [7]. In fact, serotonin, a known modulator of tension in leech muscles, affected the passive forces in all physiological muscles [13]. Overall, feeding-mediated body distension and contraction in hungry leeches, the positive autonomic musculature, and the function for pachyemia, contribute for the resemblance between the tubular body of the leech and the heart of mammals.

Previous transcriptome studies focused on specific leech tissues. The CNS and embryonic transcriptomes and 11,236 proteins of *H. medicinalis* were identified [14]. A complex cocktail of anticoagulants and other bioactive secreted proteins

including saratin, bdellin, destabilase, hirudin, decorsin, endoglucoronidase, antistatin, and eglin C were identified from the salivary transcriptome of *Macrobdella decora* [15]. In the present study, contraction in hungry leeches and extension after feeding were used as clues to examine and compare the body wall transcriptomes of *P. javanica*, *H. cavatuses*, and *H. nipponia*, based on second generation sequencing technology, aiming to provide a different perspective for leech research.

2. Materials and Methods

2.1. Leech Samples, Treatments, and RNA Extraction

Fourteen leech samples, including eight *P. javanica*, three *H. cavatuses*, and three *H. nipponia* samples, each comprising three biological replicates, were subject to three treatments, hunger, 30 min after feeding, 10 d after feeding, 20 d after feeding, 30 d after feeding, each performed in triplicate (Table 1), because during the actual feeding process, it has been observed that leeches maintain optimal viability for approximately 30 days after reaching satiety. Therefore, within a 30-day cycle, sampling and observations were conducted at 10-day intervals. These samples were collected from the south-western area of Yunnan Province, China, and starved for at least two months. Leeches subject to the feeding treatments were satiated by sucking blood from rabbit. Heads of all leeches were removed, bodies were opened, and the blood stored in their guts was washed off with deionized water and 75% ethanol.

RNA was extracted from all individuals immediately, using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To address DNA contamination and preserve RNA integrity, an optional step involves treating the extracted RNA with DNase enzyme, rapid tissue disruption using liquid nitrogen maintaining a sterile environment by using RNase-free consumables and disposable plastic ware. Following

Table 1. Samples used for transcriptome analysis.

Species	Status	Sample
P. javanica	Hunger	T2/T12
P. javanica.	30 min after fed	T3
P. javanica	10 d after fed	T5/T6
P. javanica	20 d after fed	T7
P. javanica	30 d after fed	T8/T9
H. nipponia	Hunger	T13/T14
H. nipponia	30 min after fed	T15
H. cavatuses	Hunger	T1
H. cavatuses	30 min after fed	T10/T11

Note: T2/T12, T5/T6, T8/T9, T13/T15, and T10/T11 are biological replicates; samples represent biological replicates from three leeches.

addition of Trizol reagent, efficient homogenization of the tissue sample ensures proper mixing and denaturation of proteins, facilitating efficient RNA isolation. Rapid phase separation using chloroform and prompt removal of the aqueous phase containing RNA help avoid extended exposure to RNases.

2.2. Library Preparation for Transcriptomic Analysis

For Illumina (Illumina, San Diego, CA, USA) RNA sequencing (RNA-seq), the quality and quantity of RNA was tested by an Agilent 2100 Bioanalyzer (400 $ng \cdot \mu l^{-1}$, Optical Density 260/280 = 1.8 - 2.2, RNA 28S: 18S ≥ 1.0, and RNA Integrity Number ≥ 7.0). Next, RNA was enriched using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA), and the cDNA library was built using the NEBNext mRNA Library Prep Master Mix Set for Illumina and NEBNext Multiplex Oligos for Illumina (New England Biolabs). The lengths of cDNA fragments were detected by 1% agarose gel electrophoresis, quantified using KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA), quality checked by Illuminacbot, and sequenced by Illumina HiSeq™ 2500. To mitigate potential biases associated with library preparation, random primers facilitate the unbiased synthesis of cDNA, allowing efficient capture of diverse RNA molecules. Meanwhile, the unique molecular identifiers (UMIs) were added to each individual RNA molecule before amplification. By tracking the UMIs, PCR duplicates can be identified and removed, minimizing amplification bias.

2.3. Illumina Reads Processing and De Novo Assembly

Original pair-end reads were selected based on quality scores, base distributions, data evaluations, and statistics. High-quality raw reads were separated from reads with adaptor contamination, unknown nucleotides, and low-quality reads with ambiguous sequence "Ns". High quality reads were assembled in Trinity [16]. Reads with low quality scores were filtered out to eliminate those with a high probability of sequencing errors (Q30 > 93.13%); reads below 30 bp are typically discarded; Bowtie was used to filter out rRNA or mitochondrial RNA contaminants.

2.4. Unigene Annotation and Analysis

Functional annotation was performed by sequence comparison against public databases, including the National Center for Biotechnology Information (NCBI) Non-redundant Protein database (Nr; https://www.ncbi.nlm.nih.gov/), the Swiss-Prot database (https://www.expasy.org/resources/uniprotkb-swiss-prot), the Clusters of Orthologous Groups database (COG; https://www.ncbi.nlm.nih.gov/research/cog-project/), and the Kyoto Encyclopedia of Genes and Genomes database (KEGG; https://www.genome.jp/kegg/), using the basic local alignment search tool (BLAST; E-value < 1e−5). Functional assignments were mapped onto the Gene Ontology database (GO;

https://www.geneontology.org/), and GO classification was performed using Web Gene Ontology Annotation Pilot (WEGO; https://wego.genomics.cn/). The reads were blasted to the unigene libraries by Bowtie [17], and their expression levels were estimated by RNA-seq using Expectation Maximization (RSEM) [18] and Reads Per Kilobase per Million (RPKM) mapped reads [19].

2.5. Differential Expression Analysis between Hunger and Blood-Fed Leeches

Data were separately assembled for each species, and DEGs between hunger and fed samples were detected using DESeq or EBSeq [20] [21] packages, based on a false discovery rate (FDR) < 0.01. To prevent false positive results, we used the Benjamini-Hochberg method [22] that uses the FDR value to correct the *p*-value of the original hypothesis test.

As the unique species of Hirudinidae, *Poecilobdella javanica* was chosen as representative species for studying the patterns of gene expression; samples including hungry and fed after 30 min, 10 d, 20 d, and 30 d leeches were sequenced. To examine variations in the patterns of gene expression after feeding, DEGs with |log2 fold change| ≥ 3 was selected for analysis. Comparisons between hunger and fed samples of all species were based on *t*-tests using $P < 0.05$ as the significance threshold.

2.6. Verification Analysis by Using Physiological Function Involved Gene

To verify the inferred physiological functions from the KEGG pathways enriched from DEGs between hungry and fed leeches, a batch of key genes, which can affect such physiological functions, was selected for verification analysis. In all retrieved annotated unigenes, the unigenes with highest RPKM values were select to represent expression of candidates.

For example, to verify the heart failure physiological functions which inferred from the four riched KEGG pathways, the verifying genes were choosed as: angiotensin-converting enzyme (ACE), neprilysin 1 (Nep1), neprilysin 2 (Nep2), catenin beta, extracellular matrix 3 (ECM-3), extracellular matrix alpha (ECM-A), and calmodulin (CaDK II); for verify the myocardial repair, the genes choose as: cyclin-dependent kinase 1 (CDK1), cyclin B (CCNB), cyclin D (CCND), cyclin-dependent kinase 1 (CDK5), neuregulin-1, and catenin alpha. Furthermore, physiological functions of heart repairing and excitation-contraction coupling were investigated, involving genes: protein kinase A (PKA), sorcin, NCX, FK506-binding protein 2 (FKBP2), calmodulin, titin, catenin alpha, troponin T, and troponin I.

2.7. Transcriptome Completeness Assessment

Using Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis by searching for a set of highly conserved genes present in all eukaryotic organisms and by comparing the assembled transcriptome against database of medical leech.

3. Results

3.1. Comparison of the Transcriptomes of the Three Leech Species Based on 14 Samples

Illumina RNA-seq provided 33.6 Gb for *P. javanica*, 20.3 Gb for *H. cavatuses*, and 14.58 Gb for *H. nipponia*. More than 93% of bases in *P. javanica* and *H. cavatuses* reads presented Q30 scores, whereas only 88% of bases in *H. nipponia* reads had Q30 scores. The 57,410 unigenes identified for *P. javanica* had an average length of 1413 bp, the 37,465 unigenes identified for *H. cavatuses* had an average length of 2529 bp, and the 39,812 unigenes identified for *H. nipponia* had an average length of 1012 bp (Table 2).

All unigenes identified from the three species showed significant similarity with sequences in the NR and Swiss-Prot databases (E-value < 1e−5). Some of these unigenes were annotated in the GO, COG, and KEGG databases: 33,712 (58.7%) from *P. javanica*, 22,260 (59.4%) from *H. cavatuses*, and 37,820 (95%) from *H. nipponia* (Table 3).

Table 2. Number and distribution of assembled unigenes in the three leech species.

Length (bp)	*P. javanica*	*H. nipponia*	*H. cavatuses*
200 - 300	14185	13569	8465
300 - 500	10160	8504	6903
500 - 1000	9069	6146	6146
1000 - 2000	10095	5564	7306
2000+	13895	6029	8643
Total Number	57,410	39,812	37465
Total Length (bp)	81,132,096	40,288,201	51053993
N50 Length (bp)	2749	2160	2529
Mean Length (bp)	1413.2049	1011.961	1362.711678

Table 3. Length distribution of annotated unigenes in public databases.

Database	*P. javanica*	*H. cavatuses*	*H. nipponia*
COG	11959	7779	10050
GO	14095	9449	15837
KEGG	12959	8967	14721
Swiss-Prot	25929	16915	24060
Nr	33177	21964	36395
All	33712	22260	37820

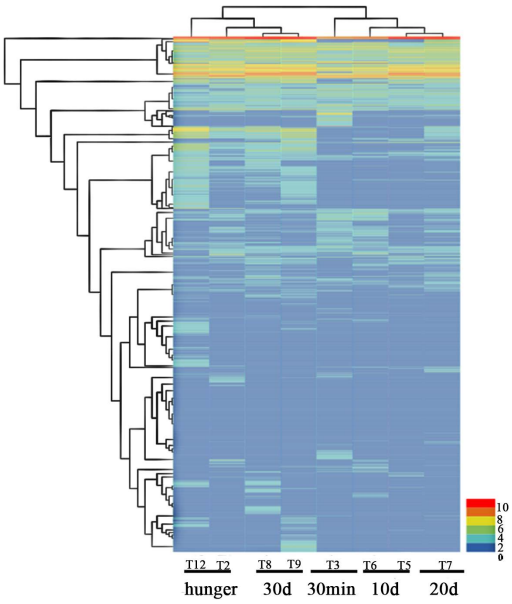
Nr, Non-redundant NCBI protein database; COG, Clusters of Orthologous Groups; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

3.2. DEG Analysis between Hunger and Blood-Fed Leeches

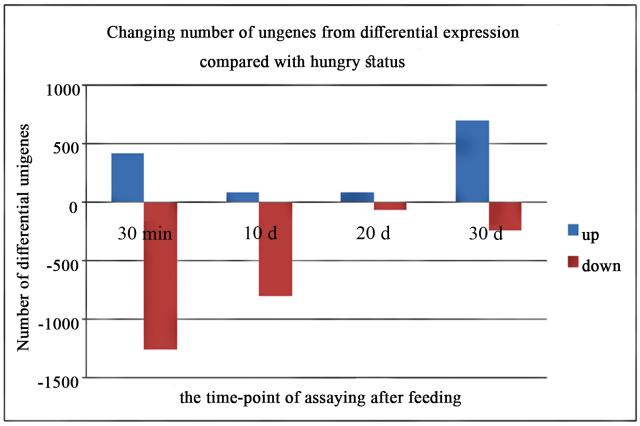
Cluster analysis of *P. javanica*, which was chosen as representative species for studying the patterns of gene expression, revealed that samples were grouped into a cluster including hungry and fed after 30 d leeches and another including fed after 30 min, 10 d, and 20 d leeches (**Figure 1(a)**). These results indicated that transcript levels changed from hunger to 30 min after feeding stages, but were restored after 30 d. Moreover, 1678, 863, 155, and 942 DEGs (|log2 fold change| ≥ 3) were obtained from the 30-min, 10-d, 20-d, and 30-d feeding groups of *P. javanica*, respectively. These results showed that the 30-min feeding group contained most DEGs while the 30-d feeding group contained the smallest number of DEGs (**Figure 1(b)**). Further analysis showed a set of unigenes up-regulated in the 30-min group (named as 30 min-up) and a set of down-regulated genes in the 30-min group, which started to up-regulate in the 10-d named as 10 d-up, which started to up-regulate in the 20-d group, named as 20 d-up, and few unigenes started to up-regulate in the 30-d (**Figure 1(b)**). The average RPKM showed that 30 min-up unigenes were down-regulated in 10 d, while 10 d-up and 20 d-up unigenes were restored in the 30-d group (**Figure 1(c)**); the few DEGs in the 30-d feeding group evidenced that most unigenes revealing variation patterns were restored to hunger status 30 d after feeding.

Highly expressed 30 min-up unigenes were not expressed or only slightly expressed in hungry leeches, while 10 d-up and 20 d-up unigenes were highly expressed in hungry leeches, as well as in 10 d and 20 d after feeding groups. Integrated function analysis based on Swiss-Prot and NR databases indicated that 20 d-up unigenes were mostly related to development and transcriptional regulation, suggesting that most of these unigenes were not involved in the stress-mediated distension regulation of the body wall. The 10 d-up unigenes, which were highly expressed in hungry leeches, are likely responsible for body shrinking in this stage, while 30 min-up unigenes are likely responsible for body distension in fed leeches. Functional annotation showed that 30 min-up and 10 d-up unigenes were mainly related to energy, anticoagulation, immunity, development, nerve system, signalling pathways, ion uptake, and transport. Among them, anticoagulation and nervous system were prominent, indicating that except anticoagulation, positive regulation for distension requires neuregulation (**Figure 1(d)**).

Therefore, the unigenes of neuregulation including gamma-aminobutyric acid (GABA), acetylcholine receptor, Rfamide, vasopressin, dopamine, and serotonin unigenes were investigated and their expression patterns were analysed by *t*-tests to evaluate differences among the three leech species. As a result, expression of Rfamide receptor decreased significantly ($P < 0.05$) and that of serotonin increased significantly ($P < 0.05$) after feeding (**Figure 2**). The FMRFamide peptides have an important role in heart regulation and body wall muscle modulation in leeches [23]. Serotonin, found in the mid body of the ganglion, is a known modulator of tension in leech muscles, affecting the passive forces



(a)



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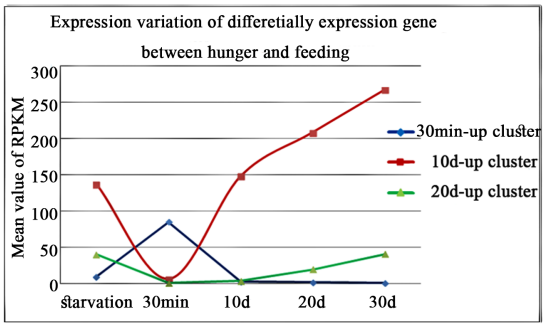

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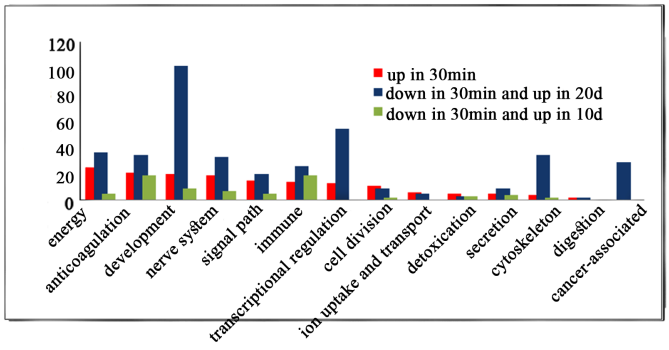

(d)

Figure 1. Comparative transcriptome analysis showing differentially expressed unigenes (DEGs) between hungry and post-30 d blood-feeding states of *P. javanica*. (a) Transcriptomic expression cluster analysis revealed two clusters—gene expression in hungry leeches and in those 30 d after feeding was similar, as well as in leeches 30 min, 10 d, and 20 d after feeding. (b - c) Expression analysis revealed three clusters of DEGs between hungry and fed leeches altered before 30 d, all of which returned to levels of expression in hungry leeches in 30 d. (d) Comprehensive functional annotation of genes in the "30 min-up", "10 d-up", and "20 d-up" unigene clusters revealed that the main DEGs were related to energy, anticoagulation, development, signal pathway, and immune.

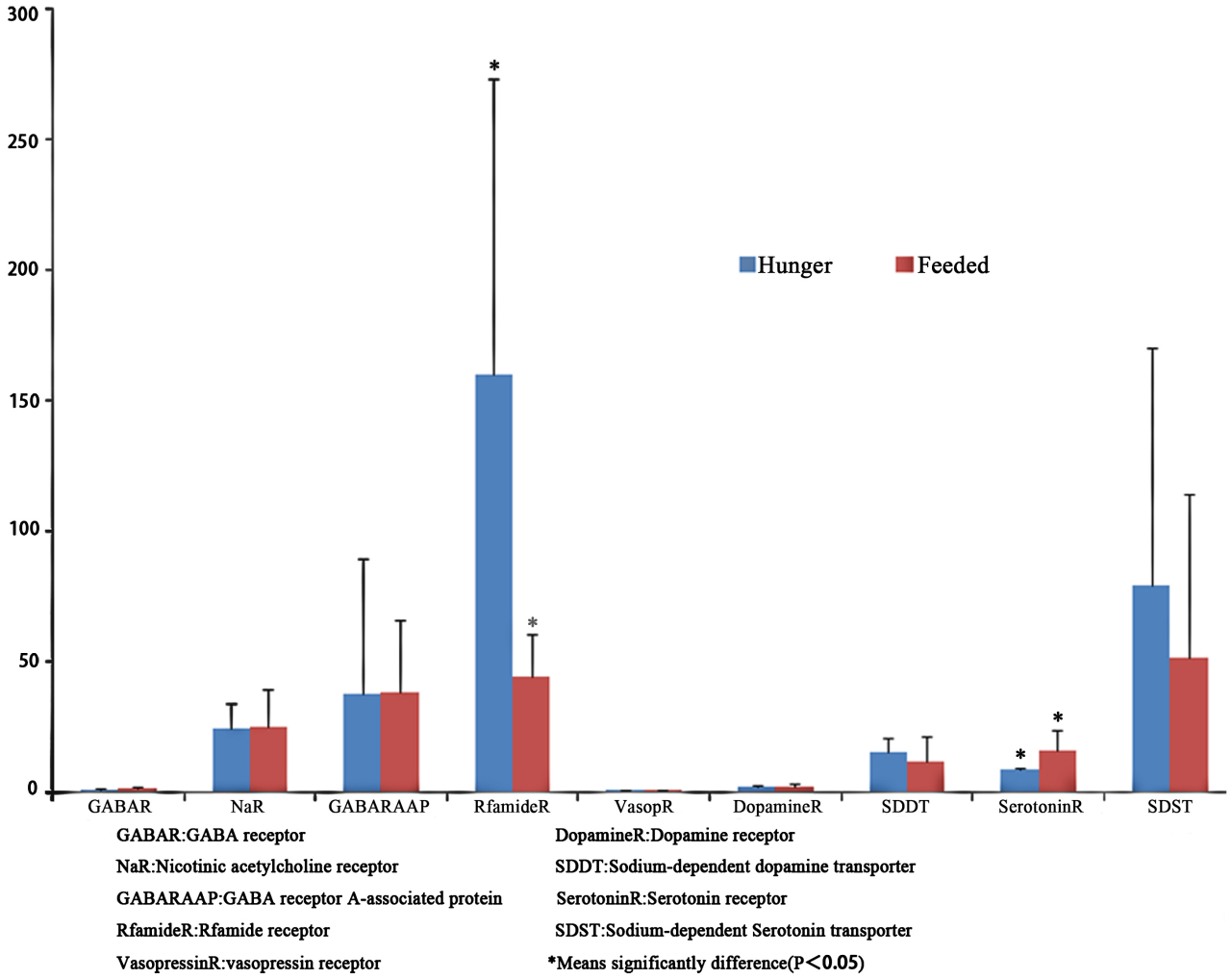

Figure 2. Expression analysis (RPKM value) of neuroregulation genes. * indicates significant differences at P < 0.05; P values correspond to comparisons via *t*-tests between samples from hungry and fed leeches (three replicates of *P. javanica*, *H. cavatuses*, and *H. nipponia*).

operating in all physiological muscles [13]. Thus, the results found here indicated that the feeding-mediated distension of the leech body wall was involving heart regulation.

3.3. KEGG Pathways Enrichment Analysis and Expression Assay of Associated Unigenes

A KEGG pathway enrichment analysis was performed for the DEGs between hungry and fed *P. javanica* (three replicates), *H. cavatuses* (two replicates), and *H. nipponia* (two replicates). The KEGG pathways displaying significant results (P < 0.05) which occur repeatedly in 3 species were chosen as a common feature. As a result, four interactive pathways at the "organismal systems" level were chosen, namely cardiac muscle contraction (CMC, hsa04260), complement and coagulation cascades (CCC, hsa04610), renin-angiotensin system (RAS, hsa04614), and hypertrophic cardiomyopathy (HCM, hsa05410) (**Table 4**).

Table 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that emerged as significantly ($P < 0.05$) enriched at least three times between starved and fed blood-sucking leeches.

Pathway	*P. javanica*			*H. cavatuses*		*H. nipponia*		Sum
	R1	R2	R3	R1	R2	R1	R2	
Environmental information processing								
ECM-receptor interaction	*	*	*	*	*	*	*	√
Organismal Systems								
MAPK signalling pathway	*	*	*	*	*			
NOD-like receptor signalling pathway		*	*					
Complement and coagulation cascades			*	*	*		*	√
Renin-angiotensin system	*	*		*	*	*	*	√
Cardiac muscle contraction			*	*	*		*	√
Osteoclast differentiation		*	*					
Vasopressin-regulated water reabsorption			*					
Long-term potentiation	*	*	*	*				
Human Diseases								
Hypertrophic cardiomyopathy (CHM)	*			*	*		*	√
Dilated cardiomyopathy	*							

Note: R: One time significantly enrichment occur between single starved and fed samples (fed at 30 d); * indicates that the pathway emerged as a significantly enriched ($P < 0.05$) following comparison; √ indicates that the pathway emerged as significantly enriched in 3 leeches at least three times.

Further expression analysis was carried out for the unigenes involved in these KEGG pathways in the three leech species. In the RAS pathway, ACE, neprilysin (MME), and kallikrein 1 (Tonin) induce vasodilation; glutamyl aminopeptidase (AP-A) and aminopeptidase N (AP-N) induce vasoconstriction. In addition, ACE and Tonin were highly expressed in three fed leeches but most in fed *P. javanica*. In addition, AP-A, and AP-N were relatively highly expressed in fed *H. cavatuses* and *H. nipponia*, suggesting that vasodilation is balanced in the two species (**Figure 3(a)**). In the CCC pathway, kallikrein and the von Willebrand factor (VWF) were highly expressed in fed leeches, which led to vasodilation, while serpin family C member 1 (SERPINC1), heparin cofactor II (SERPIND1), and alpha-2-macroglobulin (A2M) were highly expressed in hunger thereby inhibiting vasodilation (**Figure 3(b)**). For the CMC pathway, high expression of tropomyosin 1 (TPM1), TnC, TnI, and myosin induced systole, while sodium/calcium exchanger (NCX) and sodium/hydrogen exchanger (NHE) high expressions induced diastole. Thus, unigenes in the CMC pathway induced

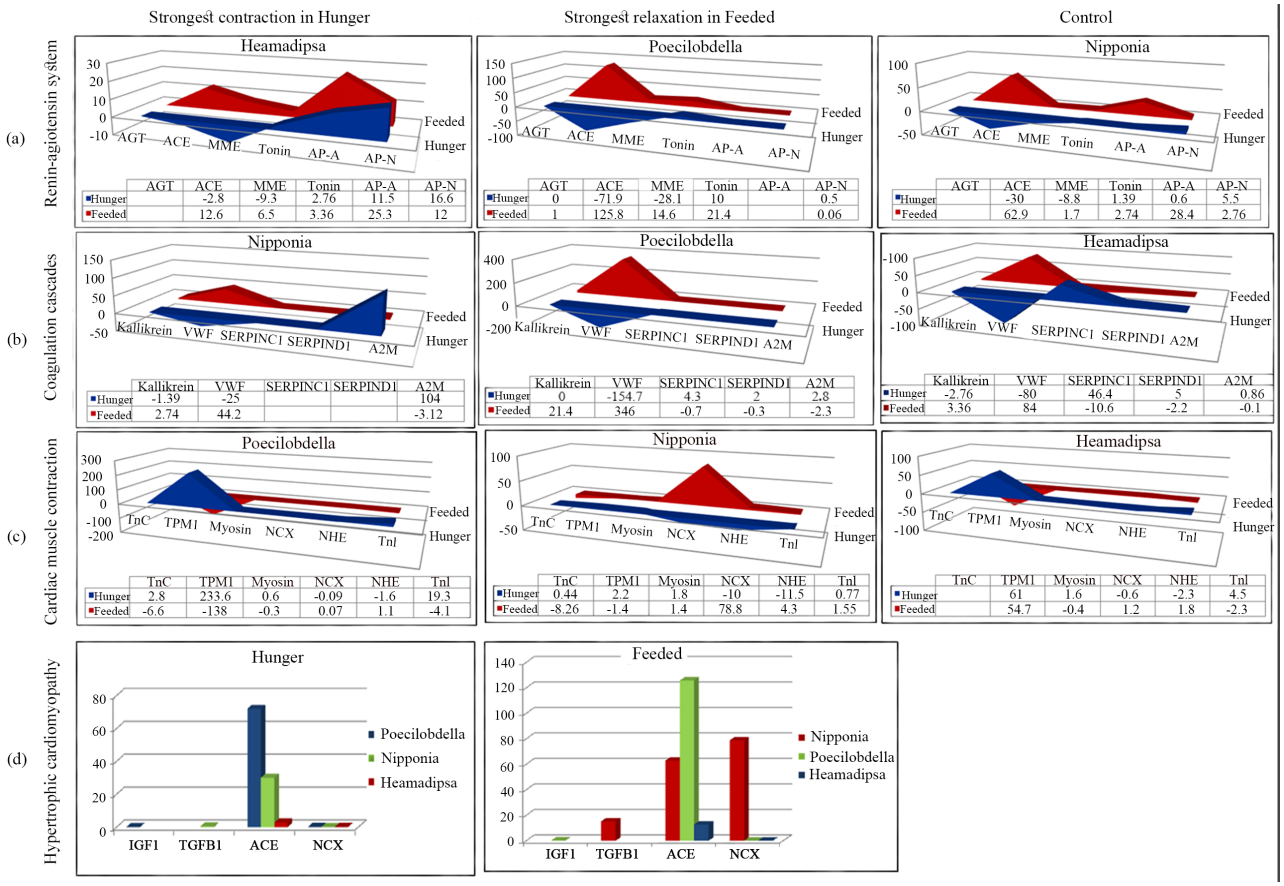

Figure 3. Expression analysis of genes from the four Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in a differential analysis between hungry and fed leeches. (a) In the renin-angiotensin system, the most strongly expressed gene from hungry *H. cavatuses* induces vasoconstriction, and that from fed *P. javanica* induces vasodilation. (b) In the coagulation cascade, the up-regulation or down-regulation of the unigenes induced vasoconstriction in hunger and vasodilation in fed *P. javanica* but genes associated with vasodilation were most strongly induced in fed *P. javanica*. (c) For cardiac muscle contraction, genes associated with systole were most strongly induced in hungry *P. javanica* and genes associated with diastole were most strongly induced in fed *H. nipponia*. (d) In the hypertropic cardiomyopathy pathway, genes associated with heart failure were most strongly induced in hungry *P. javanica* and in fed *H. nipponia*.

systole in hungry individuals. Specially, TPM1, TnI, myosin were relatively up-regulated in hungry *P. javanica* and *H. cavatuses* lead to systole; NCX in fed *H. nipponia* led to strong diastole (Figure 3(c)). For the HCM pathway, the genes inducing heart failure were investigated, and their comprehensive roles were assessed. In hungry individuals, the strongest heart failure function was found in *P. javanica*; after feeding, the species showing the strongest heart failure was *H. nipponia* (Figure 3(d)). interspecific comparison revealed that strongest heart failure was associated with the strongest systole in hungry *P. javanica* and with strongest diastole in fed *H. nipponia.*

3.4. Expression Analysis of Physiological Function Infer from KEGG Pathways

The strongest expression of unigenes related to heart failure was found in hungry *P. javanica* and in fed *H. nipponia*, similar to the results inferred from the

HCM pathway analysis (**Figure 3**, hypertrophic cardiomyopathy; **Figure 4**, heart failure). Further analyses showed that the species with low expression of genes involved in heart failure or in the HCM pathway had the strongest heart repairing activity both in hungry and fed individuals (**Figure 3**, hypertrophic cardiomyopathy; **Figure 4**, heart repair). The third verification analysis revealed that *P. javanica* had the strongest excitation-contraction coupling when hungry, while *H. cavatuses* had the strongest when fed; the strongest individuals found in excitation-contraction coupling when hungry and fed were similar to that found for hungry individuals in the CMC pathway and for fed individuals in the RAS pathway (**Figure 3** and **Figure 4**), indicating that excitation-contraction coupling in leech involves these two pathways.

Moreover, when comparing the unigenes related to the three physiological functions occurring in hunger and fed individuals from the three leech species, many unigenes always appeared simultaneously in hunger or fed individuals, and unigenes that occurred in excitation-contraction coupling were highly correlated to genes involved in heart failure (**Figure 5**).

3.5. RAS of the Three Leech Species Is Highly Regulated

In human, RAS, CMC, and HCM are the pathways underlying heart failure [24] [25]. However, the present analysis showed that CMC-annotated unigenes presented the strongest expression, which was completely consistent with the results of HCM pathway analysis or heart failure physiology, but not with the information inferred from RAS pathway analysis. To examine this inconsistency, we

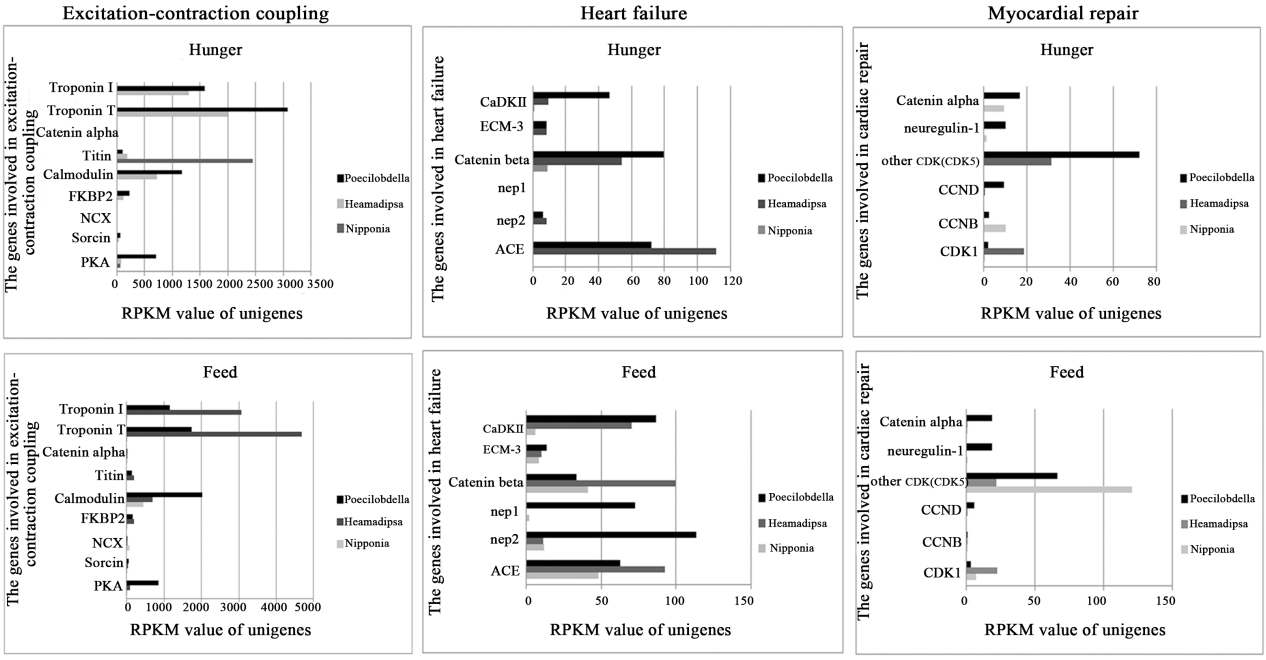

Figure 4. Unigenes with the highest expression (RPKM value) in three leech species that regulate four physiological functions—excitation-contraction coupling, heart failure, and myocardial repair—elicit similar information related to heart failure, as inferred from the four pathways prominently enriched from differentially expressed genes (DEGs) between hungry and fed leeches.

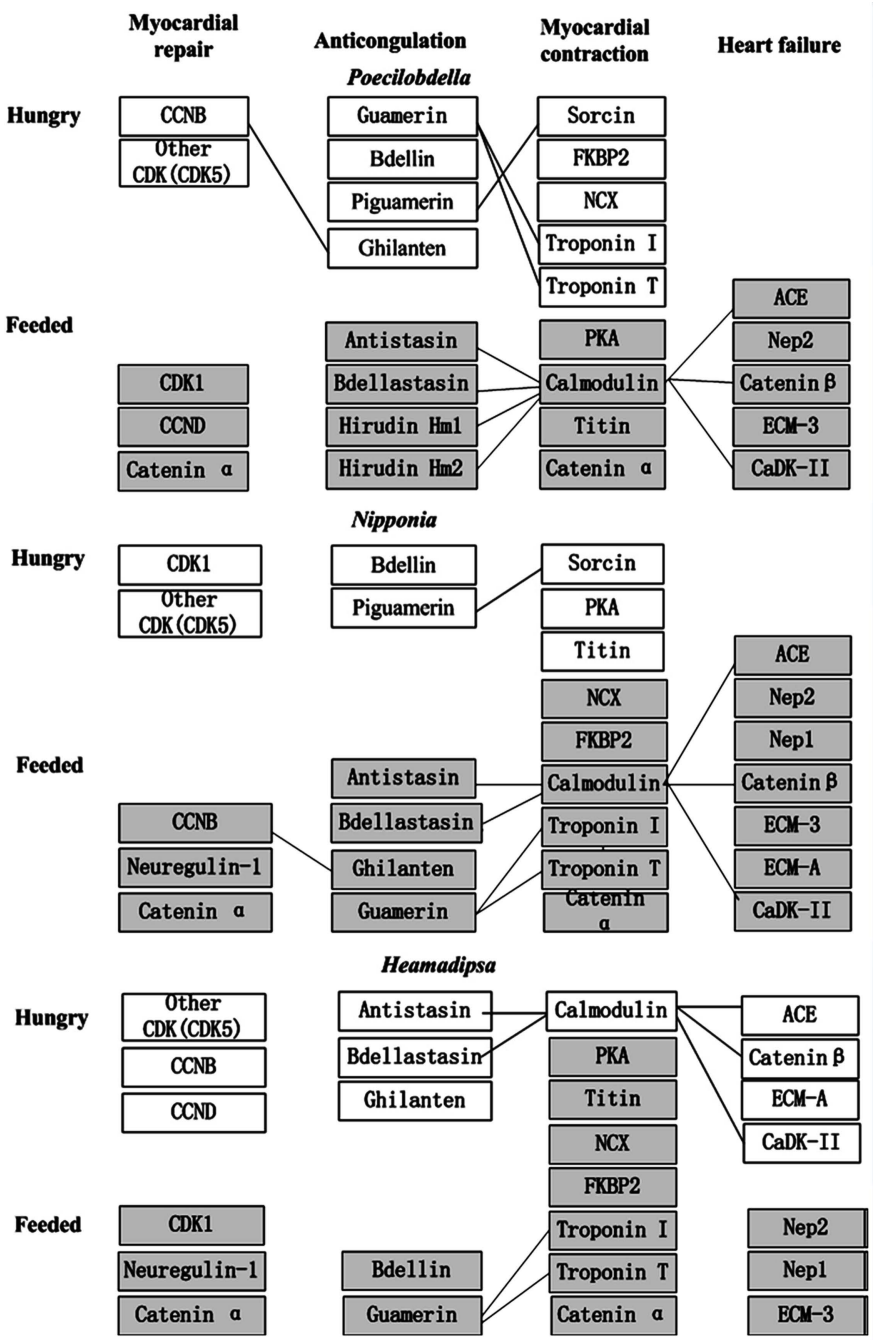

Figure 5. Correlation analysis of the genes regulating four physiological functions— excitation-contraction coupling, heart failure, and myocardial repair. Unigenes in blank boxes were up-regulated in samples from hungry leeches; those in shaded boxes were up-regulated in samples from fed leeches; unigenes whose expression did not change between hungry and fed samples are not shown. The connecting line represent pairs of unigenes that are always up-regulated regardless of feeding status, and consistent in expression profile across the three leech species.

further analysed the unigenes that could possibly regulate the RAS pathway. The results showed three regulated unigenes: angiotensin-converting enzyme-related protein (ACER), is a possible antagonist gene of ACE; angiotensin II type I re-

ceptor-associated protein (AGTRAP), a putative antagonist to import nodes gene angiotensin II type I receptor; and atrial natriuretic peptide-converting enzyme (NPCE), which regulates atrial natriuretic peptide work therefore playing an important role in the pathway (Table 5). These unigenes can regulate the RAS pathway in multiple ways (Supplementary Figure 1). While ACER and NPCE inhibited vasodilation in hungry *P. javanica* and *H. nipponia*, AGTRAP inhibited vasoconstriction in fed *H. nipponia*, which is consistent with the results elicited from the CCC pathway. Vasodilation in fed *H. cavatuses* was inhibited by NPCE.

4. Discussion

4.1. DEGs between Hungry and Fed Individuals Include Genes for Controlling Body Changes from Shrinkage to Distension

Analysis of DEGs at five time points in eight samples of *P. javanica* clearly evidenced three gene clusters: 30 min-up, 10 d-up, and 20 d-up groups. Functional annotation showed that 30 min-up include body distension of fed leech, While 10 d-up genes were highly expressed in hungry leeches from blood-sucking to 10 d, containing genes of controlling body shrink in hungry leeches. The 30 min-up gene cluster shows lower mean expression level than the 10 d-up cluster, indicate that the shrinking of hunger body stronger than positive regulation of distension.

4.2. Vasoconstriction and Systole Happen in Shrinking Leeches; Vasodilation, and Diastole Happen during Distension

Rfamide receptor decreased significantly after feeding means highly expression in hungry, as rfamide regulate heart and body shrink, indicating rfamide is the neuregulator when hunger ; serotonin, a known modulator of tension in leech muscles [22], up-regulation of serotonin when fed provided evidence that distension after feeding is regulated by serotonin.

The KEGG pathway analysis also indicated that the CMC pathway elicited systole in hungry leeches of the three species, while CCC and RAS pathways led to vasoconstriction. Vasodilation was strongest in fed *P. javanica* and *H. nipponia*, both elicited by the RAS pathway. A modulation system including ACER,

Table 5. Unigenes expressed (RPKM value) at different time points after feeding that can act as antagonists of key link proteins in renin-angiotensin regulation.

	P. javanica		*H. nipponia*		*H. cavatuses*	
	Hunger	fed	Hunger	fed	Hunger	fed
ACER	1.7	2	0.84	1.48	0	0
AGTRAP	1.5	0	1.2	9.03	0	0
NPCE	13.1	0.1	25.75	0	5.1	18.9

Note: ACER, angiotensin-converting enzyme-related protein; AGTRAP, angiotensin II type I receptor-associated protein; NPCE, natriuretic peptide-converting enzyme.

AGTRAP, and NPCE, that is antagonist to important nodes of RAS, covered the vasodilation induced by RAS whereas the vasoconstriction elicited was revealed in hungry animals. All these results showed vasoconstriction and systole in fed leeches and vasodilation and diastole in hungry leeches.

4.3. Heart Failure-Like Function Might Work as a Physiological Mechanism to Regulate Leech Body Changes

The KEGG pathway enrichment analysis indicated that the DEGs annotated in HCM, RAS, and CMC pathways were significantly expressed in the three leech species, and these pathways are correlated and involved in heat failure in human [26] [27] [28]. Strongest heart failure disclosed by RPKM value of unigene, which was associated with strong systole in hungry *P. javanica* and strongest diastole in fed *H. nipponia.*

The key gene of physiological modulation involved in heart failure were investigated by using unigenes with the highest values of RPKM. The results showed that hungry *P. javanica* individuals presented the highest expression of unigenes relative to hungry individuals from other species, while fed *H. nipponia* presented the highest expression of unigenes relative to fed individuals from other species, similar to the results obtained in HCM pathway analysis. However, unigenes were nearly similar in expression levels between hungry and fed *H. cavatuses*. These results were consistent with changes in the body wall of the three leech species, that is, *P. javanica* and *H. nipponia* varied acutely, but *H. cavatuses* only changed slightly. In addition, physiological analysis of heart repair regulating genes including CCND, CCNB, CNK1, CNK5 below [29], neuregulin-1 [30], and catenin alpha [31] showed that *H. cavatuses* was the strongest in heart repairing. Another evidence is the plentiful ECM secreted during feeding, which was verified by the ECM-receptor interaction prominently enriched in the KEGG pathway (**Table 4**) that will elicit fibrosis, as reported for the mammal heart [32].

All these results showed that leech body wall musculature is regulated by the same pathways of circulatory and excretory systems. The body cavity of leech is therefore similar to the mammal heart, which shrinks with vasoconstriction and systole like response in hunger, and distends with vasodilation and diastole like response when fed.

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Authors' Contributions

All the authors have read and approved the final manuscript. Bin Wang de-

signed, supervised all the experiments, and led the team and drafted the manuscript. Li Yang participated in all the research work, revised it, and made important contributions. Meiquan Li DeBin Wang, ZiChao Liu and Xiao Wang provided support as members of the research team, they assisted with various technical and managerial matters, including sample preparation and providing valuable suggestions for this paper.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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