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Research article

Exploring symbiont gene expression in two echinoid-associated shrimp species under host separation

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ABSTRACT

Symbiotic relationships are omnipresent and particularly diverse in the marine world. In the Western Indian Ocean, the sea urchin *Echinometra mathaei* associates with two obligate ectosymbiotic shrimp species, *Tuleariocaris holthuisi* and *Arete indicus*. These shrimps are known for their host-dependent nature. *T. holthuisi*, for example, exhibits severe host separation syndrome, showing signs of stress and rapid mortality when isolated. Specific host pigments called spinochromes seem essential for *T. holthuisi* survival. Our study employs a transcriptomic approach to assess the stress induced by host separation on these shrimps.

Using paired-end Illumina HiSeq technology, we analyzed transcriptomes of both species under three conditions: (i) symbionts on their host (CC), (ii) isolated symbionts in seawater (IC), and (iii) isolated symbionts in spinochrome-enriched seawater (IC + S).

Sequencing revealed a total of 217,832 assembled unigenes, with an N50 value of 2061 bp. Isolated *T. holthuisi* showed 16.5 % DEGs (IC/CC), reduced to 8.5 % with spinochromes (IC + S/CC), both compared to the control condition (CC). Further analyses of stress-related genes show that *T. holthuisi* expressed stress-related genes when isolated in comparison to the control (IC/CC). Notably, heat shock proteins (HSPs) were significantly upregulated in isolated *T. holthuisi*, especially without spinochromes. In contrast, *A. indicus* displayed differential expression of diverse genes, suggesting an adaptive micro-regulation mechanism to cope with isolation stress.

This study pioneers the use of NGS in exploring the transcriptomic responses of symbiotic shrimp species, shedding some light on the molecular impact of the host-separation syndrome and chemical dependencies.

1. Introduction

Most marine organisms are associated with at least one or several individual(s) belonging to another species (Castro, 1988). Symbioses are intimate and durable associations between two heterospecific organisms, typically referred to as the host (*i.e.,* the larger partner) and the symbiont (*i.e.,* the smaller one) (Paracer and Ahmadjian, 2000; Parmentier and Michel, 2013; Brasseur et al., 2018). Symbioses are generally classified into three categories - parasitism, commensalism and mutualism - which are distinguished by the effects of the symbionts on their hosts (negative, neutral and positive effects on the host, respectively) (Paracer and Ahmadjian, 2000; Parmentier and Michel, 2013). A symbiotic association can, however, switch from one category to another depending on the timing of the symbiontlife cycle (*e.g.,* most myzostomids switch from a parasitic to a commensal style during their life) or the environmental conditions (*e.g.,* bacteria in the digestive tracts may switch from a mutualistic to a parasitic lifestyle) (Parmentier and Michel, 2013). Symbionts may exhibit various degrees of host-

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specificity, which can vary significantly from living with a single host species (host-specific symbiont) to multiple host species belonging to varied taxa (opportunistic symbiont) (Eeckhaut and Jangoux, 1993; Eeckhaut and Jangoux, 1995; Eeckhaut and Jangoux, 1997; Paracer and Ahmadjian, 2000). Because symbiotic species have lived together intimately for a significant period (*i.e.*, usually over numerous generations), hosts and symbionts have co-evolved together. This coevolution involves adaptations (*e.g.,* morphological, metabolic) in symbionts, hosts and/or both. These adaptations lead to evolutionary advantages for the symbiont and/or the host and ensure, in the case of symbionts, to live in appropriate habitats over time (Eeckhaut and Jangoux, 1993; Eeckhaut and Jangoux, 1995; VandenSpiegel et al., 1998; Lanterbecq et al., 2009; Rouse et al., 2013; Terrana et al., 2016). Some symbionts may be highly dependent on their host (VandenSpiegel et al., 1998; Terrana et al., 2016; Brasseur et al., 2018) and, very often, their life cycle cannot be completed without the symbiotic association (Olsen, 1986; Baker, 2003). Those symbionts are qualified as "obligatory" (Eeckhaut, 2003; Bauer, 2023).

While possessing a large set of mechanical and chemical defenses against biotic and abiotic threats (Brasseur et al., 2017; Brasseur et al., 2018; Caulier et al., 2022), numerous echinoderms are colonized by a wide range of symbionts (Lanterbecq et al., 2010; Horká et al., 2016; Chow et al., 2021). For example, the sea urchin *Echinometra mathaei* (Blainville, 1825) can be colonized by two symbiotic shrimps, *Arete indicus* Coutière, 1903 (Alpheidae) and *Tulariocaris holthuisi* Hipeau-Jacquotte, 1965 (Palaemonidae) (Hipeau-Jacquotte, 1965; Gherardi, 1991; Marin and Anker, 2009; De Grave and Fransen, 2011; Brasseur et al., 2018) (Fig. 1). The mimetic pigmentation of both shrimps relies on spinochromes, which are specific pigment metabolites belonging to the naphthoquinone category and are specifically produced by echinoids (Brasseur et al., 2017). Indeed, mass spectrometry analyses revealed different cocktails of molecules containing at least nine different spinochromes in *E. mathaei*, five in *T. holthuisi*, and three in *A. indicus* (Brasseur et al., *2017*). Gut content and stable isotope analyses ($\delta^{15}C$, $\delta^{13}N$) demonstrated that the host integument constitutes the exclusive food of *T. holtuisi*, while *A. indicus* also feeds on other food sources (Brasseur et al., 2018). Both shrimps are attracted by their echinoid host through chemical sensing, with spinochromes serving as an attractive stimulus for host selection. When *T. holthuisi* shrimps are isolated from their hosts, specifically from the chemical environment they produce, they experience a "host separation syndrome" that results in a fast alteration of their health status associated with a visible depigmentation, loss of mobility and high mortality rates, most individuals dying within four days following the host separation. In comparison, the survival rate of *A. indicus* was less impacted by the host separation, with no significative differences observed in pigmentation or mobility over the same period (Brasseur et al., 2018). Interestingly, the host-separation syndrome is attenuated when isolated *T. holthuisi* individuals are placed in a solution of spinochromes, significantly increasing shrimp survival rates after four days of experiment (Brasseur et al., 2018). These experiments suggest that echinoid host spinochromes influence, to some extent, the physiology of the host-chemically-dependent shrimp *T. holthuisi* (Brasseur et al., *2018*).

To investigate this hypothesis, we explore the gene expression of *T. holthuisi* and *A. indicus* under various conditions, with or without a host and with or without echinoid spinochromes through comparative transcriptomic analyses.

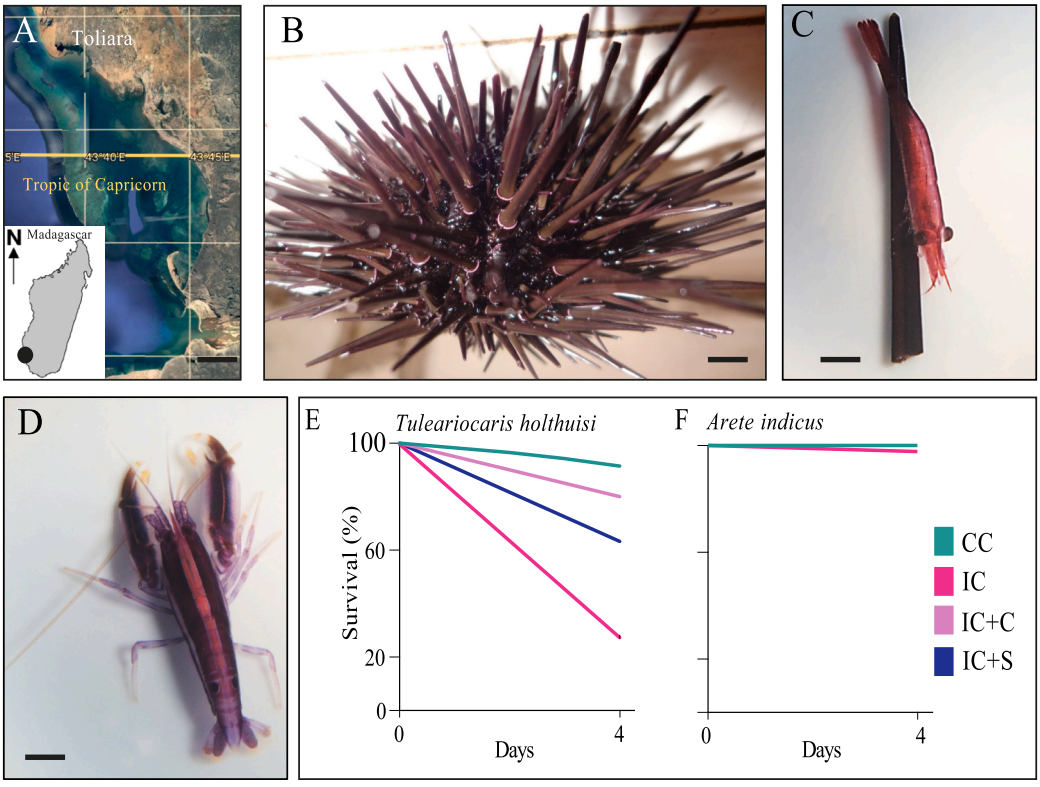

Fig. 1. Biology of the symbiosis between *Tulariocaris holthuisi*, *Arete indicus*, and *Echinometra mathaei* in the South-West of Madagascar. (A) South-West of Madagascar and Great Reef of Toliara, (B) *E. mathaei* sea urchin host, (C) *T. holthuisi* on a host spine, (D) isolated *A. indicus* (*E*-F) Survival rate of *T. holthuisi* and *A. indicus* shrimps under different conditions: control condition (CC, shrimps associated with their host); isolated condition (IC, shrimps isolated from their host); isolated shrimps in host-conditioned water (IC + C); isolated shrimps with spinochrome conditioned seawater (IC + S). (E-F) Modified from Brasseur et al., 2018. Scale bars represent 7 km in A, 1 cm in B and 0.2 cm in C and D.

2. Material and method

2.1. Sample collection

The sea urchin *Echinometra mathaei* (Blainville, 1825) and its symbionts, *Tuleariocaris holthuisi* Hipeau-Jacquotte, 1965, and *Arete indicus* Coutière, 1903, were hand-collected during low tide on the flat of the Great Reef of Toliara, Madagascar (23°23′34″S, 43°38′47″E) in November 2015 and November 2016. Originally, *E. mathaei* ($n = 254$) were sampled with *T. holthuisi* ($n = 72$) and *A. indicus* ($n = 72$) in order to perform the *in-vivo* host separation experiment. The organisms were maintained together (*i.e.,* Control Condition) in tanks supplied directly with seawater from the sea at the Halieutic Institute and Marine Sciences (IHSM) of the University of Toliara (Madagascar) for a minimum of 24 h before any experimentation occurred. All organisms that were still alive and not preserved in RNA later at the end of the experiment (*i.e.,* 90 % of the hosts and 35 % of the symbiotic shrimps) were returned to their original collection site in the lagoon.

2.2. In vivo host separation experiments

Based on Brasseur et al. (2018), the following experimental design was used: (*i*) 24 *T. holthuisi* individuals were placed on 12 *E. mathaei* for four days (control condition, CC), (*ii*) 24 *T. holthuisi* individuals were isolated in 24 vials limited by a grid (*i.e.,* allowing water exchanges) placed in a second aquarium without their host (host isolation condition, IC), (*iii*) 24 *T. holthuisi* individuals were incubated in similar vials and placed in an aquarium containing seawater with 0.5 mg/L of *E. mathaei* spinochromes crude extract from tests and spines (isolation condition + spinochromes, IC + S) (Fig. 2). The same experimental procedure was used for the species *A. indicus* (Fig. 2). The methodology associated with the spinochromes crude extraction is explained in detail by Brasseur et al. (2018). For CC and IC, the seawater was replaced every day with fresh 1 µm-filtered seawater. For IC + S, the seawater was replaced every day with fresh 1 µm-filtered seawater containing 0.5 mg/L of spinochrome extract. All experiments were performed in tanks of 14 cm × 18 cm × 15 cm containing 1 L of 1 µm-filtered seawater at 28 °C and 35 PSU of salinity mimicking the experiments of Brasseur et al. (2018). The different treatments were stopped after 4 days (96 h). At the end of this period, five shrimps taken from the pool of individuals that survived (depending on the conditions) were then randomly selected and preserved in RNA later for downstream analyses. Sexing the selected individuals was not possible for both symbiotic shrimps due to the to the lack of material on the field.

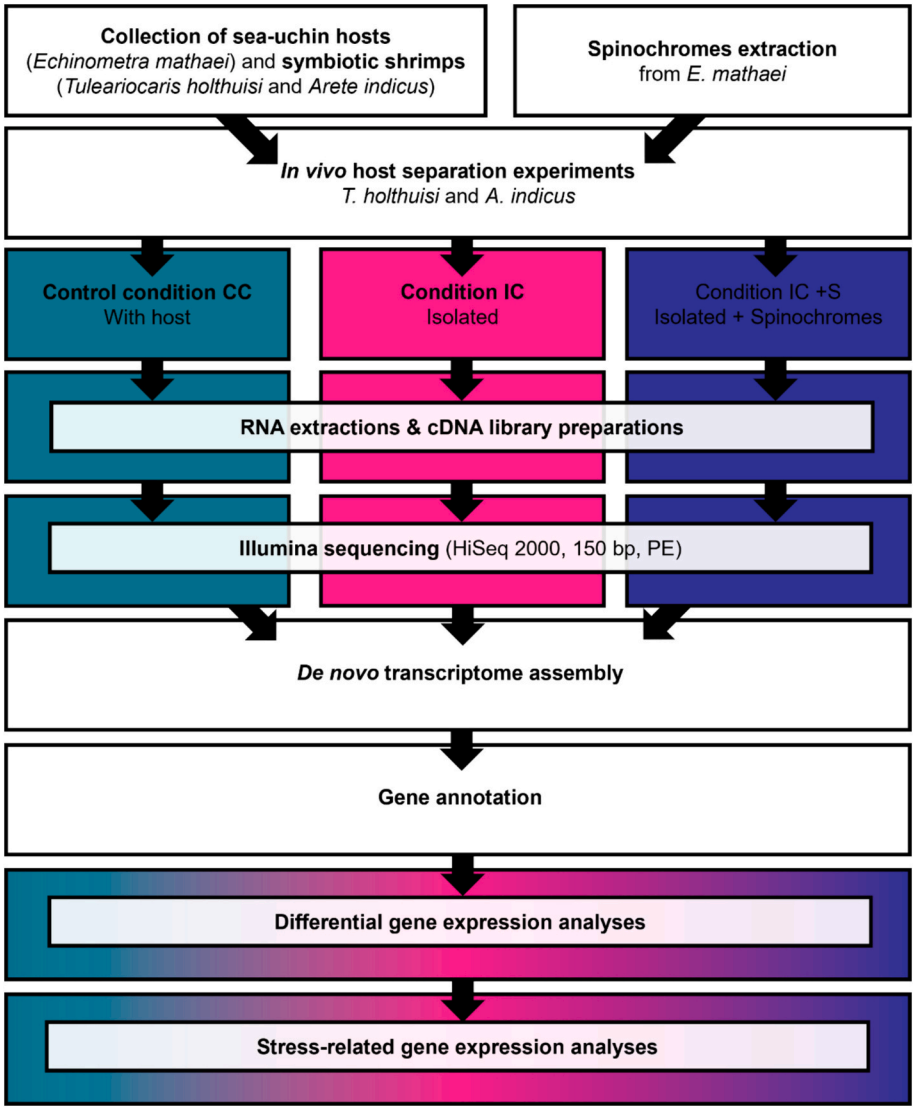

Fig. 2. Study methodological pipeline to investigate the differential gene expression associated with the host separation syndrome in *Tuleariocaris holthuisi* and *Arete indicus*, both symbionts of *Echinometra mathaei*. Bp = base pairs; PE = Paired-End sequencing.

2.3. RNA extraction, cDNA library preparation and sequencing

For each species and each treatment, five individuals were processed for RNA extractions (a total of 30 RNA extractions) and finally 3 samples per condition and per species were selected depending on the quality of the RNA extracts and were chosen for subsequent transcriptomic analyses. Whole shrimp individuals were stabilized in RNA later (Life Technologies) overnight at 4 °C following the manufacturer instructions. Due to field constraints, samples were stored at 4 °C for a few days until the return from the field mission. Subsequently, samples were transferred to our laboratory in Belgium and stored at −80 °C until RNA extraction. For RNA extractions, frozen samples were processed using the RNeasy Mini Kit (QIAGEN) following the manufacturer instructions. The quality of the RNA extracts was checked by gel electrophoresis on a 1.2 M TAE agarose gel, and by size-exclusion chromatography with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

After treatment of the total RNA with DNase I, magnetic beads with Oligo (dT) were used to isolate mRNAs. The purified mRNAs were then mixed with fragmentation buffer and fragmented into 100–400 bp pieces using divalent cations at 94 °C for 5 min. Using these fragments as a template, random hexamer primers (Illumina) were used to synthesize the first-strand cDNA, followed by the synthesis of the second-strand cDNA using RNase H and DNA polymerase I. End reparation and single nucleotide A (adenine) addition were performed on the synthesized cDNAs. Next, Illumina paired-end adapters were ligated to the ends of these 3′ adenylated short cDNA fragments. To select the proper templates for downstream enrichment, the products of the ligation reaction were purified on a 2 % agarose gel. The cDNA fragments of about 200 bp were excised from the gel. Fifteen rounds of PCR amplification were carried out to enrich the purified cDNA template using PCR primer PE 1.0 and 2.0 (Illumina Inc., San Diego, CA, USA) with Phusion DNA polymerase. Finally, the cDNA library was constructed with 200 bp insertion fragments. After validation on the Agilent Technologies 2100 Bioanalyzer, the library was sequenced using Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA), and the workflow was as follows: template hybridization, isothermal amplification, linearization, blocking, sequencing primer hybridization, and first read sequencing. After completion of the first read, the templates are regenerated *in situ* to enable a second read from the opposite end of the fragments. Once the original templates are cleaved and removed, the reverse strands undergo sequencing by synthesis. High-throughput sequencing was conducted using the Illumina HiSeq 2000 platform to generate 150 bp paired-end reads. The cDNA library preparation and sequencing were performed at Beijing Genomics Institute (BGI, Hong Kong) according to the manufacturer's instructions (Illumina, San Diego, CA). After sequencing, raw image data were transformed by base calling into sequence data, which were called raw reads and stored in the fastq format. Transcriptome quantity and quality control of reads were checked using the FastQC software (Andrews, 2010).

2.4. De novo transcriptome assembly

De novo transcriptome assembly was performed separately for *A. indicus* and *T. holthuisi* samples (Fig. 2). Before the transcriptome assembly, the raw sequences were filtered to remove the low-quality reads. The filtration steps were as follows: 1) removal of reads containing only the adaptor sequences; 2) removal of reads containing over 5 % of unknown nucleotides "N"; and 3) removal of low-quality reads (those comprising >20 % of bases with a quality value lower than 10). These filtration steps are routinely employed (Delroisse et al., 2015; Delroisse et al., 2018). The remaining clean reads were used for further analysis.

Transcriptome *de novo* assembly was carried out with short paired-end reads using the Trinity software (Grabherr et al., 2011) (version v2.0.6, parameters: min_contig_length 150, CPU 8, min_kmer_cov 3, min_glue 3, bfly_opts '-V 5, edge-thr = 0.1, stderr'). After Trinity assembly, the TGI Clustering Tool (TGICL, version: v2.0.6, parameters: -l 40 -c 10 -v 25 —O '-repeaT. stringency 0.95 -minmatch 35 -minscore 35'), followed by Phrap assembler (http://www.phrap.org) were used for obtaining distinct sequences. These sequences are defined as unigenes (*i.e.*, unique predicted transcripts generated during the process of transcriptome assembly) in the following manuscript. Unigenes can either form clusters in which the similarity among overlapping sequences is superior to 94 %, or singletons that are unique unigenes. As the length of sequences assembled is a criterion for assembly success, we calculated the size distribution of both contigs and unigenes.

BLASTx alignments (*E*-value threshold $<1e^{-5}$) between unigenes and public databases including non-redundant protein database (Nr, NCBI), non-redundant nucleotide database (Nt, NCBI), SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG), InterPro protein families and domains (InterPro), Genes Ontology (GO) and Clusters of Orthologous Genes (COG), were performed, and the best-aligning results were used to identify the sequence direction of unigenes. When results from different databases were conflicting, the priority order Nr, Swiss-Prot, KEGG, and COG was followed to decide on sequence direction for unigenes. When a unigene was unaligned in all the above databases, the ESTScan software (v3.0.2) was used to decide on its sequence direction. ESTScan produces a nucleotide sequence (5′–3′) direction and the amino sequence of the predicted coding region. The BUSCO software (Benchmarking Universal Single-Copy Orthologs, v4) was also used to evaluate the completeness of the assembled unigenes using the "Eukaryota odb" dataset (Simão et al., 2015).

To evaluate unigene expression levels, all usable reads were mapped back to the unigenes using SOAP aligner with the default settings (Li et al., 2008). For both transcriptomes, unigene expression was evaluated using the FPKM (fragments per kilobase of transcript, per million fragments sequenced) method. The FPKM value is calculated following the specific formula $FPKM = \frac{10^6 C}{NL/10^3}$ where C is the number of fragments shown as uniquely aligned to the concerned unigene, N is the total number of fragments that uniquely align any unigene, and L is the base number in the coding DNA sequence of the concerned unigene. The FPKM method integrates the influence of different gene lengths and sequencing levels on the calculation of gene expression.

2.5. Functional gene annotations

Following the pipeline described in Fig. 2, all unigenes were used for homology searches against the various databases cited above. Best annotation results were selected to annotate the unigenes (*E*-value $<1e^{-05}$).

To estimate the number of assembled transcripts that appear to be nearly full-length, the unigenes were aligned against all known proteins from the Nr database and the numbers of unique top matching proteins (E-value $<1e^{-05}$) that align across more than a certain percentage of the reference protein length were counted.

To further annotate the unigenes, the Blast2GO program was used with Nr annotation to get Gene Ontology annotation according to molecular function, biological process, and cellular component ontologies (http://www.geneontology.org/). The unigene sequences were also compared to the COG database to predict and classify possible functions (Tatusov et al., 2000). Finally, pathway assignments were performed according to the KEGG pathway database (Kanehisa et al., 2017). Potential molecular pathways involving predicting unigenes can be highlighted from KEGG pathway annotations.

2.6. Global differential gene expression analyses

NOIseq (Tarazona et al., 2011) was used to detect differentially expressed genes (Parameters: Fold Change ≥ 2.00 and Probability ≥ 0.8). Hierarchical clustering for DEGs was performed, after a $\log_{10}$

normalization, using the R function *PlotHeatMap* implemented in the web tool Metaboanalyst (Chong and Xia, 2018; Pang et al., 2022). The following R script was used: mSet, "heatmap_0_", "norm", "row", "euclidean", "ward.D","bwm", 8, "overview", T, T, NULL, T, F, T, T, T). PCA analyses were performed using the web tool MetaboAnalyst and using the following R script mSet<-Normalization(mSet, "NULL", "LogNorm", "NULL", ratio = FALSE, ratioNum = 20; mSet<-PlotPCAScree(mSet, "pca_scree_0_", 5); mSet<-PlotPCA2DScore(mSet, "pca_score2d_0_", 1,2,0.95,0,0); mSet<-PlotPCALoading(mSet, "pca_loading_0_", 1,2); mSet<-PlotPCABiplot(mSet, "pca_biplot_0_", 1,2).

To highlight the most representative differential gene expression (DEG), we created an additional filtered dataset of 100 DEGs (for all pairwise comparisons and both species) which present the smallest intra-condition mean deviation (*i.e.,* minimal standard deviation between replicates of the same condition (IC, IC + S or CC)). The selection was performed using the web tool MetaboAnalyst (http://www.metaboanalyst.ca). For visualization purposes, heatmaps of the 100 DEGs presenting the smallest intra-condition mean deviation were performed.

2.7. BLAST/ reciprocal BLAST analyses to highlight stress-related genes

Genes that are specifically expressed under stress conditions have been mined from the literature with a particular emphasis on crustacean-specific studies. Gene sequences were then obtained from open-access NCBI online databases (http://www.ncbi.nlm.nih.gov) and used for a 'tBLASTn/reciprocal BLASTx' approach. The reference sequences are listed in the Table S1 (Chen et al., 2013; Kim et al., 2017; Armstrong et al., 2019). Homologous sequences (*i.e.*, the best candidate potentially corresponding to the closest orthologous) to reference stress-related genes were found in the transcriptomes of *A. indicus* and *T. holtuisi*. The expression profiles of the genes of interest were characterized based on our differential gene expression analysis.

3. Results

3.1. Illumina sequencing and read assembly

Eighteen cDNA libraries were constructed to perform Illumina HiSeq sequencing for both shrimp species. A total of 742 million raw reads were generated. After removing the adapter sequences, low-quality sequences, and ambiguous nucleotides, we obtained a total of 642 million clean reads, representing 96 Gigabases. The GC content for the clean reads was 39 % (Table 1). The mean value of Q20 was 98.5 % and Q30 percentage was 95.5 %. After the assemblies, 217,832 unigenes were obtained, with a total length, an average length and a N50 of 225,166,173 bp, 1033 bp and 2061 bp, respectively (Table S2 and S3).

3.2. Transcriptome annotation

The length distribution of unigenes provides important information about the size and complexity of the transcriptome and can be used to determine the completeness of the assembly **(**Fig. 3**A)**. Around 36 % of unigenes had lengths between 500 and 1500 base pairs. A significant proportion of unigenes were either shorter (43 %) or longer (20.9 %) than this range. Taxonomic distribution analyses revealed that the main represented species within the unigene annotation is the highly studied amphipod species *Hyalella azteca* (29.38 %), followed by the insect model species *Zootermopsis nevadensis* (4.94 %) (Fig. 3**B**). A global annotation of assembled unigenes, referring to the process of assigning predicted functional information to the sequences generated from transcriptome assembly was conducted (Fig. 3**C**). Out of the 217,832 annotated genes, the majority found matches in various databases: 38.32 % in NCBI NR database, 31.29 % in KEGG pathway database, and 30.74 % in the SwissProt database, among others. The completeness of the transcriptome was evaluated by BUSCO (Benchmarking Universal Single-Copy Orthologs) analyses (Fig. 3D). The pooled unigenes of *T. holtuisi* and *A. indicus* transcriptomes demonstrate 98.5 % of complete (Fig. 3**C**) BUSCO (9.8 % of single and 88.6 % of duplicated copies), indicating a high level of completeness of the transcriptomes. For descriptive purposes, the 10 most expressed unigenes within the different samples and treatments were compiled in Table S4, with their predicted annotations.

Table 1
Description of the output sequenced data for *A. indicus* and *T. holthuisi* transcriptomes. i. Conditions were "CC: Control condition (with host)"; "IC: Isolation condition"; "IC+S: Isolation condition + spinochromes". R = Replicate, M = Million, Gb = Giga bases.

Sample		Total raw reads (M)	Total clean reads (M)	Total clean bases (Gb)	Clean read Q20 (%)	Clean read ratio (%)
	*A. indicus* CC, R1	42.34	36.59	5.49	98.47	86.42
	A. indicus CC, R2	42.34	36.05	5.41	98.54	85.16
	A. indicus CC, R3	41.67	35.60	5.34	98.53	85.43
	A. indicus IC, R1	40.71	36.52	5.48	98.50	89.71
	A. indicus IC, R2	43.96	36.06	5.41	98.50	82.02
	A. indicus IC, R3	42.34	36.98	5.55	98.45	87.35
	A. indicus IC + S, R1	40.71	35.81	5.37	98.55	87.96
	A. indicus IC + S, R2	38.75	31.85	4.78	98.55	82.20
	A. indicus IC + S, R3	41.93	34.59	5.19	98.57	82.51
	*T. holtuisi* CC, R1	39.97	34.53	5.18	98.54	86.38
	T. holtuisi CC, R2	39.08	35.67	5.35	98.48	91.28
	T. holtuisi CC, R3	39.08	35.68	5.35	98.54	91.31
	T. holtuisi IC, R1	40.71	36.12	5.42	98.53	88.72
	T. holtuisi IC, R2	42.34	35.87	5.38	98.36	84.72
	T. holtuisi IC, R3	39.08	35.89	5.38	98.36	91.85
	T. holtuisi IC + S, R1	43.96	36.44	5.47	98.52	82.89
	T. holtuisi IC + S, R2	42.34	36.51	5.48	98.62	86.24
	T. holtuisi IC + S, R3	40.71	35.84	5.38	98.55	88.05

3.3. Differentially expressed unigenes under host separation

Differentially expressed genes (DEGs) between the control condition (CC; *i.e.*, shrimp on its host) and the treatments were analyzed for both species (Fig. 4).

Based on Brasseur et al. (Brasseur et al., 2018) and their host-separation experimental framework, we hypothesized that the shrimp *A. indicus* exhibits reduced chemical host dependency. Short-term survival rates of *A. indicus* were not reduced when isolated from their hosts compared to shrimps that remained on the sea urchin. We then anticipated minimal differential gene expression in *A. indicus* in the following comparisons: (i) between shrimps in contact with their hosts (control; CC) and isolated shrimps (IC), (ii) between the control group (CC) and isolated shrimps treated with spinochromes (IC + S), (iii) between isolated shrimps (IC) and isolated shrimps with spinochromes (IC + S). Our findings reveal that the proportion of DEGs in *A. indicus* accounts for 7.4 %, 5.7 %, and 5 % of the total predicted unigenes for each comparison (CC *vs* IC, CC *vs* IC + S, IC *vs* IC + S), respectively.

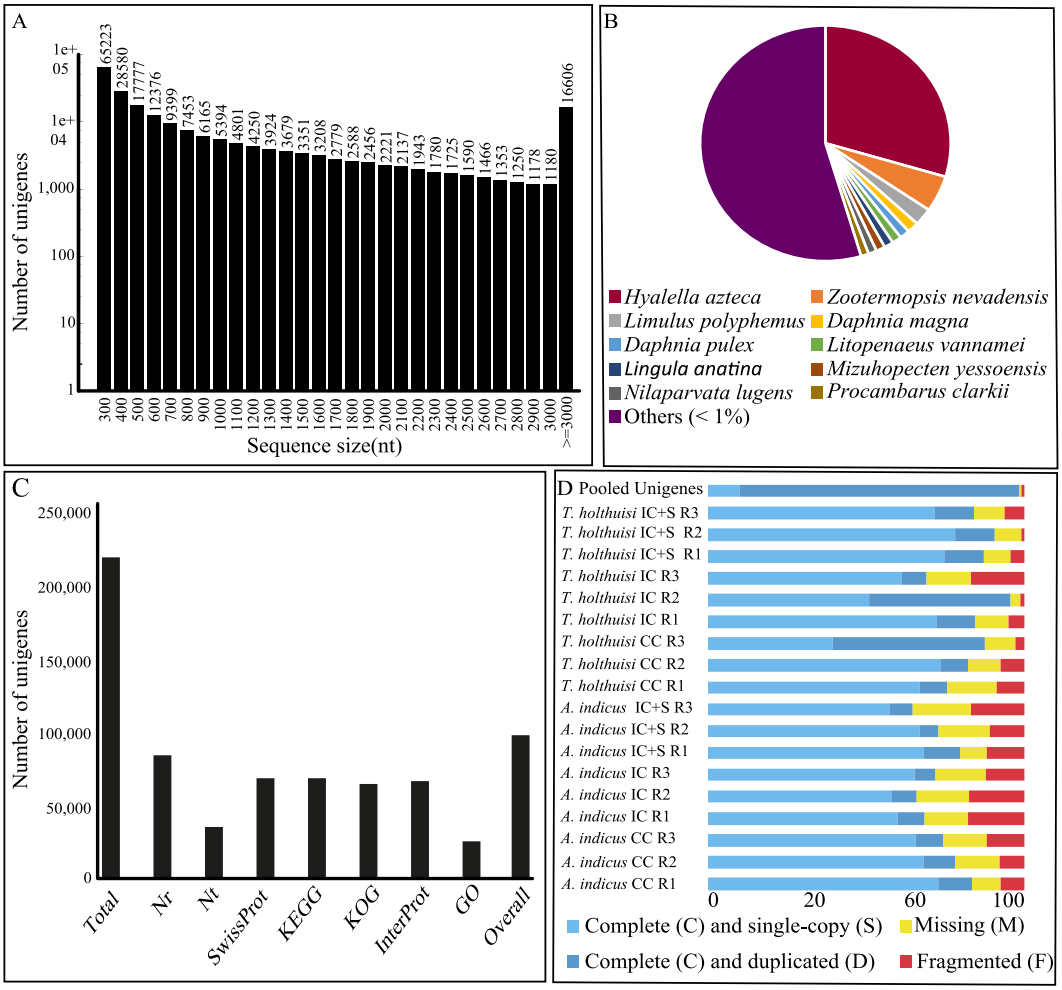

Fig. 3. (A) Length distribution of all assembled unigenes (pooled for *Tulearіocaris holthuisi* and *Arete indicus*, (B) species annotation of all assembled unigenes, (C) global annotation of all assembled unigenes, (D) Benchmarking Universal Single-Copy Orthologs (BUSCO) analyses of all isolated transcriptome unigenes (Eukaryota database). Databases include *non-redundant protein* (Nr, NCBI), *non-redundant nucleotide* (Nt, NCBI), SwissProt, *Kyoto Encyclopedia of Genes and Genomes* (KEGG), *InterPro protein families and domains* (InterPro), *Gene Ontology* (GO) and *Clusters of Orthologous Genes* (COG).

On the other hand, the shrimp species *T. holthuisi* develops a pronounced host separation syndrome (Brasseur et al., 2018). Shrimp survival was maximized when they were associated to their hosts (CC) or when they were isolated from their hosts but in presence of host spinochromes (IC + S) (Brasseur et al., 2018). We hypothesized notable variations in differential gene expression in the following comparisons: (i) between shrimps in contact with their hosts (the control; CC) and isolated shrimps (IC) and (ii) between isolated shrimps (IC) and isolated shrimps with spinochromes (IC + S). Conversely, we expected less variation when comparing control individuals (CC) with isolated shrimps with spinochromes (IC + S). For *T. holthuisi*, the proportions of DEGs are 16.5 %, 18.6 %, and 8.5 % of the total unigenes, respectively, when comparing CC with IC, IC with IC + S, and CC with IC + S (Fig. 4). Furthermore, we observed a 50 % decrease in DEG numbers in the presence of spinochromes (IC + S) compared to the control condition (CC).

We conducted a differential gene expression analysis at the individual scale (*i.e.,* biological replicate) and performed clustering of individuals based on their similarity of gene expression profiles (Fig. 5). We anticipated encountering distinct clusters for isolated individuals (IC) and the two other conditions for *T. holthuisi* (CC, IC + S) since shrimp survival was maximized in these latter two conditions. On the contrary, we hypothesized that in *A. indicus*, a clustering by condition would not be observed. However, the results of the analysis for *T. holthuisi* (Fig. 5 **A-C**) did not reveal a condition-based clustering, and our data showed wide intra-condition variability (Fig. 5 **A-C**). For *A. indicus*, we also did not observe a well-defined clustering, which may be more consistent with our expectations (Fig. 5 ***E*-G**).

For all condition comparisons, the 40 most significant differentially expressed unigenes (20 up-regulated and 20 down-regulated) are detailed in Table S5 and **Fig. S1**. In *T. holthuisi*, a comparison between control (CC) and isolated treatments (IC) revealed a significant up-regulation of genes related to the production of cuticle and chitin proteins in isolated individuals. This up-regulation concerned at least 16 unigenes (*e.g., cuticle protein 19-like*, *cuticle protein CB6*, *early cuticle protein 3*, *chitin-binding protein*, *calcification associated soluble matrix protein 2*). However, this up-regulation was mainly observed in the second replicate of *T. holthuisi* IC. The other unigenes differentially expressed in this comparison were associated with diverse functions, without highlighting a clear functional pattern. Moreover, 12 unigenes were not annotated in this comparison, with one up-regulated and 11 down-regulated in isolated individuals compared to the control condition. For this comparison, no clear replicate clustering per condition was revealed by clustering heatmap and PCA analyses (**Fig. S1**).

For *A. indicus*, the comparison between the control (CC) and the host isolation condition (IC) revealed differentially expressed unigenes with various functions. For the IC condition, we observed three up-regulated genes coding for structural proteins (*glycine-rich cell wall structural protein 1.8-like, cell wall protein AWA1-like, vegetative cell wall protein gp1-like*) and one coding for a predicted histone activity regulator (*YEATS*

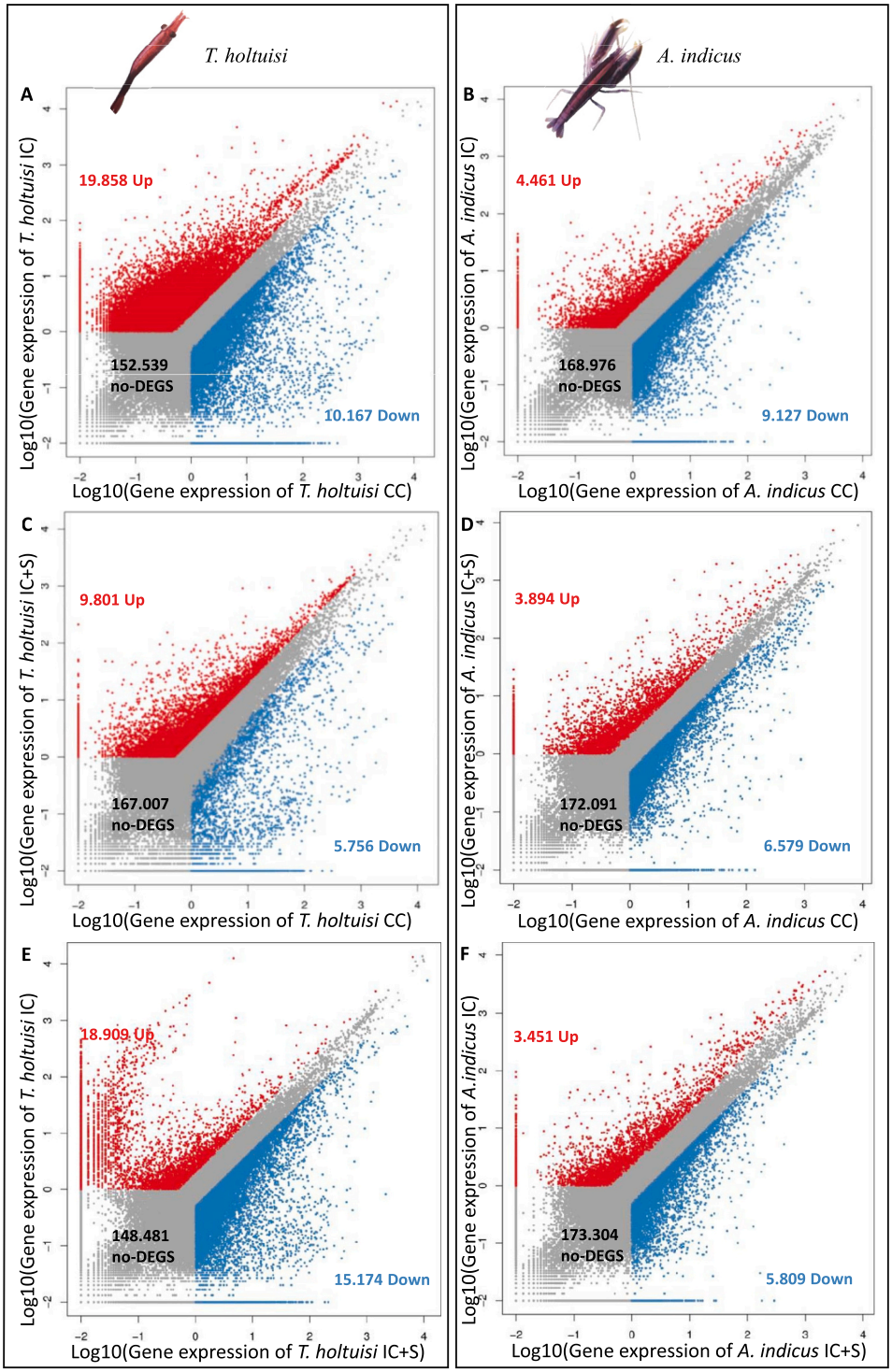

Fig. 4. Differential unigene expression for *Tulеariocaris holthuisi* (A; C; E)and *Arete indicus* (B; D; F). Conditions were "CC: Control condition (with host)"; "IC: Isolation condition"; "IC+S: Isolation condition + spinochromes". For each comparison (A and B = IC/CC; C and D = IC + S/CC and E and F = IC/IC + S), if fold change >1 = gene(s) is upregulated the first condition compared to the second condition and appears in red, fold change <−1 = gene(s) is downregulated the first condition compared to the second condition and appear in blue, and if fold change is comprised between −1 and + 1 = gene(s) is not differentially expressed in the corresponding comparison and appear in grey. The graph was generated using $\log_{10}$ (FPKM +0.01) values.

domain-containing protein 2-like). Down-regulated unigenes included, as selected examples, a cuticle-associated gene (*chitin-binding protein*), and a ligase (*ubiquitin-protein ligase*). Three unigenes known to be expressed in stress conditions (*mitochondrial heme-binding protein* that facilitates necrosis and cell death, two *cathepsins L* that are lysosomal cysteine proteases) (Chen et al., 2013; Kim et al., 2017; Armstrong et al., 2019) were also up-regulated in isolated individuals. In this comparison, twenty sequences were not annotated (seven up-regulated/ 13 down-regulated in IC). For this species, the results of the heatmap and the PCA analyses revealed a more significant clustering, especially in comparison between isolated shrimps with spinochromes (IC + S) (**Fig. S1 and Fig. S2**).

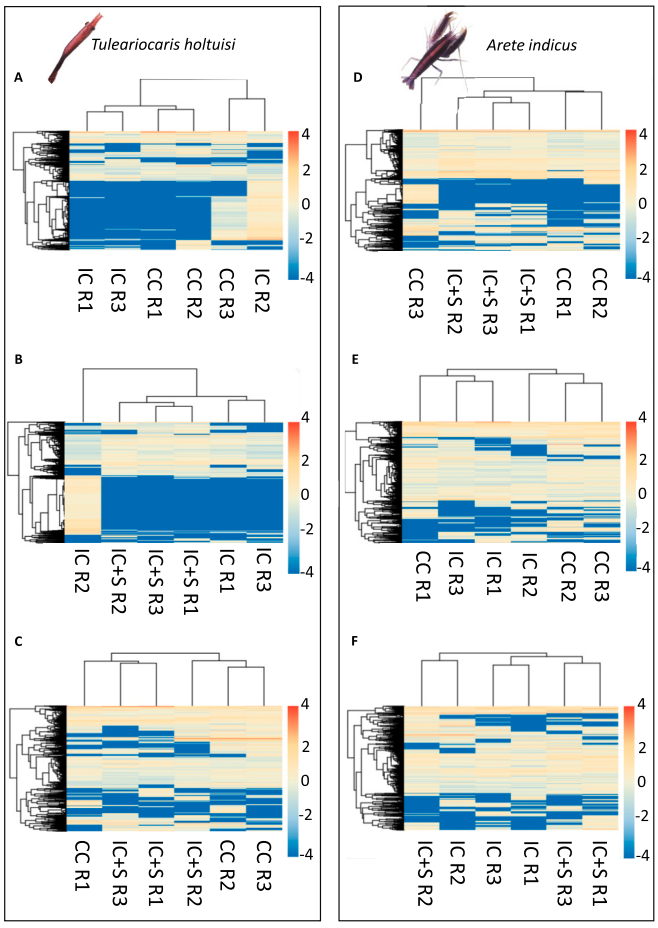

Fig. 5. Clustering heatmap of the fold change of all statistically significant DEGs in relation to the host separation and the chemical dependency for *Tulearioca ris holthuisi* (A, B and C) and *Arete indicus* (D, E and F). The hierarchical clustering analysis is made according to the expression profile. Each row represents a DEG, and each column represents a sample. For each comparison (A and D = IC/CC; B and E = IC + S/CC and C and F = IC/IC + S), the fold change is color coded from blue to red. Both species were exposed to three conditions: "CC: Control condition (with host)"; "IC: Isolation condition"; "IC+S: Isolation condition + spinochromes". The differentially expressed genes were determined in 3 independent replicates (shown as columns of the heatmap).

Based on our results and considering the strong inter-replicate/condition variability, we completed our DEG analyses by performing an additional filtering step on the DEGs. We therefore selected the 100 DEGs for all pairwise comparisons and for both species (**Fig. S2 and** Fig. 6), which present the smallest mean deviation within the same condition. Our hypothesis is that these filtered DEGs will better represent the impact of host isolation than the whole DEG dataset.

For both species, the three experimental replicates clustered consistently by treatment in all comparisons. For *T. holthuisi*, the heatmap of the top 100 differentially expressed unigenes in the CC-IC comparison presents a clear expression pattern with two main DEG clusters (Fig. 6A). In both clusters, most unigenes seemed to be up-regulated in the IC + S condition. In the first cluster (45 DEGs), the DEGs were up-regulated in IC and down-regulated in CC, and in the second cluster (55 DEGS), the DEGs were up-regulated in CC and down-regulated in IC. The majority of DEGs were not annotated in the GO database (79/100, *E* value $>10^{-5}$). The GO annotated of the remaining DEGs are various and distributed across the three main ontologies (*biological process, molecular function and cellular component*). There are eight GO affiliated to the *biological process*, including, for example, the *lipid storage* (GO:0019915) in cluster 1 and the *chitin process* (GO:0006030) found in cluster 2. The cellular component ontology included eight ontologies, such as *integral component of membrane* (GO:0016021), which contained seven different DEGs, including four in cluster 1 and 3 in cluster 2. The *molecular function* included most of the GO terms detected, with 24 different ontologies like *metal ion binding* (GO:0046872), which contained three different DEGs found in both clusters (1 and 2 respectively), *extracellular matrix structural constituent* (GO:0005201) characterized by one DEG found in cluster 2, or *GTP binding* (GO:0005525), which includes 1 DEG of cluster 1.

For *A. indicus*, four main clusters of DEGs were highlighted: (i) cluster 1 (13 DEGs) brings together DEGs up-regulated in IC and IC + S but down-regulated in CC, (ii) the second cluster included 19 DEGs up-regulated in IC but down-regulated in both CC and IC + S, (*iii*) cluster 3 gathered 23 DEGs up-regulated in CC and down-regulated in both IC and IC + S and (*iv*) the last cluster grouped 45 DEGs up-regulated in both CC and IC + S but down-regulated in IC. The majority of DEGs were not annotated in the GO database (86/100, E value $>10^{-5}$). As for *T. holthuisi,* represented GO ontologies were diverse and distributed across the three main ontologies (*biological process, molecular function and cellular component*). Twelve different GO ontologies were highlighted for the *biological process* such as *oxidation-reduction process* (GO:0055114), which contained one DEG found in cluster 1 or the *chitin metabolic process* (GO:0006030), which annotated 1 DEG in cluster 2. There were nine ontologies affiliated with the *cellular component category*, including, for example, the *integral component of membrane* (GO:0016021), which characterized three DEGs found in clusters 1, 3 and 4, or the *extracellular region* (GO:0005576), highlighted for two DEGs found in clusters 1 and 2, for example. The *molecular function* included 22 different detected ontologies such as the *glucose transmembrane transporter activity* (GO:0005355) found once in cluster 4 or the *actin-binding* (GO:0003779), which characterized one DEG of cluster 4.

3.4. Expression of stress-related genes in the host separation condition

To examine the potential differential expression of stress-related genes, genes of interest were selected based on the literature (Chen et al., 2013; Kim et al., 2017; Armstrong et al., 2019) (Table S1) and were obtained from NCBI. Using a BLAST approach, the closest corresponding orthologous genes were identified in both species, and their expression levels were visualized (Table S6 and **Fig. S3**). The selected genes of interest included *cytochrome P450* genes, *heat shock protein* genes, structural protein genes, genes linked to apoptosis and cell cycle, cellular defense effector genes, immune regulator genes, protease and protease inhibitor genes, genes involved in signal transduction, and the *crustacean hyperglycemic hormone protein* gene.

The analysis revealed that *T. holtuisi* exhibit more differential expression than *A. indicus*, with 27 DEGs in control individuals (CC) compared to isolated individuals (IC), 30 for the control individuals (CC) compared to isolated individuals with spinochromes (IC + S), and 21 for the isolated individuals (IC) compared to isolated individuals with spinochromes (IC + S) (Table S6 and **Fig. S3)**. For *A. indicus*, fewer unigenes were differentially expressed, with 20 DEGs in the CC/IC comparison, 11 DEGs in both the CC/IC + S and IC/IC + S comparisons (Table S6 and **Fig. S3)**.

When examining each unigenes individually, categories of differentially expressed genes between treatments did not reveal marked differences except for *heat shock proteins* (HSP). The categories of *cytochrome P450*, antioxidant response, apoptosis, immune regulator cell cycle, and genes involved in signal transduction, categories showed heterogeneity of expression, particularly in *T. holtuisi*, with several corresponding unigenes not being differentially expressed. Categories associated with *structural protein*, *cellular defense effector* and *protease and protease inhibitor* showed only a few sequences with differential expression in pairwise comparison. The *crustacean hyperglycemic*

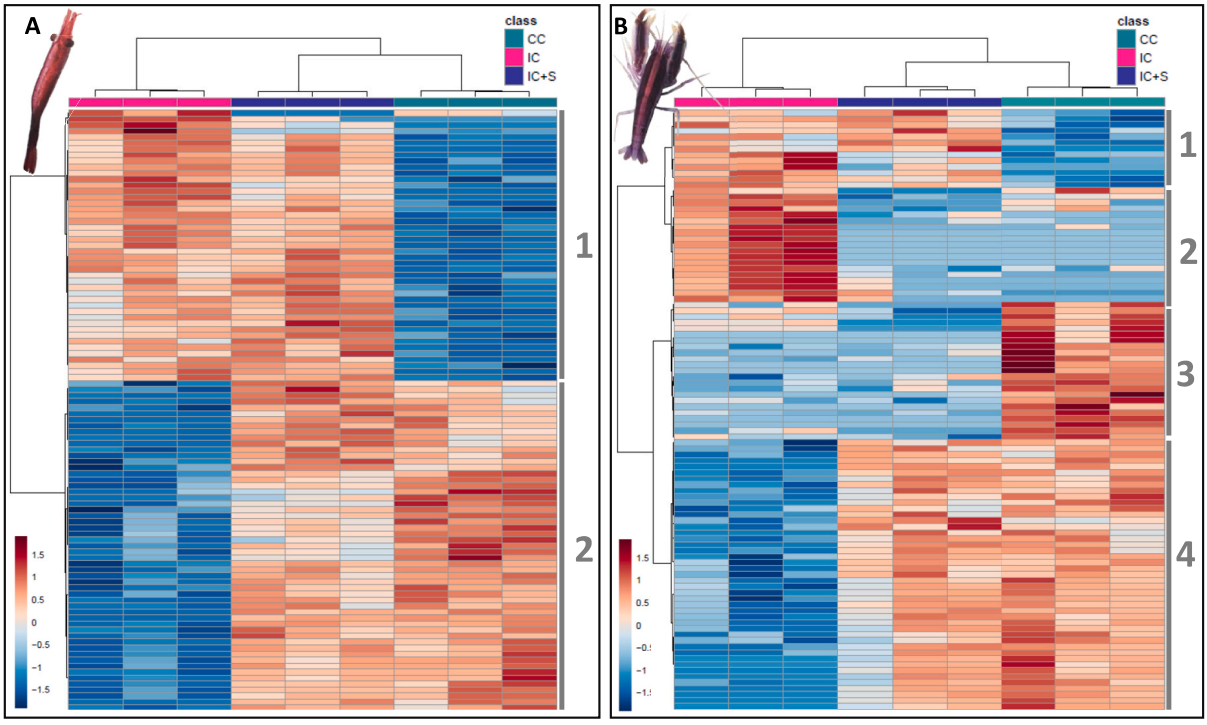

Fig. 6. Cluster heatmap of the top 100 differentially expressed unigenes expression in the context of the host separation and chemical dependency for *Tulеariocaris holthuisi* (A) and *Arete indicus* (B). Both species were exposed to three conditions: "CC: Control condition (with host)"; "IC: Isolation condition"; "IC+S: Isolation condition + spinochromes". The differentially expressed genes were determined in all independent biological replicates (shown as columns of the heatmaps). The heatmaps are based on all differentially expressed genes for every comparison (shown as a row of the heatmaps). The similarity of the gene expression patterns is indicated by Pearson's distance dendrogram at the top and left sides. For every comparison (A and D = IC/CC; B and E = IC + S/CC and C and F = IC/IC + S), the colors of the heatmap indicate the unigene expression level (log_{10} (FPKM+ 0.001)).

hormone protein, LITAF, and the signal transduction categories showed no strong expression in any treatment for both species.

Out of the nine targeted HSP unigenes, in *A. indicus*, the control individuals (CC) compared to isolated individuals (IC) highlighted three differentially expressed unigenes, and the two other comparisons showed one differentially expressed gene each (Table S6; Fig. 7**)**. For *T. holtuisi*, out of the nine selected sequences, six were mostly expressed in the IC and three in IC + S. (Table S6; Fig. 7).

The HSP heatmap (Fig. 7) indicated that the control shrimps were well grouped together, as most of the nine targeted HSP unigenes were down-regulated: this is evident for the control individuals 1 and 2, and less clear for the individual 3, where only four unigenes were down-regulated. The isolated individuals and those isolated with spinochromes were mainly clustered together (5 out of 6 individuals) except the isolated individual 2, which had five down-regulated HSP unigenes, whereas all the others showed from four to seven up-regulated HSP unigenes. Among the five individuals that exhibited up-regulation of the HSP unigenes, there was, however, no clear distinction between shrimps

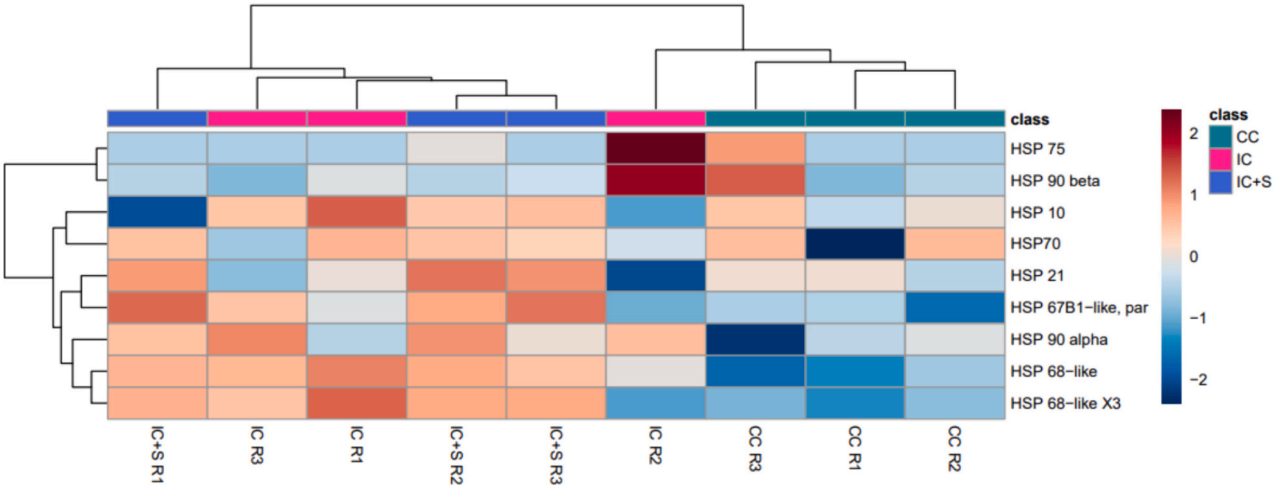

Fig. 7. Heatmap of Heat Shock Protein (HSP) in *Tulеariocaris holtuisi* subjected to host separation. Unigene annotation is shown on the right side of the heatmap. There are three conditions: "CC: Control condition (with the host)"; "IC: Isolation condition"; "IC+S: Isolation condition + spinochromes". The similarity of the gene expression patterns is indicated by Pearson's distance dendrogram at the top and left sides. The colors of the heatmap indicate the HSP unigene expression (log_{10} (FPKM+ 0.001)).

exposed to spinochromes and those that were not. We observed that HSP21 and 67B1-like genes were highly expressed in isolated individuals with spinochromes (IC + S). Differential expression was also noted between IC and CC of the HSP21 gene, with the slight up-regulation in IC. The HSP68-like, 68-like isoform X3, and HSP90 alpha genes were up-regulated in both IC and IC + S compared to the control condition, without showing a strong difference between the two treatments. The HSP75 and 90 beta genes showed higher expression in IC compared to both IC + S and the control condition, with a slight down-regulation observed in IC + S compared with the control condition.

4. Discussion

Symbiotic relationships between decapods and echinoderms are common in marine ecosystems, with many associations being obligatory for the symbionts to complete their life cycle (Castro, 1988; Gherardi, 1991; De Grave and Fransen, 2011; Horká, 2017). The shrimp *T. holthuisi* is mainly found on the sea urchin *E. mathaei*, its primary host, especially on the Great Reef of Toliara (Hipeau-Jacquotte, 1965; Brasseur et al., 2018), but it can also be found on other secondary sea urchin hosts such as *Diadema mexicanum*, *Astropyga radiata*, or *Stomopneustes variolaris* (Castro, *1971;* Bruce, *1974;* Wicksten and Hernández, 2000*;* Marin and Anker, 2009*;* Salas-Moya et al., *2021)*. The second obligate symbiont investigated here, *A. indicus*, appears to be strictly species-specific with *E. mathaei* (Gherardi, *1991;* Dabbagh et al., *2019;* Limviriyakul et al., *2020;* Ghory and Kazmi, 2021*)*, but questionable occurrences of this species have been described on *Diadema sp.* and *Heliocidaris crassispina* (Miya and Miyake, 1968*;* Ganapati and Sastry, 1972*;* Al-Kandari et al., *2020)*. Their hosts provide them with various benefits, such as a safe shelter from predators and food platforms, which may make them host-dependent (Gherardi, 1991; Brasseur et al., 2018). *T. holthuisi* feeds directly on its host, while *A. indicus* prefers seaweeds and can sweep up the crumbs from the sea urchin meal (Gherardi, 1991; Brasseur et al., 2018). They both present specific adaptations to efficiently live in symbiosis with their host, such as a specific motion behavior or a pigmentation that mimics the color of their host (Gherardi, 1991; Horká et al., 2016; Brasseur et al., 2017; Horká, 2017; Salas-Moya et al., 2021). *A. indicus* is always found grasping onto a sea urchin spine with the head pointed outwards, while *T. holthuisi* shows a similar behavior with the head directed in the opposite direction (Gherardi, 1991; Horká, 2017). *T. holthuisi* is particularly efficient in mimicry of the host spine, as its body shape and color are almost identical to the host spines (Hipeau-Jacquotte, 1965; Brasseur et al., 2017; Horká, 2017). Moreover, Brasseur et al. (2018) demonstrated that the survival of *T. holthuisi* separated from its host could be extended if it is placed in the presence of the chemical environment produced by its host, the spinochromes (Brasseur et al., 2018). Although both *A. indicus* and *T. holthuisi* are obligate and highly host-dependent symbionts, the latter has a stronger chemical dependence on its host.

Aiming to investigate the effects of host separation on both symbionts through a transcriptomic approach, this study also wants to examine the chemical dependency of *T. holthuisi* and the effect of spinochromes, which seem to attenuate the separation stress in this species (Brasseur et al., 2018). For that purpose, we compared the gene expression of both species in three conditions: unstressed symbionts living on their hosts, isolated stressed individuals, and isolated individuals in seawater presenting the chemical environment of the host, notably the spinochrome pigments.

We observed significant differences in the number of differentially expressed genes (DEGs). For *A. indicus*, the three comparisons showed a similar number of DEGs (CC/IC = 7.4 %; CC/IC + S = 5.7 % and IC/IC + S = 5 %). For *T. holthuisi*, the total predicted unigenes showed similar differential expression levels (CC/IC = 16.5 % and IC/IC + S = 18.6 %) when comparing isolated individuals with the other conditions (CC/IC + S = 8.5 %). The presence of spinochromes, however, is associated with a 50 % decrease in DEGs in the comparison between control individuals and those isolated in spinochrome-containing environment. This finding suggests that spinochromes have a significant effect at the molecular level, bringing the health status of isolated shrimp closer to that of individuals living on hosts. However, the number of DEGs remains high in other comparisons (*T. holthuisi*: CC/IC + S; *A. indicus*: CC/IC, CC/IC + S and IC/IC + S), which also suggests a transcriptomic regulation in conditions that appear less impactful (IC + S in both species and IC for *A. indicus*) in the survival experiments performed by Brasseur et al. (Brasseur et al., 2018)

Having observed certain differences in the number of DEGs according to the categories of individuals, we expected to obtain well-defined clusters depending on the condition involved, which was not often the case. We first focused on the 40 most significant expressed unigenes (20 up-regulated and 20 down-regulated). In *T. holthuisi,* no clear grouping was found in any of the three conditions which indicates a high inter-individual variability. Variations in the age, sex and proximity to the molting time could have greatly influenced the clustering of individual gene expression profiles. In filtered DEGs, *T. holthuisi* presents two clear clusters of DEGs, while *A. indicus* presents five clusters of differentially expressed genes in the IC compared to the CC. This heterogeneity of differential expression of genes in this comparison may highlight a more complex process of regulation to cope with host separation for *A. indicus* and may explain why this species does not seem to be affected by host separation. While *T. holthuisi* individuals are affected by the host separation and do not survive long, *A. indicus* may present alternative expressions of genes to cope longer with this stress. For at least a short period, this species will not appear to be impacted in their survivability by the host separation.

We then determined a list of genes expressed during stress conditions in crustaceans based on the scientific literature to investigate their expression in our study (Chen et al., 2013; Kim et al., 2017; Armstrong et al., 2019). By comparing conditions (CC, IC and IC + S), we observed that *T. holtuisi* had more differential expression, in all pairwise comparisons, than *A. indicus*. This suggests that, in all cases of isolation, with or without spinochromes, the stress genes are more differentially expressed in the species *T. holthuisi*. We then focused on heat shock proteins (HSPs) which are highly conserved and ubiquitous proteins primarily functioning as molecular chaperones (Bruce, 1974). These proteins facilitate the folding, refolding, and unfolding of partially denatured proteins, enabling protein movement across cellular compartments and membranes, as well as degradation of unstable proteins and dissolution of protein complexes and aggregates (Bruce, 1974; Li et al., 2008; Dabbagh et al., 2019; Al-Kandari et al., 2020; Limviriyakul et al., 2020; Ghory and Kazmi, 2021). Although initially discovered as proteins that respond to high thermal stress, HSPs are now known to perform a variety of other functions, and their up-regulation, especially under environmental and non-environmental stress conditions, has been described in various organisms, including crustaceans (Miya and Miyake, 1968; Bruce, 1974; Chen et al., 2013; Dabbagh et al., 2019; Limviriyakul et al., 2020). HSPs participate in various stress responses, such as oxidative stress, osmotic shock, starvation, toxic chemicals, bacterial infections, and immune reactions (Bruce, 1974; Chong and Xia, 2018; Kim et al., 2017; Ganapati and Sastry, 1972; Linden et al., 2009; Ghiselli et al., 2012; McCormick et al., 2018). HSPs are generally categorized into different families based on their molecular weights (McCormick et al., 2018).

In the current study, *T. holtuisi* showed a general up-regulation of HSPs in both isolation treatments compared to the control condition, particularly in the complete host isolation condition. Additionally, several stress-related genes that have been previously identified in crustaceans, such as the crustacean hyperglycemic hormone and antioxidant-response proteins were not differentially expressed in all pairwise comparisons for both species in these analyses. Since our stress-related gene focused only on the recovering the closest homologs in both species (i.e., potential ortholog), the selected sequences do not always correspond to the most expressed or the most differentially expressed

unigenes coding for protein homologs, which may cause a bias in our analyses.

During this study, numerous DEGs appeared not to be annotated using the classical public databases. This may be explained by the fact that the investigated species are poorly studied non-model organisms for which no genomic data is available. Stress-related genes of interest may not have been captured by the analyses. Due to technical constraints (*i.e.,* difficult field conditions in Madagascar) and to avoid RNA degradation during a potential dissection procedure, the experiments were performed on whole-body tissue of the symbionts. This is a methodological limitation as most transcriptomic stress responses are more frequently evaluated by targeting specific tissues of interest only (*e.g.,* gills (Tarazona et al., 2011; Kanehisa et al., 2017; Pang et al., 2022), muscles (Tarazona et al., 2011), or the hepatopancreas (Chong and Xia, 2018)) to avoid dilution effects (*i.e.,* potential high-coverage of read extracted from a tissue for which no physiological response is observed) or RNA contamination (*e.g.,* coming from organisms present in the digestive system). This choice was driven by two factors: first, the studied symbiotic shrimps are small (ranging from 0.6 to 1 cm), and second, no prior data was available to inform us about the most suitable tissue to focus on since the gene expression of crustacean stress-response has been more frequently evaluated on stress including salinity (Tarazona et al., 2011; Kanehisa et al., 2017), temperature (Chen et al., 2013), and various chemical exposures (Kim et al., 2017; Chong and Xia, 2018; Pang et al., 2022). In addition, due to the small size of the echinoid-associated shrimp specimens, it was impossible to determine the sex of these individuals accurately. This limitation is a common constraint encountered in studies involving small, cryptic species inhabiting complex ecosystems (Ghiselli et al., 2012). The inability to distinguish between male and female individuals may have implications for understanding the transcriptomic responses observed in this study. Sex-specific differences in gene expression can be crucial in shaping the symbiotic relationships between hosts and symbionts. Therefore, the lack of sex information in our dataset should be considered when interpreting the results and their implications in the host-separation context.

In the case of environmental stress, the energy balance of an organism can be significantly disrupted, as additional energy is required to maintain metabolic integrity and recover from stress-induced damages (Horká, 2017; Armstrong et al., 2019). Previous studies have identified two main survival strategies in response to stress: the “limiting process” and the “compensatory process” (Castro, 1971; Wicksten and Hernández, 2000;Armstrong et al., 2019; Salas-Moya et al., 2021). The limiting process occurs in low-level stress environments and relies on cellular, physiological, and behavioral mechanisms to minimize and cope with the negative impact of stress before it causes significant damage to the organisms (Castro, 1971; Armstrong et al., 2019). Although more energy-intensive, the compensatory process offsets the impact of stress, such as the production of reactive oxygen species that may damage DNA, which can lead to the death of the organism or a reduction in fitness-related functions (Armstrong et al., 2019; Salas-Moya et al., 2021). In our study, the transcriptomic approach yielded mixed results, indicating that both species are impacted by host isolation stress but with different degrees of coping. *A. indicus* can cope with this kind of stress, at least for a limited period(>four days), without a decrease in its survival rate (Brasseur et al., 2017). However, various genes with essential functions appeared differentially expressed which indicated potential micro-adaptation of the different individuals to the isolation stress. In addition, *A. indicus* is a bigger symbiont than *T. holthuisi*, and this size can play a key role in its survivability as the larger species is usually more able to cope with stressful situations (Linden et al., 2009; McCormick et al., 2018). For *T. holthuisi,* the species exhibiting a clear host-separation syndrome and a strong host chemical dependence (Parmentier and Michel, 2013), results indicated a strong transcriptomic response. In both conditions, shrimps may be stressed, but the addition of spinochromes may enable the shrimp to cope more efficiently with the isolation situation. The up-regulation of HSPs may have played a key role in the compensatory process used by the symbiont to defend itself against the stress of isolation until the stress became too severe, eventually resulting in organism death.

5. Conclusion

In this study, we present the transcriptome sequencing and analysis of two symbiotic shrimps associated with the sea urchin *E. mathaei* in the southwest of Madagascar. Our work provides the first report on the use of NGS techniques for studying the host-separation syndrome involving a new kind of host reliance: chemical dependency.

We generated a reference transcriptome for two symbiont species, *A. indicus* and *T. holtuisi*, by obtaining >96.41 clean Giga bases. For *T. holthuisi*, the results reveals a strong transcriptomic response, particularly in isolation without the addition of the chemical environment produced by the host. Based on a comparison of gene expression, genes coding for heat shock proteins were found to be differentially expressed in response to the chemical host-isolation experiment. The addition of spinochromes would enable this shrimp to better cope with the stressful situation. Similarly, *A. indicus* also showed signs of stress from host isolation, yet it demonstrated potential for coping with this stress through transcriptomic adaptation. Our findings contribute to the understanding of the host-separation syndrome and the symbiont dependence on semiochemicals produced by their host.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2024.101327.

Ethics approval

Animals used in our experiments were maintained and treated in compliance with the guidelines specified by the Belgian Ministry of Trade and Agriculture.

CRediT authorship contribution statement

Alexia Lourtie: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Igor Eeckhaut:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Guillaume Caulier:** Writing – review & editing, Validation, Methodology, Conceptualization. **Lola Brasseur:** Resources, Methodology, Conceptualization. **Jérôme Mallefet:** Writing – review & editing, Supervision. **Jérôme Delroisse:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

SRA archive files and transcriptome assemblies are publicly accessible via the NCBI SRA and TSA servers. The list of all DEGs, with their corresponding annotation, is accessible with the following link: http://www.ncbi.nlm.nih.gov/bioproject/1086254; BioProject ID PRJNA1086254.

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