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RESEARCH ARTICLE

Different transcription regulation routes are exerted by L- and D-amino acid enantiomers of peptide hormones

Moshe Tom[1,*] Chiara Manfrin[2], Alessandro Mosco[2], Marco Gerdol[2], Gianluca De Moro[2], Alberto Pallavicini[2] and Piero Giulio Giulianini[2]

ABSTRACT

Conversion of one or more amino acids in eukaryotic peptides to the D-enantiomer configuration is catalyzed by specific L/D-peptide isomerases and it is a poorly investigated post-translational modification. No common modified amino acid or specific modified position has been recognized, and mechanisms underlying changes in the peptide function provided by this conversion are not widely studied. The 72 amino acid crustacean hyperglycemic hormone (CHH) in Astacidea crustaceans exhibits a co-existence of two peptide enantiomers with either D- or L-phenylalanine as their third residue. It is a pleiotropic hormone regulating several physiological processes in different target tissues and along different time scales. CHH enantiomers differently affect time courses and intensities of examined processes. The short-term effects of the two isomers on gene expression were examined in the hepatopancreas, gills, hemocytes and muscles of the astacid *Pontastacus leptodactylus*. Gene expression in muscles and hemocytes was not affected by either of the isomers. Two modes of action for CHH were elucidated in the hepatopancreas and the gills: specific gene induction in both organs by D-CHH, and targeted attenuation caused by both enantiomers in the gills. Consequently, a two-receptor system is proposed for conveying the effect of the two CHH isomers.

KEY WORDS: *Pontastacus leptodactylus*, Crustacea, Post-translational modification, Crustacean hyperglycemic hormone (CHH), D/L-peptide enantiomers, Digital analysis of gene expression

INTRODUCTION

Conversion of an amino acid in eukaryotic peptides from the biologically dominant L-configuration to the D-configuration is a poorly studied post-translational modification. The mRNA of D/L-peptides contains codons for L-amino acids and isomerization of one of these amino acids is catalyzed post-translation by specific peptide L/D-isomerases (Bansal et al., 2008; Heck et al., 1996; Jilek et al., 2005; Shikata et al., 1995). This apparently uncommon chirality was revealed in a variety of taxa, reviewed by Kreil (Kreil, 1997) and summarized by Buczek et al. (Buczek et al., 2005) and Ollivaux et al. (Ollivaux et al., 2009). Although not confined to a particular amino acid or a specific position along the peptide, the isomerization is located near one of the peptide termini. Most eukaryotic D-isomerized peptides are toxins, exerting their effects on tissues of other species. The D-isomer is the more active isomer and often the only existing one, and synthetic L-isomers were experimentally synthesized. In most cases, synthetic or native L-isomers were not active and/or more vulnerable to degradation (Buczek et al., 2005; Erspamer, 1992; Fujimoto et al., 1991; Heck et al., 1994; Jimenéz et al., 1996; Mignogna et al., 1993). In contrast, both stereoisomers of the 39 amino acid native pair of C-type natriuretic peptides in the Australian mammal platypus (*Ornithorhynchus anatinus*) are equally active as rat uterus muscle relaxant (Torres et al., 2002) and it is the only simultaneously functioning D/L-peptide pair besides the two pairs of crustacean neurohormones belonging to the crustacean hyperglycemic hormone (CHH) family.

The CHH neuropeptide family is mainly represented in crustaceans but is also found in insects and its biological and chemical aspects were recently reviewed in relation to a variety of physiological processes (Chung et al., 2010; Dircksen, 2009; Giulianini and Edomi, 2006; Katayama et al., 2013; Webster et al., 2012). CHHs are produced in the X-organ and secreted to an adjacent hemal sinus termed the sinus gland. The X-organ is composed of neurosecretory perikarya sited in the medulla terminalis of the optic ganglion located within the crustacean eyestalk and the entire organ is termed the X-organ sinus gland (XOSG). Hence, bilateral ablation of the eyestalk eliminates CHH production and secretion. However, CHHs identical to the XOSG-secreted peptides or their similar structural isoforms are also produced in other tissues: the pericardial organ, the subesophageal ganglia and the fore- and hindgut. CHH receptors were identified in the hepatopancreas, Y-organs, gills, hindgut and abdominal muscle of a variety of crustaceans (Chung and Webster, 2006; Katayama and Chung, 2009; Kummer and Keller, 1993; Webster, 1993).

Two D/L pairs of CHH family members were elucidated in Astacidea crustaceans. One is the vitellogenesis/gonad inhibiting hormone, characterized by L- and D-tryptophan in its fourth N-terminus position (Ollivaux et al., 2006; Ollivaux et al., 2009). The other one, the target peptide of this study, is the XOSG-secreted CHH, after which the entire family was named. It is a 72 amino acid neuropeptide, characterized by six cysteines forming three intradisulfide bridges, p-glutamyl at its N-terminus and valyl amide at its C-terminus (Mosco et al., 2008). The Astacidea CHH is present in the circulation as a mix of two chiral isomers differing by the presence of D- or L-phenylalanine at the third N-terminus position in contrast to the exclusively L-CHH peptide of crabs (Chung and Webster, 1996). The two enantiomers were simultaneously discovered in *Homarus americanus* (Soyez et al., 1994) and *Procambarus clarkii* (Yasuda et al., 1994). Later, they were also identified in other astacids (Aguilar et al., 1995; Bulau et al., 2003; Serrano et al., 2003; Soyez et al., 1997). Each of the two isomers is synthesized in specific X-organ cells (Soyez et al., 1998) and the L-isomer of *H. americanus* (Soyez et al., 1997) and *Orconectes limosus* (Ollivaux and Soyez, 2000) is about three times more abundant than the D-isomer. Recently, the two CHH enantiomers of *Pontastacus leptodactylus* were chemically synthesized and the physiological effects of the

[1]Israel Oceanographic and Limnological Research, P.O.B. 8030, Haifa 31080, Israel. [2]Department of Life Sciences, University of Trieste, Via Licio Giorgieri 5, Trieste, 34127, Italy.

*Author for correspondence (tom@ocean.org.il)

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List of abbreviations

CHH	crustacean hyperglycemic hormone
CL	carapace length
ER	endoplasmic reticulum
MMI	molt mineralization index
NCBI	National Center for Biotechnology Information
PBS	phosphate-buffered saline
PCA	principal component analysis
SR	sarcoplasmic reticulum
SRA	Sequence Read Archive
TSA	transcriptome shotgun assembly
XOSG	X-organ sinus gland

recombinant proteins have been demonstrated (Lebaupain et al., 2012; Mosco et al., 2012). Access to large amounts of synthesized isomers obviates the tedious extraction and purification procedures of the native isomers from the XOSG.

Three of the functions assigned to CHH have been studied in the context of differences between the physiological activities of the two isomers. Yasuda et al. (Yasuda et al., 1994) applied the two isomers to *in vitro* incubated Y-organs, the molting hormone-producing organ, from *P. clarkii* and noticed about 10-times stronger inhibition of molting hormone synthesis by D-CHH than by the L-isomer. Serrano et al. (Serrano et al., 2003) demonstrated that, in *P. leptodactylus,* a more prominent increase of osmolarity and sodium concentration in the circulation was caused by D-CHH than by L-CHH. Finally, a different time course and intensity of increase in circulating glucose was triggered by the two isomers in *P. leptodactylus* (Lebaupain et al., 2012; Mosco et al., 2012; Serrano et al., 2003). Earlier hyperglycemia was induced by L-CHH but it lasted for a shorter period and was less intensive than that induced by D-CHH.

The narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz 1823) is the experimental species of the present study. It is a freshwater European astacid that inhabits an area between France and the Ukraine, including Great Britain (Souty-Grosset et al., 2006). Its ovarian development ranges from June to November (Hubenova et al., 2009), with mating from December to January and spawning in January. Eggs are incubated glued to the female pleopods until July (Balik et al., 2005).

The majority of the D/L-peptide pairs described to date are toxins, often lacking an L-isomer and where it does exist, the L-isomer is not active and is more sensitive to proteolysis. In contrast, CHH enantiomers are both active hormones, endogenously exerting their partially overlapping effects and distinguished by different time course and intensity of effect. The present study is aimed at comparative examination of short term effect of the two isomers on multi-gene expression profile in several *P. leptodactylus* target tissues.

RESULTS

Experimental design

The study is composed of two experiments. Experiment 1 was carried out in August 2011, using females after hatching with undeveloped ovaries and experiment 2 was carried out in October 2012, using vitellogenic females with developed ovaries. The use of vitellogenic females was dictated by the availability of individuals. The results of experiment 1 have already been published (Manfrin et al., 2013) but were partially reanalyzed here to allow comparison with experiment 2. The plan of experiment 1 is detailed in Manfrin et al. (Manfrin et al., 2013). Briefly, 2 days after removal of their eyestalks, which aimed to decrease the circulating glucose to a basal level, non-vitellogenic females at intermolt were injected with D- or L-CHH. The females were killed 1 h post injection. Two control female groups were used, sham-injected ones, which were treated similar to hormone-exposed crayfish and injected with the hormone carrier phosphate-buffered saline (PBS), and a native, non-ablated, non-injected group. Hemolymph samples were taken just before injection and 1 h post injection at time of death.

Experiment 2 differed, not only in the use of vitellogenic females, but also the eyestalks were not ablated before application of the two hormone isomers. We have noted previously that pre-injection glucose levels of non-ablated females are sufficiently low to distinguish the effect of the injected hormone. The *P. leptodactylus* females of experiment 2 were divided into three experimental groups. A control group that was sham-injected with PBS and two experimental groups injected with one of the two CHH isomers.

Morphology and physiology of the experimental females

The morphometric characteristics of the experimental females of experiment 1 and their molt stage were described previously (Manfrin et al., 2013). The average carapace length of females in experiment 1 and experiment 2 was 40.05±1 and 40.4±2.1 mm, respectively, and there was no significance difference between treatment and control groups. All analyzed females of both experiments were at intermolt. The effect of CHH on circulating glucose level and its time course upon pharmacological application of each of the isomers is well documented and was used here to ascertain the effect of the applied CHHs. In experiment 1, it was examined only 1 h after injection and its level was as expected (Manfrin et al., 2013). A broader time scale of 48 h was examined in experiment 2 and results are presented in supplementary material Fig. S1, revealing the typical time course and intensity caused by each of the applied isomers (Mosco et al., 2012).

RNA-seq analysis of the effects CHH isomers

Short sequences that directly result from next generation sequencing are termed ‘reads’ to distinguish them from the conceptual longer reads termed contigs. All *P. leptodactylus* reads available to us were deposited in the Sequence Read Archive (SRA). They include reads that were determined in the framework of the present study (SRR1258878, SRR1262377, SRR1262390, SRR1264022 and SRR1264027) and others that were determined previously, SRR629687 (Tom et al., 2013) and SRR650486 (Manfrin et al., 2013). All these reads were used for the construction of a *de novo* comprehensive transcriptome assembly. The assembly contained 106,363 contigs with an average length of 779±1053 bp deposited at the Transcriptome Shotgun Assembly (TSA acc. no. GBEI01000000) of the American National Center for Biotechnology Information (NCBI). The characteristics of the four tissue-specific reference assemblies are described in Table 1.

The samples of both experiments were uniformly labeled, with the first letter(s) designating the tissue: H, hepatopancreas; HM, hemocytes; G, gills; M, muscles. The second letter designates the treatment: D, D-CHH application; L, L-CHH application; S, sham-injected; N, native females. The first (or first and second) number designates the exposure period to the treatment (in hours) and the final number is the serial number within the group (e.g. HMD21 indicates hemocytes exposed to D-CHH for 2 h in female 1). The following RNA-seq analyses were performed: (1) re-analysis of hepatopancreatic experiment 1 expression results 1 h after exposure to CHH using the reference assembly constructed here; (2) hepatopancreatic experiment 2 expression results after exposure for 2 and 8 h (2-8); (3) experiment 2 hemocyte, gill and muscle

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Table 1. Global characteristics of the tissue-specific reference assemblies and their RNA-seq results

Tissue	*N*	Size of ref. assembly (No. of contigs)	Overall mapping (%)[a]	Mapping in ref. assembly (%)[b]	Total reads of each sample (×10^6)	Mapped by RNA-seq (%)[c]	Non-specifically mapped by RNA-seq (%)[c]
Experiment 1							
Hepatopancreas	12	21,219	71.58	98.3	11.9±4.2	53±4.7	1.9±0.5
Experiment 2							
Hepatopancreas	12	21,219	71.58	98.3	10.6±3.5	59.9±1.5	2.6±0.3
Gills	6	21,383	75.07	96.6	39.6±1.3	62.4±2.2	1.9±0.2
Muscle	6	8795	86.66	97.5	34±3.7	75.5±0.4	1.3±0.1
Hemocytes	6	20,969	69.93	96.6	39.7±4.8	56.1±0.6	2±0.2

[a]From all reads in the relevant tissue.
[b]From all mapped reads.
[c]An average of sample-specific RNA-seq mappings presented as means ± s.d.

expression results after exposure for 2 h; and (4) comparison of HN1 non-vitellogenic females with HS2-8 vitellogenic females. Analyses 1–3 were performed against sham-injected females as controls and analysis 4 against the native females as a control.

Mapping in the present context is the assignment of reads to contigs by sequence similarity. The RNA-seq mapping percentages ranged from 53 to 75% and among them, non-specific mappings ranged from 1.3 to 2.6%, which is a relatively low value (Table 1). Hence, only the profiles of unique mappings were used for the RNA-seq analyses. A contig was considered differentially expressed in comparison to the control by passing three thresholds: (a) a statistical *P* value of $P<0.05$ [not corrected for multiple testing because of the low number of samples in each of the experimental groups (2–3 samples)]; (b) fold change >|2|; and (c) at least 100 normalized reads per contig in at least one member of the compared pair. Thresholds (b) and (c) were aimed at avoiding misinterpretation of natural variability as differential expression. The characteristics of the transcripts presented below in the context of the RNA-seq analyses including their conceptual transcript and protein sequences and their protein domains are presented in supplementary material Table S1.

Hepatopancreatic gene expression

Principal component analysis (PCA) of the hepatopancreatic samples of experiment 1 and experiment 2 (Fig. 1) provides an overview of the hormonal effects on the transcript profiles as an indication of the physiological state of the hepatopancreas. D-CHH treatment in experiment 1 resulted in a clustered expression profile compared with the L-CHH-treated, the sham-injected and the native females. Vitellogenesis resulted in a high similarity among all samples in experiment 2 regardless of the treatment and exposure period. Overall differential expression was characterized, for each treatment in the two experiments, by three parameters: (1) the summed percentages of reads (from the total number of reads) that were mapped to induced or attenuated contigs; (2) the number of reads/induced or attenuated contig; and (3) the number of induced and attenuated contigs. The characterization of the overall differential expression in experiment 1 is presented in Fig. 2. The pronounced effect of D-CHH is clearly demonstrated, encompassing 23.4% induced reads and 15.6% attenuated reads over or under the percentages of the control. The induction involved only 54 genes that were highly induced as shown by the increase in the average number of reads per contig and by the high standard deviation, indicating that even fewer genes contributed most of the induction (Fig. 2B,C). D-CHH-related attenuation involved a high number of genes (1318), each of which contributed a small number of reads (Fig. 2B,C). L-CHH caused minor induction, which was considered negligible in terms of percentage reads and the number of reads per contig. The L-CHH attenuation involved only 27 contigs with a relatively high number of reads per contig (Fig. 2B,C).

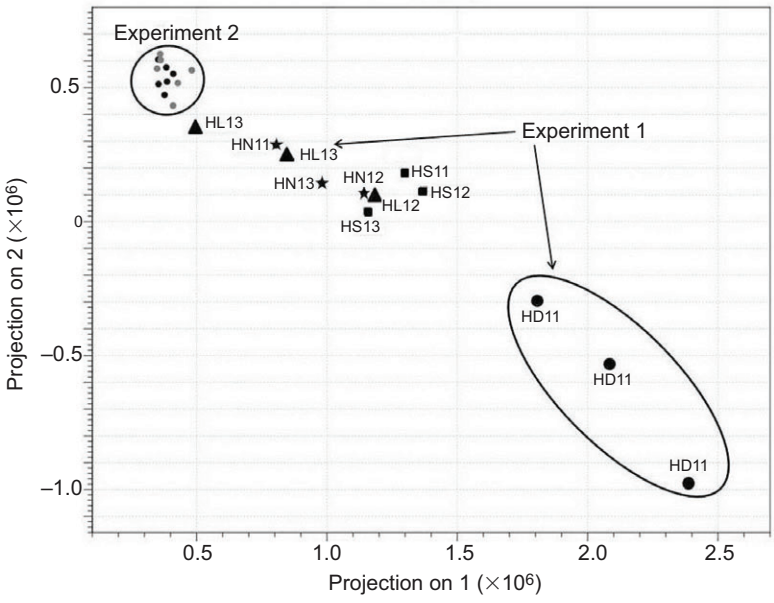

Fig. 1. Principal component analysis of hepatopancreatic expression profiles in the narrow-clawed crayfish *Pontastacus leptodactylus*. H, hepatopancreas; second letter: D, D-CHH injected; L, L-CHH injected; S, sham injected; N, native females; first number indicates exposure time (h); second number indicates serial number of the sample within a group. Experiment 2 samples are not labeled for the sake of clarity; the respective 2 h time point samples are black and the 8 h samples are gray.

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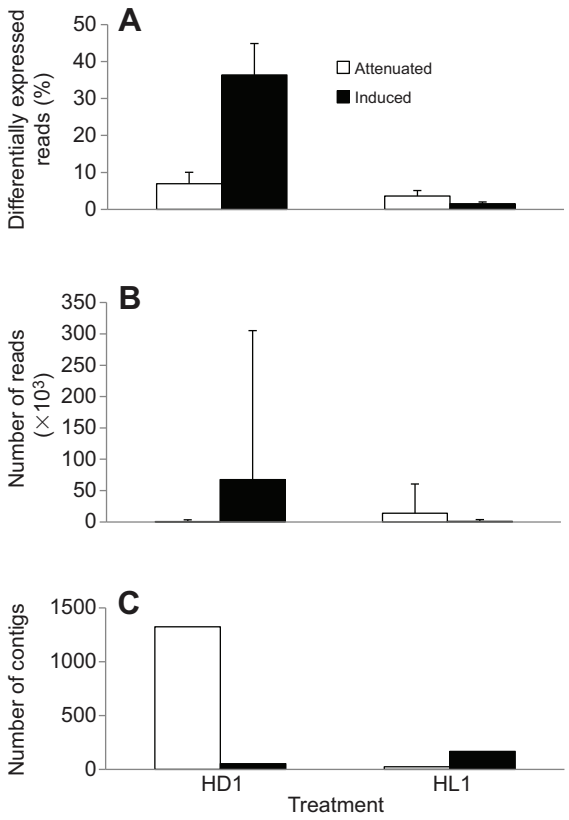

Fig. 2. Characterization of overall differential expression in *P. leptodactylus* injected with D-CHH or L-CHH. Results are from hepatopancreas of all crayfish examined in experiment 1 and represent means ± s.d. (A) Percentage of reads mapped to the differentially expressed contigs from the entire transcript population for the two treatments. (B) Mean number of of reads mapped to the contig. (C) Number of differentially expressed contigs. HD1, hepatopancreas from animals 1 h after injection with D-CHH; HL1, hepatopancreas from animals 1 h after injection with L-CHH.

The major contributing contigs (96%) to the induction caused by application of D-CHH are presented in Table 2 including three non-annotated contigs of which two are the most affected by the D-isomer. The major contributing contigs (98%) to the attenuation caused by application of L-CHH are presented in Table 3. Interestingly, two of these three attenuated contigs are also major contributors to the induction caused by application of D-CHH. Unlike experiment 1, no effect of hormone on hepatopancreatic gene expression was observed in experiment 2 (Fig. 1 and supplementary material Fig. S2).

Expression analysis of the native females of experiment 1 was compared with expression in the sham-injected females of experiment 2. The females in these two experimental groups have an intact XOSG, they were not treated with hormones and a dramatic change in the gene expression profile was observed in the hepatopancreas of vitellogenic females in comparison with non-vitellogenic ones. Around 40% of the transcript population is involved in the change in both directions. The major change is increased vitellogenin expression, constituting 8.8% of the hepatopancreatic transcript population, in comparison to 0% in the non-vitellogenic females. However, more than 3000 other contigs were also induced. It is outside the scope of the present study, which deals with the effects of CHH, to analyze in detail the conversion of the hepatopancreas into a vitellogenic organ.

Gene expression in the gills

In experiment 2, the lack of any effect of CHH on the vitellogenic hepatopancreas on one hand, but a normal CHH-affected hyperglycemia on the other hand (supplementary material Fig. S1), led us to assume that CHH has an effect in other tissues. Therefore, gene expression was examined in the muscle, the hemocytes and the gills. The characterization of overall differential gene expression in the gills upon hormonal application is presented in Fig. 3. In general, the effect of hormonal treatment here is lower by an order of magnitude than that seen in the hepatopancreas. Less than 3% of the transcriptome population is affected in any of the experimental groups (Fig. 3A) and two phenomena were distinguished. The first one is a relatively high gene induction due to application of D-CHH (elevated from 0.78 to 2.36% of the total transcript population), accompanied by a high number of reads per contig and a large standard deviation (Fig. 3B). Thirty one out of the total 118 induced contigs are responsible for 95% of the induction and the major contributors to the induction (70%) are presented in Table 4. Induction of the L-CHH-exposed individuals was negligible (Fig. 3A,B). The second phenomenon is a similar attenuation of transcripts, caused by exposure to both hormonal treatments (Fig. 3) and a considerable overlap of genes attenuated by each of the two isomers, 128 contigs out of 361 or 398 attenuated contigs by D- and L-CHH, respectively.

The 128 mutual contigs were enriched with 43 contigs that were related directly and indirectly to the actin motor system attenuated much more strongly than the average of all other attenuated contigs, 15.8- versus 2.9-fold in L-CHH-affected gills and 12- versus 2.6-fold in D-CHH-affected gills. These contigs represent 17 groups of genes and the response of their expression to the hormonal treatment is presented in Fig. 4. The actin system is the skeletal motor system but also a major component of the cytoskeleton. Hence, an effort was made to identify the source of the actin-system-related genes revealed here. Myosin heavy chain (MHC) is the major motor protein of the muscular and cytoskeletal system and the resemblance of the attenuated MHC contigs here to arthropod muscular and cytoskeletal genes was examined. In insects, using *Drosophila melanogaster* as a model, there are two different MHC genes, *zipper*, the cytoskeletal MHC and *mhc*, the

Table 2. Highly induced genes in D-CHH-treated hepatopancreas of non-vitellogenic female *Pontastacus leptodactylus*

Contig[b]	Fold change	D-CHH (%)[a]	Sham (%)[a]	Added reads (%)[a]	Annotation
Pl_CLC_4238	2.3	13.56±5.35	5.80±0.69	7.76	
Pl_ CLC_5624	2.8	8.42±3.69	2.99±0.18	5.43	
Pl_ CLC_2	7.1	6.53±2.24	0.92±0.74	5.61	α-amylase
Pl_ CLC_76	2.4	3.89±0.48	1.60±0.70	2.29	Cysteine proteinase C1A
Pl_ CLC_146	2.1	1.85±0.69	0.87±0.21	0.97	ATP synthase subunit A
Pl_ CLC_24887	2.7	0.67±0.01	0.25±0.11	0.42	

[a]Mean percentage of reads that were mapped to the specific contig ± s.d., *N*=3. [b]Designation in TSA acc. no. GBEI01000000.

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Table 3. Highly attenuated genes in L-CHH-treated hepatopancreas of non-vitellogenic female *Pontastacus leptodactylus*

Contig[b]	Fold change	L-CHH (%)[a]	Sham (%)[a]	Subtracted reads (%)[a]	Annotation
Pl_CLC_78	–2.8	1.77±0.53	4.86±1.57	–3.09	
Pl_CLC_5624	–2	1.48±0.87	2.99±0.18	–1.51	
Pl_CLC_146	–3	0.29±0.13	0.88±0.21	–0.59	ATP synthase subunit a

[a]Mean percentage of reads that were mapped to the specific contig ± s.d., *N*=3. [b]Designation in TSA acc. no. GBEI01000000.

muscular version, each producing several proteins resulting from alternative splicing of each of the two genes (George et al., 1989). Hence, the *D. melanogaster* MHC protein sequences (zipper, acc. nos. ADV37253-6, AAX52688, AAM70805, NP_523860, AAX52687, AFH08269; and mhc acc. nos. AFH03722-5, AAF53566) were compared by BLASTP to the 15 *P. leptodactylus* myosin heavy chain conceptually translated proteins and also to the protein assembly of *Daphnia pulex*, the only crustacean with a fully deciphered genome. The aims were to ascertain the existence of analogs of the two *D. melanogaster* genes in crustaceans, and to indicate the origin of the attenuated MHC contigs in *P. leptodactylus* gills. The results of the comparisons with *P. leptodactylus* MHCs are presented in Table 5, demonstrating the existence of analogs to both zipper and mhc. All the attenuated MHCs in the gills closely resembled the *D. melanogaster* mhc-like protein and one non-differentially expressed contig, Pl_CLC_2953 (supplementary material Table S1) is a zipper-like protein. Comparisons of proteins encoded by *zipper* and *mhc* with the protein assembly of *D. pulex* also revealed the two orthologs (acc. no. EFX76460 is *D. pulex* zipper-like and EFX87104-6 is mhc-like protein).

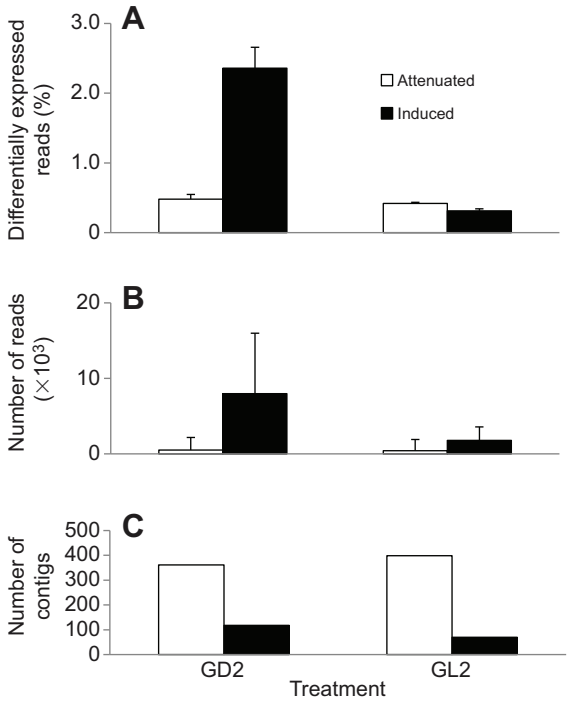

Fig. 3. Characterization of overall differential expression in the gills of *P. leptodactylus* injected with D-CHH or L-CHH. Results are from gills of all crayfish examined in experiment 2 and represent means ± s.d.
(A) Percentage of reads mapped to the differentially expressed contigs for the two treatments. (B) Mean number of reads per differentially expressed contig. (C) Number of differentially expressed contigs. GD2, gills from animals 2 h after injection with D-CHH; GL2, gills from animals 2 h after injection with L-CHH.

Gene expression in the hemocytes and muscle

A low percentage of the transcriptome population was induced or attenuated in the hemocytes and muscles upon application of the two isomers (supplementary material Figs S3, S4) and almost no apparent overall difference was observed between the effects of the two isomers. A similar hemocytic attenuation pattern resulted from application of each of the two isomers, comparable to the attenuation pattern in the gills. Forty contigs out of 155 or 156 were mutually attenuated by the two treatments, but unlike in the gills, the attenuation was mild. The muscle was the least-affected tissue and it can be concluded that muscle of vitellogenic females is not affected by CHH 2 h post injection.

DISCUSSION

The present study indicated two routes of hormonal signal delivery of the two CHH isomers by demonstrating their different effects on gene expression patterns: (1) a D-isomer-specific route, inducing target transcripts in females with non-vitellogenic hepatopancreas and in gills of vitellogenic females, whereas no such pathway was indicated for L-CHH in any of the examined tissues. (2) Attenuation of specific transcripts, mainly in the gills, similarly affected by both isomers. Specific attenuation by the L-isomer was also observed in the non-vitellogenic hepatopancreas, but no comparable attenuation by the D-isomer was found, which could be because it is masked by the high induction in this D-CHH-affected organ. The masking is assumed to be a result of the RNA-seq computational methodology, which calculates relative abundance of transcripts in the population. Hence, if the prominent induction of a few genes occurs, the proportion of many other genes in the transcript population would be smaller. Indeed, the 54 D-CHH-induced contigs increased their relative expression by 23.4%, whereas 1318 contigs were attenuated by 15.6% in comparison with the control.

In view of the dual route for signal delivery, a dual receptor system is proposed, one is D-isomer-specific and the other is used by both isomers. Signal transduction through cyclic nucleotide second messengers may be part of the signal-conveying system for each of the postulated receptor routes, contributing both to protein activation and to changes in the composition of the transcript population. The involvement of cAMP and cGMP second messengers in the signal transduction route of a CHH isomer mix was previously observed (Chung and Webster, 2006; Katayama and Chung, 2009; Sedlmeier, 1982; Sedlmeier, 1987; Sedlmeier, 1988). Also, cGMP, but not cAMP, was induced by CHH in the penaeid *Marsupenaeus japonicus* (Nagai et al., 2009).

CHH is a pleiotropic hormone with established involvement in several physiological processes in several tissues. Its regulation of glucose has been indicated in the hepatopancreas, integumentary tissue and muscles. Keller and Andrew (Keller and Andrew, 1973) examined the effect of eyestalk extraction on glucose levels in the circulation and glycogen levels in the integumentary tissue and the abdominal muscles of crustaceans. Glycogen-containing reserve cells of the hypodermal connective tissue have been located in the hypodermis (Johnson, 1980; Roer and Dillaman, 1984). The involvement of the hepatopancreas was shown by Sedlmeier

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Table 4. Highly induced genes in D-CHH-treated gills from vitellogenic female *Pontastacus leptodactylus*

Contig[b]	Fold change	D-CHH (%)[a]	Sham (%)[a]	Added reads (%)[a]	Annotation
PI_CLC_47865	2.4	0.26±0.05	0.11±0.03	0.15	
PI_CLC_6363	3.1	0.21±0.06	0.07±0.001	0.14	α-2-macroglobulin
PI_CLC_80467	2.4	0.2±0.02	0.08±0.02	0.12	
PI_CLC_4778	2	0.19±0.04	0.09±0.01	0.10	
PI_CLC_4150	2.1	0.11±0.01	0.05±0.009	0.06	Periostin
PI_CLC_11705	2.2	0.08±0.004	0.03±0.0004	0.05	Transferrin

[a]Mean percentage of reads that were mapped to the specific contig ± s.d., *N*=2. [b]Designation in TSA acc. no. GBEI01000000.

(Sedlmeier, 1987), who demonstrated depletion of glycogen upon application of CHH to *O. limosus* hepatopancreas incubated *in vitro* in the presence of prelabeled glycogen. We did not examine the integument and neither of the CHH enantiomers had an effect on muscle gene expression. CHH affected non-annotated contigs in the hepatopancreas, and even some of the annotated ones could not be confidently related at present to regulation of glucose levels. Both may be candidates to further studies. Alpha-amylase which is highly induced in the hepatopancreas 1 h post injection of D-CHH may contribute to glucose provision to the circulation. It endohydrolyzes the (1→4)-α-D-glucosidic bonds in polysaccharides containing three or more (1→4)-α-linked D-glucose units of starch and glycogen. The smallest reported α-amylase product is the disaccharide maltose and longer oligosaccharides were also reported, whereas glucose is the final product required to relate α-amylase to hyperglycemia. Several α-amylase motifs have been linked to specific substrates and products based in this multi-member family of enzymes (MacGregor et al., 2001). Nevertheless, it is not possible at this stage to finally determine the exact enzymatic activity and products of *P. leptodactylus* α-amylase. Maltase, which cleaves maltose into glucose is present in the reference assembly but was poorly induced and was not specific to D-CHH. However, it may participate in glucose production because of its constitutive presence or it may be induced later than 1 h post injection. Only up to 25% of the α-amylase content was secreted to the medium of *O. limosus* hepatopancreas incubated *in vitro* upon mixed bi-isomer CHH treatment (Sedlmeier, 1988), still leaving the reasonable possibility that this enzyme, with or without the concerted action of enzymatic partners, contributes to the rapid provision of glucose to the circulation upon exposure to D-CHH.

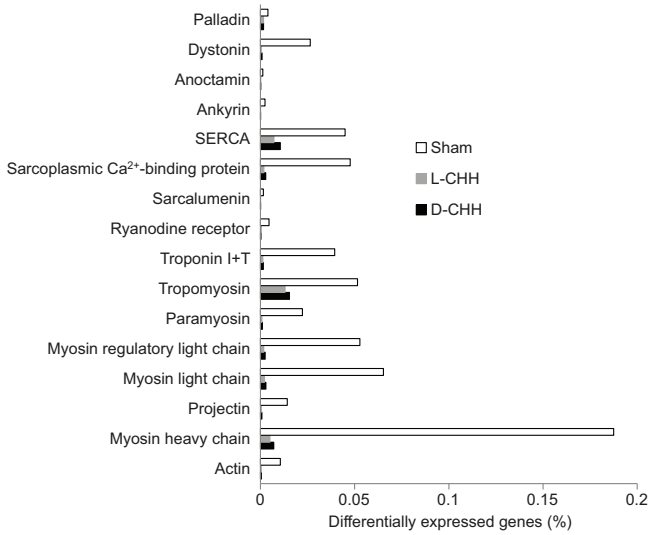

Fig. 4. Characterization of the differential expression of actin motor system genes in the gills. The percentage of the differentially expressed genes from the entire transcript population is shown for treatment with D-CHH, L-CHH and after sham injection.

An additional potential source of circulating glucose is trehalose, which was recently demonstrated to be a major circulating disaccharide in crustaceans in the hemolymph, the hemocytes and the hepatopancreas. Expression of the trehalose 6-phosphate synthase was detected in a variety of crab tissues (Chung, 2008; Shi and Chung, 2014) and trehalose level is attenuated in the circulation following eyestalk ablation (Tamone et al., 2012). However, trehalase, and also phosphotrehalase, trehalose-6-phosphate synthase and facilitated trehalose transporter Tret1, all present in the reference assembly, were poorly expressed in the hepatopancreas and their expression was not affected by the application of CHH.

The attenuation caused by L-CHH in the hepatopancreas of non-vitellogenic females revealed the characteristics of a specific effect, involving mainly three genes responsible for almost all the attenuation. Moreover, two of these genes are highly induced by D-CHH. In nature, the two isomers are simultaneously present in the hemolymph and this contradictory effect may indicate some kind of regulation. One of these genes, ATP synthase, may be fine-tuned by this system to provide the optimized ATP resources needed by the hepatopancreas at a given time point.

Another demonstrated phenomenon is the change in hepatopancreatic response to CHHs depending on the reproductive phase. The hepatopancreas was previously indicated to be a source of circulating glucose, which is affected by CHH (Sedlmeier, 1987), and it contains a glycogen reservoir (Johnson, 1980). However, the vitellogenic hepatopancreas here looks less appropriate for this task because it is devoted to vitellogenin synthesis and it is unresponsive to the influence of CHH, at least in terms of gene expression. In spite of that, the typical glycemic effect of CHH is also maintained in vitellogenic females (supplementary material Fig. S1). Gene expression in the hemocytes and the muscle, other candidates for glucose provision, was not affected by CHH 2 h after exposure. Therefore, these organs were not indicated as glucose providers to the circulation through *de novo* synthesis of proteins and the issue of a glucose-providing organ in vitellogenic females requires further study.

Gills were previously shown to be the main tissue involved in regulation of osmotic pressure and sodium ion levels in the circulation (Spanings-Pierrot et al., 2000). Generally, the effect of CHH on the gill gene expression profile encompassed, upon application of the hormones, less than 0.3% of the transcript population. This relatively minor effect compared with that of the hepatopancreas may be explained by the time course of the affected physiological processes and by the presence of intact XOSG in the experimental females. Serrano et al. (Serrano et al., 2003) demonstrated no decrease in the hemolymphatic osmotic pressure in intact females in any stage of a normal molt cycle. When the eyestalk was removed, an osmotic pressure drop was observed only after a synchronized molt event 22–23 days after eyestalk removal.

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Table 5. Comparison between *Drosophila melanogaster* zipper and mhc proteins and *Pontastacus leptodactylus* MHC contigs

Gene	Resemblance of MHC contigs (%)	MHC aligned region (%)	Resemblance of *zipper*-like contig (%)	*zipper*-like aligned region (%)
Drosophila zipper	41±10	59.6±39.6	71	68.6
Drosophila mhc	68±5.9	92.2±19.8	39	37.9

CHH isomers were applied by Serrano et al. (Serrano et al., 2003) to eyestalk-ablated individuals at that point of low osmotic pressure, whereas in experiment 2 here, the eyestalk was not removed and there was probably no osmotic pressure drop before hormonal application. In addition, increases of sodium influx and osmotic pressure were observed by Serrano et al. (Serrano et al., 2003) after 8 h, and it may be that the 2 h exposure period was too short to demonstrate a stronger effect. Of the four D-CHH-specific induced contigs in the gills in the D-CHH affected females, only α-2-macroglobulin could be related to osmotic regulation at present. This big monomeric to tetrameric molecule of 179 kDa for each monomer (Sottrup-Jensen et al., 1984) can not cross membranes and if concentrated, it increases the cellular osmotic pressure. More intriguing is the similar and specific attenuation of 43 contigs related to the actin motor system by both isomers. The similar 12- to 15-fold attenuation in the gills caused by both isomers indicates a hormonal-driven effect. However, the location of each of these genes (i.e. whether cytoskeletal or muscular) and accordingly, their function, was not resolved. The sequence of *P. leptodactylus* MHCs resemble a muscle-type sequence.

Twelve complete actin open reading frames belonging to three types of actin – skeletal, cardiac and cytoskeletal – were identified in *H. americanus* (Kim et al., 2009), but no distinction of *P. leptodactylus* actin types was found by BLASTP comparisons of the seven partial actin transcripts with those of *H. americanus*. Projectin is a long protein that binds filamentous actin and provides elasticity to muscles, and at present, it is assigned a function only in the muscular context (Ayme-Southgate et al., 2008). The modulatory genes of the actin system were studied in both the cytoskeletal and the muscular contexts. Tropomyosin isoforms participate in both cytoskeletal and muscular processes as modulators of actin–myosin binding (Gunning et al., 2005). Paramyosin is an invertebrate-specific modulatory protein of actin–myosin binding and tropomyosin, and troponin T and I modulate this binding in vertebrates (Gunning et al., 2005; Landsverk and Epstein, 2005). Palladin is part of the cytoskeletal structure, bound to filamentous actin (Dixon et al., 2008) and the ankyrin family has a general role as an adapter between a variety of integral membrane proteins and the cytoskeleton (Bennett and Baines, 2001). Dystonin is a cytoskeletal actin-binding protein (Brown et al., 1995). Genes that are effectors or are affected by the calcium second messenger system may be considered participants in the osmoregulation that controls cytoskeletal genes, although some were also assigned a function in the muscular context. The ryanodine receptor functions as a calcium release channel in both the sarcomere and the endoplasmic reticulum (ER) (Van Petegem, 2012). The sarcoplasmic calcium-binding protein is an invertebrate EF-hand-domain calcium-buffering protein. Its expression pattern has been studied in many invertebrates, including crayfish, only in the muscular context (White et al., 2011). Sarco-endoplasmic reticulum (SR) calcium ATPases (SERCAs) are membrane-bound calcium pumps that hydrolyze ATP to pump calcium either across the plasma membrane or into the ER or SR (Sanyal et al., 2005). Sarcalumenin is a calcium-binding glycoprotein found only in the lumen of the SR (Leberer et al., 1989). Anoctamin is a membrane integral calcium-affected chlorine channel and may participate in osmoregulation (Milenkovic et al., 2010). An argument against a muscular context is the absence of previous reports of muscles in crustacean gills (Goodman and Cavey, 1990; Johnson, 1980). A supporting argument for a muscular context is the high non-differential expression of this suite of contigs in the muscles, constituting almost 40% of the transcript population. However, this high expression could be due to sequence similarity between muscular and cytoskeletal actin system genes. Recently, we performed a gene expression study in the hypodermis of a related crayfish, *P. clarkii*, during premolt, induced by eyestalk removal (Tom et al. 2014). In that case, simultaneous induction of many actin-related genes preceded changes in molt-related gene expression profiles. *P. clarkii* MHCs also resembled the muscular *D. melanogaster mhc* more than the cytoskeletal *zipper* even though the hypodermis was carefully removed to avoid muscular tissue.

In view of the ambiguous function of some actin motor system proteins, we propose a working hypothesis, whereby the attenuated actin system genes all function in the cytoskeletal context and sequence separation between muscular and cytoskeletal MHCs and also other actin-system-related genes occurred later in arthropod evolution. Reorganization of cytoskeletal actin structures has been shown to alter cell shape, cellular structures and cellular interconnections, affecting both transcellular and paracellular transport of water and solutes (Castillo et al., 2002; Chou et al., 2008; Kapus and Szászi, 2006), also indicating a possible role of the actin system in CHH-controlled osmoregulation by crustacean gills.

In summary, our study indicates at least two routes of signal transduction for CHH isomers by examining their effect on gene expression: a D-CHH-specific route and another one mutual to both isomers. Alpha-amylase was shown to be part of the CHH-induced glucose provision pathway to the circulation. Proteins related to the structure and the function of the actin system were attenuated in the gills by both isomers, initially suggesting participation in the CHH-driven osmoregulation and sodium influx control. Vitellogenic hepatopancreas did not respond to *in vivo* exposure to CHH, which correlated with a prominent change in hepatopancreas function. Muscle and hemocytes were poorly affected by CHHs during the examined period of exposure to the two isomers. Hence, their short-term participation in CHH-driven processes is doubtful.

MATERIALS AND METHODS

Crayfish maintenance and experimental procedures

Adult *P. leptodactylus* females were obtained live from an Armenian freshwater source. They were kept for 2 weeks before the experiments in 120 l tanks provided with closed circuit filtered and thoroughly aerated tap water at ~19°C and were fed with fish pellets (Sera granular, Heisenberg, Germany) three times per week. No external abdominally incubated ova were observed in females participating in either of the experiments. The source recombinant CHHs and their synthesis procedure were described by Mosco et al. (Mosco et al., 2012). The technical procedure of injection into the circulation, the evaluation of circulating glucose level, the female seasonal reproductive status, and the carapace length and molt stage were detailed previously (Manfrin et al., 2013). A uniform amount of 0.5 µg CHH per female in 100 ml PBS was injected throughout this study and carrier alone was sham-injected. The glucose level was evaluated in three females

from each experimental or control group of experiment 1 upon death 1 h after injection, and from five females from each of the experimental groups of experiment 2, killed 1, 2, 4, 8, 24 and 48 h post injection. The experimental period included 10 min of anesthesia on ice before killing, to minimize the animal's suffering. Tissue pieces were dissected at the end of the experiment. The sampling location was uniform for each of the dissected tissues in all the sampled females. The carapace was removed and one lobule of the hepatopancreas was sampled from its anterior part. Extensor abdominal muscle was sampled from the anterior abdominal segment and one gill branch was removed from the mid-gill region. Hemocytes were pelleted from the sampled hemolymph. Sampled tissues were snap-frozen in liquid nitrogen and kept at −70°C for RNA extraction.

The statistical analyses and presentation of glucose levels and morphometric parameters were performed using R software, version 2.14.1 (R Development Core Team, 2011). Normality of data was checked using the Shapiro–Wilk test and the homogeneity of variance across groups was checked using the Bartlett test. Differences in glucose levels among the experimental groups were tested using the non-parametric Kruskal–Wallis rank sum test with *post hoc* Wilcoxon rank sum test pairwise comparisons with Bonferroni correction. Box and whiskers plots were drawn using the boxplot command.

RNA extraction and sequencing

RNA was extracted from the sampled tissues using the Tri Reagent RNA denaturation solution (Sigma-Aldrich) following the manufacturer's instructions. The resulting RNAs were further purified using the RNeasy kit (Qiagen) and were quantified by NanoDrop spectrophotometer (Thermo Scientific, USA). Quality was examined using capillary electrophoresis (BioAnalyzer 2100, Agilent).

An Illumina sequencer HiSeq2000 (USA) was used for all the sequencing done in this study. The selection of samples for sequencing when not all the experimental females were used was based on the intensity of the hyperglycemic response. RNA sequencing of hepatopancreatic single-end 50 bp sequences was carried out from RNAs of samples from both experiments, by the Istituto di Genomica Applicata (IGA, Udine, Italy). In experiment 1, a total of 12 samples were used, three from each experimental group: D-CHH, L-CHH, sham-injected and the native group, killed 1 h post injection (Manfrin et al., 2013). Twelve hepatopancreatic samples from females killed 2 or 8 h post injection in experiment 2 were loaded on one sequencer lane with an expected yield of ~10 million reads per sample. An RNA pool of two females was used for each sample. At a later stage, 18 additional RNA samples from muscle, gills and hemocytes of experiment 2 females, which were killed 2 h post injection were sequenced. Six samples were used per tissue, two samples per experimental group: D- and L-CHH injected, as well as the sham-injected control group. An RNA pool of two females was used for each sample. Sequencing of 50 bp paired sequences was performed at the Epigenomics Core Facility of the Weill Cornell Medical College (NY, USA). Nine samples were loaded on each lane with an expected yield of ~40 million reads per sample. Additional paired-end 100 bp reads were also sequenced by IGA (Udine, Italy) prepared from RNA extracted from the hepatopancreas (Manfrin et al., 2013) and epithelial tissue (Tom et al., 2013), and they were combined with the sequences of this study for the construction of a comprehensive transcript assembly.

Digital transcript expression analysis

Digital gene expression analysis is aimed at evaluating the relative amount of each transcript in a sampled RNA population. It requires a reference transcript sequence assembly. The processing and analysis of the raw sequences obtained for both the preparation of the transcript assembly and the digital gene expression analysis were carried out using the CLC Genomics Workbench 6.51 software (CLC Bio, Aarhus, Denmark).

Preparation of reference assemblies

The initial comprehensive contig assembly was aimed at the best possible representation of the expressed transcriptome, in terms of number and length of the obtained contigs. All available raw reads were trimmed according to base-calling quality, which also eliminates residual primers bound to the fragments as part of the sequencing procedure. Duplicates were removed from the 50 bp reads prior to the assembly and the final set included ~167 million single-end 50 bp reads, ~680 million paired-end 50 bp reads and ~442 million paired-end 100 bp reads. The reads were assembled using the automatically set-up parameters option of the CLC software and the minimum allowed length of assembled contig was set to 200 bp. The assembly obtained was further elaborated using the tools of the TSA database of the NCBI, which include additional identification of residual sequencing primers, shortening of long N-rows artificially produced by the software in poor mapping regions between paired-end reads and omission of contaminant sequences. The sample-specific 50 bp reads obtained from each of the four studied tissues were separately mapped to this assembly, resulting in 70–87% mapping of the tissue-specific reads to the initial assembly. Selection of contigs that were covered by more than 500 reads for each mapped tissue allowed the construction of four smaller tissue-specific reference assemblies. These assemblies include 96.6–98.3% of the mapping, creating a robust set of contigs, not subjected to minor random expression fluctuations or to zero counts and are the tissue-specific reference assemblies used for the following digital transcript expression analysis (Table 1).

Digital transcript expression analysis

An RNA-seq analysis was applied according to Wang et al. (Wang et al., 2009) and the CLC instructions. This type of analysis is based on mapping a sample-specific population of short reads to the contigs of a reference assembly and counting the mapped reads to each contig. The software was allowed to accept up to two mismatches per mapped reads and a reads was accepted as mapped if it matched a maximum of five contigs. Reads that were mapped to 2–5 contigs were randomly mapped to one of them and designated non-specifically mapped reads not considered in the expression analysis but used to evaluate the uniqueness of the mapping. The expression values were calculated based only on unique reads mapped to a single contig and normalized to the total reads in the sample. The value used for normalization was determined to roughly agree with the actually obtained total number of reads per sample (Table 1). Therefore, it was set to 10 million reads for the hepatopancreatic samples and to 40 million reads for the other tissues. A higher number of sample-specific reads provides more-sensitive determination of differentially expressed genes. Hence, the analyses of the gills, hemocytes and muscles are more sensitive than those of the hepatopancreas. The Baggerly's test (Baggerly et al., 2003) was used to identify statistically significant differential expression in comparison to the control group and the global similarity among transcript expression profiles of the various samples was examined by PCA (CLC instructions).

Characterization of transcript sequences

The transcripts of the comprehensive initial assembly were annotated using the TRINOTATE tool of the TRINITY software (Grabherr et al., 2011). The annotation includes: homology search to known sequence data (NCBI-BLAST) (Altschul et al., 1997), protein domain identification (HMMER/PFAM) (Punta et al., 2012) and protein signal peptide prediction and transmembrane domain identification (SignalP 4.1) (Käll et al., 2004; Petersen et al., 2011). Manual, contig-by-contig analysis was carried out for contigs of interest using the above-mentioned databases and the alignment software MEGA5.1 (Tamura et al., 2011).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.T., C.M., P.G.G. and A.M. planned and performed the gene expression experiments; M.T., C.M., M.G., G.D. and A.P. did the RNA-seq analysis; M.T. wrote the manuscript and all authors reviewed it.

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Supplementary material

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