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Diffusible gas transmitter signaling in the copepod crustacean *Calanus finmarchicus*: identification of the biosynthetic enzymes of nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S) using a *de novo* assembled transcriptome

Andrew E. Christie*, Tiana M. Fontanilla, Vittoria Roncalli, Matthew C. Cieslak, and Petra H. Lenz

Békésy Laboratory of Neurobiology, Pacific Biosciences Research Center, University of Hawaii at Manoa, 1993 East-West Road, Honolulu, HI 96822 USA

Abstract

Neurochemical signaling is a major component of physiological/behavioral control throughout the animal kingdom. Gas transmitters are perhaps the most ancient class of molecules used by nervous systems for chemical communication. Three gases are generally recognized as being produced by neurons: nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S). As part of an ongoing effort to identify and characterize the neurochemical signaling systems of the copepod *Calanus finmarchicus*, the biomass dominant zooplankton in much of the North Atlantic Ocean, we have mined a *de novo* assembled transcriptome for sequences encoding the neuronal biosynthetic enzymes of these gases, *i.e.* nitric oxide synthase (NOS), heme oxygenase (HO) and cystathionine β -synthase (CBS), respectively. Using *Drosophila* proteins as queries, two NOS-, one HO-, and one CBS-encoding transcripts were identified. Reverse BLAST and structural analyses of the deduced proteins suggest that each is a true member of its respective enzyme family. RNA-Seq data collected from embryos, early nauplii, late nauplii, early copepodites, late copepodites and adults revealed the expression of each transcript to be stage specific: one NOS restricted primarily to the embryo and the other was absent in the embryo but expressed in all other stages, no CBS expression in the embryo, but present in all other stages, and HO expressed across all developmental stages. Given the importance of gas transmitters in the regulatory control of a number of physiological processes, these data open opportunities for investigating the roles these proteins play under different life-stage and environmental conditions in this ecologically important species.

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*Correspondence to: Dr. Andrew E. Christie, Békésy Laboratory of Neurobiology, Pacific Biosciences Research Center, University of Hawaii at Manoa, 1993 East-West Road, Honolulu, Hawaii 96822 USA. Phone: 808-956-5212; FAX: 808-956-6984; crabman@pbrc.hawaii.edu.

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Keywords

nitric oxide synthase (NOS); heme oxygenase (HO); cystathionine β -synthase (CBS); Illumina sequencing; functional genomics; neurotransmitter; neuromodulator

1. Introduction

The molecules used for neurochemical signaling are large in number and diverse in structure, but can be divided into several broad categories including peptides, amines, small molecule transmitters and gas transmitters. The latter class is considered among the most ancient of these signaling molecules (*e.g.* Garthwaite, 2008), with diffusible gases being used for chemical communication in essentially all prokaryotic and eukaryotic organisms (*e.g.* Crane et al., 2010; Lloyd, 2006; Sudhamsu and Crane, 2009; Wang, 2012; Wilson et al., 2012). In nervous systems, three diffusible gases, nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S), are now generally recognized as serving roles as neurotransmitters/modulators (*e.g.* Althaus and Clauss, 2013; Barañano et al., 2001; Barañano and Snyder, 2001; Boehning and Snyder, 2003; Gadalla and Snyder, 2010; Garthwaite, 2008; Mustafa et al., 2009; Snyder and Ferris, 2000; Vandiver and Snyder, 2012).

Signaling by diffusible gases has been described as “atypical” with respect to the other commonly recognized classes of neurochemicals. Specifically, unlike peptides, amines and small molecule transmitters, gas transmitters are not stored in vesicles and are not released via exocytosis (*e.g.* Snyder and Ferris, 2000). Instead, NO, CO and H₂S are synthesized as needed, and are freely diffusible and membrane permeant once produced. Thus, the sphere of influence of a gas transmitter is not constrained by physical barriers such as desmosomes and other junctional complexes, but is limited only by its diffusion constant and life span. Given these factors, the control of gas transmitters relies largely on the fine tuned regulation of their biosynthetic enzymes (*e.g.* Mustafa et al., 2009), *i.e.* nitric oxide synthase (NOS) for NO, heme oxygenase (HO) for CO, and cystathionine β -synthase (CBS) for H₂S (*e.g.* Althaus and Clauss, 2013).

As part of an ongoing effort to identify and characterize the neurochemical signaling systems of the copepod crustacean *C. finmarchicus*, the biomass dominant zooplankton species for much of the North Atlantic Ocean (Dale et al., 2001; Head et al., 2000; Marshall and Orr, 1955; Meise and O'Reilly, 1996), we have mined a *de novo* assembled transcriptome (Christie et al., 2013a; Lenz et al., 2014) for sequences encoding putative gas transmitter biosynthetic enzymes. In addition, the proteins deduced from the identified transcripts were assessed for structural motifs characteristic of the respective enzyme family. Finally, RNA-Seq data collected from six *C. finmarchicus* developmental stages (embryo, early nauplius, late nauplius, early copepodite, late copepodite and adult) were used to map the developmental expression of the enzymes in this species. As our data will show, NOS-, HO-, and CBS-encoding transcripts were identified from our transcriptome assembly; for NOS, two different sequences were found. The proteins deduced from the identified transcripts all possess structural features characteristic of their respective family, and reverse

BLAST analyses support these enzyme family attributions. Expression mapping of the two NOS-encoding transcripts revealed one to be expressed in embryos and essentially absent in all other stages, and the other to be absent in embryos, but present in all other developmental stages. The CBS-encoding sequence was absent in embryo but expressed in all other stages, while the HO-encoding transcript was expressed throughout development. These data represent the first descriptions of gas transmitter biosynthetic enzymes in *C. finmarchicus*, suggest that these proteins are stage-specific in their expression, and lay a strong foundation for future anatomical, biochemical and physiological investigations of diffusible gas signaling in this environmentally critical copepod species.

2. Materials and methods

2.1. De novo transcriptome assembly

A *de novo* transcriptome for *C. finmarchicus* was generated as described in detail in Lenz et al. (2014). In brief, multiplexed gene libraries were prepared from RNA extracted from six developmental stages of wild-caught or laboratory cultured *C. finmarchicus*. The developmental stages included in the libraries were egg (which represents a mixture of embryonic stages), early nauplius (stages NI and NII), late nauplius (stages NV and NVI), early copepodite (stages CI and CII), late copepodite (stage CV) and adult female; all libraries were sequenced at the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA) in a single lane using an Illumina HiSeq 2000 instrument (Illumina Inc.). In total, 415,469,690 raw, 100 bp, paired-end reads were obtained. After quality filtering and trimming, the combined raw reads from all six developmental stage libraries were assembled *de novo* using Trinity 2012-03-17-IU_ZIH_TUNED software on a node of the National Center for Genome Analysis Support's (NCGAS; Indiana University, Bloomington, IN, USA) Mason Linux cluster. In total, 206,041 unique nucleotide sequences were generated using Trinity.

2.2. Transcriptome mining

Searches of the transcriptome assembly produced by Trinity were conducted using the DeCypher Tera-BLASTP algorithm on the Mount Desert Island Biological Laboratory's TimeLogic DeCypher server (MDIBL, Salisbury Cove, ME, USA; http://decypher.mdibl.org/decypher/algo-tera-blast/tera-tblastn_an.shtml) as described previously (Christie et al., 2013a, b, 2014). For all searches, the DeCypher program database was set to "6libTrinity", the combined Trinity assembly, and a known fruit fly *Drosophila melanogaster* protein was used as the query. All hits were translated and checked manually for homology to the target query. Table 1 provides the BLAST-generated E-value for each hit that was identified as encoding a putative target transcript, as well as the lengths of identified transcripts; the length of the protein deduced from each target sequence is also provided in this table.

2.3. Analyses of protein conservation and structure

Analyses of protein conservation and structure were conducted using a protocol described in detail in several recent publications (e.g. Christie et al., 2013a, b, 2014). To identify the most similar arthropod proteins to each of the *C. finmarchicus* enzymes described in this

study, the deduced *Calanus* sequence was used to query the non-redundant arthropod protein dataset (taxid:6656) curated in GenBank (excluding *C. finmarchicus* proteins, obvious partial proteins and synthetic constructs) using the blastp algorithm (Altschul et al., 1997); the results of these searches are summarized in Table 2. In addition, the identified *Calanus* NOS and HO isoforms were used as queries against selected vertebrate protein databases (*i.e.* pufferfish *Takifugu rubripes* [taxid:31033], the clawed frog *Xenopus laevis* [taxid: 8355], the chicken *Gallus gallus* [taxid:9031], and human *Homo sapiens* [taxid: 9606]) in order to determine the subfamilies into which they likely reside; these data are summarized in Table 3. All database searches were conducted on or before May 20, 2013.

To determine amino acid identity/similarity between proteins, the sequences in question were aligned using MAFFT version 7 (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>; Katoh and Standley, 2013), and amino acid identity/similarity was subsequently determined using the alignment output. Percent identity was calculated as number of identical amino acids (denoted by “*” in the MAFFT output) divided by the total number of amino acids in the longest sequence ($\times 100$). Percent similarity was calculated as number of identical and similar amino acids (the latter denoted by the “:” and “.” symbols in the protein alignment) divided by the total number of amino acids in longest sequence ($\times 100$).

Protein structural motifs were analyzed using the online program Pfam version 27.0 (<http://pfam.sanger.ac.uk/>; Punta et al., 2012). A common highlighting scheme has been used to denote functional domains in the relevant figures: nitric oxide synthase oxygenase domain, green; flavodoxin domain, dark blue; FAD-binding domain, light blue; oxidoreductase NAD-binding domain, black; heme oxygenase domain, red; pyridoxal-phosphate dependent enzyme domain, yellow; cystathionine β -synthase domain, pink.

2.4. Vetting of deduced protein sequences using publicly accessible expressed sequence tags

In an attempt to confirm the amino acid sequences of the proteins deduced from the Trinity assembly, each of the putative *Calanus* proteins was used as a query to search the extant *C. finmarchicus* ESTs (~11,000 in total; Lenz et al., 2012) curated at NCBI using the tblastn algorithm as described previously (Christie et al., 2013a, b, 2014).

2.5. Developmental expression mapping

To compare the relative levels of transcript expression in embryos, early nauplii, late nauplii, early copepodites, late copepodites, and adult females, Illumina reads from each of these developmental stages were mapped against the sequence in question using Bowtie software (Johns Hopkins University, Baltimore, MD, USA; <http://bowtie-bio.sourceforge.net/index.shtml>; Langmead et al., 2009) as described previously (Christie et al., 2013a, c, 2014). For each stage, four biological replicates (one generated from animals collected in 2011 and three from material collected in 2012), with two technical replicates for each biological replicate, were aligned against each reference sequence independently.

2.6. Statistical analyses

The mapped data were normalized by dividing the reads for each technical replicate that mapped to a given target transcript by the total number of reads in the technical replicate that mapped to the assembled transcriptome as a whole. A two-way ANOVA was used to test for differences in relative transcript expression between years and among stages on the normalized expression data to determine which stages showed significant differences between years. Differences in expression among stages were calculated for the data collected in 2012 since it included biological replicates using a one-way ANOVA followed by a Tukey's post hoc test to calculate p-values for all pair-wise comparisons using the software GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Identification and structural analysis of gas biosynthetic enzymes

3.1.1. Nitric oxide synthase (NOS)—By far the most commonly recognized gas transmitter is NO, which is produced during the conversion of L-arginine to L-citrulline via the action of NOS, a calcium/calmodulin-dependant enzyme (*e.g.* Barañano et al., 2001; Boehning and Snyder, 2003; Garthwaite, 2008; Mustafa et al., 2009; Snyder and Ferris, 2000). To identify native *C. finmarchicus* NOS-encoding transcripts, a BLAST search of the *de novo* assembled *Calanus* transcriptome described in Section 2.1 was conducted using the amino acid sequence of a *D. melanogaster* NOS isoform (Accession No. [AAF53014](#); Adams et al., 2000). Via this query, two transcripts (comp146200_c3_seq1 and comp179394_c1_seq1) were identified as encoding putative NOS homologs (Table 1); for the ease of future discussion these sequences have been named *Calanus finmarchicus* nitric oxide synthase I and II (*calfi-nos I* and *calfi-nos II*), respectively. Translation of *calfi-nos I* yielded a 1,121 amino acid, full-length NOS (Calfi-NOS I [Fig. 1 and Table 1]), with the open reading frame (ORF) bounded on both sides by stop codons (data not shown). Translation of *calfi-nos II* yielded a 1,084 amino acid, full-length protein (Calfi-NOS II [Fig. 1 and Table 1]); here too the ORF was bounded on both sides by stop codons (data not shown).

Figure 1 shows the alignment of the sequences of Calfi-NOS I and II. As can be seen from this figure, the two proteins share 46.6% identity/78.8% similarity in amino acid composition, with the only major region of deletion/insertion present at their amino termini. Structural analyses of the two Calfi-NOSs using the online program Pfam identified a one nitric oxide synthase oxygenase domain, one flavodoxin domain, one FAD-binding domain and one oxidoreductase NAD-binding domain in each protein (Fig. 1). These structural domains are similarly positioned within the two NOSs, and support these proteins being true NOS family members (Fig. 1).

3.1.2. Heme oxygenase (HO)—CO is produced during the enzymatic conversion of heme to biliverdin by members of the HO family, which, like the NOSs, are calcium/calmodulin-dependant proteins (*e.g.* Barañano et al., 2001; Barañano and Snyder, 2001; Boehning and Snyder, 2003; Mustafa et al., 2009; Snyder and Ferris, 2000). Using the sequence of *D. melanogaster* HO (Accession No. [AAF54680](#); Adams et al., 2000) as the

query sequence, a single *C. finmarchicus* transcript (comp27018_c0_seq1; named here *Calanus finmarchicus* heme oxygenase or *calfi-ho* [Table 1]) was identified as encoding a putative HO homolog (Fig. 2A). Translation of *calfi-ho* yielded a 287 amino acid, putative full-length HO isoform (Calfi-HO; Fig. 2A and Table 1), with the 3' end of the putative ORF terminating in a stop codon, but none identified at the 5' end of the transcript (data not shown). Analysis of Calfi-HO using Pfam identified a single heme oxygenase domain in this protein (Fig. 2A), which is highly suggestive of it being a true member of the HO family.

3.1.3. Cystathionine β -synthase (CBS)—While multiple enzymatic pathways have been shown to produce H₂S as a byproduct (e.g. Gadalla and Snyder, 2010; Mustafa et al., 2009; Vandiver and Snyder, 2012), production of this gas in the nervous system stems largely from the synthesis of cystathionine from homocysteine and cysteine via CBS, a calcium/calmodulin independent enzyme (e.g. Gadalla and Snyder, 2010; Mustafa et al., 2009; Vandiver and Snyder, 2012). Via a query using the sequence of a *D. melanogaster* CBS isoform (Accession No. **AAF50862**; Adams et al., 2000), one *C. finmarchicus* transcript (comp15975_c0_seq1; named here *Calanus finmarchicus* cystathionine β -synthase or *calfi-cbs* [Table 1]) was identified as encoding a putative CBS homolog (Fig. 2B). Translation of *calfi-cbs* yielded a 579 amino acid, putative full-length CBS protein (Calfi-CBS; Fig. 2B and Table 1), with the ORF bounded on its 3' but not 5' end by a stop codon (data not shown). Pfam analysis of Calfi-CBS predicts this protein to possess one pyridoxal-phosphate dependent enzyme domain and one cystathionine β -synthase domain (Fig. 2B), both of which are consistent with it being a true CBS family member.

3.2. Expressed sequence tag support for deduced protein sequences

As the transcriptome used in our study was assembled *de novo* from over 400,000,000 raw, 100 bp, paired end reads (Lenz et al., 2014), it almost certainly contains some assembly artifacts that may manifest themselves in errors in deduced amino acid sequences. To help vet the sequence assignments of the NOS, HO and CBS proteins presented here, each was used to query the extant *C. finmarchicus* ESTs curated in GenBank for transcripts encoding identical/highly similar proteins. While small in number compared to our transcriptome (~11,000 ESTs in total vs. over 200,000 unique sequences), essentially all of these ESTs were produced using single-pass Sanger sequencing (Lenz et al., 2012), and thus are not subject to assembly errors. While no ESTs were identified as encoding proteins identical to Calfi-NOS I, Calfi-NOS II or Calfi-HO (data not shown), one was identified (Accession No. **ES237695** [BLAST score = 461, E-value = 6e-161]) that encodes a partial protein vetting the N-terminus of Calfi-CBS. As can be seen from Figure 3, Calfi-CBS and the 224 amino acid partial protein deduced from **ES237695** are identical in sequence over their region of overlap, which represents approximately 40% of the amino acids present in Calfi-CBS.

3.3. Phylogenetic comparisons of *Calanus* gas-producing enzymes with those of other arthropods and selected vertebrates

3.3.1. Arthropod proteins—While the data presented earlier strongly support Calfi-NOS I, Calfi-NOS II, Calfi-HO and Calfi-CBS being members of the NOS, HO and CBS families, respectively, reverse BLAST analyses were conducted using each protein to further confirm this hypothesis. Specifically, each of the *Calanus* proteins was used as a query

sequence to search the non-redundant arthropod proteins curated at NCBI for the protein sets most similar to them; the top 10 BLAST hits for each query are summarized in Table 2.

Using the amino acid sequence of Calfi-NOS I as the query, an isoform of NOS from the beetle *Tribolium castaneum* (Accession No. **XP_967195**) was returned as the top BLAST hit (Table 2). The same *T. castaneum* NOS isoform was also the top BLAST hit when Calfi-NOS II was used as the query sequence (Table 2). Comparisons of the amino acid sequences of Calfi-NOS I and II with that of the *Tribolium* protein revealed 37.2% identity/68.7% similarity between the former pair (alignment not shown), and 35.1% identity/70.8% similarity between the latter (alignment not shown). Interestingly, while a number of crustacean isoforms of NOS have been deposited at NCBI, none were among the top 10 BLAST hits for Calfi-NOS I, and just two, *Gecarcinus lateralis* NOS (Accession No. **AAT46681**; Kim et al., 2004) and *Litopenaeus vannamei* NOS (Accession No. **ADD63793**; Yao et al., 2010), were among the top 10 hits returned when Calfi-NOS II was used as the query sequence (Table 2).

The search of the non-redundant arthropod proteins using Calfi-HO as the query returned insect heme oxygenase isoforms as the top 10 BLAST hits for this protein (Table 2). The top hit, a hypothetical protein from mosquito *Anopheles darlingi* (Accession No. **EFR29866**) shares 40.1% identity/67.6% similarity with Calfi-HO (alignment not shown). As with the search using Calfi-NOS I as the query, no crustacean sequences were among the top 10 hits generated using Calfi-HO (Table 2), though only one such sequence, **EFX8559** from the water flea *Daphnia pulex* (Colbourne et al., 2011), is currently present in the database.

In contrast to searches using the *Calanus* NOS and HO proteins, a reverse BLAST using the sequence of Calfi-CBS did return a crustacean sequence as the top hit (Table 2), specifically a hypothetical protein from *D. pulex* (Accession No. **EFX67333**; Colbourne et al., 2011); the *Calanus* and *Daphnia* proteins share 59.2% identity/81.7% similarity in amino acid composition (alignment not shown). In addition two of the remaining nine top hits were also from crustaceans, including another hypothetical *D. pulex* protein (Accession No. **EFX64847**; Colbourne et al., 2011), the second hit, and a CBS isoform from the parasitic copepod *Lepeophtheirus salmonis* (Accession No. **ABU41040**; unpublished submission), the fourth hit (Table 2).

Taken collectively, the results of all our reverse BLAST analyses strongly support our hypotheses that Calfi-NOS I and II are true members of the NOS family, and that Calfi-HO and Calfi-CBS are authentic HO and CBS family members, respectively.

3.3.2. Vertebrate sequences—In many vertebrate species, multiple isoforms of NOS and HO are present, with some constitutively active and others inducible (e.g. Mustafa et al., 2009). Often the different isoforms are tissue specific, with the neuronal isoform of NOS being NOS-1 or nNOS, and the neural isoform of HO being HO-2 (e.g. Mustafa et al., 2009). As a first step in assessing which of the vertebrate isoforms the deduced *Calanus* sequences are most closely related to, each *C. finmarchicus* protein was used to query the sequences of selected vertebrates curated at NCBI. The species targeted were the pufferfish *T. rubripes*, the clawed frog *X. laevis*, the chicken *G. gallus*, and human *H. sapiens*. With

the exception of the *Gallus* protein search, nNOS was returned as the top BLAST hit using Calfi-NOS I as the query sequence (Table 3); inducible NOS (iNOS) was returned as the most similar *Gallus* NOS isoform (Table 3). In searches using Calfi-NOS II as the query, nNOS was the top BLAST hit for both *T. rubripes* and *X. laevis* (Table 3), while iNOS was the top return in from both the chicken and human datasets (Table 3). For the searches using Calfi-HO as the query, HO-2s were returned as the most similar protein for all species except the pufferfish, and here the HO returned was not annotated as to its subtype (Table 3). Thus, it appears that the two *Calanus* NOSs are more similar to the vertebrate neuronal or inducible NOS subgroups than they are to endothelial NOSs, and that Calfi-HO is most similar to members of the vertebrate neuronal HO-2 subgroup.

3.4. Developmental expression of NOS-, HO- and CBS-encoding transcripts

To assess the expression of *calfi-nos I*, *calfi-nos II*, *calfi-ho* and *calfi-cbs*, Illumina RNA-Seq reads from six *C. finmarchicus* developmental stages (embryo, early nauplius, late nauplius, early copepodite, late copepodite, and adult female) were mapped against each of their respective transcript sequences. Normalized data from samples collected in both 2011 and 2012 are shown in Figure 4. As can be seen from this figure, there were no significant differences between years, except for expression of *calfi-nos II* and *calfi-cbs* in the early nauplii (Fig. 4, arrows). For these two transcripts, expression in 2011 was significantly higher than in 2012. Comparisons among stages indicate that expression of *calfi-nos I* was significantly higher in the embryos than in all other stages (Fig. 4A and Table 4). Interestingly, the expression pattern for *calfi-nos II* is markedly different from that of *calfi-nos I*, with expression levels significantly lower in the embryo and adult females than in the late nauplii, early copepodites and late copepodites (Fig. 4B and Table 4). Expression of *calfi-ho* also showed stage-specific expression levels (Fig. 4C and Table 5): highest expression was observed in the adult females, which was significantly different from all other stages. Embryos and late copepodites had intermediate expression of *calfi-ho*, and the lowest expression of this transcript was observed in the nauplii and early copepodites (Fig. 4C and Table 5). Expression of *calfi-cbs* was lowest in the embryos (where it was essentially absent), while expression in the other stages was similar, except for the late nauplius groups (Fig. 4D and Table 5).

4. Discussion

4.1. In silico identification of diffusible gas transmitter-producing enzymes in *Calanus* using a de novo assembled transcriptome

In much of the North Atlantic Ocean, *C. finmarchicus* serves as the primary consumer of phytoplankton, as well as the primary food source for a large number of commercially important fish (e.g. Cohen and Lough 1983; Kiørboe et al., 1988; Kristiansen et al., 2008; Purcell and Grover, 1990; Taggart et al., 1994). Recently there have been shifts in the geographic range of this species, with global warming cited as one factor contributing to this change in distribution (e.g. Helaouët et al., 2011; Pepin and Head, 2009; Pepin et al., 2011; Reygondeau and Beaugrand 2011; Speirs et al., 2006). Due to the altered population dynamics of *C. finmarchicus* in some areas, there have been concurrent changes in the populations of organisms that utilize them as prey. For example, recent large-scale declines

in cod populations in the North Sea have been attributed to reductions in *Calanus* abundance (Beaugrand et al., 2002, 2003). Given these and other issues, it has become critically important to understand how *C. finmarchicus* adapts to environmental change physiologically (e.g. Hansen et al., 2003; Hirche et al., 1997). Unfortunately, the small size of this crustacean does not lend itself well to standard physiological manipulations, and there have been few resources available to study the physiological ecology of it at the molecular level. In an effort to remedy the latter shortcoming, we recently undertook a large-scale sequencing of the *C. finmarchicus* transcriptome using the Illumina platform, and *de novo* assembled the resultant raw reads using the software program Trinity (Lenz et al., 2014). This effort has produced a transcriptome for *Calanus* that consists of over 200,000 unique sequences, and includes material collected from six different developmental stages (Lenz et al., 2014). This resource has provided us with a platform to initiate targeted protein discovery in *C. finmarchicus*, including the identification and characterization of a large number of neuropeptide precursors and receptors (Christie et al., 2013a) and amine biosynthetic enzymes (Christie et al., 2014), which is part of an ongoing effort to identify and characterize all of the neurochemical signaling systems of this species.

In the study presented here, we have focused on the targeted identification of the transcripts and proteins responsible for the generation of diffusible gas transmitters, specifically NOS, HO and CBS, the neuronal biosynthetic enzymes for NO, CO and H₂S, respectively. Using the known proteins of *Drosophila* as our templates, *Calanus* transcripts encoding homologs of all three enzymes were identified, two for NOS and one each for HO and CBS. The protein deduced from each transcript appears to be a full-length sequence, and each possesses the structural hallmarks of its respective enzyme family. These data are the first identifications of the protein components of gasotransmitter signaling systems in *C. finmarchicus*. While other crustacean NOSs have been described (e.g. Inada et al., 2010; Kim et al., 2004; Labbé et al., 2009; Li et al., 2012; McCoole et al., 2012; McDonald et al., 2011; Rodríguez-Ramos et al., 2010; Yao et al., 2010), to the best of our knowledge, there is only one other report describing a crustacean HO (McCoole et al., 2012), and no studies describing crustacean CBS homologs have been published.

4.2. Comparative studies suggest diverse physiological roles for NO, CO and H₂S in *Calanus*

While the functional roles played by diffusible gases in *C. finmarchicus* are unknown, anatomical, biochemical and physiological studies conducted in other species suggest they will ultimately be found to be numerous and diverse. With respect to NO, work done on other crustaceans has long implicated this gas as a neuromodulator (e.g. Aonuma et al., 2000; Aonuma and Newland, 2001, 2002; Christie et al., 2003; Lee et al., 2000; Mahadevan et al., 2004; Scholz et al., 2001, 2002; Schuppe and Newland, 2004; Stein et al., 2005). In addition, there is growing evidence for the involvement of NO in the control of bioluminescence (Krönström et al., 2007), color change (Filgueira et al., 2010; Milograna et al., 2012; Vargas et al., 2008), immune response (e.g. Fu et al., 2010; Jiang et al., 2006; Labbé et al., 2009; Rodríguez-Ramos et al., 2011; Yeh et al., 2006), molting/metamorphosis (Kim et al., 2004; Lee et al., 2007; McDonald et al., 2010; Zhang et al., 2012), and neurogenesis (Benton et al., 2007). As additional studies are conducted on *Calanus*, it will

be interesting to see which of these roles NO serves in this species, and as many of the above mentioned functions appear to be conserved across the Crustacea, it is possible that all are roles served by this gas in *C. finmarchicus*.

In contrast to NO, little is currently known about the physiological roles played by CO in crustaceans, or for that matter, any arthropod species. To the best of our knowledge, there are only two studies examining CO in crustaceans. In one report, the distribution of CO-producing neurons in the stomatogastric nervous system of the crayfish *Cherax quadricarinatus* was assessed using immunohistochemistry directed against HO (Christie et al., 2003); the distribution of immunolabeling in this species suggests a neuromodulatory role for the gas in this portion of the nervous system (Christie et al., 2003). In the other study, a gene encoding HO was identified and characterized from *D. pulex* (McCoole et al., 2012). The studies focused on CO in insects are equally scant (Cui et al., 2008; Knipp and Bicker, 2009), but what can be said from them is that CO is likely to play a key role in developmental control, including the regulation of nervous system development. As additional studies are conducted on *C. finmarchicus*, it will be interesting to see if in this species too, CO functions as a neuromodulator and/or plays a role in developmental control.

As for CO, essentially nothing is known on the physiological roles served by H₂S in crustaceans. In the one study that has been done, an immunohistochemical analysis of the distribution of CBS in the central nervous system of the crab *Hemigrapsus sanguineus*, a neuromodulatory role for the gas was proposed (Kotsyuba, 2011). In insects too, little work has focused on the physiological functions of H₂S, though a study of CBS suggests a role for it in aging, where it has been proposed as a positive regulator of longevity (Kabil et al., 2011). Whether or not these roles are served by H₂S in *Calanus* remains an open question.

4.3. Stage specificity of *Calanus* gas transmitters

As just discussed, the roles played by gas transmitters in *C. finmarchicus* are currently unknown, though homology to other crustaceans and insects suggests that each is likely multifunctional. Our RNA-Seq mapping of the transcripts encoding the two NOSs, HO and CBS across six *Calanus* developmental stages (embryo, early nauplius, late nauplius, early copepodite, late copepodite and adult), suggest that regardless of the function(s) served by each gas, there is likely stage-specific regulation ongoing in this species. For example, NO produced by Calfi-NOS I would almost exclusively be restricted to the embryo, while that generated by Calfi-NOS II would be largely excluded from this stage, but would provide the majority of gas output in nauplii, copepodites and adults. Given that our reverse BLAST analyses against selected vertebrate sequences suggest the Calfi-NOS I may be the homolog of vertebrate neuronal NOS and Calfi-NOS II the homolog of the vertebrate inducible isoform, it is possible that their expression patterns correlate to the need for increased neurogenesis (via Calfi-NOS I) in the embryo, where development is occurring within the protection of the egg, but after hatching, and thus direct exposure to pathogens in the environment, there is a need for induction of iNOS (Calfi-NOS II) due to increased immune challenge. Clearly this is all speculation at present, but as the regulation of gas transmitters lies largely in the fine tuned modulation of their biosynthetic enzymes, the molecular tools

provided by the identification and characterization of the transcripts and proteins identified here positions the field to begin asking these and other functional questions in this species.

4.4. Conclusions and future directions

Recently we generated a 206,041-sequence transcriptome for the copepod *C. finmarchicus* using Illumina sequencing and Trinity software *de novo* assembly (Lenz et al., 2014). Here, this resource has been used to mine for and characterize the transcripts and proteins that are putatively involved in the biosynthesis of the diffusible gases NO, CO and H₂S. These data contribute significantly to our ongoing effort to fully describe the neurochemical signaling systems of *C. finmarchicus*, the biomass dominant zooplankton in the North Atlantic Ocean, complementing and augmenting our earlier studies on peptidergic and aminergic signaling in this ecologically important species (Christie et al., 2013a, 2014). As the costs of high throughput sequencing continue to decrease, and the number of freely available tools to assemble and analyze these types of data increase, the production and mining of *de novo* transcriptome assemblies should become a standard method for protein discovery in crustaceans, as well as many other non-model organisms.

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References

- Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferreira S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Sidén-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA,

- Weinstock GM, Weissenbach J, Williams SM, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC. The genome sequence of *Drosophila melanogaster*. Science. 2000; 287:2185–2195. [PubMed: 10731132]
- Althaus M, Clauss WG. Gasotransmitters: novel regulators of ion channels and transporters. Front. Physiol. 2013; 4:27. [PubMed: 23437016]
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389–3402. [PubMed: 9254694]
- Aonuma H, Nagayama T, Takahata M. Modulatory effects of nitric oxide on synaptic depression in the crayfish neuromuscular system. J. Exp. Biol. 2000; 203:3595–3602. [PubMed: 11060220]
- Aonuma H, Newland PL. Opposing actions of nitric oxide on synaptic inputs of identified interneurons in the central nervous system of the crayfish. J. Exp. Biol. 2001; 204:1319–1332. [PubMed: 11249841]
- Aonuma H, Newland PL. Synaptic inputs onto spiking local interneurons in crayfish are depressed by nitric oxide. J. Neurobiol. 2002; 52:144–155. [PubMed: 12124752]
- Barañano DE, Ferris CD, Snyder SH. Atypical neural messengers. Trends Neurosci. 2001; 24:99–106. [PubMed: 11164940]
- Barañano DE, Snyder SH. Neural roles for heme oxygenase: contrasts to nitric oxide synthase. Proc. Natl. Acad. Sci. USA. 2001; 98:10996–11002. [PubMed: 11572959]
- Beaugrand G, Reid PC, Ibanez F, Lindley JA, Edwards M. Reorganization of North Atlantic marine copepod biodiversity and climate. Science. 2002; 296:1692–1694. [PubMed: 12040196]
- Beaugrand G, Brander KM, Lindley JA, Souissi S, Reid PC. Plankton effect on cod recruitment in the North Sea. Nature. 2003; 426:661–664. [PubMed: 14668864]
- Benton JL, Sandeman DC, Beltz BS. Nitric oxide in the crustacean brain: regulation of neurogenesis and morphogenesis in the developing olfactory pathway. Dev. Dyn. 2007; 236:3047–3060. [PubMed: 17948307]
- Boehning D, Snyder SH. Novel neural modulators. Annu. Rev. Neurosci. 2003; 26:105–131. [PubMed: 14527267]
- Christie AE. Crustacean neuroendocrine systems and their signaling agents. Cell Tissue Res. 2011; 345:41–67. [PubMed: 21597913]
- Christie AE, Edwards JM, Cherny E, Clason TA, Graubard K. Immunocytochemical evidence for nitric oxide- and carbon monoxide-producing neurons in the stomatogastric nervous system of the crayfish *Cherax quadricarinatus*. J. Comp. Neurol. 2003; 467:293–306. [PubMed: 14608595]
- Christie AE, Fontanilla TM, Nesbit KT, Lenz PH. Prediction of a putative *Calanus finmarchicus* (Crustacea, Copepoda) circadian signaling system using a *de novo* assembled transcriptome. Comp. Biochem. Physiol. Part D Genomics Proteomics. 2013b; 8:165–193. [PubMed: 23727418]
- Christie AE, Fontanilla TM, Roncalli V, Cieslak MC, Lenz PH. Identification and developmental expression of the enzymes responsible for dopamine, histamine, octopamine and serotonin biosynthesis in the copepod crustacean *Calanus finmarchicus*. Gen. Comp. Endocrinol. 2014; 195:28–39. [PubMed: 24148657]
- Christie AE, Roncalli V, Lona PB, McCoole MD, King BL, Bucklin A, Hartline DK, Lenz PH. *In silico* characterization of the insect diapause-associated protein couch potato (CPO) in *Calanus finmarchicus* (Crustacea: Copepoda). Comp. Biochem. Physiol. Part D Genomics Proteomics. 2013c; 8:45–57. [PubMed: 23262277]
- Christie AE, Roncalli V, Wu LS, Ganote CL, Doak T, Lenz PH. Peptidergic signaling in *Calanus finmarchicus* (Crustacea, Copepoda): *In silico* identification of putative peptide hormones and their receptors using a *de novo* assembled transcriptome. Gen. Comp. Endocrinol. 2013a; 187:117–135. [PubMed: 23578900]
- Cohen RE, Lough RG. Prey field of larval herring *Clupea harengus* on a continental shelf spawning area. Mar. Ecol. Prog. Ser. 1983; 10:211–222.
- Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, Bauer DJ, Cáceres CE, Carmel L, Casola C, Choi JH, Dettler JC, Dong Q, Dusheyko S, Eads BD, Fröhlich T, Geiler-Samerotte KA, Gerlach D, Hatcher P, Jogdeo S,

- Krijgsveld J, Kriventseva EV, Kültz D, Laforsch C, Lindquist E, Lopez J, Manak JR, Muller J, Pangilinan J, Patwardhan RP, Pitluck S, Pritham EJ, Rechtsteiner A, Rho M, Rogozin IB, Sakarya O, Salamov A, Schaack S, Shapiro H, Shiga Y, Skalitzy C, Smith Z, Souvorov A, Sung W, Tang Z, Tsuchiya D, Tu H, Vos H, Wang M, Wolf YI, Yamagata H, Yamada T, Ye Y, Shaw JR, Andrews J, Crease TJ, Tang H, Lucas SM, Robertson HM, Bork P, Koonin EV, Zdobnov EM, Grigoriev IV, Lynch M, Boore JL. The ecoresponsive genome of *Daphnia pulex*. *Science*. 2011; 331:555–561. [PubMed: 21292972]
- Crane BR, Sudhamsu J, Patel BA. Bacterial nitric oxide synthases. *Annu. Rev/Biochem*. 2010; 79:445–470.
- Cui L, Yoshioka Y, Suyari O, Kohno Y, Zhang X, Adachi Y, Ikehara S, Yoshida T, Yamaguchi M, Taketani S. Relevant expression of *Drosophila* heme oxygenase is necessary for the normal development of insect tissues. *Biochem. Biophys. Res. Commun*. 2008; 377:1156–1161. [PubMed: 18983822]
- Dale T, Kaartverdt S, Ellertsen B, Amundsen R. Large-scale oceanic distribution and population structure of *Calanus finmarchicus*, in relation to physical environment, food and predators. *Mar. Biol*. 2001; 139:561–574.
- Filgueira D, Guterres LP, Votto AP, Vargas MA, Boyle RT, Trindade GS, Nery LE. Nitric oxide-dependent pigment migration induced by ultraviolet radiation in retinal pigment cells of the crab *Neohelice granulata*. *Photochem. Photobiol*. 2010; 86:1278–1284. [PubMed: 21091482]
- Fu LL, Shuai JB, Xu ZR, Li JR, Li WF. Immune responses of *Fenneropenaeus chinensis* against white spot syndrome virus after oral delivery of VP28 using *Bacillus subtilis* as vehicles. *Fish Shellfish Immunol*. 2010; 28:49–55. [PubMed: 19800009]
- Gadalla MM, Snyder SH. Hydrogen sulfide as a gasotransmitter. *J. Neurochem*. 2010; 113:14–26. [PubMed: 20067586]
- Garthwaite J. Concepts of neural nitric oxide-mediated transmission. *Eur. J. Neurosci*. 2008; 27:2783–2802. [PubMed: 18588525]
- Hansen BW, Marker T, Andreassen P, Arashkewich E, Carlotti F, Lindeque P, Tande KS, Wagner M. Differences in life-cycle traits of *Calanus finmarchicus* originating from 60°N and 69°N, when reared in mesocosms at 69°N. *Mar. Biol*. 2003; 142:877–893.
- Head EJH, Harris LR, Campbell RW. Investigations on the ecology of *Calanus* spp. in the Labrador Sea. I. Relationship between the phytoplankton bloom and reproduction and development of *Calanus finmarchicus* in spring. *Mar. Ecol. Prog. Ser*. 2000; 193:53–73.
- Helaouët P, Beaugrand G, Reid PC. Macrophysiology of *Calanus finmarchicus* in the North Atlantic Ocean. *Prog. Oceanogr*. 2011; 91:217–228.
- Hirche H-J, Meyer U, Niehoff B. Egg production of *Calanus finmarchicus*: effect of temperature, food and season. *Mar. Biol*. 1997; 127:609–620.
- Inada M, Mekata T, Sudhakaran R, Okugawa S, Kono T, El Asely AM, Linh NT, Yoshida T, Sakai M, Yui T, Itami T. Molecular cloning and characterization of the nitric oxide synthase gene from kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol*. 2010; 28:701–711. [PubMed: 20109558]
- Jiang G, Yu R, Zhou M. Studies on nitric oxide synthase activity in haemocytes of shrimps *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* after white spot syndrome virus infection. *Nitric Oxide*. 2006; 14:219–227. [PubMed: 16403661]
- Kabil H, Kabil O, Banerjee R, Harshman LG, Pletcher SD. Increased transsulfuration mediates longevity and dietary restriction in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 2011; 108:16831–16836. [PubMed: 21930912]
- Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol*. 2013; 30:772–780. [PubMed: 23329690]
- Kim HW, Batista LA, Hoppes JL, Lee KJ, Mykles DL. A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis*. *J. Exp. Biol*. 2004; 207:2845–2857. [PubMed: 15235013]
- Kjørboe T, Munk P, Richardson K, Christensen V, Paulsen H. Plankton dynamics and larval herring growth, drift, and survival in a frontal area. *Mar. Ecol. Prog. Ser*. 1988; 44:205–219.

- Knipp S, Bicker G. Regulation of enteric neuron migration by the gaseous messenger molecules CO and NO. *Development*. 2009; 136:85–93. [PubMed: 19019991]
- Kotsyuba EP. Cystathionine β -synthase in the central nervous system of the shore crab *Hemigrapsus sanguineus*. *Dokl. Biol. Sci.* 2011; 437:94–96. [PubMed: 21562955]
- Kristiansen T, Lough RG, Werner FE, Broughton EA, Buckley LJ. Individual-based modeling of feeding ecology and prey selection of larval cod on Georges Bank. *Mar. Ecol. Progr. Ser.* 2009; 376:227–243.
- Krönström J, Dupont S, Mallefet J, Thorndyke M, Holmgren S. Serotonin and nitric oxide interaction in the control of bioluminescence in northern krill, *Meganyctiphanes norvegica* (M. Sars). *J. Exp. Biol.* 2007; 210:3179–3187. [PubMed: 17766295]
- éLab P, McTaggart SJ, Little TJ. An ancient immunity gene duplication in *Daphnia magna*: RNA expression and sequence analysis of two nitric oxide synthase genes. *Dev. Comp. Immunol.* 2009; 33:1000–1010. [PubMed: 19416737]
- Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009; 10:R25. [PubMed: 19261174]
- Lee CY, Zou HS, Yau SM, Ju YR, Liao CS. Nitric oxide synthase activity and immunoreactivity in the crayfish *Procambarus clarkii*. *Neuroreport*. 2000; 11:1273–1276. [PubMed: 10817606]
- Lee SG, Bader BD, Chang ES, Mykles DL. Effects of elevated ecdysteroid on tissue expression of three guanylyl cyclases in the tropical land crab *Gecarcinus lateralis*: possible roles of neuropeptide signaling in the molting gland. *J. Exp. Biol.* 2007; 210:3245–3254. [PubMed: 17766302]
- Lenz PH, Roncalli V, Hassett RP, Wu L, Cieslak MC, Hartline DH, Christie AE. *De novo* assembly of a transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) – the dominant zooplankton of the North Atlantic Ocean. *PLoS One*. 2014 In press.
- Lenz PH, Unal E, Hassett RP, Smith CM, Bucklin A, Christie AE, Towle DW. Functional genomics resources for the North Atlantic copepod, *Calanus finmarchicus*: EST database and physiological microarray. *Comp. Biochem. Physiol. Part D Genomics Proteomics*. 2012; 7:110–123. [PubMed: 22277925]
- Li S, Zhang Z, Li C, Zhou L, Liu W, Li Y, Zhang Y, Zheng H, Wen X. Molecular cloning and expression profiles of nitric oxide synthase (NOS) in mud crab *Scylla paramamosain*. *Fish Shellfish Immunol.* 2012; 32:503–512. [PubMed: 22200639]
- Lloyd D. Hydrogen sulfide: clandestine microbial messenger? *Trends Microbiol.* 2006; 14:456–462. [PubMed: 16908154]
- Mahadevan A, Lappé J, Rhyne RT, Cruz-Bermúdez ND, Marder E, Goy MF. Nitric oxide inhibits the rate and strength of cardiac contractions in the lobster *Homarus americanus* by acting on the cardiac ganglion. *J. Neurosci.* 2004; 24:2813–2824. [PubMed: 15028775]
- Marshall SM, Orr AP. On the biology of *Calanus finmarchicus* VIII. Food uptake, assimilation and excretion in adult and stage V *Calanus*. *J. Mar. Biol. Ass. UK*. 1955; 34:495–529.
- McCoole MD, D'Andrea BT, Baer KN, Christie AE. Genomic analyses of gas (nitric oxide and carbon monoxide) and small molecule transmitter (acetylcholine, glutamate and GABA) signaling systems in *Daphnia pulex*. *Comp. Biochem. Physiol. Part D Genomics Proteomics*. 2012; 7:124–160. [PubMed: 22305610]
- McDonald AA, Chang ES, Mykles DL. Cloning of a nitric oxide synthase from green shore crab, *Carcinus maenas*: a comparative study of the effects of eyestalk ablation on expression in the molting glands (Y-organs) of *C. maenas*, and blackback land crab, *Gecarcinus lateralis*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 2011; 158:150–62. [PubMed: 20959144]
- Meise CJ, O'Reilly JE. Spatial and seasonal patterns in abundance and age-composition of *Calanus finmarchicus* in the Gulf of Maine and on Georges Bank: 1977–1987. *Deep-Sea Res. II*. 1996; 43:1473–1501.
- Milograna SR, Bell FT, McNamara JC. Signaling events during cyclic guanosine monophosphate-regulated pigment aggregation in freshwater shrimp chromatophores. *Biol. Bull.* 2012; 223:178–191. [PubMed: 23111130]
- Mustafa AK, Gadalla MM, Snyder SH. Signaling by gasotransmitters. *Sci. Signal.* 2009; 2:re2. [PubMed: 19401594]

- Pepin P, Head EJH. Seasonal and depth-dependent variations in the size and lipid contents of stage 5 copepodites of *Calanus finmarchicus* in the waters of the Newfoundland Shelf and the Labrador Sea. *Deep Sea Res. I.* 2009; 56:6989–1002.
- Pepin P, Parrish CC, Head EJH. Late autumn condition of *Calanus finmarchicus* in the northwestern Atlantic: evidence of size-dependent differential feeding. *Mar. Ecol. Prog. Ser.* 2011; 423:155–166.
- Punta M, Coggill PC, Eberhardt, R, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD. The Pfam protein families database. *Nucleic Acids Res.* 2012; 40:D290–301. [PubMed: 22127870]
- Purcell JE, Grover JJ. Predation and food limitation as a cause of mortality in larval herring at a spawning ground in British Columbia. *Mar. Ecol. Prog. Ser.* 1990; 59:55–61.
- Reygondeau G, Beaugrand G. Future climate-driven shifts in distribution of *Calanus finmarchicus*. *ChangeGlobal Change Biol.* 2011; 17:756–766.
- Rodríguez-Ramos T, Carpio Y, Bolívar J, Espinosa G, Hernández-López J, Gollas-Galván T, Ramos L, Pendón C, Estrada MP. An inducible nitric oxide synthase (NOS) is expressed in hemocytes of the spiny lobster *Panulirus argus*: cloning, characterization and expression analysis. *Fish Shellfish Immunol.* 2010; 29:469–479. [PubMed: 20580828]
- Rodríguez-Ramos T, Carpio Y, Ramos L, Pons T, Farnós O, Iglesias C, Sánchez de Melo I, Ramos Y, Pendón C, Estrada MP, Bolívar J. New aspects concerning to the characterization and the relationship with the immune response in vivo of the spiny lobster *Panulirus argus* nitric oxide synthase. *Nitric Oxide.* 2011; 25:396–406. [PubMed: 21971212]
- Scholz NL, de Vente J, Truman JW, Graubard K. Neural network partitioning by NO and cGMP. *J. Neurosci.* 2001; 21:1610–1618. [PubMed: 11222651]
- Scholz NL, Labenia JS, de Vente J, Graubard K, Goy MF. Expression of nitric oxide synthase and nitric oxide-sensitive guanylate cyclase in the crustacean cardiac ganglion. *J. Comp. Neurol.* 2002; 454:158–167. [PubMed: 12412140]
- Schuppe H, Newland PL. Nitric oxide modulates presynaptic afferent depolarization of mechanosensory neurons. *J. Neurobiol.* 2004; 59:331–342. [PubMed: 15146549]
- Snyder SH, Ferris CD. Novel neurotransmitters and their neuropsychiatric relevance. *Am. J. Psychiatry.* 2000; 157:1738–1751. [PubMed: 11058466]
- Speirs DC, Gurney WSC, Heath MR, Horbelt W, Wood SN, de Cuevas BA. Ocean-scale modeling of the distribution, abundance, and seasonal dynamics of the copepod *Calanus finmarchicus*. *Mar. Ecol. Prog. Ser.* 2006; 313:173–192.
- Stein W, Eberle CC, Hedrich UB. Motor pattern selection by nitric oxide in the stomatogastric nervous system of the crab. *Eur. J. Neurosci.* 2005; 21:2767–2781. [PubMed: 15926924]
- Sudhamsu J, Crane BR. Bacterial nitric oxide synthases: what are they good for? *Trends Microbiol.* 2009; 17:212–218. [PubMed: 19375324]
- Taggart, CT.; Lochmann, SE.; Griffin, DA.; Thompson, KR.; Maillet, GL. Abundance distribution of larval cod (*Gadus morhua*) and zooplankton in a gyre-like water mass on the Scotian Shelf. In: Watanabe, Y.; Yamashita, Y.; Oozeki, Y., editors. *Survival Strategies in Early Life Stages of Marine Resources: Proceedings of an International Workshop, Yokohama, Japan, 11-14 October 1994.* Balkema Press; Rotterdam, The Netherlands: 1996. p. 127-145.
- Vandiver M, Snyder SH. Hydrogen sulfide: a gasotransmitter of clinical relevance. *J Mol Med (Berl).* 2012; 90:255–263. [PubMed: 22314625]
- Vargas MA, Cruz BP, Maciel FE, Geihs MA, Cousin JC, Trindade GS, Baisch AL, Allodi S, Nery LE. Participation of nitric oxide in the color change induced by UV radiation in the crab *Chasmagnathus granulatus*. *Pigment Cell Melanoma Res.* 2008; 21:184–191. [PubMed: 18426411]
- Wang R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol. Rev.* 2012; 92:791–896. [PubMed: 22535897]
- Wilson JL, Jesse HE, Poole RK, Davidge KS. Antibacterial effects of carbon monoxide. *Curr. Pharm. Biotechnol.* 2012; 13:760–768. [PubMed: 22201612]
- Yao CL, Ji PF, Wang ZY, Li FH, Xiang JH. Molecular cloning and expression of NOS in shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 2010; 28:453–460. [PubMed: 20026409]

- Yeh FC, Wu SH, Lai CY, Lee CY. Demonstration of nitric oxide synthase activity in crustacean hemocytes and anti-microbial activity of hemocyte-derived nitric oxide. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 2006; 144:11–17. [PubMed: 16516518]
- Zhang Y, He LS, Zhang G, Xu Y, Lee OO, Matsumura K, Qian PY. The regulatory role of the NO/cGMP signal transduction cascade during larval attachment and metamorphosis of the barnacle *Balanus* (= *Amphibalanus*) *amphitrute*. *J. Exp. Biol.* 2012; 215:3813–3822. [PubMed: 22855617]

***Highlights**

- Transcripts encoding *Calanus* NOS, HO and CBS proteins were identified
- All deduced enzymes appear to be full-length homologs of vertebrate neural isoforms
- *Calanus* CBS is the first crustacean member of this enzyme family to be identified
- The identified *Calanus* HO is the second known crustacean member of this family
- RNA-Seq mapping suggests stage-specific expression of gas transmitter transcripts

Calfi-NOS-I	MSSSVEMVSTKTRVDSGIGEPISSSSSSPGLAVCPFGFGKKVAKKTTLADIGFKIIQELR
Calfi-NOS-II	M-----TQGSDDSDGIEDQVSSK-----QENQ
	* : : **** : ** :
Calfi-NOS-I	KSKKKPQTILWRDDSAHSDILHRRNRKKMPCDTPDKMASAMNIGPANAPLPRDQMLQQA
Calfi-NOS-II	TTTKKPVIIYDWRDDGGISDTLNKSDRPLICERGRCLGSKMNMTPMKNPLPLDKAVAQA
	..*** ** ..** : : : : : : : : : : : : : : : : *
Calfi-NOS-I	EDFIKEYQDRENHEEPGVCKEERTVEVMRQLKSVESYTLTEDELTWAGARTAWNRASRCS
Calfi-NOS-II	KDFLKQYVADTTNHEVPEKSFQERQADVADLREKKTIDLTDELTWAGARMAWRNAPRCP
	:**:* ** * ** * : : ** : : : : : * ** * ** * ** * ** * ** *
Calfi-NOS-I	ARIWKLRVLDCRNATDSGDMFTAICKHLDISLNGGAIEPCITIFRERRSGQHLRNVN
Calfi-NOS-II	ARVVWKKLVDFRRHIDNTDDMFATMEHLDFSNNGGNIRPAISLFRQLPGQKDFRVVN
	::*:* ** * : : ** * * : ** * * ** * * ** * : ** * : ** * ** *
Calfi-NOS-I	NLLAFAGYEQEDGSIIGDPASVELTQVCESLGWVGGGQWDLPLVLSGEDGVPHWYEI
Calfi-NOS-II	GLITGFAAYEQEDGSIIGDPSSGLTKFVKLGWKGKGGQDFPLPMVFNAGADGIPHYDI
	..* : ** * ** * ** * ** * : ** : : ** * * ** * : ** * : ** * ** *
Calfi-NOS-I	PEKYCILVNIKHPTIEAISKMLKWIIPMVSNMMEYVGGMQFTAAPFGWYOGTEIANR
Calfi-NOS-II	PEKYLLRIKIQHPTVPGIDSLNLEWFGLPVGSMMFECCGQIQAAPFAGWYQGSSEVSR
	*** * : : ** * : . : : ** * : * ** * * ** * : ** * : ** * : ** * *
Calfi-NOS-I	DLLDQRYNLEPLGQAMGLDMSNTTMKDVVSLELNRAVLESYKAGVSIVDHYTQAE
Calfi-NOS-II	DLLDIQRYNLEPLGMAMGLDMSNTTLWKDEVALELNRAVLSYKAGVSMVDHFTQAD
	*** : ** * ** * ** * ** * ** * : ** * : ** * : ** * : ** * : ** * :
Calfi-NOS-I	QFIEHMKSEHKVRGGCPADWVWVPPQSGALVPTFHQEMIRYHLSPSYEQDRPWHTY
Calfi-NOS-II	QFMDHLAETKERGGCPADWVWVPPQSGSLTSTFHQEMVNYHLSPSYEQDKPYETYGR
	**::* : * * ** * ** * ** * ** * : . : ** * : . : ** * ** * : ** * :
Calfi-NOS-I	NRRKISFRSVAWSVLFWNSIYKKMLTARKKVTVLYGTETGSSKRYAKQAEVFSSTFNS
Calfi-NOS-II	EQSKKSFRSVAWSVMMWTSLSFKMEKKRKQVTFYSSSETGTAKKYAKQAEFSLSYKTS
	: : * ** * ** * : * : ** * : * ** * : * : ** * : * : ** * : * : ** * :
Calfi-NOS-I	LLALNSEGLYREIMSSDMCLFISSTFGNGEAPIMAESFNQELNAKLSKNEENS
Calfi-NOS-II	IFSLDDENVVSNLAQCHLALVIVSTFGNGEPPMSRPYNNKLTGIVERYQDNGEQTLAG
	: : : * : . : : : : * ** * ** * * : : : * : . : : : : :
Calfi-NOS-I	-EWFTRLPYGVFGLGSSAYPNIAAFGKFVDASLEKLGVRRIIPVGVGDELKQKGSFKVW
Calfi-NOS-II	MEPAKKIYFSTFGLGSSAYPKFAFGQHLDTSYDILGFORVLRFEAGDELKQKGSFKVW
	* : : : : . : ** * ** * : ** * : * ** * : . : ** * : ** * : ** * *
Calfi-NOS-I	IQQAFLAADMFDITLSEKMLQALKVKMETGKQDFRWVQSSSTDVSLYNTLSINHGRI
Calfi-NOS-II	VKKLEFTASLKVEVQ-APTSYLES- - - - -SAKKRFKWTLSHKKQSKSLNECLSEFHGKV
	: : * * : : : : : : : : * : : * : : * : : * : : * : : * : : * : : *
Calfi-NOS-I	MVELTLKQKNSLHEVAGESRALLDFFPDDASNRLSYNAGDHLGICPSNENEDVAFIKE
Calfi-NOS-II	AQDFTLTKRSHLHKEEKEPKTLQVDFSDN- - -SDVSYDPGDHLSIFPRNETRKVEYLKS
	: : ** : . : * : : * : * : * : : : : ** : ** * * * ** * : : *
Calfi-NOS-I	HLLDNPPSDMPLLFQKCNVWTVDDDFPKHLYHELLTYFADLKRVPQDVLALFLAHA
Calfi-NOS-II	RMNNPPSDKLVTLQVYHDFWENAEDLPSEVLFDLLYYFLDINMVPSQEMMDVFANAA
	: : : ** * : : * : . : * : : * : : : : : : : : ** * * : : : * : *
Calfi-NOS-I	TSKADRESLYTLVNDWEVYEAWRKEETTLCTFREFPSSVRISQAQVIGMLRIIQRNLSI
Calfi-NOS-II	TDKDKETLTLFLAQDDVSYQEWLEESKVCETLRDFESISLSATLVGYLTVIKPRRYSI
	* * * ** * * : * : * : . : ** * : * : : * : : * : : * : : * : * *
Calfi-NOS-I	ASAPSKGRVSLVGVVEYKTPLDFFPKLGLCTGELLQASLGTKIPGFFRPA--VSFSLPKD
Calfi-NOS-II	ASSPQGRNLSLVGMVEYQTKTGLTKGLATGNLNDMQIRTSLPGFIKYANKVHFRLEPD
	** : . : ** * : ** * : . : * ** * : : : * : ** * : * : * : * : *
Calfi-NOS-I	THKPVLMVGAGSGIAPFRGFQERQLQSAKGLPVGATSLFYGCRVPGLDLRDETMLAN
Calfi-NOS-II	PSWPIIMIGAGSGIAPFRGFWMRFQEQQDGYEVGKTLIFYGCRKSMNLFKNETESASK
	* : : ** * ** * ** * * * * * * * * * : : : ** * : : : : : : *
Calfi-NOS-I	KQGLFS--WALSLLTGGSLEFERYTALSRAPGTSRRYVQDLLVEEGARVWKLWQEQGGS
Calfi-NOS-II	SNQSLSSLRWMMQCSDNKLLDFEREVAFSREPGQPKQYVQNLTIDKAAKIYDLWQKGGY
	: : * * : . : ** * : * : ** * : : ** * : : : : : : ** * : ** *
Calfi-NOS-I	LYVCGKISMAAGVCEGLENICQEFGGISRKEAKELDKMRAEGRYQEDIFGNH
Calfi-NOS-II	TYICGKIQAEEVQAIETLTHLGNMDKATATATLEDMRKLGRYQEDIFG--
	: : ** * ** * * : : : : : : : * : * : ** * ** * ** *

Figure 1.

Alignment and predicted functional domains of *Calanus finmarchicus* nitric oxide synthase I and II (Calfi-NOS I and II). In the line immediately below each sequence grouping, “*” indicates amino acids that are identical in the two proteins, while “.” and “:” denote amino acids that are similar in structure between the two sequences. In this figure, the nitric oxide synthase oxygenase, flavodoxin, FAD-binding and oxidoreductase NAD-binding domains identified by Pfam analyses are highlighted in green, dark blue, light blue and black, respectively.

A. Amino acid sequence and structural domains of *Calanus finmarchicus* heme oxygenase

MEQENTDEKVEDEKDDKRKELFSQRMRAATRNHNRSALVNAGLVMTSDDNVWAEGLLVFYEIFKFLEESLAAHSDSLIGDLLIPGMA
 RTEALESDLAHYLGDKWKGYYVRPEVQSYLAHLKKLDKENPYLLIPYIYHLYMGLFSGGQVLRATRFLSMSSISGSGAGGDEEDGDKPGN
 SVTTYGDVTIGSLKKQLRKAINDLADELDVETKEAILVESVAVFEWNNVIIGSVKGVDRVLKRRLLKLLIAVILLLLFLFAYVFKSASEI
 EIEGDFVSPGPIAGAEI

B. Amino acid sequence and structural domains of *Calanus finmarchicus* cystathionine β -synthase

MSPVAITEEPMNGIPKVELKIPIGPITAGRGRKKSQASSKKDNGCPMGFVRPDLTSRCTWIPNGTKPDLQKGTQGPHTIDKSKDRMQIM
 PDVLFNIGNTPLVRINKISKSAGLKCDDLAKCEFFNAGGSVKDRIALRMVEDAEKCGMLKAGDTIIIEPTSGNTGIGLALAAVKGYRCII
 VLPEKMSNEKVATLRALGAEIVRTPTSASWDSPEHSISVAQRLLAEVPNSIILDQYRNPGNPLAHYDTTAEIILEQCNGKVDMMVMGAGT
 GGTISGIGRKLKEKCEPCIVVGVDPLGSILAEPENLNRSDDVTYYDVEGTGYDFIPTVLDRTVVVDKWKIKSGDKESFIMARRLIREEGLLCG
 GSSGATMACAVQAAMDLPKQKCVALLADGVRNYMTKFLDDQWLADRDIIELNDEMNTNWAAEKVSSELSAPLSILPSVAVEHAIDIMV
 KEGFDQLPVITESGKIIGVATMGSLKAKLLKGINATDPVEAATYTQFKKLNLDTTLSKLNRIIDTEHFALVVHNQRLYTSDKNIQEREV
 IVGIVTDIDLLQYIARRESGSSPDGSGSISPRNSQKGAN

Figure 2.

Calanus finmarchicus hemoxygenase (Calfi-HO) and cystathionine β -synthase (Calfi-CBS) deduced proteins and functional domains. (A) Calfi-HO. In this panel, the heme oxygenase domain identified by Pfam analysis is highlighted in red. (B) Calfi-CBS. In this panel, the pyridoxal-phosphate dependent enzyme and cystathionine β -synthase domains identified by Pfam analysis are highlighted in yellow and pink, respectively.

Calfi-CBS ES237695	MSPVAITEEPMNGIPKVELKIPITAGRGRKKSQASSKKDNGCPMGFVRPDLTSRCT MSPVAITEEPMNGIPKVELKIPITAGRGRKKSQASSKKDNGCPMGFVRPDLTSRCT *****
Calfi-CBS ES237695	WIPNGTKPDLQKGTQGPHTIDKSKDRMQIMPDVLFNIGNTPLVRINKISKSAGLKCDLLA WIPNGTKPDLQKGTQGPHTIDKSKDRMQIMPDVLFNIGNTPLVRINKISKSAGLKCDLLA *****
Calfi-CBS ES237695	KCEFFNAGGSVKDRIALRMVEDAEKCGMLKAGDTIIPTSGNTGIGLALAAAVKGYRCII KCEFFNAGGSVKDRIALRMVEDAEKCGMLKAGDTIIPTSGNTGIGLALAAAVKGYRCII *****
Calfi-CBS ES237695	VLPEKMSNEKVATLRALGAEIVRTPTSASWDSPESHISVAQRLLAEVPNSIILDQYRNP VLPEKMSNEKVATLRALGAEIVRTPTSASWDSPESHISVAQRLL----- *****
Calfi-CBS ES237695	NPLAHYDTTAEIILEQCNGKVDMMVMGAGTGGTISGIGRKLKEKCECIVVGVDPLGSIL -----
Calfi-CBS ES237695	AEPENLNRSDVITYYDVEGTGYDFIPTVLDRTVVDKWKIKSGDKESFIMARRLIREEGLLCG -----
Calfi-CBS ES237695	GSSGATMACAVQAAMDLPKQKCVALLADGVRNYMTKFLDDQWLADRDIIELNDEMTNWW -----
Calfi-CBS ES237695	AEEKVSSLELSAPLSILPSVAVEHAIDIMVKEGFDQLPVITESGKIIGVATMGSLKAKLL -----
Calfi-CBS ES237695	KGKINATDPVEAATYTQFKKLNLDTTLSKLNRIILDEHFALVVHNQRLYTSKNIQEREV -----
Calfi-CBS ES237695	IVGIVTDIDLLQYIARRESGSSPDGSGSISPRNSQKGAN -----

Figure 3.

Confirmation of protein sequence of *Calanus finmarchicus* cystathionine β -synthase (Calfi-CBS) using publicly accessible expressed sequence tags (ESTs). To increase our confidence in the deduced amino acid sequence of Calfi-CBS, this protein was used to query the extant *C. finmarchicus* ESTs curated at NCBI for identical sequences. As can be seen here, the BLAST search using Calfi-CBS identified one EST (Accession No. **ES237695**) that encodes an amino (N)-terminal partial protein homolog. Calfi-CBS and the EST-deduced partial protein are identical in amino acid sequence in their region of overlap, thus vetting the N-terminal portion of full-length protein.

Figure 4.

Expression patterns of gasotransmitter enzyme-encoding transcripts across six *Calanus finmarchicus* developmental stages in material collected in 2011 (black bars) and 2012 (gray bars) based on RNA-Seq data. Expression levels are presented as normalized counts per million reads that mapped to each target transcript sequence in embryo, early nauplius (stages NI-II), late nauplius (stages NV-NVI), early copepodite (stages CI-II), late copepodite (stage CV) and adult female (stage CVI). (A) *Calanus finmarchicus* nitric oxide synthase I (Calfi-NOS I)-encoding transcript. (B) *Calanus finmarchicus* nitric oxide

synthase II (Calfi-NOS II)-encoding transcript. **(C)** *Calanus finmarchicus* heme oxygenase (Calfi-HO)-encoding transcript. **(D)** *Calanus finmarchicus* cystathionine β -synthase (Calfi-CBS)-encoding transcript. Significant differences in stage-specific expression between years are indicated by arrows (panel **A**: early nauplius; panel **D**: early nauplius). Significant differences among stages are indicated by small letters over the gray bars (2012): the same letter indicates that two or more stages have similar expression levels (p-values given in Tables 4 and 5). Error bars in the 2012 samples represent standard deviation of the mean of three biological replicates (two technical replicates each); no error bars are given for the 2011 data as there is only a single biological sample for each stage.

Table 1

Putative *Calanus finmarchicus* gas transmitter biosynthetic enzyme-encoding transcripts and proteins identified via *in silico* transcriptome mining

Query protein	<i>Calanus</i> transcript/protein identifications					
	Transcripts				Proteins	
	Trinity ID Number	Name	E-value	Length [*]	Name	Length [†]
Nitric oxide synthase (NOS)	comp146200_c3_seq1	<i>calfi-nos I</i>	1.0e-110	3612	Calfi-NOS I	1121
	comp179394_c1_seq1	<i>calfi-nos II</i>	1.7e-100	3775	Calfi-NOS II	1084
Heme oxygenase (HO)	comp27018_c0_seq1	<i>calfi-ho</i>	1.2e-029	1002	Calfi-HO	287
Cystathionine β -synthase (CBS)	comp15975_c0_seq 1	<i>calfi-cbs</i>	1.8e-124	2680	Calfi-CBS	579

* Length in nucleotides

[†] Length in amino acids

Table 2

blastp analyses of putative *Calanus finmarchicus* gas transmitter biosynthetic enzymes vs. all NCBI curated non-redundant arthropod proteins*

Calanus query	Top 10 blastp arthropod protein hits				
	Accession No.	Species	Protein description	BLAST score	E-value
Calfi-NOS I	XP_967195	<i>Tribolium castaneum</i>	similar to nitric oxide synthase	747	0.0
	Q26240	<i>Rhodnius prolixus</i>	nitric oxide synthase, salivary gland	731	0.0
	EFZ14055	<i>Solenopsis invicta</i>	hypothetical protein SINV_03517	723	0.0
	BAH14964	<i>Gryllus bimaculatus</i>	nitric oxide synthase	721	0.0
	BAF63161	<i>Luciola cruciata</i>	nitric oxide synthase	717	0.0
	XP_003708103	<i>Megachile rotundata</i>	nitric oxide synthase, salivary gland-like	710	0.0
	EG162001	<i>Acromyrmex echinator</i>	nitric oxide synthase, salivary gland	708	0.0
	XP_003247939	<i>Acyrtosiphon pisum</i>	nitric oxide synthase, salivary gland-like isoform	711	0.0
	XP_003698873	<i>Apis florea</i>	nitric oxide synthase, salivary gland-like	709	0.0
	XP_002003249	<i>Drosophila melanogaster</i>	GH17812	713	0.0
Calfi-NOS II	XP_967195	<i>Tribolium castaneum</i>	similar to nitric oxide synthase	706	0.0
	EFZ14055	<i>Solenopsis invicta</i>	hypothetical protein SINV_03517	697	0.0
	BAH14964	<i>Gryllus bimaculatus</i>	nitric oxide synthase	694	0.0
	Q26240	<i>Rhodnius prolixus</i>	nitric oxide synthase, salivary gland	689	0.0
	BAF63161	<i>Luciola cruciata</i>	nitric oxide synthase	686	0.0
	AAT46681	<i>Gecarcinus lateralis</i>	nitric oxide synthase	687	0.0
	BAF63160	<i>Luciola lateralis</i>	nitric oxide synthase	683	0.0
	NP_001161704	<i>Nasonia vitripennis</i>	nitric oxide synthase	682	0.0
	ADD63793	<i>Litopenaeus vannamei</i>	nitric oxide synthase	677	0.0
	XP_003247939	<i>Acyrtosiphon pisum</i>	nitric oxide synthase, salivary gland-like isoform	676	0.0
Calfi-HO	EFR29866	<i>Anopheles darlingi</i>	hypothetical protein AND_00886	214	2e-066
	XP_003703646	<i>Megachile rotundata</i>	heme oxygenase 1-like	210	8e-065
	XP_001848782	<i>Culex quinquefasciatus</i>	heme oxygenase 1	206	4e-063
	XP_003490927	<i>Bombus impatiens</i>	heme oxygenase 1-like	199	2e-060
	XP_001658955	<i>Aedes aegypti</i>	hypothetical protein AaeL_AAE008136	197	4e-060

Top 10 blastp arthropod protein hits					
Calanus query	Accession No.	Species	Protein description	BLAST score	E-value
	XP_318426	<i>Anopheles gambiae</i>	AGAP003975-PA	197	8e-060
	XP_002429049	<i>Pediculus humanus corporis</i>	heme oxygenase, putative	196	2e-059
	XP_003397030	<i>Bombus terrestris</i>	heme oxygenase 1 -like	196	4e-059
	EG169271	<i>Acromyrmex echinator</i>	heme oxygenase 2	195	9e-059
	EFN82497	<i>Harpegnathos saltator</i>	heme oxygenase 2	193	3e-058
Calfi-CBS	EFX67333	<i>Daphnia pulex</i>	hypothetical protein DAPPUDRAFT_218706	670	0.0
	EFX64847	<i>Daphnia pulex</i>	hypothetical protein DAPPUDRAFT_333777	658	0.0
	XP_001659242	<i>Aedes aegypti</i>	cysteine synthase	553	0.0
	ABU41040	<i>Lepeophtheirus salmonis</i>	cystathionine-beta-synthase	537	0.0
	XP_001863044	<i>Culex quinquefasciatus</i>	cysteine synthase	523	1e-178
	EFZ14120	<i>Solenopsis invicta</i>	hypothetical protein SINV_06230	522	2e-178
	XP_001600075	<i>Nasonia vitripennis</i>	cystathionine-beta-synthase-like isoform 1	515	6e-176
	XP_003424942	<i>Nasonia vitripennis</i>	cystathionine-beta-synthase-like isoform 2	516	1e-175
	XP_003424943	<i>Nasonia vitripennis</i>	cystathionine-beta-synthase-like isoform 3	514	2e-175
	XP_003400432	<i>Bombus terrestris</i>	cystathionine-beta-synthase-like isoform 1	513	3e-175

* Excluding C. finmarchicus proteins, obvious partial proteins and synthetic constructs.

blastp analyses of putative *Calanus finmarchicus* nitric oxide synthase (NOS) and heme oxygenase (HO) isoforms vs. all non-redundant proteins from selected vertebrates

Table 3

Calanus query	Top blastp protein hit				
	Target species	Accession No.	Protein description	BLAST score	E-value
Calfi-NOS I	<i>Takifugu rubripes</i>	AAL82736	neuronal nitric oxide synthase	723	0.0
	<i>Xenopus laevis</i>	NP_001079155	nitric oxide synthase 1 (neuronal)	708	0.0
	<i>Gallus gallus</i>	NP_990292	nitric oxide synthase, inducible	711	0.0
	<i>Homo sapiens</i>	NP_000611	nitric oxide synthase, brain isoform 1	709	0.0
Calfi-NOS II	<i>Takifugu rubripes</i>	XP_003975239	nitric oxide synthase, brain-like	674	0.0
	<i>Xenopus laevis</i>	AAI70183	NOS1-A protein	677	0.0
	<i>Gallus gallus</i>	NP_990292	nitric oxide synthase, inducible	661	0.0
	<i>Homo sapiens</i>	AAC83553	inducible nitric oxide synthase	666	0.0
Calfi-HO	<i>Takifugu rubripes</i>	O73688	heme oxygenase	79.3	1e-017
	<i>Xenopus laevis</i>	NP_001085675	heme oxygenase 2	72.0	7e-015
	<i>Gallus gallus</i>	XP_414960	heme oxygenase 2	65.1	4e-012
	<i>Homo sapiens</i>	NP_002125	heme oxygenase 2	72.0	1e-013

* Excluding obvious partial proteins and synthetic constructs.

Table 4

Matrix showing significance levels of pair-wise comparisons of *calanus finmarchicus nitric oxide synthase I* (*calfi-nos I*; top portion of matrix) and *calanus finmarchicus nitric oxide synthase II* (*calfi-nos II*; bottom portion of matrix) expression between stages for samples collected in 2012 using a one-way ANOVA (followed by Tukey's post hoc test).

<i>calfi-nos I</i>						
	Embryo	Early nauplius	Late nauplius	Early copepodite	Late copepodite	Adult female
Embryo		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Early nauplius	0.9		~1.0	~1.0	~1.0	~1.0
Late nauplius	<0.0001	<0.0001		~1.0	~1.0	0.9
Early copepodite	<0.0001	0.0009	0.0410		~1.0	0.9
Late copepodite	0.0005	0.05	0.0002	0.09		0.8
Adult female	0.7	0.7	<0.0001	0.0001	0.0046	
<i>calfi-nos II</i>						

Statistically significant values are shown in red font.

Table 5

Matrix showing significance levels of pair-wise comparisons of *calanus finmarchicus* heme oxygenase (*calfi-ho*; top portion of matrix) and *calanus finmarchicus* cystathionine β -synthase (*calfi-cbs*; bottom portion of matrix) expression between stages for samples collected in 2012 using a one-way _ANOVA (followed by Tukey's post hoc test).

<i>calfi-ho</i>						
	Embryo	Early nauplius	Late nauplius	Early copepodite	Late copepodite	Adult female
Embryo		0.003	0.02	0.02	0.2	0.02
Early nauplius	0.02		0.9	~1.0	0.2	<0.0001
Late nauplius	<0.0001	0.0001		~1.0	0.6	<0.0001
Early copepodite	0.002	0.3	0.008		0.5	<0.0001
Late copepodite	0.0006	0.2	0.004	~1.0		<0.0005
Adult female	0.02	~1.0	0.0001	0.4	0.2	
<i>calfi-cbs</i>						

Statistically significant values are shown in red font.