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Identification and developmental expression of the enzymes responsible for dopamine, histamine, octopamine and serotonin biosynthesis in the copepod crustacean *Calanus finmarchicus*

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Abstract

Neurochemicals are likely to play key roles in physiological/behavioral control in the copepod crustacean *Calanus finmarchicus*, the biomass dominant zooplankton for much of the North Atlantic Ocean. Previously, a *de novo* assembled transcriptome consisting of 206,041 unique sequences was used to characterize the peptidergic signaling systems of *Calanus*. Here, this assembly was mined for transcripts encoding enzymes involved in amine biosynthesis. Using known *Drosophila melanogaster* proteins as templates, transcripts encoding putative *Calanus* homologs of tryptophan-phenylalanine hydroxylase (dopamine, octopamine and serotonin biosynthesis), tyrosine hydroxylase (dopamine biosynthesis), DOPA decarboxylase (dopamine and serotonin biosynthesis), histidine decarboxylase (histamine biosynthesis), tyrosine decarboxylase (octopamine biosynthesis), tyramine β-hydroxylase (octopamine biosynthesis) and tryptophan hydroxylase (serotonin biosynthesis) were identified. Reverse BLAST and domain analyses show that the proteins deduced from these transcripts possess sequence homology to and the structural hallmarks of their respective enzyme families. Developmental profiling revealed a remarkably consistent pattern of expression for all transcripts, with the highest levels of expression typically seen in the early nauplius and early copepodite. These expression patterns suggest roles for amines during development, particularly in the metamorphic transitions from embryo to nauplius and from nauplius to copepodite. Taken collectively, the data presented here lay a strong foundation for future gene-based studies of aminergic signaling in this and other copepod species, in particular assessment of the roles they may play in developmental control.

Keywords

tryptophan-phenylalanine hydroxylase (TPH); tyrosine hydroxylase (TH); DOPA decarboxylase (DDC); histidine decarboxylase (HDC); tyrosine decarboxylase (TDC); tyramine β-hydroxylase (TβH); tryptophan hydroxylase (TRH); Illumina sequencing; functional genomics

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1. Introduction

One major class of molecules used by nervous systems for chemical communication are amines. In crustaceans, four amines are generally recognized, dopamine, histamine, octopamine and serotonin (Christie, 2011), each of which is produced via the enzymatic processing of an amino acid substrate. While regulation of aminergic signaling systems can occur at many levels, modulation of the transcription, translation, and degradation of the enzymes involved in their biosynthesis is a major component in their control.

In arthropods, both dopamine and octopamine are produced from the amino acid phenylalanine in a three-step process (*e.g*. Coleman and Neckameyer, 2005; Monastirioti, 1999). The first step in the biosynthesis of both of these amines involves the enzymatic conversion of phenylalanine to tyrosine via the action of tryptophan-phenylalanine hydroxylase (TPH). To produce dopamine, tyrosine is subsequently converted to L-3,4-dihydroxyphenylalanine (L-DOPA) via the action of tyrosine hydroxylase (TH), and then to dopamine by DOPA decarboxylase (DDC). For the production of octopamine, tyrosine is converted by tyrosine decarboxylase (TDC) to tyramine, which in turn is converted to octopamine via the action of tyramine β-hydroxylase (TβH). To produce histamine, the amino acid histidine is decarboxylated via a reaction catalyzed by histidine decarboxylase (HDC) (*e.g*. Stuart, 1999). The amino acid tryptophan is the initial substrate for the production of serotonin. This amino acid is converted to 5-hydroxytryptophan via either TPH or tryptophan hydroxylase (TRH), which in turn is converted to serotonin by DDC (*e.g*. Coleman and Neckameyer, 2005; Monastirioti, 1999).

As part of an ongoing effort to identify and characterize the neurochemical signaling systems of the copepod crustacean *Calanus finmarchicus* (Christie et al., 2013a, 2013b), the biomass dominant zooplankton for much of the North Atlantic Ocean (Dale et al., 2001; Marshall and Orr, 1955; Meise and O'Reilly, 1996), we have mined a *de novo* assembled transcriptome for sequences encoding the enzymes responsible for the generation of dopamine, histamine, octopamine and serotonin. This study complements and augments an earlier report describing transcripts/proteins that contribute to peptidergic signaling in this ecologically important species (Christie et al., 2013a). As our data will show, *C. finmarchicus* transcripts putatively encoding TPH, TH, DDC, TDC, TβH, HDC, and TRH were identified. Reverse BLAST and domain analyses of the proteins deduced from the identified transcripts show that they possess sequence homology to and the structural hallmarks of their respective enzyme families. In addition, RNA-Seq profiling of the identified transcripts across *C. finmarchicus* development (embryo, early nauplius, late nauplius, early copepodite, late copepodite, and adult), revealed peaks in expression in the early nauplius and early copepodite stages, which suggests the amines may play roles in the metamorphic transitions between embryo/nauplius and nauplius/copepodite in this species. Collectively, the data presented in this study not only provide the first descriptions of amine biosynthetic enzymes in *C. finmarchicus*, but also lay a strong foundation for future gene-based studies of aminergic signaling in this species, including possible neurochemical roles for the amines in metamorphic control.

2. Materials and methods

2.1 *De novo* transcriptome assembly

A *de novo* transcriptome for *C. finmarchicus* was generated as described in detail in Christie et al. (2013a). In brief, multiplexed gene libraries were prepared from RNA extracted from six developmental stages of wild-caught or laboratory cultured *C. finmarchicus:* egg (which represents a mixture of embryonic stages; cultured), early nauplius (stages NI and NII; cultured), late nauplius (stages NV and NVI; cultured), early copepodite (stages CI and CII;

cultured), late copepodite (stage CV; wild-caught) and adult female (wild-caught). 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 base pair (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 (Grabherr et al., 2011) 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 >300 bp in length 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 in several recent publications (Christie et al., 2013a, 2013b). For all searches, the DeCypher program database was set to the combined Trinity assembly and a known fruit fly *Drosophila melanogaster* protein was used as the query sequence. All hits were translated (Supplemental Figure 1) and checked manually for homology to the query protein. Table 1 provides the BLAST-generated E-value (the number of alignments expected by chance that have the same score as the alignment) for each hit that was identified as encoding a putative target transcript, as well as the length 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 workflow described in Christie et al. (2013a, 2013b). To identify the proteins most similar to each of the *C. finmarchicus* amine biosynthetic enzymes identified in this study, the deduced *Calanus* sequence was used to query the annotated protein dataset present in FlyBase (version FB2013_01), as well as the non-redundant arthropod protein dataset (taxid:6656) curated in GenBank (excluding *C. finmarchicus* proteins, obvious partial proteins, synthetic constructs, and provisional/unannotated hypothetical protein sequences); for both searches the blastp algorithm was used (Altschul et al., 1997). The results of these searches, including BLAST scores (the similarity between the queried sequence and the found sequence) and E-values (see Section 2.2 for description), are summarized in Tables 2 and 3.

To determine amino acid identity/similarity between proteins, the sequences in question were aligned using MAFFT version 7 (http://align.bmr.kyushu-u.ak.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 the number of identical amino acids (denoted by "*" in the MAFFT output) divided by the total number of amino acids in the longest sequence (x100). Percent similarity was calculated as the 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 the longest sequence (x100). In the MAFFT output ":" indicates that one of the following strong groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY or FYW (Kazutaka Katoh, personal communication). The "." symbol in MAFFT indicates full conservation of one of the following weaker groups: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM or HFY (Kazutaka Katoh, personal communication).

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 Figures 1 and 2 and Supplemental Figure 2: ACT, green; biopterindependent aromatic amino acid hydroxylase, yellow; pyridoxal-dependent decarboxylase conserved, blue; DOMON, pink; copper type-II ascorbate-dependent monooxygenase N-terminal, red; copper type-II ascorbate-dependent monooxygenase C-terminal, dark red.

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 GenBank using the tblastn algorithm; see Christie et al. (2013a, 2013b) for details.

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 in Christie et al. (2013a, 2013c). For five stages, 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. For one stage grouping, early copepodites, we had three biological replicates (one from 2011 and two from 2012), which were also included in the analyses.

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 mapped reads in the technical replicate (Christie et al., 2013a). A two-way ANOVA was used to test for differences in relative transcript expression between years and among stages. Since both variables showed significant differences, a Bonferroni test was used to identify which stages showed significant differences in expression between years. Differences in expression among stages were calculated for the data collected in 2012 only, since it included biological and technical replication. Here, we used a one-way ANOVA followed by a Bonferroni post hoc test to calculate p-values for all pair-wise comparisons; GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses.

3. Results

3.1. Discovery of aminergic biosynthetic enzymes using a *de novo* assembled transcriptome

3.1.1. Dopamine biosynthesis—As described in Section 1, dopamine is synthesized from phenylalanine via the actions of the enzymes TPH, TH and DDC. Using known *Drosophila* proteins as query sequences, transcripts encoding putative *Calanus* homologs of each enzyme were identified within our *de novo* assembled transcriptome; these sequences are described in turn below.

3.1.1.1. Tryptophan-phenylalanine hydroxylase (TPH): Using *D. melanogaster* phenylalanine hydroxylase (Accession No. **CAA66797**; Ruiz-Vázquez et al., 1996) as the query sequence, a single, 1,531 bp *C. finmarchicus* transcript (comp34563_c3_seq1; named here *calanus finmarchicus tryptophan-phenylalanine hydroxylase* or *calfi-tβh*) was identified

as encoding a putative TPH homolog (Table 1). Translation of *calfi-tβh* yielded a 443 amino acid, full-length TPH isoform (Calfi-TPH; Fig. 1A and Table 1), with the open reading frame (ORF) bounded on both sides by stop codons (Supplemental Fig. 1A).

Alignment of the *D. melanogaster* query protein and Calfi-TPH revealed the two proteins share 65.3% identity/87.6% similarity in amino acid composition (Fig. 2). Structural analysis of the *Drosophila* protein using the online program Pfam identified a single biopterin-dependent aromatic amino acid hydroxylase domain within the sequence (Fig. 2); this domain was also identified by Pfam within Calfi-TPH (Figs. 1 and 2), as was a single ACT domain (Figs. 1 and 2). The biopterin-dependent aromatic amino acid hydroxylase domains are identically positioned in the two proteins and are nearly identical in amino acid composition (Fig. 2). Interestingly, while not identifed as an ACT domain, the amino acids in the *Drosophila* protein are also nearly identical to those corresponding to this motif in Calfi-TPH (Fig. 2). These data support Calfi-TPH being a member of the tryptophan-phenylalanine hydroxylase family.

3.1.1.2. Tyrosine hydroxylase (TH): Using *D. melanogaster* TH (Drome-TH; Accession No. **CAA53802**; Neckameyer and Quinn, 1989) as the query sequence, a single, 1,932 bp *C. finmarchicus* transcript (comp541083_c3_seq1; named here *calanus finmarchicus tyrosine hydroxylase* or *calfi-th*) was identified as encoding a putative TH homolog (Table 1). Translation of *calfi-th* yielded a 516 amino acid, full-length TH isoform (Calfi-TH; Fig. 1B and Table 1), with the ORF bounded on both sides by stop codons (Supplemental Fig. 1B).

Alignment of Drome-TH and Calfi-TH revealed the two proteins share 48.6% identity/ 82.6% similarity in amino acid composition (Supplemental Fig. 2A). Pfam analysis of the two proteins identified a single biopterin-dependent aromatic amino acid hydroxylase domain within each sequence (Fig. 1B and Supplemental Fig. 2A); the location of this domain was the same within the two proteins, and the amino acid composition of the motif is highly conserved between the two THs (Supplemental Fig. 2A). These data support Calfi-TH being a member of the tyrosine hydroxylase family.

3.1.1.3. DOPA decarboxylase (DDC): Using an isoform of *D. melanogaster* DDC (Drome-DDC; Accession No. **AAF53763**; Adams et al., 2000) as the query sequence, a single, 1,560 bp *C. finmarchicus* transcript (comp374436_c0_seq5; named here *calanus finmarchicus dopa decarboxylase* or *calfi-ddc*) was identified as encoding a putative DDC homolog (Table 1). Translation of *calfi-ddc* yielded a 487 amino acid, putative full-length DDC isoform (Calfi-DDC; Fig. 1C and Table 1), with the ORF bounded on its 3’ but not 5’ end by a stop codon (Supplemental Fig. 1C).

The alignment of Drome-DDC and Calfi-DDC revealed the two proteins share 60.0% identity/84.6% similarity in amino acid composition (Supplemental Fig. 2B). Pfam analysis of the two proteins identified a single pyridoxal-dependent decarboxylase conserved domain within each sequence (Fig. 1C and Supplemental Fig. 2B), the location and amino acid sequence of which is highly conserved between the two DDCs (Supplemental Fig. 2B). These data support Calfi-DDC being a member of the DOPA decarboxylase family.

3.1.2. Histamine biosynthesis

3.1.2.1. Histidine decarboxylase (HDC): As described in Section 1, histamine is produced from the amino acid histidine in a one-step reaction involving the enzyme HDC. Using an isoform of *D. melanogaster* HDC (Drome-HDC; Accession No. **AAF58823**; Adams et al., 2000) as the query sequence, a single, 2,659 bp *C. finmarchicus* transcript (comp175390_c0_seq2; named here *calanus finmarchicus histidine decarboxylase* or *calfi-*

hdc) was identified as encoding a putative HDC homolog (Table 1). Translation of *calfi-hdc* yielded a 724 amino acid, full-length HDC isoform (Calfi-HDC; Fig. 1D and Table 1), with the ORF bounded on both sides by stop codons (Supplemental Fig. 1D).

Alignment of the Drome-HDC and Calfi-HDC revealed the two proteins share 49.9% identity/71.9% similarity in amino acid composition (Supplemental Fig. 2C). Pfam analysis of the two proteins identified a single pyridoxal-dependent decarboxylase conserved domain within each sequence (Fig. 1D and Supplemental Fig. 2C), the locations and the amino acid sequences of which are nearly identical in the two HDCs (Supplemental Fig. 2C). These data support Calfi-HDC being a member of the histidine decarboxylase family.

3.1.3. Octopamine biosynthesis—Octopamine, like dopamine, is synthesized from phenylalanine in a three-step process. As with dopamine, TPH is the first enzyme in the biosynthesis of octopamine, with TDC and TβH catalyzing the two remaining reactions. The identification of a *Calanus* TPH-encoding transcript is described in Section 3.1.1.1, while those encoding *C. finmarchicus* TDC and TβH are presented below.

3.1.3.1. Tyrosine decarboxylase (TDC): Using *D. melanogaster* TDC (Drome-TDC; Accession No. **AAM70810**; Adams et al., 2000) as the query sequence, a single, 2,127 bp *C. finmarchicus* transcript (comp374436_c0_seq1; named here *calanus finmarchicus tyrosine decarboxylase* or *calfi-tdc*) was identified as encoding a putative TDC homolog (Table 1). Translation of *calfi-tdc* yielded a 617 amino acid, putative full-length TDC isoform (Calfi-TDC; Fig. 1E and Table 1), with the ORF bounded on its 3' but not 5' end by a stop codon (Supplemental Fig. 1E).

Alignment of the Drome-TDC and Calfi-TDC revealed the two proteins share 46.7% identity/74.6% similarity in amino acid composition (Supplemental Fig. 2D). Pfam analysis of the two proteins identified a single pyridoxal-dependent decarboxylase conserved domain within each sequence (Fig. 1E and Supplemental Fig. 2D); in both TDCs, the locations and amino acid sequences of this motif are nearly identical (Supplemental Fig. 2D). These data support Calfi-TDC being a member of the tyrosine decarboxylase family.

3.1.3.3. Tyramine β-hydroxylase (TβH): Using *D. melanogaster* TβH (Drome-TβH; Accession No. **AAO41640**; Adams et al., 2000) as the query sequence, a single, 2,122 bp *C. finmarchicus* transcript (comp975856_c0_seq1; named here *calanus finmarchicus tyramine β-hydroxylase* or *calfi-tβh*) was identified as encoding a putative TβH homolog (Table 1). Translation of *calfi-tβh* yielded a 672 amino acid, putative full-length TβH isoform (Calfi-TβH; Fig. 1F and Table 1), with the ORF bounded on its 3' but not 5' end by a stop codon (Supplemental Fig. 1F).

Alignment of the Drome-TβH and Calfi-TβH revealed the two proteins share 36.0% identity/65.5% similarity in amino acid composition (Supplemental Fig. 2E). Pfam analysis of the two proteins identified one DOMON, one copper type-II ascorbate-dependent monooxygenase N-terminal, and one copper type-II ascorbate-dependent monooxygenase C-terminal domain within each sequence (Fig. 1F and Supplemental Fig. 2E). The location of each of these domains is similar in the two proteins (Supplemental Fig. 2E). Likewise, the amino acid composition of each of the domains is highly conserved between the two TβHs (Supplemental Fig. 2E). These data support Calfi-TβH being a member of the tyramine β-hydroxylase family.

3.1.4. Serotonin biosynthesis—As described in Section 1, serotonin is synthesized from tryptophan via the actions of TPH or TRH and DDC. Discovery of the *Calanus* transcript encoding the former protein is discussed in Section 3.1.1.1, while discovery of a

C. finmarchicus DDC-encoding sequence is discussed in Section 3.1.1.3. The identification of a TRH-encoding transcript is provided below.

3.1.4.2. Tryptophan hydroxylase (TRH): Using *D. melanogaster* TRH (Drome-TRH; Accession No. **AAF47444**; Adams et al., 2000) as the query sequence, a single, 1,976 bp *C. finmarchicus* transcript (comp441733_c0_seq2; named here *calanus finmarchicus tryptophan hydroxylase* or *calfi-trh*) was identified as encoding a putative TRH homolog (Table 1). Translation of *calfi-trh* yielded a 541 amino acid, full-length TRH isoform (Calfi-TRH; Fig. 1G and Table 1), with the ORF bounded on both sides by stop codons (Supplemental Fig. 1G).

Alignment of the Drome-TRH and Calfi-TRH revealed the two proteins share 44.7% identity/76.9% similarity in amino acid composition (Supplemental Fig. 2F). Pfam analysis of the two proteins identified a single biopterin-dependent aromatic amino acid hydroxylase domain within each sequence (Fig. 1G and Supplemental Fig. 2F), which is similarly positioned and highly conserved in amino acid composition between the two TRHs (Supplemental Fig. 2F). These data support Calfi-TRH being a member of the tryptophan hydroxylase family.

3.2. Reverse BLAST analyses using deduced protein sequences

To confirm the annotations attributed to the putative aminergic biosynthetic enzyme-encoding transcripts described in Section 3.1, each deduced protein was used to query the extant annotated proteins in FlyBase and the extant non-redundant arthropod proteins in GenBank for the most similar sequences. As can be seen in Table 2, for the searches of FlyBase, an isoform of the protein to which the *Calanus* sequence was annotated was identified as the most similar protein for each query. For example, the query of FlyBase using Calfi-TPH returned Henna (FlyBase No. **FBpp0306707**), a synonym for TPH, as the most similar protein. Likewise, the query using Calfi-TH returned Pale (FlyBase No. **FBpp0076665**), a synonym for TH, as most similar to this *Calanus* sequence. While not the case here, we have noted that for some targeted protein discoveries, a *Drosophila* member of an unrelated family is returned as the top FlyBase hit (*e.g.* (6-4)-photolyase rather than cryptochrome for *C. finmarchicus* protein initially identified using *D. melanogaster* cyptochrome as the query; Christie et al., 2013b), which immediately calls into question the functional attribution ascribed to the query sequence. Like the reciprocal BLASTs conducted against FlyBase, those directed against the non-redundant arthropod proteins curated at GenBank typically identified proteins from the same family attributed to the *Calanus* query as the most similar arthropod sequence (Table 3), again supporting our functional attributions, *e.g.* TβH from the cockroach *Periplaneta americana* (Accession No. **AFO63077**; Chatel, Murillo, Bourdin, Quinchard, Picard and Legros, unpublished direct submission) for Calfi-TβH and tryptophan 5-hydroxylase 1 from the ant *Harpegnathos saltator* (Accession No. **EFN85713**; Bonasio et al., 2010) for Calfi-TRH. Taken collectively, the reverse BLASTs conducted against FlyBase and GenBank support the functional attributions given to the transcripts/proteins described in Section 3.1.

3.3. Expressed sequence tag support for deduced protein sequences

The *C. finmarchicus* transcriptome used here for protein discovery was assembled from over 400,000,000 paired-end reads (100-bp long). As the software used to assemble the reads is not infallible, there may be errors in some output sequences. If such errors occur in protein coding regions or result in frame shifts upstream of ORFs, then there would be a high likelihood of spurious protein predictions from the misassemblies. Thus, to strengthen our confidence in the sequences of the proteins presented in Section 3.1, each protein was BLASTed against the *C. finmarchicus* ESTs curated in GenBank. The extant *Calanus* ESTs,

while small in number relative to the sequences present in our Trinity assembly, are single-pass sequences (Lenz et al., 2012), and thus are not subject to assembly errors. Via this protocol, EST support for the deduced amino acid sequences of Calfi-TH, Calfi-TDC and Calfi-TRH was obtained (Table 4). However, no ESTs were identified that encoded any portion of Calfi-TPH, Calfi-DDC, Calfi-HDC, or Calfi-TβH (Table 4). An example of the EST vetting of one of the deduced *Calanus* amine biosynthetic enzymes, Calfi-TDC, is shown in Figure 3. As can be seen from this figure, the protein deduced from **FK041551** is a 232 amino acid, partial protein that differs from an internal portion of the amino (N)-terminus of Calfi-TDC at just two residues (99.1% identity), both conservative amino acid substitutions (Fig. 3). While the agreement between EST sequences and those derived from the *de novo* assembled transcriptome increases our confidence in the predicted proteins, it also illustrates the need for deep sequencing in order to obtain the predicted complement of full-length protein sequences.

3.4. Expression mapping of aminergic signaling transcripts

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 the transcripts encoding Calfi-TPH (Fig. 4A), Calfi-TH (Fig. 4B), Calfi-DDC (Fig. 4C), Calfi-HDC (Fig. 4D), Calfi-TDC (Fig. 4E), Calfi-TβH (Fig. 4F) and Calfi-TRH (Fig. 4G) to determine their patterns of developmental expression. Normalized data from samples collected in both 2011 (gray bars) and 2012 (black bars) are shown in Figure 4, and as can be seen from this figure, significant differences between years were noted for 10 of the 42 comparisons (arrows in Fig. 4). In all cases, the differences between years were in either the early or late nauplii, and with the exception of *calfi-th, calfi-hdc* and *calfi-trh* in the late nauplii, expression in 2012 was significantly higher than in 2011. The factor(s) responsible for the variation seen between these 2011 and 2012 samples remain unknown, but as both stages were laboratory cultured in eachyear, environmental variables (*e.g*. ocean temperature, salinity, etc) seem unlikely to be major contributors.

Comparisons among stages for the 2012 data (Fig. 4 and Supplemental Fig. 3), for which both biological and technical replication was included, show a common pattern of expression for all of the enzyme-encoding transcripts except *calfi-tph* (Fig. 4A). Specifically, *calfi-th, calfi-ddc, calfi-hdc, calfi-tdc, calfi-tβh and calfi-trh* all showed a low level of relative expression in the embryo, high relative expression in the early nauplius and early copepodite, and intermediate expression levels the late nauplius, late copepodite and adult (Fig. 4B–G). For *calfi-tph*, an increase in relative expression was noted between the embryo and early nauplius, with relative expression levels subsequently decreasing through the early copepodite stage and then being maintained at this lower level in the late copepodite and adult (Fig. 4A).

4. Discussion

4.1. *In silico* characterization of neurochemical signaling systems in *C. finmarchicus*

Large-scale shifts in the geographic range of *C. finmarchicus*, presumably due at least in part to rising ocean temperature (Helaouët et al., 2011; Pepin and Head, 2009; Pepin et al., 2011; Reygondeau and Beaugrand 2011; Speirs et al., 2006), have resulted in an increased focus on understanding how this species adapts to its environment physiologically. As part of this ongoing effort, we have generated a transcriptome for *Calanus* that consists of over 200,000 unique sequences (Christie et al., 2013a) and are currently using this resource as a platform for targeted gene/protein discovery (Christie et al., 2013a, 2013b). Among our ongoing projects is the identification and characterization of the neurochemical signaling

systems of *C. finmarchicus* (Christie et al., 2013a), which are likely key components in the control systems that allow this species to adapt physiologically to environmental challenges.

In a recent publication, we described the peptidergic systems of *C. finmarchicus* (Christie et al., 2013a). In this study, evidence for over twenty neuropeptide families was presented (Christie et al., 2013a). Here, we have expanded this work by identifying the transcripts and deducing the proteins responsible for amine biosynthesis. Specifically, the amino acid sequences of known *Drosophila* amine biosynthetic enzymes were used to query our *C. finmarchicus* transcriptome for sequences encoding proteins implicated in the production of dopamine, histamine, octopamine and serotonin. Via this approach, putative *Calanus* transcripts encoding TPH (dopamine, octopamine and serotonin biosynthesis), TH (dopamine biosynthesis), DDC (dopamine and serotonin biosynthesis), HDC (histamine biosynthesis), TDC (octopamine biosynthesis), TβH (octopamine biosynthesis) and TRH (serotonin biosynthesis) were identified. Each transcript appears to encode a full-length enzyme, and reverse BLAST and domain analyses confirm that these proteins possess sequence homology to and structural hallmarks of their respective enzyme families. Collectively, the data described here are the first identifications of the gene/protein components of aminergic signaling systems in *C. finmarchicus*. Moreover, while a number of other crustacean amine biosynthetic enzymes have been identified, only those of the cladoceran *Daphnia pulex* were among the top arthropod BLAST hits returned when the *Calanus* sequences were used to query the extant GenBank database, and here for just four of the seven queries, with but two as top 5 hits. This finding mirrors that seen for the peptidergic signaling systems of *Calanus* (Christie et al., 2013a), and reinforces the hypothesis that *C. finmarchicus* may occupy a position in arthropod phylogeny that is at or near the point where the crustacean and insect lineages diverged (*e.g.* Andrew, 2011; Andrew et al., 2012; Strausfeld and Andrew, 2011). If this hypothesis continues to be supported as sequence data for the Crustacea are expanded (it is currently less specious than that for the hexapods), *C. finmarchicus* may be a key species for studying the evolution of neurochemical signaling within the Arthropoda.

4.2. Developmental acquisition of aminergic systems in *C. finmarchicus* and a possible role for them in metamorphic transitions

Currently, little work has focused on the developmental acquisition of aminergic systems in crustaceans, and the few studies that are extant have focused on nervous systems, primarily those of decapods, in particular members of the Astacidea. Immunohistochemical mapping of aminergic profiles in the nervous systems of lobsters (*Homarus americanus* and/or *Homarus gammarus*) and crayfish (the parthenogenetic variant of *Procambarus fallax*, *i.e.* Marmorkrebs) suggest each amine to be initially expressed during embryonic development, with serotonin expression likely occurring early in embryogenesis, and the others in mid- to late embryonic life (Beltz et al., 1990; Cournil et al., 1995; Kilman et al., 1999; Le Feuvre et al., 2001; Pulver et al., 2003; Richards et al., 2003; Rieger and Harzsch, 2008; Schneider et al., 1996). For histamine and serotonin, the embryonic expression patterns are reported to be largely stable through neural developmet (Beltz et al., 1990; Rieger and Harzsch, 2008), whereas acquisition of the full complement of dopaminergic and octopaminergic neurons appears to be a more protracted event (Cournil et al., 1995; Schneider et al., 1996). Regardless, the early appearance of amines in crustacean nervous systems relative to other neuroactive compounds, *e.g.* many peptides (*e.g.* Beltz et al., 1990), suggests roles for them in the developmental control of this and other (see Section *4.3*) organ systems (*e.g.* Beltz et al., 2001; Benton and Beltz, 2001; Benton et al., 1997; Sullivan et al., 2000).

Our developmental profiling of the *C. finmarchicus* transcripts encoding the biosynthetic enzymes for dopamine, histamine, octopamine and serotonin suggests that the initial

acquisition of all four amines occurs at least by early naupliar life, as the highest levels of expression for each of the enzyme-encoding transcripts in this species was observed at this time point. As in the decapods nervous systems, this developmental peak in expression occurs earlier than was seen for many of the peptide/peptide receptor-encoding transcripts in *Calanus* (Christie et al., 2013a), and is suggestive that the amines may play a regulatory role in development control in this species too.

An interesting phenomenon that was noted for each of the amine biosynthetic enzyme-encoding transcripts except *calfi-tph* was the presence of two peaks in expression, one in the early nauplius and the other in the early copepodite. For most of the sequences, the differences in expression seen at these stages were significant compared to the other developmental time points that were examined. The early nauplius and the early copepodite stages represent two major transitions in the life history of *C. finmarchicus*. The transition from the embryo to the nauplius represents the activation of the sensory motor system to allow for swimming and escape responses (Bradley et al., 2013), but not yet feeding (Mauchline, 1998). In copepods, the transition from nauplius to copepodite involves a major change in body plan, including changes in the nervous system (Wilson and Hartline, 2011). Thus, it is possible that some or all of the amines may play key neurochemical roles in controlling these events, potentially including neurogenesis, for which at least serotonin has been implicated in decapods (Benton et al., 2008; Sandeman et al., 2009; Zhang et al., 2011).

4.3. Other roles for biogenic amines in *C. finmarchicus*

The acquisition of aminergic systems in *C. finmarchicus* appears to occur early in development and suggests a possible role for amines in the development and/or reorganization of the nervous system. While the distributions of aminergic neurons have not yet been mapped across development in *Calanus*, those expressing dopamine, histamine and serotonin have been examined in adults (Hartline and Christie, 2010). For these three amines, the distributions of labeled profiles were consistent with them serving as locally-released neuromodulators (Hartline and Christie, 2010), *i.e*. present, and presumably released, within central neuropil. For serotonin, and possibly dopamine, putative endocrine-like structures were also labeled in the nervous system, suggesting neurohormonal release is also possible (Hartline and Christie, 2010).

The specific roles served by locally-released and/or circulating amines in *Calanus* are currently unknown. However, work done on other vertebrate and invertebrate species, including numerous decapod crustaceans, suggests that they will ultimately be found to be both numerous and diverse. For example, in other crustaceans, dopamine, histamine, octopamine and serotonin have all been shown to affect rhythmic behaviors (*e.g*. locomotion, heartbeat and foregut movements) by acting directly on neural circuits and/or at neuromuscular junctions (*e.g*. Battelle and Kravitz, 1978; Callaway and Stuart, 1999; Christie et al., 2004; Djokaj et al., 2001; Flamm and Harris-Warrick, 1986a, 1986b; Florey and Rathmayer, 1978; Fort et al., 2004; Glusman and Kravitz, 1982; Harzsch and Glötzner, 2002; Johnson and Harris-Warrick, 1990, 1997; Johnson et al., 1993, 1994, 1995; Jorge-Rivera et al., 1998; Kravitz et al., 1980; Kvarta et al., 2012; Kwiatkowski et al., 2013; McCoole et al., 2011; Mulloney and Hall, 1991; Peck et al., 2006; Pulver et al., 2003; Rieger and Harzsch, 2008). In addition, dopamine, octopamine and serotonin have all been shown to play roles in the control of crustacean osmoregulation (*e.g*. Lohrmann and Kamemoto, 1987; Morris and Ahern, 2003; Morris et al., 2000), with the latter two amines also key components in the control of aggression/social dominance (*e.g*. Edwards and Kravitz, 1997; Huber, 2005; Huber et al., 2001; Kravitz, 2000; Panksepp et al., 2003; Pedetta et al., 2010; Sosa and Baro, 2002), and dopamine implicated in immune function (*e.g*. Chang et al.,

2007; Cheng et al., 2005; Li et al., 2005). Clearly much work will be needed to assess the roles of the amines in these and other actions in *Calanus*, but as additional studies are conducted, it will be interesting to see how their functions in *C. finmarchicus* correspond to those described for decapod crustaceans given differences in lifestyle (planktonic vs. benthic) and the evolutionary relationship between copepods and members of the Malacostraca.

4.4. Conclusions and future directions

Using a well-vetted bioinformatics workflow, we have mined for and characterized the transcripts and proteins that are likely responsible for the synthesis of dopamine, histamine, octopamine and serotonin in the copepod *C. finmarchicus*. These data complement and augment those previously described for the peptidergic systems of this species (Christie et al., 2013a), which is the biomass dominant zooplankton in the North Atlantic Ocean. Our ultimate goal is to characterize all of the neurochemical signaling systems of *Calanus*, which will allow for genebased studies of physiological adaptation, including the contributions of neurochemical signaling, in this ecologically critical species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Calanus* transcripts encoding amine biosynthetic enzymes were identified
- Dopamine, histamine, octopamine and serotonin pathways proteins were deduced
- Expression mapping showed all amines are produced early in development
- Two peaks in expression were noted (*i.e.* early nauplius and early copepodite)
- Expression peaks suggest hormonal roles for amines in metamorphic transitions

A. *Calanus finmarchicus* tryptophan-phenylalanine hydroxylase

```
MMNVDMEVEPTIMEGQFIKEAKNAAKSVNIIFSLNEAVGALAESLKIFKKHQVNLLHIESRSSIRVPGYEFMVEVDSTVGDIEGALENIKQESSYFQ
IITRNYKDNAEAVPWFPRKIQDLDKFANQILSYGSELESDHPGFTDQVYRARRKEFADIAFHYKHGTPIPNVSYTEQEKGVWKVVFNKLTRLYKTHA
CYEHNHVFPLLEENCGYSADEIPQLQTVSDFLKSVTGFQLRPVAGLLSSRDFLAGLAFRVFHSTQYIRHHTRPNYTPEPDVCHELLGHVPLFADPAF
AQFSQEIGLASLGASDDYIEKLATCYWFTVEFGVTRQHGQLKAYGAGLLSSFGELEWCLSGKPELRSFDPEKTGLQKYPITEHQPIYFVTESFESAK
QKMIDFAATIPRPFGVRYDPYTQTIQLLDSKRQIQKLITNINSEIRTLIDAFNKF
```

B. *Calanus finmarchicus* tyrosine hydroxylase

```
MTQGENDSSVPNGQMERREREPMKRSYSVENGYTGKTRSLVGDAKFEKLVRRESRLSFFNQESQDIQELSEDEAILMESLQDEAANSKIKLSLLIKL
SNAVLGMTSIVKTVEVQDGNLVHLETREPKAGGLENVVMEVLLTVEIVKDRILQLISQVKTREHVQHVKAVGCKRATVKGVWFPRHISELDNCNHIL
TKFEPELDMNHPGWSDGEYRARRKMIADNSFNYRHGDKIPTIEYTKEEIATWDAVYAKVFELLPDRASSVHRKYLSIMEKECGFGLGKIPQLEDVSN
FLKKSSGFSLRPAAGLLTARDFLASLAFRVFQCTQYVRHHTSPHYSPEPDLLHELIGHTPIFADPSFAQFSQEIGLASLGASDEEIEKLATVYWFTV
EFGLCKENGSIRAYGAGLLSSYGELLHALSQKESVEYRAFDPASASVQEYDDQAYQDIYYIAESFEDAKAKLRVWIGENLGRQFTVRYLPLTQSIEV
VDSLESAADIIEEMKLQVNQLSSAFEQIGRK
```

C. *Calanus finmarchicus* DOPA decarboxylase

```
MTSSEEFRKFGTAMINYVADYLDNIRDRPVVPQVSPGYLAKMIPTTAPEKAEDWKAVMEDIEKVIMPGVTHWHHPQFHAYFPTANSYPGIVADILSG
AIACIGFSWIASPACTELEMVTMDWLGKMLNLPKEFLFESKGHGGGVIQGTASEATLVAVLSARARAVRQYKAEHPDDNLEDGLIMSKLICYGSKQA
HSSVERAGLLAGVKMRLLEPDQDFSLTGDILSSAIAEDREKGLIPFCLISTLGTTSSCAYDHILSLGPVCREEGLWMHVDAAYAGSSFICPEFRPLL
DGVELAESFNFNPHKWMLVNFDCSAMWVRDSRLIVDAFNVDPMYLKHRHQGEIPDYRHWHIPLGRRFRSLKLWFVMRLYGVEGLQQHIRTQVKLAEQ
FADLVAKDSRFEMPVPPTMGLVCFRLKASNAVNETLNKNINDTGKIHITPSMIREKYILRFAVCSRFTVLSDITLAWQEVLDQVQSITMVKQVDTEK
ME
```

D. *Calanus finmarchicus* histidine decarboxylase

```
MEAAEYRVKGKEMVDYIADYLENIRERRVFPDVKPGYLRELLPDAAPHEGEEFDKIFADIERVIMPGVTHWQSPHMHAYFPALNSYPSLLGDMLADG
INCLGFTWASSPACTELESLVMDWLGQAIGLPAEFLHKTQGSLGGGVIQTTASESTFVCLLAGRTEAIKRFQTMFPDIEDAEINSRLVGYCSDQAHS
SVEKAGLIGLVKLRYIESDENLSLRGDKLKEAIKTDRENGLVPFFLCATLGTTGACAFDNLQELGPICQEEQMWLHVDAAYAGSAFVCPEFRKWMKG
IEFADSIAFNPSKWLMVHFDCTAMWVKNSRSLHRTFNVEPLYLQHENTGLAIDYMHWQVPLSKRFRALKLWFVLRNYGISGLQKMIRENVRLAQKFE
AMVRSDNRFEIPAARHMGLIVFRLRGECSMTEKLLKKLNSSGKMHAVPCCIKGKYVIRFTITSQRTTAQDLTRDWAIIRSTATDVLEEYGIVSTNRK
RVPLKEIKEKNASFGTSLLLANIGGNSPISPKIVDGSFAALFETDDVVVDFSKKLKNMQKDIHSNSTRSQATRRVRGMMMSGKQYSLDSRIDLVEAV
QASQDEEDQENVTRTQDKEDTGGPVTAGRGRSLSVVENSEVVGEVGMVLRKARAAQMAIPEGEAEDENGVSLTTLLQPSQTATIAQMMKQLDLNLSN
GSSSEQEIKEAFIEFYKLFDDFGIIDKNRYENINNWYEPEHRMKT
```

E. *Calanus finmarchicus* tyrosine decarboxylase

```
MDGQEFKVRGRQMVDYIIEYLEDIETRRVTPAIEPGYLSELIPASAPHDPEPWEDVMKDVDEKIMVGMTHWQHPRFHAYFPAGNSFPSILADMLSGA
IGCVGFSWAAAPSCTELETIMLDWLGKMVGLPPQFLSQSPGSRGGGVIQASASECVLDCLLAARAQAIRALKVKMGDQVEDTVLLSKLMAYCSKEAH
SCVEKAAMIGFVKLRILEPDDHSVLRGARLKEAMMEDIENGLYPFFVSTTLGTTGCCAFDRLDEIGPVCKEMGAWLHVDASYAGSAFICPEFRPLLN
GVEYAQSFNMNPNKWMLVNFDCSTMWVTDRYKLTQALVVDPLYLQHSYSESAIDYRHWGIPLSRRFRSLKLWFVIRNFGVSGLQEYVRNHCRLAKVF
EAKVEADTRFRVMNDVKVGLVCFRLFGSNQLNQKLLTTINASGKLHMVPASVNDYFVIRFCVCAQSATEEDIDIAWKIISEIASEVLKTMQAEEQSE
DREEDDVVKAMQKKSSETLEHKRSFFVRMVSDPKLYNPKIVKIDRRQKLQNMGVTSDCSDISPMHSWVSWPLAVLVQGVEIDEVSMRFRNWNTTLSL
SPHQASSSSSTSSSPRNGLKALEDENVTENVTKAK
```

F. *Calanus finmarchicus* tyramine β-hydroxylase

```
MTNRAYLFLLCLMLLVKTSSQFSLKLGKQILLDWEVDRIEQIARIQVRATLGPQDWVGVGFSDYGSLTGADMCLLWRDWKGGTKLADINTDEKSLVV
EDVKNDCVGAKWRKKIVADGVEEVEYMYSRKLDTCDEMDYKILEGTTHIVWALGRGPLYGISGVNISDSATVDTGMTRFRFLGVSLESLDESVVPYS
ITSKDVELPPVDTMYWCSVHKLDNQFESKHHVVQYEADISPGSEDVVHHMEVFHCPSQESGEFPVWSGSCSDENAPKELTQCKKVLAAWAIGAGPFT
YPEQTGMAIGGNDFHPFVMLEVHYNNPDKKSGIIDSSGIKFHITPNLRPHDAGIMELGLIYNNWMAIPPHATQFPLTGTCVPQCTAVGLPQKGIVVF
GSQLHTHGTGTQVQTTVLRNGKEHLLNEDRHYSTHFQEIRVLSKQVSVLPGDALITTCKYSTTERTNITLGGFGFTEEMCVNYIHYYPRVQLEVCKS
SIDRKQLYDYFNELNVDEDQNTDSNKSIEDNYHSICWNKKRTNELKQFYQKGTLNMQCMMGGGESFPGRWENIPQPHVKESYKLSNPCTEESKHKDN
DDAHSDIAYDPLVTNTEEDDTHRMMSLRKKKNFFGNSYADDWSDYTNWGVDKRTSNLPEKHHEDTQSHLLNTKRSGMEHVHMGKRRLGSM
```

G. *Calanus finmarchicus* tryptophan hydroxylase

```
MNTSGKGLVALYLHKRGEQWMVQQIKDNEEEQKLASLKQGGIYHFRSTNGVFKTAVIFPLHKRMAGLSKALRVFEDKNMNLVHIESRLAKGTKDQYE
IYLEIDSETSKDWNHIQQVIDTLRGIDLGPRDVEPVWKRNSFDCVDMISFPKAIGELDNCQKVLVYGTDLDADHPGFKDPVYRKRRQFFGDLAMIFK
YGQQIPRVKYTTVEIETWGRILKKLRELHIKYACKQFLDNWSELEQFCGYREDNIPQLEDINRYLRSKTGFQIRPVAGYLSPRAFLAGLAFRVFHCT
QYIRHSSDPFYTPEPDCCHELLGHMPLFADANFAQFSQEIGLASLGASEEEIAKLVSCYFFTVEFGLCREGDEMKVYGAGLLSSAAELQYVMSGVES
GQLPLLELKSDAVFTAEMMVTTYQKQYFFTETMDNAKEFVRDIANGVNRPFSVRYNPYTQTIEVLNNSDKILDMAKELRGDLCIVANALKKVQERDG
EIDPDIIANFLTAGLDITPYGSRCTTPVHSPPLDTNSPDSSPAHTGNQLRVPFAPR
```

Figure 1.

Deduced amino acid sequences of *Calanus finmarchicus* amine biosynthetic enzymes and their functional domains as predicted by the online program Pfam. (**A**) *Calanus finmarchicus* tryptophan-phenylalanine hydroxylase. (**B**) *Calanus finmarchicus* tyrosine hydroxylase. (**C**) *Calanus finmarchicus* DOPA decarboxylase. (**D**) *Calanus finmarchicus* histidine decarboxylase. (**E**) *Calanus finmarchicus* tyrosine decarboxylase. (**F**) *Calanus finmarchicus* tyramine β-hydroxylase. (**G**) *Calanus finmarchicus* tryptophan hydroxylase. Highlighting code for functional domains identified by Pfam: ACT, green; biopterin-dependent aromatic amino acid hydroxylase, yellow; pyridoxal-dependent decarboxylase

conserved, blue; DOMON, pink; copper type-II ascorbate-dependent monooxygenase N-terminal, red; copper type-II ascorbate-dependent monooxygenase C-terminal, dark red.

Figure 2.

Alignment of *Drosophila melanogaster* tryptophan-phenylalanine hydroxylase (Drome-TPH; Accession No. **CAA66797**) with a putative *Calanus finmarchicus* homolog (Calfi-TPH) deduced from the Trinity *de novo* transcriptome assembly. 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, ACT and biopterin-dependent aromatic amino acid hydroxylase domains identified by Pfam analyses are highlighted in green and yellow, respectively.

Figure 3.

Confirmation of protein sequence of *Calanus finmarchicus* tyrosine decarboxylase (Calfi-TDC) using publicly accessible expressed sequence tags (ESTs). To increase our confidence in the deduced amino acid sequences of the amine biosynthetic enzymes reported in this study, each protein was used to query the extant *C. finmarchicus* ESTs curated at GenBank for identical sequences. As can be seen here, the BLAST searches using Calfi-TDC identified one EST, **FK041551**, as encoding partial protein homologs of the full-length sequence; the overlapping regions of the two proteins differ at just two positions (black highlighting).

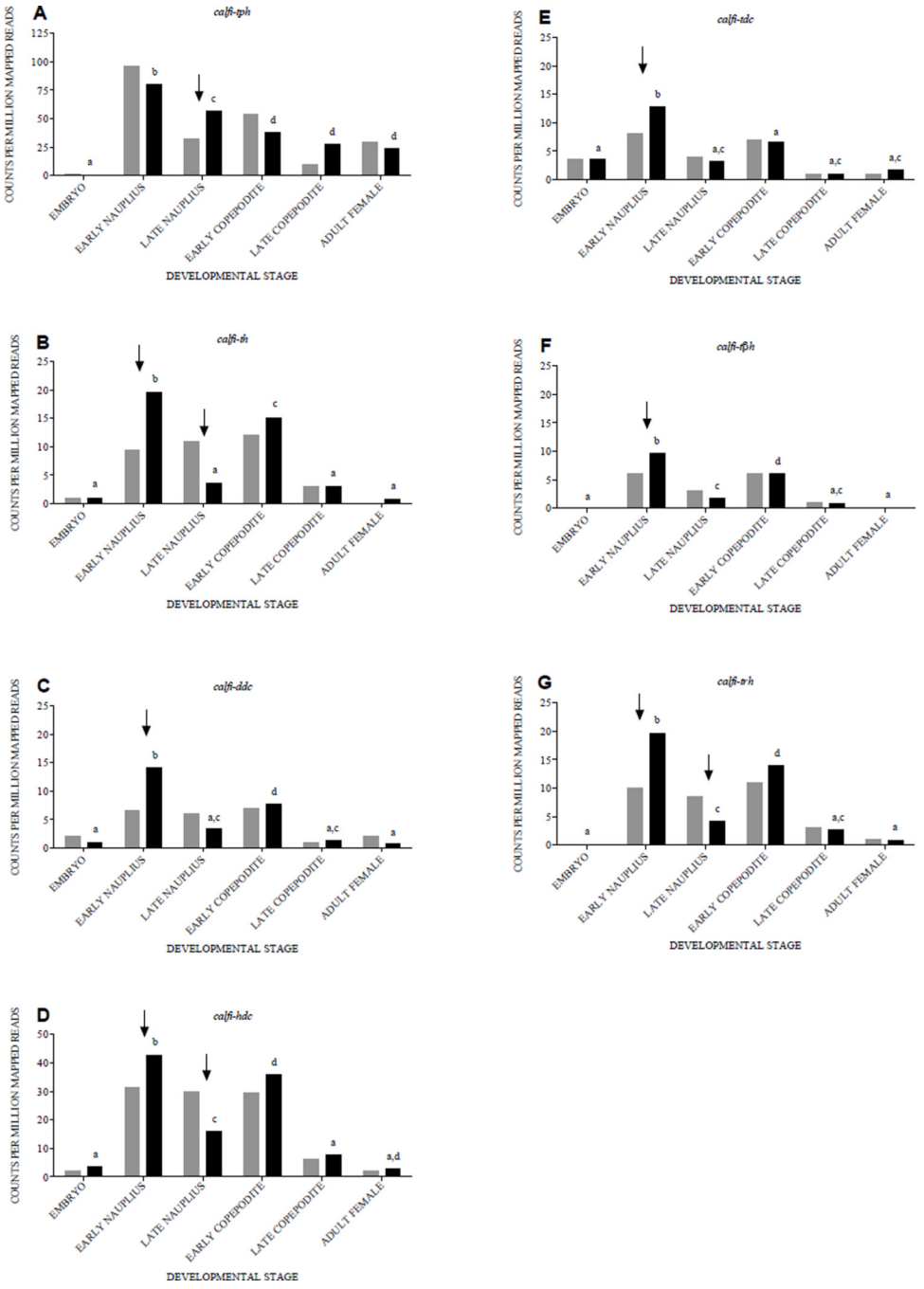

Figure 4.

Expression patterns of amine biosynthetic enzyme-encoding transcripts across six *Calanus finmarchicus* developmental stages in material collected in 2011 (grey bars) and 2012 (black 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 tryptophan-phenylalanine hydroxylase (calfi-tph)*. (**B**) *calanus finmarchicus tyrosine hydroxylase (calfi-th)*. (**C**) *calanus finmarchicus dopa decarboxylase (calfi-ddc)*. (**D**) *calanus finmarchicus*

histidine decarboxylase (calfi-hdc). (**E**) *calanus finmarchicus tyrosine decarboxylase (calfi-tdc)*. (**F**) *calanus finmarchicus tyramine β-hydroxylase (calfi-tβh)*. (**G**) *calanus finmarchicus tryptophan hydroxylase (calfi-trh)*. Significant differences in stage-specific expression between years are indicated by arrows. Significant differences among stages are indicated by small letters over the black bars (2012): the same letter indicates that two or more stages have similar expression levels (p-values given in Supplemental Fig. 3).

Table 1

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

	***Calanus* transcript/protein identifications**					
	Transcripts				**Proteins**	
Query protein	**Trinity ID Number**	**Name**	**E-value**	**Length***	**Name**	**Length†**
Tryptophan-phenylalanine hydroxylase[1,3,4]	comp34563_c3_seq1	*calfi-tph*	2.9e-175	1531	Calfi-TPH	443
Tyrosine hydroxylase[1]	comp541083_c3_seq1	*calfi-th*	5.1e-139	1932	Calfi-TH	516
DOPA decarboxylase[1,4]	comp374436_c0_seq5	*calfi-ddc*	1.7e-168	1560	Calfi-DDC	487
Histidine decarboxylase[2]	comp175390_c0_seq2	*calfi-hdc*	2.3e-230	2659	Calfi-HDC	724
Tyrosine decarboxylase[3]	comp374436_c0_seq1	*calfi-tdc*	6.7e-154	2127	Calfi-TDC	617
Tyramine β-hydroxylase[3]	comp975856_c0_seq1	*calfi-tβh*	4.1e-110	2122	Calfi-TβH	672
Tryptophan hydroxylase[4]	comp441733_c0_seq2	*calfi-trh*	2.8e-133	1976	Calfi-TRH	541

*Length in nucleotides

†Length in amino acids

[1]Dopamine biosynthesis

[2]Histamine biosynthesis

[3]Octopamine biosynthesis

[4]Serotonin biosynthesis

Table 2

BLAST analyses* of putative *Calanus finmarchicus* amine biosynthetic enzymes vs. all annotated *Drosophila melanogaster* proteins in FlyBase

	Top Drosophila protein hit			
Calanus query	**FlyBase No.**	**Protein description**	**BLAST score**	**E-value**
Calfi-TPH	FBpp0306707	Henna†, isoform C	629	3e-180
Calfi-TH	FBpp0076665	Pale‡, isoform A	482	5e-136
Calfi-DDC	FBpp0288730	DOPA decarboxylase, isoform D	593	2e-169
Calfi-HDC	FBpp0304256	Histidine decarboxylase, isoform B	833	0.0
Calfi-TDC	FBpp0085475	Tyrosine decarboxylase 2, isoform A	734	0.0
Calfi-TβH	FBpp0089042	Tyramine β-hydroxylase, isoform B	385	1e-106
Calfi-TRH	FBpp0072534	Tryptophan hydroxylase, isoform A	498	1e-140

*All searches conducted on or before June 1, 2013.

†A synonym for tryptophan-phenylalanine hydroxylase.

‡A synonym for tyrosine hydroxylase.

Abbreviations: Calfi-TPH, *Calanus finmarchicus* tryptophan-phenylalanine hydroxylase; Calfi-TH, *Calanus finmarchicus* tyrosine hydroxylase; Calfi-DDC, *Calanus finmarchicus* DOPA decarboxylase; Calfi-HDC, *Calanus finmarchicus* histidine decarboxylase; Calfi-TDC, *Calanus finmarchicus* tyrosine decarboxylase; Calfi-TβH, *Calanus finmarchicus* tyramine β-hydroxylase; Calfi-TRH, *Calanus finmarchicus* tryptophan hydroxylase.

Table 3

blastp analyses* of putative *Calanus finmarchicus* amine biosynthetic enzymes vs. all GenBank curated non-redundant arthropod proteins†

	Top 10 blastp arthropod protein hits				
***Calanus* Query**	**Accession No.**	**Species**	**Protein description**	**BLAST score**	**E-value**
Calfi-TPH	AAM28250 AAT78350 EFR29984 BAJ83477 EFZ21127 ADM33594 BAE66652 EHJ64587 AFL02790 EFN61629	*Aedes aegypti* *Armigeres subalbatus* *Anopheles darlingi* *Gryllus bimaculatus* *Solenopsis invicta* *Bombyx mori* *Papilio xuthus* *Danaus plexippus* *Polyrhachis vicina* *Camponotus floridanus*	Phenylalanine hydroxylase Phenylalanine hydroxylase Hypothetical protein AND_000708 Phenylalanine-tryptophan hydroxylase Hypothetical protein SINV_04627 Phenylalanine hydroxylase Phenylalanine hydroxylase Phenylalanine hydroxylase Phenylalanine hydroxylase Protein henna	646 645 643 643 642 642 640 639 637 632	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0
Calfi-TH	ABQ95974 CAA53802 AAW47987 EEB18101 EFX75078 ACU77882 BAJ07593 BAJ07587 BAE43824 BAF32574	*Tribolium castaneum* *Drosophila melanogaster* *Apis mellifera* *Pediculus humanus* *corporis* *Daphnia pulex* *Tenebrio molitor* *Papilio polytes* *Papilio machaon* *Papilio xuthus* *Mythimna separata*	Tyrosine hydroxylase Pale, isoform A Tyrosine hydroxylase Tyrosine 3-monooxygenase Hypothetical protein DAPPUDRAFT_323742 Tyrosine hydroxylase Tyrosine hydroxylase Tyrosine hydroxylase Tyrosine hydroxylase Tyrosine hydroxylase type 2, brain form	518 515 514 514 513 512 495 494 493 489	1e-177 8e-177 1e-176 5e-176 5e-176 3e-175 4e-168 5e-168 2e-167 1e-166
Calfi-DDC	BAJ83478 BAJ07594 BAJ07588 BAE43825 BAB68545 AAR23825 BAB68549 EFA03481 ABU25222 AAK48988	*Gryllus bimaculatus* *Papilio polytes* *Papilio machaon* *Papilio xuthus* *Mamestra brassicae* *Antheraea pernyi* *Mythimna separata* *Tribolium castaneum* *Tribolium castaneum* *Bombyx mori*	Aromatic L-amino-acid decarboxylase DOPA decarboxylase DOPA decarboxylase DOPA decarboxylase DOPA decarboxylase DOPA-decarboxylase DOPA decarboxylase Hypothetical protein TcasGA2_TC 13480 DOPA decarboxylase DOPA decarboxylase	650 647 645 645 644 640 640 638 637 636	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0

Calanus Query	Top 10 blastp arthropod protein hits				
	Accession No.	**Species**	**Protein description**	**BLAST score**	**E-value**
Calfi-HDC	EFX79676	*Daphnia pulex*	Hypothetical protein	876	0.0
	EDS34715	*Culex quinquefasciatus*	D APPUDRAFT_304481	865	0.0
	EAA14857	*Anopheles gambiae*	Aromatic amino acid	854	0.0
	EFN88410	*Harpegnathos saltator*	decarboxylase	848	0.0
	EG158389	*Acromyrmex echinatior*	AGAP009001-AP	847	0.0
	EFZ20538	*Solenopsis invicta*	Histidine decarboxylase	838	0.0
	EDX06463	*Drosophila simulans*	Histidine decarboxylase	842	0.0
	AGB93389	*Drosophila melanogaster*	Hypothetical protein SINV_06729	832	0.0
	CAA49989	*Drosophila melanogaster*	GD10721	840	0.0
	EDW32340	*Drosophila persimilis*	Histidine decarboxylase, isoform C	839	0.0
			Histidine decarboxylase GL11582		
Calfi-TDC	EEB13798	*Pediculus humanus corporis*	Aromatic-L-amino-acid decarboxylase	791	0.0
	EFA10348			791	0.0
	EDS34186	*Tribolium castaneum*	Hypothetical protein	772	0.0
	EAT45251	*Culex quinquefasciatus*	TcasGA2_TC012567	766	0.0
	EDW89072	*Aedes aegypti*	Aromatic-L-amino-acid decarboxylase	764	0.0
	AAM70812	*Drosophila yakuba*		764	0.0
	EDV38125	*Drosophila melanogaster*	Aromatic-L-amino-acid-decarboxylase	764	0.0
	EDV59802	*Drosophila ananassae*		764	0.0
	EDW61660	*Drosophila erecta*	GE24598	763	0.0
	EAL24715	*Drosophila virilis*	Tyrosine decarboxylase 2	761	0.0
		Drosophila pseudoobscura pseudoobscura	GF13800		
			GG10812		
			GJ20190		
			GA15851		
Calfi-TβH	EEB12867	*Pediculus humanus corporis*	Dopamine beta-hydroxylase precursor	536	0.0
	AFO63077			526	8e-178
	AFO63079	*Periplaneta americana*	Tyramine beta hydroxylase	526	1e-177
	AFO63076			526	1e-177
	AFO63080		Tyramine beta hydroxylase	525	4e-177
	EFA00428		Tyramine beta hydroxylase	520	3e-175
	EFZ13313	*Tribolium castaneum*	Similar to tyramine beta hydroxylase	512	8e-172
	EFN89747	*Solenopsis invicta*		489	8e-164
	EFX67831	*Harpegnathos saltator*	Hypothetical protein SINV_01889	489	2e-163
	EGI65668	*Daphnia pulex*	Dopamine beta-hydroxylase	487	9e-163
		Acromyrmex echinatior	Hypothetical protein DAPPUDRAFT_1839		
			Tyramine beta-hydroxylase		

Calanus Query	Top 10 blastp arthropod protein hits				
	Accession No.	**Species**	**Protein description**	**BLAST score**	**E-value**
Calfi-TRH	EFN85713	*Harpegnathos saltator*	Tryptophan 5-hydroxylase	551	0.0
	BAJ83476	*Gryllus bimaculatus*	Tryptophan hydroxylase	543	0.0
	EFN68680	*Camponotus floridanus*	Tryptophan 5-hydroxylase	526	4e-180
	EFA01002	*Tribolium castaneum*	Similar to AGAP006020-PA	522	3e-179
	BAK19479	*Gryllus bimaculatus*	Tryptophan hydroxylase long	523	2e-178
	EHJ65579	*Danaus plexippus*	variant	514	2e-175
	EEB18903	*Pediculus humanus*	Hypothetical protein KGM_15172	507	1e-173
	EAA11742	*corporis*	Tryptophan 5-hydroxylase,	509	1e-173
	EFX86644	*Anopheles gambiae*	putative	496	7e-170
	EDX08760	*Daphnia pulex*	AGAP006020-PA	498	3e-169
		Drosophila simulans	Hypothetical protein DAPPUDRAFT_44381		
			GD13494		

*All searches conducted on or before June 1, 2013.

†Excluding *C. finmarchicus* proteins, obvious partial proteins, obvious provisional proteins and synthetic constructs. Crustacean hits are shown in red font.

Abbreviations: Calfi-TPH, *Calanus finmarchicus* tryptophan-phenylalanine hydroxylase; Calfi-TH, *Calanus finmarchicus* tyrosine hydroxylase; Calfi-DDC, *Calanus finmarchicus* DOPA decarboxylase; Calfi-HDC, *Calanus finmarchicus* histidine decarboxylase; Calfi-TDC, *Calanus finmarchicus* tyrosine decarboxylase; Calfi-TβH, *Calanus finmarchicus* tyramine β-hydroxylase; Calfi-TRH, *Calanus finmarchicus* tryptophan hydroxylase.

Table 4

Expressed sequence tag (EST) support for deduced *Calanus finmarchicus* amine biosynthetic enzyme protein sequences

	Expressed sequence tags				
***Calanus* query**	**Accession No.**	**E-value**	**Protein length***	**Location on query**	**% identity to query**
Calfi-TPH	-	-	-	-	-
Calfi-TH	EH666558	8e-68	130	N	94.6
Calfi-DDC	-	-	-	-	-
Calfi-HDC	-	-	-	-	-
Calfi-TDC	FK042551	3e-158	232	I	99.1
Calfi-TβH	-	-	-	-	-
Calfi-TRH	EL585923	1e-141	200	N	98.0

Abbreviations: Calfi-TPH, *Calanus finmarchicus* tryptophan-phenylalanine hydroxylase; Calfi-TH, *Calanus finmarchicus* tyrosine hydroxylase; Calfi-DDC, *Calanus finmarchicus* DOPA decarboxylase; Calfi-HDC, *Calanus finmarchicus* histidine decarboxylase; Calfi-TDC, *Calanus finmarchicus* tyrosine decarboxylase; Calfi-TβH, *Calanus finmarchicus* tyramine p-hydroxylase; Calfi-TRH, *Calanus finmarchicus* tryptophan hydroxylase; N, amino-terminal partial protein; I, internal fragment; C, carboxy-terminal partial protein.

* In amino acids.