Mass spectrometry profiling and quantitation of changes in circulating hormones secreted over time in *Cancer borealis* hemolymph due to feeding behavior

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Abstract

The crustacean stomatogastric ganglion (STG) is a valuable model for understanding circuit dynamics in neuroscience as it contains a small number of neurons, all easily distinguishable and most of which contribute to two complementary feeding-related neural circuits. These circuits are modulated by numerous neuropeptides, with many gaining access to the STG as hemolymph-transported hormones. Previous work characterized neuropeptides in the hemolymph of the crab *Cancer borealis* but was limited by low peptide abundance in the presence of a complex biological matrix and the propensity for rapid peptide degradation. To improve their detection, a data-independent acquisition (DIA) mass spectrometry (MS) method was implemented. This approach improved the number of neuropeptides detected by approximately twofold and showed greater reproducibility between experimental and biological replicates. This method was then used to profile neuropeptides at different stages of the feeding process, including hemolymph from crabs that were unfed, or 0 min, 15 min, 1 h, and 2 h post-feeding. The results show differences both in the presence and relative abundance of neuropeptides at the various time points. Additionally, 96 putative neuropeptide sequences were identified with de novo sequencing, indicating there may be more key modulators within this system than is currently known. These results suggest that a distinct cohort of neuropeptides provides modulation to the STG at different times in the feeding process, providing groundwork for targeted follow-up electrophysiological studies to better understand the functional role of circulating hormones in the neural basis of feeding behavior.

Keywords Crustacean · Neuropeptides · Data-independent acquisition mass spectrometry (DIA MS) · Peptidomics · Peptide hormone · Hemolymph

Introduction

The decapod crustacean stomatogastric ganglion (STG), either completely isolated or when still connected to the rest of the stomatogastric nervous system, has long been used to study neural circuit dynamics [1]. This ganglion is composed of only ~25–30 neurons, depending on the species, all of which are readily accessible and easily distinguishable, enabling a fully realized connectome [2, 3]. In the crab *Cancer borealis*, 22 of the 26 STG neurons contribute to the gastric mill (chewing) and pyloric (pumping and filtering of chewed food) circuits [4]. The STG circuits are modulated by many neurotransmitters, including numerous neuropeptides, that are released from sensory neurons and descending projection neurons [3, 5]. These circuits are also influenced by neuropeptides released as circulating hormones from neurosecretory tissue [6–10]. These neurosecretory tissues include predominantly the pericardial organ (PO), located laterally on either side of the heart, and the sinus glands (SGs) found in the eyestalks [11, 12].

Molecules released from neurosecretory tissue are transported throughout the body via the hemolymph. Hemolymph, a combination of blood and lymph, acts as circulatory fluid providing oxygen and nutrients to various parts

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of the body. As decapod crustaceans have an open circulatory system, this transportation is done through a pressure differential in which the heart contracts to push hemolymph to various tissues that are bathed in the fluid [13]. The contents of hemolymph are complex, containing enzymes, proteins and protein fragments, lipids, metabolites, and neuropeptides [14]. Through the hemolymph, hormones released from the POs and SGs gain access to the STG via the dorsal ophthalmic artery, in which the STG is located, thereby providing modulation to the circuits therein.

Neuropeptides are short chains of amino acids synthesized from preprohormones that act most commonly on GTP-binding protein-coupled receptors (GPCRs) [15–19]. They are the most diverse class of signaling molecules, responsible for modulating many different functions within the nervous system. They are also present in numerous isoforms, and while these isoforms sometimes differ by only a single amino acid, they can have either the same or distinct actions. Furthermore, neuropeptides are present in low abundances in vivo and are prone to rapid degradation by extracellular peptidases, making their study challenging [19]. As a result, the role(s) played by most peptides in modulating biological activity remains to be determined.

Many recent developments have improved our ability to study neuropeptides. While targeted assays such as histology, nuclear magnetic resonance (NMR), and antibody assays can provide substantial detail about individual neuropeptides, mass spectrometry (MS) has been particularly valuable for large-scale profiling of neuropeptides in biological samples. It is advantageous for untargeted, discovery-based experiments, as not only can it identify large numbers of neuropeptides in a single analysis, but it does not require prior knowledge about neuropeptide identities. This is particularly advantageous for model organisms without a completely sequenced genome, as is the case with *Cancer borealis* [20]. With the use of sophisticated de novo sequencing software, information about the sequence of neuropeptides can be obtained from MS analyses, facilitating the discovery of neuropeptides not previously identified or characterized within these organisms.

Previous studies have investigated the role of neuropeptides in *C. borealis* tissue and hemolymph due to various perturbations, such as stress caused by alterations in salinity [21], hypoxia [22], temperature [23], and pH [24], leading to information regarding how neuropeptides are involved in adaptations of the animal. There have also been studies investigating the role of neuropeptides in feeding, focusing on the tissue involved in this process [12, 25, 26]. However, there has been relatively little research done into the contents of hemolymph that influence feeding-related activity [27]. Moreover, these previous studies have mostly been limited to two discrete conditions (e.g., unfed and fed). However, feeding is a dynamic and cyclic process. Rather than simply involving a before and after state, there are numerous stages that a crab

undergoes throughout the process of food intake [28]. Therefore, investigating multiple time points during the feeding process would provide a more complete understanding of how circulating molecules influence the feeding-related circuits in the STG.

Profiling neuropeptides in hemolymph is particularly challenging. Compared to tissue, hemolymph neuropeptides occur in even lower concentrations in vivo, often ~1 to 100 pM, and are in the presence of numerous interfering artifacts such as high salt, lipids, enzymes, and protein fragments [14]. Rapid and sophisticated sample preparation is required prior to MS analysis, as hemolymph clots substantially after withdrawal from the animal without proper treatment [29]. Furthermore, with the complexity of hemolymph samples, sufficiently sensitive MS acquisition methods are necessary to detect neuropeptides in the presence of other interfering biological species.

To address these challenges and better understand the influence of circulating neuropeptide hormones on the STG, this study uses a label-free data-independent acquisition (DIA) MS approach to profile and quantify relative changes in abundance between neuropeptides in hemolymph at five time points over the course of feeding: before feeding and 0 min, 15 min, 1 h, and 2 h post-feeding. Additionally, de novo sequencing is employed to identify putative novel neuropeptides that may also be implicated in some aspect(s) of the feeding process.

Materials and methods

Chemicals and materials

ACS-grade formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Amicon Ultra 10 kDa 0.5 mL molecular weight cutoff (MWCO) devices and 10 μL C18 ziptips were purchased from Merck Millipore (Billerica, MA, USA). Acidified methanol was prepared using 90/9/1 water/methanol/acetic acid. ACS-grade solvents were used for sample preparation, and Optima-grade solvents were used for MS analysis.

Animals and feeding experiments

No institutional approval is required for working with invertebrates, and all experiments were performed under national or local guidelines and regulations. Male Jonah crabs, *Cancer borealis*, were purchased from the Fresh Lobster Company, LLC (Gloucester, MA, USA) and housed in artificial seawater tanks maintained at 12–13 °C with an alternating 12-h light/dark cycle. Animals were allowed to adjust to their environment for 2 weeks without food prior to feeding experiments. For feeding experiments, crabs were given 4 g of thawed

tilapia. Time after feeding was counted from when the crab finished eating, which typically took 10 to 15 min. The following time points were analyzed: unfed (crab was not given tilapia), and 0 min, 15 min, 1 h, and 2 h post-feeding. After the appropriate elapsed duration for each time point, crabs were cold-anesthetized in packed ice for 10 min. A 1-mL plastic syringe with a 23-gauge needle was used to withdraw 1 mL of hemolymph from the open chamber near the heart via the soft leg joint of the first pereiopod (i.e., leg closest to the claw). Hemolymph was immediately added to an equal volume of chilled acidified methanol containing 1×10^{-9} M bradykinin as an internal standard. A total of 6 biological replicates from distinct animals were performed. All hemolymph was withdrawn from the crabs at approximately the same time of day (9:00 am–11:00 am local time).

Sample preparation

Neuropeptide extraction from hemolymph was performed immediately after hemolymph withdrawal from animals. Samples were vortexed, bath-sonicated for 5 min, and centrifuged at 16.1 relative centrifugal force (rcf) for 5 min. The supernatant from each sample was collected, and 0.5 mL of acidified methanol was added to the pellet. The pellet was then manually homogenized and again vortexed, sonicated, and centrifuged. The resulting supernatant was collected, and the process was repeated for a total of 3 extractions. The combined supernatant was evaporated in a speedvac on medium heat and reconstituted in 1 mL 70/30 water/methanol.

Each sample was then separated into 2 aliquots of 0.5 mL and subjected to molecular weight cutoff (MWCO). For MWCO of hemolymph, the device was rinsed with 0.2 mL of 0.1 M NaOH by centrifuging at 14 rcf for 4 min. The device was then rinsed with 0.5 mL of 50/50 methanol/water for 8 min at 14 rcf. The hemolymph sample was then loaded into the device and centrifuged at 14 rcf until most of the liquid passed through the filter. The device was then rinsed with 0.1 mL 70/30 water/methanol for 20 min at 14 rcf, and the flow-through from the sample and rinse were combined and evaporated in the speedvac on medium heat.

Samples were reconstituted in 45 μL 0.1% FA and divided into 3 aliquots of 15 μL. Each aliquot was desalted with C18 ziptips following package instructions. The eluted samples were then evaporated in the speedvac on medium heat and stored at −80 °C until MS analysis.

Mass spectrometry analysis

Samples were constituted in 12 μL of 0.1% FA and 2 μL per technical replicate was loaded onto a 14-cm self-packed C18 column (75 μm inner diameter, 1.7 μm particle size) for LC separation on a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA) with a flow rate of 0.300 μL/min. Water with 0.1% FA (A) and acetonitrile with 0.1% FA (B) were used as mobile phases, and the following gradient was used for separation: 0–1 min 3–10% B; 1–90 min 10–35% B; 90–92 min 35–95% B; 92–102 min 95% B; 102–105 min 95–3% B; 105–120 min 3% B. The LC system was coupled online to a Thermo Scientific Q Exactive instrument (Thermo Scientific, Bremen, Germany) with a nano-ESI source. MS spectra were acquired with positive ESI, 30 eV collision energy, 70,000 resolution for MS scans, and 17,500 resolution for MS/MS scans. Twenty MS/MS spectra were acquired from 400 to 800 m/z per cycle with a DIA method using 20 m/z isolation width, as optimized previously [30].

Data analysis

Raw MS files were converted to the vendor-neutral mzXML format using MSConvert [31] and processed with DIA-Umpire [32] using the default parameters. Output files from DIA-Umpire were converted from mgf to mzXML using MSConvert and processed in PEAKS 7.0 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) with a list of 915 known crustacean neuropeptides used as the database (available at the following link: https://www.lilabs.org/resources) [33]. The following parameters were specified for PEAKS de novo and database searches: no enzyme cleavage specified, instrument orbi-orbi, HCD fragmentation, and precursor correction enabled. The following modifications were included in the search parameters: amidation, pyroglutamate, dehydration, and oxidation. All other parameters were set to the default. Search results were exported from PEAKS for subsequent MS1 quantitation with Skyline with Orbitrap settings, unspecific enzymatic cleavage, and all other parameters set to default. Statistical analysis was performed with Perseus, including log2(x) transformation, imputation from normal distribution, one-way ANOVA, and Tukey HSD post hoc test. For identification of putative novel neuropeptides, de novo sequences from PEAKS were filtered for an average local confidence (ALC) score threshold of 75% or greater and were manually searched for characteristic neuropeptide sequence motifs [11].

Results and discussion

The STG circuits are active throughout the feeding process and are modulated by both locally released neuropeptides and circulating peptide hormones [1, 34]. This research implemented a DIA MS method to detect and relatively quantify neuropeptides in hemolymph at several different feeding-related time points.

Assessment of DIA method

Neuropeptide studies are uniquely challenging due to their low abundance in vivo, rapid degradation, multiple family members, and high variability. These challenges are exacerbated with hemolymph samples because neuropeptides are present at even lower in vivo concentrations in the presence of numerous interfering artifacts (e.g., proteins, lipids, and enzymes). As a result, previous studies detecting neuropeptides in hemolymph have had limited success. In order to improve coverage and reproducibility of neuropeptides in these samples, a DIA method that had previously been optimized for neuropeptides in tissue samples was applied to the hemolymph samples [30]. Because this was the first time using this method for crustacean fluid samples, a comparison was performed between the DIA method and the typical data-dependent acquisition (DDA) method normally used for the analysis of these samples.

For the comparison, 6 mL of hemolymph was pooled from 6 crabs and aliquoted into 6 separate samples for LC-MS/MS analysis. Of these aliquots, 3 were analyzed with a conventional top-10 DDA method as is normally done and 3 were analyzed with a DIA method. When the methods were compared, the DIA method enabled detection of more than twice as many neuropeptides (DDA: ~44 peptides; DIA: ~97 peptides; Fig. 1A). Furthermore, nearly all the neuropeptides detected with DDA (~86%) were also detected with DIA, confirming that the DIA method did not miss a substantial number of the DDA-detected neuropeptides (Fig. 1A). This result demonstrates that DIA is a suitable replacement for DDA in analyzing hemolymph samples and shows promise at providing a more complete analysis of the neuropeptides present.

In addition to detecting more neuropeptides, the DIA method provided improved reproducibility in neuropeptide detection. Reproducibility is a challenge with hemolymph analysis, possibly caused by the contents continuously circulating throughout the animal, leading to fluctuation in relative peptide abundances, particularly in relation to other molecules in the sample, as well as more complex composition. When detection relies on relative abundance of an ion, as is the case with DDA, these variations have a considerable impact on what is detectable in each sample [35]. With DIA, the same ions are fragmented in each run regardless of abundance [35]. As a result, DIA offers better reproducibility. Figure 1B shows the overlap in detected neuropeptides with DDA and DIA between experimental replicates that originate from the same hemolymph sample. Figure 1C shows the overlap in detected neuropeptides in biological replicates, originating from hemolymph samples from different individuals. As can be seen in both cases, the percentage of neuropeptides detected consistently in all 3 replicates is greater in the DIA samples than in the DDA samples (Fig. 1B: DIA, 52%; DDA, 16%; Fig. 1C:

DIA, 46%; DDA, 9%), demonstrating the improved robustness and reliability of the DIA method. Based on these comparisons, it was determined that the DIA method was more suitable for hemolymph analysis and was used below to analyze feeding-induced changes.
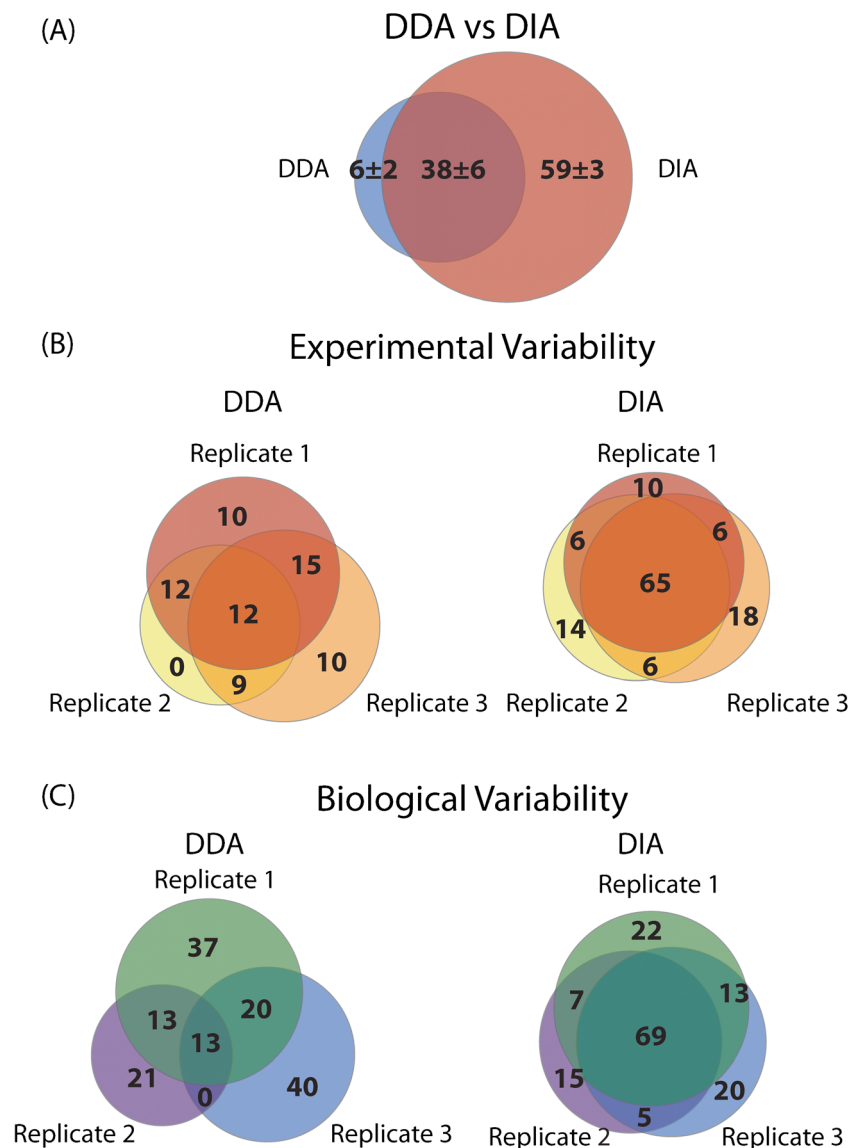
Changes in relative abundance of neuropeptides in hemolymph throughout the feeding process

Neuropeptide contents were profiled at each time point over the course of feeding from 0 min to 2 h post-feeding, as well as before feeding (unfed). While there were many neuropeptides whose presence was limited to particular post-feeding time points, there were other neuropeptides that were present at all 5 time points but with varied abundance (Fig. 2). For example, the A-type allatostatin YSKFNFGLamide (m/z 974.509) exhibited a statistically significant decrease 15 min, 1 h, and 2 h post-feeding compared to 0 min after feeding (Fig. 2A). The B-type allatostatin TNWNKFQGSWamide (m/z 1266.600) was comparably present from the unfed to the 1-h post-fed time point, but exhibited a significant increase at the 2-h post-feeding time point relative to the unfed condition (Fig. 2B), suggesting either a sudden increased rate of release or reduction in enzymatic cleavage. There was a similar increased abundance of the CPRP peptide EKLLSSISPSSTPLGFLSQDHSVN (m/z 2543.230) from 1 h to 2 h post-feeding (Fig. 2C). The Orcokinin NFDEIDRSGSFGFV (m/z 1502.691: Fig. 2D) and the RFamide YSQVSRPRFamide (m/z 1138.612: Fig. 2E) both decreased in abundance from earlier to later time points, while a HIGSLYRamide precursor-related peptide, HFGSLLKSPSYRAISIPamide (m/z 1872.050), increased 1 h post-feeding compared to unfed (Fig. 2F). The function of these peptides is not known, but the time point-dependent changes in abundance suggest that they play a feeding-related role. Additionally, 2 cryptocyanin peptides showed changes, with one of them (KIFEPLRDKN, m/z 1259.711) showing decreased abundance from 15 min to 1 h post-feeding and the other (KIFEPLVA, m/z 916.550) showing an increase at 2 h post-feeding (Fig. 2G, H). These latter two peptides are considered to be degradation products, but their feeding-induced changes may be indicative of a functional role of either the product peptide or the parent peptide that shows the same time-dependent abundance changes [36].

Changes in the presence of neuropeptides in hemolymph at various stages of feeding

In addition to quantitative changes in abundance, the presence of neuropeptides at different time points may also be indicative of a functional role. A total of 217 neuropeptides were detected in at least half of the biological replicates (n = 3/6) at one or more time points, a substantial improvement over the

Fig. 1 Comparison of data-dependent acquisition (DDA) and data-independent acquisition (DIA) methods for detecting neuropeptides in *C. borealis* hemolymph, including (A) comparison of number of detected neuropeptides (± standard deviation) with each method (n = 3), (B) comparison of overlap of detected neuropeptides between experimental replicates of identical hemolymph samples (n = 3), and (C) comparison of overlap of detected neuropeptides between biological replicates originating from distinct animals (n = 3)

(A) DDA vs DIA

DDA 6±2 38±6 59±3 DIA

(B) Experimental Variability

DDA / DIA

(C) Biological Variability

DDA / DIA

previous profiling study [36]. Of the detected neuropeptides, 120 were identified at all five time points. These neuropeptides are similar to ones previously identified in hemolymph or neurosecretory tissue [26, 36]. The families of neuropeptides detected in all time points are shown in Fig. 3B, which indicates the family and corresponding number of isoforms detected. A large portion of these have been previously characterized as hormones, such as diuretic hormones (DH), pigment-dispersing hormones (PDH), and crustacean hyperglycemic hormone (CHH) fragments and its propeptide precursor-related peptides (CPRP). DH peptides are extensively characterized in insects, including in insect hemolymph. DH homologs present in crustaceans, such as the isoforms identified here, are present in neuroendocrine and cardiac tissue and influence the lobster *Homarus americanus* cardiac neuromuscular system [37–39]. PDH peptides are also present in numerous isoforms, many of them acting as

circulating hormones in many species, so it is unsurprising that they are well-represented in the *C. borealis* hemolymph. However, the previously studied PDH peptide was not reported to be biologically active within the *C. borealis* STG [40], but PDH peptides have a well-defined role in circadian rhythms in insects [41, 42]. CHH corresponds to a superfamily of large peptides with a variety of functions, such as carbohydrate metabolism and hydration [43]. However, as the CHH peptides identified in this study are all fragments of intact CHH isoforms, it cannot be determined if intact CHH is present and biologically active in hemolymph or if the detected peptides are degradation products. While CPRPs are derived from the same preprohormone as CHH, nothing is currently known about their function, though they are known to be present in hemolymph [11, 44].

Many of the neuropeptides present at all time points are commonly identified in the pericardial organs, such as those
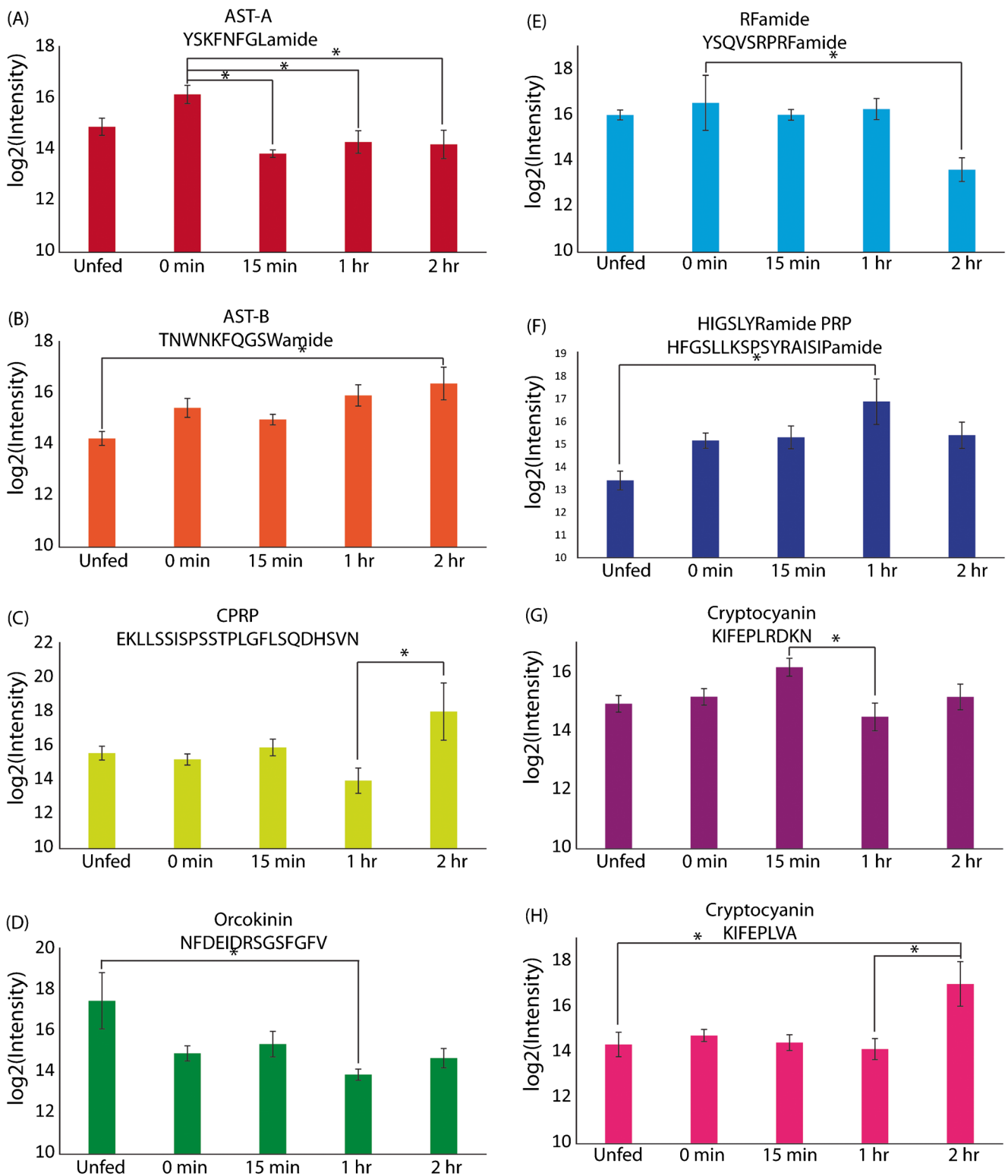
Fig. 2 Relative changes in abundance of neuropeptides across all 5 time points for 8 different neuropeptides, * indicates statistical significance (p-value < 0.05 based on one-way ANOVA followed by Tukey HSD post hoc test), error bars indicate standard error of the mean (SEM) (n = 6)

belonging to the allatostatin, RFamide, and pyrokinin families [12]. This finding is to be expected, as hemolymph was sampled from the open chamber near the heart, into which the

neuron terminals in the pericardial organs release their neuropeptides. Other notable neuropeptide families identified across all time points include those containing

Family Distribution of Neuropeptides Detected in Hemolymph
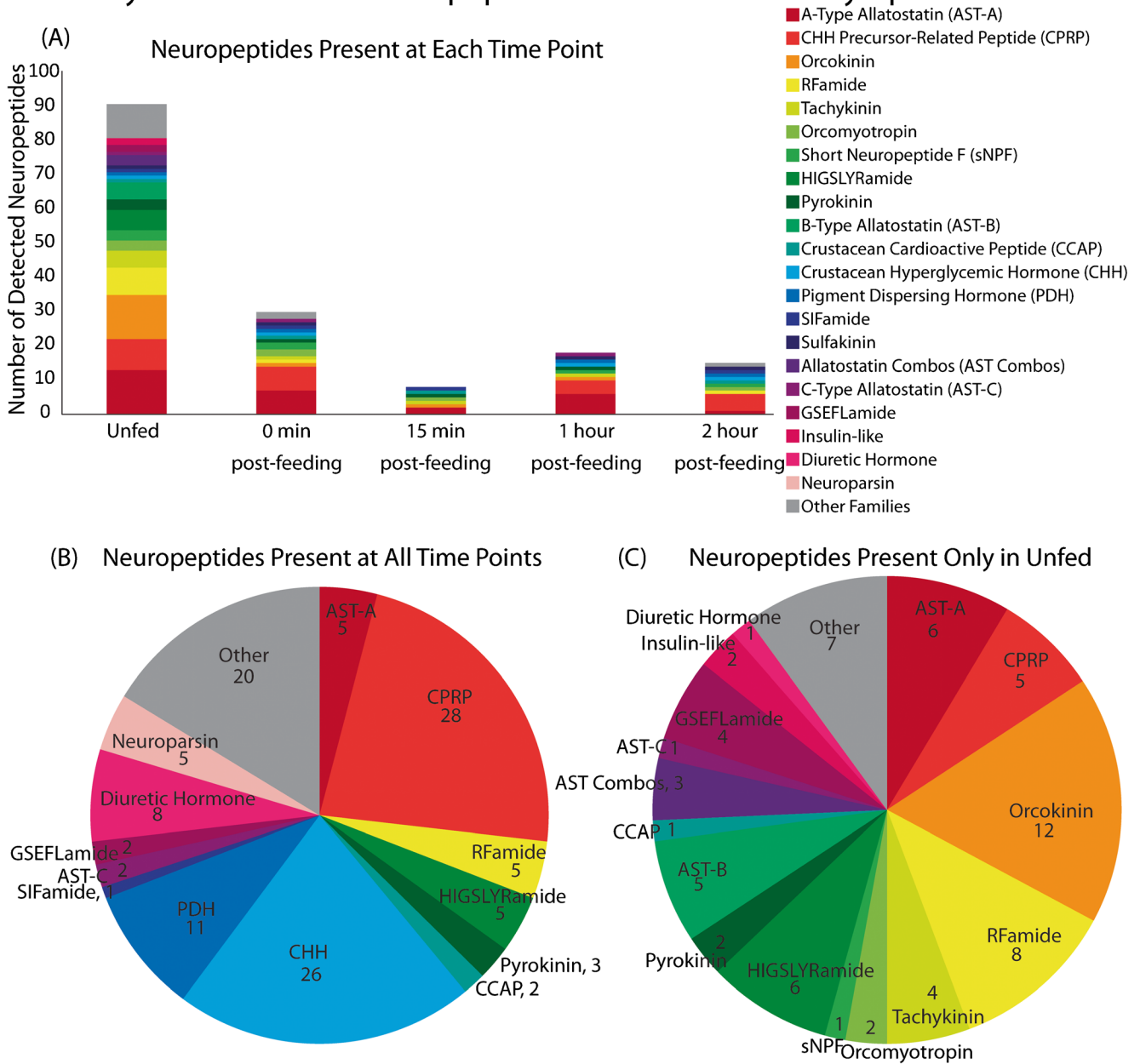
Fig. 3 Distribution of neuropeptide families detected in each sample of hemolymph, including (A) distribution of families detected at each time point, excluding neuropeptides detected at all time points; (B) family distribution of neuropeptides detected in all time points; and (C) family distribution of neuropeptides detected only in unfed hemolymph (n = 6). Abbreviations are defined in the top right corner. Numbers indicate the number of family members.

HIGSLYRamide and Neuroparsin, whose functions are not currently known, and peptides related to those present in the propeptide precursor for crustacean cardioactive peptide (CCAP), which has well-established modulatory actions both in the heart and in the STG (Fig. 3A, B) [8, 9, 45].

The remaining neuropeptides that were detected varied between time points. Figure 3A shows the number of neuropeptides per peptide family detected at the various time points, with those detected in all time points excluded. Interestingly, samples from unfed crabs had by far the largest number of

detected neuropeptides (~90 neuropeptides in unfed compared to less than 40 in the other time points). Many of the neuropeptides unique to the unfed time point are A-, B-, or C-type allatostatin peptides. In previous electrophysiological studies, neuropeptides from these families slowed/inhibited the pyloric rhythm [46–49], and so their absence at the onset of feeding may be related to this function. The other families prominent in unfed hemolymph include the orcokinin, tachykinin, and pyrokinin families, which may seem surprising because they all contain isoforms that modulate the feeding-related

STG circuits [50–52]. However, these isoforms may well continue to influence the STG circuits in the unfed condition because the pyloric rhythm is active nearly continuously, while the gastric mill rhythm is active episodically even when feeding has not recently occurred [28, 53, 54].

There were also neuropeptides that appeared exclusively at other notable time points. For example, the orcomyotropin peptide FPAFTTGFGSH (m/z 1168.542) was only present at the time point 0 min after feeding, suggesting that a short-lasting release of this peptide is triggered by the act of consumption. At least one orcomyotropin peptide family member has an excitatory role in modulating hindgut contractions [55]. A C-type allatostatin precursor-related peptide (MFAPLSGLPGNLRTI, m/z 1586.873) was detected only 0 min and 1 h after feeding, and a RFamide peptide (HDLVQVFLRFamide, m/z 1271.64) was detected at 0 min and 2 h after feeding. The presence of these neuropeptides only at certain time points suggests that they have feeding stage-specific roles in the feeding process. A complete list of neuropeptides and the time points at which they were detected is shown in Supplementary Information (ESM) Table S1, with their relative abundance values and statistical information shown in Table S3 (see ESM).

Discovery of putative novel neuropeptides

In addition to identifying many previously reported neuropeptides in hemolymph, the enhanced detection capabilities of the DIA method enabled the discovery of putative novel neuropeptides. A total of 96 putative sequences were identified with de novo sequencing that have not previously been reported in the literature but possess characteristic sequence motifs of common neuropeptide families. Some of the common families include three types of allatostatin, A-type (motif: -YXFGLamide), B-type (motif: -WX_6Wamide), and C-type (motif: -PISCF), as well as pyrokinin (motif: -FXPRLamide), RFamide (motif: -RFamide), RYamide, and tachykinin (motif: -FLGMRamide) [11]. A summary of all the families is shown in Table 1, along with how many sequences were identified in samples from each time point. A complete list of all sequences is shown in ESM Table S2, and MS/MS spectra of each sequence are shown in Figs. S1–S96 (see ESM). Interestingly, in contrast to the above-reported finding for previously identified peptides (Fig. 3), more novel sequences were identified in samples from post-feeding time points than in the unfed sample. This finding is likely a consequence of previous studies focusing on the hemolymph of either control crabs or crabs exposed to other perturbations, such as pH stress [24]. These peptides may only be released into the hemolymph after feeding, indicating potential feeding-related functions. Most of these peptides correspond to the RFamide and RYamide families, which are diverse families that already contain many known isoforms [11].

Table 1 Number of putative novel neuropeptide sequences belonging to each family identified and the time point at which they were identified. As some sequences were detected at multiple time points, the final column indicates the total number of putative novel neuropeptides detected from each family

Family	Post-feeding time point					Total
	Unfed	0 min	15 min	1 h	2 h	
A-type Allatostatin	1	2	5	6	3	15
B-type Allatostatin	0	2	0	1	2	5
C-type Allatostatin	7	8	3	2	7	16
Cryptocyanin	0	0	0	3	2	5
Insect Kinin	1	0	0	1	2	3
Pyrokinin	0	0	0	1	0	1
RFamide	3	5	5	5	8	21
RYamide	7	13	6	5	2	30
SIFamide	1	0	2	2	0	4
Tachykinin	0	0	1	0	0	1

There were also many putative new neuropeptides corresponding to novel A- and C-type allatostatins. The sequences of all these newly identified sequences will need to be confirmed with follow-up analyses and their biological activity tested for specific functions before any conclusive statements can be made. However, the large number of neuropeptides that were able to be identified demonstrates both the capabilities of this method and the large degree of complexity of the neuropeptidomic profile of crustacean circulating fluid.

Conclusion

This study examined the neuropeptide content of hemolymph and how it changes over the course of the feeding process in *C. borealis*, including changes in the presence and relative abundance of neuropeptides. By implementing a DIA MS analysis, a greater number of neuropeptides were able to be reproducibly detected, compared to a conventional DDA MS analysis. With the improved method, a total of 217 neuropeptides were detected in hemolymph samples, a larger number than had previously been achieved in a single study. Of these, 120 were consistently detected across all 5 time points. The rest were present only at certain time points, suggesting a time point-specific action on the feeding process. Of the 120 neuropeptides detected across all 5 time points, 8 showed relative changes in abundance that were statistically significant at specific time points. Collectively, these results suggest that a cohort of neuropeptide hormones influence the feeding process and may have a functional role on the STG circuits. Additionally, the DIA analysis with de novo sequencing revealed 96 putative neuropeptides, at least some of which may

also influence the STG. Follow-up studies confirming the sequence of these neuropeptides and determining their electrophysiological actions can be performed to enable a more detailed understanding of the functional role of these neuropeptides. Overall, this study identifies neuropeptides that may be implicated in hormonal modulation of the STG circuits and uncovers more of the complexity of the crustacean neuropeptidome.

Declarations

No institutional approval is required for working with invertebrates, and all experiments were performed under national or local guidelines and regulations.

Conflict of interest The authors declare no competing interests.

Code availability In-house crustacean neuropeptide database is publicly available at the following link: https://www.lilabs.org/resources

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