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In silico identification of neuropeptide genes encoded by the genome of *Crassostrea virginica* with a special emphasis on feeding-related genes

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ABSTRACT

Suspension-feeding bivalves, including the oyster *Crassostrea virginica*, use mucosal lectins to capture food particles. For instance, oysters can increase the transcription of these molecules to enhance food uptake. However, the regulatory processes influencing food uptake remain unclear although likely involve neuropeptides. Information on the neuropeptidome of *C. virginica* is limited, hindering the comprehension of its physiology, including energy homeostasis. This study explored the genome of *C. virginica* to identify neuropeptide precursors in silico and compared these with orthologs from other mollusks. A special focus was given to genes with potential implication in feeding processes. qPCR was used to determine the main organs of transcription of feeding-related genes. To further probe the function of target neuropeptides, visceral ganglia extracts and synthetic NPF were injected into oysters to evaluate their impact on genes associated with feeding and energy homeostasis. A total of eighty-five neuropeptides genes were identified in *C. virginica* genome. About 50 % of these are suggested to play a role in feeding processes. qPCR analyses showed that visceral ganglia and digestive system are the main organs for the synthesis of feeding-related neuropeptides. Further, results showed that the transcription of several neuropeptide genes in the visceral ganglia, including NPF and insulin-like peptide, increased after starvation. Finally, the injection of visceral ganglia extracts and synthetic NPF increased the transcription of a mucosal lectin and a glycogen synthase, known to be involved in food capture and glucose storage. Overall, this study identifies key genes regulating oyster physiology, enhancing the understanding of the control of basic physiological mechanisms in *C. virginica*.

1. Introduction

Within coastal and estuarine ecosystems of the east cost of the United States, the eastern oyster *Crassostrea virginica* is important both for its environmental impact and socio-economical value. Shellfish, and most specifically oysters, represent a major part of marine resources in the US. In 2021, the eastern oyster landings in the US amounted to over $190 million representing more than 8700 tons (NOAA, 2020). Known as ecosystem engineers, oysters provide crucial ecosystem services including enhancing water quality via the control of phytoplankton populations, controlling nutrient cycling, and providing critical habitat (Grabowski and Peterson, 2007).

Despite the large number of studies on *C. virginica* and most specifically on their physiology (e.g., reproduction, immunity, feeding process), the nervous system of this species and its neuropeptidome, have received little attention as compared to other molluscan species (Galtsoff, 1964). This may be due to the difficulty in studying target tissues, as oyster visceral and cerebral ganglia are less accessible than those of their gastropod congeners (Galtsoff, 1964), and their neural cells are also much smaller than the ones from *Aplysia* sp. for example, which are known to exceed 1 mm in length (Moroz and Kohn, 2010). The lack of information on neuropeptides in *C. virginica*, and in bivalves in general, dramatically limits the comprehension of physiological processes and most particularly their regulation.

One of the most critical physiological functions in animals is the feeding process since it provides the organism with energy required for all other activities. Bivalves are suspension-feeders that use their ciliated gills to filter water and capture particles. Food particles are then transported toward the mouth to be ingested while undesired particles are rejected in the form of pseudofeces. As a matter of fact, through their

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suspension-feeding process, these organisms are confronted with a wide range of suspended material, including a usually high proportion of non-nutritive material (e.g., silt) and debris, and live plankton of varying nature, sizes and quality. Consequently, and instead of bulk ingestion of suspended material that can quickly saturate their digestive system, suspension feeding bivalves have developed strategies to enhance the nutritive value of ingested material and optimize energy gain (Pales Espinosa and Allam, 2013). This elaborate ability to select food particles represents an adaptation to various constraints faced by bivalves including a relatively dilute diet with a large proportion of non-nutritive particles that can easily saturate mucociliary transport of food particles, and a discontinuous and energetically-costly intracellular digestion. Therefore, particle selection probably evolved as a means to supply the digestive diverticula (blind digestive sacs), and their associated phagocytic cells, with the most nutritive material for intracellular digestion.

Among several mechanisms advanced over the last century to explain the ability for bivalve to sort particles, recent studies demonstrated that biochemical cues play a primary role in mediating this selection process (Beninger and Decottignies, 2005), providing a novel conceptual framework for unraveling the mechanisms used by suspension feeding bivalves to optimize foraging. Thus, particle sorting in bivalves (including among species with contrasting architectures of the feeding organs) was shown to be mediated by specific interactions (i.e., carbohydrate-protein interactions) between the surfaces of food particles and the mucus covering the feeding organs, triggering the assignment of individual particles to acceptance or rejection tracts. More specifically, mucus covering the feeding organs of the eastern oyster *C. virginica* was shown to contain lectins (a group of sugar-binding proteins that specifically and reversibly bind to carbohydrates including those covering living cells) that differentially bind microalga cell surface carbohydrates, triggering selection (Pales Espinosa and Allam, 2018).

Previous modeling work by Willows (Willows, 1992) showed that bivalves can modulate food particle sorting and/or enhance their filtration rates in order to improve net energy gain and maintain a constant metabolic digestive investment. This interpretation was comforted by experimental findings obtained by Bayne and Svensson (Bayne and Svensson, 2006) that showed changes in pre-ingestive selection in the Sydney rock oyster *Saccostrea glomerata* in order to fulfil the animal's nutritional demands. Similar results were obtained in the blue mussel (*Mytilus edulis*) where significant regulation of pre-ingestive selection throughout an annual cycle has been reported, in conjunction with changes in energetic demands (Pales Espinosa and Allam, 2013). Increases in pre-ingestive selection were positively correlated with increases in the transcription of mucosal lectins in the feeding organs. Furthermore, particle capture, selection efficiency and transcription of lectins in the feeding organs of mussels increased during the spawning and post spawning periods when animals display the lowest levels of storage tissue and energy reserve (Berthelin et al., 2000). Finally, the transcription levels of mucosal lectins in mussels (MeML or *M. edulis* mucosal lectin, Pales Espinosa et al., 2010) and oysters (*C. virginica* mucosal lectins including CvML3914) increased following starvation, suggesting that both bivalves regulate these molecules to enhance food uptake (Jing et al., 2011; Pales Espinosa et al., 2010). While evidence suggests that sorting by suspension feeding bivalves is modulated via the regulation of the transcription of mucosal lectins, the upstream control of food uptake itself is very likely regulated by neurohormonal processes that maintain energetic homeostasis, as shown in all invertebrates investigated so far, including mollusks (Wang et al., 2017).

In mollusks, glycogen is the main form of glucose storage and plays a key role as energy resource for maintenance and gametogenesis (Berthelin et al., 2000). Glycogen storage and utilization mechanisms have been found to be under the control of diverse factors, including neuropeptides (Bigot, 2012; Favrel and Mathieu, 1996). Among these molecules, the neuropeptide F (NPF) plays a crucial role in regulating physiological processes, such as feeding behavior and metabolism (Fadda et al., 2019; Wang et al., 2017). It functions similarly to its homolog, neuropeptide Y (NPY), in vertebrates, influencing energy balance by, for example, regulating glucose homeostasis (Fadda et al., 2019).

Nevertheless, pathways involved in the regulation of feeding mechanisms in bivalves are not understood. Specifically, the link between a "low level of fuel" in the organism and the increase in lectin production leading to the activation of particle capture is missing. The control of energy allocation is vital in marine bivalves because they are exposed to large fluctuations of environmental parameters, including food availability. The identification and, more importantly, the functional characterization of factors regulating food uptake in mollusks are crucial for a better understanding of the ecology and physiology of these organisms.

In this study, the genome of *C. virginica* was interrogated to identify putative neuropeptide genes. A special focus was given to genes with potential function in feeding processes and a subset of these genes was chosen for further experimentations. Thus, the main organs of production of these genes were determined using quantitative PCR. Further, synthetic neuropeptide (NPF) and visceral ganglia extracts were injected into oysters to evaluate their effect on the regulation of genes involved in feeding processes and energy homeostasis.

2. Materials and methods

2.1. Identification of neuropeptide genes encoded by the genome of Crassostrea virginica

Neuropeptide sequences described in other mollusk species (In et al., 2016; Stewart et al., 2014; Veenstra, 2010; Zatylny-Gaudin et al., 2016; Zhang et al., 2018) were catalogued and used to query the translated protein database obtained from the *C. virginica* genome (GCA_002022765.4).

The cDNA and deduced amino acid sequences were analyzed by BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) and the presence of signal peptides and the location of their cleavage site, were predicted using the SignalP 5.0 Server (https://services.healthtech.dtu.dk/services/SignalP-5.0/). Multiple alignments were performed using Clustal omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). In addition, the same protein database was screened for the presence of repetitive GKK, GKR, GRK and GRR motifs and recurrent small peptides that could indicate putative neuropeptides (Stewart et al., 2014). The cleavage sites in precursors were also predicted using NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py). Finally, visceral ganglia transcriptomic data (Pales Espinosa et al., unpublished) were also interrogated to confirm the presence of the in silico predicted genes.

To compare the neuropeptide genes (NPG) found in *C. virginica* with the ones existing in related species, we used a previous clustering (Farhat et al., 2022) generated with OrthoFinder (Emms and Kelly, 2019) on nineteen bivalvia proteomes including two strains of *Mercenaria mercenaria* (GCA_021730395.1 and GCA_014805675.1), *Ruditapes philippinarum* (GCA_014805675.1), *Cyclina sinensis* (GCA_012932295.1), *Lutraria rhynchaena* (GCA_008271625.1), *Archivesica marissinica* (GCA_014843695.1), *Dreissena rostriformis* (GCA_007657795.1), *Sinonovacula constricta* (GCA_007657795.1), *Anadara broughtonii* (no accession number), *Crassostrea gigas* (GCA_902806645.1), *Crassostrea virginica* (GCA_002022765.4), *Ostrea edulis* (GCA_947568905.1), *Saccostrea glomerata* (GCA_003671525.1), *Mizuhopecten yessoensis* (GCA_002113885.2), *Pecten maximus* (GCA_902652985.1), *Modiolus philippinarum* (GCA_002080005.1), *Limnoperna fortunei* (GCA_003130415.1), *Mytilus coruscus* (GCA_011752425.2) and *Mytilus galloprovincialis* (GCA_900618805.1) with the addition of an outgroup species from the Gastrodopa class, *Aplysia californica* (GCA_000002075.2). From this clustering we selected all orthogroups having at least one sequence described as neuropeptide in the literature

(In et al., 2016; Stewart et al., 2014; Zhang et al., 2018). Identified neuropeptides were characterized (sequence information) and a select number of these was used to assess their regulation in response to starvation.

2.2. Animals

Adult oysters (86 ± 12 mm in length, mean ± standard deviation), *C. virginica*, were obtained from a commercial source (Islip, NY) in April 2021. Their external shell surface was scrubbed to remove mud and marine life. Oysters were then acclimated in the laboratory for ten days (salinity of 28, 15 °C) and fed daily (5 % dry weight) using DT's Live Marine Phytoplankton supplemented with fresh cultures of microalgae (*Tisochrysis lutea*, *Tetraselmis chui*, and *Chaetoceros muelleri)* grown in f/2 media at 15 °C under a 14-h light:10-h dark photoperiod (Pales Espinosa and Allam, 2013).

2.3. Starvation experiment

Before the starvation experiment (T0), a subsample of 12 oysters were dissected. Shells were notched ventro-posteriorly and hemolymph (~5 ml) was collected from the adductor muscle using a 26-gauge needle. Hemolymph was then individually placed in ice-cold 15 ml tubes and centrifuged (800 x*g* for 15 min at 4 °C) to pellet hemocytes. Further, visceral ganglia, gills, labial palps, mantle, digestive gland, and esophagus were dissected and separately collected. All samples were immediately flash frozen and maintained at −80 until RNA extraction. Additional oysters ($n = 30$) were submitted to two different treatments (i.e., fed and unfed). For each treatment, 15 oysters were equally divided into three aquariums (triplicates). The first group (fed) received the same food as described for the acclimation period while the 2nd group did not receive any food. After seven days (T7), oysters ($n = 12$) were collected from each treatment and dissected as described for T0.

2.4. NPF and visceral ganglia extract injection experiment

Oysters ($n = 20$) were starved for three days to stimulate (based on preliminary experiments) the production of putative feeding-related neuropeptides. After three days, mantle (negative control) and visceral ganglia (positive control) from the twenty oysters were dissected, pooled according to their nature, and maintained on ice for the entire procedure. The two group of tissues (~100 mg) were then separately crushed with 1 ml of sterile artificial seawater (ASW) and centrifuged (3000 x*g*, 30 min, 4 °C). Supernatants were collected and filter sterilized (0.22 μm syringe filters). A 25 μl aliquot of each extract was used to determine protein concentrations with a Pierce BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) as per manufacturer's recommendations. Extracts were then diluted with sterile ASW to a protein concentration of 0.5 mg/ml and maintained at −80 °C until use, typically within 24 h.

Another batch of oysters ($n = 48$) was then randomly subdivided into four different groups (12 oysters per group) and placed in four aquariums per group (3 oysters per tank) and fed as described for the acclimation (Section 2.2.). Oysters from the first group were injected with 100 μl of ASW while those from the second group were injected with 100 μl of mantle extract. Both group 1 and 2 served as controls. Oysters from the 3rd and 4th groups were injected with 100 μl of either visceral ganglia extract or synthetic NPF (XP_022307882.1, NDSLLPPSRPSSFRSPGQLRQYLKALNDYYAIVGRPRF-NH2, Biomatik, Canada, 50 μg in 100·μl of sterile ASW). Each group received one injection per day for three days. After four days, labial palps (organs associated with glycogen storage and lectin transcription) were dissected and immediately flash frozen and stored at −80 °C until RNA extraction. No mortality was observed during the experiment.

2.5. Real-time PCR analysis

Nine NPG with diverse putative function, including involvement in feeding processes (Table 1), were selected for further analysis. In addition, the transcription of three lectins known to be regulated after starvation (Pales Espinosa and Allam, 2013) and the transcription of the glycogen synthase and glycogen phosphorylase, which are both known to be involved in glucose homeostasis (Bacca et al., 2005), were also studied after the injection of synthetic NPF and tissue extracts.

RNA was extracted from frozen tissues using the Monarch® Total RNA Miniprep kit (New England BioLabs, Inc.) following the manufacturer's protocol. cDNA was generated from extracted mRNA with M-MLV reverse transcriptase (Promega) and used as template with each set of primers indicated in Table 2. Relative quantification of transcripts from each target gene was carried out in 10-μl reactions with Takyon Low Rox SYBR 2× MasterMix blue dTTP (Eurogentec), 100 nM final primer concentration and 5 ng of RNA-equivalent cDNA under thermal profiles given in Table 2. The PCR reactions were performed using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Each run was followed by a melting curve program for quality control. PCR efficiency (typically from 90 to 110 %) was determined for each primer pair using the slopes of standard curves obtained from serial dilutions of cDNA. The correct amplification products were confirmed using gel electrophoresis. Transcription levels of each gene were normalized to the 18S rRNA gene (ΔCt, calculated as Ct of target gene – Ct of housekeeping gene) and relative transcript levels were calculated using the (2-ΔΔCt) method (Livak and Schmittgen, 2001). Data obtained from Real-Time PCR analysis were subjected to Student *t*-test or 1-way ANOVA followed by SNK test (SigmaStat, version 3.1). Differences were considered significant at $p < 0.05$.

3. Results and discussion

A total of eighty-five neuropeptide genes, including duplicated genes and isoforms, were identified in the genome of *C. virginica* (Table 3, Supplementary data 1 and Supplementary data 2) which corroborates previous work on mollusks (Stewart et al., 2014; Zhang et al., 2018). Although it cannot be ruled out that some of the duplicated genes may result from potential errors in genome assembly, each duplicated gene was carefully examined for its position on the genome and for its transcription, thereby supporting their legitimacy. Gene duplication in mollusks is a relatively common (Farhat et al., 2022) and important evolutionary process, although its frequency may vary between species. It contributes to genetic diversity, functional innovation, and adaptability, especially in response to environmental challenges. Consequently, gene duplications are more frequent in certain gene families, such as those involved in shell formation, immune responses, and stress tolerance (Farhat et al., 2022). The current study demonstrated that some neuropeptides also undergo gene duplication events.

Given the nature of these small molecules and the difficulty of retrieving them from in silico data, it is expected that the actual number of neuropeptides in *C. virginica* could be higher. Indeed, nineteen additional neuropeptide genes, found in several bivalves, were not found in the *C. virginica* genome (Supplementary data 3). For example, this is the case of GNamide, LRFNVamide or MIP. The absence of previously identified neuropeptides in the *C. virginica* genome could reflect a genuine lack or, alternatively, may result from bioinformatic errors. For instance, some neuropeptides may be highly specific to a particular taxonomic group (e.g., genus, order) and could be truly absent in *C. virginica*. That's the case of the cerebrin and the pleurin that do not seem to be present in any *Ostreida* genome (e.g., *C. gigas*, *O. edulis*, *S. glomerata*). Furthermore, it cannot be ruled out that the genomes of other bivalves used to catalog neuropeptides in this study may contain errors, particularly if a neuropeptide was found exclusively in one species. That's for example the case of the LRYamide that was only found in the scallop *P. yessoensis* while it was absent in other pectinid species

Table 1
Neuropeptides involved or suggested to be involved in feeding processes in invertebrates.

Generic name	Activity	Species	References
ACHATIN	Activate the feeding circuit and induce egestive activity	*A. californica* (Gastropod)	Bai et al., 2013
ALLATOSTATIN A /BUCCALIN-like	Inhibit gut motility, activate gut amylase secretion, reduce food intake	*B. germanica* (Insect)	Aguilar et al., 2003
	Stimulate the copulatory canal contractility	*C. aspersum* (Gastropod)	Stewart et al., 2016
ALLATOSTATIN B /WWamide	Reduces the pyloric muscle network frequency	*C. borealis* (crustacean)	Fu et al., 2007
ALLATOSTATIN C	Myoregulatory activity, control of coordinated movements during feeding	*Hydra* sp. (Cnidaire)	Alzugaray et al., 2016
ALLATOTROPIN	Myoregulatory activities associated with meal ingestion and digestion	*H. plagiodesmica* (Cnidaire)	Alzugaray et al., 2013
BURSICON alpha	Mediates metabolic homeostasis	*Drosophila* (Insect)	Scopelliti et al., 2019
CCK/SK	Stimulate the digestion (gut motility, enzyme and hormone secretion)	*P. maximus* (Bivalve)	Donval and Bellon, 1992, Nagabhushanam et al., 1995
	Decrease the frequency of the spontaneous contractions of hindgut	*C. gigas* (Bivalve)	Schwartz et al., 2019
FEED CIRCUIT ACTIVATING PEPTIDE (FCAP)	Induce rhythmic feeding motor programs, suggested to initiate food induced arousal	*A. californica* (Gastropod)	Sweedler et al., 2002
FMRFamide	Induce digestive enzyme secretion	*P. maximus* (Bivalve)	Favrel et al., 1994
	Induce digestive enzyme secretion	*L. stagnalis* (Gastropod)	Santama et al., 1994
	Modulate feeding motor program and induce egestive activity	*A. californica* (Gastropod)	Vilim et al., 2010
GGNamide	Induce gut and esophagus contraction	*E. foetida* (Annelid)	Oumi et al., 1995
INSULIN-like peptide - Cv-ILP	Putative role in energy storage: glycogen metabolism (ILP, QCW06538.1)	*P. fucata martensii* (Bivalve)	Zhang and He, 2020
	Putative role in digestive processes	*C. gigas* (Bivalve)	Cherif-Feildel et al., 2019
INSULIN-like peptide - Cv-ILP3	Putative role in reproduction	*C. gigas* (Bivalve)	Cherif-Feildel et al., 2019
INSULIN-like peptide - Cv-ILP4	Putative role in larval development	*C. gigas* (Bivalve)	Cherif-Feildel et al., 2019
LFRFamide	Increase the frequency, tonus and amplitude of rectal contractions	*S. officinalis* (Cephalopod)	Zatylny-Gaudin et al., 2010
	Putative role in energy resources (metabolism)	*L. stagnalis* (Gastropod)	Hoek et al., 2005
	Putative role in the coordination of nutrition, energy storage and metabolism	*C. gigas* (Bivalve)	Bigot et al., 2014
	Induced spawning but suppressed food intake	*H. discus hannai* (Gastropod)	Yoon et al., 2022
NPF/NPY	Putative regulation of energy homeostasis	*C. gigas* (Bivalve)	Bigot, 2012
	Modulate feeding motor program and induce egestive activity	*A. californica* (Gastropod)	Jing et al., 2007, Zhang et al., 2018
OPIOID-like	Putative role in the functional regulation of the digestive system	*C. farreri* (Bivalve)	Sun et al., 2018
PKYMDT/SPTR-GF	Modulate feeding motor program	*A. californica* (Gastropod)	Jing et al., 2007, Zhang et al., 2018
PEDAL PEPTIDE/ ORCOKININ-like type A, B, C	Myotropic function (stomach, gut)	*A. rubens* (Echinoderm)	Lin et al., 2018
	Myotropic function	*O. limosus* (Crustacean)	Stangier et al., 1992
sCAP	Control the muscles involved in feeding and gut motility	*M. mercenaria* (Bivalve)	Candelario-Martinez et al., 1993
TACHYKININ	Putative role in the regulation of feeding	*C. gigas* (Bivalve)	Dubos et al., 2018

(NCBI).

Alternatively, it is not impossible that the *C. virginica* genome may contain errors that prevent the identification of neuropeptides known to be present in closely related species. A striking example of this possibility is the absence of the Egg-Laying Hormone (ELH) in the *C. virginica* genome, while ELH was found in several mollusks (Veenstra, 2010; Zatylny-Gaudin et al., 2016; Zhang et al., 2018), including in members of the *Ostreidae* family (*Magallana angulata*, *Saccostrea cucullata* and *Saccostrea echinata*). ELH is a neuropeptide that has been well-studied in species like *Aplysia*, where it triggers egg release (Stuart and Strumwasser, 1980). Along with ELH, it is noteworthy that the adipokinetic hormone (AKH) is also absent from the *C. virginica* genome, despite being present in other oyster species, including *Magallana gigas* (a.k.a. *C. gigas*, Dubos et al., 2017) and *S. cucullata*. AKH is a neuropeptide that regulates energy mobilization in invertebrates, particularly during periods of increased metabolic demand. Despite these notable absences, eighty-five NPGs have been identified in silico, many of which have also been found to be transcribed.

Probing transcriptomic datasets (RNA sequencing) generated from *C. virginica* visceral ganglia confirmed the transcription of all of these genes except the bursicon alpha (LOC111102137) and the insulin-like peptides (LOC111099742, LOC111102913, LOC111130821), which may be produced in other tissues. Based on published information, these genes have been classified into two groups: one including neuropeptides demonstrated or suspected to be involved in feeding processes (e.g., myoregulation, enzymatic regulation), and the second group encompasses neuropeptides with other or unknown functions. An ortholog identification of twenty-one molluscan genomes, including *C. virginica*, was also conducted (Supplementary data 4).

Table 2
Primers and PCR conditions used in this study. The asterisk denotes primers (one NPF, one ILP) that did not produce amplicon. PCR conditions for 18S rRNA (housekeeping gene) varies according to the gene analyzed.

Gene	Forward Sequence (5′-3′)	Reverse Sequence (3′-5′)	Temperature (°C) and time (s)				Amplicon size
			Denaturation	Annealing	Extension	Cycles	
PKYMDT LOC111114656	GCCTATAGATGTCGCCAGGG	AACTTCCGGGTTGACCTTCC	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	154
NPF * LOC111113871 XP_022307875.1	GTAATGGTGATGAAAATACGAGGT	GGGAGTCATTTCCCAGGA					
NPF LOC111113871 XP_022307882.1	AATCGGAACACATGAAAGGT	ATTGGCGTAGCTGTCCTG	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	153
NPY LOC111114944 XP_022309209.1	CCCCAGCGGATTCCAAAACT	GACCTCCGACTTCACACTCA	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	130
LFRF LOC111119307 XP_022315073.1 LOC111103730 XP_022292882.1	AGAGGCGGTCAAATCCTGTG	ACAGAGCACCCCTCTTACCA	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	117
FMRF LOC111110267 XP_022302400.1	CGACTGTTACGAGTACCCCG	TGTCATCACTGTTGCGTCCA	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	146
ILP LOC111105634 XP_022295716.1	ACTATAAACATGGTCAAATTGGAC	AGACAGGATGTCCGTCAGAG	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	131
ILP * LOC111105634 XP_022295717.1	GGACGTGTTTACTGATTAGGTCA	AGACAGGATGTCCGTCAGAG					
BUR LOC111114850 XP_022309064.1	GGTGTGTCTGTCCACTGCTT	TAGAGCGATTTGGCGCGTTA	95 °C, 30s	60 °C, 30s	72 °C, 30s	35	106
GS (Glycogen synthase like) LOC111135290	GGACCGTCAGATCTATCACC	GGCCTCTACACCTGTGATTT	95 °C, 30s	53 °C, 30s	72 °C, 30s	40	97
GP (Glycogen phosphorylase) LOC111125461	GGCAGCTCCTGGCTATCA	GGTACACCACCTTCAGACG	95 °C, 30s	53 °C, 30s	72 °C, 30s	40	110
CvML LOC111101523	ATGACTACATCAAGGAGGGC	CGCAGATGTACTGTCCG	95 °C, 30s	55 °C, 30s	72 °C, 30s	35	186
CvML3914 LOC111115169	CCACATAGCAAACCTCATTAAAC	AATCTGAAGCACATGGGTC	95 °C, 15 s	62 °C, 20s	72 °C, 18 s	40	236
CvML3912 LOC111112474	GTTCCTGGCAAATTTTATGCGAA	AATGAAAGCCGCAGAATCGG	95 °C, 15 s	62 °C, 25 s	72 °C, 20s	35	349
18S rRNA	CGCCGGCGACGTATCTTTCAA	CTGATTCCCCGTTACCCGTTA					92

3.1. Neuropeptides with demonstrated or suspected feeding-related function

3.1.1. Achatin

Achatin is a tetrapeptide first identified in the snail *Achatina fulica* (GFAD, Kamatani et al., 1989) and described as a neuromodulator and neurotransmitter. In the gastropod *Aplysia californica*, achatin-like (GFFD) was shown to activate the feeding circuit and induce egestive activity (Bai et al., 2013). Achatin-like genes were also found in other mollusks, including cuttlefish (GSWN, GSWD, GFGD; Zatylny-Gaudin et al., 2016), scallops and oysters (GFWD; Stewart et al., 2014; Zhang et al., 2018). In *C. virginica*, the achatin precursor was identified as a 134-amino acid protein encoding a 24-residue signal peptide and three copies of GFWD. Looking at the orthogroups, the achatin precusor from *C. virginica* does not belong to any defined gene family, suggesting that the overall sequences could not be annotated or have diverged between different species.

3.1.2. Allatostatins

Allatostatins type A (a.k.a. Buccalin), type B, (a.k.a. WWamide), and type C (AstC) are a diverse group of neuropeptides originally characterized in arthropods and also found in mollusks (Stewart et al., 2014; Veenstra, 2010; Zhang et al., 2018). These peptides are involved in a variety of feeding-related functions in arthropods, including feeding choices (Hentze et al., 2015). In mollusks however, the function of these peptides still needs to be clarified. Orthogroups showed that two Cv_allatostatin A precursor genes (LOC111135918 and LOC111123000) encode two similar precursors (XP_022342145.1 and XP_022320818.1) that clusters with proteins from *Ostreida*. They encode a 258-amino acid protein, including a 25-residue signal peptide, ten peptides with C-terminal Lamide (ALDPYSFYGGLamide, GLDRYNFFGGLamide, FVGGLamide) and a 17-residue peptide (ANLSPEDDVDSDGNLLQ), referred to as LDA (a.k.a. love dart allohormone), thought to increase paternity success in the garden snail *Cornu aspersum* (Stewart et al., 2016).

Allatostatin B has been found to significantly increase food intake, fat storage and body weight in the insect *Drosophila* (Min et al., 2016). In the mussel *Mytilus edulis*, this neuropeptide regulates the contraction of the anterior byssus retractor muscle (Minakata et al., 1993). Cv_Allatostatin B gene (LOC111118931) encoding one precursor (XP_022314361.1) clusters with proteins from other members of the Ostreida order. Cv_Allatostatin B precursors encode a 262-amino acid peptide including a 22-residue signal peptide and eight WWamide peptides (e.g., DNNWNQFPAWamide, WSSLSAWamide, GWNALTTWamide).

Finally, Allatostatin C has been suggested to have a myoregulatory activity in cnidarian, playing a role in the control of coordinated movements during feeding (Alzugaray et al., 2016). Cv_Allatostatin C precursor gene (LOC111137637) encodes a 107-amino acid peptide (XP_022344895.1) including a 27-residue signal peptide and a predicted Allatostatin C peptide (RSHIQCLVNVIACY). Cv_Allatostatin C precursor gene was found in a same orthogroup as thirteen other molluscan proteins.

Table 3
List of Neuropeptides retrieved from the genome of *Crassostrea virginica*. Superscript numbers (1 to 9) associated with Gene ID indicate gene duplicates.

Gene ID	Generic name	Description
LOC111116753	ACHATIN	uncharacterized protein LOC111116753
LOC111135918[1]	ALLATOSTATIN A /BUCCALIN-like	Buccalin-like
LOC111123000[1]	ALLATOSTATIN A /BUCCALIN-like	buccalin-like
LOC111118931	ALLATOSTATIN B /WWamide	uncharacterized protein LOC111118931
LOC111137637	ALLATOSTATIN C	uncharacterized protein LOC111137637
LOC111124198	ALLATOTROPIN	surfeit locus protein 1-like
LOC111124199	ALLATOTROPIN	uncharacterized protein LOC111124199
LOC111126501[2]	ALLATOTROPIN	surfeit locus protein 1-like
LOC111127303[2]	ALLATOTROPIN	surfeit locus protein 1-like
LOC111133878	APGWamide	APGW-amide-related neuropeptide-like isoform X2
LOC111137306	BURSICON ALPHA1	norrin-like
LOC111102137	BURSICON ALPHA2	bursicon-like
LOC111114850	BURSICON BETA	partner of bursicon-like
LOC111119833	CALCITONIN-like	uncharacterized protein LOC111119833 isoform
LOC111100620	CCAP	uncharacterized protein LOC111100620 isoform
LOC111126434	CCK/SK	uncharacterized protein LOC111126434 isoform
LOC111137287	CONOPRESSIN	terepressin/terephysin-like
LOC111102200	ELEVENIN	uncharacterized protein LOC111102200
LOC111132053	FEED CIRCUIT ACTIVATING PEPTIDE (FCAP)	feeding circuit activating peptides-like
LOC111110267	FMRFamide	FMRF-amide neuropeptides-like
LOC111122611	FxRIamide	FMRF-amide neuropeptides-like
LOC111137593	FYFY	uncharacterized protein LOC111137593 isoform X1
LOC111102718[3]	GGNamide	uncharacterized protein LOC111102718 isoform X2
LOC111102813[3]	GGNamide	uncharacterized protein LOC111102813 isoform X1
LOC111102549[4]	GNQQNxP	uncharacterized protein LOC111102549
LOC111104426[4]	GNQQNxP	uncharacterized protein LOC111104426
LOC111122224	GnRH-like	prepro-gonadotropin-releasing hormone-like protein
LOC111128799	GPA2/GPB5	thyrostimulin beta-5 subunit-like
LOC111130827	GPA2/GPB5	thyrostimulin alpha-2 subunit-like
LOC111102913	INSULIN-like peptide - Cv-ILP	probable insulin-like peptide 3
LOC111105634	INSULIN-like peptide - Cv-ILP	insulin-like growth factor I
LOC111101835	INSULIN-like peptide - Cv-ILP3	con-Ins Im2-like
LOC111101100	INSULIN-like peptide - Cv-ILP4	con-Ins Im2-like
LOC111099742[5]	INSULIN-like peptide - Cv-ILP7	probable insulin-like peptide 7
LOC111130821[5]	INSULIN-like peptide - Cv-ILP7	probable insulin-like peptide 7
LOC111135239	LASGLVamide	buccalin-like
LOC111103730[6]	LFRFamide	FMRF-amide neuropeptides-like
LOC111119307[6]	LFRFamide	FMRF-amide neuropeptides-like
LOC111126954	LRFamide	uncharacterized protein LOC111126954
LOC111113361	LUQIN	uncharacterized protein LOC111113361
LOC111113216	MYOMODULIN	myomodulin neuropeptides 2-like
LOC111128069[7]	Neuropeptide prohormone-4-like	Neuropeptide prohormone-4-like isoform X1
LOC111128152[7]	Neuropeptide prohormone-4-like	neuropeptide prohormone-4-like isoform X2
LOC111130993	Neuropeptide-like protein 29 isoform X3	Neuropeptide-like protein 29 isoform X3
LOC111113871	NPF	pro-neuropeptide Y-like isoform
LOC111114944	NPY	pro-neuropeptide Y-like
LOC111123190	OPIOID-like	uncharacterized protein LOC111123190 isoform
LOC111133193[8]	PEDAL PEPTIDE/ ORCOKININ-like type A	uncharacterized protein LOC111133193
LOC111133493[8]	PEDAL PEPTIDE/ ORCOKININ-like type A	uncharacterized protein LOC111133493
LOC111133660	PEDAL PEPTIDE/ ORCOKININ-like type B	uncharacterized protein LOC111133660
LOC111136946	PEDAL PEPTIDE/ ORCOKININ-like type C	uncharacterized protein
LOC111133984	PFGx8amide	uncharacterized protein LOC111133984
LOC111134026	PFGx8amide	uncharacterized protein LOC111134026
LOC111114656	PKYMDT/SPTR-GF	uncharacterized protein LOC111114656
LOC111104271	RxIamide	uncharacterized protein LOC111104271
LOC111133394	sCAP	uncharacterized protein LOC111133394 isoform
LOC111103719[9]	TACHYKININ	uncharacterized protein LOC111103719
LOC111101576[9]	TACHYKININ	uncharacterized protein LOC111101576
LOC111130401	VAKKSPH	uncharacterized protein LOC111130401 isoform
LOC111119480	Wx3Yamide	uncharacterized protein LOC111119480
LOC111116753	ACHATIN	uncharacterized protein LOC111116753

3.1.3. *Allatotropin*

The neuropeptide Allatotropin was first purified from the moth *Manduca sexta* and found to stimulate the production of the juvenile hormone responsible for metamorphosis in insects (Kataoka et al., 1989). Allatotropin-like peptides have then been found in other invertebrates including mollusks (Zatylny-Gaudin et al., 2016). The presence of Allatotropin receptors has also been reported in cnidarian. In this case, ligand-receptor interactions have been suggested to play a role in myoregulatory activities associated with meal ingestion and digestion (Alzugaray et al., 2013). Ortholog search identified four Cv_Allatotropin genes (LOC111124198, LOC111124199, LOC111126501 and LOC111127303) that cluster with nineteen molluscan orthologs. These genes encode 4 proteins (i.e., XP_022322767.1, XP_022322768.1, XP_022326896.1 and XP_022328114.1) even though only 2 (i.e., XP_022322767.1 and XP_022322768.1) encode the Allatotropin peptide precursors. Only the precursor XP_022322767.1 present a 35-residue signal peptide and both genes encode a GFRQSIVDRMGHGFamide peptide.

3.1.4. *Bursicon alpha (α) and beta (β)*

Bursicon is a glycoprotein hormone, consisting of an α- and β-subunit. This hormone is responsible for the hardening and tanning of the cuticle (Fraenkel and Hsiao, 1965). It plays a major role in wing extension in arthropods and was found to regulate major life transition (e.g., hatching, metamorphosis) in ecdysozoans (Zieger et al., 2021). Further, it was demonstrated in *Drosophila* sp. that Bursicon α can limit

the production of adipokinetic hormone (AKH), a hormone typically secreted during energy-demanding conditions in insects (Scopelliti et al., 2019). Ortholog search showed that the Cv_Bursicon α1, α2 and β genes (LOC111137306, LOC111102137 and LOC111114850 respectively), encode one precursor each (XP_022344437.1, XP_022290495.1 and XP_022309064.1, respectively). Cv_Bursicon α1, α2 and β cluster with six, twenty and eleven molluscan proteins, respectively. In *C. virginica*, the three bursicon precursors (α1, α2 and β) present signal peptides and eleven highly conserved cysteines (Supplementary data 2 and 5) for Bursicon beta).

3.1.5. Cholecystokinin (CCK)

Cholecystokinin (CCK) is a member of the gastrin/cholecystokinin (G/CCK) neuropeptide family originally identified in mammals (Ivy and Oldberg, 1928). These peptides, characterized by a common amidated C-terminal tetrapeptide sequence (WMDFamide), are associated with gastrointestinal processes and known to stimulate digestion. CCK-like peptides have been identified in several bivalve species (Stewart et al., 2014; Zatylny-Gaudin et al., 2016; Zhang et al., 2018), including in the king scallop *Pecten maximus* where they increase after a meal (Donval and Bellon, 1992) and stimulate the production of digestive enzymes. Cv_CCK gene (LOC111126434) presents two isoforms (XP_022326792.1 and XP_022326793.1) that cluster with eleven other molluscan proteins. In *C. virginica*, these isoform precursors encode a 25- and 37-signal peptide respectively, and two amidated peptides (pQGAWDY-DYGLGGGRFamide and FDYSFGGGRWamide), identical to their two counterparts found in *C. gigas* (Cragi-CCK1 and Cragi-CCK2; Schwartz et al., 2018). Interestingly, Cragi-CCK1 was found to decrease the frequency of the spontaneous contractions of oyster hindgut, but not Cragi-CCK2.

3.1.6. Feeding circuit-activating peptide (FCAP)

Feeding circuit-activating neuropeptides (FCAP) were first identified from the cerebral-buccal connective tissue of *Aplysia californica* and found to induce feeding motor programs, a series of procedures organized into a correct sequence to perform a movement. FCAP precursors have also been found in other mollusks, including scallops and oysters (Stewart et al., 2014; Zhang et al., 2018). In bivalves, FCAP mature peptides present a X_4LGGX_6 conserved pattern. Ortholog search showed that the Cv_FCAP gene (LOC111132053) clusters with three proteins from other *Ostreida* species. In *C. virginica*, the predicted FACP precursor encodes a 423-amino acid peptide, including a 21-residue signal peptide and fifteen predicted FCAPs (ALSSLGGLSLHGY, GLSSLGGFSLHGY and GLSSLGGMSLHGGFS).

3.1.7. FMRFamide-related peptides

The FMRFamide peptides have been identified and studied in several mollusk species (Zatylny-Gaudin and Favrel, 2014), and are also present in invertebrates (Veenstra, 2011), and mammals (Raffa, 1988). These peptides are part of the bigger group FaRPs (FMRFamide-related peptides) involved in a multitude of physiological processes including cardiac activity (Jakobs and Schipp, 1992), feeding processes, glucose homeostasis and reproduction (Favrel et al., 1994). Orthogroups showed that the Cv_FMRF gene-coding protein (LOC111110267) clusters with eighteen molluscan proteins. In *C. virginica*, the predicted FMRFamide precursor gene encodes a 24-residue signal peptide and eighteen copies of FMRFamide, two copies of FLRFamide, and several other peptides, including the decapeptides ALSGDHYIRFamide and the DPKDDRFMERFVRNamide (Supplementary data 2 and 5).

3.1.8. GGNamide

The GGNamides have been initially identified in annelids (Oumi et al., 1995) and are characterized as myoactive peptides, able to regulate gut and esophagus contraction (Niida et al., 1997). GGNamide have also been found in mollusks including the limpet *Lottia gigantea* (Veenstra, 2010). In addition, GGNamide has been found to be overexpressed in egg-laying females of the cuttlefish *S. officinalis* (Zatylny-Gaudin et al., 2016). Ortholog search showed that two genes code for Cv_GGNamide (LOC111102718 and LOC111102813) and each of them encodes two identical precursors (XP_022291284.1, XP_022291399.1 and XP_022291398.1, XP_022291283.1). These four precursors were found to cluster with fifteen other molluscan proteins. The Cv_GGNamide precursors encode a 26- and a 27-residue signal peptide, depending on the duplicates, followed by an unchanged predicted AKCKGPWANHMCFGGNamide, similar to the one found in *O. edulis* (Boutet et al., 2022).

3.1.9. Insulin-Like peptides (ILPs or IRPs)

In invertebrates, insulin-like peptides (ILPs) were first discovered in the clam *Mya arenaria* (Collip, 1923) and then found in various invertebrates (Matsunaga et al., 2017; Perillo and Arnone, 2014). These peptides are members of the large insulin gene family that encodes structurally related, yet distinct ILPs. Despite their diversity, the amino-acid residues that are primordial for the basic conformation of ILPs (cysteines, cleavage sites) are conserved. Thus, ILPs are made of two chains, A and B, linked by disulphide bonds (Cherif-Feildel et al., 2019; Matsunaga et al., 2017). In mollusks, ILPs display three (type γ, involving six cysteines) or four (type β, involving eight cysteines) disulphide bonds (Cherif-Feildel et al., 2019). The biological functions of ILPs in invertebrates include the regulation of growth, fat storage, resistance to starvation and fecundity. More specifically in mollusks, ILPs have been found or suggested to play a role in growth, energy storage, development, digestive processes and reproduction (Cherif-Feildel et al., 2019; Zhang and He, 2020). In *C. virginica*, four insulin-like peptides were identified. They include: 1) Cv-ILP (type γ, CC(3×)C(8×)C, identified under LOC111102913 and LOC111105634 which are likely duplicate genes, each coding for two isoforms: XP_022291566.1 and XP_022291565.1; XP_022295717.1 and XP_022295716.1, respectively); 2) Cv-ILP3 (type β, C(1×)CC(3×)C(8×)C, LOC111101835 coding for the precursor XP_022290164.1); 3) Cv-ILP4 (type β, C(1×)CC(3×)C(8×)C, LOC111101100 coding for the precursor XP_022289108.1); and 4) Cv-ILP7 (type β, CC(4×)C(8×), LOC111099742 and LOC111130821, duplicate genes, each coding for one precursor: XP_022286880.1 and XP_022333774.1). Ortholog search showed that the two Cv_ILP proteins cluster with ten others molluscan orthologs (Supplementary data 2, 4 and 5) while Cv_ILP3 and Cv_ILP4 proteins cluster with each other and thirty-nine molluscan orthologs. Finally, the two Cv_ILP7 proteins cluster with three orthologs mostly from *Ostreida* species.

3.1.10. LFRFamide

The LFRFamide family peptides are present in several members of the Mollusca, including bivalves (Stewart et al., 2014; Zhang et al., 2018) and cephalopods (Zatylny-Gaudin et al., 2010). LFRFamide precursor encodes one or several copies of diverse LFRFamides (Zatylny-Gaudin and Favrel, 2014) demonstrated or suggested to be involved in feeding processes, energy storage and reproduction (Zatylny-Gaudin et al., 2010). Ortholog search showed that two Cv_LFRFamide genes (LOC111103730 and LOC111119307) encode two identical precursors (XP_022292882.1 and XP_022315073.1) that cluster with nine others molluscan orthologs. The Cv_LFRFamide precursor encodes a 23-residue signal peptide and six LFRFamide peptides, including five hexapeptides ((G/S)(S/A)LFRFamide) and one 13-residue peptide (SVDNDKPHTPFRFamide). Sequence alignments confirm similarity to LFRFamide precursors found in *C. gigas* (Supplementary data 4 and 5).

3.1.11. NPF/NPY

Neuropeptides F (NPFs) are very similar to the NPY found in vertebrates. NPF peptides are usually long (up to forty residues) and possess a C-terminal tyrosine-amide (Fa) instead of a phenylalanine-amide (Ya) present in the NPY. As reported by Fadda et al. (Fadda et al., 2019), the nomenclature of the NPF/NPY system is not always clear (e.g., confusion between NPF and NPY), which could lead to misinterpretation. In

mollusks, NPF was identified in the snails *L. stagnalis* (de Jong-Brink et al., 1999), in the cuttlefish *S. officinalis* (Zatylny-Gaudin et al., 2016) and in several bivalve species (Bigot, 2012; Zhang et al., 2018). Molluscan NPF seem to play crucial roles in the coordination of feeding, reproduction and energy homeostasis (Zatylny-Gaudin and Favrel, 2014). Ortholog search showed that Cv_NPF gene (LOC111113871) encodes two precursors (XP_022307875.1 and XP_022307882.1) that cluster with 24 molluscan orthologs. In *C. virginica*, the two precursors encode a signal peptide followed by the same peptide (NDSLLPPSRPSSFRSPGQLRQYLKALNDYYAIVGRPRFamide). The two *C. virginica* NPF are highly similar to several mollusks, including *C. gigas*.

In addition to the NPF, one NPY harboring a C-terminal phenylalanine-amide (Ya) was also found in the *C. virginica* genome. Ortholog search showed that Cv_NPY gene (LOC111114944) encodes a precursor (XP_022309209.1) that only clusters with two orthologs from *C. gigas* and *O. edulis*. The Cv_NPY precursor encodes a 27-residue signal peptide followed by a SPLAVDPDLEMMALVPPSRPSGFQNFQEMQ-RYLMKLNRFYNMMSRPRYamide (Supplementary data 2 and 5).

3.1.12. Opioid-like

In vertebrates, opioids, including endomorphins (YP(W/F)amide), dynorphin (dynomorphin A, YGGFLRRI) and enkephalins (Leu-enkephalin, YGGFL or Met-enkephalin, YGGFM), are endogenous peptides that bind to receptors localized in the brain and induce diverse effects (Akil et al., 1984). Opioids and opioid-like peptides have been described in several invertebrate species, including insects, annelids and mollusks (Leung and Stefano, 1984; Stefano et al., 1998). Interestingly, these peptides have been found in neural ganglia and circulating hemocytes of *M. edulis* (Leung and Stefano, 1984; Stefano et al., 1998). Opioids are known to play a major role in motivation, response to stress and the control of food intake (Nagabhushanam et al., 1995). For example, the administration of morphine to the slug *L. maximus* (starved or satiated) initiate a feeding response (Kavaliers et al., 1985). Ortholog search showed that the Cv_opioid-like (LOC111123190) encodes two highly similar precursors (XP_022321067.1 and XP_022321066.1) that cluster with eighteen others molluscan orthologs. The two precursors encode a signal peptide and a 102-residue peptide containing six different opioid-like peptides harboring a C-terminal amidation (e.g., YGRLGFGGSRGFLYamide, FWRLGVKGSNGFLYGamide).

3.1.13. Pedal peptide/Orcokinin-like

The myotropic peptide orcokinin, also named pedal peptide, has been identified in the crayfish *Orconectes limosus* as a stimulator of the frequency and the amplitude of the gut contraction (Stangier et al., 1992). Orcokinin-like peptides have also been found in several invertebrates (Nathoo et al., 2001), including mollusks (Stewart et al., 2014; Veenstra, 2010; Zhang et al., 2018). Orcokinin-like precursors can code for three different types of orcokinins, namely type A, B and C (Veenstra, 2010), all found in *C. virginica*. Indeed, ortholog search showed that two Cv_Orcokinin-like type A genes (LOC111133193 and LOC111133493) encode two similar precursors (XP_022337035.1 and XP_022337651.1) that cluster with four orthologs from *Ostreida* and *Orcida*. These two type A precursors encode a 25-residue signal peptide followed by ten orcokinin-like peptides. Ortholog search showed that the Cv_Orcokinin-like type B gene (LOC111133660) encodes two similar precursors (XP_022337905.1 and XP_022337904.1) that do not cluster with other orthologs. These two precursors encode a 23-residue signal peptide and seventeen orcokinin-like peptides, including two copies of GYDSIGGSRGLQGFN and three copies of NYDSIGGSRGLSGFV and SYDSIGGSRGLQGFN. Interestingly, two of these peptides (e.g., NYD-SIGGSRGLQG and GYDSISGSRGMKGFN) are also found in *C. gigas*. Ortholog search showed that the Cv_Orcokinin-like type C gene (LOC111136946) encodes a precursor (XP_022343838.1) that clusters with two orthologs from *O. edulis* and *S. glomerata*. It encodes a 25-residue signal peptide and eleven orcokinin-like peptides (e.g., TMDSIYNRPGIFGGLamide).

3.1.14. PKYMDT/SPTR-GF

The PKYMDT/SPTR-GF (SPTR gene family) precursor was first identified in the gastropod L. *stagnalis* and where it encodes two peptide hormones suggested to modulate serotonin-induced spiking activity of the serotonergic cerebral cell (involved in feeding motor program) of the snail (Koert et al., 2001). Ortholog search showed that the Cv_PKYMDT/SPTR-GF gene (LOC111114656) encodes a precursor (XP_022308767.1) that clusters with fourteen orthologs from various molluscan species (Supplementary data 4). In *C. virginica*, the PKYMDT/SPTR-GF precursor encodes a 23-residue signal peptide, a 17-amino acid amidated peptide, followed by a 58-amino acid peptide that contains a conserved region PKYMDT and a C-terminally amidated peptide (GHLRI-CIRRSGSRIVPYPCFRamide, Supplementary data 5).

3.1.15. sCAP

Small cardioactive peptides, type A (sCAP$_A$) and B (sCAP$_B$), are part of a group of peptides that controls muscle movement in gastropods, including in feeding processes and gut motility (Perry et al., 1999). In bivalves, these peptides have been found to have similar functions where they regulate feeding and digestive activities (Candelario-Martinez et al., 1993). Ortholog search showed that the Cv_sCAP gene (LOC111133394) encodes four precursors (XP_022337462.1 (X1), XP_022337463.1 (X2), XP_022337460.1 (X3), and XP_022337461.1 (X4)) that cluster with orthologs from *C. gigas* (NP_001292304.1) and *O. edulis* (g1117.t1). The precursor X1 and X2 encode one sCAP$_B$ (APGYQFPRLG) and additional C-terminally amidated peptides (e.g., PLQFPRLGamide, GIQFPRLamide and SILHamide). The precursor X3 encodes one sCAP$_B$ (APKYFYFPRMamide), one sCAP$_A$ (SAFYFPRMamide) and a carboxy-terminal peptide enriched in six conserved cysteines residues. Finally, the precursor X4 only encodes two peptides (GIQFPRLamide and SILHamide).

3.1.16. Tachykinin

Tachykinin peptides are a large group of neuropeptides distributed in both vertebrates and invertebrates, including mollusks (Dubos et al., 2018; Stewart et al., 2014; Veenstra, 2010; Zatylny-Gaudin et al., 2016). They are involved in diverse functions reviewed by Nässel et al., (Nässel et al., 2019). For example, in insects, tachykinin is thought to be involved in the regulation of foraging and social behaviors (Takeuchi et al., 2004). Ortholog search showed that two Cv_Tachykinin genes (LOC111103719 and LOC111101576) encode identical precursors (XP_022292873.1 and XP_022289818.1) that cluster with three orthologs, two from *C. gigas* and one from *O. edulis*. Each precursor produces three different heptapeptides (FGFAPMRamide, ARFFGLRamide, FRFTALRamide) similar to those described in *C. gigas* (Dubos et al., 2018). In this particular oyster, a tachykinin precursor is highly expressed in the visceral ganglia of starved animals, suggesting its implication in the regulation of feeding processes.

3.2. Neuropeptides with other or unknown functions

3.2.1. APGWamide

The tetrapeptide APGWamide has been identified in several mollusks (Favrel and Mathieu, 1996; Stewart et al., 2014) where it has been found to be involved in the regulation of reproduction (Li et al., 1992). For example, the injection of synthetic APGWamide in the Sydney rock oyster (*S. glomerata*) advances conditioning and gonadal maturation and triggers spawning in ripe animals (In et al., 2016). Ortholog search showed that Cv_APGW gene (LOC111133878) encodes two similar isoforms (XP_022338293.1 and XP_022338292.1) that cluster with three orthologs from *Ostreida*. The Cv_APGW precursor gene generates a 242-amino acid peptide, including a 20-amino acid signal peptide and four tetrapeptide KPGWamide, two RPGWamide, two APGWamide, two SPGWamide, as well as a putative 45-amino acid long C-terminal peptide.

3.2.2. Calcitonin-like

Calcitonin is a hypocalcaemic hormone that, in humans, is produced by the thyroid gland and reduces the level of calcium in the blood (Carter and Schipani, 2006). Calcitonin-like precursors have also been described in bivalves and most specifically in the mantle of *C. gigas*, an organ involved in shell biomineralization (Schwartz et al., 2019). Ortholog search showed that the Cv_Calcitonin-like gene (LOC111119833) codes for two slightly different precursors (XP_022316062.1 and XP_022316070.1) that cluster with forty others molluscan orthologs.

3.2.3. CCAP

Crustacean cardioactive peptide (CCAP) was first described in the shore crab *Carcinus maenas* as a heartbeat regulator hormone (Stangier et al., 1987). In mollusks, CCAPs have also been identified as peptides containing two conserved cysteines and often harboring a C-terminal amidation (In et al., 2016; Stewart et al., 2014; Zatylny-Gaudin et al., 2016). Synthetic CCAP peptide have been found to trigger spawning in ripe Sydney rock oysters (In et al., 2016). Ortholog search showed that the Cv_CCAP gene (LOC111100620) present two isoforms (XP_022288389.1 and XP_022288390.1) that cluster with twelve orthologs from several orders, including the *Ostreida* and *Mytilida*. In *C. virginica*, the two predicted CCAP precursors code for the same 23-amino acid signal peptide and two peptides, VFCNGFFGCSNamide and LFCNQGGCFamide.

3.2.4. Conopressin

Conopressin, isolated from the snail L. *stagnalis*, is a nanopeptide containing consensus amino acids of the Oxytocin/Vasopressin superfamily peptides: Cys^{1}, Asn^{5}, Cys^{6}, Pro^{7}, Gly^{9}; and a C-terminal amidation (Kawada, 2016). In mollusks, conopressin has been found to regulate sexual behavior in *Lymnaea* sp. (Van Kesteren et al., 1995) and to reduce gill activity in *Aplysia* sp. (Martinez-Padrón et al., 1992). Ortholog search showed that the Cv_Conopressin gene (LOC111137287) clusters with twenty-one orthologs from several molluscan species. In *C. virginica*, the precursor encodes a 164-amino acid peptide, including a 32-residue signal peptide directly followed by a conserved conopressin peptide, CFIRNCPPGamide and a Hormone_5 domain (neurohypophysial hormone family whose main representatives are oxytocin and vasopressin).

3.2.5. Elevenin (Neuropeptide L11)

Elevenin precursor was first identified in the neurons L11 of the abdominal ganglion of *Aplysia californica* (Taussig et al., 1984). It was also found as a mature product in the hemolymph of *S. officinalis* (Zatylny-Gaudin et al., 2016). Recently, elevenin precursors and cognate receptors have been identified in insects, including the planthopper *Nilaparvata lugens*, where it was shown to regulate the cuticle melanization process (Uchiyama et al., 2018). Ortholog search showed that the Cv_Elevenin gene (LOC111102200), coding for the protein XP_022290563.1, clusters with fourteen orthologs from thirteen bivalve species. In *C. virginica*, the precursor encodes a 134-amino acid peptide, including a 21-residue signal peptide and a putative mature peptide FCELKPFARRCMGIAA. The function of this peptide in mollusks is yet to be defined.

3.2.6. FxRIamide

FxRIamide peptides, also referred to xSSFxRIamide, have been described in several invertebrates including mollusks (Kuroki et al., 1993; Stewart et al., 2014). In this last group (e.g., *L. stagnalis*), FxRIamides have been suggested to regulate muscle activity (Kuroki et al., 1993). Ortholog search showed that Cv_FxRIamide clusters with four orthologs from bivalves including members of *Ostreida* and *Arcida*. In *C. virginica*, the FxRIamide precursor encodes a 20-residue signal peptide and seventeen FxRIamide peptides, including eleven copies of ASGFFRIamide, two copies of SKGFFRIamide and single copies of other variants of FxRIamides (AFFRIamide, HFFRIamide, AKGFFRIamide, VPTSAFMRIamide, QHLLQSLVSDPYYRNamide and IQQSSFIRIamide).

3.2.7. FYFY

The FYFY peptide was recently identified in silico in *M. yessoensis* (Zhang et al., 2018). Ortholog search showed that the Cv_FYFY gene (LOC111137593) encodes two closely related precursors (XP_022344820.1 and XP_022344821.1) that cluster with fourteen others molluscan orthologs. In *C. virginica*, the FYFY precursor encodes a 24-residue signal peptide and a putative GRGSFRKFYFYK peptide. The function of the resulting peptide is yet to be defined.

3.2.8. GNQQNxP

The GNQQNxP peptide was recently identified in silico in *M. yessoensis* and confirmed by mass spectrometry analysis (SEEGIGFGNQQNLPKI, Zhang et al., 2018). Due to its high expression in the eyes, this peptide has been suggested to be involved in the functioning of scallop eye. In *C. virginica*, two genes are coding for Cv_GNQQNxP (LOC111102549 and LOC111104426) and each one of them encodes one precursor (XP_022291050.1 and XP_022294073.1) and both are almost identical to each other. Ortholog search showed that the two precursors were found to cluster with twenty-one others molluscan orthologs. In *C. virginica*, the GNQQNxP precursors encode an 18-residue signal peptide, a putative AQEVMMFGNQQNKPRI peptide and possible additional peptides since several cleavage sites have been identified on the sequence. Similar predicted peptides have also been found in the oyster *C. gigas*, *O. edulis* and *S. glomerata*.

3.2.9. GnRH-like

The gonadotropin-releasing hormone (GnRH) is a common and central hormone in the control of the reproductive function in vertebrates (Dufour et al., 2020). It has also been identified in several mollusk species and was found to be involved in multiple functions, including the stimulation of spermatogonia cell division, oocytes proliferation, spawning (Funayama et al., 2019; Treen et al., 2012). Ortholog search showed that Cv_GnRH gene (LOC111122224) encodes one precursor (XP_022319572.1) that clusters with five others molluscan orthologs (e.g., *Ostreida* and *Pectenida* species). In *C. virginica*, GnRH gene encodes a 90-amino acid precursor, including a 24-residue signal peptide and a conserved sequence of 11-residues (QNYHFSNGWQPamide). Interestingly, the GnRH-like peptide in *C. virginica*, *C. gigas* and *S. glomerata* are identical.

3.2.10. GPA2/GPB5

The GPA2/GPB5 heterodimer belongs to a family of hormones, the glycoprotein cysteine knot-forming heterodimers, consisting of an α (GPA2) and β (GPB5) subunits. In invertebrates, GPA2/GPB5 has been involved in ionic and osmotic balance (Paluzzi et al., 2014) and in reproduction (Rocco et al., 2019). In *C. virginica*, Cv_GPA2 gene (LOC111128799) encodes two isoform precursors (XP_022330363.1 and XP_022330362.1) that cluster with twenty-four others molluscan orthologs. Further, the Cv_GPB5 gene (LOC111130827) encodes one precursor (XP_022333778.1) and has been found to cluster with fifteen others molluscan orthologs. In *C. virginica*, GPA2 and GPB5 precursors encode 122- and 133-residues, respectively. They both contain 18- and 19-residue signal peptides and ten conserved cysteines.

3.2.11. LASGLVamide

The LASGLVamide neuropeptides were identified in silico in the mollusks *L. gigantea* (Veenstra, 2010), *C. gigas* (Stewart et al., 2014), *S. glomerata* (In et al., 2016) and confirmed as mature peptides in *M. yessoensis* (Zhang et al., 2018). The biological function of these peptides is still unknown. Ortholog search showed that the Cv_LASGLVamide gene (LOC111135239) encodes four identical precursors that only cluster with four orthologs from *C. gigas*. In *C. virginica*, the LASGLVamide precursor encodes a 28-residue signal peptide, thirteen

decapeptides XXDX(I/L)ASGL(V/I)amide (e.g., QFDRLASGLIGamide) and three other peptides.

3.2.12. LRFamide

The LRFamide neuropeptides were recently identified in silico in the scallop *M. yessoensis* but not confirmed by mass spectrometry (Zhang et al., 2018). Ortholog search showed that the Cv_LRFamide gene (LOC111126954) encodes a precursor (XP_022327616.1) that clusters with nine others molluscan orthologs, including *Ostreida*, *Pectinida* and *Mytilida*. In *C. virginica*, cleavage prediction indicates that the LRFamide precursor encodes a 28-residue signal peptide and a putative SPQVRMPSLRFamide or MPSLRFamide. The biological function of these peptides is still to be determined.

3.2.13. Luqin

The luqin was initially identified in the neurons of *A. californica* (Shyamala et al., 1986) and has also been reported in echinoderms, annelids and arthropods (Tensen et al., 1998). If luqin was shown to exhibit myoexcitatory (cardiac and penis retractor muscles) function in the snail *Achatina* (Fujimoto et al., 1990), it has been also suspected to play a role in the control of reproduction and the regulation of water and ion balance in several mollusks (Tensen et al., 1998). Ortholog search showed that the Cv_Luqin gene (LOC111113361) encodes three similar precursors (XP_022307301.1, XP_022315265.1 and XP_022315274.1) that cluster with twenty others molluscan orthologs. In *C. virginica*, luqin precursors were identified as a 110/116-amino acid protein containing a 23/29-amino acid signal peptide (depending on the precursors) and encoding for the predicted mature peptide APQWRPQGRFGa. The same luqin precursor also encodes another predicted 14-amino acid peptide VCVESNVPGLYKCY (Stewart et al., 2014).

3.2.14. Myomodulin

The myomodulin neuropeptides have been identified in the gastropod *A. californica* in which they control buccal muscle contraction (Cropper et al., 1991). In another snail, *L. stagnalis*, myomodulins have been found to be involved in the modulation of muscle activities of the penis complex (van Golen et al., 1996), and likely controlling reproduction. These peptides have also been reported in cephalopods (Zatylny-Gaudin et al., 2016) and in bivalves, including *C. gigas*, *P. fucata* (Stewart et al., 2014), and *M. yessoensis* (Zhang et al., 2018). Ortholog search showed that the Cv_Myomodulin gene (LOC111113216) encodes one precursor (XP_022307028.1) that clusters with nineteen molluscan orthologs. In *C. virginica*, the myomodulin precursor encodes a 19-residue signal peptide, four GMPMLRLamides, one copy of several variants GGLSMLRLamide, GLQMLRLamide, QPAAESNamide and an additional peptide.

3.2.15. Neuropeptide prohormone-4-like (PH-4)

Neuropeptide prohormone-4-like (PH-4) has been found in crustaceans (Ventura et al., 2014) and was associated with the venom of several sea snails (Robinson et al., 2017). In *C. virginica*, two genes encoding for Cv_PH-4 are present (LOC111128069 and LOC111128152) and each of them encodes two precursors, a long precursor (XP_022329187.1 and XP_022329331.1, respectively) and a short precursor (XP_022329332.1 and XP_022329188.1, respectively). Ortholog search showed that the four precursors have been found to cluster with twenty-five molluscan orthologs. In *C. virginica*, the PH-4 precursor encodes a 192/220-residue depending on the precursor (short or long). A 24-amino-acid signal peptide has been predicted for the shorter precursors while no cleavage site has been predicted for the longest precursor. The four precursors further encode a 15-amino acid peptide (MSVDFSRLRKPYLLP) delimitated by a KR cleavage site, a cysteine rich low-density lipoprotein (LDL) receptor class A domain and an extended cysteine free region. The exact function of this peptide in bivalves is still unknown.

3.2.16. Neuropeptide-like protein 29 (nlp)

The neuropeptide-like protein 29 (*nlp*) is part of the larger family called neuropeptide-like proteins identified in the nematode *C. elegans* (Nathoo et al., 2001). The members of this family are expressed in neural cells and found to have neuromodulatory and antimicrobial functions (Couillault et al., 2004). The Cv_*nlp* gene (LOC111130993) encodes two identical precursors (XP_022334013.1 and XP_022334011.1) that embed a 16-amino acid signal peptide followed by several peptides including a C-terminal amidation. The function of the resulting peptides is yet to be defined.

3.2.17. PFGx8amide

PFGx8amide neuropeptide gene was identified in two oyster species *P. fucata* and *C. gigas* (Stewart et al., 2014). A variant, PFVx7amide, has also been found in the scallop *M. yessoensis* (Zhang et al., 2018). Ortholog search showed that two Cv_PFGx8-amide genes (LOC111133984 and LOC111134026) encode two highly similar precursors (XP_022338436.1 and XP_022338499.1) that cluster with three orthologs from the *Ostreida*. The Cv_PFGx8amide precursors encode a 21-residue signal peptide and eleven PFGx8amide peptides, among which seven contain a C-terminal amidation (PFGGVYGRSYQVamide). The function of the resulting peptides is yet to be defined.

3.2.18. RxIamide

RxIamide peptides are a group of neuropeptides identified in *C. gigas* by Stewart et al. (Stewart et al., 2014). Ortholog search showed that the Cv_RxIamide gene (LOC111104271) encodes a precursor (XP_022293840.1) that clusters with two orthologs identified in *C. gigas* and *O. edulis*. The Cv_RxIamide precursor encodes a 23-residue signal peptide, seven RxI peptides containing a C-terminal amidation (e.g., AVNDPVDLRNIamide, LGVDFRNIamide). The function of these peptides is not known.

3.2.19. VAKKSPH

A precursor of the VAKKSPH neuropeptide and a mature peptide have been recently identified in the ganglia of the scallop *M. yessoensis* (Zhang et al., 2018). Ortholog search showed that the Cv_VAKKSPH genes (LOC111130401) encode a long (XP_022333159.1) and a short (XP_022333160.1) precursor that cluster with twenty-three orthologs from various mollusks. In *C. virginica*, the long precursor codes for a VAKKSPH peptide (KSLVAKKSPHMV) whereas the short precursor does not, due to a gap in the sequence. Interestingly, this characteristic (the deletion of the portion coding for the peptide) is also found in *C. gigas* and *M. yessoensis*. The function of these peptides has not been identified yet.

3.2.20. WX_3Yamide

The neuropeptide WX_3Yamide was first identified in *C. gigas* and *P. fucata* (Stewart et al., 2014). Ortholog search showed that the Cv_WX_3Yamide gene (LOC111119480) encodes a precursor (XP_022315389.1) that clusters with three orthologs from the *Ostreida*. The Cv_WX_3Yamide precursor encodes a 24-residue signal peptide and three C-terminally amidated octopeptides (GQEWAIAYamide, ASPGWTISYamide and QQGWHIAYamide) also found in the three other oyster species, with one minor modification in *O. edulis*. The function of these peptides has not been identified yet.

3.3. NPGs tissue location and gene transcription in response to starvation

In this study, several NPGs have been further investigated because their homologs in other invertebrates, including in mollusk species, have been described to be involved in the feeding mechanism (Table 1). These investigations included the evaluation of changes in gene transcription following starvation. Out of the nine selected NPGs, one NPF (LOC111113871) and one ILP (LOC111105634, XP_022295717.1) were not successfully amplified by PCR (Table 2). Further analysis of the

genome annotation showed that these 2 sequences could have been incorrectly annotated (limited RNASeq coverage), suggesting that these genes were not found to be expressed in this experiment.

Six NPGs (PKYMDT/SPTR-GF, a second NPF, NPY, LFRF, FMRF, and BUR, Table 2 and Supplementary data 1) were significantly expressed in the visceral ganglia of oysters as compared to the other tissues (i.e., digestive gland, esophagus, labial palps, gills, mantle and hemocytes, Fig. 1) while ILP was expressed in the visceral ganglia as well as in tissues associated with the digestive tract (digestive gland, esophagus and labial palps). Based on this information, the transcription of these seven NPGs was evaluated in the visceral ganglia and the digestive gland of oysters submitted to starvation.

The transcription of all NPGs significantly increased in the visceral ganglia after seven days of starvation, except for LFRF where the increase was not significant (Fig. 2). In the digestive gland, the transcription of PKYMDT/SPTR-GF and LFRF significantly increased after 7 days of starvation, while the transcription of NPF significantly decreased (Fig. 2). The increase in the transcription of NPGs following starvation suggests the involvement in feeding processes and energy homeostasis. The case of NPF, and more specifically the contrasting expression after starvation in the visceral ganglia and the digestive gland, is more intriguing and needs to be further explored using other method such as RNAseq to evaluate the whole neuropeptidome landscape during starvation. It is important to note that NPF expression is primarily localized in the visceral ganglia, rather than in the digestive gland (nearly 10-fold higher in the ganglia). Additionally, for RNA extraction, the entire visceral ganglia tissue is used, while only a small portion of the digestive gland is collected and processed. Therefore, slight differences in tissue sampling between different individuals may introduce some error if NPF expression is specific to particular areas of the digestive gland that could be under-sampled in some individuals. More details about NPF regulation and function are given in paragraph 3.4.

While the role of RFamides (e.g., LFRF, FMRF, NPF) in feeding processes has been documented in mollusks (Bigot, 2012; Zatylny-Gaudin and Favrel, 2014), this is not the case for the PKYMDT/SPTR-GF and bursicon. Recent research on the gastropod *A. californica* reported the presence of a PKYMDT/SPTR-GF precursor encoding for a signal peptide and two apSPTR-GF-DPs (Zhang et al., 2018). Further functional investigations in the same species demonstrated that one of the peptides (i.e., apSPTR-GF-DP2) was able to modulate the duration of the protraction phase of the feeding motor program. Consequently, a similar function implying the modulation of muscular activity during feeding processes could be hypothesized in bivalves.

Bursicon is another example of NPG whose functional role has not been elucidated in mollusks. In arthropods however, bursicon has been found to play a major role in the regulation of shedding of the cuticle and as a regulator of energy homeostasis, by modulating the production of adipokinetic hormone (AKH) involved in the mobilization and utilization of endogenous energy reserves (Scopelliti et al., 2019). Accordingly, it could be suggested that, in bivalve mollusks, bursicon could play a role similar to the one found in arthropods by regulating enzymes involved in energy mobilization, including the glycogen phosphorylase, an enzyme that catalyzes glycogen into glucose and liberates stored energy.

In addition to the visceral ganglia, the digestive system has also been found to express a high level of an insulin-like peptide (ILP), a member of a large family of neuropeptides that can regroup up to six members in mollusks (Cherif-Feildel et al., 2019). In the current study, ILP has been found in the visceral ganglia and the digestive system of *C. virginica*, and more specifically in the digestive gland, the esophagus and the labial palps. Similarly, the orthologous gene of Cv-ILP in *C. gigas* (Cg-ILP) was found to be expressed in the same organs as *C. virginica,* as well as in the gonad (Cherif-Feildel et al., 2019). Further, the location of Cg-ILP in the digestive system and the upregulation of an ILP receptor (CIR) in the digestive gland of fasted oysters, lead to the conclusion that this gene could be involved in digestive processes (Cherif-Feildel et al., 2019). In our study, the transcription of Cv-ILP in the visceral ganglia significantly increased with starvation supporting its involvement in feeding mechanisms or energy homeostasis. For example, it could be hypothesized that ILP plays a role of hunger signal to stimulate food intake, similarly to the ghrelin, a "hunger hormone" described in vertebrates, from mammalian to amphibian (Kaiya et al., 2011) but absent from invertebrates. In addition, another possible function for Cv-ILP could be energy maintenance. Thus, it has been shown that recombinant ILP from the oyster *P. fucata martensii* (ortholog of Cv-ILP) increased the RNA transcription of two enzymes, GSK3 (Glycogen synthase kinase-3) and PP1 (Protein phosphatase 1), both of which being involved in the control of glycogen metabolism (Zhang and He, 2020).

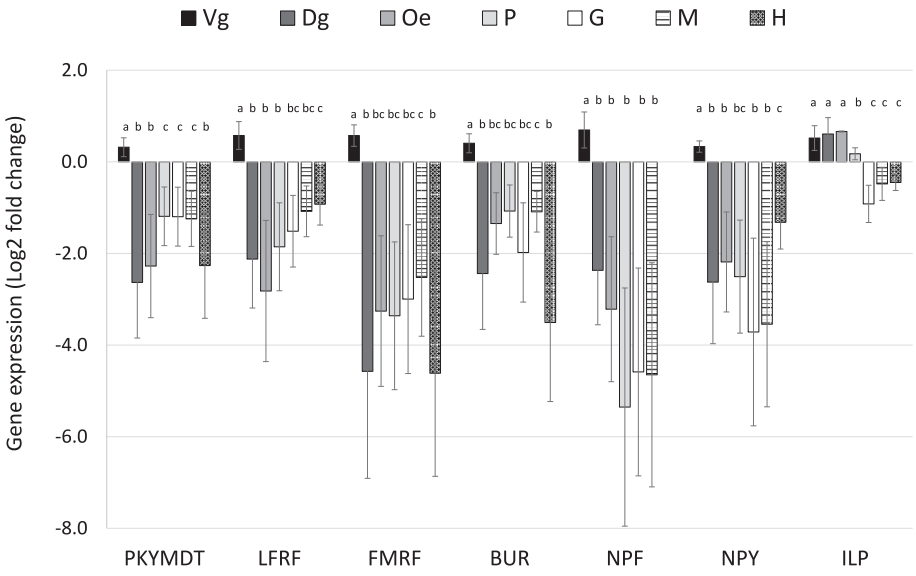

Fig. 1. Genes expression of selected neuropeptides in different oyster tissues. Data are expressed using the 2−ΔΔCt method and gene expression levels were normalized to the housekeeping gene (18S rRNA gene). Each bar represent the mean and standard deviation of 12 individuals. Letters indicate significant differences between tissue for a given neuropeptide (1-way ANOVA followed by SNK, p-value < 0.05). Visceral ganglia (Vg), digestive gland (Dg), oesophagus (Oe), labial palps (P), gills (G), mantle (M) and hemocytes (H). Liste of genes is provided in Table 3.

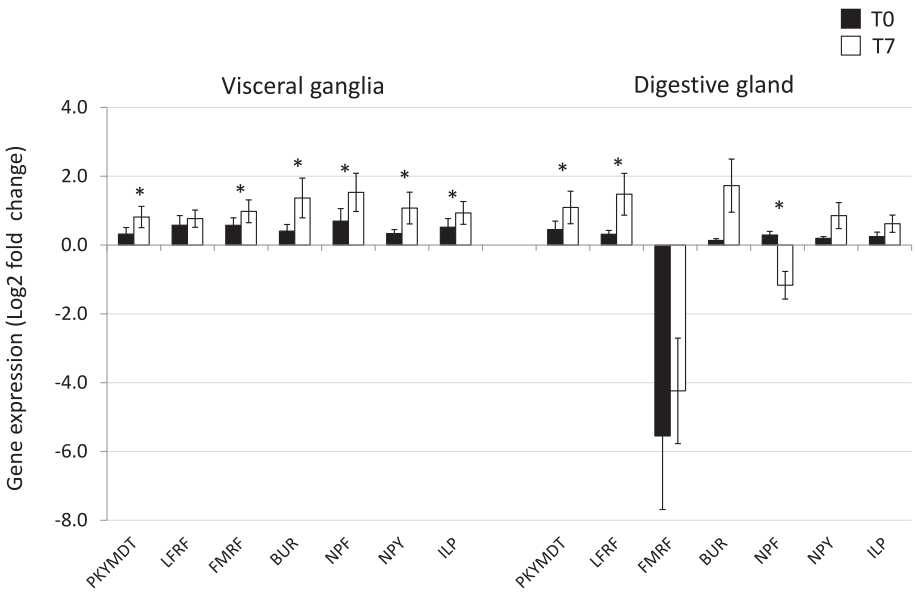

Fig. 2. Genes expression of selected neuropeptides in visceral ganglia and digestive gland after 7 days starvation. Data are expressed using the 2−ΔΔCt method and gene expression levels were normalized to the housekeeping gene (18S rRNA gene). Each bar represent the mean and standard deviation of 12 individuals. The asterisks indicate significant differences between bars (Student t-test, p-value < 0.05). The list of genes is provided in Table 3.

3.4. Transcription of selected genes in response to NPF and tissue extract injection

In the current study, the injection of visceral ganglia extracts and synthetic NPF were found to significantly increase the transcription level of the lectin CvML3914 and the glycogen synthase in the labial palps but not the other tested genes (i.e., glycogen phosphorylase, CvML, and CvML3912) while the control treatments (injection with ASW or mantle extract) had no effect on the transcription of these genes (Fig. 3). CvML3914 is an oyster mucosal lectin involved in the capture of food particles (Pales Espinosa and Allam, 2018). The transcription of CvML3914 was previously shown to increase in pallial organs (i.e., labial palps and gills) in response to starvation, allowing oysters to capture food more efficiently and help the bivalve to maintain energy homeostasis. The second regulated gene, the glycogen synthase, is an enzyme that catalyzes the transfer of glucose from UDP-glucose to a terminal branch of glycogen, therefore contributing to glucose storage pathways. Injection of either visceral ganglia extracts or synthetic NPF increases the transcription of CvML3914 and glycogen synthase which indicate that NPF, and possibly other NPGs produced by the visceral ganglia, may control feeding processes and energy homeostasis in oyster. In fact, the NPY/NPF genes have been found to play an important role in the feeding behavior in vertebrates (Clark et al., 1984), but also in invertebrates (Fadda et al., 2019; Kiris et al., 2004), including mollusks. For example, starvation of the oyster *C. gigas* (Bigot, 2012) or the clam *R. phillipinarum* (Wang et al., 2017) induces an increase of the transcription of NPY/NPF genes in the visceral ganglia, in agreement with our results (see section 3.2.). Further, it was also demonstrated that the injection of synthetic NPY/NPF has an impact on food intake in several species. For instance, NPY injection increases food intake and growth of

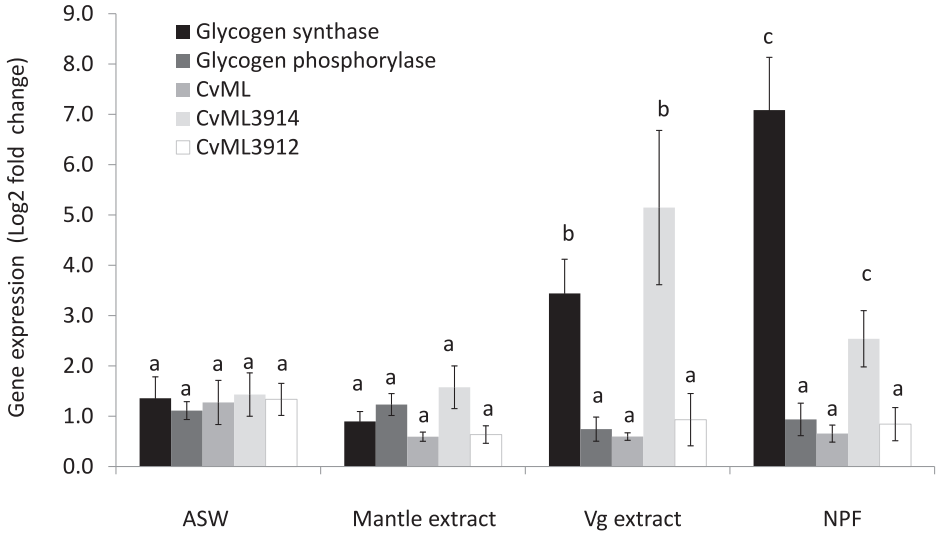

Fig. 3. Expression of genes (glycogen synthase, glycogen phosphorylase, three mucosal lectins CvML, CvML3914, CvML3912) in the labial palps after the injection of tissues extracts and synthetic NPF. Data are expressed using the 2−ΔΔCt method and gene expression levels were normalized to the housekeeping gene (18S rRNA gene). Each bar represent the mean and standard deviation of 12 individuals. For a given gene, letters indicate significant difference between treatment (ANOVA test followed by SNK, p < 0.05).

rats (Clark et al., 1984), and shrimp (Kiris et al., 2004). If in vertebrates the orexigenic effect of NPY/NPF has been clearly demonstrated, this is not the case in mollusks. Thus, the injection of synthetic rp-NPF to the clam *R. phillipinarum* was shown to increase the filtration rate and possibly food uptake (Wang et al., 2017). In another study using the Pacific abalone *Haliotis discus hannai*, the injection of Hdh-NPF peptide increased food uptake while the knockdown of Hdh-NPF transcription, using gene silencing, decreased food consumption (Kim et al., 2021). As a divergent example, the injection of ApNPY to the gastropod *Aplysia californica* not only reduced food intake, but also slowed down ingestion rate (Jing et al., 2007). It is also noteworthy that delivering LyNPY to the snail *Lymnaea stagnalis* does not seem to affect food intake (de Jong-Brink et al., 1999). Despite these inconsistent findings in mollusks, our results support the involvement of NPF/NPY in the control of feeding processes and energy homeostasis in the eastern oyster.

4. Conclusion

In this study, we generated the first repertoire of neuropeptide genes in the eastern oyster, a commercially and ecologically important species in the US. A total of eighty-five neuropeptide genes were identified in silico and a majority of these were confirmed at the transcriptomic level. Results strongly suggest or confirm the involvement of several of these genes in feeding processes, including a member of the NPF/NPY family. Indeed, the injection of synthetic NPF in *C. virginica* leads to the increase of two important genes (i.e., a mucosal lectin and the glycogen synthase) implicated in feeding processes, energy storage and more broadly in energy homeostasis.

The number of neuropeptide genes found in *C. virginica* is comparable to what has been described in other mollusks but it is probable that more of these molecules have yet to be identified and characterized. Because neuropeptides are known to drive and modulate a multitude of physiological processes, identifying their specific action through functional assays is required. In an era where functional genomics and gene manipulation approaches are more attainable, the new information presented here have the potential to unravel obscure aspects of the biology and physiology of the eastern oyster with obvious basic and applied implications.

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CRediT authorship contribution statement

Emmanuelle Pales Espinosa: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Sarah Farhat:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Bassem Allam:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing or financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111792.

Data availability

Data will be made available on request.

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