General and Comparative Endocrinology 332 (2023) 114183

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Unravelling the neuropeptidome of the ornate spiny lobster *Panulirus ornatus*: A focus on peptide hormones and their processing enzymes expressed in the reproductive tissues

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A R T I C L E I N F O

Keywords:
Adipokinetic hormone/corazonin-like peptide
Neuropeptide
Decapod
Prohormone convertase

A B S T R A C T

Neuropeptides are commonly produced in the neural tissues yet can have effects on far-reaching targets, with varied biological responses. We describe here the neuropeptidome of the ornate spiny lobster, *Panulirus ornatus*, a species of emerging importance to closed-system aquaculture, with a focus on peptide hormones produced by the reproductive tissues.

Transcripts for a precursor to one neuropeptide, adipokinetic hormone/corazonin-related peptide (ACP) were identified in high numbers in the sperm duct of adult spiny lobsters suggesting a role for ACP in the reproduction of this species. Neuropeptide production in the sperm duct may be linked with physiological control of spermatophore production in the male, or alternatively may function in signalling to the female.

The enzymes which process nascent neuropeptide precursors into their mature, active forms have seldom been studied in decapods, and never before at the multi-tissue level. We have identified transcripts for multiple members of the proprotein convertase subtisilin/kexin family in the ornate spiny lobster, with some enzymes showing specificity to certain tissues. In addition, other enzyme transcripts involved with neuropeptide processing are identified along with their tissue and life stage expression patterns.

1. Introduction

Neuropeptides, or peptide hormones, evoke responses that are varied and can have fundamental consequences by altering the behaviour or physiology of an individual. Usually produced by the tissues of the nervous system following a change detected in the external environment or in the internal milieu, neuropeptides are initially formed as a prohormone precursor which undergoes processing into the mature, active peptide (Christie et al., 2010). Subsequently, the active form of a peptide will bind to a receptor (usually from the G-protein coupled receptor (GPCR) family) to initiate a cascade within the target cells (Caers et al., 2012; Mykles et al., 2010). The target cells will then provide a response that will either maintain homeostasis or allow a new state to be acquired.

From the crustacean perspective, neuropeptides have been studied for a range of reasons. For example, in economically important species such as *Sagmariasus verreauxi* (Ventura et al., 2014), *Panulirus interruptus* (Ye et al., 2015), *Macrobrachium rosenbergii* (Suwansa-ard et al., 2015), *Scylla paramamosain* (Bao et al., 2015), *Cherax quadricarinatus* (Nguyen et al., 2016), *Nephrops norvegicus* (Nguyen et al., 2018), and *Eriocheir sinensis* (Liu et al., 2019), neuropeptides have been identified for the advancement of aquacultural practices. Crustacean neuropeptides can also be investigated for the greater understanding of ubiquitous processes found in all animals, for example to study disease (Buchberger et al., 2020) and neural circuitry (Marder and Bucher, 2007; Skiebe, 2001), although their roles in controlling moulting (Mykles, 2021; Oliphant et al., 2018) and aspects of reproduction (Chandler et al., 2016; Ventura et al., 2009; Ventura et al., 2012) are also becoming well defined.

The central nervous system in crustaceans comprises the supra-esophageal (brain), thoracic and eyestalk ganglia which are major sites for neuropeptide production (Hopkins, 2011), although peripheral tissues may also be important sites for their transcription, translation and processing (Christie et al., 2010). Other neuroendocrine organs, where

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https://doi.org/10.1016/j.ygcen.2022.114183
Received 14 June 2022; Received in revised form 21 November 2022; Accepted 24 November 2022
Available online 28 November 2022
0016-6480/

neuron terminals can access the haemolymph, include: the pericardial organ, the anterior cardiac plexus and the anterior commissural organ – the latter two being associated with the stomatogastric nervous system (Hsu et al., 2006). Expression patterns for the site of production of particular neuropeptides can vary between species.

The first neuropeptide sequenced in crustaceans was red pigment concentrating hormone (RPCH) (Fernlund and Josefsson, 1972), related to the pigmentary-effector hormones (PDH) (Rao and Riehm, 1989) and similarly, the receptor for RPCH was the first GPCR to be characterised pharmacologically in a crustacean (Marco et al., 2017). The octapeptide structure of RPCH is conserved across crustacean species (Gaus et al., 1990), and is related to adipokinetic hormones (AKHs) (Gade, 2004; Gade and Marco, 2009; Kaufmann et al., 2009) from insects. Other neuropeptides related to RPCH are corazonin (Veenstra, 1989) and the more recently discovered adipokinetic hormone/corazonin-like peptide (ACP) (Hansen et al., 2010), together making up a group with similar structures yet very different functions that are related to the reproductively relevant gonadotropin releasing hormone family (GnRH). Members of the AKH/RPCH family may themselves have roles in reproduction, for example, RPCH precursor transcripts are present in the ovary of the green shore crab, *Carcinus maenas* (Alexander et al., 2018) and RPCH may be involved in the production of methyl farnesoate in some species of crustaceans (Rao and Riehm, 1989).

Another important neuropeptide found in crustaceans, the insulin-like androgenic gland hormone (IAG) (Mareddy et al., 2011; Ventura et al., 2009), is produced by the androgenic gland situated at the base of the fifth walking leg in male decapods, and is involved with the production and maintenance of male characteristics. Therefore, peptides involved in controlling aspects of reproduction are not produced solely in the central nervous system of crustaceans. Other neuropeptides with roles in crustacean reproduction include: crustacean female sex hormone (CFSH) (Jiang et al., 2020; Zmora and Chung, 2014) and vitellogenesis inhibiting hormone/gonad inhibiting hormone (VIH/GIH) (De Kleijn et al., 1998; De Kleijn et al., 1992), although these neuropeptides can also produce responses that are not directly related to maturation or maintenance of the reproductive tissues. Maturation and maintenance of gonads and secondary sexual characteristics and reproductive behaviour are all important in ensuring that reproduction occurs successfully and these can be controlled or governed by neuropeptide action. With that in mind, we sought to identify neuropeptide precursor transcripts in different tissues of the ornate spiny lobster, *Panulirus ornatus*, as a species of interest to the development of on-shore aquaculture systems. Recently, transcriptomes were produced for nineteen different tissues from *P. ornatus* (Ventura et al., 2020). Factors relating to the sexual development of this species were identified and mapped to their sites of production in different tissues, including IAG, RPCH and CFSH as well as members of the crustacean hyperglycemic hormone (CHH) family. The transcriptomes of *P. ornatus* embryo (Lewis et al., 2022) and larval stages (Hyde et al., 2020b) have also been produced with those studies focusing on changes in neuropeptide expression that happen during development of this species. In the present study, we mine the transcriptome of *P. ornatus* produced by Ventura et al. (2020) and demonstrate the expression pattern of additional precursor neuropeptides in adult and juvenile tissues, with a focus on those neuropeptides that are present in tissues related to reproduction such as the ovary, oviduct, testis and different regions of the sperm duct. Identification of neuropeptides in these reproductive tissues could lead to strategies for manipulating sexual development in these animals for aquacultural purposes.

As well as the expression of neuropeptide precursors themselves, enzymes which are involved with their production are helpful to understanding the significance of the neuropeptidome: these enzymes which ultimately permit the processing of the precursor into mature neuropeptides. For example, enzymes that are involved with processing particular neuropeptides that regulate moulting or germ cell maturation in crustaceans would be fundamental for the processes of growth or fecundity, respectively, and may be of interest in aquaculture. The maturation of neuropeptides involves several steps occurring in the endoplasmic reticulum, *trans* golgi network and secretory vesicles (De Haes et al., 2015): removal of the signal peptide by signal peptide peptidase (SPP), cleavage of the nascent precursor peptide by prohormone convertase (PC), trimming of the remaining C-terminal residues by carboxypeptidase (CP), amidation of the C-terminal glycine residue by peptidylglycine-α-amidating monooxygenase (PAM) (or by peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine-α-amidating lyase (PAL) at least in *Drosophila*: Kolhekar et al., 1997), and finally cyclization of the first glutamine residue to pyroglutamic acid by glutaminyl cyclase (QC) (Fricker, 2005; Hook et al., 2008). Other processes are involved in the maturation of some neuropeptides, for example the sulfation of tyrosines in sulfakinin (Nachman et al., 1986).

The first step in neuropeptide precursor processing involves proprotein convertases (PCs), the "master switches" of the cell, which are therapeutic targets in diseases such as cancer and endocrinopathies (Klein-Szanto and Bassi, 2017). Seven members of the proprotein convertase subtisilin kexin superfamily, Ca^{+} dependent serine proteases involved with processing peptides (including growth factors, receptors, cell adhesion molecules and hormones) into their active forms, are currently known in mammals (Thomas, 2002) and vary in their C-terminal regions (Zhou et al., 1999). Two additional members of the PC family have been discovered in mammals, which have slightly different functions to the other PCs (Seidah et al., 2008). Recently, the presence of PCs in invertebrate groups have been examined in more detail, with differences highlighted in their functions between vertebrates and invertebrates, as well as differences in the variety of PCs found even within the insect family (Fritzsche and Hunnekuhl, 2021).

Other peptides are involved with the regulation of PCs themselves such as 7B2, which is involved with the regulation of a neuropeptide PC, named PC2, in mammals (Westphal et al., 1999), *Caenorhabditis elegans* (Lindberg et al., 1998) and *Drosophila melanogaster* (Hwang et al., 2000). In crustaceans, there has been limited work on the enzymes which process neuropeptides, and they have not been looked at previously in any tissues other than neural tissues to our knowledge. PC2 has been identified in a handful of decapod species (Tangprasittipap et al., 2012; Toullec et al., 2002) and recently PC2 along with other biosynthetic enzymes have been identified in *Homarus americanus* (Christie et al., 2015) thus it is an emerging field for crustacean research. In the present study, as well as identifying the neuropeptide precursor transcripts and their distribution within *P. ornatus*, we report the occurrence of neuropeptide processing enzyme transcripts for the first time in this species.

2. Materials and methods

2.1. Transcriptome mining

A transcriptomic library was produced previously by Ventura et al. (2020) for multiple tissues taken from adult and immature ornate spiny lobsters (*Panulirus ornatus*: three males and three females for each life stage). The tissues used were: testis (3 immature, 3 mature), ovary (3 immature, 3 mature), hepatopancreas (2 for immature male, 3 for mature male, 3 each for immature and mature females) and samples from one adult of each sex of eyestalk, brain, thoracic ganglia, antennal gland, sperm duct (proximal, medial and distal), oviduct, gill (anterior and posterior), heart, midgut, hindgut, tail muscle, epidermal tissue, fat tissue and hemocytes. The FASTQ files are available in the NCBI SRA database (PRJNA903480). The neuropeptide precursor sequences are presented in Supplementary Table S2.

2.2. RT PCR and qPCR validation of ACP expression

Quantitative PCR was carried out to validate the unexpectedly high *in silico* expression observed for ACP in the sperm duct. Tissues were dissected from three male adult lobsters (1.5 to 2 kg) obtained from the

Institute for Marine and Antarctic Studies (IMAS) in 2021. The tissues were snap frozen, shipped on dry ice to the University of the Sunshine Coast and stored at −80 °C. RNA was subsequently extracted from the brain, eyestalk, thoracic ganglia, testis, antennal gland, testis, tail muscle, hepatopancreas and medial sperm duct (n = 3 for each tissue) using RNAzolRT (Sigma) according to the manufacturer's instruction with the addition of BME to RNAzolRT. Synthesis of cDNA was then carried out (Tetro cDNA kit, Bioline) using 1 μg RNA (Ventura et al., 2015) and random hexamers. Reverse transcription PCR and qPCR were carried out using ACP specific primers (Table 1). For qPCR, forward and reverse primers were designed (Roche Assay Design Centre website) and mixed with FastStart Universal Probe Master (Rox; Roche Diagnostics GmbH), cDNA and the appropriate Universal ProbeLibrary Probe (Roche). Primers for TRL-18S were used as control to normalise the relative target gene expression and cDNA was diluted 1:100 for the 18S amplification. The formula $2^{-\Delta\Delta CT}$ was applied, where CT was the cycle threshold before the fluorescence related to amplicon concentration plateaued. Each sample was run in duplicates and assessed for reliable reproducibility by assuring <1 CT difference in each duplicate. The relative expression was normalised based on the 18S expression which was used to calculate the relative expression of ACP in selected tissues.

2.3. Annotation of neuropeptide processing enzymes

Peptide sequences of known processing enzymes from arthropod species were obtained from NCBI and used to blast the *P. ornatus* transcriptome using a tBLASTN search on CrustyBase (Hyde et al., 2020a). The protein sequences of the putative processing enzyme transcripts from *P. ornatus* were examined further by phylogeny (MEGA 7.0.21 (Kumar et al., 2016)) and by inspection of their domain architecture (SMART https://smart.embl-heidelberg.de/) and through analysis of relevant matches following protein BLAST of all NCBI non-redundant protein sequences. For the phylogenetic analyses, amino acid sequences were aligned by ClustalW and a Neighbour-Joining tree was constructed using partial deletion of any gaps in the data, and evolutionary distances calculated using the Poisson correction method with bootstrap tests using 1000 replicates performed (MEGA7).

For PC1/3, furin1 and furin2, amino acid sequences from invertebrate species collated recently by Fritzsche and Hunnekuhl (2021) were used to identify potential *P. ornatus* furin sequences. 18 contigs from *P. ornatus* with similarity to the furin1 amino acid sequence of *Tribolium castaneum* were identified using a tBLASTN search on CrustyBase. These sequences were aligned using MEGA7 using ClustalW alongside amino acid sequences from other invertebrates including crustacean species identified from blast searches on NCBI. The clustering of these sequences visualised using a neighbour-joining tree as described above with known or presumed furin1, furin2, PC2 and PC1/3 sequences from other species allowed identification in *P. ornatus*. Accession numbers for the sequences used to construct the tree are available in Supplementary Table S1 and nucleotide and peptide sequences of *P. ornatus* sequences for the processing enzymes are available in Supplementary Table S3.

Table 1
Primer sequences used in this study.

Primer name	Primer sequence	Amplicon size	qPCR Probe #
qPCR primers:			
qTRL_ACP_F_21	TAGGCTGGCAAGTGATGACA	73	21
qTRL_ACP_R_21	CGGGAGAAGGTGATCTGTG	73	21
qTRL_18S_F_49	AACGGACTTGACGGTTGGTT	70	49
qTRL_18S_R_49	CTGTTCGGAGCCTGACAGAA	70	49
RT-PCR primers:			
Po-ACP_F_526	CTAGACACACCAGCACTCCA	526	–
Po-ACP_R_526	GGAGGAGGTGTGAGAGGATG	526	–
TRL_18S_F	GGTGCATGGCCGTTCTTA	95	–
TRL_18S_R	ATCAGGGTCCCTCGACTAGTTA	95	–

For other processing enzymes involved with neuropeptide maturation, homologue nucleotide sequences from *Homarus americanus* (Christie et al., 2018) were obtained from NCBI, translated using Expasy Translate (https://web.expasy.org/translate/) and submitted to a tBLASTN search of *P. ornatus* adult tissues using CrustyBase as mentioned previously. The top hits were assessed for redundancies in the dataset by alignment of the amino acid sequences using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

The digital expression patterns of the processing enzymes were identified in metamorphic life stages and embryonic stages of *P. ornatus* using transcriptomes generated previously by Hyde et al. (2019) and Lewis et al. (2022) respectively.

2.4. Mapping analyses of transcripts

To validate the digital expression quantification of particular genes of interest, in particular ACP and PC2, unassembled read libraries (FASTQ files) of specific tissues, such as the medial sperm duct and eyestalk, were mapped to the particular nucleotide sequences (Supplementary Table S2 or Supplementary Table S3) using CLC Genomics Workbench 8.0.3 (CLC; Qiagen, Chadstone, VIC, Australia). The parameters used were: similarity fraction 0.9 and map-randomly.

2.5. Statistical analysis

All data are represented as mean values ± standard error of the mean (SEM). The $2^{-\Delta\Delta CT}$ values following qPCR analysis were subjected to Kruskal-Wallis test to identify significant differences between the relative ACP expression in the different tissues at $P < 0.05$ (SPSS).

3. Results

3.1. Neuropeptide identification and expression pattern

Sixty-nine neuropeptide precursor transcripts were identified in *P. ornatus* (Fig. 1). The brain, eyestalk and thoracic ganglia, as components encompassing the nervous system, exhibited the highest expression for most of those neuropeptide precursors as expected. Neuropeptide precursors which had particularly high expression of transcripts (over 10,000 RPKM) in the neural tissues were: crustacean hyperglycaemic hormone-1 (CHH-1), moult-inhibiting hormone-3 (MIH-3), neuroparsin-2 (NP-2), neuroparsin-4 (NP-4), neuropeptide F (NPF2), proctolin, prohormone-4 and SIFamide. NP-2 precursor transcripts were found at the highest level of all neuropeptides (136506 and 140,901 RPKM in male and female brain respectively). CHH-1 and MIH-3 precursor transcripts were found produced almost exclusively in the eyestalk, although low levels (120 RPKM) were found in the female antennal gland, anterior gill (107 RPKM) and tail muscle (210 RPKM) and in the male midgut (133 RPKM). Some peptide hormone precursor transcripts were almost ubiquitous throughout the animal: nesfatin, NP-1, NP-2 (although not in the antennal gland), NP-4, pigment dispersing hormone 3 (PDH3), phoenixin and prohormone-4 (although not in the hepatopancreas). Trissin and vasopressin precursor mRNA were found at very low amounts in most tissues, but were not at all in the heart and midgut. The gills, as well as the heart and midgut seem to produce certain neuropeptide precursors at high levels. For example, precursors of NP-1 and NP-4 were produced at high amounts in the anterior gill, with NP-4 additionally produced in the heart, midgut and hindgut. NP-2 RNA was present in the epithelial tissue of both sexes, as well as being produced in the neural tissues.

3.2. Sex-biased expression of neuropeptides in the neural tissues

Approximately half of the neuropeptide precursor transcripts had higher expression in the males than in the females, when considering the brain tissue (Fig. 1). Transcripts for calcitonin-like neuropeptide,

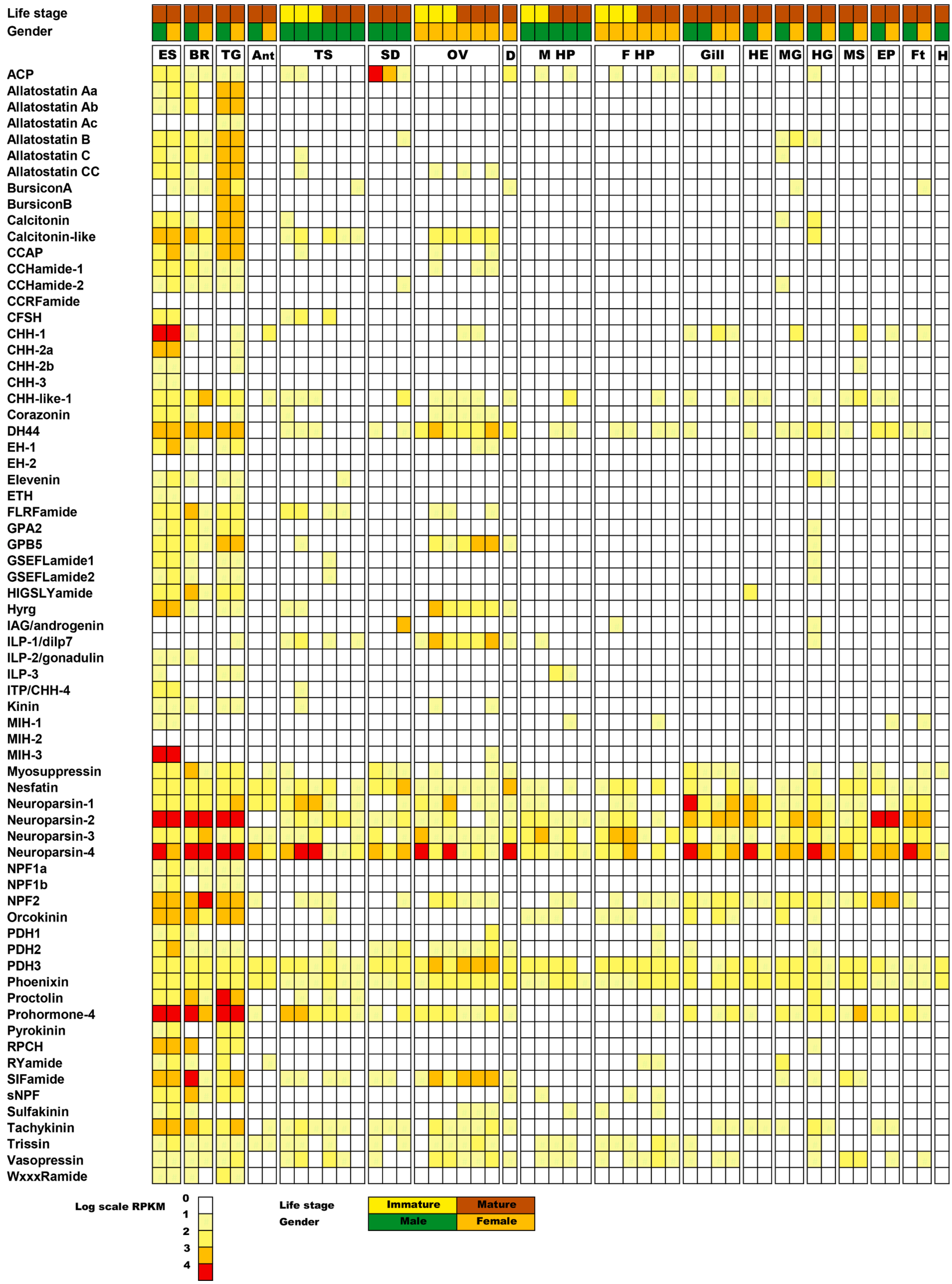

Fig. 1. Digital expression pattern of neuropeptides across tissues in the ornate spiny lobster, *Panulirus ornatus* using a multi-tissue transcriptome from Ventura et al. (2020). Expression in reads per kilobase million reads (RPKM) represented by gradient shading using a log scale. The tissues are: eyestalk (ES), brain (BR), thoracic ganglia (TG), AnG (antennal gland), testis (TS), sperm duct (proximal, medial, distal, SD), ovary (OV), oviduct (D), male hepatopancreas (M HP), female hepatopancreas (F HP), gill (anterior and posterior), heart (HE), midgut (MG), hindgut (HG), tail muscle (MS), epidermis (EP), fat tissue (Ft), hemocytes (H). CHH-1 to 3, CHH-like-1, CFSH, IAG, ILP-1, ILP-2, GPA2, GPB5, MIH-1 to 3 and RPCH expression patterns have been shown previously (Ventura et al., 2020). Sequences for neuropeptide precursors are presented in Supplementary Table S2.

FLRFamide, HIGSLYamide, myosuppressin, orcokinin, proctolin, prohormone-4, RPCH, SIFamide, sNPF and tachykinin precursors were higher in the male than in female brains (Fig. 1). CFSH transcripts were more highly expressed in the female than in the male eyestalk, although present at only low levels in the testis and nowhere else (Fig. 1). Indeed, 12 of the neuropeptide precursor transcripts found in the eyestalk were elevated in the female whilst only 1 (NP-4) was higher in males than in females.

3.3. Neuropeptide expression in the reproductive tissues

The expression pattern of neuropeptide precursor transcripts was examined in the testis and ovary of both adult and immature *P. ornatus* to identify any reproductively-relevant peptide hormones. The sperm duct (proximal, medial and distal) and oviduct of mature individuals were additionally used for transcriptomic analysis in this species. In the ovary, NP-4 was the main neuropeptide precursor transcript produced, particularly in immature females and it was in addition found in the oviduct at far higher levels than any other neuropeptide (Fig. 1). PDH3 and ILP-1/dilp7 transcripts were expressed in the ovary at higher levels than in the neural tissues, while other neuropeptide precursor RNA of note that were present in the ovary included calcitonin-like neuropeptide, diuretic hormone (DH44), GPB5, hyrg, and SIFamide (Fig. 1).

Transcripts of precursors for prohormone-4, NP-4 and NP-1 were more highly expressed in the testis of immature spiny lobsters, with lower amounts present in the testis of mature *P. ornatus*. Prohormone-4 and NP-4 precursors were expressed to a far greater extent in the neural tissues than in the testis, while for NP-1, expression was comparable to that seen in the thoracic ganglia, brain and eyestalk of both sexes with surprisingly high levels produced in the male anterior gill (Fig. 1).

Two neuropeptide precursors were expressed at over 1000 RPKM in the oviduct: nesfatin and NP-4. The three regions of the sperm duct did not express many neuropeptide precursors at all, with transcript amounts of nesfatin (in distal sperm duct) comparable to those in the oviduct, and NP-4 at lower levels than in other tissues (Fig. 1). Most notably and intriguingly, the expression of adipokinetic hormone/corazonin related peptide (ACP) precursor was far higher (10286 RPKM) in the proximal sperm duct, and medial sperm duct (9746 RPKM) than in any of the neural tissues (151, 39, 15 RPKM in the male eyestalk, brain and thoracic ganglia, respectively). For this reason, we decided to delve further into the presence of the ACP precursor peptide mRNA in the sperm duct of *P. ornatus*.

3.4. Validation of ACP expression pattern

Using RT-PCR and qPCR, the high level of expression of ACP precursor in the sperm duct that was seen in the digital expression map was validated alongside ACP expression in selected other tissues as a comparison (Fig. 2 and Supplementary Information Figure S1). The ACP precursor amplicon was present to a lesser extent in the eyestalk, brain, thoracic ganglia and not at all in the antennal gland or hepatopancreas of male spiny lobsters, using RT-PCR (Supplementary Information Figure S1). The differences in ACP precursor expression of the tissues were significant ($H_7 = 17.55$, $P = 0.014$) with medial sperm duct and thoracic ganglia both expressing higher levels of ACP precursor transcript than hepatopancreas, antennal gland and tail muscle following qPCR (Fig. 2). In addition, mapping of the raw reads was carried out using the ACP nucleotide sequence as a reference, indicating that there were considerably more reads and therefore likely to be higher expression of the ACP precursor in the medial and proximal regions of the sperm duct than in any of the other tissues (Supplementary Information Figure S6).

3.5. Processing enzymes

To understand whether precursor neuropeptides, or prohormones, can be processed in tissues other than the neural tissues, we identified putative enzymes that may coordinate some aspects of post-translational modification in *P. ornatus* (Figs. 3 and 4) and examined their expression patterns in adult tissues, embryos and metamorphic larval stages (Figs. 5–8). Putative sequences were identified for enzymes thought to be involved in the hormone maturation process in *P. ornatus* such as SPP, PC2, PC1/3, CP, PHM, PAL and QC. Domain structure summaries for these factors are available in Supplementary Information Figures S2 and S3. A neighbour-joining tree was used to classify putative members of the proprotein convertase/furin family identified in *P. ornatus*, as well as related fragments (Fig. 3), with domains present that support their identification (Fig. 4 and Supplementary Information Figure S2). Signal peptides were identified in all prohormone convertase sequences except the fragments of two PC7-like peptide precursors (Supplementary Information Figure S2).

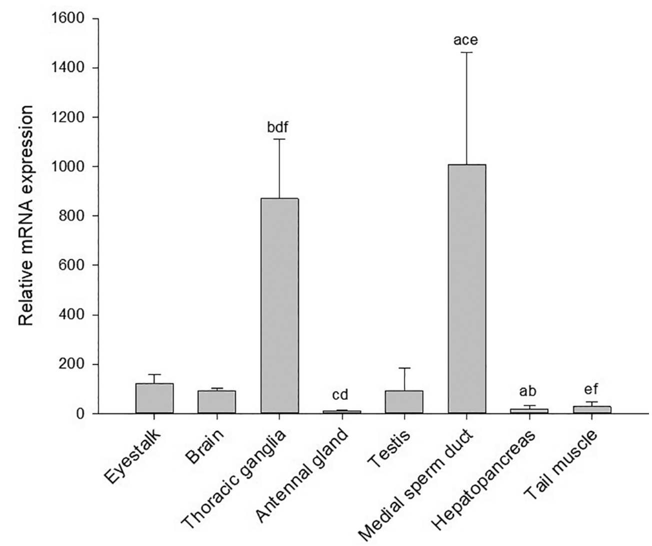

Fig. 2. Tissue specific expression of ACP in adult male *Panulirus ornatus* using quantitative PCR. Data represent mean +/- SEM, n = 3 for all tissues except testis (n = 2). Matching letters indicate a significant difference at $P < 0.05$ following a Kruskal-Wallis test (SPSS).

One PC2 peptide transcript was identified, one PC1/3 peptide transcript, and two more transcripts which show similarity to PC7 and PC1/3 and aligned with a 'furin-like protease' from *Homarus americanus* were found (Fig. 3). Four isoforms of furin 1 were found, each with varying lengths and different locations of their regions of low complexity, furin-like cysteine regions (FU domains) and transmembrane domains and were designated furin1 v1, v2, v3 and v4 (Supplementary Information Figure S2 and Table S2). *P. ornatus* possesses one furin 2 sequence, which contained an extended furin-like cysteine region in comparison to the furin 1 isoforms. PC1/3, PC2, furin 1 and furin 2 sequences identified in *P. ornatus* contained the S8 pro-domain, Peptidase_S8 and P_proprotein domains, with transmembrane regions only present in the furin and potential PC7 peptide sequences (Supplementary Information Figure S2). Two *P. ornatus* putative PC7 sequences were found which clustered with PC7 sequences from other species including three from vertebrate species, and six from other crustacean species identified using an NCBI BLAST (Fig. 3). The expression patterns and domain architecture for putative PC7 sequences from different crustacean species were examined using Crustybase and consistently comprised of a peptidase S8 domain upstream of a P proprotein domain, but without the prosegment found in the other prohormone convertases. The putative PC7 fragments were expressed mainly in the neural tissues and some chemosensory tissues of the crustaceans at low levels, and in the ovary of *P. ornatus* (data not shown).

In the other enzyme types, signal peptides were only identified in the

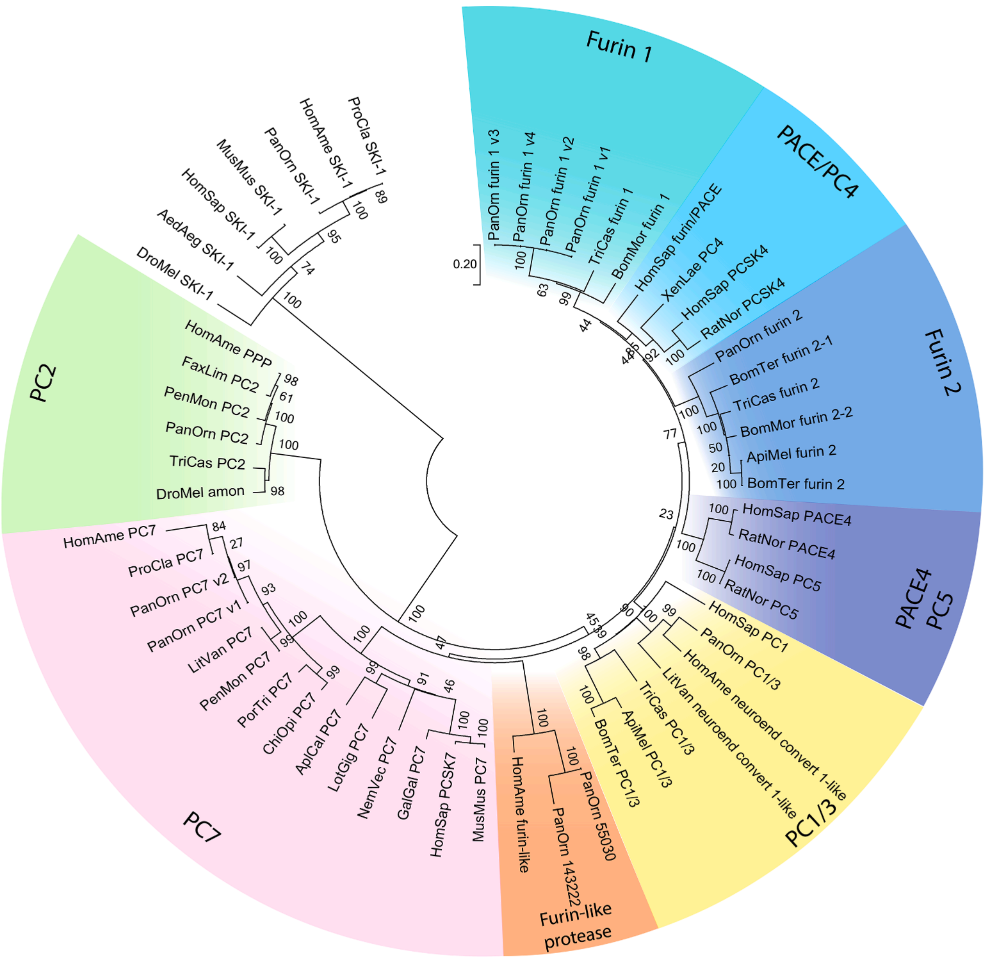

Fig. 3. Neighbour-joining tree of putative prehormone convertase enzyme genes of *Panulirus ornatus* and those of other species, with a focus on decapod crustaceans. Amino acid sequences were aligned by ClustalW and the tree was constructed using partial deletion of any gaps in the data (MEGA7). Bootstrap percentages (1000 replicates) are indicated. PanOrn = *Panulirus ornatus*, TriCas = *Tribolium castaneum*, ApiMel = *Apis mellifera*, BomTer = *Bombus terrestris*, XenLae = *Xenopus laevis*, HomSap = *Homo sapiens*, RatNor = *Rattus norvegicus*, DroMel = *Drosophila melanogaster*, PenMon = *Penaeus monodon*, FaxLim = *Faxonius limosus*, HomAme = *Homarus americanus*, LitVan = *Litopenaeus vannamei*, MusMus = *Mus musculus*, GalGal = *Gallus gallus*, ProCla = *Procambarus clarkii*, NemVec = *Nematostella vectensis*, LotGig = *Lottia gigantea*, ChiOpi = *Chionoecetes opilio*, PorTri = *Portunus trituberculatus*, AedAeg = *Aedes aegypti*, AplCal = *Aplysia californica*. Neuroend convert1-like = neuroendocrine convertase 1-like. Some sequences were used from Fritzsche and Hunnekuhl (2021), *e.g. Lottia gigantea*, *Nematostella vectensis*, *Tribolium* and other insect species, as well as some SKI-1 sequences. Crustacean species sequences were identified from blast searches in NCBI. SKI-1 was used as an outgroup (subtisilin/kexin isozyme 1).

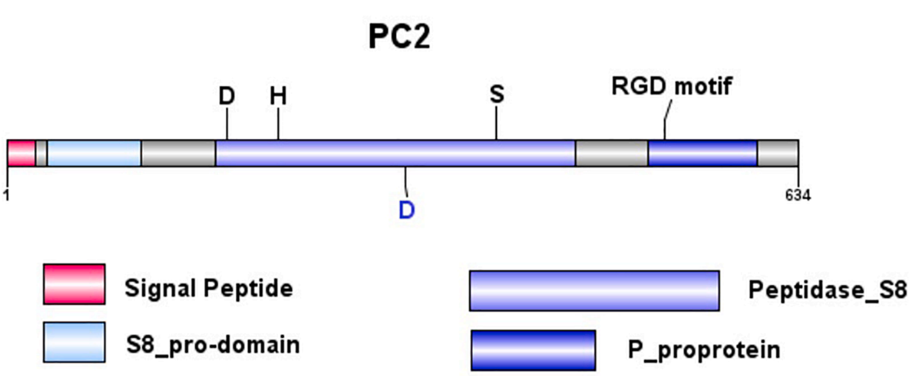

Fig. 4. Domain structure of putative proprotein convertase PC2 from *Panulirus ornatus*. Signal peptide, S8 pro-domain, Peptidase S8 and P proprotein domains were identified using SMART. The position of the catalytic triad residues (D, H and S) are indicated above the diagram along with the RGD motif. The position of the D (Asp) residue shown in blue may be the residue of the oxyanion hole. The positions of these residues was found by comparison with those of *Penaeus monodon* in Tangprasittipap et al. (2012). Diagram was drawn using DOG 2.0 (Ren et al., 2009).

PAL1 isoforms, and in PAL2 but not in CP, QC or PHM. The protein 7B2 is thought to be involved with the production and inhibition of PC2 and a sequence for 7B2 was identified in *P. ornatus* which was very similar to that from *H. americanus*. A second protein, proSAAS, is involved with PC1/3 in some species, however no transcripts matching proSAAS could be identified in the spiny lobster.

3.6. Expression patterns of processing enzymes in P. ornatus tissues, embryo and larval stages

SPP, CP, QC, PHM and PAL1a and 1b RNA were ubiquitously expressed throughout adult tissues at low levels in *P. ornatus* (Fig. 5). PC2 transcripts were abundant in the thoracic ganglia however few reads mapped to the medial sperm duct (Fig. 6A, B). Thus, it is unlikely that PC2 was present in the medial sperm duct of *P. ornatus*. Transcripts of 7B2, a protein associated with the regulation of PC2, were moderately highly expressed in the neural tissues but low elsewhere (Fig. 5), providing greater support for the importance of PC2 only in the neural tissues.

Other enzymes involved with general precursor processing such as SPP, CP, QC and the amidating enzymes PHM, PAL1 and PAL2, were expressed in the medial sperm duct (Fig. 5, Supplementary Figure S5). Most importantly, this region of the sperm duct was missing PC2, PC1 and furin2 (Fig. 5, Fig. 6B and Supplementary Figure S5). The only prohormone convertase detected in *P. ornatus* medial sperm duct appeared to be furin1 following additional mapping of furin1 v1

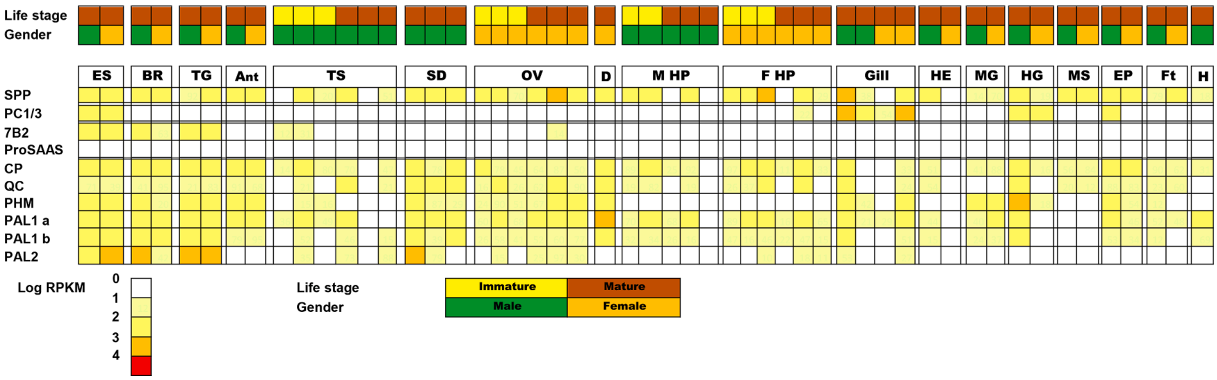

Fig. 5. Multi tissue expression patterns of processing enzymes in *Panulirus ornatus*. Digital expression pattern of processing enzymes and related proteins across tissues in the ornate spiny lobster, *P. ornatus* using a transcriptome from Ventura et al. (2020). Expression is provided in reads per kilobase per million reads (RPKM) represented by gradient shading. The tissues are: eyestalk (ES), brain (BR), thoracic ganglia (TG), AnG (antennal gland), testis (TS), sperm duct (proximal, medial, distal, SD), ovary (OV), oviduct (D), male hepatopancreas (M HP), female hepatopancreas (F HP), gill (anterior and posterior), heart (HE), midgut (MG), hindgut (HG), tail muscle (MS), epidermis (EP), fat tissue (Ft), hemocytes (H). Sequences for the processing enzymes are presented in Supplementary Table S3.

Fig. 6. Mapping graphs demonstrating the number of raw reads in selected tissues from adult *Panulirus ornatus* which map to either prohormone convertase 2 (PC2) or furin1 v1 nucleotide sequences. FASTQ libraries from the thoracic ganglia were mapped against *PC2* (A) whilst those from the medial sperm duct were mapped against either *PC2* (B) or *furin1 v1* (C).

(Fig. 6C).

During development of *P. ornatus*, many of the processing enzymes gradually increase their expression, for example PC2 and to a lesser extent PC1/3 (Fig. 7A, B). PHM and PAL transcript expression follow a similar trend (Fig. 7C, D). The transcript for PC2, once reaching a high level of expression at the end of embryogenesis and following hatching, exhibits a reduction in expression to reach a minimum level at the gut retraction phase prior to molting into the puerulus (Fig. 8A). PC1/3 similarly shows reduced expression following gut retraction, but continues this low level of expression through the puerulus phase before dramatically increasing as a juvenile (Fig. 8B). The other processing enzymes are fairly linear during the larval stages, with the exception of signal peptide peptidase (SPP), which spikes at gut retraction and in the pigmented puerulus (Fig. 8D). PAL2 shows a similar expression pattern to PC2 (Fig. 8C).

4. Discussion

4.1. Neuropeptides in the neural and reproductive tissues of Panulirus ornatus

In the present study we have used a multi-tissue approach to studying the expression pattern of precursor neuropeptide transcripts in *P. ornatus*, a decapod species important to aquaculture. As expected, transcripts encoding neuropeptides were expressed to the greatest extent in the brain, eyestalk and thoracic ganglia, fitting with the

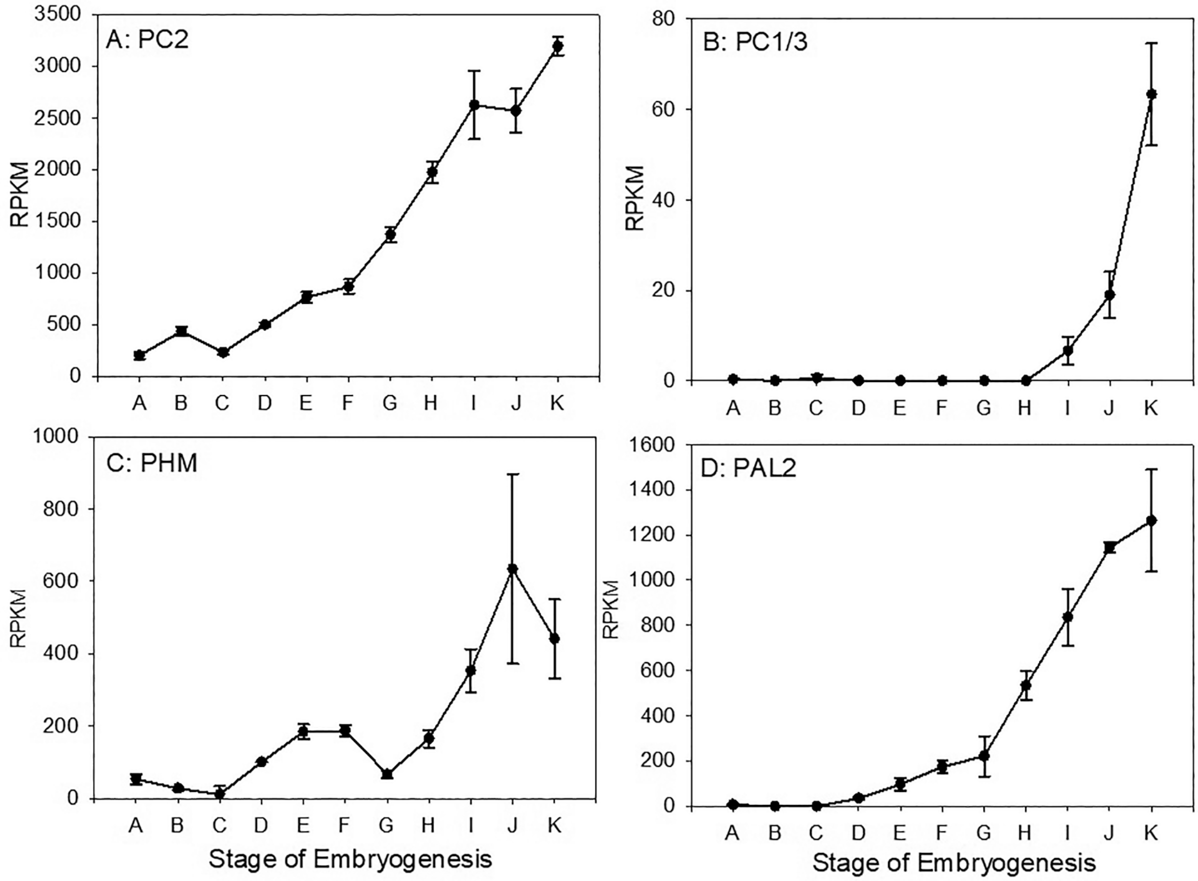

Fig. 7. Embryo stage expression patterns of selected processing enzymes in *Panulirus ornatus* using a transcriptome produced by Lewis et al. (2022). Embryo stages taken every-three days from fertilization until hatching are represented by the letters A-K where A = day 0 and K = day 30. A: Prohormone convertase 2 (PC2), B: prohormone convertase 1/3 (PC1/3), C: PHM and D: PAL2. N = 3 for each embryo stage.

generally accepted model of neuropeptide/peptide hormone production in neural tissues for endocrine or paracrine functioning.

Neuropeptide mRNA expression patterns in neural tissues can vary between the sexes in crustaceans. In the Eastern rock lobster, *S. verreauxi*, expression of EH-1 transcripts were elevated in the male neural tissues compared with females (Ventura et al., 2014), however they were higher in female *P. ornatus* in the present study. There can therefore be differences between even closely related species in the neuropeptide transcript expression patterns of different sexes, or perhaps differences in the moult stage of the individuals examined since EH regulates ecdysis in crustaceans (Zhao et al., 2022). Orcokinin transcripts were more abundant in male *P. ornatus* brains than in females, similar to that seen in *S. verreauxi* (Ventura et al., 2014). Orcokinin has a role in reproduction in insects, specifically an inhibitory role in courtship by male *Drosophila melanogaster* (Silva et al., 2021) and control of vitellogenesis and oocyte maturation in the cockroach, *Blatella germanica* (Ons et al., 2015). In crustaceans, orcokinin has a myotropic role in the hindgut of the crayfish, *Orconectes limosus* (Stangier et al., 1992) and interestingly it is present in the olfactory system of *Procambarus clarkii* (Yasuda-Kamatani and Yasuda, 2006) indicating a behavioural role in crustaceans too.

Some previous studies have examined the neuropeptide repertoire of different crustacean species in reproductive tissues as well as those from the brain and eyestalk. In the redclaw crayfish, *Cherax quadricarinatus*, transcripts encoding several neuropeptides thought to be involved with reproduction were found in tissues other than the eyestalk and central nervous system (Nguyen et al., 2016). Crustacean cardioactive peptide (CCAP), myosuppressin, pyrokinin and SIFamide were found in the ovary of *C. quadricarinatus* along with members of the neuroparsin (NP) family (Nguyen et al., 2016). In the present study, NP-4 precursors were more abundant than any other neuropeptide across multiple tissues, including the *P. ornatus* ovary and oviduct. This is perhaps not surprising, given the role of neuroparsins in vitellogenesis and gonad development in insects (Badisco et al., 2011), and in crustaceans (Kyei Amankwah et al., 2020). Indeed, in the mud crab, *Scylla paramamosain*, a neuroparsin inhibits vitellogenin synthesis although the neuroparsin mRNA was present at higher levels in the nervous tissues than in the ovary of this crab species (Liu et al., 2020). The ovary of *P. ornatus* additionally expressed mRNA of pigment dispersing hormone (PDH), a neuropeptide which has isoforms found in the ovary of other crustacean species (Huang et al., 2014) and which may have a role in reproduction (Rotllant et al., 2018). SIFamide precursor transcripts, which were found in the ovary of both *C. quadricarinatus* (Nguyen et al., 2016) and now *P. ornatus*, have been linked to aggression (Vázquez-Acevedo et al., 2009), olfaction (Yasuda-Kamatani and Yasuda, 2006) and visual processing (Polanska et al., 2007) in crustacean brains. In the mud crab, *S. paramamosain*, SIFamide in the brain is upregulated during the early vitellogenic stage (Bao et al., 2015).

In both the distal sperm duct and oviduct, transcripts for the nesfatin precursor were more abundant than in the neural tissues of *P. ornatus*. Nesfatin was previously identified in *P. ornatus* larval stages where the expression peaked during the gut retraction phase before metamorphosis of the non-feeding phyllosoma phase into the puerulus (Hyde et al., 2020b). Although nesfatin has been associated mainly with hunger in vertebrates (Stengel et al., 2011), there have been associations made between this neuropeptide and reproductive tissues (Gao et al., 2016) or reproductive processes (Gonzalez et al., 2012). Interestingly, nesfatin is an unusual neuropeptide which, along with phoenixin, may

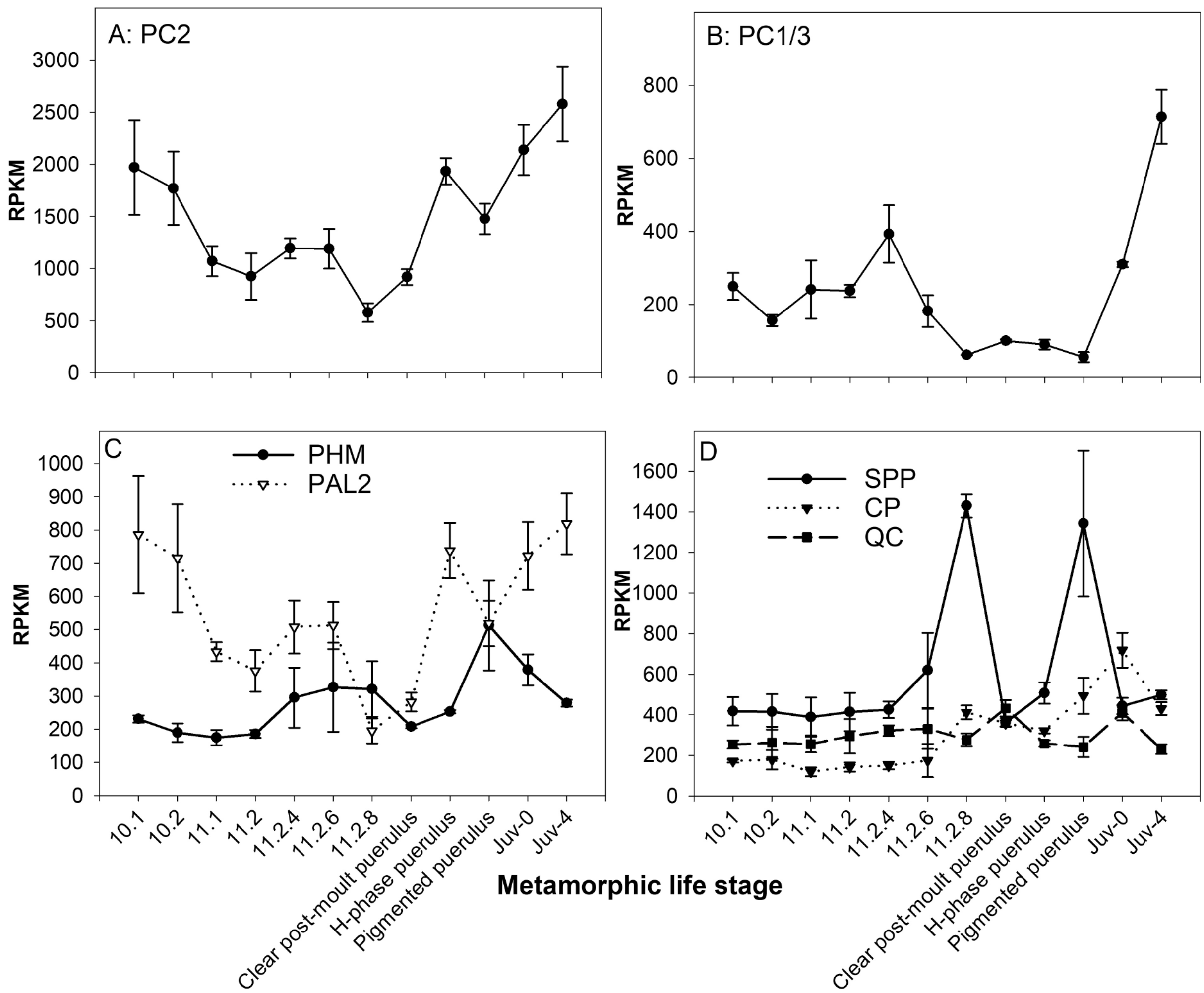

Fig. 8. Larval stage expression patterns of selected processing enzymes in *Panulirus ornatus* using a transcriptome produced by Hyde et al. (2019). Phyllosoma stages are represented by 10.1 to 11.2.8 as described by Smith et al. (2009); gut retraction occurs prior to metamorphosis into the puerulus stage (11.2 to 11.2.8); two juvenile stages were sampled at 0 and 4 days post metamorphosis from puerulus. A: Prohormone convertase 2 (PC2); B: PC1/3; C: PHM and PAL2; D: SPP, CP and QC at different metamorphic larval stages. Mean ± SEM are shown at each developmental stage (N = 3).

have evolved prior to neurons (Yañez-Guerra et al., 2022). Phoenixin was recently discovered for the first time in crustaceans (Nguyen et al., 2018) and is of interest since one of the many functions of this hypothalamic neuropeptide is related to oocyte maturation in vertebrates as reviewed by (Billert et al., 2020). In *P. ornatus*, phoenixin transcripts were present in most ovary samples at higher levels than in the neural tissues in the present study, as well as being expressed in the ovary of the Norway lobster, *N. norvegicus* (Nguyen et al., 2018) implying perhaps a function in female crustacean reproduction.

4.2. Adipokinetic hormone/corazonin-like peptide in the sperm duct of *Panulirus ornatus*

Exceptionally high expression of one particular neuropeptide transcript, the ACP precursor, in the proximal and medial regions of the sperm duct indicated a potentially novel involvement in reproduction by this peptide hormone precursor. The proximal and medial regions of the sperm duct in lobsters are made up of muscle fibre layers alongside blood vessels and it is within the proximal region that the spermatozoa are enclosed in spermatophores (Comeau and Benhalima, 2018). In the Norwegian lobster, *N. norvegicus*, the medial sperm duct (or middle vas deferens) has increased musculature compared with more proximal regions, and passes the spermatophore to the distal sperm duct (Rotllant et al., 2012).

Neuropeptides, or their precursors, which are associated with the sperm duct of crustaceans have seldom been described before. One exception is the presence of an abalone egg-laying hormone (aELH)-like peptide in the male reproductive system and spermatophore of *Macrobrachium rosenbergii*, which has sequence similarity to diuretic hormone 44 (DH44) (Kruangkum et al., 2019). In the silkworm, *Bombyx mori*, neurons innervating the sperm ducts and accessory glands were found to produce neuropeptide precursors of five different neuropeptides: calcitonin-like diuretic hormone, allatotropin and allatotropin-like peptides, allatostatin C and myoinhibitory peptides which were involved with stimulation and inhibition of seminal vesicle contractions (Čižmár et al., 2019). It is possible that the ACP precursor transcript identified in the present study may therefore be produced within nerve cells close to the sperm duct and is regulating muscle contractions involved with spermatophore release. Lipid metabolism has been verified as a function of ACP in locust muscle cells (Hou et al., 2021), and adipokinetic functions for ACP have been documented for the prawn *Macrobrachium rosenbergii* (Suwansa-ard et al., 2016) but not the crab *Carcinus maenas* (Alexander et al., 2018). Interestingly, the AKH homologue, RPCH, has roles in pyloric contractions and therefore it is not unforeseeable that the precursor ACP transcripts found in the proximal and medial sperm duct of *P. ornatus* may be involved in contractions of the muscles found in this tissue (Nusbaum and Marder, 1988).

The unique function of the sperm duct in packaging and transporting the spermatogonia for delivery to the female for reproduction to occur leads to other potential functions for ACP/ACP precursor. Following release from the sperm duct cells, the ACP/ACP precursor could be transferred *via* the seminal fluid to the female and may be involved in signalling to alter her behaviour following copulation, similar to the function of sex peptide in *Drosophila melanogaster* (Kubli and Bopp, 2012). Indeed, the closely-related AKH is one of the peptides transferred from male to female during courtship in the mosquito *Aedes albopictus* (Boes et al., 2014).

4.3. Neuropeptide processing enzymes

In order to carry out the functions suggested for ACP and other neuropeptides expressed in the reproductive tissues of *P. ornatus*, the precursor would be processed post-translationally by various enzymes. Therefore, it became important to identify whether the processing enzymes required were present in the regions of the sperm duct, and in other tissues where neuropeptide precursor transcripts were found.

Examining ACP specifically, once the precursor has been translated into mRNA, five steps involving different processing enzymes are thought to be involved in producing the active peptide, as suggested for *H. americanus* (Christie et al., 2015). Following cleavage of the signal peptide, PCs cleave 14 amino acids from the comparatively larger remaining peptide of unknown function. PCs, CP, PHM, PAL and QC enzymes are thereafter important in producing the mature peptide ready for secretion. We identified homologues for each of these enzymes in *P. ornatus* and their expression patterns in the adult tissues, embryo stages and metamorphic life stages in this species of spiny lobster.

As embryogenesis progresses in *P. ornatus*, most neuropeptide precursors gradually increase in expression, particularly between days 18 to 30 when hatching occurs (Lewis et al., 2022). In the present study, we established that there is a concomitant increase in expression of the processing enzymes involved with neuropeptide maturation during embryo development in this species of spiny lobster. During development of the larvae of *P. ornatus* there is a decrease in expression of most neuropeptide precursors between metamorphosis from phyllosoma into the non-feeding puerulus stage when the gut retracts and extensive remodelling of the neural tissues occurs (Hyde et al., 2020b). Many of the enzymes that are likely to be responsible for processing these neuropeptides during the metamorphic stages show a similar reduction in expression to their potential substrates, particularly in the cases of PC2, PC1/3, PAL2 and SPP, before increasing once more in expression in the juvenile stages. The corresponding changes in expression of the processing enzymes with the neuropeptide precursors could indicate a feedback system is in place to regulate expression of the processing enzymes required.

4.4. Proprotein convertases in Panulirus ornatus

Studies on decapod proprotein convertases (PCs) have identified the presence of PC2 in the eyestalk ganglia of the crayfish *O. limosus* (Toullec et al., 2002) and American lobster *H. americanus* (Christie et al., 2018), as a major site for the production of neuropeptides in decapods. Studies on the substrates of PC2 have been carried out in the model fly *D. melanogaster*, where PC2 (encoded by the *amontillado* gene in this species) is likely to be involved with the processing of multiple neuropeptide precursors including corazonin (Wegener et al., 2011) and AKH (Rhea et al., 2010). PC2 is likely to be involved with production of ACP in crustaceans, since the precursor peptide contains a typical cleavage site for this enzyme (Veenstra, 2000).

The number and variety of PCs varies between invertebrate and vertebrate species, and between proto- and deuterostomes. For example, *D. melanogaster* possesses 3 PCs (PC2 and two furins), while mice and humans possess 7 (Seidah and Prat, 2012), and *C. elegans* has at least 4 (Thacker and Rose, 2000). Other invertebrates contain multiple PCs (Fritzsche and Hunnekuhl, 2021; Hammond et al., 2019), with PC1/3 present in all insect groups apart from dipterans (Fritzsche and Hunnekuhl, 2021). Most invertebrate groups examined previously have PC1/3, PC2, furin1 and furin2 although few possess PC7 in their repertoire (Fritzsche and Hunnekuhl, 2021). Some studies have shown that PCs probably have similar targets to one another (Turpeinen et al., 2011) therefore it seemed prudent to identify other PCs as well as PC2 in *P. ornatus* to determine neuropeptide processing capability in the different tissues of this species.

Some functional studies in invertebrates have demonstrated specific roles for PCs. For example, PC1/3 and PC2 knockdown produced different effects on growth, moulting and fertility in the beetle, *Tribolium castaneum* (Fritzsche and Hunnekuhl, 2021). Both of these PCs are found in the neural tissues of mammals, while others such as furin are more widespread. In addition to PC2, *P. ornatus* orthologues for each of the following were identified: furin1, furin2 (related to the *T. castaneum* furin1 and furin2 amino acid sequences respectively), and a putative orthologue of the PC1/3 enzyme, related closely to neuroendocrine convertase 1-like enzymes from *L. vannamei* and *H. americanus* and which clustered with PC1/3 from *T. castaneum*. Two putative PC transcripts were identified which clustered between the PC1/3 and PC7 enzyme groups, and were similar to a furin-like protease from *Homarus americanus*. The presence of putative PC7 transcripts in *P. ornatus* is notable, since insect species examined so far do not seem to possess this prohormone convertase (Fritzsche and Hunnekuhl, 2021). PC7 has a role in memory in mice (Wetsel et al., 2013) possibly through involvement with processing the neuropeptide cholecystokinin (Anyetei-Anum et al., 2017) and is critical for brain development in *Xenopus laevis* (Senturker et al., 2012), but any function for PC7 in invertebrates remains to be discovered. In terms of reproduction-related prohormone convertases, in mammals, a PC designated PC4 is thought to be involved with fertility due to the expression of PC4 in the testis and sperm (Gyamera-Acheampong and Mbikay, 2009) however a PC4 homolog was not identified in the ornate spiny lobster.

The presence of such a complement of different prohormone convertase enzymes in *P. ornatus* may imply similar but perhaps non-overlapping roles comparable to that seen in functional studies using *T. castaneum* (Fritzsche and Hunnekuhl, 2021). In the present study, there were marked differences between the expression pattern of PC1/3 compared with the other PCs, with PC1/3 present almost exclusively in the eyestalk and gill tissues. There is some evidence from *D. melanogaster* that PCs other than PC2 may be involved in processing neuropeptides as described by Pauls et al. (2014), although PC2/AMON is involved in cleavage of the majority studied, for example in the fruit fly midgut (Reiher et al., 2011). Perhaps other PCs such as the furin homologues identified may be responsible for cleaving ACP in the sperm duct, since additional mapping analysis did not indicate the presence of PC2 in this tissue. Alternatively, perhaps the ACP precursor could be transported to the female to undergo processing, as postulated to occur for egg-laying hormone in crustaceans (Liu et al., 2009).

5. Conclusion

Using a transcriptomic approach, we have quantified the expression of neuropeptide precursors and their processing enzymes in a wide range of tissues in the ornate spiny lobster. One neuropeptide precursor, for ACP, had high expression in an unexpected tissue: the sperm duct. The expression of ACP precursor mRNA was validated in this tissue, although a likely ACP-cleaving enzyme, PC2, was absent from the sperm duct following mapping analyses of the tissue transcriptome. Further investigations into the purpose of ACP in the sperm duct of *P. ornatus* would be useful to determine whether interventions using this neuropeptide, or others identified in the reproductive tissues in the present study could be applicable to the aquaculture industry.

CRediT authorship contribution statement

Susan Glendinning: Investigation, Writing – original draft, Data curation. **Quinn P. Fitzgibbon:** Writing – review & editing. **Gregory G. Smith:** Writing – review & editing, Funding acquisition. **Tomer Ventura:** Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Australian Government with funding from the Australian Research Council (http://www.arc.gov.au/) Industrial Transformation Research Hub (project number IH190100014). The views expressed herein are those of the authors and are not necessarily those of the Australian Government or Australian Research Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2022.114183.

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