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Corresponding Author: Prof. Abigail Elizur, PhD

Corresponding Author's Institution: Univeristy of the Sunshine Coast

First Author: Vu V In, PhD

Order of Authors: Vu V In, PhD; Nikoleta Ntalamagka; Wayne O'Connor, PhD; Tainfang Wang, PhD; Daniel Powell; Scott Cummins, PhD; Abigail Elizur, PhD

Suggested Reviewers: Natasha Carr
Natasha.Carr@csiro.au
Expert in mollusc endocrinlogy and bioinformatics. Works on Abalone genomics and ELH

John Hyde PhD
NOAA
john.hyde@noaa.gov
Expert in abalone and oyster reproduction and genetics

Carlo Di Cristo
niversità degli Studi del San..., Benevento · Department of Science and Technology (DST) Follow
Expert in cephalopod neuropeptides

David Raftos PhD
david.raftos@mq.edu.au
oyster expert

Opposed Reviewers:

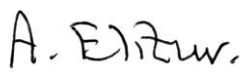
Dear Editor, 7 May 2016

I am pleased to submit our manuscript "Reproductive neuropeptides that stimulate spawning in the Sydney Rock Oyster (Sacco*strea glomerata*)" for your consideration for publication in Peptides.

In this original work we have isolated transcripts coding for neuropeptides from the gonad and ganglia, of the Sydney Rock Oyster, verified their presence using an MS analysis, and investigated their biological activity in the context of spawning induction as well as advancement of reproductive development in the oyster. We are aware that there are many studies today, which report a transcriptome analysis and identification of putative peptides, however in this case we have used *in-vivo* assays and identified peptides which offer an exciting application for the aquaculture industry.

I look forward to your response,

Kind regards



Abigail Elizur

Reproductive neuropeptides that stimulate spawning in the Sydney Rock Oyster (Sacco*strea glomerata*)

Vu Van In[a,b], Nikoleta Ntalamagka[a], Wayne O'Connor[a,c], Tianfang Wang[a], Daniel Powell[a], Scott F. Cummins[a] and Abigail Elizur[a,d]

[a] Centre of Genetics, Ecology and Physiology, University of the Sunshine Coast, Maroochydore, Queensland 4558, Australia

[b] Northern National Broodstock Center for Mariculture, RIA1, Catba Islands, Haiphong, Vietnam

[c] Industry and Investment NSW, Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, NSW, 2316, Australia

[d] Corresponding author at: Centre of Genetics, Ecology and Physiology, Faculty of Science Health and Education, University of the Sunshine Coast, Locked Bag 4, Maroochydore Dc, QLD 4558, Australia. Tel.: +61 7 54594813. E-mail address: AElizur@usc.edu.au

Abstract

The Sydney Rock Oyster, *Saccostrea glomerata,* is a socioeconomically important species in Australia, yet little is known about the molecular mechanism that regulates its reproduction. To address this gap, we have performed a combination of high throughput transcriptomic and peptidomic analysis, to identify genes and neuropeptides that are expressed in the key regulatory tissues of *S. glomerata*; the visceral ganglia and gonads. Neuropeptides are known to encompass a diverse class of peptide messengers that play functional roles in many aspects of an animal's life, including reproduction. Approximately 28 neuropeptide genes were identified, primarily within the visceral ganglia transcriptome, that encode precursor proteins containing numerous neuropeptides; some were confirmed through mass spectral peptidomics analysis of the visceral ganglia. Of those, 28 bioactive neuropeptides were synthesized, and then tested for their capacity to induce gonad development and spawning in *S. glomerata*. Egg laying hormone, gonadotropin-releasing hormone, APGWamide, buccalin, CCAP and LFRFamide were neuropeptides found to trigger spawning in ripe animals. Additional testing of APGWa and buccalin demonstrated their capacity to advance conditioning and gonadal maturation. In summary, our analysis of *S. glomerata* has identified neuropeptides that can influence the reproductive cycle of this species, specifically by accelerating gonadal maturation and triggering spawning. Other molluscan neuropeptides identified in this study will enable further research into understanding the neuroendocrinology of oysters, which may benefit their cultivation.

Keywords: Molluscs, Egg-laying hormone, Gonadotropin-releasing hormone, Mass spectrometry, Neuropeptides, *Saccostrea glomerata*, Reproduction.

Background

The Sydney Rock Oyster, *Saccostrea glomerata,* is one of the most ecologically and commercially important species of the oyster family (*Ostreidae*) in Australia. In the wild, it dominates sheltered shorelines of intertidal and immediate subtidal regions along the Eastern Australian coast. It also forms the basis of an extensive oyster industry in South-East Queensland and New South Wales. *S. glomerata* production is one of the oldest aquaculture industries in Australia and its current production has reached 7,793,390 dozen oysters (at farm gate), valued at around $34.7 million for 2014/2015 (Trenaman et al., 2015).

Critical to the production and marketing of *S. glomerata* is their physical and reproductive condition. *S. glomerata* is a protandric species, where the gonad condition cycles are broadly understood, and to some extent can be manipulated through gross environmental changes (O'Connor et al., 2008a, O'Connor et al., 2008b). However, our understanding of the molecular and biochemical processes underpinning changes in gonadal condition, as well as our capacity to monitor these changes, is limited. Thus, our ability to manipulate maturation and spawning in *S. glomerata* is also limited. The identification of neuropeptides that may regulate *S. glomerata* reproduction provides an important research area, which should contribute to our understanding of *S. glomerata* biology as well as help to facilitate hatchery production (Dove and O'Connor, 2009, Nell, 2006).

Neuropeptides are produced and released by neurons through a regulated secretory pathway (Burbach, 2011). They represent a highly diverse and multifunctional group of signalling molecules that include hormones, neurotransmitters and neuromodulators (Conzelmann et al., 2013, Stewart et al., 2014). Their roles in the molluscan physiology, behaviour and reproduction are well established (Fricker, 2012, Morishita et al. 2010), and includes APGWamide, gonadotropin releasing hormone (GnRH), and egg-laying hormone (ELH), which have each been investigated through *in vitro* and *in vivo* studies. These neuropeptides are generated from precursor sequences (Morishita et al., 2010, Nuurai et al., 2010, Cummins et al., 2011).

In the oysters, it has been demonstrated that the tetrapeptide APGWa plays a role in the reproduction of *Crassostrea gigas* female oysters, where it can induce *in vitro* adductor

muscle contraction followed by oocyte release (Bernay et al., 2006). GnRH is a well-known reproductive regulator in vertebrates, but has also been found in the CNS of bivalve molluscs (Pazos and Mathieu, 1999, Nakamura et al., 2007). GnRH-like peptides have been identified in the visceral, cerebral and pedal ganglia of scallops (*Patinopecten yessoensis*), the oyster (*C. gigas*) and the pearl oyster (*P. fucata*) (Treen et al., 2012, Bigot et al., 2012, Stewart et al., 2014). In support of their role in reproduction, there exists a highly expressed orthologue of the GnRH receptor in mature gonads of *C. gigas*, (Morishita et al., 2010). Also, *in vitro* trials in *C. gigas* and the mussel (*Mytilus edulis*) show that GnRH stimulates proliferation of gonadal cells (Pazos and Mathieu, 1999). Finally, the ELH is a well-known egg laying inducer in the aquatic snails *Aplysia and Lymnaea* (Strumwasser et al., 1987, Conn and Kaczmarek, 1989, Smit, 1998), and its gene sequence has been identified in *C. gigas* and *P. fucata* (Stewart et al, 2014). Numerous other molluscan neuropeptides exist, although their role in reproduction is less well known.

The main objective of the present study was to identify neuropeptide genes that may be important in regulating *S. glomerata* reproduction. We focused on the neural (visceral) and gonad tissues through transcriptome and peptidomic analysis and then performed *in vivo* maturation and spawning bioassays to elucidate potential reproduction-associated roles.

Materials and methods

Experimental Animals

Animals for tissue collection for RNA - Wild live adult *S. glomerata* were obtained from Port Stephens Fisheries Research Institute, New South Wales (PSFI). The stage of gonadal development of each individual was determined (stages I-V) as described by Dinamani (1974). Gonad and visceral ganglia tissues were isolated from each individual within three out of five gonadal development stages: 1) stage I - Ripening; 2) stage II - Fully ripe and 3) Stage III - Post spawning (20-25 oysters/each stage, N=70) in July, 2012. Tissues for gonad and ganglia from males and females were kept separately at -80^{o}C until used for total RNA and peptide extraction.

Animals for physiological bioassays - Live adult *S. glomerata* from wild and hatchery lines were obtained from either local retail outlets on the Sunshine coast, QLD (for bioassays carried out at University of the Sunshine Coast, USC) or from PSFI (for those carried out at PSFI). Animals were acclimatized in culture tanks for at least 24 h and fed with algae before used for the experiments.

Transcriptomics and peptidomics

The overall procedure applied in this study is shown in **Figure 1**.

RNA extraction and transcriptome sequencing

Total RNA was isolated using TRIsure™ Reagent (Bioline USA Inc.) following the manufacturer's specifications. The quality and concentration of the total RNA were checked by gel electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Total RNA of each sex was pooled separately from all developmental stages (stages I-V). Twenty micrograms of total RNA of each tissue were freeze dried and sent to BGI for *de novo* transcriptome sequencing (HighSeq 2000, Illumina, San Diego, CA), assembly and functional annotation. *De novo* assemblies were performed by SOAPdenovo software using trimmed reads from the Illumina sequencing. The assembler was run with the parameter sets as following: SeqType, fq; minimum kmer coverage = 4; minimum contig length of 100 bp; group pair distance = 250.

Bioinformatics analyses

To identify target sequences, gender-specific transcriptomes for the gonadal and visceral ganglia of *S. glomerata* were imported into the CLC Main Workbench (v7.0.2; CLC-bio, Denmark). Previously identified molluscan neuropeptide sequences were then queried (tBLASTn) against the transcriptomes. To complement this, Open Reading Frames (ORF) were retrieved from the *S. glomerata* databases and screened for signal sequences using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/). The presence of recurrent KK; KR; RK cleavage sites was identified using NeuroPred (Web-based software on http://neuroproteomics.scs.illinois.edu/neuropred.html). Multiple sequence alignments were

done by MEGA software version 6.06 (Tamura et al., 2013). Derived and amino acid sequences were aligned guided by chain cleavage sites and conserved cysteines (Brunak et al., 1991). Domain graph 2.0 and Miktex-2.9 were used to build the peptide schematics and sequence alignments, respectively. Data of other species used for alignment of amino acid sequences and schematic diagrams was obtained from the supplementary list of neuropeptides provided by Stewart et al. (2014). Web-based Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to estimate percentage of identity between *S. glomerata* peptide amino acid sequences and other mollusc species.

Reverse phase-high performance liquid chromatography (RP-HPLC)

The collected ganglia tissues were homogenized on ice in a solution of 0.1% Trifluro-acetic acid (TFA – Solution A), with subsequent sonication consisting of three times 30 s pulses separated by 20 seconds. The homogenized tissues were then centrifuged at 16,000 rpm for 20 minutes at 4°C and the supernatants were collected. This process was repeated with the pellet leftover. The extracted peptide mixture was analysed by RP-HPLC (PerkinElmar series 200 pump/autosampler, Flexar PDA detector and Chromera v3.2 software). The total collected peptides from the extractions were loaded on the HPLC. Samples were separated and eluted with a protocol of 100% to 40% solution A at a flow rate of 1 mL/min over 20 min for the synthetic peptides and 60 min for the extracted peptide mixture. Eluted compounds were detected at wavelengths of 210 nm and 280 nm. Mobile phases used were solution A (0.1% TFA) and solution B (0.1% TFA in acetonitrile). A total of 12 fractions were collected in 5 min intervals for further analysis by mass spectroscopy (MS). Control synthetic peptides were tested in RP-HPLC and observed to elute at 42.5% acetonitrile for GLDRYSFMGGI-NH_2; 43.5% acetonitrile for GMPMLRL-NH_2; 42% acetonitrile for MRYFL-NH_2; and 58.5% acetonitrile for RPGW-NH_2. Five-minute fractions were lyophilised and resuspended in 1% formic acid for MS analysis.

Mass spectrometry analysis (LC-MS/MS analysis) and protein identification

Resuspended HPLC fractions were analyzed by LC-MS/MS on a Shimadzu Prominance Nano HPLC (Japan) coupled to a Triple Tof 5600 mass spectrometer (ABSCIEX, Canada) equipped with a nano electrospray ion source. The protocol has been detailed elsewhere [25]. Briefly, approximately 6 µL of each extract was injected and de-salted on the trap column

before entering a nano HPLC column (Agilent Technologies, Australia) for mass spectrometry analysis. The mass spectrometer acquired 500 ms full scan TOF-MS data followed by 20 by 50 ms full scan product ion data. Full scan TOFMS data was acquired over the mass range 350-1800 and for product ion MS/MS 100-1800. Ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. The data were acquired and processed using Analyst TF 1.5.1 software (ABSCIEX, Canada).

Fragmentation data was analysed by PEAKS v6.0 (BSI, Canada) software. Sequences of peptides were determined by comparing the fragmentation patterns with those predicted from the *S. glomerata* transcriptomes. Search parameters were as follows: no enzyme was used; variable modifications included methionine oxidation, conversion of glutamine/glutamate to pyroglutamic acid, deamidation of asparagine and peptide amidation. Precursor mass error tolerance was set to 0.1 Da and a fragment ion mass error tolerance was set to 0.1 Da. *de novo* sequencing, database search and characterising unspecific post-translational modifications (PTMs) were used to maximise the identifications; false discovery rate (FDR) was set to ≤ 1%, and the individual peptide ion score [-10*Log(p)] was calculated accordingly, where p is the probability that the observed match is a random event.

Physiological bioassays

To investigate possible roles of identified neuropeptides in *S. glomerata* reproduction, 28 neuropeptides were selected and synthesized by China Peptides Co. Ltd for *in vivo* bioassays.

Bioassay 1 - Spawning bioassay: Farmed *S. glomerata* (grown out from wild spat) were purchased from a retail outlet on the Sunshine Coast. They were held in seawater for at least 24 h before the assay. Ten oysters examined showed a fully developed gonad and the sex ratio was approximate 50% males and 50% females. The oysters were relaxed by immersion in $MgCl_26H_2O$, 50g/L, for 4 h for the valves to open to enable the injection of peptides (40 µg/peptide/oyster). Synthesized peptides were pooled into nine different groups based on their derivation from precursors that often contains several bioactive peptides. Forty µg of each bioactive peptide within one peptide treatment group were pooled into 10 µL of distilled water (volume designed for one injection). Distilled water was used as negative control, and 10 µL serotonin (5HT), 50mM, was used as positive control, since it has been shown to induce

spawning in several bivalve species (Matsutani and Nomura, 1987, Gibbons and Castagna, 1984, Ram et al., 1993). Peptides were injected into the adductor muscle of *S. glomerata* (n=10/group) with a Hamilton syringe (10R-GT syringe).

After each injection, oysters were placed individually into 540 mL plastic containers, which were randomly distributed to avoid any environmental influence. Electric fans were used to ventilate the containers. Spawning and fecundity were determined by screening for the presence of eggs in each container 12 h post-injection using a stereoscope (Motic SMZ140-Labled).

Bioassay 2 - Maturation bioassay: This bioassay was designed to investigate the involvement of identified neuropeptides at earlier developmental stages of *S. glomerata* gonad by implantation cholesterol pellets that contained 50 µg (0.05mg) of either APGWa or buccalin and neuropeptide-free implants for negative control. Pellets were implanted into the adductor muscle using a chip implant applicator (Trovan Ltd. Germany). In total, three treatments, including blank (negative control; n=24), APGWa (n=24) and buccalin (n=24) were tested. *S. glomerata* were obtained from PSFI. To enable implantation, oysters were relaxed by immersion into $MgCl_26H_20$ (50g/L).

Fifty cholesterol implants (size: 1.5x 3 mm, weight: 5 mg) were made using a mixture containing 230 mg cholesterol + 15 µL copha + 10 mg peptides [(0.05 mg RPGWa + 0.05 mg KPGWa + 0.05 mg SPGWa + 0.05 mg APGWa)/each implant x 50 implants = 10 mg)] were used. Each final implant weighed 5 mg and contained 0.2 mg of peptides. Blank implants were made using the above mixture minus the peptides. The same procedures were applied to make 50 buccalin implants that contain four distinct buccalin, 0.05 mg each (ALDRYSFFGGL-NH_2, ALDKYGFFGGI-NH_2, GLDRYSFMGGI-NH_2, GLDRYSFMGGI-NH_2 and GLDRYGFAGSL-NH_2).

Only oysters with spent gonads were used for this Bioassay. Ten oysters were sacrificed at the start to confirm their gonad-spent condition and determine sex ratio, and approximate 90% of them showed their gonad at totally spent stage, the rest were at ripening stage (Stage I, Dinamani, 1974) with sex ratio of approximate 50% male and 50% female. Implanted oysters were then reared in three tanks containing 200L of seawater at room temperature (24-25 ^{0}C), 24 oysters per tank with 100% water exchange every two days. Oysters were fed three times per day with a mixture of algae (*Chaetoceros muelleri*; *Isochrysis* sp. and *Pavlova lutheri*) to maintain 10^5 algal cells/mL. Oysters from each group (n=24) were sampled at four weeks post-

implantation for determination of condition index (CI) and gamete quality by fertilization efficiency. For CI estimate, oysters were weighed individually for whole weight, then sacrificed and weight for shell and soft tissue (meat weight) was measured for condition index as described by (Lawrence and Scott, 1982):

$$CI = \frac{Softtissueweight(g)}{Wholeweight(g) - Shellweight(g)} x1000$$

To assess gamete quality, fertilization rate was determined, where eggs and sperm were stripped from the aforementioned oysters and stored individually in separate 540 mL plastic containers containing 250 mL filtered seawater. To make sure the egg density was even, the same volume of water (250 mL) and eggs was used for fertilization. Male gonads were kept on ice before use for fertilization in order to keep good quality of sperm. For egg fertilization, three fully ripe males and five females were selected from farmed oysters from an outside population as a reference group and their sperm and eggs were striped and held separately. Before use, reference sperm or eggs were mixed thoroughly. The same volume of sperm or eggs was used for each experiment. Eggs and sperm were mixed gently and left at room temperature (24-25^{o}C) for 3-4 h (**Figure** B), ventilated by electric fan. Fertilization was determined by microscopic observation of cell division, while unfertilized eggs tended to be degraded or partially damaged or broken. Count of fertilized eggs was done for 30 oysters, 10 for each treatment including negative control, APGWa and buccalin.

Data on condition index and fertilization rates was statistically analysed using one-way ANOVA followed by Tukey's test on SPSS 22 (SPSS statistics 22, 2013).

Results

Transcriptome summary

Visceral ganglion RNA-seq provided 58 million nucleotide reads (SRAXXXXXX – to be added upon acceptance), that were assembled into 124,250 and 75,122 contigs, and 68,271 and 41,686 unigenes for male and female, respectively. Gonad RNA-seq provided 37 million nucleotide reads (SRAXXXXXX – to be added upon acceptance), that were assembled into 100,109 and 85,668 contigs, and 47,033 and 37,54 unigenes for male and female, respectively.

From the unigene database, open reading frames were obtained then used for BLAST identification of mollusc neuropeptides.

Neuropeptides identified from transcriptomes and LC-MS/MS analysis

In total, 28 neuropeptide precursors, including 11 putative full-length and 17 putative partial-length neuropeptide precursors were identified within the gender-specific visceral ganglia and gonad transcriptomes (File S1). From these precursors, numerous bioactive neuropeptides are predicted to be released. The majority of neuropeptides were identified from the visceral ganglia transcriptome (22 neuropeptides) and less from the gonad transcriptome (12 neuropeptides) (Table 1). A few neuropeptides were identified within only one sex. Although the majority of previously known molluscan neuropeptides were identified, no ELH or GnRH peptide precursor was found by LC-MS/MS. However, the ELH and GnRH precursors were identified within additional transcriptomic data, (Ertl et al. 2016), and a draft *S. glomerata* genome, respectively. Several of the identified neuropeptides were synthesised for the *in vivo* bioassay (**Table 1**).

To investigate the presence of neuropeptides, peptides were extracted from the visceral ganglia of female *S. glomerata* and separated by RP-HPLC (**Figure 2**). Fractions were collected between 5-65 minutes for LC-MS/MS analysis. Neuropeptides derived from 11 precursors were identified (**Table 1**), including APGWamide, Buccalin, FCAP, FMRFamide, FXRlamide, GGN, Myoinhibitin, Myomodulin 2, RxIamide, SCAP and Tachykinin. The APGWa neuropeptide precursor was identified through 2 matching peptides, both present with collected HPLC fraction 2 (**Figure 2B**). The buccalin neuropeptide precursor was identified through 3 matching peptides, distributed throughout fractions 2, 3 and 4 (**Figure 2B)**.

Egg Laying Hormone (ELH)

The *S. glomerata* ELH (Sg-ELH) transcript encodes a 169-residue precursor protein that is likely cleaved to produce two separate bioactive ELH peptides: ELH1 and ELH2 (**Table 1, Figure 3A**). Comparison with other known oyster ELH **(Figure 3A)** shows that the Sg-ELH precursor shares more similarity with *C. gigas* ELH (Cg-ELH; 64.4%) than *P. fucata* ELH (Pf-ELH; 40.0%). High identity is found within the bioactive neuropeptide regions (**Figure 3B**); 88.8% with Cg-ELH1, 2 and 53.8% with Pf-ELH1,2.

Gonadotropin Releasing Hormone (GnRH)

A precursor of *S. glomerata* GnRH (Sg-GnRH) was identified that contains an N-terminal signal peptide, one bioactive GnRH peptide, and a GnRH-associated peptide (**Figure 3C**). Sg-GnRH shares high similarity within the bioactive GnRH peptide with other oysters (**Figure 3D**); it is identical between Sg- GnRH and Cg-GnRH, while there is only one amino acid difference with Pf-GnRH.

APGWa

A full-length *S. glomerata* APGWa (Sg-APGWa) was identified that contains 250 amino acids and is predicted to be cleaved to release 10 copies of four different bioactive peptides, including the amidated tetrapeptides: *1)* APGWa (x3), *2)* KPGWa (x3), *3)* SPGWa (x1) and *4)* RPGWa (x3) (**Figure 4A**). The precursor Sg-APGWa is most conserved with other oyster APGWa precursors in the tetrapeptide regions (>90% identity with Cg-APGWa and Pf-APGWa). Two Sg-APGWa were confirmed by MS (IKSFVDKRRP and RAPGWGKRSEMEKR), detected in fraction 2 (**Figure 4B**).

Buccalin

A full-length buccalin precursor (Sg-buccalin) was identified that consists of 265 amino acids, and predicted to be processed to release nine distinct bioactive amidated peptides: *1)* ALDRYSFFGGLa, *2)* ALDKYGFFGGIa, *3)* ALDRYGFAGSLa, *4)* GLDRYNFFGGIa, *5)* GLDRYGFAGSLa, *6)* GLDRYSFMGGIa, *7)* KLDRFGFMGGLa and *8)* LDSHRFFGGLa and *9)* RLDSHRFFGGLa (**Figure 4C**). Its precursor shows high identity with other oysters, however only within bioactive regions and with overall identity of 80% and 84% with Cg-buccalin Cr-buccalin, respectively (**Figure 4D**). MS peptides matched to transcriptomic Sg-buccalin in three segments; RYGFAGSLGKR (fractions 2 and 4), RYGFAGSLGKRfALDRYGFIGSLGKR (fraction 2), and GKRRLDSHRFFGGLGKRAADQYENQG (fraction 3) (**Figure 2A, B**)

Other neuropeptides

S. glomerata neuropeptide precursor proteins such as, allatotropin, conopressin, GGN, GPA2, LFRYamide, NPY and PKYMDT were also identified. While the PKYMDT precursors contain a single putative bioactive peptide, others such as CCAP, LASGLVamide, LFRFamide, LRNFVamide and pedal precursor contain at least two predicted bioactive peptides (**Table 1**).

Alignment of the *S. glomerata* neuropeptide precursors with other mollusc species (e.g. *C. gigas*, *P. fucata*, *Aplysia californica* and *Lottia gigantea)* confirms the identification of Allatotropin, CCAP, LFRFa and LRNFVa (**Figure 5**).

In vivo bioassays

Bioassay 1 - Spawning bioassay: Synthesized neuropeptides were injected into female SRO and the results are presented in **Table 2**. Five peptide-treated groups, including ELH, GnRH, APGWa, buccalin, CCAP and LFRFa (Groups 1, 2, 3, 4 and 6) plus 5HT (positive control) show a significantly higher percentage of spawned females (70-100%) than the untreated, negative control (20%). Other peptide-treated groups showed no significant spawning response (17-50%). The positive control, serotonin, did result in a significant spawning response.

Bioassay 2 – Maturation bioassay: Results of buccalin and APGW neuropeptide implantation using cholesterol pellets, for assessing the impact of sustained release of the peptides on gonad maturation, are shown in **Figure 6**. There was a significant difference in gonad maturation conditions between control (blank implants) and the neuropeptide-implanted treatments (buccalin and APGWa) at four weeks post-implantation, based on condition index and fertilization. The gonad of the neuropeptide-implanted oysters appeared far more developed, as shown by a large milky appearance, compared to the relatively small gonads of control oysters (**Figure 6A**). Moreover, condition index of both APGWa and buccalin treatments was significantly higher than control ($P<0.05$). Similarly, the fertilisation rate of oysters treated with neuropeptide implants was higher than the control (87.4% for buccalin and 91.3% for APGWa *vs* 53.5% for control) (**Figure 6B**). In addition, females were found to be dominant after 4 weeks of peptide treatment (Female/total oysters: 15/19 and 13/18 in buccalin and APGWa, respectively *vs* 10/19 in the control).

Discussion

In this study, we have undertaken transcriptome sequencing from male and female *S. glomerata*, which provided a database of over 100,000 unigenes from visceral ganglion and over 84,000 unigenes from gonads. This enabled the identification of 28 putative neuropeptide precursors that are likely to be proteolytically processed to release numerous bioactive

neuropeptides. Thereafter, we have investigated their potential role in the regulation of *S. glomerata* reproduction.

Although ELH and GnRH are abundant in molluscan neural tissues e.g. visceral, cerebral or pedal ganglia (Morishita et al., 2010, Brown and Mayeri, 1989), they are also reported to be present within other tissues such as mantle, gills, adductor muscle and gonad, or even the hemolymph (Treen et al., 2012, Bigot et al., 2012). In this study, we targeted the visceral ganglia and gonad, yet the Sg-ELH and Sg-GnRH were not detected. Their absence in these tissues could be explained by either no or low levels of expression. *S. glomerata* also contains another major ganglia, the cerebral ganglia, which was not analysed. Due to the physical proximity of the visceral ganglia with the gonad, it was assumed to be most likely for neuroendocrine signalling, and indeed many of the other neuropeptides were detected in it. Fortunately, a transcriptome generated from mixed *S. glomerata* tissues (hemolymph, mantle, gill, gonad, digestive tissue and adductor muscle) enabled the identification of ELH and GnRH, although it is unclear which tissue and what sex contained the transcripts (Ertl et al., 2016). ELH is known to be involved in the reproduction of gastropods (Morishita et al., 2010, Li et al., 1999), were it induces egg laying behaviour in sexually mature *Aplysia* (Scheller et al., 1983, Nambu and Scheller, 1986, Nagle et al., 1988). However, there is no report on the function of ELH as an oyster spawn inducer. As a first step towards understanding its potential role in oyster reproduction, we initially analysed the Sg-ELH precursor's primary sequence, showing that its organization is similar to that of ELH from other oysters, where it contains two ELH-like peptides (ELH1 and ELH2) within the same precursor. Their high level of identity with respective Cg-ELH1 and ELH2 confirms a critical regulatory role in *S. glomerata*. Moreover, our spawning bioassay (Bioassay 1) did show that ELH1 and ELH2 could induce spawning in 70% of the females within 12 h post-peptide injection. This result provides a first insight into the role of ELH in an oyster, as an egg-laying (spawning) hormone.

GnRH is a well-studied reproductive neuropeptide in vertebrates and there has been accumulating evidence for GnRH peptides in mollusc species (Tsai and Zhang, 2008). In bivalves, GnRH peptides were found in visceral ganglia of both sexes in the scallop *P. yessoensis* and *C. gigas* (Bigot et al., 2012, Treen et al., 2012, Stewart et al., 2014). GnRH tends to be strictly conserved within the bioactive GnRH region, even between invertebrates and chordates (Tsai, 2006, Stewart et al., 2014). In this study, we found that the Sg-GnRH peptide was identical to

the Cg-GnRH peptide. The GnRH peptide induced 86% of mature *S. glomerata* females to spawn. Its direct role may be to regulate gamete proliferation or maturation, as has been demonstrated in *P. yessoensis,* where *in vitro* application of mammalian GnRH and extracts of scallop cerebral and pedal ganglia could stimulate spermatogonial proliferation (Nakamura et al., 2007). The presence of GnRH receptors in mature gonads of *M. edulis* (Pazos and Mathieu, 1999) and *C. gigas* (Morishita et al., 2010) also provides evidence for a reproductive role for GnRH in these species. Our preliminary analysis of the *S. glomerata* gonads did not find a GnRH receptor.

The APGWamide was first identified in the ganglia of the gastropod *Fusinus ferrugineus* (Kuroki et al., 1990) and later in other gastropods e.g. *Lymnaea stagnalis*, *A. californica* and *L. gigantea* (Veenstra, 2010, Smit et al., 1992, Fan et al., 1997) and oysters e.g. *C. gigas* (Bernay et al., 2006, Stewart et al., 2014). Similar to the *C. gigas*, we found the Sg- APGWa in the visceral ganglia. APGWa precursors tend to be conserved among mollusc species in both organization and number of bioactive peptides (approximately 8-10 repeats) (Stewart et al., 2014, Fan et al., 1997, York et al., 2012, Veenstra, 2010, Smit et al., 1992). However, we found that the Sg-APGWa precursor has greater diversity in the types of tetrapeptides than *C. gigas* and *P. fucata* (i.e. four of each APGWa, KPGWa, SPGW and RPGWa in *S. glomerata vs* three of each APGWa, KPGWa, RPGWa). Meanwhile, gastropods typically have only one type of tetrapeptide in their precursors, such as APGWa in *Haliotis asinina* (York et al., 2012), *L. gigantea* (Veenstra, 2010) and *A. californica* (Fan et al., 1997). Conservation of Sg-APGWa within the bioactive regions was high, showing 90% identity within *C. gigas* or *P. fucata*. This similarity suggests a similar role for APGWa in oysters.

APGWa's role in reproduction is well established in molluscs, including its involvement as a male reproductive stimulant through activation of genital eversion in *L. stagnalis* (Koene, 2010). Also, it can activate spermiation in *Helix aspersa* and induce male spawning in *H. asinina* (Chansela et al., 2008). In *C. gigas* females, APGWa regulates egg transportation and spawning (Bernay et al., 2006). Moreover, APGWa present in sperm may act as a pheromone to trigger female spawning when females come into contact with the sperm (Bernay et al., 2006). In *S. glomerata*, we confirm a role for APGWa in regulating female oyster spawning, showing 100% bioactivity (see Bioassay 1).

APGWa appears to not only induce spawning in *S. glomerata*, but also stimulates *S. glomerata* gonad development and maturation. In *S. glomerata*, APGWa-treated oysters obtained a significantly higher gonad condition index and fertilisation rate than the control (peptide-free implantation). So far, the four tetrapeptide types identified within the precursor (APGWa, KPGWa, SPGWa and RPGWa) were used in combination in the assay, therefore, the role of each individually in *S. glomerata* reproduction is still unknown.

We identified a *S. glomerata* buccalin gene as well as various peptides that match the Sg-buccalin precursor. Buccalin was first identified in *A. californica* (Cropper et al., 1988), and then later found in other molluscs, (reviewed by Morishita et al. (2010)). Buccalin is known as a multifunctional peptide, having a role in muscle contraction and regulating neuromuscular transmission during feeding behaviours in *Aplysia* (Miller et al., 1993, Cropper et al., 1988). Recently, buccalin peptides were found in ganglia of the oysters *C. gigas* and *P. fucata* (Stewart et al., 2014), however, their functional role in oysters was still unknown prior to this investigation. In this study, buccalin induced spawning in all female *S. glomerata* tested in Bioassay 1 and advanced gonad development, from spent gonad stage to fully developed condition in Bioassay 2.

The dominance of females both in the buccalin and APGWa treatments in the implantation bioassay (more than 70% females in buccalin and APGWa *vs* about 50% in Control) warrants further investigation into their potential role in sex change in oysters.

Conclusions

Transcriptome analysis of *S. glomerata* has revealed 28 neuropeptide precursors, from which 11 neuropeptides could be confirmed by peptidomics MS analysis within the visceral ganglia. The synthesized ELH, GnRH, APGWa, buccalin, CCAP and LFRFa induced spawning in ripe wild-caught oysters. APGWa and buccalin enhanced gonad development, and increased the efficiency of *S. glomerata* gamete fertilisation. This is the first report on neuropeptides identified from *S. glomerata* transcriptomes that have a regulatory role in oyster reproduction.

Acknowledgements

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Supplementary data

File S1. Amino acid sequences of Sydney rock oyster, *Saccostrea glomerata* and other molluscan neuropeptides used for schematic diagrams and alignments.

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Figure legends

Figure 1. Workflow of transcriptomics and peptidomics experiments to identify neuropeptides of *S. glomerata*. Position of the visceral ganglia and gonad is shown in schematic [modified from (Paul, 1964)].

Figure 2**.** RP-HPLC chromatogram and identification of neuropeptides extracted from visceral ganglia of female *S. glomerata***.**

Figure 3**.** Identification and characterization of *S. glomerata* ELH precursor in comparison with ELH from other oysters. (A) Amino acid sequences: signal peptides in yellow, bioactive peptides in grey where ELH1 is located before ELH2, amidation sites in aqua and cleavage sites in red. (B) Alignment of ELH1 and ELH2 sequences: blue shading represents conservation of amino acid. (C) Schematic diagrams illustrating the organisation of ELH precursors, (*C. gigas* and *P. fucata* precursor sequences were obtained from a supplementary file provided by Stewart et al. (2014)).

Identification and characterization of *S. glomerata* GnRH precursor in comparison with other oysters. (A) Amino acid sequences: signal peptides in yellow, bioactive peptides in grey, amidation sites in aqua and cleavage sites in red; (B) Alignment of bioactive sequences: blue shading represents conservation of amino acid; (C) Schematic diagrams illustrating the organisation of precursors, (*C. gigas* and *P. fucata* precursor sequences were obtained from a supplementary file provided by Stewart et al. (2014)).

Figure 4. Identification and characterization of *S. glomerata* APGWa precursor in comparison with other oysters. (A) Amino acid sequences: signal peptides in yellow, cleavage sites in red and bioactive peptides in grey, amidation sites in aqua; *S. glomerata* APGWa precursor shows peptide fragments identified by MS/MS, including the fraction (numbers on the scale bar) they were present. (B) Schematic diagrams illustrating the organisation of precursors, (*) Peptides synthesized for bioassay. APGWa precursors of *C. gigas* and *P. fucata* were obtained from a supplementary file provided by Stewart et al. (2014).

Identification and characterization of *S. glomerata* buccalin precursor in comparison with other oysters. (A) Amino acid sequence: signal peptides in yellow, bioactive peptides in grey, amidation sites in aqua and cleavage sites in red. *S. glomerata* Buccalin precursor shows peptide fragments identified by MS/MS, including which fractions (numbers on the scale bar) they were present; (B) Schematic diagrams illustrating the organisation of precursors. (*)

Peptide synthesized for bioassays. Buccalin precursor sequences of *C. gigas* and *C. rhizophorae* oyster were obtained from a supplementary file provided by Stewart et al. (2014).

Figure 5. Identification and characterization of other *S. glomerata* neuropeptides. Schematic diagrams show the organisation of neuropeptide precursors and multiple sequence alignment of bioactive peptide between mollusc species. Blue shading represents conservation of amino acid. Precursor sequences of neuropeptide of other molluscs were obtained from a supplementary file provided by Stewart et al. (2014).

Figure 6. Condition index and fertilization rate of *S. glomerata* oysters treated with buccalin, APGWa or neuropeptide-free (blank) implants four weeks post-implantation. (A) Photos show representation of typical gonad condition for each treatment at four weeks post-implantation. (B) Graph shows condition index (n=18). (C) Graph shows fertilization rate (n=10). Different letters (a, b and c) on top of the column bars indicate significant differences ($p<0.05$).

Tables

Table 1. Summary of neuropeptides deduced from the *S. glomerata* transcriptomes.

No	Name of peptides	Synthesised peptides	Full length of precursors	Signal peptide (aa)	Amidation	No. of bioactive peptides	Length (aa)	Mass spectroscopy (MS)	Gonad		Visceral Ganglia	
									Male	Female	Male	Female
1	Allatotropin			19		1	107					
2	APGWamide			20		10	250					
3	Buccalin			25		9	265					
4	CCAP			28		3	146					
5	Conopressin			30		1	262					
6	ELH1,2			19		2	107					
7	FCAP					10	192					
8	FMRFamide			24		20	359					
9	FxRlamide					4	86					
10	GGN			27		1	135					
11	GnRH			24		1	73					
12	GPA2					1	55					
13	Insulin					2	176					
14	LASGLVamide			22		10	238					
15	LFRFamide			23		5	159					
16	LFRYamide			26		1	90					
17	LRNFVamide			24		10	210					
18	Myoinhibitin			16		3	142					
19	Myomodulin 2			25		2	120					
20	Myomodulin 3					5	86					
21	NPY					1	63					
22	Opioid					2	70					
23	Pedal peptide					5	293					
24	PKYMDT			23		1	123					
25	RxIamide					8	124					
26	SCAP			21		4	181					
27	Tachykinin			23		2	158					
28	WWamide					5	165					

Table 1. Results of female spawning bioassay 1 using synthetic peptides. Peptide in the same group number were mixed.

No	Group	Treatment	Sequence	% Female spawned
	Control	Distilled water	*Negative control*	20
		Serotonin (50mM)	*Positive control*	90
1	1	ELH1	GRLSLTADLRSLARMLEAHRKRYLASRSPYDSIRKKLFKF-NH_2	70
2		ELH2	Pyr-QRLSVNGALSSLADMLAASGRQRMRSEMEINRQRLFGL-NH_2	
3	2	GnRH	Pyr-QNYHFSNGWQP-NH_2	86
4	3	APGWamide	RPGW-NH_2	100
5			KPGW-NH_2	
6			SPGW-NH_2	
7			APGW-NH_2	
8	4	Buccalin	ALDRYSFFGGL-NH_2	100
9			ALDKYGFFGGI-NH_2	
10			GLDRYSFMGGI-NH_2	
11			GLDRYGFAGSL-NH_2	
12	5	Allatotropin	GFRQSIVDRMGHGF-NH_2	17
13		LASGLVamide	MLDRVGSGFI	
14			LDRLSMGLL	
15			YFDRLSSGFI	
16			RFDRLGSGFI	
17	6	CCAP	GICPYWGC-NH_2	78
18			LFCNFGGCFN-NH_2	
19			SLPLKTRFLMR-NH_2	
20		LFRFamide	GMPMLRL-NH_2	
21	7	LFRYamide	LRYFI-NH_2	50
22			MRYFL-NH_2	
23			MRYFLGKRTRYFL-NH_2	
24	8	LRNFVamide	LRYFI-NH_2	44
25			MRYFL-NH_2	
26			DGTRYFL-NH_2	
27	9	Pedal	IPYMYNNYRYGTHGLFA	38
28			SFDSIAHSGRFGVFS	

Figure
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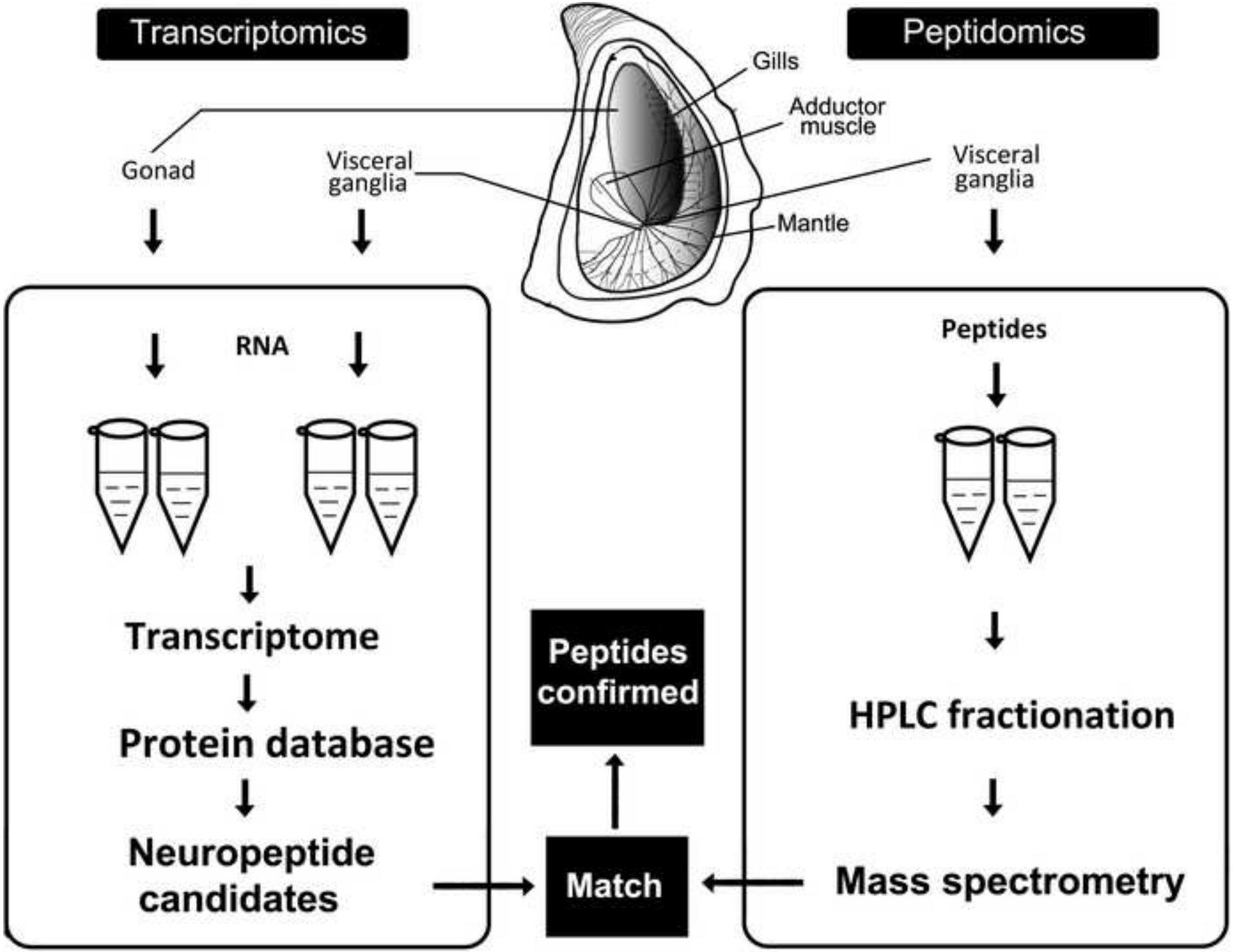

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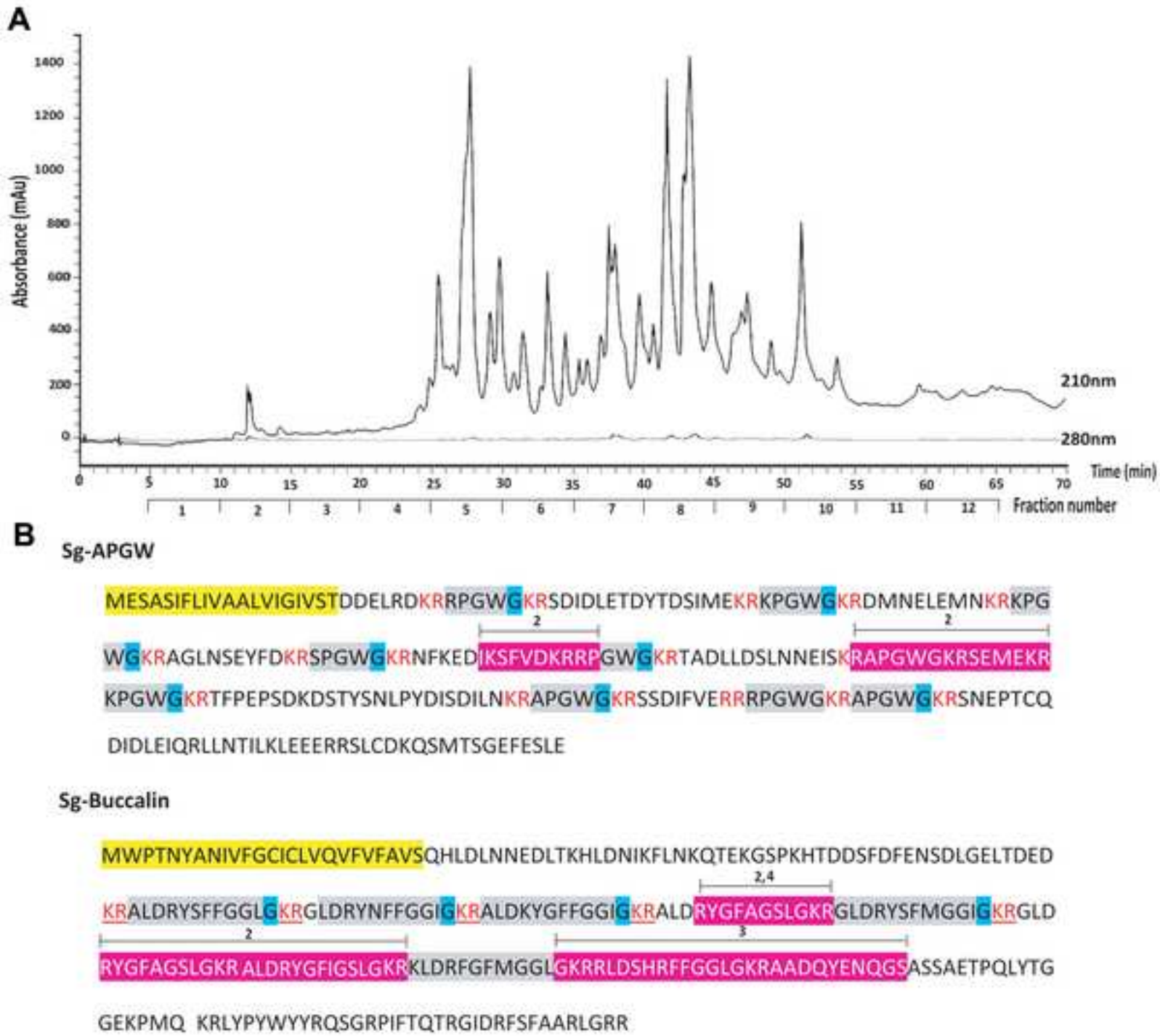

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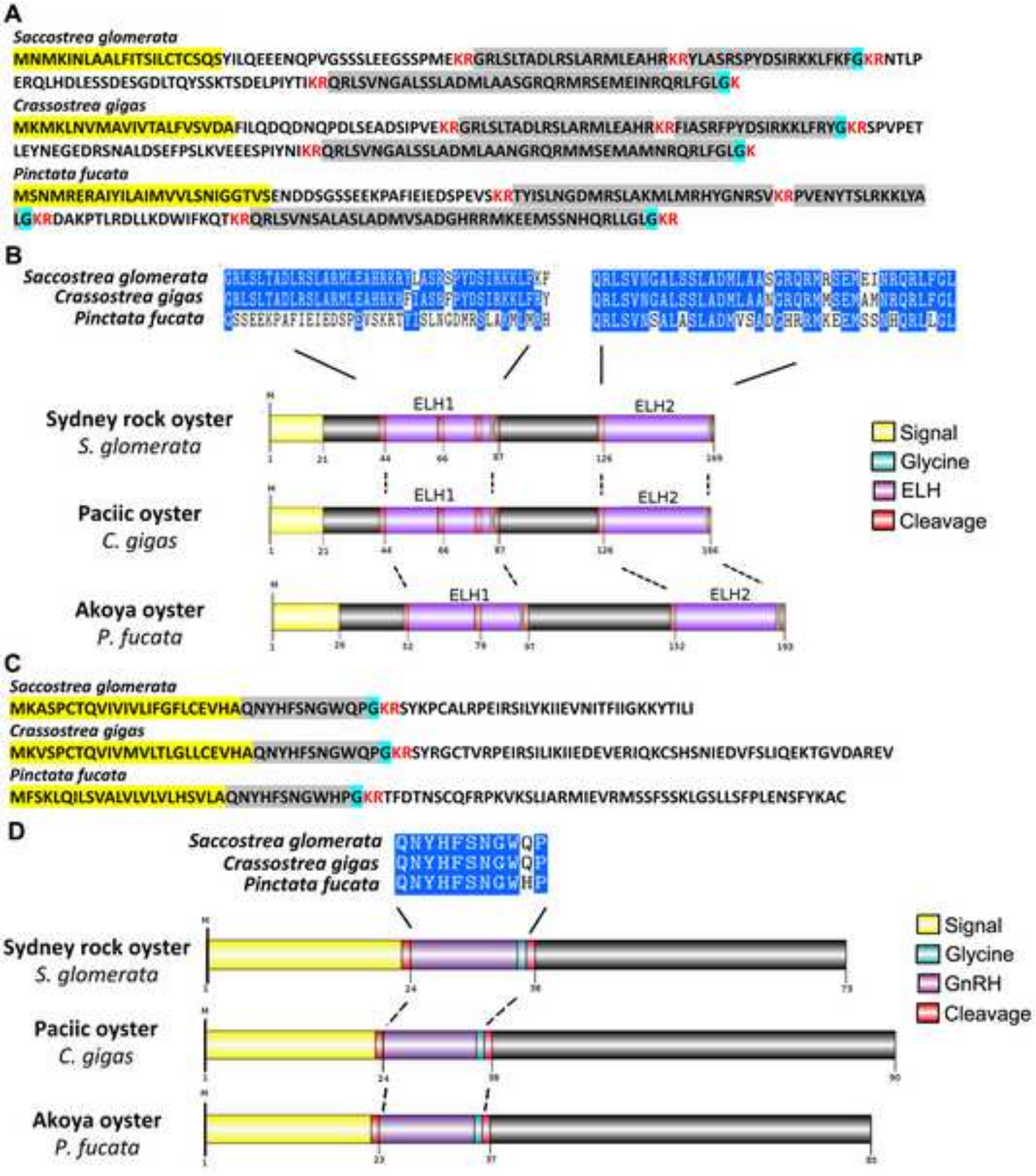

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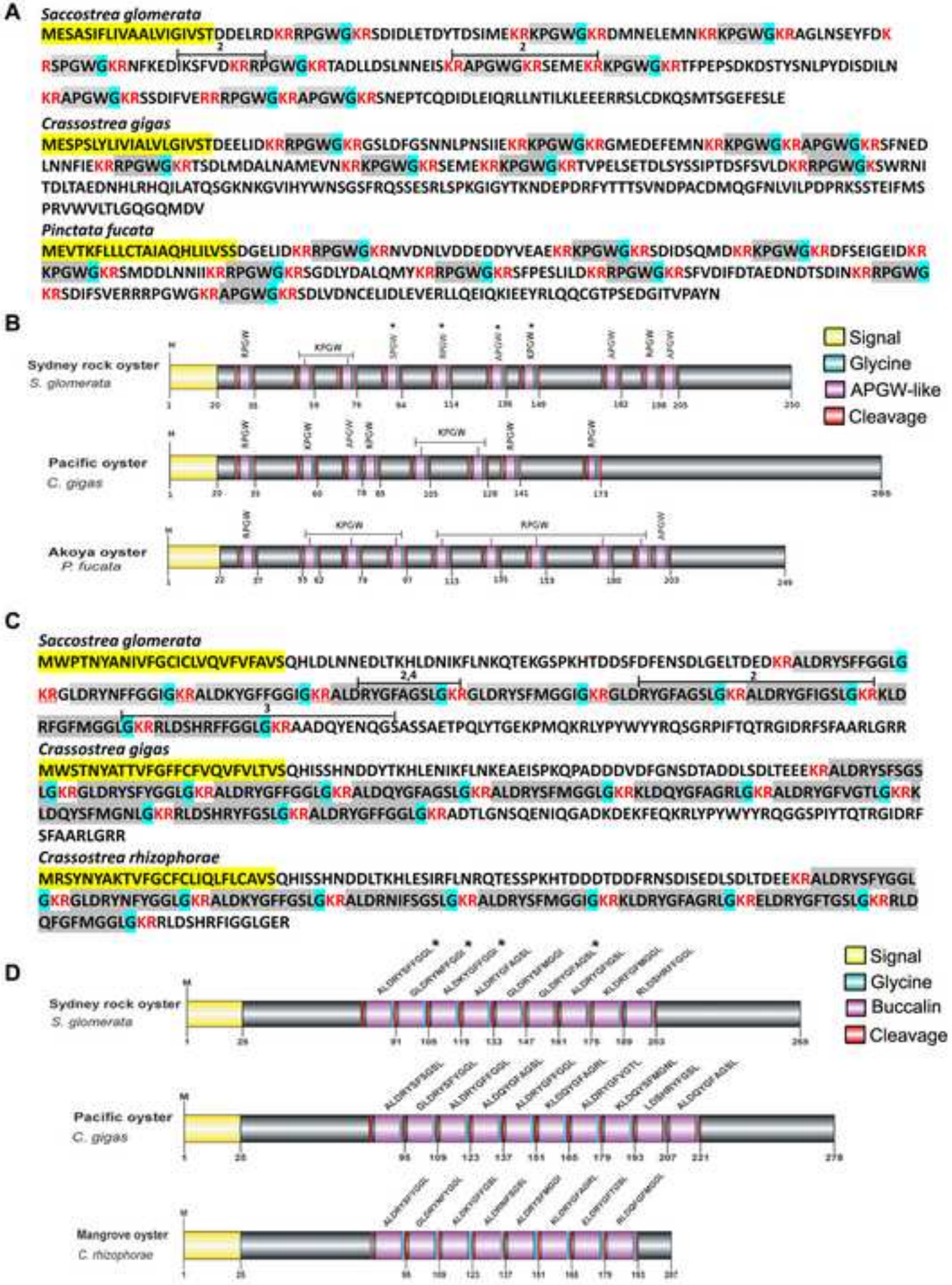

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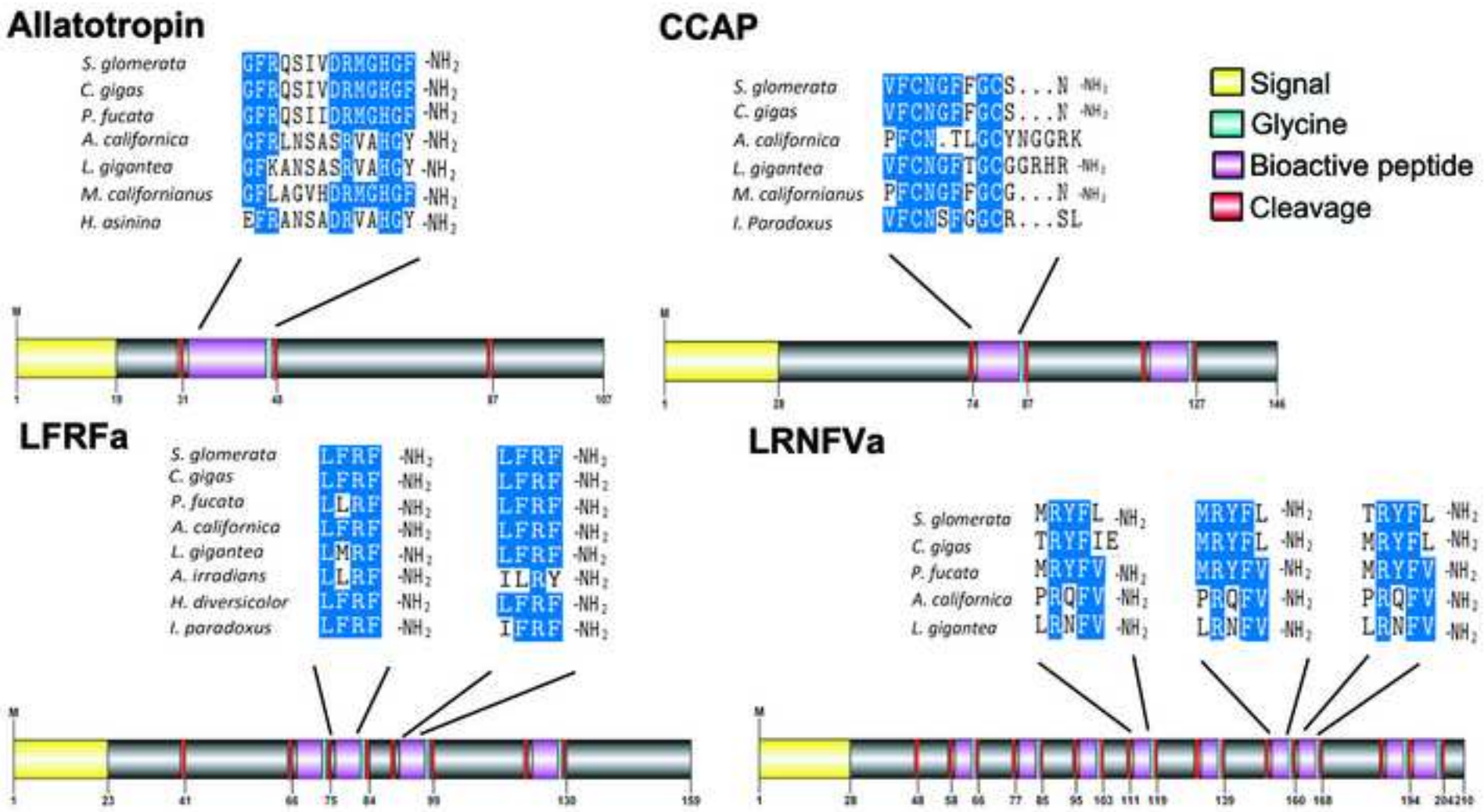

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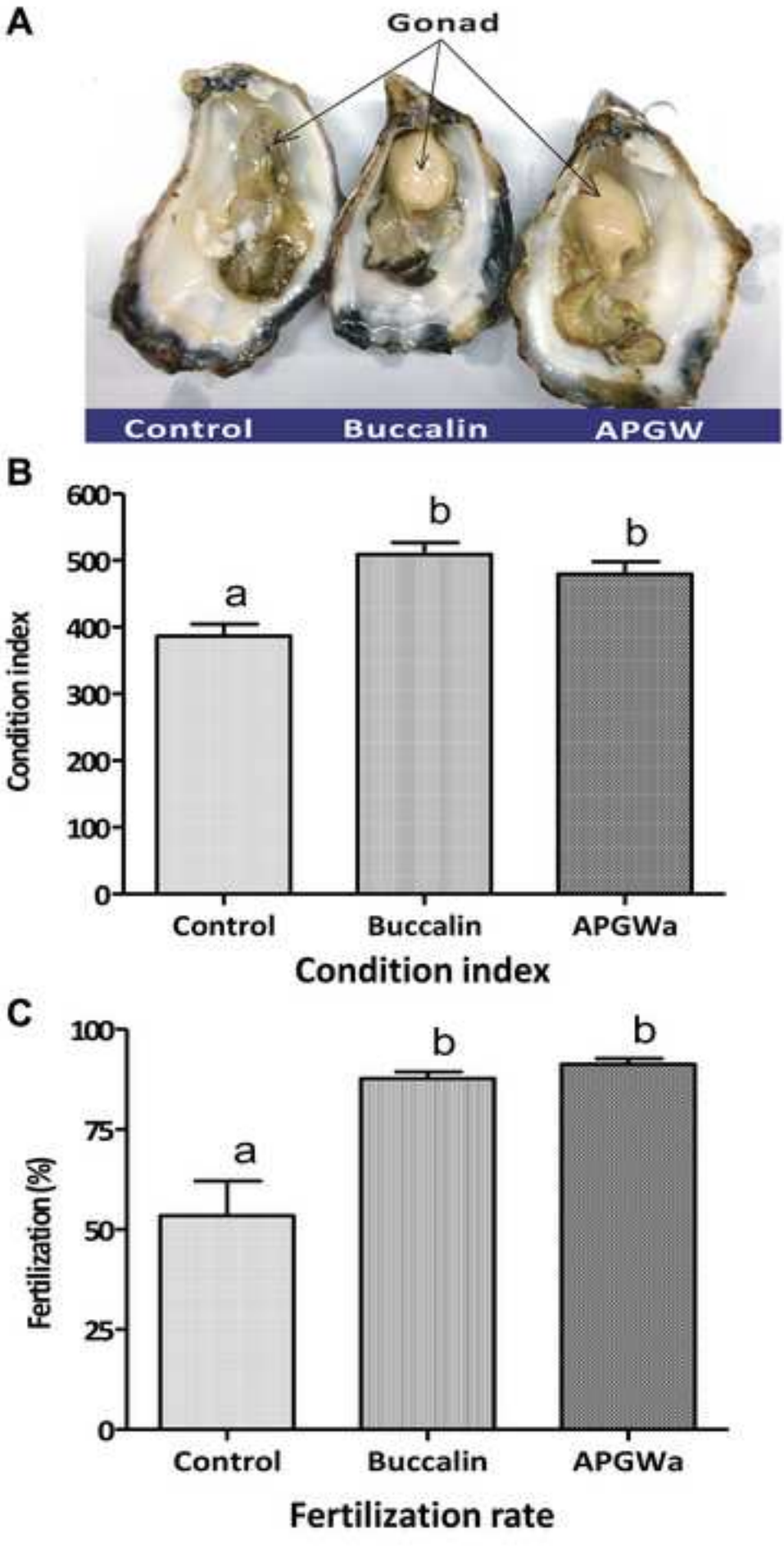

E-component(s)

Click here to download E-component(s): Supplementary 1 - list of neuropeptides.txt