Understanding conditioning of Sydney Rock Oysters

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Non-Technical Summary

Reproductive development is determined by environmental cues, which are perceived by the oyster's nervous system and mediated by neuro peptides. This project set out to identify Sydney Rock Oyster neuropeptides that affect reproduction in order to develop technologies for improving reproductive condition and spawning. New generation high throughput technologies were employed to sequence genes that are expressed in the ganglia, (which is the equivalent to the oyster's central nervous system) and the gonads. We have identified a suite of peptides, which based on studies in other species, are likely to have a role in reproduction, and tested some of these for their capacity to induce a physiological response and spawning. We have confirmed a set of peptides that cause spawning, but still need to establish their roles in advancing conditioning.

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PROJECT OBJECTIVES:

1. Characterise the transcriptome and peptidome of SRO at different levels of conditioning.
2. Identify genes and peptides that are relevant to SRO conditioning.
3. Test the in vivo effects of identified peptides on SRO conditioning.
4. Apply the knowledge obtained to achieve reliable and consistent conditioning in SRO.

OUTCOMES ACHIEVED

New hormonal options for the induction of spawning and possibly conditioning in oysters

LIST OF OUTPUTS PRODUCED

1. The transcriptome and peptidome of the SRO gonad and visceral ganglia.
2. Identified series of putative peptides associated with reproduction.
3. Ten new microsatellites for SRO

ACKNOWLEDGEMENTS

We are grateful to the Australian Seafood CRC, the FRDC, Oysters Australia, NSW DPI and The University of the Sunshine Coast for supporting this project. Mr In Vu is supported by an Australian Centre for International Agricultural Research John Allwright PhD fellowship.

1. Introduction and Background

Critical in the production and marketing of Sydney Rock Oysters (SRO) is their physical and reproductive condition. While cycles in SRO condition are broadly understood, and to some extent can be manipulated through gross environmental changes, our understanding of the biochemical processes underpinning changes and our capacity to monitor these changes is limited. The process of controlling maturation in SRO is poorly understood. This is a challenge particularly for the SRO aquaculture industry in New South Wales (NSW) that uses oysters that have been selectively bred for fast growth and winter mortality resistance (Nell *et al.* 2000). Compared with their wild counterparts, these selectively bred SRO lines can devote more energy to growth and as a result they can have both lower condition indices at a given time and can take significantly longer to move from spawned or quiescent condition to reach reproductive maturity. This results in lines which exhibit clear differences in reproductive behaviour, and therefore their capacity to be artificially propagated (Dove and O'Connor, 2012). These differences also have marked impacts on marketability and have been an impediment to industry adoption of selectively bred stock in some estuaries (Dove and O'Connor, 2011). This research was developed in response to the challenges that the New South Wales SRO aquaculture industry is currently facing. The strong demand for the selectively bred SRO has created both challenges and opportunities for the entire industry. Oysters in ripe (ready to spawn) reproductive condition are required for hatchery production; however, it is also at such condition when the oysters' market price is at its highest. The challenge for the SRO aquaculture industry is heightened by the observed deviation of the reproductive behaviour of the selectively bred SRO from their wild counterpart.

The NSW SRO aquaculture industry was consulted when we first developed this proposal. The research relates to completed FRDC funded projects to Wayne O'Connor that enabled the development of a reliable hatchery production technology and utilisation of genetic markers for the

selectively bred SRO. It further relates to a current Seafood CRC project (2009/743) in response to the oyster industries' need for breeding programs that focus on quality and market appeal, in order to increase competitiveness alongside imported and alternative products.

We used transcriptomics and proteomics to identify functional neuropeptides, which will lead to better understanding of the outcomes of 2009/743 through helping to unravel the relative importance of environmental influences on reproductive behaviour. For instance, now knowing what reproductive peptides are important we can examine if there are critical periods in which the environment influences reproductive behaviour and can such periods alone be manipulated. Presumably sex determination occurs at an early stage of the cycle and our studies and wealth of genes and peptides could both pinpoint the time and its duration. It is desirable to increase the number of females, as they are a limiting factor in egg production. Currently a commercial batch could require 800 million eggs, for which about 80 females would be needed. When the sex ratio cannot be controlled, 300-400 oysters need to be conditioned to ensure 80 females are available. Reducing this number can save significant efforts in feeding and maintaining such a large number of conditioned oysters. By having the molecular tools we can determine if it may only be necessary to manipulate oysters for a brief period and return them to the field for nature to do the work. We won’t get the fine level of resolution to address these sorts of questions from the current fieldwork in 2009/743.

Neuropeptides encompass a diverse class of cell signalling molecules that are produced and released from neurons through a regulated secretory route (Burbach 2011). They may function as hormones, transmitters and modulators. Conventional methods of neuropeptide characterization have involved their purification directly from neural-associated tissues in conjunction with the analysis of corresponding gene expression. Identification of cross-species neuropeptide conservation has typically relied on the use of antibody probes that bind to homologs. More recently, with the relative ease to which nucleic acids can be sequenced using next-generation techniques,

high throughput genome mining approaches can enable researchers to identify putative neuropeptides, even within non-model animal species. Thus, genome mining combined to high throughput peptidomic analysis has become a valuable tool for peptide discovery and has been used effectively in a number of species for which a genome is available.

The owl limpet, *Lottia gigantea,* is a marine gastropod that has emerged as a molluscan genome model following the recent sequencing of its relatively small genome. Data mining of the *L. gigantea* genome has revealed around 59 genes that encoded for putative neuropeptides, most of which had been previously identified in other molluscs, including Ala-Pro-Gly-Trp-NH_2 (APGWamide), egg laying hormone (ELH) and the Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Veenstra 2010). Some had not been identified in molluscs, yet share homologs with *Drosophila*, such as the putative proctolin homolog PKYMDT and allatostatin C or are believed to derive from neuropeptides with an early origin from either the eumetazoan ancestor or the bilaterian ancestor (Jekely 2013; Mirabeau & Joly 2013). Whether or not they function in a similar way to their vertebrate counterparts has been largely unexplored.

Recently, genome sequence assemblies and annotations became available for *Pinctada fucata* and *Crassostrea gigas,* providing an excellent opportunity to characterize the repertoire of oyster neuropeptides. The *P. fucata* draft genome (approximately 40x coverage) predicts 23,257 complete gene models and includes genes associated with shell biomineralization (Takeuchi *et al.* 2012). Analysis of the highly polymorphic *C. gigas* genome and transcriptomes revealed an extensive set of genes that provides a rare glimpse of how *C. gigas* responds to environmental stress, and adapt to near environments, as well as giving insight perspective into the molecular mechanism of shell formation, development and reproduction (Zhang *et al.* 2012).

In this study, we interrogated the transcriptomes of the Sydney Rock Oyster, *Saccostrea glomerata,* to identify putative neuropeptide genes that may be of importance in regulating oyster reproductive activity and behaviour. To help support gene predictions, we performed peptidomics of *S. glomerata* ganglia. Among those identified are candidates involved in reproduction (eg. egg-laying hormone and gonadotropin-releasing hormone).

1.1 Need

The Sydney Rock Oyster (SRO) aquaculture industry is the largest and oldest aquaculture industry in NSW with annual revenue of approximately A$35 million (Trenamen, 2011). SRO breeding was initiated in 1990 through mass selection aiming to develop faster growing, winter mortality resistant lines (Nell et al. 2000). After five generations the average time to market size reduced by more than 12 months (Nell & Perkins 2005) and disease resistant lines were available. In 2004, the first progeny from 5th generation fast growth lines were distributed to oyster farmers in NSW. In comparison to wild-caught oysters, these stocks demonstrated clear differences in reproductive behaviour, which affected marketability in various estuaries throughout NSW (Dove and O'Connor, 2011). While industry demand for selectively bred SRO remains strong (2011/2012 spat sales exceed 20,000,000 and will form the basis of more than 20% of total production), these changes pose both challenges and opportunities for the entire SRO production cycle from the hatchery, where oysters in "ripe" reproductive condition are required for spawning, through to market where reproductive condition is a major determinant in "saleability".

With the progression of the SRO breeding program from 3 "base" mass selected lines to 120 pair-mated families, investigation into the biology behind the altered condition index of selectively bred oysters is of great importance. Initially, there is a fundamental need for reproductive concurrency among lines to allow breeding. Secondly, genetic variability in reproductive condition offers the opportunity to manage marketability through the selection of lines whose characteristics suit market requirements.

1.2 Objectives

1. Characterise the transcriptome and peptidome of SRO at different levels of conditioning.
2. Identify genes and peptides that are relevant to SRO conditioning.
3. Test the in vivo effects of identified peptides on SRO conditioning.

4. Apply the knowledge obtained to achieve reliable and consistent conditioning in SRO.

Methods

Rationale

The brain of molluscs is made up of a series of ganglia, which are the primary release site for major regulatory factors that play critical roles in reproductive and homeostatic processes. By using a suite of advanced transcriptomic and peptidomic approaches, we sought to discover those fundamental reproduction genes and peptides involved in maturation and spawning, and apply them to improve the conditioning of SRO in a commercial context.

Animal and tissue collection

Live adult Sydney Rock Oyster were obtained from local markets (Port Stephens, New South Wales and Sunshine Coast, QLD) and acclimatized in culture tank for 24 h before tissue collection. The stage of gonadal development of each individual was determined (Dinamani, 1974). Tissues, including ganglia and gonad were collected from animals from 18-20 July 2012 at various stages of gonadal development (20-25 oysters/each stage, N=70) and kept at -80^{o}C until total RNA preparation and peptide extraction.

RNA preparation, illumina sequencing and transcriptome assembly

Total RNA from gonad and ganglia tissue was extracted using Trisure isolation reagent (Bioline USA Inc.) following the manufacturer's protocol. The quality and concentration of total RNA was checked by gel electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). To identify different expressed genes between male and female oysters, total RNA of each sex was pooled separately. 20 µg of total RNA of each tissue was dried by freeze drier at -54^{o}C in vacuum condition before sending to BGI, Hong Kong. However, for total RNA of ganglia, liquid nitrogen was applied to keep total RNA of ganglia cold during transportation to BGI in order to get adequate quality for transcriptome. Samples were then purified and enriched by Oligo-dT, then fragmented into small fragments. The suitable fragments were

selected and reversed-transcribed into double stranded cDNAs. The cDNA libraries were constructed by PCR amplification using random hexamer primed cDNA synthesis. Finally, the samples were sequenced using an Illumina HiSeq^{TM} 2000 instrument (Illumina Inc.). De-novo assemblies were performed by SOAPdenovo software using trimmed reads from 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.

Gene and peptide identification

To identify target sequences, gender-specific transcriptomes for the gonad and visceral ganglia of the *S. glomerata* were imported into the CLC Main Workbench (v7.0.2; CLCbio, Denmark). Previously identified molluscan neuropeptide sequences were then queried (tBLASTn) against the transcriptomes. To compliment this, ORF retrieved from the databases were screened for signal sequences and for presence of recurrent KK; KR; RK; RR cleavage sites. Multiple sequence alignments were created with the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Tamura et al. 2013). Derived and actual amino acid sequences were aligned, guided by chain cleavage sites and conserved cysteines, where necessary intron donor/acceptor splice sites were identified using NetGene2 (Brunak et al. 1991). Signal sequences and cleavage sites (>0.5 confidence) were identified by alignments with other mollusc sequences and predicted through SignalP 4.0 (Bendtsen et al. 2004) and NeuroPred (Southey et al. 2006).

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

The collected tissues were homogenized on ice in a solution of 0.1% trifluroacetic acid (TFA), with subsequent sonication consisting of three 30 s pulses separated by 20 s whilst being kept on ice. The homogenized tissues were than centrifuged at 16,000 rpm for 20 min at 4°C and the supernatant was collected. This process was repeated with the pellet left over.

The extracted peptide mixture, along with synthetic peptides (GLDRYSFMGGI-NH_2, GMPMLRL-NH_2, MRYFL-NH_2 and RPGW-NH_2;

ChinaPeptides Co. Ltd.) were analyzed by RP-HPLC (PerkinElmar series 200 pump/autosampler, Flexar PDA detector and Chromera v3.2 software). The synthetic peptides were prepared in 0.1% TFA to allow 20 µg to be loaded onto the column, whereas the total collected peptides from the extractions were loaded. Samples were separated on an Agilent Zorbax 300 SB-C18 column (internal diameter 4.8 mm x 150 mm and particle size of 5µm) 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 0.1% TFA for solution A and 0.1% TFA in acetonitrile (ACN) for solution B. Fractions were collected in 5 min intervals for further analysis by mass spectroscopy.

Mass spectrometry analysis

MS analysis was carried out on a Triple Tof 5600 mass spectrometer (ABSCIEX, Canada) equipped with a nano electrospray ion source. The ionspray voltage was set to 2400V, declustering potential (DP) 100V, curtain gas flow 25, nebuliser gas 1 (GS1) 12 and interface heater at 150^{o}C. The mass spectrometer acquired 500ms full scan TOF-MS data followed by 20 by 50ms full scan product ion data in an Information Dependant Acquisition, IDA, mode. 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, ms/ms spectra of the resultant 20 most intense ions. Fragmentation patterns were used to determine the sequence of the peptide, corresponding to the in-house SRO protein libraries derived from transcriptome analysis.

***In-vivo* and *in-vitro* bioassays**

SRO samples were purchased from a retail outlet on the Sunshine Coast. They were allowed to recover in seawater for at least 24 h before the testing. For *in-vivo* bioassay, animals will be anaesthetized with $MgCl_2 6H_2O$ 50g/L to relax and therefore their valves were open to permit the introduction of peptides (40µg/oyster). For *in-vitro* bioassay, changes in a piece of an ovary

from one oyster for each bioassay were observed under microscopes and compared to the controls after applying peptides on a piece of an ovary in 0.5 mL 30ppt artificial sea water (ASW). Serotonin (5HT) 50mM and ASW were used as positive and negative control respectively. Synthetized peptides were pooled into groups based on their precursors. In addition, gonad and ganglia extract were used as crude peptides by gathering ganglion and gonadal tissues from 5 different oysters to be homogenized in 500µL of ASW and collected supernatant after centrifuging at 12.000 rpm, 4^{o}C for 10min.

Phosphorylated Extracellular Signal-regulated Kinase (P-ERK*)*

Western Blot was used to detect Phosphorylated Extracellular Signal-regulated Kinase (P-ERK) in gonad tissues after peptides were applied to a piece of an ovary in ASW. A peptide hormone may have its effect on the development and maturation of oyster through ERK pathway where it binds to the receptor on the membrane and phosphorylates ERK in an activation process, which culminates in the nuclear DNA expressing a protein and producing changes in the cell. ERK has been found to specifically regulate thymocyte maturation (Pagès et al., 1999). For this assay, peptides were synthesised based on a list of potential peptides found from the SRO transcriptome data (China peptides Co. Ltd) and applied to the oysters. Proteins from tissues tested were extracted and purified by electrophoresis on Amersham ECL gel 10% (EG Healthcare #28990155) at 160V for 50 min, it was then transferred to Western blot membrane (Biorad #170-4158) by Trans-Blot Turbo Transfer System (#690BR2839). The membranes were run through 3 washes in PBS in 0.1% Tween (PBS-T), block in 5% skim milk in PBST, 1st antibodies for total ERK (T-ERK) and phosphorylated ERK (P-ERK) for overnight at 4^{o}C, 3 washes in PBS-T, 2nd antibody for 1 h at 4^{o}C, 3 washes, dry out and scanning on an Odyssey CLx (LI-COR) on Image studio Ver 2.0 software.

Results

In silico gene and peptide identification from transcriptomes

We have identified genes encoding putative full-length or partial-length neuropeptide precursors within the gender-specific gonad and visceral ganglia transcriptomes of the *S. glomerata* (**Table 1**). Numerous bioactive neuropeptides are predicted to be released from these precursors.

Within the gonad transcriptomes, full-length sequences were identified for insulin, LFRYamide, myomodulin 2 and NPY1, and partial-length sequences were identified for allatotropin, buccalin, CCAP, GGN, LFRFamide, LRNFVamide, pedal peptide and tachykinin. All of these were identified in the male specific transcriptome, however only LFRYamide was identified in the female specific transcriptome.

Within the visceral ganglia transcriptomes, full-length gene sequences were identified for allatotropin, APGWamide, buccalin, CCAP, conopressin, FMRFamide, GGN, LASGLVamide, LFRFamide, LFRYamide, LRNFVamide, myoinhibitin, myomodulin 3, opioid, PKYMDT, SCAP, tachykinin and WWamide, and partial-length gene sequences were identified for GPA2 and RxIamide. All of these were identified in the male-specific transcriptome, however, LASGLVamide and myomodulin 3 were not identified in the female-specific transcriptome.

The egg laying hormone (ELH) was not identified within SRO gonad or visceral ganglia transcriptomes. An ELH gene was identified from a combined tissue SRO transcriptome (developed as part of CRC project 2011/718), encoding a 169-residue precursor protein that is likely cleaved to produce two ELH peptides (**Figure 1**). Comparison with other oyster species, *C.gigas* and *P. fucata*, indicates high similarity, but less with non-oyster species. Synthetic peptides were produced for SRO ELH1 and ELH2 for *in-vivo* bioassay.

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 (**Figure 2A**). RP-HPLC separation of peptides extracted from the visceral ganglia of female *S. glomerata* showed numerous peaks following linear increase of acetonitrile from 0% to 60% over 60 min (**Figure 2B**). Five-minute fractions were taken for LC-MS/MS analysis and those identified represented in red in **Table 1**. Besides these, numerous other gene products were identified.

Other candidate neuropeptides were searched through *in silico* searches of the SRO transcriptomes. To achieve this, precursor proteins predicted from the SRO visceral ganglia transcriptomes were screened for signal peptide and presence of cleavage sites (KK; KR; RK; RR). We have identified approximately 2,352 female and 3,843 male precursor proteins that are likely processed into peptides (over 30,000 peptides) and released from visceral ganglia.

Table 1. Neuropeptides identified in the gender-specific gonad and visceral ganglia transcriptomes of the *Saccostrea glomerata*, and within combined tissues (Other). Black, identified in the transcriptome only; Red, identified in the transcriptome and peptide confirmed by mass spectroscopy.

Neuropeptide	Gonad		Visceral		Other
	Male	Female	Male	Female	M/F
Allatotropin					
APGWamide					
Buccalin					
CCAP					
Conopressin					
ELH					
FACP					
FMRFamide					
FxRIamide					
GGN					
GPA2					
Insulin					
LASGLVamide					
LFRFamide					
LFRYamide					
LRNFVamide					
Myoinhibitin					
Myomodulin 2					
Myomodulin 3					
NPY					
Opioid					
Pedal peptide					
PKYMDT					
RxIamide					
SCAP					
Tachykinin					
WWamide					

Saccostrea glomerata

ELH1

MNMKINLAALFITSILCTCSQSYILQEEENQPVGSSSLEEGSSPMEKRGRLSLTADLRSLARMLEAHRKRYLASRSPYDSIRKKLFKFGKRNTLPERQLHDLESSDESGDLTQYSSKTSDEL

PIYTIKRQRLSVNGALSSLADMLAASGRQRMRSEMEINRQRLFGLGK

ELH2

Crassostrea gigas

ELH1

MKMKLNVMAVIVTALFVSVDAFILQDQDNQPDLSEADSIPVEKRGRLSLTADLRSLARMLEAHRKRFIASRFPYDSIRKKLFRYGKRSPVPETLEYNEGEDRSNALDSEFPSLKVEEESPI

YNIKRQRLSVNGALSSLADMLAANGRQRMMSEMAMNRQRLFGLGK

ELH2

Pinctata fucata

ELH1

MSNMRERAIYILAIMVVLSNIGGTVSENDDSGSSEEKPAFIEIEDSPEVSKRTYISLNGDMRSLAKMLMRHYGNRSVKRPVENYTSLRKKLYALGKRDAKPTLRDLLKDWIFKQTKRQRL

SVNSALASLADMVSADGHRRMKEEMSSNHQRLLGLGKR

ELH2

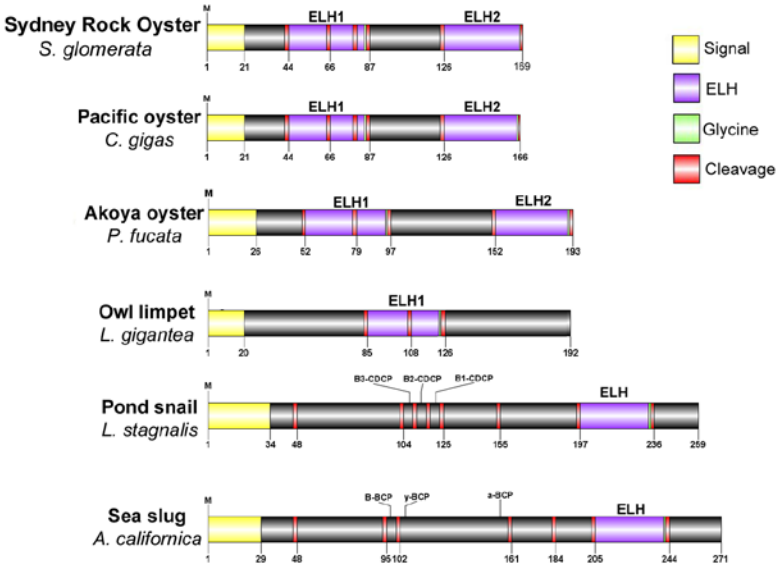


Figure 1. *Saccostrea glomerata* egg laying hormone (ELH) and comparison with other oyster ELH.

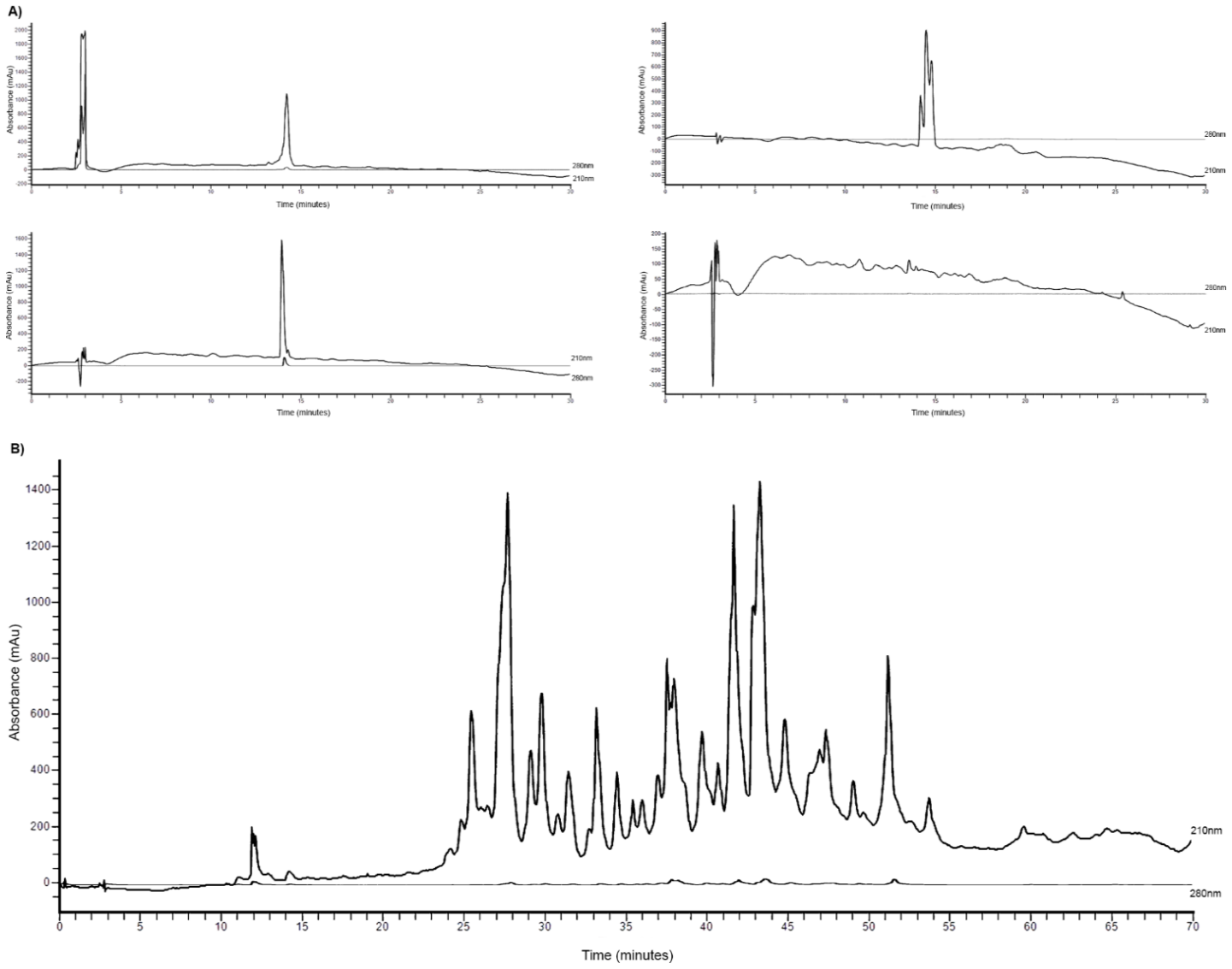


Figure 2. Isolation of female *Saccostrea glomerata* visceral ganglia peptides for MS analysis. **A)** Chromatograph of synthetic peptides, with UV detection at wavelengths of 210nm and 280nm. Top-left: GLDRYSFMGGI-NH_2; top-right: GMPMLRL-NH_2; bottom-left: MRYFL-NH_2; bottom-right: RPGW-NH_2. **B)** Chromatograph of peptides extracted from the visceral ganglia of female *S. glomerata*, at wavelengths of 210nm and 280nm.

In-vitro bioassay

We have the tested synthesised peptides by pooling peptides in groups as follows:

Table 2. List of synthetized peptide groups

No	Sequences	Genes	Group
1	GICPYWGC-NH_2	CCAP like -	1
2	LFCNFGGCFN-NH_2		
3	SLPLKTRFLMR-NH_2		
4	GMPMLRL-NH_2	LFRFFamide like-	
5	LRYFI-NH_2	Hypothetical protein captedraft 221538	2
6	MRYFL-NH_2		
7	MRYFLGKRTRYFL-NH_2		
8	IPYMYNNYRYGTHGLFA	Pendal like –	3
9	SFDSIAHSGRFGVFS		
10	RPGW-NH_2	APGWAmide like –	4
11	KPGW-NH_2		
12	SPGW-NH_2		
13	APGW-NH_2		
14	GFRQSIVDRMGHGF-NH_2	Allatoropin like –	5
15	MLDRVGSGFI	LASGLV like-	
16	LDRLSMGLL		
17	YFDRLSSGFI		
18	RFDRLGSGFI		
19	LRYFI-NH_2	LRFNamide –	6
20	MRYFL-NH_2		
21	DGTRYFL-NH_2		
22	ALDRYSFFGGL-NH_2	Pedal like – Unigene10913_srof2	7
23	ALDKYGFFGGI-NH_2		
24	GLDRYSFMGGI-NH_2		
25	GLDRYGFAGSL-NH_2		
26	GRLSLTADLRSLARMLEA HRKRYLASRSPYDSIRKKL FKF-NH_2	ELH1	8
27	Pyr-QRLSVNGALSSLADMLAA SGRQRMRSEMEINRQRL FGL-NH_2	ELH2	
28	Pyr-QNYHFSNGWQP-NH_2	GnRH	9

The photos taken after 30, 60 and 180 min after application show that 5HT has clear effect on egg maturation which caused germinal vesicle migration

(GVM) and germinal vesicle membrane breakdown (GVBD) after 30 min or longer whereas it is still unclear whether other treatments have any kind of effect on ovarian maturation except G5 showing some GVM (Fig 3 & 4).

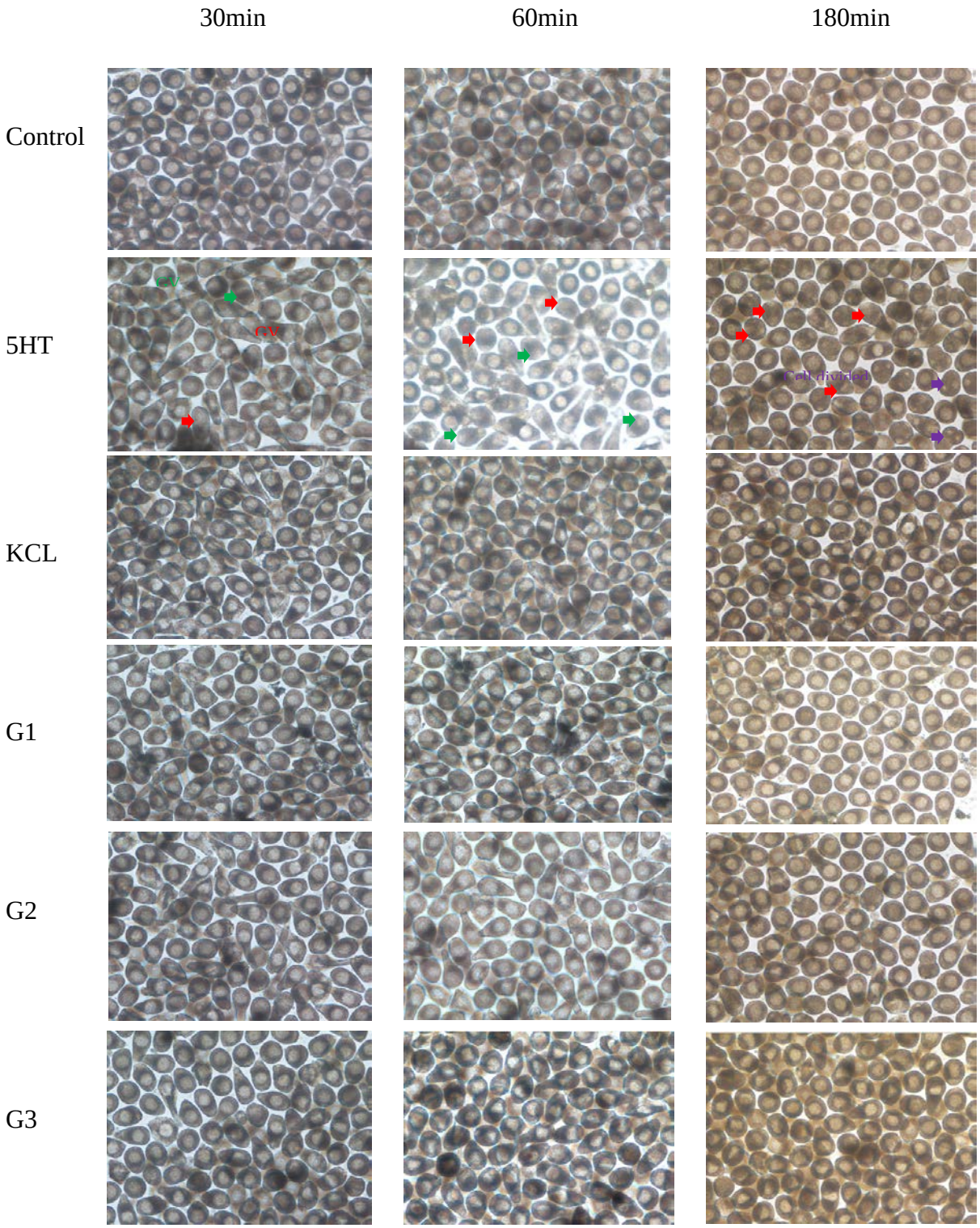
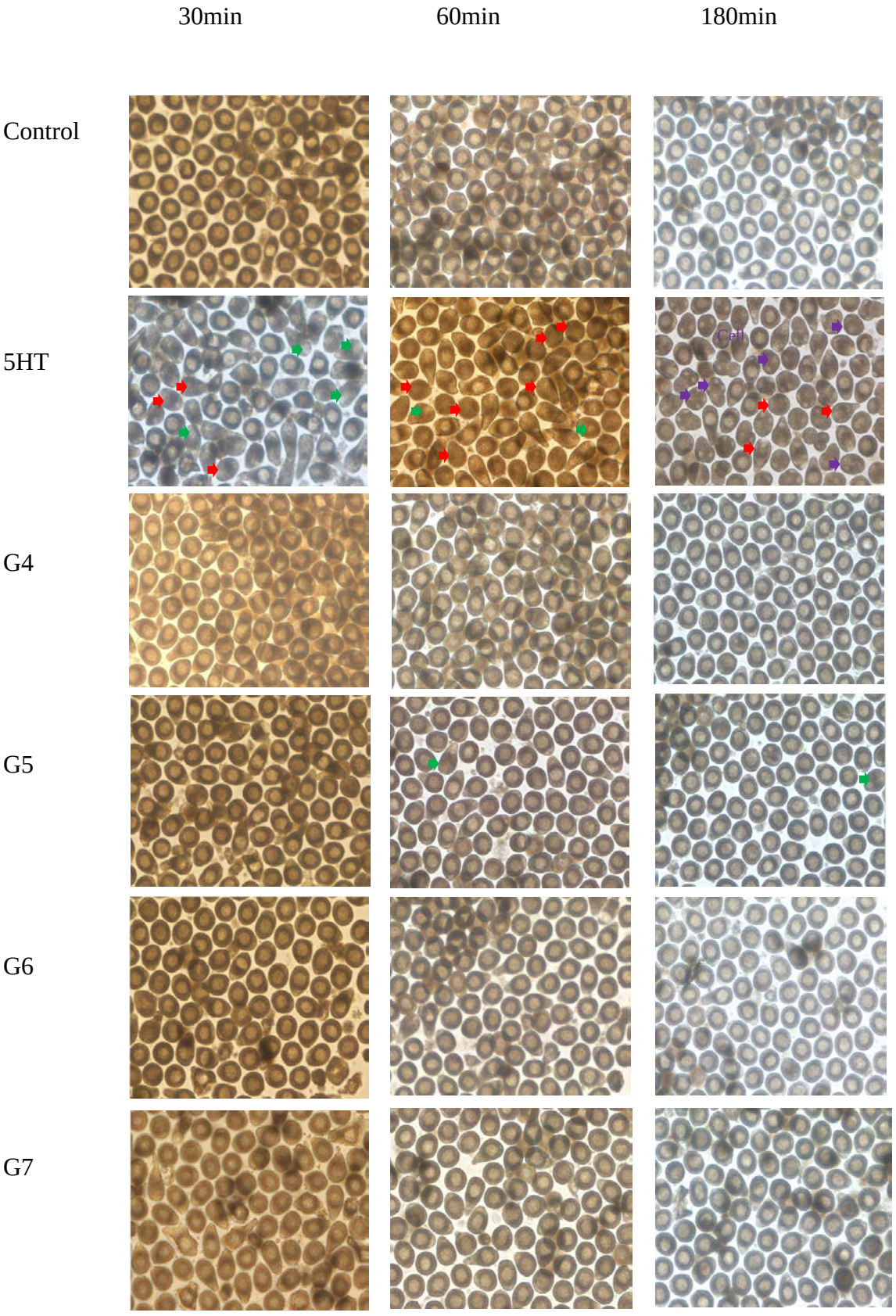


Figure 3. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides. Changes in fully developed ovarian samples after treatment with synthesized peptides from group 1 (G1), group 2 (G2) group 3 (G3), 5HT and KCl (Green for Germinal vesicle migration (GVM), Red for Germinal vesicle migration and breakdown (GVMB), Purple for cell division)


30min
60min
180min
Control
5HT
G4
G5
G6
G7

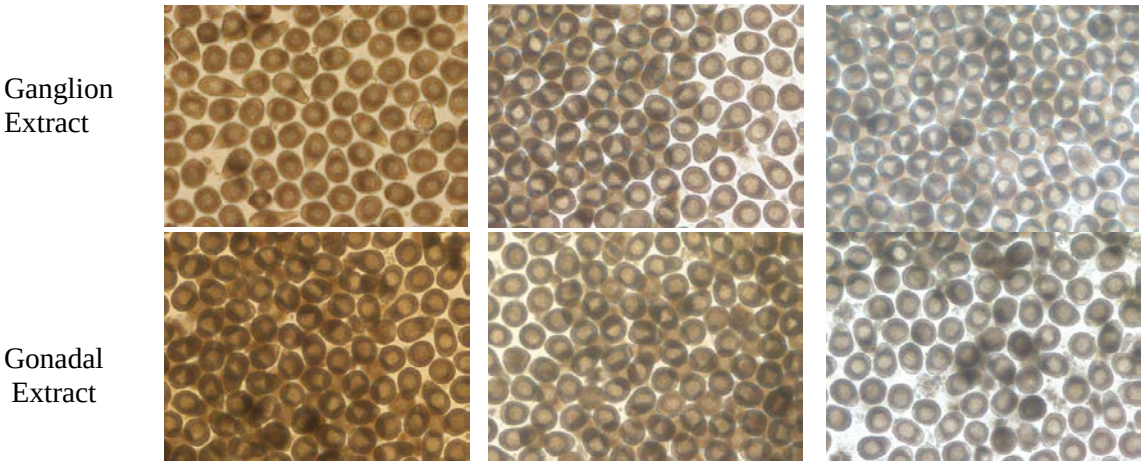


Figure 4. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides. Changes in fully developed ovarian tissue after treatment with synthesized peptides (Groups 4-7), ganglion and gonadal extracts (Green for GVM, Red for GVMB, Purple for cell division)

Assessment of biological activity using signal transduction

We did Western Blot (WB) for total ERK and phosphorylated ERK with 2-3 replicates for each treatment. Data from WB (Fig 5, 6, 7 and 8) shows that 5HT was clearly positive in all bioassays. Similarly, G1 looks significantly different compared to the negative control (Fig 6), other treatments for G1, 2, 3 and 7 (Fig 6, 7 and 8) and ELH show some effect. These were then tested *in-vivo*.

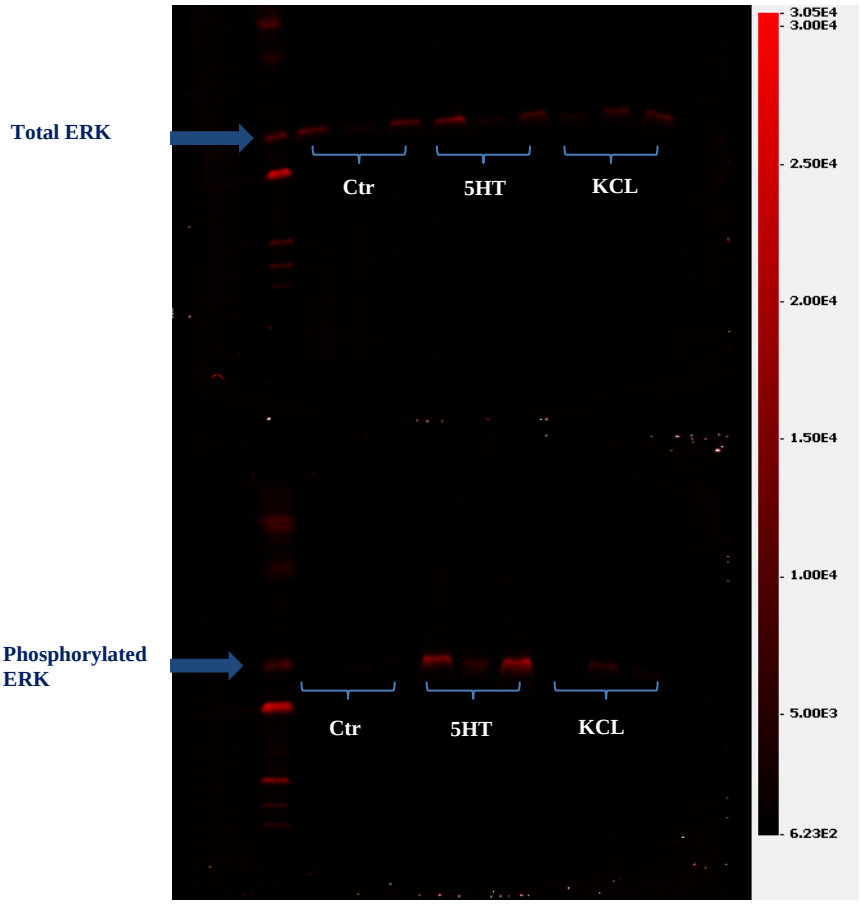


Figure 5. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides through assessment of phosphorylation. Total and phosphorylated ERK from *in-vitro* bioassay using negative control (Ctr), positive control (5HT) and KCl

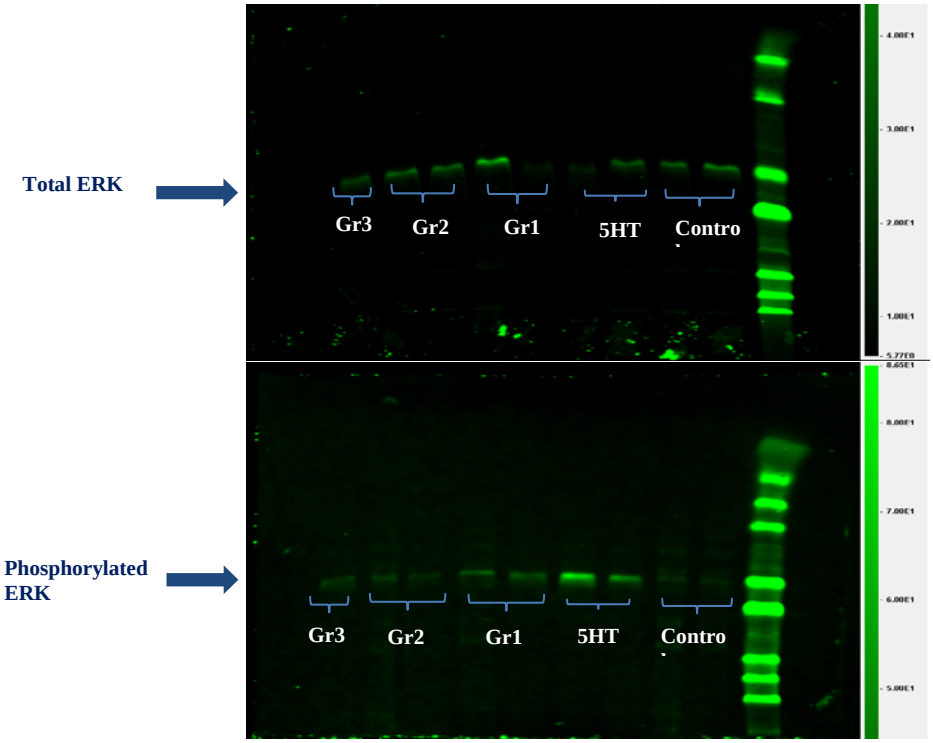


Figure 6. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides through assessment of phosphorylation. Total and phosphorylated ERK from *in-vitro* bioassay examining peptides from groups 1, 2, 3 5HT and negative control.

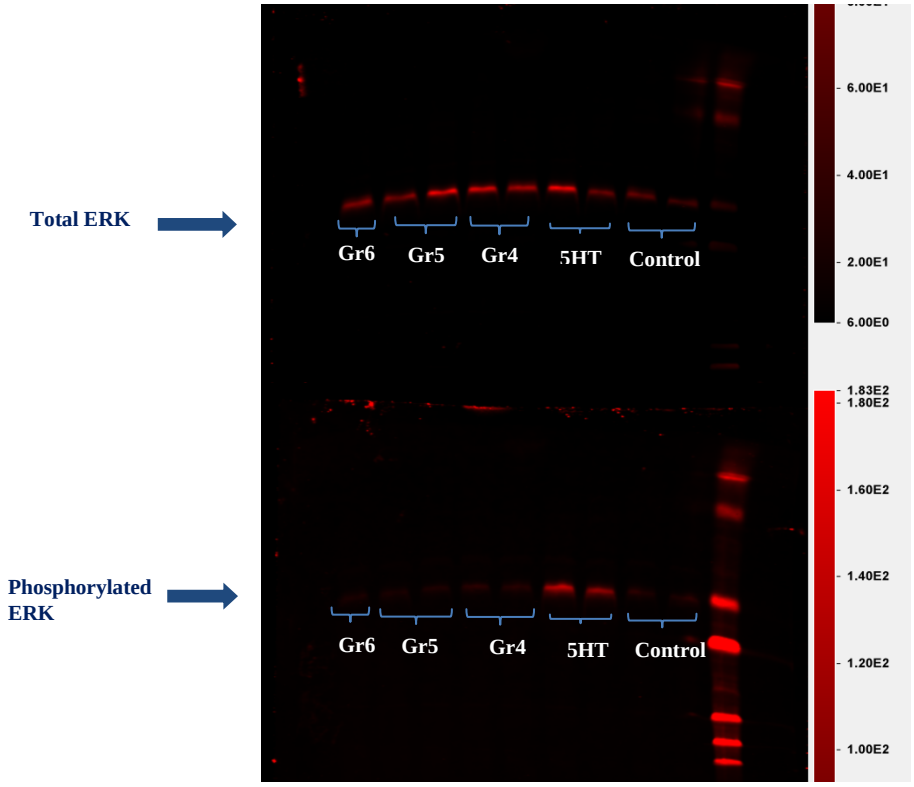


Figure 7. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides through assessment of phosphorylation. Total and phosphorylated ERK from *in-vitro* bioassay examining peptides from groups 4, 5, 6 5HT and negative control.

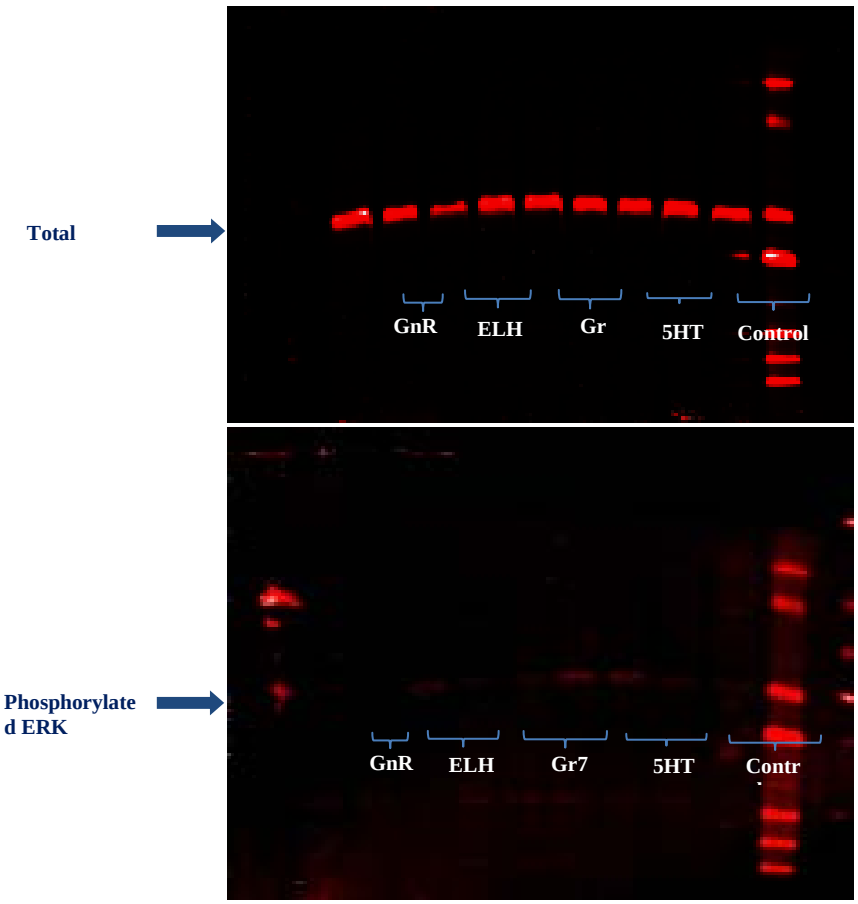


Figure 8. Ovarian *in-vitro* bioassay to assess biological activity of SRO neuropeptides through assessment of phosphorylation. Total and phosphorylated ERK from bioassay examining GnRH, ELH, 5HT and negative control.

In-vivo bioassay

Twenty-eight different synthetic peptides were tested. Two to five peptides were grouped together for the test based on their precursors. Each injection included 40µg of individual peptide in the group and made up to 10µL with distilled water. Distilled water and 50mM Serotonin using the same volume (10µL) for each injection were used for negative and positive control respectively. The SRO were kept individual in separate containers, 10 oysters for each treatment.

Figure 9. Left- In Vu and Nikoleta Ntalamagka. Right- 110 tubs each with individual oysters. Spawning was assessed 6 and 24 h post treatment by looking for eggs in the container.

Table 3. List of synthetic peptides and controls used for the *in-vivo* bioassay. 40ug of each peptide were injected into each oyster (n=10). The % females that spawned within 6 or 24 hours (combined) in response to the treatment is presented.

No	Group	Names	Sequences	% Female spawned
1		Distilled water	*For negative control*	20
2		Serotonin 50mM	*For positive control*	90
3	1	ELH1	GRLSLTADLRSLARMLEAHRKRYLASRSPYDSIRKKLFKF-NH_2	70
4		ELH2	Pyr-QRLSVNGALSSLADMLAASGRQRMRSEMEINRQRLFGL-NH_2	
5	2	GnRH	Pyr-QNYHFSNGWQP-NH_2	86
6	3	CCAP like	GICPYWGC-NH_2	78
7			LFCNFGGCFN-NH_2	
8			SLPLKTRFLMR-NH_2	
9		CP-1672W	GMPMLRL-NH_2	
10	4	Hypothetical protein captedraft 221538	LRYFI-NH_2	50
11			MRYFL-NH_2	
12			MRYFLGKRTRYFL-NH_2	
13	5	Pendal like	IPYMYNNYRYGTHGLFA	38
14			SFDSIAHSGRFGVFS	
15	6	APGWAmide like	RPGW-NH_2	100
16			KPGW-NH_2	
17			SPGW-NH_2	
18			APGW-NH_2	
19	**7**	Allatoropin like	GFRQSIVDRMGHGF-NH_2	17
20		LASGLV like	MLDRVGSGFI	
21			LDRLSMGLL	
22			YFDRLSSGFI	
23			RFDRLGSGFI	
24	8	LRFNamide	LRYFI-NH_2	44
25			MRYFL-NH_2	
26			DGTRYFL-NH_2	
27	9	Pedal like	ALDRYSFFGGL-NH_2	100
28			ALDKYGFFGGI-NH_2	
29			GLDRYSFMGGI-NH_2	
30			GLDRYGFAGSL-NH_2	

Data from table shows that Serotonin was a good positive control with 90% of females tested experienced spawning (one died before the 24 h assessment point). Peptides in groups 2,3,6, and 9 show spawning induction activity *in-vivo* with 86, 78, 100, and 100 % females spawning. Gonad tissues were also collected for histology work (Bouin's) and Western blot for P-ERK calculation, however the results for those assays will not be included in this report but published in a manuscript currently in preparation.

4. Discussion

We have identified genes encoding putative full-length or partial-length neuropeptide precursors from the *S. glomerata* transcriptome databases (**Table 1**). Numerous peptides are released from these precursors, some of which were confirmed by mass spectrometry analysis. For some of the neuropeptides, defined roles in reproduction and growth have been established and will be discussed in the context of the newly identified oyster sequences. The peptidomic approach confirmed the expression and the actual processing of about half of the predicted peptides.

Neuropeptide precursors were identified in *S. glomerata* for allototropin and conopressin. The allatotropin peptide stimulates synthesis of juvenile hormones in insects (Kataoka *et al.* 1989), where it has been most well studied. *Sg* allatotropin precursors can be cleaved to produce a 14 amino acid allatotropin peptide, GFRQSIVDRMGHGF-NH_2, which is identical to that found in the *C. gigas*. It was tested as part of group 7 and did not induce spawning. Another gene, *Sg-conopressin* encodes a single vasopressin-related conopressin precursor. In *Lymnaea*, conopressin controls sexual behavior (Van Kesteren *et al.* 1995). This peptide has not been tested yet.

Other neuropeptide precursors were identified in *S. glomerata* for NPY and LFRFamide. Multiple sequence alignment between the oyster putative neuropeptide precursors with previously identified homolog sequences in mollusc confirms high identity within the bioactive peptide sequences and variability outside these regions. RFamides, which feature a C-terminal sequence –RFa, constitute one of the largest families of neuropeptides and include the FMRFa and LFRFa (Cropper *et al.* 1994; Hoek *et al.* 2005; Moulis 2006; Walker *et al.* 2009). The *Sg-LFRFa* gene encode for precursors that contain multiple basic GXLL/FRFa peptides. In *L. stagnalis*, LFRFa peptides inhibit electrical activity of neuroendocrine cells that control either growth and metabolism or reproduction (Hoek *et al.* 2005). However, in the cephalopod, *Sepia officinalis*, LFRFa activity is predominantly targeted to the rectum, where it increases the frequency, tonus and amplitude of rectal contractions

(Zatylny-Gaudin *et al.* 2010). In our study the LRFN family (group 8) did not induce significant spawning.

In oysters, synchronized gonad maturation and spawning is thought to occur via environmental cues, including tides and pheromonal cues released by conspecifics that initiate endogenous cues (Galtsoff 1938). The gonadotropin-releasing hormone (GnRH) and ELH peptides have been most well studied in relation to endogenous reproduction hormones. Although much is known about the role of GnRH in vertebrate reproduction (Roch *et al.* 2011), we are only just beginning to explore its function in molluscs. In molluscs, a GnRH was first identified in *Aplysia* (Zhang *et al.* 2008) and it was later determined that although administration of synthetic GnRH could stimulate behaviours such as inhibition of feeding, no effects on ovotestis, reproductive tract, egg-laying or penile eversion was observed (Zhang *et al.* 2008). However, in the scallop *Patinopecten yessoensis*, mammalian GnRH can stimulate spermatogonial proliferation (Nakamura *et al.* 2007). Two GnRH-related peptides were later found by mass spectrometry of the *C. gigas* visceral ganglia, and gene expression analysis demonstrated a correlation with reproduction and nutritional status (Bigot *et al.* 2012). The *Sg-GnRH* and gene identified in the present study encodes for a precursor, which includes the conserved 11 residue GnRH-like peptide identical to that found in *C. gigas* and differ at only residue 10 in *P. fucata* (ie. His for *P. fucata* and Gln for *S. glomerata*). GnRH has induced spawning in 86% of the oysters in this study (group 2) and is certainly a peptide which warrants further research.

The egg-laying hormone has received significant attention due to the nature of its function in eliciting egg laying in the sea slug *Aplysia,* from which it was first discovered (Strumwasser *et al.* 1987). Its gene structure is typical of many molluscan neuropeptides in that it is derived from a precursor that gets modified by processing enzymes, such as prohormone convertase. The *Sg-ELH* gene encodes a 169 amino acid preprohormone consisting of a signal peptide and two ELH-like peptides (**Fig 1**). ELH induced spawning in 70% of the oysters in this study, and will also be studied further.

A glycoprotein family known as cysteine knot-forming heterodimers consisting of alpha- (GPA) and beta-subunits (GPB) are evolutionarily conserved (Sellami *et al.* 2011). In vertebrates the heterodimer is called

thyrostimulin, composed of GPA2 and GPB5. Homologs occur in arthropods, nematode, cnidarians, and molluscs implying that this neurohormone system existed prior to the emergence of bilateral metazoans (Paluzzi *et al.* 2014). The GPA2 of the GPA2/GPB5 dimer was identified in *S. glomerata.*

Although a function for these proteins in molluscs is currently unknown, in insects, studies using the mosquito (*Aedes aegypti*) suggest that GPA2/GPB5 participates in ionic and osmotic balance, since it appears to inhibit natriuresis and promote kaliuresis (Paluzzi *et al.* 2014).

Full and partial-length *S. glomerata* genes were also identified that encoded peptides with identity to buccalins, CCAP FCAP, which resulted in spawning in 78% of the *S. glomerata* females, GGNamide LASGLVamide, (which didn't induce spawning), LFRYa, myomodulin 2, Pedal peptide, which induced spawning in 100% of the females, sCAP (small Cardio Active Peptide)/pyrokinin-like peptides, tachykinins and the NdWFamide. APGWAmide like peptides also induced spawning in 100% of the *S. glomerata* females. Our testings were done using groups of peptides derived from the same protein, and the following studies would test each of those peptides independently, to identify the active one.

Conclusions

In this study we described the identification of putative oyster neuropeptides using *in silico* transcriptome database searches. The results clearly demonstrate that neuropeptide genes are conserved in bivalves, however, there are distinct differences with other molluscs. Despite a sessile mode of life and thus less intricate patterns of behavioural events, oysters have obviously retained a repertoire of neuropeptides with a complexity similar to that of other mollusc classes. The number of peptides predicted in our study supports the power of genome mining for neuropeptide gene discovery, and provides a strong foundation for future *in silico* investigations within oysters. Further research is additionally needed to validate peptide predictions through gene expression analysis as well as peptide expression identification using

mass spectrometry approaches with other endocrine tissues and at different stages of development and metabolic states.

5. Benefits and Adoption

Through this study we have identified peptides that induce spawning in SRO, and these can be used immediately by the industry where control of spawning is required to undertake specific activities - controlled crosses are an obvious example. From a commercial perspective it offers the chance to spawn only those animals needed and not resort to strip spawning which kills the animals. Hence commercial adoption is available to any farmer interested to explore these peptides. The project would certainly benefit from further R&D, as detailed below.

6. Further Development

We have identified peptides that induce spawning, however have not yet assessed their effect on oyster conditioning, or followed their expression in oysters at different developmental stages to assess their individual roles. This needs to be explored in order to offer an improved solution for securing appropriate conditioning and spawning in oyster lines that experience difficulties. Further work to develop tailored treatments for conditioning, spawning and sex control are still required, and further investment in the scale of a few tens of thousands of dollars will be needed to:

1. Carry out a molecular analysis of the profiles of peptides at different condition, gonadal development and sex determination stages so efficient and specific treatments can be designed.
2. Further test and optimise the treatments with the identified peptides. This will need to be done where access to a large number of animals at different stages of conditioning and from different genetic backgrounds are available.

Both of these can achieved within a two-year period.

7. Planned Outcomes

Public Benefit Outcomes

The genomic data obtained in this study will be published and the results available to the public. We expect the knowledge obtained to be of general

interest and result in improved strategies for controlling reproductive development in oysters.

Private Benefit Outcomes

These findings will lead to reduced costs and increases in the reliability of hatchery operations; this in turn reduces costs for farmers, increases the diversity of families hatcheries can produce (remembering there are up to 7 "types" of oysters demanded from hatcheries in NSW) and increases farmer faith in the reliability of hatchery production and therefore the likelihood of adopting selectively bred seed. If we can control reproduction - it could also open the path to simultaneously conditioning various lines in common systems, which would further reduce costs.

Linkages with CRC Milestone Outcomes

This project links with CRC milestone outcomes 1.3 - Removal or reduction of key production and 1.3.1 - New genetic tools developed for genetic constraints in existing aquaculture systems.

8. Conclusion

This study demonstrates the power of genomics and how we can start to harness the animals' own physiological cues to obtain better control over reproduction, in this case, but most likely this can be extended to growth and other traits. We have set the foundations for bio-discovery of active peptides, identified a suite of peptides which show biological activity in the context of reproduction which now can be further optimised in collaboration with the industry.

This approach can be extended to other species to address production bottlenecks and achieve a better balance between environmental and physiological cues.

This program is also a valuable adjunct to CRC 2009/743 "Incorporation of selection for reproductive condition, marketability and survival into a breeding strategy for Sydney Rock Oysters and Pacific Oysters", which sought to develop selection methods to enhance reproductive conditioning,

marketability and survival and to develop oyster families which increasingly display these features. 2009/743 confirmed variability in the reproductive behaviour among SRO families and has provided a platform for ongoing selection. It has however also highlighted challenges arising from such variability for ongoing breeding programs and reinforced the need for greater understanding of the mechanisms underlying reproductive behaviour and the capacity to manipulate reproductive condition.

While 2009/743 sought to address the practical and logistical challenges associated with unravelling genetic and environmental influences on reproductive behaviour of the various SRO family lines through appropriately designed field trials, this program offers a complimentary and perhaps a more direct approach to the assessment of genetic differences affecting reproductive responses. There is a clear role here for the use of the transcriptome and proteome to refine and better understand the outcomes of 2009/743 as well as modify the approach taken in future studies.

In the first instance we are now on the path to the determining critical periods in which the environment influences reproductive behaviour and can be manipulated. Sex ratios in SRO are both variable and unpredictable. Presumably sex determination occurs at an early stage of the cycle and our studies could both pinpoint the time and its duration. If this can be achieved it could be possible to influence sex ratios to suit production. Commonly, younger oysters first mature as males. Increasing the likelihood of females at first maturity could reduce generation times for breeding programs. Ensuring a balanced sex ratio reduces the numbers of oysters required for spawning and reduces costs associated with conditioning oysters for spawning – now allowance is required for highly skewed sex ratios. However, once access to a minimum number of males for diversity has been achieved, females are commonly more desirable as egg numbers are almost invariably a greater constraint than sperm numbers. Deliberate manipulation of the sexes additionally opens the path to separate conditioning of males and females, which could also reduce the likelihood of unplanned and uncontrolled spawning.

This study provides the initial understanding of transcriptomic and proteomic responses required to encourage laboratory based studies in which the relative importance of particular environmental variables is assessed, free of the multitude of confounding factors ever-present in field evaluations. Critically, the knowledge gained here will be of significant relevance to ongoing SRO research in which the “omics” will be used to assess genetic responses to other selection pressures. Large studies are underway using the genome, transcriptome and proteome to investigate responses to disease. Disease is often inextricably linked with reproductive behaviour, for instance high mortality often occurring at or shortly after the reproductive peak (Summer mortality in PO). Understanding reproductive changes is therefore essential to unravelling differences in genetic response to disease from fundamental changes in reproductive behaviour.

While not the aim of this study but still an important side product, the development of the microsatellite suite undertaken in the current program has had both immediate and long term benefits for SRO research in general. The suite has been used to provide valuable insights into the status of current breeding lines and the management practices available to ensure continued diversity. In the future it will also assist in developing studies to assess population differences and translocation responses, as well as future planned assessments of genetic transfer between cultured and wild populations.

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