Evolution and comparative physiology of luqin-type neuropeptide signalling

Luis Alfonso Yañez-Guerra[1,2], Maurice R. Elphick[1]

1. Queen Mary University of London, School of Biological & Chemical Sciences, Mile End Road, London, E1 4NS, UK

2. Present address, University of Exeter, Living Systems Institute, North Park Road, Exeter, EX4 4QD, UK

Correspondence should be addressed to:

Dr. Luis A. Yañez Guerra: L.Yanez-Guerra@exeter.ac.uk

or

Prof. Maurice R. Elphick: m.r.elphick@qmul.ac.uk

Key words

Luqin, cardio-excitatory peptide, RYamides, RWamides, neuropeptide evolution, G-protein coupled receptors.

Abstract

Luqin is a neuropeptide that was discovered and named on account of its expression in Left Upper Quadrant cells of the abdominal ganglion in the mollusc *Aplysia californica*. Subsequently, luqin-type peptides were identified as cardio-excitatory neuropeptides in other molluscs and a cognate receptor was discovered in the pond snail *Lymnaea stagnalis.* Phylogenetic analyses have revealed that orthologs of molluscan luqin-type neuropeptides occur in other phyla; these include neuropeptides in ecdysozoans (arthropods, nematodes) that have a C-terminal RYamide motif (RYamides) and neuropeptides in ambulacrarians (echinoderms, hemichordates) that have a C-terminal RWamide motif (RWamides). Furthermore, precursors of luqin-type neuropeptides typically have a conserved C-terminal motif containing two cysteine residues, although the functional significance of this is unknown. Consistent with the orthology of the neuropeptides and their precursors, phylogenetic and pharmacological studies have revealed that orthologous G-protein coupled receptors mediate effects of luqin-type neuropeptides in spiralians, ecdysozoans and ambulacrarians. Luqin-type signalling originated in a common ancestor of the Bilateria as a paralog of tachykinin-type signalling but, unlike tachykinin-type signalling, luqin-type signalling was lost in chordates. This may largely explain why luqin-type signalling has received less attention than many other neuropeptide signalling systems. However, insights into the physiological actions of luqin-type neuropeptides (RYamides) in ecdysozoans have been reported recently, with roles in regulation of feeding and diuresis revealed in insects and roles in regulation of feeding, egg laying, locomotion and lifespan revealed in the nematode *Caenorhabditis elegans*. Furthermore, characterisation of a luqin-type neuropeptide in the starfish *Asterias rubens* (phylum Echinodermata) has provided the first insights into the physiological roles of luqin-type signalling in a deuterostome. In conclusion, although luqin was discovered in *Aplysia* over thirty years ago, there is still much to be learnt about luqin-type neuropeptide signalling. This will be facilitated in the post-genomic era by the emerging opportunities for experimental studies on a variety of invertebrate taxa.

Introduction

Neuropeptides are evolutionarily ancient neuronal signalling molecules that typically exert their effects on target cells by binding to cognate G-protein coupled receptors (GPCRs) (Jékely et al., 2018; Elphick et al., 2018). Phylogenetic studies have revealed that the evolutionary origin of at least thirty neuropeptide signalling systems can be traced back to the urbilaterian common ancestor of deuterostomes and protostomes. However, some neuropeptide signalling systems have been lost in one or more bilaterian phyla/sub-phyla (Jékely, 2013; Mirabeau and Joly, 2013; Elphick et al., 2018). Luqin-type neuropeptide signalling, which is the focus of this review, is one of the bilaterian neuropeptide signalling systems that have been lost in chordates/vertebrates. This may in part explain why less is known about luqin-type neuropeptide signalling than other bilaterian neuropeptide systems that have been retained in vertebrates. Nevertheless, several advances in our knowledge of the evolution and comparative physiology of luqin-type signalling in invertebrates have been made recently and therefore writing of this the first review article on luqin-type neuropeptide signaling is timely.

The neuropeptide luqin and its cognate G-protein coupled receptor were first discovered in molluscs (Shyamala et al., 1986; Fujimoto et al., 1990; Tensen et al., 1998). Subsequently luqin-like neuropeptides known as RYamides, on account of a C-terminal Arg-Tyr-NH_2 motif, were discovered in the arthropod *Cancer borealis* (Li et al., 2003). Furthermore, receptors for RYamides were identified in the fruitfly *Drosophila melanogaster* and in the red flour bettle *Tribolium castaneum* (Collin et al., 2011; Ida et al., 2011). In 2013, evidence that molluscan luqin-type signalling and arthropod RYamide-type signalling are orthologous was reported. Thus, use of pairwise-based clustering methods revealed that luqin precursors and RYamide precursors form part of the same protein cluster (Jékely, 2013). Furthermore, phylogenetic analysis of G-protein coupled neuropeptide receptors revealed that molluscan luqin receptors are orthologues of arthropod RYamide receptors (Mirabeau and Joly, 2013). In addition, these two studies also reported for the first time the discovery of a luqin-type signalling system in the nematode *Caenorhabditis elegans*, which has subsequently been functionally characterised experimentally (Ohno et al., 2017; Jékely, 2013; Mirabeau and Joly, 2013).

Importantly, phylogenetic analysis of transcriptome/genome sequence data has revealed that whilst luqin-type signalling has been lost in vertebrates and other chordates (urochordates, cephalochordates), genes encoding luqin-type precursors and receptors are present in ambulacrarian deuterostomes (echinoderms and hemichordates) (Mirabeau and

Joly, 2013; Jékely, 2013). Thus, it was established for the first time that the evolutionary origin of luqin-type neuropeptide signalling predates the divergence of protostomes and deuterostomes, but with differential loss in the deuterostome branch of the Bilateria. Luqin-type neuropeptides in ambulacrarians are characterised by a predicted C-terminal RWamide motif (Elphick and Mirabeau, 2014; Rowe et al., 2014; Semmens et al., 2016). Furthermore, biochemical characterisation of luqin-type neuropeptide signalling in the starfish *Asterias rubens* (phylum Echinodermata) demonstrated that a neuropeptide with the confirmed structure EKGRFPKFMRW-NH_2 acts as a ligand for two luqin-type receptors in this species (Yañez-Guerra et al., 2018). Thus, the luqin-type neuropeptides in spiralian protostomes, the luqin-type RYamides in ecdysozoan protostomes and the luqin-type RWamides in ambulacrarian deuterostomes have been unified as members of a bilaterian family of neuropeptides (Jékely, 2013; Mirabeau and Joly, 2013; Yañez-Guerra et al., 2018). It is against the backdrop of these important recent findings that we review here our knowledge of the evolution and comparative physiology of luqin-type neuropeptide signalling.

The discovery and functional characterisation of luqin-type neuropeptide signalling in molluscs

The discovery of the neuropeptide luqin was enabled by identification of transcripts expressed in the L5 neuron of the mollusc *Aplysia californica*. This was decades before the development of contemporary single cell transcriptomic methodologies and was facilitated by the large size of the cell body of the L5 neuron (Shyamala et al., 1986). Analysis of the expression of the cardio-excitatory neuropeptide FMRFamide in the central nervous system of *A. californica* using antibodies to FMRFamide revealed immunostaining in many neurons, including the L5 neuron that is located in the upper quadrant of the left abdominal ganglion (Brown et al., 1985; Schaefer et al., 1985). However, it was found that the FMRFamide gene is not expressed in the L5 neuron. Therefore, to determine the identity of neuropeptide(s) responsible for FMRFamide-like immunoreactivity in L5, transcripts expressed in this neuron were sequenced. Sequencing of a transcript named L5-67 revealed that it encodes a 112 amino acid residue protein comprising an N-terminal signal peptide followed by a neuropeptide with a predicted C-terminal RFamide motif, which therefore could be cross-reactive with FMRFamide antibodies (Shyamala et al., 1986). Subsequent biochemical analysis revealed that processing of this precursor gives rise to the amidated decapeptide APSWRPQGRF-NH_2, which was named luqin because it is expressed in the Left Upper Quadrant cells of the abdominal ganglion (Aloyz and DesGroseillers, 1995). Furthermore, a

76 amino acid peptide corresponding to the region of the precursor protein C-terminal to the luqin neuropeptide was also detected and named proline-rich mature peptide (PRMP) (Aloyz and DesGroseillers, 1995). PRMP contains two cysteines separated by a 10 amino acid-residue sequence and subsequent studies have revealed that this is an evolutionarily conserved feature of luqin-type precursor proteins (Figure 1) (Yañez-Guerra et al., 2018; Jékely, 2013; Mirabeau and Joly, 2013).

In addition to luqin and PRMP, a mass spectrometric survey of the left upper quadrant neurons revealed that two other peptides are derived from the *A. californica* luqin precursor, which were named luqin-B and luqin-C (Li et al., 1998). The luqin-B fragment contains part of the mature luqin neuropeptide and the luqin-C fragment contains a shorter version of PRMP (Li et al., 1998), but it is not known if PRMP, luqin-B or luqin-C are biologically active molecules. However, it has been shown that alternative splicing by exon skipping of one of the exons of the gene encoding the luqin precursor results in a frame shift and production of a precursor protein comprising the complete mature amidated decapeptide luqin and a short C-terminal region that does not contain the two cysteines characteristic of PRMP. This suggests that PRMP may not be essential for biosynthesis of the mature luqin neuropeptide. Still, from a functional perspective it is noteworthy that whilst the full-length transcript is widely expressed in *A. californica*, the alternative transcript is specifically expressed in the kidney (Angers and DesGroseillers, 1998).

Sequencing of transcripts encoding luqin-type precursors and mass spectrometric identification of neuropeptides derived from them has revealed a high level of sequence conservation of luqin-type neuropeptides in molluscs. For example, in the giant snail *Achatina fullica* a luqin-type peptide was identified as an amidated undecapeptide SGQSWRPQGRF-NH_2, the C-terminal region of which (underlined) is identical to *Aplysia* luqin (Fujimoto et al., 1990), and in the pond snail *Lymnaea stagnalis* the luqin-type peptide TPHWRPQGRF-NH_2 was identified. It is noteworthy that the luqin precursors in *A. californica, L. stagnalis, A. fullica* comprise a single luqin-type neuropeptide, whereas in some other gastropod molluscs, such as the eastern mudsnail *Ilyanassa obsoleta* and the freshwater snail *Biomphalaria glabrata,* the precursor comprises two luqin-type neuropeptides (Figure 1). The first insight into the characteristics of luqin receptors was made with the discovery that the luqin-type peptide TPHWRPQGRF-NH_2 is a ligand for an orphan G protein-coupled receptor, GRL106, in the pond snail *Lymnaea stagnalis.* It was also reported that this receptor is closely related to vertebrate tachykinin receptors and the *Drosophila* neuropeptide-Y-type receptor (Tensen et al., 1998).

Insights into the physiological roles of luqin-type neuropeptides were facilitated by the identification of a luqin-type neuropeptide in the snail *A. fullica* – a pulmonate gastropod. The peptide was discovered on account of its bioactivity as a cardioactive peptide that causes an increase in the frequency of beating when applied to auricle preparations from *A. fullica*. Hence, this peptide was named *Achatina* cardioexcitatory peptide (ACEP) (Fujimoto et al., 1990). However, ACEP also affects other muscle systems in *A. fullica,* increasing the amplitude of tetanic contraction of the penis retractor muscle and buccal muscle in response to electrical stimulation. Furthermore, ACEP was found to induce depolarisation and rhythmic firing of a motor neuron (B4) that innervates buccal muscle (Fujimoto et al., 1990), an effect indicative of a physiological role in regulation of feeding behaviour. Consistent with findings from *A. fullica*, the luqin-type peptide identified as a ligand for the *Lymnaea* receptor GRL106 was also found to be cardioexcitatory in this pulmonate gastropod species. Hence the peptide was named *Lymnaea* cardioexcitatory peptide (LyCEP). Furthermore, the sequence similarity that LyCEP and ACEP share with *Aplysia* luqin was noted (Tensen et al., 1998). In accordance with the cardioexcitatory actions of LyCEP, immunohistochemical analysis revealed that LyCEP-immunoreactivity is present in nerve fibres ending in the pericardial cavity of the heart, indicating that the peptide is released into the pericardial cavity as a neurohormone (Tensen et al., 1998). However, LyCEP is not, as its name implies, specifically a cardioactive peptide because it is also expressed in nerve fibres associated with inhibition of the egg-laying hormone-producing caudodorsal cells in *L. stagnalis.* Accordingly, transcripts encoding the LyCEP receptor are present in the caudodorsal cells and LyCEP causes hyperpolarisation of these cells *in vitro* (Tensen et al., 1998).

Analysis of the expression of the luqin precursor in the ophistobranch gastropod *A. californica* revealed transcripts in approximately 100 neurons of the central nervous system, but predominantly in neurons that innervate the circulatory and reproductive systems. In peripheral tissues, transcripts were detected in the intestine and in the kidneys (Giardino et al., 1996). Whole mount immunolabelling experiments with an antibody directed against the luqin precursor revealed immunoreactive fibres in different regions of the circulatory system of *Aplysia,* including the auricle, the ventricle and the aorta. In the reproductive system, immunoreactive fibres were detected in the small and large hermaphroditic ducts and in the ovotestis (Giardino et al., 1996). The kidney also displayed immunoreactivity, located on the inner surface of the kidney wall. Strong immunoreactivity was also seen in neurites located in a large nerve associated with muscles of the renal pore, a sphincter that controls urine efflux (Angers et al., 2000). Altogether, these findings suggest roles of luqin-type peptides in

regulation of the reproductive and circulatory systems, in fluid mobilization, and water homeostasis in gastropod molluscs.

Whilst what is known about luqin expression and function in molluscs is largely based on experimental studies on selected gastropod species, as detailed above, genes/transcripts encoding luqin-type precursors have been identified in other gastropod species and in species belonging to other molluscan classes, including Bivalvia, Scaphopoda, Cephalopoda, Monoplacophora, Polyplacophora, Chaetodermomorpha and Neomeniomorpha (De Oliveira et al., 2019). Thus, the occurrence of luqin-type neuropeptide precursors throughout the phylum Mollusca has been established, providing a basis for extending analysis of luqin-type neuropeptide function beyond gastropods to other classes.

Luqin-type signalling in annelids

Luqin-type precursors with a similar organisation to those in molluscs have been identified in annelids. Analysis of genomic sequence data from *Capitella teleta* revealed the occurrence of a luqin-type precursor comprising the predicted mature peptide QFAWRPQGRF-NH_2 (Veenstra, 2011) (Figure 1). Later, a partial precursor transcript was identified in the annelid *Platynereis dumerilii* and the structure of the luqin-type peptide derived from this precursor was confirmed as WRPQGRF-NH_2 using mass spectrometry (Conzelmann et al., 2013). Furthermore, a transcript encoding a luqin-type receptor was identified in *P. dumerilii* and pharmacological characterisation of this receptor demonstrated that the luqin-type peptide WRPQGRF-NH_2 acts as a ligand for this receptor (Bauknecht and Jékely, 2015). Currently, there are no data available that provide insights into the physiological roles of luqin-type neuropeptides in annelids.

Luqin-type signalling in other spiralians

Analysis of genome/transcriptome sequence data has revealed the occurrence of genes/transcripts encoding luqin-type precursors in a variety of species belonging to the phylum Platyhelminthes. For example, in the planarian *Schmidtea mediterranea* an expanded family of genes encoding four luqin-type precursors have been identified (Koziol et al., 2016). The predicted neuropeptides derived from these precursors share sequence similarities with luqin-type neuropeptides from molluscs and annelids, including a C-terminal RFamide motif and the N-terminal motif WRPQ, which is conserved with only conservative substitutions (Figure 1). Interestingly, only two of the four precursors have a C-terminal pair

of cysteines separated by ten amino acids, which is typically a conserved feature of luqin-type precursors in other phyla (Koziol et al., 2016). The functional significance of the loss of this feature in two precursors is unknown, but it may be reflective of a loss of selection pressure after gene duplications gave rise to the four precursor genes.

Genes encoding luqin-type precursors have also been identified in parasitic platyhelminths, including the fox tapeworm *Echinococcus multilocularis*, the salmon fluke *Gyrodactlyus salaris*, the rodent tapeworm *Hymenolepis microstoma*, the broad fish tapeworm *Diphyllobothrium latum* and the cestode *Mesocestoides corti* (Koziol et al., 2016). In all these species a single luqin-type precursor comprising a single predicted neuropeptide was identified, with the peptides having the N-terminal motif WRPH that is similar to the WRPQ motif that is a feature of luqin-type neuropeptides in molluscs and annelids. Interestingly, however, the luqin-type peptides in these species are positioned in the C-terminal region of the precursor protein. This contrasts with precursors in other taxa, where luqin-type peptides are located N-terminally and proximal to the signal peptide. Furthermore, none of the luqin-type precursors in the parasitic platyhelminth species listed above have a C-terminal pair of cysteines separated by ten amino acids. Thus, the luqin-type precursors identified in in parasitic platyhelminthes are highly divergent by comparison with other luqin-type precursors. This makes sequence alignment difficult and for this reason luqin-type precursors from parasitic platyhelminths are not included in Figure 1 but instead they are shown in Supplementary Figure 1B. Nevertheless, evidence that luqin-type neuropeptides in parasitic platyhelminths are functional can be found in the identification of genes encoding candidate luqin-type receptors; for example, in *E. multilocularis* (Koziol et al., 2016). Furthermore, a comprehensive analysis of GPCRs encoded in the genome of the parasitic helminth *Fasciola hepatica* has revealed the presence of a receptor that is clearly an orthologue of the luqin-type receptor from the annelid *P. dumerilii* and the RYamide receptor from *D. melanogaster* (McVeigh et al., 2018). Further studies are now needed to confirm the predicted luqin-type ligand-receptor partners in platyhelminths and to investigate the expression and pharmacological actions of luqin-type neuropeptides in platyhelminths.

Luqin-type precursors have also been identified in the brachiopod *Lingula anatina* and in the nemertean *Lineus longissimus* (bootlace worm) (De Oliveira et al., 2019). In both species, a single luqin-type precursor was identified that contains a predicted mature neuropeptide with the C-terminal sequence WRPQGRF-NH_2, which is the same motif identified in molluscan and annelid luqin-type peptides. The precursors identified in these species also have the typical C-terminal region containing the two cysteines separated by ten

amino acid residues (Figure 1). Furthermore, two proteins in *L. anatina* have been annotated as luqin-type receptors (XP_013402794.1, XP_013402807.1).

RYamides: luqin-type neuropeptides in arthropods

Arthropodan neuropeptides with a C-terminal RYamide motif (RYamides) were first identified in the decapod *Cancer borealis* by *de novo* post source decay sequencing of peptides in extracts of the pericardial organs of this species. Five different peptides were identified, all of them sharing the conserved C-terminal motif FXXXRY-NH_2, where X is variable (Li et al., 2003). RYamides sharing the same C-terminal motif were subsequently identified by analysis of tissue extracts from other decapods, including *Cancer productus* (Fu et al., 2005), *Pugettia producta* (Stemmler et al., 2007), *Carcinus maenas* (Ma et al., 2009), and in the Pacific white shrimp *Litopenaeus vannamei* (Ma et al., 2010).

Genes/transcripts encoding precursors of RYamides have been identified in several crustacean species, including the water flea *Daphnia pulex* (Dircksen et al., 2011), the isopod *Proasellus cavaticus* (Christie, 2017), the red swamp crayfish *Procambarus clarkii* (Veenstra, 2015), the Australian crayfish *Cherax quadricarinatus* (Nguyen et al., 2016) and the freshwater amphipod *Hyalella Azteca* (Christie et al., 2018). Interestingly, while most of these precursors comprise two RYamides, the *D. pulex* and *P. clarkii* precursors comprise three predicted RYamides (Supplementary Figure 1A). In the case of the *D. pulex* RYamide precursor, mass spectrometric analysis of brain tissue enabled identification of two of the three RYamides predicted to be derived from this precursor. The first peptide, located immediately after the signal peptide, was identified in its post-translationally modified form as pQTFFTNGRY-NH_2, with both C-terminal amidation and N-terminal conversion of a glutamine residue to pyroglutamate. The second RYamide predicted to be derived from this precursor was not detected by mass spectrometry. Two different forms of the third RYamide peptide were detected - the twenty-seven residue peptide SGNGGIVLGNSELDAR<u>R</u>NPERFFIGSRY-NH_2 and a C-terminal fragment of this peptide (NPERFFIGSRY-NH_2) generated by cleavage at the underlined arginine residue in the longer peptide (Dircksen et al., 2011).

Genes/transcripts encoding precursors of RYamides have also been identified in a variety of insects, including six *Drosophila* species, the red flour beetle *Tribolium castaneum*, the silkworm *Bombyx mori*, the honey bee *Apis mellifera*, the pea aphid *Acyrthosiphon pisum*, the yellow fever mosquitoes *Aedes aegypti* and *Culex pipiens* and the stick insect

Carausius morosus (Hauser et al., 2010; Liessem et al., 2018), and these typically comprise two predicted RYamides. Interestingly, in the RYamide precursor of the parasitic wasp *Nasonia vitripennis* paracopy expansion has occurred to give rise to a precursor comprising seven predicted RYamide-type neuropeptides (Supplementary Figure 1A). However, only one of these peptides has thus far been characterised biochemically by mass spectrometry (Hauser et al., 2010).

The first arthropod RYamide receptors to be characterised were from *T. castaneum* and *D. melanogaster*. In the case of the red flour beetle *T. castaneum,* a receptor was identified (GenBank Accession Number: HQ709383) and shown to be activated in a dose-dependent manner by the two RYamide peptides derived from the *T. castaneum* RYamide precursor (Collin et al., 2011). In *D. melanogaster*, the RYamide receptor was characterised independently by two laboratories, revealing that the two RYamides derived from the *D. melanogaster* RYamide precursor activate, in a dose-dependent manner, a receptor encoded by the gene CG5811 (Ida et al., 2011; Collin et al., 2011). Ida et al. (2011) also reported that injection of RYamide-1 suppresses the proboscis extension reflex (PER) in the blowfly *Phormia regina,* indicating a physiological role in regulation of feeding behaviour (Ida et al., 2011). Subsequent experimental studies on this species revealed the presence of twenty-six RYamide-immunoreactive neurons in the brain and showed that injection of RYamide-1 or -2 has no effect on the volume of sucrose solution intake when feeding occurs but causes a reduction in the percentage of flies exhibiting the PER. Furthermore, injection of RYamide-1 was found to cause a significant decrease in the responsiveness to sucrose solution of sugar receptor neurons located on the labellum of the proboscis. Thus, it was concluded that RYamides suppress feeding motivation and sucrose responsiveness in the blow fly *Phormia regina* (Maeda et al., 2015). Analysis of the expression of the RYamide precursor in the silkworm *Bombyx mori* using mRNA *in situ* hybridisation revealed expression in the brain, terminal abdominal ganglion and midgut. In the larval and adult brain four to seven pairs of RYamide precursor-expressing neurons were identified in the protocerebrum and tritocerebrum. Expression was also revealed in a pair of posterior dorsomedial neurons in the terminal abdominal ganglion of adults and larvae. Lastly, RYamide precursor expression was revealed in enteroendocrine cells of the anterior midgut of larvae, pupae and adult specimens of *B. mori.* This pattern of expression indicates that RYamides are involved in regulation of feeding and digestion in *B. mori* (Roller et al., 2016).

Consistent with the hypothesis that RYamides may also regulate feeding behaviour in other arthropods, use of quantitative real-time PCR revealed that expression of the RYamide

precursor gene is significantly downregulated in the brain after starvation in the decapod *Marsupenaeus japonicus* (kuruma shrimp). Furthermore, injection of RYamides into the muscle of juvenile *M. japonicus* caused suppression of food intake in some experiments, but this was not consistently reproducible (Mekata et al., 2017). Foregut activity in decapods is controlled by motor neurons in the stomatogastric ganglion (STG), the activity of which is regulated by a variety of different neuropeptide types (Marder and Bucher, 2007). To gain further insights into the complexity of neuropeptide signalling in the STG, transcriptomic analysis of the STG in the crab *C. borealis* revealed the presence of forty-six transcripts encoding receptors for twenty-seven different neuropeptide types, but interestingly RYamide receptor transcripts were not detected. Consistent with this finding, *in vitro* application of RYamides to *C. borealis* STG preparations had no consistent modulatory effect on the motor outputs of the ganglion (Dickinson et al., 2019). Thus, further studies are now required to investigate the mechanisms by which RYamides may affect feeding in decapod crustaceans.

RYamides are not only involved in regulation of feeding behaviour in arthropods. Analysis of RYamide expression in several *Drosophila* species revealed that the RYamide precursor gene is expressed in two abdominal neurons of the adult central nervous system that project to the rectal papillae, organs that mediate water re-absorption in flies (Veenstra and Khammassi, 2017). Consistent with this expression pattern, injection of female mosquitoes with RYamides delays postprandial diuresis (Veenstra and Khammassi, 2017). Thus, it appears that RYamides may act as regulators of urine production in insects. Interestingly, this is consistent with immunohistochemical evidence of a similar role in the mollusc *Aplysia*, where luqin-immunoreactivity has been localised in a nerve associated with muscles of the renal pore, a sphincter that controls urine efflux (Angers et al., 2000) (see above).

Detailed functional characterisation of luqin-type signalling in the nematode *Caenorhabditis elegans*

In 2003 Keating et al. reported an extensive functional analysis of GPCRs in the nematode *C. elegans*, employing use of RNA interference (RNAi) methods to knockdown GPCR gene expression. Included in this study was the gene *Y59H11AL.1* (also now known as *npr-22*) and a phylogenetic analysis revealed that the protein encoded by *Y59H11AL.1* is most closely related to the *L. stagnalis* luqin receptor GRL106. However, both the receptor encoded by *Y59H11AL.1* and GRL106 were classified by the authors under the general

descriptor of 'tachykinin-like receptors' (Keating et al., 2003). Subsequently, efforts were made to identify a neuropeptide(s) that act as a ligand for the *Y59H11AL.1*-encoded GPCR (NPR-22) by screening a library of synthetic neuropeptides predicted from analysis of the sequences of *C. elegans* neuropeptide precursors (Mertens et al., 2006). It was discovered that FMRFamide related peptides derived from the FLP-7 precursor can activate NPR-22, with the peptide FLP-7.3 (SPMERSAMVRF-NH_2) being the most potent, albeit with an EC_{50} of ~1 μM (Mertens et al., 2006). The authors also noted that NPR-22 (Y59H11AL.1) is closely related to the *D. melanogaster* receptor CG5811. However, at the time the peptides that act as ligands for CG5811 were unknown and it was not until five years later that it was discovered that CG5811 is a RYamide receptor (Ida et al., 2011; Collin et al., 2011).

In 2013, an extensive phylogenetic analysis of GPCRs and neuropeptide precursors identified a luqin-type precursor in the nematode *C. elegans* (Mirabeau and Joly, 2013). Furthermore, alignment of the *C. elegans* precursor with luqin-type precursors from other taxa revealed many conserved features. Thus, the precursor comprises two putative luqin-type peptides that have an RYamide motif and the C-terminal region of the precursor contains two cysteines, which are separated by eight amino acid residues (Mirabeau and Joly, 2013). The spacing of the two cysteines residues in the *C. elegans* luqin-type precursor is atypical of luqin-type precursors where, as highlighted above and shown in Figure 1, the two cysteine residues are usually separated by ten residues. However, this feature of the *C. elegans* luqin-type precursor is probably a derived characteristic because in another nematode species, the parasite *Trichuris suis*, the luqin-type precursor has two C-terminal cysteines separated by ten residues (Figure 1) (Yañez-Guerra et al., 2018).

Recently, a detailed analysis of the phylogenetic relationships of luqin-type receptors revealed that the *C. elegans* receptor NPR-22 (Y59H11AL.1) is an ortholog of the *L. stagnalis* luqin receptor (GRL106) and the *D. melanogaster* receptor CG5811 and belongs to a clade of luqin-type receptors that is quite distinct from the closely related tachykinin-type receptors and neuropeptide-Y-type receptors (Yañez-Guerra et al., 2018) (Figure 2). Furthermore, the neuropeptides LURY-1.1 (PALLSRY-NH_2) and LURY-1.2 (AVLPRY-NH_2) derived from the *C. elegans* luqin/RYamide precursor (LURY-1) were shown to act as ligands for NPR-22 at nanomolar concentrations (Ohno et al., 2017). Additionally, the authors showed that, as reported previously by Mertens et al. (2006), the FLP-7.3 peptide (SPMERSAMVRF-NH_2) derived from the FLP-7 precursor also acts as a ligand for NPR-22, but only at micromolar concentrations (Ohno et al., 2017). Therefore, it is likely that LURY-1.1 and LURY-1.2 are the natural ligands for NPR-22 and the ability of FLP-7 derived-

peptides to activate NPR-22 at micromolar concentrations may be an *in vitro* pharmacological and non-physiological phenomenon due to the presence of a C-terminal RYamide-like RFamide motif in these peptides. However, evidence that NPR-22 mediates effects of FLP-7 derived peptides *in vivo* has been reported in an investigation of neuroendocrine mechanisms of serotonin-induced fat loss in *C. elegans* (Palamiuc et al., 2017).

Having identified the molecular components of a luqin/RYamide-type neuropeptide signalling system in *C. elegans*, Ohno *et al*. (2017) performed a detailed functional characterisation of this signalling system. Analysis of the expression of the LURY-1 precursor and NPR-22 in *C. elegans* revealed that LURY-1 is expressed by the pharyngeal neurons M1 and M2, which regulate feeding in *C. elegans*. Thus, the M1 neuron stimulates spitting behaviour whereas the M2 neuron stimulates pharyngeal pumping (Bhatla et al., 2015). With a much wider pattern of expression, NPR-22 is expressed in head muscles, I2 pharyngeal neurons, feeding pacemaker MC neurons, the RIH neuron, the interneurons AIA and AUA, the ASK neurons, the ASI neurons, a few B-type motoneurons in the posterior ventral nerve cord, pharyngeal muscles, body wall muscles, the intestine and a few unidentified cells anterior to the nerve ring (Palamiuc et al., 2017; Ohno et al., 2017).

To gain insights into the physiological roles of LURY-1-derived peptides, the *lury-1* gene was overexpressed in *C. elegans*, with several phenotypes being observed. First, there was an increase in the number of unlaid eggs in the uterus but the rate of egg-laying was not affected, indicating that the rate of ovulation is normal but egg-laying is facilitated and embryos are laid prematurely. Accordingly, microinjection of synthetic LURY-1 peptides (10 µM) caused the same phenotype. Second, pharyngeal pumping, which is required for food intake, was reduced and microinjection of synthetic LURY-1 peptides (10 µM) caused the same phenotype. Third, adult lifespan was extended by as much as 21-50%. Finally, a reduction in locomotor activity was observed (Ohno et al., 2017). Importantly, all of these phenotypes were largely suppressed by the deletion of *npr-22*, indicating that LURY-1 peptides act upstream of NPR-22. Furthermore, these findings were consistent with a previous analysis of *npr-22* knockdown, which revealed a phenotype in which animals have a reduced body size and brood size (Ceron et al., 2007). More specifically, cell-specific rescue experiments indicated that NPR-22 acts in the feeding pacemaker MC neurons to control feeding and lifespan and NPR-22 acts upstream of the serotonin-uptaking RIH neuron to control egg-laying (Ohno et al., 2017). The authors conclude that food-evoked activation of

the pharynx triggers MC neurons to release of LURY-1 peptides, which then act as hormones via NPR-22-dependent mechanisms to control feeding, egg-laying and roaming behaviour (Ohno et al., 2017). Thus, use of *C. elegans* as a model experimental system has provided the first whole-animal perspective on the physiological/behavioural roles of luqin-type neuropeptide signalling.

RYamide-type neuropeptide precursors in other ecdysozoans

Analysis of transcriptome sequence data from the penis worm *Priapulus caudatus* (phylum Priapulida) revealed the existence of a luqin-type neuropeptide precursor in this species (Yañez-Guerra et al., 2018). This precursor comprises two putative luqin-type neuropeptides with a C-terminal RYamide motif and has the conserved C-terminal region with two cysteines separated by ten amino acid residues (Figure 1). Interestingly, the *P. caudatus* neuropeptides share similarities with both arthropod/nematode RYamides and spiralian luqins. Thus, whilst the C-terminal RYamide motif is a conserved feature of this neuropeptide family in ecdysozoans, the N-terminal region of the *P. caudatus* luqin-type neuropeptides shares more sequence similarity with mollusc/annelid luqins than with arthropod/nematode RYamides. For example, the N-terminal sequence QWRP in one of the *P. caudatus* RYamides is also a feature of several molluscan luqin-type peptides (Figure 1). These 'intermediate' characteristics of the *P. caudatus* RYamides are also reflected in a phylogenetic analysis of the relationships of luqin/RYamide-type precursors, where the *P. caudatus* RYamide precursor is not positioned in a clade comprising arthropod/nematode precursors, as would be expected based on animal phylogenetic relationships, but instead it is positioned at the base of a clade comprising mollusc/annelid precursors. This suggests that the *P. caudatus* RYamide precursor may have retained many of the ancestral characteristics of protostome luqin-type precursors, whereas the arthropod/nematode luqin-type precursors appear to be more divergent (Yañez-Guerra et al., 2018). In *P. caudatus*, two candidate receptors for luqin-type neuropeptides have been identified based on their phylogenetic relationship with luqin-type receptors that have been characterised in other protostomes (Figure 2) (Yañez-Guerra et al., 2018). Experimental studies are now needed to determine if the two *P. caudatus* luqin-type neuropeptides are effective as ligands for both receptors or if the receptors exhibit preferential ligand binding. Furthermore, experimental studies are needed to investigate the physiological roles of luqin-type signalling in priapulids.

Analysis of the genome sequences of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus* (phylum Tardigrada) has revealed the occurrence of luqin-type precursors and receptors in both of these species (Koziol, 2018). One luqin-type precursor was identified in *H. dujardini* and two luqin-type precursors were identified in *R. varieornatus* (Koziol, 2018). The predicted neuropeptides derived from these precursors have a C-terminal RYamide motif, consistent with other members of this neuropeptide family in ecdysozoans. However, atypical of ecdysozoan luqin-type precursors, the tardigrade precursors comprise only one predicted neuropeptide (Figure 1). The C-terminal region of the precursor contains two cysteines separated by ten amino acid residues in *H. dujardini* and in one of the two precursors from *R. varieornatus*, whilst the second precursor in *R. varieornatus* lacks the second cysteine (Figure 1). Genes encoding a single luqin-type receptor have been identified in both *H. dujardini* and *R. varieornatus* (Koziol, 2018) but the ligand-binding properties of these receptors remain to be investigated experimentally. Furthermore, nothing is known about the physiological roles of luqin-type neuropeptides in tardigrades and so this will be an interesting area for investigation in the future, particularly in the context of their remarkable capacity to withstand extreme environmental conditions, including radiation tolerance, desiccation and both high and low temperature and pressure (Kamilari et al., 2019; Jönsson et al., 2019; Jönsson, 2019).

Discovery of luqin-type signalling in ambulacrarian deuterostomes reveals the urbilaterian origin of this neuropeptide signalling system

The discovery of precursors of luqin-type peptides in deuterostomian invertebrates was first reported in 2013. They were identified in the hemichordate *Saccoglossus kowalevskii* and in the echinoderm *Strongylocentrotus purpuratus,* which was facilitated by the presence of the aforementioned conserved C-terminal region containing two cysteines separated by ten amino acid residues (Jékely, 2013). The *S. purpuratus* and *S. kowalevskii* precursor proteins comprise the putative neuropeptides EIRSPGGKPHKFMRW-NH_2 and EGSNTFLRW-NH_2, respectively. Thus, the presence of a C-terminal FXRW-NH_2 motif (where X is L or M) was identified as a characteristic feature of luqin-type peptides in the ambulacrarian clade of the deuterostomes (Elphick and Mirabeau, 2014).

Through analysis of transcriptome/genome sequence data, luqin-type neuropeptide precursors have subsequently been identified in other echinoderm classes, including Holothuroidea (sea cucumbers), Asteroidea (starfish or sea stars) and Ophiuroidea (brittle stars). In the sea cucumbers *Apostichopus japonicus*, *Holothuria glaberrima*, *Holothuria*

scabra and *Holothuria leucospilota* the precursor comprises a single neuropeptide with same predicted structure in all four species - KPYKFMRW-NH_2 (Suwansa-Ard et al., 2018; Chieu et al., 2019; Rowe et al., 2014; Chen et al., 2019). Luqin-type precursors identified in the starfish species *A. rubens* and *Acanthaster planci*, comprise a single putative neuropeptide with the amino acid sequence EKGRFPKFMRW-NH_2 and EEKTRFPKFMRW-NH_2 respectively (Semmens et al., 2016; Smith et al., 2017). Ophiuroid luqin-type precursors also comprise a single putative neuropeptide, which has the predicted sequence QGFNRDGPAKFMRW-NH_2 in *Ophionotus victoriae*, QGFNRGEGPAKFMRW-NH_2 in *Ophiopsila aranea* and QGFSRDGPAKFMRW-NH_2 *Amphiura filiformis* (Zandawala et al., 2017). Thus, the C-terminal motif KFMRW-NH_2 appears to be a conserved feature of luqin-type neuropeptides in echinoderms.

A large-scale analysis of the phylogenetic distribution of G-protein coupled neuropeptide receptors in bilaterian animals revealed the presence of luqin-type receptors in ambulacrarians (hemichordates and echinoderms) (Mirabeau and Joly, 2013), consistent with the identification of luqin-type neuropeptide precursors in these taxa. However, luqin-type receptors were not identified in vertebrates or other chordates (urochordates and cephalochordates) and accordingly luqin-type neuropeptide precursors have not been identified in these taxa. Thus, it was concluded that the evolutionary origin of luqin-type receptors can be traced to the common ancestor of protostomes and deuterostomes, but with subsequent loss in the chordate lineage (Mirabeau and Joly, 2013). Furthermore, the phylogenetic analysis of neuropeptide receptor relationships reported by Mirabeau and Joly (2013) revealed that luqin-type receptors are paralogues of tachykinin-type receptors and this finding was confirmed recently by a phylogenetic analysis specifically focused on luqin-type receptors and closely related neuropeptide receptors (Yañez-Guerra et al., 2018) (Figure 2). Thus, it can be inferred that gene duplication in a common ancestor of the Bilateria gave rise to the paralogous luqin-type and tachykinin-type signalling systems, but with subsequent loss of luqin-type signalling in chordates (Figure 3).

A detailed analysis of luqin-type receptors in ambulacrarians revealed the presence of four genes/transcripts in the hemichordate *S. kowalevskii* and two genes/transcripts in the echinoderms *S. purpuratus* (sea urchin) and *A. rubens* (starfish) that encode members of this family of neuropeptide receptors (Yañez-Guerra et al., 2018). Furthermore, the starfish *A. rubens* was selected a model experimental system in which to functionally characterise luqin-type neuropeptide signalling for the first time in a deuterostome. A cDNA encoding the *A. rubens* luqin-type precursor ArLQP was cloned and the structure of the mature peptide

517 derived from this precursor was determined using mass spectrometry as a 12 amino acid
518 residue peptide that is C-terminally amidated - EEKTRFPKFMRW-NH_2 (ArLQ). Cloning,
519 sequencing and heterologous expression of cDNAs encoding two *A. rubens* luqin-type
520 receptors (ArLQR1 and ArLQR2) facilitated testing of synthetic ArLQ as a candidate ligand
521 for these receptors. This revealed that ArLQ is a potent ligand for both ArLQR1 and
522 ArLQR2, with EC_{50} values of 2.4×10^{-8} M and 7.8×10^{-10} M, respectively (Yañez-Guerra et
523 al., 2018).
524 To gain insights into the physiological roles of luqin-type neuropeptide signalling in
525 *A. rubens*, mRNA *in situ* hybridisation methods were employed to investigate the expression
526 pattern of ArLQP in adult starfish. ArLQP-expressing cells were revealed in the central
527 nervous system, including the circumoral nerve ring and the radial nerve cords. However,
528 expression was limited to the ectoneural region, which contains sensory and interneurons,
529 with no expression detected in motoneuron cell bodies located in the hyponeural region.
530 ArLQP-expressing cells were also revealed in starfish locomotor organs - tube feet -
531 specifically located in close proximity to the basal nerve ring in the disk region. Lastly, in the
532 digestive system, ArLQP-expressing cells were revealed in the cardiac stomach and pyloric
533 stomach (Yañez-Guerra et al., 2018). Efforts to generate antibodies to ArLQ were also made
534 to enable immunohistochemical analysis of ArLQ expression in *A. rubens*, but these were
535 unsuccessful. Nevertheless, informed by the pattern of ArLQP expression revealed by use of
536 mRNA *in situ* hybridisation, synthetic ArLQ was tested as a potential myoactive peptide on
537 *in vitro* preparations of tube feet and cardiac stomach. No effects on cardiac stomach
538 preparations were observed but, interestingly, ArLQ was found to cause dose-dependent
539 relaxation of the tube foot preparations (Yañez-Guerra et al., 2018). Furthermore, the
540 relaxing effect of ArLQ was similar in potency and magnitude to SALMFamide-2 (S2), a
541 neuropeptide that has been identified and functionally characterised previously as a
542 myorelaxant in *A. rubens* (Elphick et al., 1991; Melarange and Elphick, 2003). Clearly,
543 further studies are now needed to gain broader insights into the physiological roles of luqin-
544 type neuropeptides in starfish and other echinoderms. Further investigation of the
545 physiological roles of luqin-type neuropeptide signalling in echinoderms could also be
546 extended beyond adult animals to the free-swimming larval stage of these animals. Detailed
547 anatomical analyses of neuropeptide precursor gene expression in larvae of *A. rubens* and *S.*
548 *purpuratus* have been reported recently (Mayorova et al., 2016; Wood et al., 2018) but these
549 studies did not incorporate analysis of the expression of luqin-type precursors. Therefore, this

is also an important objective for future work on luqin-type neuropeptide signalling in echinoderms.

Luqin-type neuropeptide signalling in xenacoelomorphs: receptors with missing ligands

The phylum Xenacoelomorpha comprises an assemblage of marine worms that have a simple body plan without a through-gut (Gavilán et al., 2019; Hejnol and Pang, 2016; Telford and Copley, 2016). They are of particular interest for evolutionary studies because of controversy regarding their phylogenetic position in the animal kingdom. On the one hand they have been placed as a sister group to all other bilaterian animals [Nephrozoa hypothesis] (Rouse et al., 2016; Cannon et al., 2016) . Alternatively, they are considered to be closely related to the Ambulacraria, forming a clade known as the Xenambulacraria [Xenambulacraria hypothesis] (Bourlat et al., 2006; Philippe et al., 2011). The most recent analysis of the phylogenetic position of xenacoelomorphs, including a strategy devoted to mitigate the effects of systematic errors, has supported the Xenambulacraria hypothesis (Philippe et al., 2019).

Analysis of transcriptome sequence data from thirteen xenacoelomorph species revealed the occurrence of luqin-type receptors in this phylum (Thiel et al., 2018). Transcripts encoding luqin-type receptors were identified in species belonging to each of the three xenacoelomorph sub-phyla; Xenoturbellida *(Xenoturbella bocki),* Nemertodermatida (*Nemertoderma westbladi*) and Acoela (*Hofstenia miamia)*. Furthermore, phylogenetic analysis revealed that these receptors form part of a clade of luqin-type receptors that include spiralian luqin receptors and ecdysozoan RYamide receptors. Interestingly, the xenacoelomorph luqin-type receptors are positioned within a branch that also includes ambulacrarian luqin-type receptors (Thiel et al., 2018). Therefore, this may be additional evidence in support of the Xenambulacraria hypothesis. Thus far, precursors of luqin-type neuropeptides have yet to be identified in xenacoelomorphs and so this represents an important objective for future work. In particular, it would be interesting to determine the C-terminal motif of luqin-type neuropeptides in xenacoelomorphs. If the peptides have a C-terminal RWamide motif, which is a characteristic of ambulacrarian luqin-type neuropeptides, then this would be further evidence of a close relationship between xenacoelomorphs and ambulacrarians. Furthermore, discovery of luqin-type neuropeptides in xenacoelomorphs would provide a basis for functional investigation of physiological roles of these neuropeptides in this phylum.

General conclusions and speculations

The discovery and naming of luqin as a neuropeptide that is expressed in Left Upper Quadrant cells of the abdominal ganglion of the mollusc *Aplysia* was perhaps a rather esoteric beginning to a new field of neuropeptide research. However, gradually over a period of more than three decades it has become apparent that in fact this finding has broad relevance to neuropeptide signalling in bilaterian animals, with the notable exception of chordates. Although the name luqin was highly specific in its derivation, it nevertheless provides a useful generic name for the neuropeptide family. Thus, it is preferable to RFamides, RYamides or RWamides because these C-terminal motifs are not unique to or even generally applicable to the neuropeptide family as whole. Therefore, our recommendation is that all members of this neuropeptide family are referred to as 'luqins', recognising of course that the derivation of the name is meaningless beyond *Aplysia*.

Comparison of the sequences of luqin-type precursors has revealed variability in the number of luqin-type neuropeptides derived from these proteins. All the luqin-type precursors identified thus far in ambulacrarians comprise a single luqin-tye neuropeptide and this is also a feature of some luqin-type precursors in spiralians and ecdysozaons (Figure 1). Therefore, it could be inferred that this may reflect the characteristics of the luqin-type precursor in the common ancestor of the Bilateria. However, the existence of precursors that comprise two luqin-type neuropeptides is a feature of many ecdysozoans and some spiralian species, indicating perhaps that this characteristic has evolved independently in both lineages (Figure 1). Further expansions in the number of luqin-type neuropeptides derived from precursor proteins are seen in some crustacean species; for example, in *D. pulex* and *P. cavaticus,* where there are three predicted mature peptides (Christie, 2017; Dircksen et al., 2011) (Supplementary Figure 1A). However, the most extreme example is seen in the wasp *N. vitiprenis*, where the precursor comprises seven predicted luqin-type neuropeptides (Hauser et al., 2010) (Supplementary Figure 1A). The functional significance of this expansion in some arthropods is as yet unknown. Furthermore, the existence of multiple genes encoding luqin-type neuropeptides is a feature of some platyhelminth species (Koziol et al., 2016).

Thus far, genes/transcripts encoding luqin-type precursors and/or luqin-type receptors have been discovered in all of the non-chordate bilaterian phyla that have been investigated, but not in chordates (Figure 3). The loss of LQ-type signalling in chordates is of interest from a functional perspective, as discussed below. However, loss of LQ-type signalling in chordates has also influenced efforts to classify neuropeptide receptors. Hence, the original

618 classification of the *C. elegans* and *L. stagnalis* LQ-type receptors as 'tachykinin-like
619 receptors' (Keating et al., 2003). More recently, in a phylogenetic analysis of gonadotropin-
620 inhibitory hormone (GnIH)-type signalling it was concluded that GnIH-type receptors have a
621 'strong evolutionary relationship' with the *C. elegans* receptor NPR-22 (Ubuka and Tsutsui,
622 2018). This conclusion was a consequence of a phylogenetic analysis that was restricted to
623 comparison of human GnIH-type receptors with a variety of *C. elegans* neuropeptide
624 receptors, whilst more comprehensive phylogenetic analyses clearly show that the *C. elegans*
625 receptor NPR-22 is a LQ-type receptor (Ohno et al., 2017; Yañez-Guerra et al., 2018).
626 Whilst genes/transcripts encoding luqin-type precursors and/or luqin-type receptors
627 have been discovered in all of the non-chordate bilaterian phyla that have been investigated,
628 there are still many phyla that remain to be examined and so we cannot rule out the
629 possibility that luqin-type neuropeptide signalling has been lost in some non-chordate phyla.
630 Nevertheless, based on what it currently known, the loss of luqin signalling in chordates
631 appears to be singular and therefore notable. Why then was luqin signalling lost in the
632 chordates? To address this question, we need to consider, firstly, what is known about the
633 physiological roles of luqin-type neuropeptide signalling and, secondly, the paralogous
634 relationship of luqin-type signalling with tachykinin-type signalling.
635 Although luqin-type signalling was first discovered and then functionally
636 characterised in molluscs, it was the recent detailed analysis of this signalling system in the
637 nematode *C. elegans*, employing use of reverse genetic techniques, that has provided the
638 most comprehensive insights into the physiological roles of luqin-type neuropeptide
639 signalling. Furthermore, the findings from *C. elegans* provide a broad functional context for
640 comparison with experimental findings from other less intensely studied taxa (Figure 4).
641 A key finding from *C. elegans* was that luqin-type neuropeptides are secreted by a
642 pair of pharyngeal neurons (M1 and M2) and act as hormones to suppress feeding (Ohno et
643 al., 2017). It is noteworthy, therefore, that luqin-type RYamides suppress feeding motivation
644 and sucrose responsiveness in an insect (Maeda et al., 2015) and brain expression of the
645 luqin-type RYamide precursor gene is significantly downregulated after starvation in a
646 crustacean (Mekata et al., 2017). Thus, in ecdysozoans there is evidence of an evolutionarily
647 conserved role of luqin-type neuropeptide signalling as an inhibitory regulator of feeding in
648 association with changes in food availability. Further studies are now required to investigate
649 if luqin-type neuropeptides also regulate feeding activity in spiralians and ambulacrarians.
650 Nevertheless, it is noteworthy that in the mollusc *A. fullica* luqin has excitatory effects on
651 buccal neurons and muscles (Fujimoto et al., 1990) and in the starfish *A. rubens* a luqin-type

neuropeptide precursor is expressed in the cardiac stomach, a region of the digestive system that is everted when starfish feed (Yañez-Guerra et al., 2018).

Luqin-type neuropeptides also regulate egg laying in *C. elegans*, acting via the luqin-type receptor NPR-22 upstream of the serotonin-uptaking RIH neuron. Thus, by comparsion with wild-type animals, mutant worms lacking expression of the luqin-type LURY-1 precursor or NPR-22 exhibit reduced egg-laying during a period of refeeding after starvation (Ohno et al., 2017). Accordingly, detection of luqin-immunoreactivity in nerve fibres associated with the hermaphroditic ducts and the ovotestis and in nerve fibres associated with inhibition of the egg-laying hormone-producing caudodorsal cells in *L. stagnalis*, which express luqin-type receptor transcripts (Tensen et al., 1998), suggest that luqin-type signalling may likewise regulate egg laying in molluscs.

Overexpression of luqin-type neuropeptides in *C. elegans* produced a phenotype where worms exhibited reduced roaming activity and, importantly, this was not observed in animals lacking the luqin-type receptor NPR-22. It was concluded that this action may reflect a physiological role of luqin signalling in attenuating locomotory activity when food is abundant (Ohno et al., 2017). It is noteworthy, therefore, that the relaxing effect of the luqin-type neuropeptide ArLQ on starfish tube feet may likewise be consistent with a physiological role in inhibitory regulation of locomotor activity in an echinoderm (Yañez-Guerra et al., 2018). Investigation of the effects of ArLQ on locomotor activity will therefore be an interesting objective for future studies, employing use of methods that have been reported recently to examine the effects of other neuropeptides on starfish locomotor activity (Tinoco et al., 2018).

The discovery that luqin-type signalling is paralogous to tachykinin-type signalling was based on phylogenetic analysis of the relationships of G-protein coupled neuropeptide receptors in the Bilateria (Yañez-Guerra et al., 2018; Thiel et al., 2018; Mirabeau and Joly, 2013) (Figure 3). Therefore, it can be inferred that duplication of genes encoding a common ancestral neuropeptide precursor and receptor occurred in a common ancestor of the Bilateria to give rise to the paralogous luqin-type and tachykinin-type signalling systems. Analysis of the phylogenetic distribution of tachykinin-type signalling indicates that it has been retained in all bilaterian phyla that have been analysed (Mirabeau and Joly, 2013; Elphick et al., 2018).

A possible explanation for the loss of a neuropeptide signalling system in a taxon could be functional redundancy with respect to a paralogous signalling system. However, given i). the length of time elapsed since the gene duplications that gave rise to the

686 paralogous luqin-type and tachykinin-type signalling systems is likely to be in excess of 650
687 million years, based on the estimated time of divergence of protostomes and deuterostomes
688 (Erwin et al., 2011) and ii). the preservation of both signalling systems in most bilaterian
689 phyla that have been analysed, it seems unlikely that this explains the loss of luqin signalling
690 in chordates. Further insights may be obtained as part of a broader investigation of the
691 functional significance of the loss of bilaterian neuropeptide signalling systems. Examples of
692 other neuropeptide types that have been lost in chordates include the leucokinin-type and
693 pigment dispersing factor (PDF)-type signalling systems (Mirabeau and Joly, 2013) whilst
694 more specifically corazonin-type signalling has been lost in vertebrates and urochordates but
695 not in cephalochordates (Zandawala et al., 2018; Tian et al., 2016). Comparisons with these
696 neuropeptide systems may therefore be informative in future efforts to draw general
697 conclusions on the evolutionary and functional significance of neuropeptide loss in chordates.

Acknowledgements

LAYG was supported by a Leverhulme Trust grant (RPG-2016-353) and by a PhD studentship awarded by the Mexican Council of Science and Technology (CONACyT studentship no. 418612) and Queen Mary University of London. MRE was supported by grants from the Biotechnology and Biological Sciences Research Council (BB/M001644/1) and the Leverhulme Trust (RPG-2016-353). We thank Michael Crossley (University of Sussex, UK), Marycruz Flores-Flores (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico), Marina Ezcurra (University of Kent, UK) and Ray Crundwell (Queen Mary University of London, UK) for providing copyright free photographs of *Lymnaea stagnalis*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Asterias rubens*, respectively.

Figure legends.

Figure 1. Alignment of the N-terminal neuropeptide-containing and C-terminal regions of luqin-type precursor proteins in bilaterians. Conserved residues are highlighted in black or grey. The C-terminal residues of the luqin-type neuropeptides and species names are highlighted in phylum-specific colours: red (Mollusca), pink (Annelida), orange (Platyhelminthes, Brachiopods and Nemerteans), green (Arthropoda), purple (Nematoda), yellow (Priapulida and Tardigrada), light blue (Echinodermata) and dark blue (Hemichordata). Species names are as follows: Acal (*Aplysia californica*), Aful (*Achatina fulica*), Cgig (*Crassostrea gigas*), Iobs (*Ilyanasa obsoleta*), Bgla (*Biomphalaria glabrata*), Ctel (*Capitella teleta*), Smed (*Schmidtea mediterranea*), Lana (*Lingula anatina*), Llon (*Lineus longissimus),* Dmel (*Drosophila melanogaster*), Aaeg (*Aedes aegypti*), Tcas (*Tribolium castaneum*), Cqua (*Cherax quadricarinatus*) Tsui (*Trichuris suis*), Cele (*Caenorhabditis elegans*), Pcau (Priapulus caudatus), Hduj (*Hypsibius dujardini*), Rvar (*Ramazzottius varieornatus),* Arub (*Asterias rubens*), Ovic (*Ophionotus victoriae*), Ajap (*Apostichopus japonicus*), Spur (*Strongylocentrotus purpuratus*), Skow (*Saccoglossus kowalevskii*). The sequences used for this alignment were reported in (Koziol et al., 2016; Koziol, 2018; Yañez-Guerra et al., 2018; De Oliveira et al., 2019).

Figure 2. Phylogenetic tree showing the occurrence and relationships of luqin/RYamide-type receptors and tachykinin-type receptors in bilaterians. The tree comprises two distinct receptor clades – luqin/RYamide-type receptors and the paralogous tachykinin-type receptors, with thyrotropin-releasing hormone (TRH)-type receptors included as an outgroup. Taxa are colour-coded and SH-aLRT support (Guindon et al., 2010) [1000 replicates] for clades is represented with coloured stars, as explained in the key. Species in which the peptide ligands that activate luqin/RYamide-type receptors or tachykinin-type receptors have been identified experimentally are shown with blue lettering. Species names are as follows: Aaeg (*Aedes aegypti*), Acal *(Aplysia californica)*, Apis *(Acyrthosiphon pisum)*, Arub *(Asterias rubens)*, Cele *(Caenorhabditis elegans)*, Cint (*Ciona intestinalis*), Ctel *(Capitella teleta)*, Dmel *(Drosophila melanogaster)*, Hduj (*Hypsibius dujardini),* Hsap (*Homo sapiens*), Lana (*Lingula anatina*), Lsta (*Lymnaea stagnalis*), Obim (*Octopus bimaculoides*), Ovul (*Octopus vulgaris*), Pcau (*Priapulus caudatus*), Pdum (*Platynereis dumerilii*), Rvar (*Ramazzottius varieornatus),* Skow (*Saccoglossus kowalevskii*), Spur (*Strongylocentrotus purpuratus*), Tcas *(Tribolium castaneum)*, Tsui (*Trichuris suis*), Tpse

(*Trichinella pseudospiralis*), Uuni (*Urechis unicinctus*). This figure is a modified version of a tree reported previously in (Yañez-Guerra et al., 2018), with the addition of published luqin-type receptor sequences from the tardigrades *H. dujardini* and *R. varieornatus* (Koziol, 2018) and the brachiopod *L. anatina* (XP_013402794.1, XP_013402807.1) and tachykinin-type receptor sequences from the nematodes *C. elegans* (tk-1; C38C10.1) (Mirabeau and Joly, 2013) and *T. spiralis* (KRY8989.1). The alignment was performed using MUSCLE (Edgar, 2004) [sixteen iterations] and the trimming was made using BMGE (Criscuolo and Gribaldo, 2010) [standard automatic trimming]. The tree was generated in W-IQ-tree (Trifinopoulos et al., 2016) using the Maximum likelihood method with automatic selection of the substitution model. The branch support analysis used was SH-aLRT (Guindon et al., 2010) with 1000 iterations.

Figure 3. Phylogenetic diagram showing the occurrence of luqin-type neuropeptide signalling in the Bilateria. The phylogenetic tree shows relationships of selected bilaterian phyla. The phyla in which luqin-type precursors and luqin-type receptors have been identified are labelled with purple-filled boxes. The number in the precursor box indicates how many luqin-type neuropeptides are known or predicted to be derived from the precursor protein. The inclusion of a plus symbol in the receptor boxes indicates that the peptide ligand(s) that activates the receptor has been determined experimentally. Note the loss of the luqin-type signalling system in the chordate lineage, which is signified by the red cross and the white-filled boxes. Note that Xenacoelomorpha are not included in this diagram because of the controversy regarding the phylogenetic position of this phylum. However, as discussed in this review, luqin-type receptors have been identified in xenacoelomorphs but the precursors of peptides that act as ligands for these receptors have yet to be identified. The cladogram depicting of bilaterian relationships is based on a recent phylogenetic study reported by Laumer et al. (Laumer et al., 2019).

Figure 4. Summary of the properties and functions of luqin-type neuropeptide signalling in species belonging to four phyla. A. Mollusca (*Lymnaea stagnalis*), B. Arthropoda (*Drosophila melanogaster*), C. Nematoda (*Caenorhabditis elegans*), D. Echinodermata (*Asterias rubens*). The photographs of the animals shown in A, B, C and D were taken by Michael Crossley (University of Sussex, UK), Marycruz Flores-Flores (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico),

776 Marina Ezcurra (University of Kent, UK) and Ray Crundwell (Queen Mary University of
777 London, UK).
778

References

Aloyz, R. S., and DesGroseillers, L. (1995). Processing of the L5-67 precursor peptide and characterization of LUQIN in the LUQ neurons of Aplysia californica. *Peptides* 16, 331–338. doi:10.1016/0196-9781(94)00140-5.

Angers, A., and DesGroseillers, L. (1998). Alternative splicing and genomic organization of the L5-67 gene of Aplysia californica. *Gene* 208, 271–277.

Angers, A., Zappulla, J. P., Zollinger, M., and DesGroseillers, L. (2000). Gene products from LUQ neurons in the abdominal ganglion are present at the renal pore of Aplysia californica. *Comp Biochem Physiol B, Biochem Mol Biol* 126, 435–443.

Bauknecht, P., and Jékely, G. (2015). Large-Scale Combinatorial Deorphanization of Platynereis Neuropeptide GPCRs. *Cell Rep* 12, 684–693. doi:10.1016/j.celrep.2015.06.052.

Bhatla, N., Droste, R., Sando, S. R., Huang, A., and Horvitz, H. R. (2015). Distinct neural circuits control rhythm inhibition and spitting by the myogenic pharynx of c. elegans. *Curr Biol* 25, 2075–2089. doi:10.1016/j.cub.2015.06.052.

Bourlat, S. J., Juliusdottir, T., Lowe, C. J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E. S., Thorndyke, M., Nakano, H., Kohn, A. B., et al. (2006). Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* 444, 85–88. doi:10.1038/nature05241.

Brown, R. O., Gusman, D., Basbaum, A. I., and Mayeri, E. (1985). Identification of Aplysia neurons containing immunoreactive FMRFamide. *Neuropeptides* 6, 517–526.

Cannon, J. T., Vellutini, B. C., Smith, J., Ronquist, F., Jondelius, U., and Hejnol, A. (2016). Xenacoelomorpha is the sister group to Nephrozoa. *Nature* 530, 89–93. doi:10.1038/nature16520.

Ceron, J., Rual, J.-F., Chandra, A., Dupuy, D., Vidal, M., and van den Heuvel, S. (2007). Large-scale RNAi screens identify novel genes that interact with the C. elegans retinoblastoma pathway as well as splicing-related components with synMuv B activity. *BMC Dev Biol* 7, 30. doi:10.1186/1471-213X-7-30.

Chen, M., Talarovicova, A., Zheng, Y., Storey, K. B., and Elphick, M. R. (2019). Neuropeptide precursors and neuropeptides in the sea cucumber Apostichopus japonicus: a genomic, transcriptomic and proteomic analysis. *Sci. Rep.* 9, 8829. doi:10.1038/s41598-019-45271-3.

Chieu, H. D., Suwansa-Ard, S., Wang, T., Elizur, A., and Cummins, S. F. (2019). Identification of neuropeptides in the sea cucumber Holothuria leucospilota. *Gen Comp Endocrinol* 283, 113229. doi:10.1016/j.ygcen.2019.113229.

Christie, A. E. (2017). Neuropeptide discovery in Proasellus cavaticus: Prediction of the first large-scale peptidome for a member of the Isopoda using a publicly accessible transcriptome. *Peptides* 97, 29–45. doi:10.1016/j.peptides.2017.09.003.

Christie, A. E., Cieslak, M. C., Roncalli, V., Lenz, P. H., Major, K. M., and Poynton, H. C. (2018). Prediction of a peptidome for the ecotoxicological model Hyalella azteca (Crustacea; Amphipoda) using a de novo assembled transcriptome. *Mar Genomics* 38, 67–88. doi:10.1016/j.margen.2017.12.003.

Collin, C., Hauser, F., Krogh-Meyer, P., Hansen, K. K., Gonzalez de Valdivia, E., Williamson, M., and Grimmelikhuijzen, C. J. P. (2011). Identification of the Drosophila and Tribolium receptors for the recently discovered insect RYamide neuropeptides. *Biochem Biophys Res Commun* 412, 578–583. doi:10.1016/j.bbrc.2011.07.131.

Conzelmann, M., Williams, E. A., Krug, K., Franz-Wachtel, M., Macek, B., and Jékely, G. (2013). The neuropeptide complement of the marine annelid Platynereis dumerilii.

BMC Genomics 14, 906. doi:10.1186/1471-2164-14-906.
Criscuolo, A., and Gribaldo, S. (2010). BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol Biol* 10, 210. doi:10.1186/1471-2148-10-210.
Dickinson, P. S., Hull, J. J., Miller, A., Oleisky, E. R., and Christie, A. E. (2019). To what extent may peptide receptor gene diversity/complement contribute to functional flexibility in a simple pattern-generating neural network? *Comp Biochem Physiol Part D Genomics Proteomics* 30, 262–282. doi:10.1016/j.cbd.2019.03.002.
Dircksen, H., Neupert, S., Predel, R., Verleyen, P., Huybrechts, J., Strauss, J., Hauser, F., Stafflinger, E., Schneider, M., Pauwels, K., et al. (2011). Genomics, transcriptomics, and peptidomics of Daphnia pulex neuropeptides and protein hormones. *J Proteome Res* 10, 4478–4504. doi:10.1021/pr200284e.
Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32, 1792–1797. doi:10.1093/nar/gkh340.
Elphick, M. R., and Mirabeau, O. (2014). The Evolution and Variety of RFamide-Type Neuropeptides: Insights from Deuterostomian Invertebrates. *Front Endocrinol (Lausanne)* 5, 93. doi:10.3389/fendo.2014.00093.
Elphick, M. R., Mirabeau, O., and Larhammar, D. (2018). Evolution of neuropeptide signalling systems. *J Exp Biol* 221. doi:10.1242/jeb.151092.
Elphick, M. R., Price, D. A., Lee, T. D., and Thorndyke, M. C. (1991). The SALMFamides: a new family of neuropeptides isolated from an echinoderm. *Proc Biol Sci* 243, 121–127. doi:10.1098/rspb.1991.0020.
Erwin, D. H., Laflamme, M., Tweedt, S. M., Sperling, E. A., Pisani, D., and Peterson, K. J. (2011). The Cambrian conundrum: early divergence and later ecological success in the early history of animals. *Science* 334, 1091–1097. doi:10.1126/science.1206375.
Fujimoto, K., Ohta, N., Yoshida, M., Kubota, I., Muneoka, Y., and Kobayashi, M. (1990). A novel cardio-excitatory peptide isolated from the atria of the African giant snail, Achatina fulica. *Biochem Biophys Res Commun* 167, 777–783.
Fu, Q., Kutz, K. K., Schmidt, J. J., Hsu, Y.-W. A., Messinger, D. I., Cain, S. D., la Iglesia, H. O. de, Christie, A. E., and Li, L. (2005). Hormone complement of the Cancer productus sinus gland and pericardial organ: an anatomical and mass spectrometric investigation. *J Comp Neurol* 493, 607–626. doi:10.1002/cne.20773.
Gavilán, B., Sprecher, S. G., Hartenstein, V., and Martinez, P. (2019). The digestive system of xenacoelomorphs. *Cell Tissue Res*, 1–14. doi:10.1007/s00441-019-03038-2.
Giardino, N. D., Aloyz, R. S., Zollinger, M., Miller, M. W., and DesGroseillers, L. (1996). L5-67 and LUQ-1 peptide precursors of Aplysia californica: distribution and localization of immunoreactivity in the central nervous system and in peripheral tissues. *J Comp Neurol* 374, 230–245. doi:10.1002/(SICI)1096-9861(19961014)374:2<230::AID-CNE6>3.0.CO;2-3.
Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59, 307–321. doi:10.1093/sysbio/syq010.
Hauser, F., Neupert, S., Williamson, M., Predel, R., Tanaka, Y., and Grimmelikhuijzen, C. J. P. (2010). Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp Nasonia vitripennis. *J Proteome Res* 9, 5296–5310. doi:10.1021/pr100570j.
Hejnol, A., and Pang, K. (2016). Xenacoelomorpha's significance for understanding bilaterian evolution. *Curr Opin Genet Dev* 39, 48–54. doi:10.1016/j.gde.2016.05.019.

879 Ida, T., Takahashi, T., Tominaga, H., Sato, T., Kume, K., Ozaki, M., Hiraguchi, T., Maeda,
880 T., Shiotani, H., Terajima, S., et al. (2011). Identification of the novel bioactive
881 peptides dRYamide-1 and dRYamide-2, ligands for a neuropeptide Y-like receptor in
882 Drosophila. *Biochem Biophys Res Commun* 410, 872–877.
883 doi:10.1016/j.bbrc.2011.06.081.
884 Jékely, G. (2013). Global view of the evolution and diversity of metazoan neuropeptide
885 signaling. *Proc Natl Acad Sci U S A* 110, 8702–8707. doi:10.1073/pnas.1221833110.
886 Jékely, G., Melzer, S., Beets, I., Kadow, I. C. G., Koene, J., Haddad, S., and Holden-Dye, L.
887 (2018). The long and the short of it - a perspective on peptidergic regulation of
888 circuits and behaviour. *J Exp Biol* 221. doi:10.1242/jeb.166710.
889 Jönsson, K. I. (2019). Radiation tolerance in tardigrades: current knowledge and potential
890 applications in medicine. *Cancers (Basel)* 11. doi:10.3390/cancers11091333.
891 Jönsson, K. I., Holm, I., and Tassidis, H. (2019). Cell biology of the tardigrades: current
892 knowledge and perspectives. *Results Probl Cell Differ* 68, 231–249. doi:10.1007/978-
893 3-030-23459-1_10.
894 Kamilari, M., Jørgensen, A., Schiøtt, M., and Møbjerg, N. (2019). Comparative
895 transcriptomics suggest unique molecular adaptations within tardigrade lineages.
896 *BMC Genomics* 20, 607. doi:10.1186/s12864-019-5912-x.
897 Keating, C. D., Kriek, N., Daniels, M., Ashcroft, N. R., Hopper, N. A., Siney, E. J., Holden-
898 Dye, L., and Burke, J. F. (2003). Whole-genome analysis of 60 G protein-coupled
899 receptors in Caenorhabditis elegans by gene knockout with RNAi. *Curr Biol* 13,
900 1715–1720. doi:10.1016/j.cub.2003.09.003.
901 Koziol, U. (2018). Precursors of neuropeptides and peptide hormones in the genomes of
902 tardigrades. *Gen Comp Endocrinol* 267, 116–127. doi:10.1016/j.ygcen.2018.06.012.
903 Koziol, U., Koziol, M., Preza, M., Costábile, A., Brehm, K., and Castillo, E. (2016). De novo
904 discovery of neuropeptides in the genomes of parasitic flatworms using a novel
905 comparative approach. *Int J Parasitol* 46, 709–721. doi:10.1016/j.ijpara.2016.05.007.
906 Laumer, C. E., Fernández, R., Lemer, S., Combosch, D., Kocot, K. M., Riesgo, A., Andrade,
907 S. C. S., Sterrer, W., Sørensen, M. V., and Giribet, G. (2019). Revisiting metazoan
908 phylogeny with genomic sampling of all phyla. *Proc Biol Sci* 286, 20190831.
909 doi:10.1098/rspb.2019.0831.
910 Liessem, S., Ragionieri, L., Neupert, S., Büschges, A., and Predel, R. (2018). Transcriptomic
911 and Neuropeptidomic Analysis of the Stick Insect, Carausius morosus. *J Proteome*
912 *Res* 17, 2192–2204. doi:10.1021/acs.jproteome.8b00155.
913 Li, L., Kelley, W. P., Billimoria, C. P., Christie, A. E., Pulver, S. R., Sweedler, J. V., and
914 Marder, E. (2003). Mass spectrometric investigation of the neuropeptide complement
915 and release in the pericardial organs of the crab, Cancer borealis. *J Neurochem* 87,
916 642–656.
917 Li, L., Moroz, T. P., Garden, R. W., Floyd, P. D., Weiss, K. R., and Sweedler, J. V. (1998).
918 Mass spectrometric survey of interganglionically transported peptides in Aplysia.
919 *Peptides* 19, 1425–1433.
920 Maeda, T., Nakamura, Y., Shiotani, H., Hojo, M. K., Yoshii, T., Ida, T., Sato, T., Yoshida,
921 M., Miyazato, M., Kojima, M., et al. (2015). Suppressive effects of dRYamides on
922 feeding behavior of the blowfly, Phormia regina. *Zoological Lett.* 1, 35.
923 doi:10.1186/s40851-015-0034-z.
924 Ma, M., Bors, E. K., Dickinson, E. S., Kwiatkowski, M. A., Sousa, G. L., Henry, R. P.,
925 Smith, C. M., Towle, D. W., Christie, A. E., and Li, L. (2009). Characterization of the
926 Carcinus maenas neuropeptidome by mass spectrometry and functional genomics.
927 *Gen Comp Endocrinol* 161, 320–334. doi:10.1016/j.ygcen.2009.01.015.
928 Ma, M., Gard, A. L., Xiang, F., Wang, J., Davoodian, N., Lenz, P. H., Malecha, S. R.,

Christie, A. E., and Li, L. (2010). Combining in silico transcriptome mining and biological mass spectrometry for neuropeptide discovery in the Pacific white shrimp Litopenaeus vannamei. *Peptides* 31, 27–43. doi:10.1016/j.peptides.2009.10.007.

Marder, E., and Bucher, D. (2007). Understanding circuit dynamics using the stomatogastric nervous system of lobsters and crabs. *Annu Rev Physiol* 69, 291–316. doi:10.1146/annurev.physiol.69.031905.161516.

Mayorova, T. D., Tian, S., Cai, W., Semmens, D. C., Odekunle, E. A., Zandawala, M., Badi, Y., Rowe, M. L., Egertová, M., and Elphick, M. R. (2016). Localization of Neuropeptide Gene Expression in Larvae of an Echinoderm, the Starfish Asterias rubens. *Front Neurosci* 10, 553. doi:10.3389/fnins.2016.00553.

McVeigh, P., McCammick, E., McCusker, P., Wells, D., Hodgkinson, J., Paterson, S., Mousley, A., Marks, N. J., and Maule, A. G. (2018). Profiling G protein-coupled receptors of Fasciola hepatica identifies orphan rhodopsins unique to phylum Platyhelminthes. *Int. J. Parasitol. Drugs Drug Resist.* 8, 87–103. doi:10.1016/j.ijpddr.2018.01.001.

Mekata, T., Kono, T., Satoh, J., Yoshida, M., Mori, K., Sato, T., Miyazato, M., and Ida, T. (2017). Purification and characterization of bioactive peptides RYamide and CCHamide in the kuruma shrimp Marsupenaeus japonicus. *Gen Comp Endocrinol* 246, 321–330. doi:10.1016/j.ygcen.2017.01.008.

Melarange, R., and Elphick, M. R. (2003). Comparative analysis of nitric oxide and SALMFamide neuropeptides as general muscle relaxants in starfish. *J Exp Biol* 206, 893–899.

Mertens, I., Clinckspoor, I., Janssen, T., Nachman, R., and Schoofs, L. (2006). FMRFamide related peptide ligands activate the Caenorhabditis elegans orphan GPCR Y59H11AL.1. *Peptides* 27, 1291–1296. doi:10.1016/j.peptides.2005.11.017.

Mirabeau, O., and Joly, J.-S. (2013). Molecular evolution of peptidergic signaling systems in bilaterians. *Proc Natl Acad Sci U S A* 110, E2028–37. doi:10.1073/pnas.1219956110.

Nguyen, T. V., Cummins, S. F., Elizur, A., and Ventura, T. (2016). Transcriptomic characterization and curation of candidate neuropeptides regulating reproduction in the eyestalk ganglia of the Australian crayfish, Cherax quadricarinatus. *Sci. Rep.* 6, 38658. doi:10.1038/srep38658.

Ohno, H., Yoshida, M., Sato, T., Kato, J., Miyazato, M., Kojima, M., Ida, T., and Iino, Y. (2017). Luqin-like RYamide peptides regulate food-evoked responses in C. elegans. *elife* 6. doi:10.7554/eLife.28877.

De Oliveira, A. L., Calcino, A., and Wanninger, A. (2019). Extensive conservation of the proneuropeptide and peptide prohormone complement in mollusks. *Sci Rep* 9, 4846. doi:10.1038/s41598-019-40949-0.

Palamiuc, L., Noble, T., Witham, E., Ratanpal, H., Vaughan, M., and Srinivasan, S. (2017). A tachykinin-like neuroendocrine signalling axis couples central serotonin action and nutrient sensing with peripheral lipid metabolism. *Nat Commun* 8, 14237. doi:10.1038/ncomms14237.

Philippe, H., Brinkmann, H., Copley, R. R., Moroz, L. L., Nakano, H., Poustka, A. J., Wallberg, A., Peterson, K. J., and Telford, M. J. (2011). Acoelomorph flatworms are deuterostomes related to Xenoturbella. *Nature* 470, 255–258. doi:10.1038/nature09676.

Philippe, H., Poustka, A. J., Chiodin, M., Hoff, K. J., Dessimoz, C., Tomiczek, B., Schiffer, P. H., Müller, S., Domman, D., Horn, M., et al. (2019). Mitigating Anticipated Effects of Systematic Errors Supports Sister-Group Relationship between Xenacoelomorpha and Ambulacraria. *Curr Biol* 29, 1818–1826.e6. doi:10.1016/j.cub.2019.04.009.

Roller, L., Čižmár, D., Bednár, B., and Žitňan, D. (2016). Expression of RYamide in the

nervous and endocrine system of Bombyx mori. *Peptides* 80, 72–79. doi:10.1016/j.peptides.2016.02.003.

Rouse, G. W., Wilson, N. G., Carvajal, J. I., and Vrijenhoek, R. C. (2016). New deep-sea species of Xenoturbella and the position of Xenacoelomorpha. *Nature* 530, 94–97. doi:10.1038/nature16545.

Rowe, M. L., Achhala, S., and Elphick, M. R. (2014). Neuropeptides and polypeptide hormones in echinoderms: new insights from analysis of the transcriptome of the sea cucumber Apostichopus japonicus. *Gen Comp Endocrinol* 197, 43–55. doi:10.1016/j.ygcen.2013.12.002.

Schaefer, M., Picciotto, M. R., Kreiner, T., Kaldany, R. R., Taussig, R., and Scheller, R. H. (1985). Aplysia neurons express a gene encoding multiple FMRFamide neuropeptides. *Cell* 41, 457–467.

Semmens, D. C., Mirabeau, O., Moghul, I., Pancholi, M. R., Wurm, Y., and Elphick, M. R. (2016). Transcriptomic identification of starfish neuropeptide precursors yields new insights into neuropeptide evolution. *Open Biol* 6, 150224. doi:10.1098/rsob.150224.

Shyamala, M., Fisher, J. M., and Scheller, R. H. (1986). A neuropeptide precursor expressed in Aplysia neuron L5. *DNA* 5, 203–208. doi:10.1089/dna.1986.5.203.

Smith, M. K., Wang, T., Suwansa-Ard, S., Motti, C. A., Elizur, A., Zhao, M., Rowe, M. L., Hall, M. R., Elphick, M. R., and Cummins, S. F. (2017). The neuropeptidome of the Crown-of-Thorns Starfish, Acanthaster planci. *J Proteomics* 165, 61–68. doi:10.1016/j.jprot.2017.05.026.

Stemmler, E. A., Bruns, E. A., Gardner, N. P., Dickinson, P. S., and Christie, A. E. (2007). Mass spectrometric identification of pEGFYSQRYamide: a crustacean peptide hormone possessing a vertebrate neuropeptide Y (NPY)-like carboxy-terminus. *Gen Comp Endocrinol* 152, 1–7. doi:10.1016/j.ygcen.2007.02.025.

Suwansa-Ard, S., Chaiyamoon, A., Talarovicova, A., Tinikul, R., Tinikul, Y., Poomtong, T., Elphick, M. R., Cummins, S. F., and Sobhon, P. (2018). Transcriptomic discovery and comparative analysis of neuropeptide precursors in sea cucumbers (Holothuroidea). *Peptides* 99, 231–240. doi:10.1016/j.peptides.2017.10.008.

Telford, M. J., and Copley, R. R. (2016). Zoology: war of the worms. *Curr Biol* 26, R335–7. doi:10.1016/j.cub.2016.03.015.

Tensen, C. P., Cox, K. J., Smit, A. B., van der Schors, R. C., Meyerhof, W., Richter, D., Planta, R. J., Hermann, P. M., van Minnen, J., Geraerts, W. P., et al. (1998). The lymnaea cardioexcitatory peptide (LyCEP) receptor: a G-protein-coupled receptor for a novel member of the RFamide neuropeptide family. *J Neurosci* 18, 9812–9821.

Thiel, D., Franz-Wachtel, M., Aguilera, F., and Hejnol, A. (2018). Xenacoelomorph neuropeptidomes reveal a major expansion of neuropeptide systems during early bilaterian evolution. *Mol Biol Evol* 35, 2528–2543. doi:10.1093/molbev/msy160.

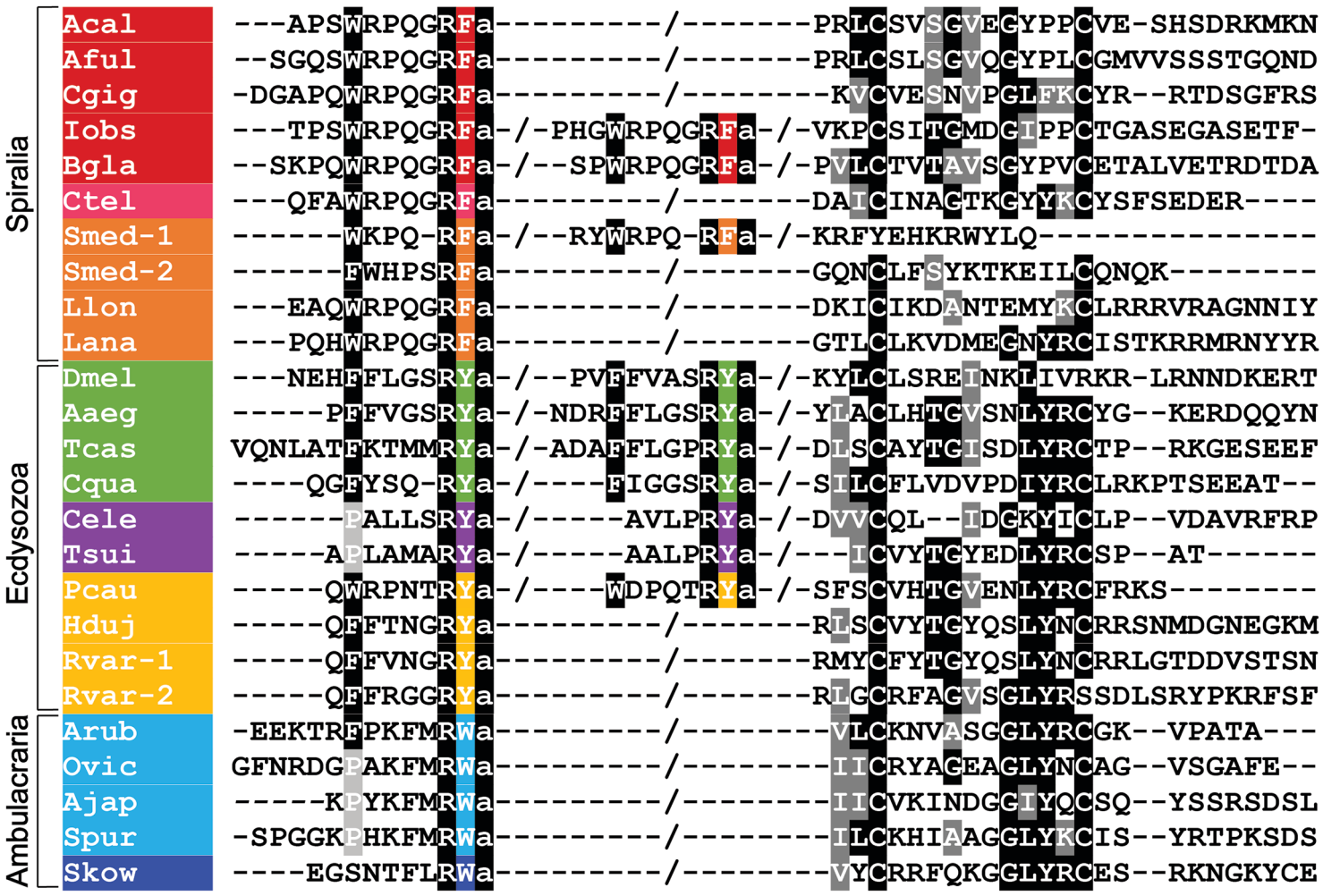
Tian, S., Zandawala, M., Beets, I., Baytemur, E., Slade, S. E., Scrivens, J. H., and Elphick, M. R. (2016). Urbilaterian origin of paralogous GnRH and corazonin neuropeptide signalling pathways. *Sci. Rep.* 6, 28788. doi:10.1038/srep28788.

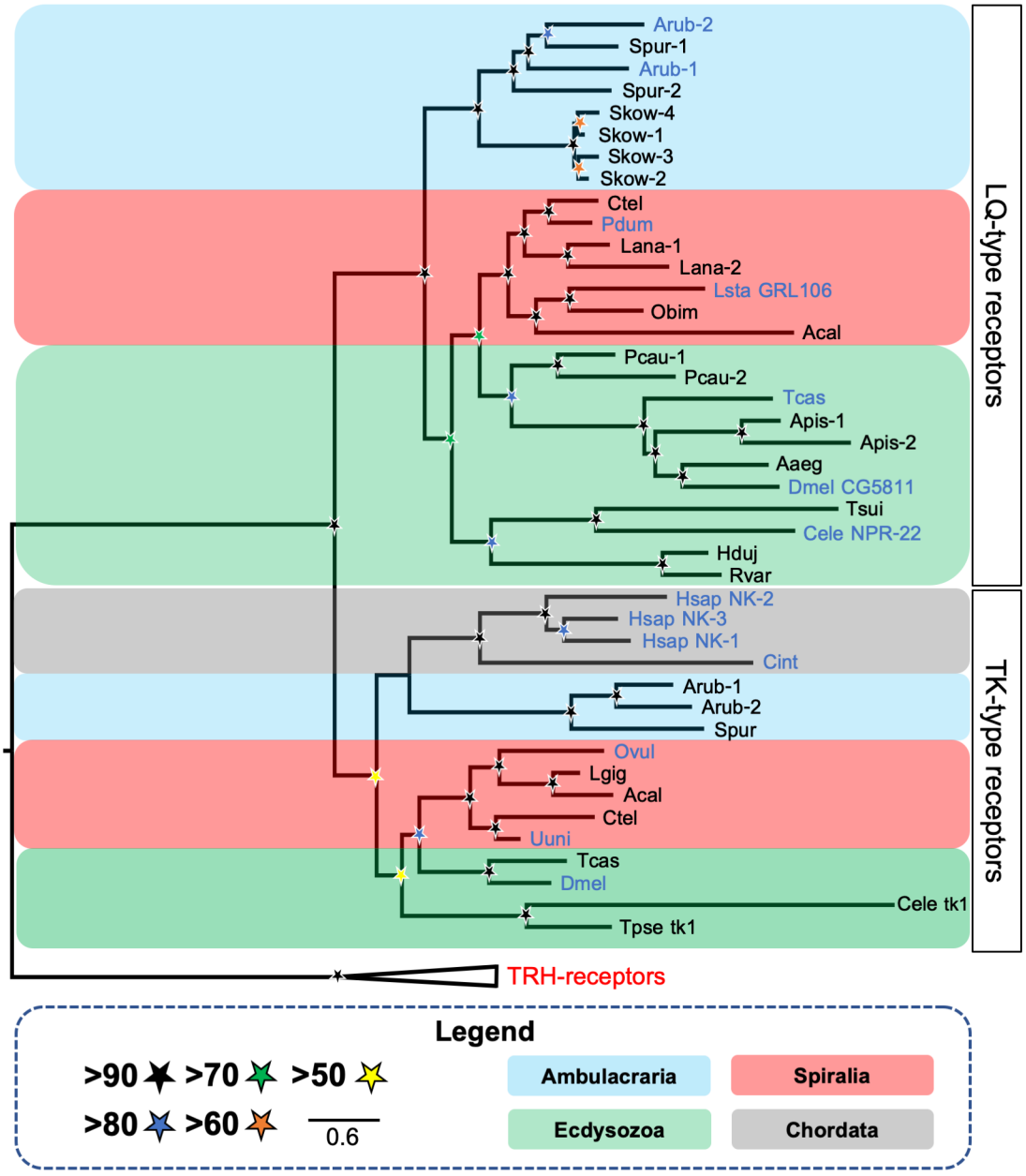
Tinoco, A. B., Semmens, D. C., Patching, E. C., Gunner, E. F., Egertová, M., and Elphick, M. R. (2018). Characterization of ngffyamide signaling in starfish reveals roles in regulation of feeding behavior and locomotory systems. *Front Endocrinol (Lausanne)* 9, 507. doi:10.3389/fendo.2018.00507.

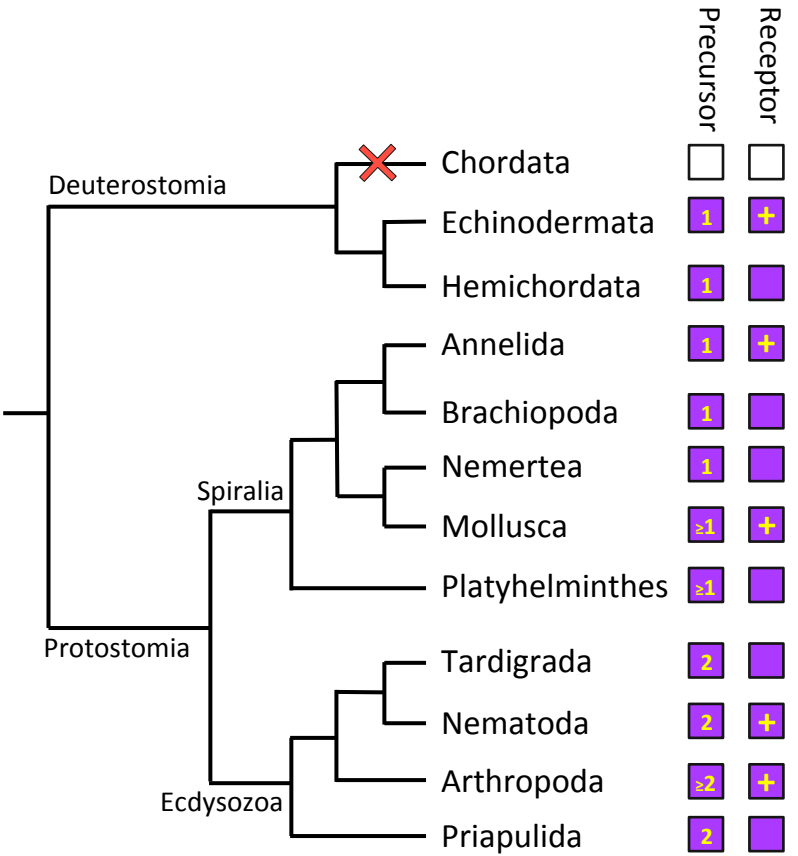
Trifinopoulos, J., Nguyen, L.-T., Haeseler, A. von, and Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res* 44, W232–5. doi:10.1093/nar/gkw256.

Ubuka, T., and Tsutsui, K. (2018). Comparative and Evolutionary Aspects of Gonadotropin-Inhibitory Hormone and FMRFamide-Like Peptide Systems. *Front Neurosci* 12, 747.

doi:10.3389/fnins.2018.00747.
Veenstra, J. A. (2011). Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of Capitella teleta and Helobdella robusta. *Gen Comp Endocrinol* 171, 160–175. doi:10.1016/j.ygcen.2011.01.005.
Veenstra, J. A. (2015). The power of next-generation sequencing as illustrated by the neuropeptidome of the crayfish Procambarus clarkii. *Gen Comp Endocrinol* 224, 84–95. doi:10.1016/j.ygcen.2015.06.013.
Veenstra, J. A., and Khammassi, H. (2017). Rudimentary expression of RYamide in Drosophila melanogaster relative to other Drosophila species points to a functional decline of this neuropeptide gene. *Insect Biochem Mol Biol* 83, 68–79. doi:10.1016/j.ibmb.2017.03.001.
Wood, N. J., Mattiello, T., Rowe, M. L., Ward, L., Perillo, M., Arnone, M. I., Elphick, M. R., and Oliveri, P. (2018). Neuropeptidergic Systems in Pluteus Larvae of the Sea Urchin Strongylocentrotus purpuratus: Neurochemical Complexity in a "Simple" Nervous System. *Front Endocrinol (Lausanne)* 9, 628. doi:10.3389/fendo.2018.00628.
Yañez-Guerra, L. A., Delroisse, J., Barreiro-Iglesias, A., Slade, S. E., Scrivens, J. H., and Elphick, M. R. (2018). Discovery and functional characterisation of a luqin-type neuropeptide signalling system in a deuterostome. *Sci Rep* 8, 7220. doi:10.1038/s41598-018-25606-2.
Zandawala, M., Moghul, I., Yañez Guerra, L. A., Delroisse, J., Abylkassimova, N., Hugall, A. F., O'Hara, T. D., and Elphick, M. R. (2017). Discovery of novel representatives of bilaterian neuropeptide families and reconstruction of neuropeptide precursor evolution in ophiuroid echinoderms. *Open Biol* 7. doi:10.1098/rsob.170129.
Zandawala, M., Tian, S., and Elphick, M. R. (2018). The evolution and nomenclature of GnRH-type and corazonin-type neuropeptide signaling systems. *Gen Comp Endocrinol* 264, 64–77. doi:10.1016/j.ygcen.2017.06.007.


Spiralia
Ecdysozoa
Ambulacraria
Acal ---APSWRPQGRFa---------/-------PRLCSVSGVEGYPPCVE-SHSDRKMKN
Aful --SGQSWRPQGRFa---------/-------PRLCSLSGVQGYPLCGMVVSSSTGQND
Cgig -DGAPQWRPQGRFa---------/--------KVCVESNVPGLFKCYR--RTDSGFRS
Iobs ---TPSWRPQGRFa-/-PHGWRPQGRFa-/-VKPCSITGMDGIPPCTGASEGASETF-
Bgla --SKPQWRPQGRFa-/--SPWRPQGRFa-/-PVLCTVTAVSGYPVCETALVETRDTDA
Ctel ---QFAWRPQGRFa---------/-------DAICINAGTKGYYKCYSFSEDER----
Smed-1 ------WKPQ-RFa-/--RYWRPQ-RFa-/-KRFYEHKRWYLQ---------------
Smed-2 ------FWHPSRFa---------/-------GQNCLFSYKTKEILCQNQK--------
Llon ---EAQWRPQGRFa---------/-------DKICIKDANTEMYKCLRRRVRAGNNIY
Lana ---PQHWRPQGRFa---------/-------GTLCLKVDMEGNYRCISTKRRMRNYYR
Dmel ---NEHFFLGSRYa-/--PVFFVASRYa-/-KYLCLSREINKLIVRKR-LRNNDKERT
Aaeg -----PFFVGSRYa-/-NDRFFLGSRYa-/-YLACLHTGVSNLYRCYG--KERDQQYN
Tcas VQNLATFKTMMRYa-/-ADAFFLGPRYa-/-DLSCAYTGISDLYRCTP--RKGESEEF
Cqua ----QGFYSQ-RYa-/----FIGGSRYa-/-SILCFLVDVPDIYRCLRKPTSEEAT--
Cele ------PALLSRYa-/-----AVLPRYa-/-DVVCQL--IDGKYICLP--VDAVRFRP
Tsui -----APLAMARYa-/-----AALPRYa-/---ICVYTGYEDLYRCSP--AT------
Pcau -----QWRPNTRYa-/----WDPQTRYa-/-SFSCVHTGVENLYRCFRKS--------
Hduj -----QFFTNGRYa---------/-------RLSCVYTGYQSLYNCRRSNMDGNEGKM
Rvar-1 -----QFFVNGRYa---------/-------RMYCFYTGYQSLYNCRRLGTDDVSTSN
Rvar-2 -----QFFRGGRYa---------/-------RLGCRFAGVSGLYRSSDLSRYPKRFSF
Arub -EEKTRFPKFMRWa---------/--------VLCKNVASGGLYRCGK--VPATA---
Ovic GFNRDGPAKFMRWa---------/--------IICRYAGEAGLYNCAG--VSGAFE--
Ajap -----KPYKFMRWa---------/--------IICVKINDGGIYQCSQ--YSSRSDSL
Spur -SPGGKPHKFMRWa---------/--------ILCKHIAAGGLYKCIS--YRTPKSDS
Skow ----EGSNTFLRWa---------/--------VYCRRFQKGGLYRCES--RKNGKYCE


Arub-2
Spur-1
Arub-1
Spur-2
Skow-4
Skow-1
Skow-3
Skow-2
Ctel
Pdum
Lana-1
Lana-2
Lsta GRL106
Obim
Acal
Pcau-1
Pcau-2
Tcas
Apis-1
Apis-2
Aaeg
Dmel CG5811
Tsui
Cele NPR-22
Hduj
Rvar
Hsap NK-2
Hsap NK-3
Hsap NK-1
Cint
Arub-1
Arub-2
Spur
Ovul
Lgig
Acal
Ctel
Uuni
Tcas
Dmel
Cele tk1
Tpse tk1
TRH-receptors
LQ-type receptors
TK-type receptors
Legend
>90
>70
>50
>80
>60
0.6
Ambulacraria
Spiralia
Ecdysozoa
Chordata


Precursor
Receptor
Deuterostomia
Protostomia
Spiralia
Ecdysozoa
Chordata
Echinodermata
Hemichordata
Annelida
Brachiopoda
Nemertea
Mollusca
Platyhelminthes
Tardigrada
Nematoda
Arthropoda
Priapulida
1
+
1
1
+
1
1
≥1
+
≥1
2
2
+
≥2
+
2

A

Mollusca

Lymnaea stagnalis

The first receptor for LQ-type peptides (GLR106) was discovered in this species and is activated by a peptide with a C-terminal RFamide motif. This peptide is expressed in nerve fibres terminating in the pericardial cavity and it has a cardioexcitatory effect. Conversely, the peptide has an inhibitory effect on GLR106-expressing caudodorsal cells that regulate egg-laying in *L. stagnalis* (Tensen et al., 1998).

B

Arthropoda

Drosophila melanogaster

Gene CG5811 encodes a receptor for two LQ-type peptides with a C-terminal RYamide motif in this species. LQ-type peptides are expressed in neurons that project to organs (rectal papillae) that regulate water re-absorption. In other insects LQ-type peptides are expressed more widely and regulate feeding, so loss of function in *D. melanogaster* is inferred (Ida et al., 2011, Veenstra & Khammassi, 2017).

C

Nematoda

Caenorhabditis elegans

NPR-22 has been identified as the receptor for two LQ-type peptides with a C-terminal RYamide motif in this species. LQ-type signalling has been functionally characterised in *C. elegans* in more detail than in any other species, revealing evidence of physiological roles in regulation of feeding and body size, egg-laying and roaming behaviour (Ohno et al., 2017).

D

Echinodermata

Asterias rubens

Two proteins (ArLQR1, ArLQR2) have been identified as receptors for a LQ-type peptide (ArLQ) with a C-terminal RWamide motif in this species. The ArLQ precursor is expressed in the nervous and digestive systems and in tube feet. ArLQ causes relaxation of tube feet, indicating a role in inhibitory regulation of these locomotory organs (Yañez-Guerra et al., 2018).

A

Daphnia pulex RYamide precursor

MARKESVFWLFCTLALMMSVVLVDAQTFFTNGRYGKRSEVRSRVASRSADERFFGGPRFGRSGNGGIVLGNSELDARNPERFFI
GSRYGKRSEMEQIVPSPQVDESTSNSQEKETFLECNPIGIEQLYHCIERLKSAHHFDLMQHQQV

Proasellus cavaticus RYamide precursor

MFFLRSFLLLVALGTLEMTLGQGFYSTRYGKRTNDASSSSNTGYENEGHSGFYANRYGRSSDLPEIKIRSSRFIGGSRYGKRST
TTPEGDLPRNIDGDTFDCVMVNSPSLYRCLRKSFQPSEETIN

Zootermopsis nevadensis RYamide precursor

MASASSVVILIMLVTCSLVTLALSAQFYTSGRYGKRDLAQRSMFWSGSRYGRSSGGGGGRRQGGNNPVEVAVRNDRFFIGSRYG
KRSEEPLTTTDETVGVLVPTEDTNSQVACMYTGVANLYRCYKRKGNSSEDASSEHE

Nasonia vitripennis RYamide precursor

MISSSRKIRRVSDYLKLDKLIVWLWISGIFLTLVSSQDNFYASGRFGKRKYALSMSQIPLCSKFDRSEDRSAGNSLKDSSLFSS
ARFGRSEDRNTGNSLRDSSSFFPARYGRSEDRSTGNSLRDSSSFFPARFGRSEDRSTGNSLKDSSSFSPARYGRSEDRSSGNSL
KESSFFSPGRYGRSEGHKNPKELPKFFEIKPRVDQFFIGSRYGKRSLSMLEPQPPLEALHNQRFEAAIDYLDRIKQNLAEAEEI
EDETRDASRDDELVEAIYPNDYTGLSKI

B

Echinococcus multilocularis Luqin-type precursor

MRGTFISILTLFYFASSLRLHDYDGEAEPTVSAATPVGEAEDDIDFFPPRYGVAKRYPLLSDLDEGMMIENPYWAKEISRRSPQ
FAWRPHSRFGRR

Echinococcus granulosus Luqin-type precursor

MRGTFISLLTLFYFASSLRLHDYDGEAEPAVSAATPVGEAEDDIDSLPPRYRVAKRYPLLSDLDEEMMIENPYWAKEISRRSPQ
FAWRPHSRFGRR

Taenia solium Luqin-type precursor

MRATCISLLTLFYFASSLRLHDLEGPVEPTVSSAMPVGEPEDDIDFPLPSRYRLAKRYPHLSNFDEEMVIGNPYWGKEISRRSP
QFAWRPHSRFGRR

Mesocestoides corti Luqin-type precursor

MVSCLTETSVILMVLLCFASALRLREPEADLDDEDIDFVVPPPLRFHKRFPMMGEFGDGAMWSPMGEREVARRSAHFAWRPHSR
FGRR

Supplementary Figure 1. Luqin-type precursor proteins with atypical characteristics. Luqin-type precursors typically comprise one or two luqin-type neuropeptides, which are located immediately after the N-terminal signal peptide, and a pair of cysteine residues separated by ten amino acid residues located in the C-terminal region of the precursor (see Figure 1 of this paper). The sequences of luqin-type precursors with atypical characteristics are shown in this figure, with the signal peptide shown in blue, potential mono/dibasic cleavage sites shown in green and predicted neuropeptides shown in red. Peptide products of the precursors that have been identified using mass spectrometry are highlighted in yellow. Where a C-terminal pair of cysteine residues separated by ten amino-acid residues are present, this region of the precursor is underlined.

A. Arthropod RYamide precursors that contain more than two luqin-type neuropeptides. The RYamide precursor in the crustacean *D. pulex* comprises three predicted neuropeptides, two of which have been identified by mass spectrometry. The first peptide is located immediately after the signal peptide and is subject to post-translational conversion of the C-terminal glycine to an amide group (as in other luqin-type peptides) and conversion of the N-terminal glutamine residue to pyroglutamate. Two different forms of the third neuropeptide are detected by mass spectrometry - a twenty-seven residue peptide and a C-terminal fragment of this peptide generated by cleavage at the highlighted arginine residue in the longer peptide. The precursor has a canonical C-terminal pair of cysteine residues separated by ten amino-acid residues. The RYamide precursors in the crustacean *Proasellus cavaticus* (isopod) and in the insect *Zootermopsis nevadensis* (termite) also comprise three putative luqin-type neuropeptides but the structures of these peptides have not been confirmed using mass spectrometry. The RYamide precursor in the insect *Nasonia vitripennis* (parasitoid wasp) is remarkable in comprising seven putative luqin-type neuropeptides, but only the first of these has been identified using mass spectrometry. The six other putative luqin-type neuropeptides are bounded by predicted mono/dibasic cleavage sites and also contain potential internal monobasic cleavage sites. Note also that the precursor lacks a C-terminal region containing the two cysteines separated by ten amino acid residues.

B. **Luqin-type precursors with a C-terminally located neuropeptide in parasitic platyhelminths.** The sequences of the luqin-type precursors from four cestode species are shown and they all have the atypical characteristics of comprising a single luqin-type neuropeptide located at the C-terminus and lacking a C-terminal pair of cysteines separated by ten amino acid residues. In spite of these differences at the precursor level, the neuropeptides derived from these precursors exhibit a high level of sequence conservation with luqin-type neuropeptides from other spiralians. Thus, the WRPHSRF-NH_2 motif in these peptides is structurally similar to the WRQPGRF-NH_2 motif of luqin-type neuropeptides in molluscs, annelids, brachiopods and nemerteans. The sequence data and mass spectroscopic data shown in this figure have been reported in the following publications: (27,33,34,38,45).