Expression of the neuropeptides RFamide and LWamide during development of the coral *Acropora millepora* in relation to settlement and metamorphosis

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**ABSTRACT**

Neuropeptides play critical roles in cnidarian development. However, although they are known to play key roles in settlement and metamorphosis, their temporal and spatial developmental expression has not previously been characterized in any coral. We here describe *Acropora millepora* LWamide and RFamide and their developmental expression from the time of their first appearance, using *in situ* hybridization and FMRFamide immunohistochemistry.

**AmRFamide** transcripts first appear in the ectoderm toward the oral end of the planula larva following blastopore closure. This oral bias becomes less apparent as the planula develops. The cell bodies of *AmRFamide*-expressing cells are centrally located in the ectoderm, with narrow projections to the mesoglea and to the cell surface. As the planula approaches settlement, *AmRFamide* expression disappears and is undetectable in the newly settled polyp. Expressing cells then gradually reappear as the polyp develops, becoming particularly abundant on the tentacles.

**AmLWamide** transcripts first appear in ectodermal cells of the developing planula, with minimal expression at its two ends. The cell bodies of expressing cells lie just above the mesoglea, in a position distinct from those of *AmRFamide*-expressing cells, and have a narrow projection extending across the ectoderm to its surface. *AmLWamide*-expressing cells persist for most of the planula stage, disappearing shortly before settlement, but later than *AmRFamide*-expressing cells. As is the case with *AmRFamide*, expressing cells are absent from the polyp immediately after settlement, reappearing later on its oral side. *AmLWamide* expression lags that of *AmRFamide* in both its disappearance and reappearance. Antibodies to FMRFamide stain cells in a pattern similar to that of the transcripts, but also cells in areas where there is no expression revealed by *in situ* hybridization, most notably at the aboral end of the planula and in the adult polyp. Adult polyps have numerous staining cells on the tentacles and oral discs, as well as an immunoreactive nerve ring around the mouth. There are scattered staining cells in the coenosarc between polyps and staining cells are abundant in the mesenterial filaments. The above results are discussed in the context of our knowledge of the behavior of coral planulae at the time of their settlement and metamorphosis.

Corals are facing multiple environmental threats, and these results both highlight the need for, and bring us a step closer to, a mechanistic understanding of a process that is critical to their survival.

1. **Introduction**

Neuropeptides are signalling molecules with important functions throughout the animal kingdom. They may, in fact, be among the oldest neural signalling molecules, given their important role in controlling the behavior of cnidarians. The structure and function of two classes of neuropeptide, RFamide and LWamide, have been particularly well studied, and they have been found in the nervous systems of members of all four major cnidarian classes.

The active forms of LWamide and RFamide are produced from prepropeptides which are incorporated into endoplasmic reticulum, where they are converted into propeptides. From there they move to...
the Golgi apparatus where they undergo post-translational modifications such as endoproteolysis and C-terminal amidation to produce several much smaller active peptides, the activity of which can be, and probably is, controlled at several levels: transcription, translation, and amidation (Plickert et al., 2004).

LWamides and RFamides have diverse roles in cnidarians (reviewed in Takahashi and Takeda, 2015) but in this paper we will focus on their function in settlement and metamorphosis. The roles of both peptides in these processes are best understood in the hydrozoan Hydractinia echinata (reviewed in Müller and Leitz, 2002; Seipp et al., 2010) where, immediately preceding metamorphosis, LWamide expression in neurons disappears as the LWamide is released, thus triggering metamorphosis. RFamide too, disappears from Hydractinia neurons shortly before settlement and metamorphosis (Plickert, 1989; Seipp et al., 2010). Of particular relevance in the context of metamorphosis is the finding by Katsukura et al. (2003) that RFamides act antagonistically to LWamides in Hydractinia, inhibiting metamorphosis induced by LWamides or other inducers of metamorphosis. The action of RFamide is downstream of LWamide release, presumably on target cells directly mediating metamorphosis, since RFamide can also block metamorphosis induced by exogenous LWamide (Katsukura et al., 2003).

Understanding coral neuropeptides and their roles in settlement and metamorphosis is currently of particular importance due to the threats posed to coral reefs worldwide by global warming, ocean acidification and pollution. The early stages of development of the reef building coral Acropora millepora have previously been described (Ball et al., 2002; Grasso et al., 2008; Hayward et al., 2015) and gene expression changes at the time of settlement and metamorphosis have been characterized (Grasso et al., 2011; Hayward et al., 2011; Meyer et al., 2011; Siboni et al., 2014; Strader et al., 2018).

Iwao et al. (2002) tested the effect of several GLWamide peptides on larvae of nine species of Acropora and found that one, the Hydra peptide Hym-248 (EPLPGLWa), induced metamorphosis in all of them, but not in other corals (Isopora brueggemannii, Montipora sp., Astreopora myriophthalma, Merulina ampliata, or Goniathea retiformis). Erwin and Szmant (2010) subsequently obtained similar results with Caribbean corals; of six cnidarian peptides tested, none produced metamorphosis in Orbicella (Montastraea) faveolata or Favia fragum and only Hym-248 induced metamorphosis in Acropora palmata. The sequences of Acropora LWamides were unknown at the time of these studies. Both Meyer et al. (2011) and Grasso et al. (2011) have also used Hym 248 to drive A. millepora planulae directly into metamorphosis without an intermediate settlement phase. In contrast to the Acropora results, all of the LWamides tested produced metamorphosis in Hydractinia (Gajewski et al., 1996; Takahashi et al., 1997) (Table 1)

Antibodies to FMRFamide have been widely used for studying cnidarian nervous systems (e.g. Grimmelikhuijzen and Spencer, 1984; Koizumi and Bode, 1986; Mackie et al., 2003; summarized in Grimmelikhuijzen and Westfall, 1996). However, in both Hydractinia (Plickert et al., 2004) and Nematostella (Marlow et al., 2009), the antibody staining pattern differed markedly from the distribution of the mRNA revealed by in situ hybridization (ISH). The number of ISH-positive cells was 3–5X greater than the number of immunoreactive cells in Hydractinia, leading to the conclusion that some cells did not "contain detectable quantities of amidated and thus bioactive peptide" (Plickert et al., 2004). In Nematostella, by contrast, more cells were immunoreactive than were seen with ISH, but the antibody did not label ISH-positive cells in the tentacles. It was hypothesized that the antibody was "crossreactive with a wide variety of neuropeptides, which may not include the antho-RFamide gene identified " (Marlow et al., 2009).

In this paper, we report the predicted amino acid sequences of the Acropora millepora LWamide and RFamide precursor proteins, describe the developmental expression of the corresponding transcripts (hereafter referred to as AmLWamide and AmRFamide) from the time that they are first detectable by in situ hybridization, and relate these findings to larval settlement and metamorphosis. In addition we describe the development of FMRFamide immunoreactivity, providing the first morphological description of the nervous system in an adult coral polyp.

2. Materials and methods

2.1. Coral material

Staged A. millepora embryos and adult tissues were collected during mass spawnings at Magnetic Island or Orpheus Island, Queensland, Australia between 1993 and 2015. Development took place under field conditions, so larvae at the same time post fertilization in different years varied somewhat in the extent of development. Additionally, the quality of fixation varied from year to year. For these reasons, larvae of approximately the same morphology, but from several spawning seasons, were routinely mixed together when doing hybridizations or immunohistochemistry in an effort to sample all developmental stages. Fixation was for 10–60 min in 3.7% formaldehyde (1 + 9 dilution of Sigma F-1635) in Millipore-filtered (0.22 μm) sea water buffered to pH 8 with HEPES buffer. After repeated rinses in sea water, specimens were dehydrated through a graded series of methanol and stored at −20 °C in absolute methanol until used. In the populations of A. millepora that we have studied, adult polyps are extended mainly at night and generally withdraw in response to even minor disturbances in their vicinity. The adult polyps that we obtained

| Name   | Sequence       | From          | In Hydractinia (Leitz and Lay, 1995) | in 9 Acropora sp. (Iwao et al., 2002) | in Acropora palmata (Erwin and Szmant, 2010) | in Acropora millepora (Grasso et al., 2011) | in Acropora millepora unpublished |
|--------|----------------|---------------|-------------------------------------|--------------------------------------|---------------------------------|-------------------------------------|---------------------------------|
| Hym-53 | NPTGGLW-NH₂    | Hydra         | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |
| Hym-54 | GPMTGGLW-NH₂   | Hydra         | ✓                                   | X                                   | ✓                               | ✓                                   | ✓                               |
| Hym-248| EPLPGLW-NH₂    | Hydra         | ✓                                   | ✓                                   | ✓                               | ✓                                   | ✓                               |
| Hym-331| GPPGGLW-NH₂    | Hydra         | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |
| MMA    | pEQPGLW-NH₂    | Anthopleura   | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |
| He-LWamide I | pERPPIGLW-NH₂ | H. echinata | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |
| He-LWamide II | PKPGLW-NH₂ | H. echinata | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |
| Am-GLWamide | GPPGGLW-NH₂ | A. millepora | ✓                                   | X                                   | X                               | ✓                                   | ✓                               |

Table 1

Only one of the peptides that produced metamorphosis in Hydractinia had the same effect in Acropora. The sequences of these peptides are shown. The data for compiling this table came from the following papers: Leitz and Lay (1995), Gajewski et al. (1996), Takahashi et al. (1997), Iwao et al. (2002) and Erwin and Szmant (2010). Informal tests using GPPGGLW-NH₂, one of the peptides encoded by the A.millepora LWamide gene, revealed some speeding up of settlement behavior, but this was minor compared to the dramatic effect of Hym 248.
were from a branch of a post-spawning colony placed undisturbed in filtered sea water in a darkened room for several hours. Under red light, formaldehyde was then gradually added to the vessel holding the branch over a period of hours until the polyps were no longer able to withdraw. The branch was then dehydrated and stored in absolute methanol. For in situ or antibody studies, individual extended polyps were dissected from the branch.

2.2. Sequence discovery and in situ hybridization

High quality genomes and transcriptomes are available for two Acropora species, A. millepora (Bioproject ID PRJNA473876; Moya et al., 2012) and A. digitifera (Shinzato et al., 2011). Predicted Acropora RFamide and LWamide precursor transcripts were identified with tblastn using known cnidarian preproteptides of RFamide and LWamide (Leviev et al., 1997; Darmer et al., 1991, 1998; Yum et al., 1998; Schmutzler et al., 1992, 1994; Reinscheid and Grimmelikhuijzen, 1994; Grimmelikhuijzen et al., 1996). Searches for single and repetitive occurrences of dipeptide motifs contained in characterized cnidarian neuropeptides (e.g.,“RF”) in conjunction with the residue necessary for amidation (G) and potential cleavage sites (K/R) were also carried out. After filtering for hits which had the capacity to contain an open reading frame of at least 50 codons, and for secreted proteins (presence of a signal peptide at the N-terminus) only single predicted transcripts corresponding to the RFamide and LWamide precursors, in addition to a putative RPamid transcript were identified.

Primers flanking the predicted complete open reading frames were used to amplify products from first-strand oligo dT-primed cDNA prepared from planulae. PCR products were ligated into pGEM-T Easy (Promega). Plasmids were sequenced using vector and internal primers with Big Dye Terminator v. 3.1 (Applied BioSystems) and the reactions were run on an ABI 3730 sequencer at the Biomolecular Resource Facility (John Curtin School of Medical Research, Australian National University). Digoxigenin-labelled antisense RNA probes were produced by run-off transcription from linearised plasmid templates using DIG RNA Labelling Mix (Sigma) and T7 (Roche) or SP6 (Promega) RNA polymerase. The in situ hybridization protocol has previously been described (Hayward et al., 2001). In brief, material stored at −20 °C in absolute methanol was rehydrated to PBS. It was then transferred to RIPA solution (Rosen and Beddington, 1993) overnight at room temperature with gentle rotation for delipidification. Delipidification was completed by ethanol dehydration to xylene for several hours, followed by rehydration to PBS, pre-hybridization and hybridization at 55 °C for a minimum of 48 h followed by repeated washes in hybridization wash. Detailed recipes for hybridization solution and hybridization wash are given by Kucharski et al. (2000).

After repeated PBT (1XPBS; 0.1% Tween-20) washes specimens were treated with anti-Dig-AP FAB (Roche) at 1:1800 for 2 h at RT and washed in PBT, washed briefly in Tris pH9.5, and developed with BCIP/NBT substrate (Vector Kit SH 5400). The reaction was stopped by washing in PBT. For double in situ a similar procedure was followed. The RFamide probe was labelled with fluorescein UTP (Roche) and the LWamide probe with DIG UTP, and the two probes were added to the hybridization solution simultaneously. After hybridization and washing the specimens were treated first with anti-Dig AP FAB (Roche) 1:2000, then developed with BCIP/NBT with 5% polyvinyl acetate in the developing solution to produce a purple stain. Development was stopped by washing in PBT, followed by dehydration through a glycerol series and mounting and photography in 90% glycerol.

For double in situ/antibody preparations BCIP/NBT in situ were carried out as described above for single in situ. Once the in situ procedure was complete, anti-FMRFamide staining was carried out with the reagents and concentrations described below.

2.3. Antibody staining

Embryos were gradually rehydrated from 100% methanol to PBT. After repeated washes, fixed tissues were pre-blocked in 5% normal goat serum (NGS) in PBT. Three different anti-RFamide antibodies were tried at various times: 146 III, raised in rabbit against RFamide [Arg-Phe-amide], 1:1000, [Grimmelikhuijzen (1985)]; Immunostar, Cat No 20091, raised in rabbit against synthetic FMRFamide coupled to bovine thyroglobulin [BTg] with FNPS, 1:400–1:800; and Peninsula Labs, Cat No T-4322, IHC 8755, raised in rabbit against FMRFamide [H-Phe-Met-Arg-Phe-NH2], 1:500–1:1500. Most of the results presented here are with the Peninsula antibody. After 12–16 h in primary antibody at 4 °C, the tissues were washed for 2–3 h in at least 4 changes of PBT. The secondary antibody was goat anti-rabbit CY5 (1:200) (Jackson Immunoresearch) in which specimens were incubated for 2 h at room temperature or overnight at 4 °C. After approximately 2 h of washing with at least 4 changes of PBT preparations were dehydrated through a glycerol series and mounted in 90% glycerol/PBS. They were then photographed on a Zeiss Axioplan II with or without Apotome or on a Leica SP2 laser scanning spectral confocal microscope. Images are presented in black and white, as collected, rather than false colored, for better visualisation of fine details. Some images, as noted in the figure captions, consist of multiple focal planes flattened using “Z-stack” in Image J.

3. Results

3.1. Neuropeptide precursors and their processing

The search strategy described above led to the identification of only a single AmRFamide transcript in A. millepora. The predicted 280 residue AmRFamide precursor protein (Fig. 1a) carries an N-terminal signal peptide (green) for translocation to the endoplasmic reticulum and contains thirteen putative RFamide peptides of the form XGRFG (highlighted in blue). These sequences are followed by an Arg residue, which is a potential cleavage site (Sossin et al., 1989; Devi, 1991), processing at which would permit amidation of the C-terminal Gly residue by peptidylglycine a-amidating monooxygenase (Eipper et al., 1992; Attenborough et al., 2012). The ten most C-terminal of the RFamide precursors are preceded by acidic residue(s). Proteolytic processing at acidic residues has been postulated for RFamide precursors in the sea anemones Calliactis and Anthopleura and in the sea pansey Renilla (Darmer et al., 1991; Schmutzler et al., 1992; Reinscheid and Grimmelikhuijzen, 1994). In four cases, the putative peptide has an
3.2. Expression of RFamide transcripts

RFamide transcript expression is first apparent in scattered ectodermal cells as the early planula starts to elongate after blastopore closure (Fig. 2a). Expressing cells are initially concentrated toward the oral end of the developing planula and their number rises along with the surface area (Fig. 2a-d). As the planula develops, the oral bias in expression is less apparent (Fig. 2e). Transverse sections (Fig. 2f,g) clearly reveal that the cell bodies of RFamide-expressing cells are located centrally in the ectoderm, with extensions projecting to the surface of the planula and to the mesoglea. As elongation continues, the aboral end becomes thicker and expressing cells become less abundant (Fig. 2h-i). Immediately after settlement no expressing cells are present on the newly settled polyp (Fig. 2j). Later, as the polyp starts to rise from the basal plate, scattered expressing cells become apparent on the developing tentacles (Fig. 2k,l). As the polyp continues to mature, expressing cells become steadily more abundant on the oral side (Fig. 2l), but are absent aborally, as shown in the oral/aboral pairing (Fig. 2m,n,m*). As the tentacles become apparent, expressing cells become even more abundant on them (Fig. 2m,n). Our attempts at in situ hybridization on adult tissue have failed, for this and all other genes. However, transcriptomic data (Moya et al., 2012) indicate that RFamide transcripts are present in the adult stage.

3.3. Expression of LWamide transcripts

AmLWamide expression appears in ectodermal cells in the early planula, with minimal expression at the two ends, particularly orally (Fig. 3a-d). Transverse (Fig. 3e,f) and longitudinal (Fig. 3g) sections confirm that expressing cells are limited to the ectoderm and that their cell bodies lie close to the mesoglea, extending long projections to the surface of the larva (Fig. 3e-g). Expressing cells persist for most of the planula stage (Fig. 3h-i) before starting to disappear just before settlement (Fig. 3j). No expressing cells are apparent in the newly settled primary polyp but later, as the polyp begins to rise above the basal plate, scattered cells begin to appear (Fig. 3k). Post-settlement expression is limited to the oral side of the polyp, as is apparent from the oral/aboral pairs in Fig. 3l/l* and Fig. 3m/m*. Although our attempts at in situ hybridization on adult tissue have failed, the transcriptome results of Moya et al. (2012) indicate that LWamide transcripts are present in adults.

3.4. Two color double labelling in situ hybridization clarifies the temporal and spatial relationships between the peptide expressing cells

The double labelling in situ hybridization results shown in Fig. 4a-c confirm the differing morphologies of the AmLWamide- and AmRFamide-expressing cells and demonstrate that the two peptides are not co-expressed. Consistent with the results of the single in situ, the nuclei of the cells expressing RFamide are mostly located approximately halfway across the ectoderm, while those of cells expressing LWamide are located directly above the mesoglea. The image of a larva undergoing metamorphosis shown in Fig. 4d shows that AmLWamide expression is still occurring in the unmetamorphosed aboral end, which represents the remaining portion of the planula larva, while it is missing from the metamorphosing oral end. Fig. 4e is a somewhat more advanced specimen, as is apparent from the more complete differentiation of the oral end. A few scattered AmLWamide-expressing cells, and even fewer AmRFamide-expressing cells, are still present in the aboral end, while AmRFamide-expressing cells are now abundant in the metamorphosed oral end. Thus, although the expression of both neuropeptide precursor transcripts fades at the onset of metamorphosis, and subsequently re-appears, AmRFamide expression both disappears and reappears first at the time of settlement and metamorphosis.

Table 2
Conserved putative peptides from the N-terminal portion of the RFamide precursor proteins of Acropora, Calliactis (Darmer et al., 1991) and Anthopleura (Schmutzler et al., 1992).

| Species       | Position | Sequence     |
|--------------|----------|--------------|
| Acropora     | 62       | PQYWKGRFS    |
|              | 97       | PQYWKGRFS    |
|              | 43       | PQYWKGRFS    |
|              | 54       | PQYWKGRFS    |
|              | 69       | PQYWKGRFS    |
| Calliactis   | 76       | PQYWKGRFS    |
|              | 54       | PQYWKGRFS    |
|              | 99       | PQYWKGRFS    |
| Anthopleura  | 109      | PQYWKGRFS    |
|              | 119      | PQYWKGRFS    |
|              | 155      | AQYWKGRFS    |
|              | 173      | PQYWKGRFS    |
| Consensus    | 155      | AQYWKGRFS    |

N-terminal Gln residue which, if converted to a pyroglutamic acid residue would result in AnthoRFamide (Glu-Gly-Arg-Phe-amide), the biologically active peptide isolated from Anthopleura (Grimmelikhuizen and Graff, 1986). In eight cases, the canonical AnthoRFamide sequence is N-terminally extended by one or two amino acids. Subsequent processing may remove these amino acids; alternatively, these may represent RFamide variants. The most N-terminal of the candidate RFamide precursors has the divergent sequence AFVPGQRFS.

In the N-terminal part of the precursor protein, two other potential peptides are present, (highlighted in yellow in Fig. 1a), one of which is flanked by acidic residues while the other is preceded by an acidic residue and followed by a basic residue. Motifs very similar to these are present in the N-terminal region of the RFamide precursors in both Calliactis (Darmer et al., 1991) and Anthopleura (Schmutzler et al., 1992); an alignment of these with the Acropora sequences is shown in Table 2. A search of publicly available cnidarian transcriptome assemblies revealed that related potential peptides are present at similar positions in the RFamide precursor proteins from Anthozoa but not in those from Hydrozoa. While it is not known if these are processed to mature peptides, the maintenance of similar sequences in both sea anemones and corals implies conservation of function.

Only a single AmLWamide transcript was identified. As in the case of the AmRFamide precursor protein, the 210 amino acid AmLWamide precursor protein has an N-terminal signal peptide, directing it to the endoplasmic reticulum. It contains five putative LWamide precursor sequences (highlighted in blue in Fig. 1b), all of which are followed by a basic residue (Arg); cleavage at this site allows the conversion of the C-terminal glycine to an amide group. Three of the putative peptide sequences (highlighted in blue in Fig. 1b), all of which are followed by a basic residue (Arg); cleavage at this site allows the conversion of the C-terminal glycine to an amide group. Three of the putative peptide sequences (highlighted in blue in Fig. 1b), all of which are followed by a basic residue (Arg); cleavage at this site allows the conversion of the C-terminal glycine to an amide group. Three of the putative peptide sequences (highlighted in blue in Fig. 1b), all of which are followed by a basic residue (Arg); cleavage at this site allows the conversion of the C-terminal glycine to an amide group.

Species | Position | Sequence
--- | --- | ---
Acropora | 62 | PQYWKGRFS
 | 97 | PQYWKGRFS
 | 43 | PQYWKGRFS
 | 54 | PQYWKGRFS
 | 66 | PQYWKGRFS
 | 76 | PQYWKGRFS
Calliactis | 54 | PQYWKGRFS
 | 99 | PQYWKGRFS
 | 69 | PQYWKGRFS
 | 79 | PQYWKGRFS
 | 89 | PQYWKGRFS
 | 99 | PQYWKGRFS
 | 109 | PQYWKGRFS
 | 119 | PQYWKGRFS
 | 136 | PQYWKGRFS
 | 155 | AQYWKGRFS
 | 173 | PQYWKGRFS
Anthopleura | 155 | AQYWKGRFS
Consensus | 155 | AQYWKGRFS

Table 2
Developmental expression of the RFamide gene as revealed by in situ hybridization. (a) Expression is first apparent in scattered ectodermal cells as the embryo begins to elongate shortly after blastopore closure. (b-d) As development continues, more and more expressing cells appear, with expression biased toward the oral end of the embryo. (e) In a slightly older planula the oral bias of expression is less apparent. (f) A transverse section of a planula reveals that the expressing cells are strictly ectodermal as none are evident in the endoderm. (g) A blowup of a portion of the embryo shown in (f) demonstrates that the cell bodies are mid-ectodermal, with narrow projections to the surface and to the mesoglea. (h-i) By the late planula stage expressing cells begin to disappear, particularly from the aboral end, which thickens as the planula approaches settlement. (j) Expressing cells are entirely absent from the newly settled polyp. (k) As the primary polyp begins to develop, expressing cells appear in the oral ectoderm. (l) As the polyp grows older the number of expressing cells on the oral side continues to rise (l-n), but there are no expressing cells on the aboral side (m*, which is the aboral side of m).
3.5. Development of FMRFamide immunoreactivity

Isolated immunoreactive cells first appear post gastrulation as the spherical embryo starts to elongate into a pear-shaped planula (Hayward et al., 2001; de Jong et al., 2006). Fig. 5a shows a planula when FMRFamide immunoreactivity is near its peak, and Fig. 5b a planula as it begins to fade. Note the greater brightness and density of immunoreactive cells toward the aboral end. Fig. 5c illustrates the resumption of FMRFamide immunoreactivity on the oral side of a polyp post-settlement. Note that at this stage immunoreactive cells are fairly evenly distributed. In a somewhat older polyp (Fig. 5d), immunoreactivity becomes concentrated in the tentacles. The nerve net as
Fig. 3. Developmental expression of the LWamide gene as revealed by in situ hybridization. (a) AmLWamide expression appears shortly after gastrulation in the ectoderm of the early planula larva as it begins to elongate in the oral-aboral axis. (b-d) As elongation continues the number of expressing cells increases, especially along the central portion of the cylindrical embryo. Expressing cells are totally absent from the oral end of the embryo and rare at the extreme aboral end. (e) is a transverse section of a planula, showing that expressing cells are fairly equally distributed around the circumference in the ectoderm and are completely absent from the central lipid-filled endoderm. (i) blow-up of a portion of (e) showing that the cell bodies of the AmLWamide-expressing cells are located directly above the mesoglea, in contrast to the AmRFamide-expressing cells shown in Fig. 2g which have their cell bodies mid-ectoderm, with finer processes extending to the mesoglea and to the cell surface. (g) longitudinal bisection of a planula larva confirming the absence of expressing cells from the two ends of the planula ectoderm and from the endoderm. The darker out-of-focus spots in the central part of the embryo are expressing cells showing through the partially cleared endoderm from the ectoderm of the opposite side of the embryo. (h-i) The central belt of expressing cells persists as the planula grows older. (j) Although late stage embryos are usually elongate, they can be highly variable in their morphology. We believe that this is a late stage planula due to the thickened aboral end and well developed pharynx. (k) scattered expressing cells begin to reappear on the oral side of post-settlement polyps. (I, l*) micrographs of the oral (l) and aboral (l*) sides of a slightly older polyp. Expressing cells are present only on the oral (polyp) side. The out-of-focus purple spots seen in (l*) are showing through the partially cleared polyp from the oral side. (m, m*) Another oral (m)/aboral (m*) pair. Expressing cells are abundant on the tentacles of the polyp, but are absent aborally (m*).

3.6. Comparison of the anti-RFamide antibodies and of the antibody and in situ results

The anti-RFamide and two anti-FMRFamide antibodies tested all stained a basically similar pattern of elongate cells extending across the ectoderm with a centrally located nucleus and with projections along the mesoglea. However, there were a few differences between the results obtained with the three antibodies. Firstly, the Grimmelikhuijzen antibody appeared to stain significantly fewer cells than either of the FMRFamide antibodies. Secondly, the Peninsula antibody in general gave a cleaner staining pattern, while the Immunostar antibody stained numerous small cytoplasmic bodies in addition to the staining pattern shown by the other two (not shown).

The antibody staining and in situ hybridization results are inconsistent in several ways. Firstly, there is strong FMRFamide immunoreactivity in areas where transcript levels are low or undetectable; for example, at the aboral end of the planula (compare Fig. 2h,i and Fig. 5a). Secondly, in the polyp there are FMRFamide immunoreactive cells in tissue that is in the process of becoming the calcicoblast layer (not shown), where transcripts are not detected (Fig. 2m). Finally, a double in situ hybridization/antibody preparation shows that FMRFamide immunoreactive cells are apparently distinct from those expressing AmRFamide transcripts (Fig. 5f).

3.7. FMRFamide immunoreactivity in the adult polyp

Although, for technical reasons, we could not detect transcripts in adult material, we have detected considerable FMRFamide immunoreactivity, as shown in Fig. 6. Fig. 6a shows 2 polyps connected by coenosarc with abundant immunoreactivity on the tentacles and oral discs and with scattered immunoreactive cells between the polyps. Both polyps have a strongly immunoreactive neural ring around the mouth, with that of the lower polyp shown at higher magnification in Fig. 6b. Fig. 6c shows immunoreactive fibers in the region of the pharynx. Part of the nerve net connecting the two polyps is shown in Fig. 6d. Another densely innervated structure is the mesenterial filament shown in Fig. 6e, which has immunoreactive sensory cells on one side and a large bundle of immunoreactive neurons on the other.

4. Discussion

To the best of our knowledge this paper marks the first description of the development of a coral nervous system using neuron specific
labels. The aboral epidermis of several corals was hypothesized to contain a “nerve cell layer” by workers in the late 19th and early 20th century (summarized in Harrison and Wallace, 1990), but this was not confirmed by the electron microscope study of Vandermeulen (1974) on planulae of Pocillopora damicornis. It is difficult to relate our findings to these histological descriptions and there are probably differences between coral species, so our findings may not be generalisable beyond the genus Acropora. Some morphological and developmental features of the A. millepora nervous system are shared with other cnidarian nervous systems, but others are unique, as discussed below.

The Acropora genome codes for surprisingly few neuropeptides in comparison to other cnidarians such as Hydra (reviewed in Takahashi and Takeda, 2015). We have found only single transcripts encoding the RFamide and LWamide precursor (Fig. 1) plus one putative RPamide. The annotation of the genome of Acropora digitifera (Shinzato et al.,...
describes a second RFamide transcript. However, it is a highly repetitive sequence and we find no evidence for such a transcript in *A. millepora*.

### 4.1. RFamide and LWamide expression patterns

The distribution of cells expressing AmRFamide has a definite oral bias from the time of their first appearance, and the absence of expressing cells from the aboral end at metamorphosis is striking. The number of expressing cells per unit area and the apparent intensity of expression both decrease after the mid-planula stage. RFamide expression, as detected with the antibodies, differs considerably from this pattern in several ways. Firstly, expressing cells appear to be both more abundant per unit area and more intensely expressing at the aboral end, including in areas where transcripts are not apparent, as indicated in Fig. 5a. This aboral immunoreactivity is still apparent in the future calicoblast layer post settlement at a time when no transcripts are detectable by *in situ* hybridization.

Dual *in situ* hybridization/antibody preparations such as that shown in Fig. 5f, provide no indication of co-expression of transcript and protein, although we cannot rule it out. There are two important considerations in evaluating the antibody results. Firstly, the antibodies may only recognize peptides once they are processed and amidated (Plickert et al., 2004), by which time transcripts may not be present, and secondly the stability of the peptides is not known.

*AmRFamide*-expressing cells are abundant in the oldest post-
settlement polyps studied (e.g., Fig. 2m) but we have been unable to detect the transcript in adult polyps in spite of repeated attempts. This is presumably due to technical problems since the transcriptome results of Moya et al. (2012) indicate the presence of AmRFamide transcripts at this stage. However, staining with an anti-FMRFamide antibody reveals extensive innervation of the oral disc, with a neural ring surrounding the mouth, such as has been reported in several other cnidarians, and is assumed to reflect the overall organisation of the nervous system. In addition there are immunoreactive cells between polyps.

In contrast to AmRFamide transcripts, which show a clear oral bias in early planulae, AmLWamide-expressing cells tend to be concentrated in a central belt around the planula. Comparison of planulae at the same developmental stage and double in situ of partially settled planulae revealed that AmRFamide transcripts both disappear and reappear before those of AmLWamide. For example, by comparing the planula shown in Fig. 4d, where AmLWamide is undetectable in the metamorphosed oral end, with the only slightly older specimen in Fig. 4e, it is apparent that AmRFamide-expressing cells are already abundant in the portion of the latter that has metamorphosed, while no AmLWamide-expressing cells are present in the comparable portion of Fig. 4d. As was the case for AmRFamide, AmLWamide-expressing cells were abundant a few days post-settlement, but could not be detected in the adult: the transcriptome results of Moya et al. (2012), however, indicate that they are present.

4.2. Comparison to Nematostella and other cnidarians

Nematostella vectensis is commonly viewed as a representative cnidarian and it has the best characterized cnidian nervous system, at least at the molecular level (reviewed by Rentzach et al., 2017). However, while Nematostella is a good model for studying some aspects of anthozoan development, it is less suitable for others. For example, genes involved in controlling aspects of early development in Acropora are relatively similar in their expression patterns to their orthologs in Nematostella (Hayward et al., 2015), but there are major differences in development of the nervous systems of the two species, as discussed below.

The first difference relates to the lack of a dramatic metamorphosis during the development of Nematostella, which rather than undergoing the type of dramatic morphological transition seen in A. millepora, changes gradually from planula to polyp as the tentacles develop and the developing polyp spends more time on the bottom (Hand and Uhlinger, 1992). Both species have a period when settled individuals can move if the site originally chosen seems unsuitable. However, this period is relatively short in Acropora but apparently indefinite in Nematostella. Thus, although many papers have been published on aspects of development of the Nematostella nervous system, and several studies have investigated LWamide- and RFamide-expressing neurons (e.g., Nakanishi et al., 2012; Watanabe et al., 2014; Nakanishi and Martindale, 2018), none has mentioned a disappearance and reappearance of neuropeptide expression comparable to that described here for Acropora. In contrast, and in spite of the fact that the hydrozoan Hydrocynthia is in a different cnidian class, the disappearance and reappearance of RFamide and LWamide at the time of metamorphosis, as described by Plickert et al. (2004) is remarkably similar to what we see in Acropora, with RFamide disappearing and reappearing first. Katsukura et al. (2003) established that RFamides act antagonistically to LWamides and “downstream of LWamide release, presumably directly on target cells mediating metamorphosis”. This then would be consistent with what we see in Acropora, with RFamide disappearing first, allowing LWamide to promote metamorphosis and then reappearing to act antagonistically to the action of LWamide.

Secondly, A. millepora appears to lack an endodermal nervous system, at least as judged by the two neuropeptides characterized here. This is in contrast to Nematostella, where the presence of an endodermal nervous system is well documented, as detailed below, but is not surprising when one examines the anatomy of the lecithotrophic A. millepora planula, where the endoderm consists mainly of large, lipid-filled cells, with only a thin layer of smaller morphologically diverse cells directly below the mesoglea. RFamide itself is probably not an adequate indicator for the presence/absence of an endodermal nervous system, as cells expressing it in other species are found mainly in the ectoderm. However, in spite of the apparent absence from the endoderm of NvRFamide transcripts, as revealed by in situ hybridization, Marlow et al. (2009) reported FMRFamide-immunoreactive neurons there, in contrast to the situation in Acropora where there was no sign of endodermal FMRFamide immunoreactivity. The situation is quite different with respect to LWamide, where both Watanabe et al. (2014) and Nakanishi and Martindale (2018) report the presence of endodermal LWamide expression from mid-planula.

A third difference is that we see no evidence for so called “ganglion cells”, with cell bodies on the mesoglea and lateral projections, but none to the surface of the animal, such as have been documented in Hydra and some other cnidarians including Nematostella. The two types of peptidergic cells reported here do have distinct morphologies, with the cell bodies of AmLWamide-containing cells located on the mesoglea while the cell bodies of the AmRFamide-containing cells are located midway across the ectoderm, but we failed to identify any additional cell types.

4.3. How complete a picture of the nervous system do we have from the work presented here?

In this and all previous studies where RFamide in situ and anti-FMFamide staining have been compared there have been discrepancies, so how complete a picture of the development of the Acropora nervous system do we have from the work reported here and how representative of the rest of the nervous system is the behavior of the AmRFamide- and AmLWamide-expressing neurons? An approach taken to this problem in some previous studies has been to compare FMRFamide immunoreactivity to that seen with anti-tubulin. In Aglantha Mackie et al. (2003) found that FMRFamide-expressing neurons constituted only a small portion of larger nerve bundles. Garm et al. (2007) used a similar approach with anti-detyrosinated tubulin and anti-FMRFamide to demonstrate that there was only a small population of FMRFamide-expressing neurons in a much larger nerve bundle. A similar result was obtained by Watanabe et al. (2014) who found, using an NvElav1, neuron-specific transgenic reporter line, that only 10% of neurons in the late planula expressed either RFamide or LWamide. Nevertheless, in the first two cases, FMRFamide immunoreactivity reflected the position, but not the extent, of innervation. In an attempt to establish the situation in Acropora we tested a number of anti-tubulin antibodies against both the tyrosinated and untyrosinated forms of tubulin, with equivocal results in that none of the resulting staining was definitely neural.

4.4. Topics for future investigation

In addition to the unexplained relative distribution of transcripts and peptides, there are physiological/behavioral results where the role of the nervous system remains to be clarified. This is particularly so in the light of the recent findings of Nakanishi and Martindale (2018) in Nematostella where the NvGLWamide gene was knocked out using CRISPR. Lack of this gene delayed, but did not prevent, metamorphosis and this delay could be overcome by addition of external peptide. This finding raises multiple questions for future investigation. Firstly, in Nematostella, where metamorphosis is gradual, is there a time when LWamide disappears and reappears as it does in Hydractinia and Acropora? Secondly, is there a role for RFamide in this process and if so, what is it? Before the publication of the Nakanishi and Martindale (2018) paper the striking parallels between Hydractinia and Acropora...
in terms of peptide disappearance, metamorphosis, and peptide reappearance suggested to us that similar processes and interactions were occurring in Acropora. However, the Nematostella findings may suggest otherwise. An assumption of similarity between Hydractinia and Acropora has some logical merit given their similarly dramatic metamorphosis processes, compared with the gradual process in Nematostella.

However, Nematostella and Acropora are more closely phylogenetically related than either is to Hydractinia, so on that basis one might expect Nematostella and Acropora to be more similar physiologically. Possibly supporting this conclusion is our unpublished finding that GPPGLW-NH₂, which is one of the peptides coded for by the Acropora transcript, provides only a slight increase in the speed of settlement and metamorphosis processes, compared with the gradual process in Nematostella.

Finding raises two further questions. Firstly, what is the receptor that binds the "foreign" peptide (Hym-248) and, secondly, are some of the other peptides produced from the Acropora transcript more effective than GPPGLW-NH₂ in producing metamorphosis? As shown in Table 1, multiple peptides sharing the sequence GLW-NH₂ have been tested, yet Hym-248 appears to be uniquely effective among a number of related sequences in producing a dramatic effect in multiple Acropora species. So, there is still a great deal to be learned about neurotransmitters and the role of the nervous system in the settlement and metamorphosis of Acropora and other corals.

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Competing interests

No competing interests declared.

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