Gonadotropic activity of a second relaxin-type peptide in starfish

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Abbreviations: 1-MeAde, 1-methyladenine; ASW, artificial seawater; CD, circular dichroism; f_H, a-helix content; FOR, Forcipulatida; GPCR, G protein-coupled receptor; GSS, gonad-stimulating substance; PAX, Paxillosida; RGP, relaxin-like gonad-stimulating peptide; RLP2, relaxin-like peptide 2; SRA, Sequence Read Archive; VAL, Valvatida.

Abstract

In starfish, a relaxin-like gonad-stimulating peptide (RGP) acts as a gonadotropin that triggers gamete maturation and spawning. In common with other relaxin/insulin superfamily peptides, RGP consists of an A- and a B-chain, with cross-linkages mediated by one intra- and two inter-chain disulfide bonds. In this study, a second relaxin-like peptide (RLP2) was identified in starfish species belonging to the orders Valvatida, Paxillosida, and Forcipulatida. Like RGP, RLP2 precursors comprise a signal peptide and a C-peptide in addition to the A- and B-chains. However, a unique cysteine motif [CC-(3X)-C-(10X)-C] is present in the A-chain of RLP2, which contrasts with the cysteine motif in other members of the relaxin/insulin superfamily [CC-(3X)-C-(8X)-C]. Importantly, *in vitro* pharmacological tests revealed that *Patiria pectinifera* RLP2 (Ppe-RLP2) and *Asterias rubens* RLP2 (Aru-RLP2) trigger shedding of mature eggs from ovaries of *P. pectinifera* and *A. rubens*, respectively. Furthermore, the potencies of Ppe-RLP2 and Aru-RLP2 as gonadotropic peptides were similar to those of Ppe-RGP and Aru-RGP, respectively, and the effect of RLP2 exhibited partial species-specificity. These findings indicate that two relaxin-type peptides regulate spawning in starfish and therefore we propose that RGP and RLP2 are renamed RGP1 and RGP2, respectively.

Keywords: Gonadotropin; Relaxin-like gonad-stimulating peptide; Relaxin-like peptide; Spawning-inducing activity; Starfish

1. Introduction

The functional properties of a gonadotropic peptide in starfish known as gonad-stimulating substance (GSS) have been known since the 1960s (Kanatani and Shirai, 1967,1969; Mita, 2019). However, it was not until 2009 that GSS was purified from the radial nerve cords of the blue bat star *Patiria pectinifera* and identified as a heterodimeric peptide, comprising A- and B-chains with cross-linkages mediated by one intra-chain and two inter-chain disulfide bonds (Mita et al., 2009). The A-chain contains a cysteine motif [CC-(3X)-C-(8X)-C], which is a signature sequence of the relaxin/insulin/insulin-like growth factor (IGF) superfamily. More specifically, phylogenetic sequence analysis incorporating precursor sequences from vertebrates and protostome invertebrates revealed that the starfish peptide is a member of the relaxin-type peptide family (Mita et al., 2009, 2015; Lin et al., 2017). Hence, GSS was renamed relaxin-like gonad-stimulating peptide (RGP) (Mita, 2016, 2019, 2023). Furthermore, *P. pectinifera* RGP (Ppe-RGP) was the first gonadotropic hormone that triggers final gamete maturation and ovulation to be identified in an invertebrate. The Ppe-RGP precursor comprises not only the A- and B-chains, but also an N-terminal signal peptide and the C-peptide, which is located between the A- and B-chains. Thus, mature Ppe-RGP is produced from its precursor protein by elimination of the signal peptide and C-peptide and formation of intra-chain and inter-chain disulfide bridges within and between the A- and B-chains. Orthologs of PpeRGP have also been identified in other starfish, including species belonging to the orders Valvatida, Forcipulatida, Paxillosida, Platyasteroidea, Spinulosida, and Valatida (Mita, 2013, 2016; Semmens et al., 2016; Lin et al., 2017; Smith et al., 2017; Mita et al., 2015, 2020a, 2022; Katayama and Mita, 2023; Mita, 2023).

Recently, the precursor of a second relaxin-like peptide (RLP2) was identified in the common European starfish *Asterias rubens* (Aru-RLP2) by analysis of neural transcriptome sequence data (Semmens et al., 2016). Furthermore, analysis of the Sequence Read Archive

(SRA) has revealed the occurrence of precursors of Aru-RLP2-like peptides in other starfish species (Veenstra, 2021; Mita 2023). It was also reported that RGP/RLP2-type precursors are closely related to IGF-type precursors (Veenstra, 2021), but this interpretation of an evolutionary relationship was influenced by omission of vertebrate and protostome relaxin-type precursors from the phylogenetic analysis performed. ~~However,~~ Currently, nothing is known about the physiological roles of RLP2 in starfish and, in particular, if it has gonadotropic activity like RGP. Here, we have analysed and compared the sequences of RLP2 precursors in several starfish species, including *A. rubens*, *P. pectinifera*, *Patiria miniata*, *Certonardoa semiregularis*, *Acanthaster* cf. *solaris*, *Astropecten scoparius*, *Astropecten duplicatus*, *Aphelasterias japonica*, and *Marthasterias glacialis*. Furthermore, we have synthesized Aru-RLP2 and *P. pectinifera* RLP2 (Ppe-RLP2) and investigated if these peptides induce oocyte maturation and spawning of ovaries.

2. Materials and methods

2.1. Animals

No permit is required for collecting starfish *P. pectinifera* in Japan and there are no laws either allowing or forbidding collection of adult *P. pectinifera*. Adult specimens of *P. pectinifera* were collected from Yokosuka (Kanagawa Prefecture, Japan), Ushimado (Okayama Prefecture, Japan) and Asamushi (Aomori Prefecture, Japan) during the reproductive season. Adult specimens of *A. rubens* were obtained during the reproductive season from a fisherman based at Whitstable (Kent, UK).

2.2. Reagents

1-Methyladenine (1-MeAde) was purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Seawater used for spawning assays was modified van't Hoff's

97 artificial seawater (ASW) adjusted to pH 8.2 with 20 mM borate buffer (Kanatani and Shirai,
98 1970).
99
100 *2.3. Identification of orthologs of Aru-RLP2 in other starfish species*
101 Orthologs of Aru-RLP2 were identified by BLAST analysis against Trinity
102 (https://github.com/trinityrnaseq) assembled contig sequences with transcriptome sequence
103 data from *P. pectinifera* (SRA: SRR8627925), *P. miniata* (SRR2454338), *C. semiregularis*
104 (SRR16157127), *A.* cf. *solaris* (SRR8613694), *A. scoparius* (SRR12736189), *A. duplicatus*
105 (SRR28443238), *A. japonica* (SRR16157129), and *M. glacialis* (ERR6436374) using Aru-
106 RLP2 (GenBank: KT601729) as a query.
107 The ExPASy translate tool (http://web.expasy.org/translate/) was used to determine the
108 RLP2 precursor protein sequence in *P. pectinifera* (Ppe-RLP2), *P. miniata* (Pmi-RLP2), *C.*
109 *semiregularis* (Cse-RLP2), *A.* cf. *solaris* (Aso-RGP2), *A. scoparius* (Asc-RLP2), *A.*
110 *duplicatus* (Adu-RLP2), *A. japonica* (Aja-RLP2), and *M. glacialis* (Mgl-RLP2) and SignalP
111 6.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide.
112
113 *2.4. Phylogenetic analysis of RLP2 precursors*
114 Phylogenetic analysis of the relationships of *P. pectinifera*, *P. miniata*, *C. semiregularis*,
115 *A.* cf. *solaris*, *A. scoparius*, *A. duplicatus*, *A. rubens*, *A. japonica*, and *M. glacialis* RLP2
116 precursor proteins was performed using the function "build" of ETE3 3.1.2 (Huerta-Cepas et
117 al., 2016) as implemented on the GenomeNet (Https://www.genome.jp/tools/ete/). The RGP
118 precursor of *P. pectinifera* (Ppe-RGP) was used as the outgroup. A tree was constructed using
119 fasttree with slow NNI and MLACC=3 (to make the maximum-likelihood NNIs more
120 exhaustive) (Price et al., 2010) and default settings.
121

2.5. RGP and RLP2 synthesis

Aru-RGP, Aru-RLP2, Ppe-RGP, and Ppe-RLP2 were synthesized in accordance with the method for synthesizing insulin-like peptides as described previously (Katayama and Mita, 2016; Katayama et al., 2019; Mita et al., 2019). In brief, A- and B-chains were prepared by the ordinary 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis. Three disulfide bonds were regioselectively formed by dimethyl sulfoxide (DMSO) oxidation, S-pyridylsulfenyl-directed thiolysis and iodine oxidation reactions. MALDI-TOF mass spectra were recorded using an Autoflex spectrometer (Brucker). Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl solution at 150°C for 2 h in a vacuum-sealed tube. The purity of the synthesized peptides was over 90%.

2.6. Analysis of the structures of Ppe-RGP, Ppe-RLP2, Aru-RGP, and Aru-RLP2

Circular dichroism (CD) spectra of synthetic Ppe-RGP, Ppe-RLP2, Aru-RGP, and Aru-RLP2 at a concentration of 35.1 μM were measured using a Jasco J-820 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature with a 2-mm path length cell using phosphate buffer (50 mM, pH 7.0) as a solvent. The α-helical content (f_H) of each synthetic peptide was calculated by determining molar ellipticity ([θ]) at 222 nm using the following formula: $f_H = -([\theta]_{222} + 2340)/30300$ (Chen and Yang, 1971; Chen et al., 1972).

Three-dimensional (3D) structure models of synthetic Ppe-RGP, Ppe-RLP2, Aru-RGP, and Aru-RLP2 were also predicted using AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk) with default settings. The structural template consisting of sequences combined with the signal peptide to B-chain and the C-peptide to A-chain in RGP was automatically set by the software to the solution structure of human insulin or IGF (PDB code, 2GF1).

147

148 *2.7. Effect of synthetic Ppe-RGP, Ppe-RLP2, Aru-RGP, and Aru-RLP2 on ovarian fragments*

149 To assay RLP2-type and RGP-type peptides for gonadotropic activity, sexually matured
150 females with a constant sensitivity to hormonal treatments were inspected for their sensitivity
151 to 1-MeAde treatment using isolated ovarian fragments. Individuals whose ovaries exhibited
152 spawning with 1-MeAde at concentrations of 0.1 µM or less were selected. The ovaries were
153 excised from specimens of *P. pectinifera* and *A. rubens* and cut into small fragments
154 (approximately 5 mm long) containing only a few lobes using scissors. Then gonadotropic
155 assays were carried out as described previously by Shirai (1986). Briefly, serial dilutions of
156 synthetic Ppe-RGP, Ppe-RLP2, Aru-RGP, or Aru-RLP2 at concentrations ranging from 0.31
157 nM to 20 nM were prepared in ASW and were aliquoted at volumes of 200 µl into wells of an
158 80-well plate (Agglutination tray MODEL T-2, TOMY, Tokyo, Japan). The ovarian
159 fragments were then added to each well of the plate and after 1 h incubation at 17–20°C, the
160 samples were examined to determine whether or not spawning had occurred, but without the
161 observer being blind to the treatment. The following scoring system, as described previously
162 (Shirai, 1986), was used: (+++) spawning occurred and most of the oocytes were ~~had~~
163 matured; (++) about 50% of the oocytes were ~~had~~ matured, (+) a few oocytes were ~~had~~
164 matured; and (−)~~:~~ no spawning had occurred. The scores were converted to numerical values
165 (+++ = 100; ++ = 67; + = 33; − = 0) so that the median effective concentration (EC_{50}) could
166 be determined graphically in a semi-quantitative manner. Means ± standard error of mean
167 (SEM) were determined from three or four separate assays using ovaries from three or four
168 different animals.

169

170 **3. Results and Discussion**

171 Analysis of transcriptome sequence data enabled identification of cDNAs encoding

orthologs of the Aru-RLP2 precursor in *P. pectinifera, P. miniata, C. semiregularis, A.* cf. *solaris, A. scoparius, A. duplicatus, A. japonica,* and *M. glacialis* (Fig. S1). The translated RLP2 precursor sequences comprise an N-terminal signal peptide, followed by the B-chain and C-peptide, with the A-chain at the C-terminus (Fig. 1A). Two proteolytic sites (Lys-Arg) are located between the B-chain and the C-peptide and between the C-peptide and the A-chain. The positions of two cysteine residues in the B-chain and four cysteine residues in the A-chain were consistent among the nine RLP2 precursors. These findings indicate that RLP2, similar to RGP, is derived from its precursor protein by removing the signal peptide and C-peptides after two disulfide bonds are formed between the A- and B-chains and one intra-chain disulfide bond is formed within the A-chain (Fig. 1B).

A phylogenetic tree was constructed using the RLP2 precursor sequence from each of the starfish species analysed (Fig. 1C), revealing that i). Ppe-RLP2, Pmi-RLP2, Aso-RLP2, and Cse-RLP2 of the order Valvatida, ii). Asc-RLP and Adu-RLP2 of the order Paxillosida, and iii). Aru-RLP2, Aja-RLP2 and Mgl-RLP2 of the order Forcipulatida are positioned in three distinct branches of the tree (Fig. 1C). Furthermore, the topology of the tree shows that RLP2 precursors in the orders Paxillosida and Valvatida are more closely related to each other than to RLP2 precursors in the order Forcipulatida, which is consistent with starfish phylogeny (Linchangoco et al., 2017; Musacchia et al., 2017; Mita et al., 2020a). Furthermore, these findings indicate that RLP2 may occur as a paralog of RGP throughout the class Asteroidea. However, it remains to be determined when the gene duplication that gave rise to RGP and RLP2 occurred during evolution.

Although the cysteine residue at the C-terminus of the A-chain of RLN2 is consistent with that of RGP, RLP2 does not contain the cysteine motif [CC-(3X)-C-(8X)-C] in the A-chain that is a characteristic feature of the relaxin/insulin superfamily, including RGP (Fig. 1B). In contrast, RLP2 has a modified cysteine motif [CC-(3X)-C-(10X)-C] in the A-chain.

According to the 3D models of RLP2 and RGP generated here, the tertiary structures of the A- and B-chains of Ppe-RLP2 and Aru-RLP2 are very similar to those of Ppe-RGP and Aru-RGP (Fig. 2B). Previous studies have shown that the B-chain is important for RGP binding to its receptor(s) (Mita et al., 2019, 2020b, 2020c). If the A-chain in RGP and RLP2 plays the role of retaining the three-dimensional structure of the B-chain, it may not be important that the cysteine motif of [CC-(3X)-C-(8X)-C] in RGP is replaced with [CC-(3X)-C-(10X)-C] in RLP2. Furthermore, CD spectra also revealed that the three-dimensional structures of Ppe-RLP2 and Aru-RLP2 are similar to those of Ppe-RGP and Aru-RGP (Fig. 2C). The CD spectra of Ppe-RLP2 and Aru-RLP2 were slightly different from those of Ppe-RGP and Aru-RGP between 190 and 200 nm, but they were very similar at wavelengths above 200 nm. The percentage of α-helices were also similar in RGP and RLP2, ranging from 30.0% in Ppe-RGP, to 28.7% in Ppe-RLP2, to 25.5% in Aru-RGP and to 20.0% in Aru-RLP2 (Fig. 2C).

Investigation of the effects of RLP2 revealed that mature eggs were discharged by ovarian fragments from *P. pectinifera* or *A. rubens* about 30 min after administration of Ppe-RLP2 or Aru-RLP2, respectively (Fig. 2D). This indicates that not only RGP but also RLP2 acts as a gonadotropic peptide in starfish. In the case of *P. pectinifera* ovaries, the peptide concentration required for 50% spawning-inducing activity (EC_{50}) was 0.89 ± 0.10 nM for Ppe-RGP and 0.93 ± 0.10 nM for Ppe-RLP2 (Table 1). Thus, the EC_{50} values were almost the same for PpeRGP and PpeRLP2. In contrast, EC_{50} values in *A. rubens* ovaries were 2.0 ± 0.5 nM for Aru-RGP and 4.8 ± 1.2 nM for Aru-RLP2. However, as these EC_{50} values were calculated using a semi-quantitative method, this two-fold difference in EC_{50} values may not reflect significant differences physiologically. Although Aru-RGP and Aru-RLP2 did not induce spawning in *P. pectinifera* ovaries, Ppe-RGP ~~could~~ did stimulate spawning in *A. rubens* ovaries. Furthermore, the EC_{50} of Ppe-RGP was 22 ± 3 nM, approximately 10 times higher than that of Aru-RGP, which although determined semi-quantitatively likely reflects a

222 significant difference in potency.

223 It has been demonstrated that RGP (GSS) stimulates the target cells, ovarian follicle cells,
224 to produce the maturation-inducing hormone (MIH), 1-MeAde (Hirai and Kanatani, 1971;
225 Hirai et al., 1973; Mita et al., 2009). RGP acts on its receptors on the surface of follicle cells
226 and induces an increase in intracellular cyclic AMP level through activation of G-proteins and
227 adenylyl cyclase (Mita and Nagahama, 1991). This indicates that, consistent with the
228 occurrence of G-protein coupled relaxin receptors in vertebrates, the RGP receptor in *P.*
229 *pectinifera* is a G protein-coupled receptor (GPCR). Accordingly, a candidate GPCR for RGP
230 has been identified in *P. pectinifera* (Mita et al., 2020c). It has been demonstrated in
231 mammals that the B-chain of relaxin and related peptides plays an important role in binding to
232 the receptor (Bathgate et al., 2012, 2018; Patil et al., 2017). In contrast, neither RGP nor
233 RLP2 sequence possess the vertebrate 'relaxin-specific receptor-binding cassette' [R-(3X)-R-
234 (2X)-I/V], a distinct and well-conserved feature of the relaxin B-chains (Büllesbach et al.,
235 1998, 2000, 2005). A comparison of the amino acid sequences of the middle region of the B-
236 chains have previously shown that residues of the 'receptor binding cassette correspond to
237 D^{B6}, M^{B10}, and F^{B13} for Ppe-RGP and E^{B7}, M^{B11} and Y^{B14} for Aru-RGP (Mita et al., 2020b).
238 Similarly, it is possible based on our 3D-models that amino acid residues A^{B10}, D^{B14}, and L^{B17}
239 for Ppe-RLP2 and L^{B13}, Y^{B17}, and V^{B20} for Aru-RLP2 are involved in receptor binding (Fig.
240 2B). Furthermore, Ppe-RGP induced spawning in ovaries of *A. rubens* as well as *P.*
241 *pectinifera*. In contrast, Aru-RGP and Aru-RLP2 did not induce spawning in the ovaries of *P.*
242 *pectinifera*. This suggests that the effect of RLP2, like RGP (Chaet, 1966a, 1966b; Noumura
243 and Kanatani, 1963; Mita et al., 2020b), on gamete shedding is partially species-specific.

244 Recently, it was revealed that a GPCR identified in *P. pectinifera* responds to Ppe-RGP
245 but not to *A. amurensis* RGP (Aam-RGP, = Aru-RGP) (Mita et al., 2020c). It seems likely
246 that the species-specificity observed with RGP is due to the steric hindrance of the A-chain

247 amino acid residues in Aru-RGP when interacting with the Ppe-RGP receptor (Mita et al.,
248 2020b). In fact, Pro^{A17} of Ppe-RGP and Arg^{A18} of Aru-RGP are located near the B-chain (Fig.
249 2B). The Pro^{A17} of Ppe-RGP is consistent with that of Ppe-RLP2, whereas in Aru-RLP2 the
250 corresponding residue is Ile^{A17} (Figs. 2A and B). Proline is the secondary amine that forms
251 the pyrrolidine loop. In contrast, the side chains of arginine and isoleucine are [$(H_2N)(HN)$-
252 $CN-(H)(CH_2)_3$-] and [$CH_3-CH_2-(CH_3)CH$-], respectively, and these side chains are larger than
253 that of proline. Thus, the side chain of amino acids in the A-chain of Aru-RGP and Aru-RLP2
254 may impair binding to the Ppe-RGP receptor. On the contrary, Aru-RGP receptors, which
255 respond to Ppe-RGP as well as Aru-RGP, appear to be more tolerant to structural differences
256 in these peptides.
257 In this study, both Aru-RLP2 and Ppe-RLP2 were shown to induce oocyte maturation and
258 ovulation in the ovaries of *A. rubens* and *P. pectinifera*, respectively. This strongly suggests
259 that RLP2, like RGP, has a physiological role as a gonadotropic hormone in starfish.
260 Therefore, we propose that RGP is renamed as RGP1 and RLP2 is renamed as RGP2.
261 However, it remains to be determined whether RLP2 acts on the same cognate receptor as
262 RGP. Although it has been shown that the concentration of RGP is highest in the radial nerve
263 cords of starfish (Kanatani and Ohguri, 1966; Kanatani, 1985; Mita and Katayama, 2018), it
264 is also expressed in other parts of the body, including the arm tips, tube feet (Lin et al., 2017),
265 coelomocytes (Jönsson et al., 2022), digestive system and gonoducts (Feng et al., 2023).
266 Furthermore, because of their proximity to the gonads, it has been proposed that the
267 gonoducts may be the physiological source of RGP that triggers spawning in starfish, whilst
268 RGP expressed in other parts may be involved in regulation of other physiological/behavioral
269 processes (Feng et al., 2023). Currently, little is known about the expression pattern of RGP2
270 in starfish and therefore addressing this issue will be an important objective for future
271 research. Furthermore, it would be interesting to compare the expression and secretion

mechanisms of RGP1 and RGP2 during the breeding season. Further studies on RGP2 will surely provide important insights into the complex neurohormonal mechanisms that control reproduction in the class Asteroidea.

Author Contributions

All authors had full access to all the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design*: Masatoshi Mita, Maurice R. Elphick, and Hidekazu Katayama. *Acquisition of data*: Masatoshi Mita (identification and analysis of orthologs of Aru-RLP2 in other starfish and bioassay of RGP and RLP2), Yuling Feng (identification and analysis of orthologs of Aru-RLP2 in other starfish and bioassay of RGP and RLP2), Victor M. Piñon Gonzalez (bioassay of RGP and RLP2), and Hidekazu Katayama (synthesis and analysis of RGP and RLP2). *Analysis and interpretation of data*: Masatoshi Mita, Yuling Feng, and Hidekazu Katayama. *Drafting of the manuscript*: Masatoshi Mita, Maurice R. Elphick, and Hidekazu Katayama. *Study supervision*: Masatoshi Mita.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in this article.

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312 **Supplementary data:** Figure S1: Coding DNA sequences (CDS) of RLP2 precursors in
313 various species of starfish.
314

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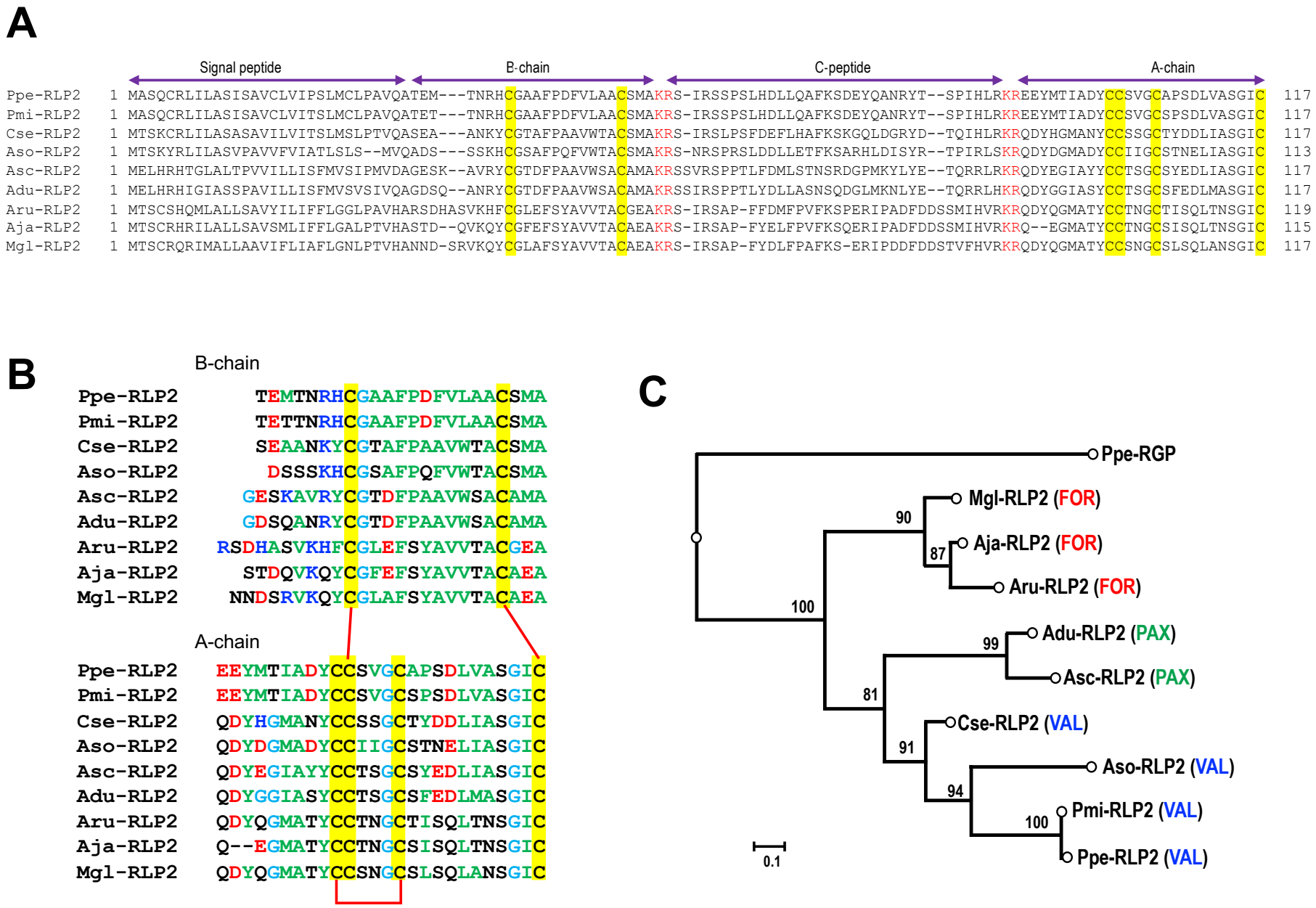
Figure captions

Fig. 1. Multiple sequence alignment and phylogenetic tree of relaxin-like peptide 2 (RLP2) in starfish species. (A) Alignment of the precursor sequences of RLP2 in *Patiria pectinifera* (Ppe-RLP2), *Patiria miniata* (Pmi-RLP2), *Certonardoa semiregularis* (Cse-RLP2), *Acanthaster* cf. *solaris* (Aso-RLP2), *Astropecten scoparius* (Asc-RLP2), *Astropecten duplicatus* (Adu-RLP2), *Asterias rubens* (Aru-RLP2), *Aphelasterias japonica* (Aja-RLP2), and *Marthasterias glacialis* (Mgl-RLP2). Characters shown in red indicate basic dipeptides (Lys, Arg) that are predicted sites of proteolytic cleavage. Cysteine residues are highlighted in yellow. The possible deduced cleavage sites of the signal peptide were predicted by SignalP 6.0. (B) A- and B-chain sequences of Ppe-RLP2, Pmi-RLP2, Cse-RLP2, Aso-RLP2, Asc-RLP2, Adu-RLP2, Aru-RLP2, Aja-RLP2, and Mgl-RLP2. Cysteine residues are highlighted in yellow, and disulfide bonds are shown with solid dark lines. To illustrate the conserved features, the amino acid types are color coded according to their properties, with basic residues in blue (Arg, Lys and His), acidic residues in red (Glu and Asp), hydrophobic residues in green (Ala, Val, Ile, Phe, Trp, Tyr, Pro and Met), hydrophilic in black (Ser, Thr, Asn and Gln), and glycine in light blue. (C) Phylogenetic tree showing relationships of RLP2 precursors from *P. pectinifera*, *P. miniata*, *C. semiregularis*, *A.* cf. *solaris*, *A. scoparius*, *A. duplicatus*, *A. rubens*, *A. japnoca*, and *M. glacialis*. The RGP precursor of *P. pectinifera* (Ppe-RGP) was used as the outgroup. Tree reconstruction was performed using the function "built" of ETE3 3.1.2 (Huerta-Cepas et al., 2016). The tree was constructed using fasttree with slow NNI and MLACC = 3 (to make the maximum-likelihood NNIs more exhaustive) (Price et al., 2010) and 1000 replicates for SH-aLRT. The number located beside each branch is the bootstrap score. Horizontal lines indicate evolutionary distance. Abbreviations in parentheses represent the taxonomic order of the species: FOR, Forcipulatida; PAX,

480 Paxillosida; and VAL, Valvatida.

481

482 **Fig. 2.** Comparison of the structural characteristics of relaxin-like gonad-stimulating peptide
483 (RGP) and relaxin-like peptide 2 (RLP2), and their gonadotropic activities in *Patiria*
484 *pectinifera* (Ppe-RGP and Ppe-RLP2) and *Asterias rubens* (Aru-RGP and Aru-RLP2). (A)
485 The heterodimeric structures of Ppe-RGP (a), Ppe-RLP2 (b), Aru-RGP (c) and AruRLP2 (d).
486 To illustrate the conserved features, the amino acid types are color coded according to their
487 properties, with basic residues in blue (Arg, Lys and His), acidic residues in red (Glu and
488 Asp), hydrophobic residues in green (Ala, Val, Ile, Phe, Trp, Tyr, Pro and Met), hydrophilic
489 residues in black (Ser, Thr, Asn and Gln), and glycine in light blue. The cysteine residues are
490 highlighted in yellow. Disulfide bridges are shown in black. (B) Three-dimensional (3D)
491 structure models of Ppe-RGP (a), Ppe-RLP2 (b), Aru-RGP (c) and AruRLP2 (d). The side
492 chains of selected amino acids in the B-chain shown and labelled are potentially involved in
493 binding to a receptor(s). Each 3D structure model was produced using the AlphaFold Protein
494 Structure Database (https://alphafold.ebi.ac.uk/). (C) Circular dichroism (CD) spectra of Ppe-
495 RGP (a), Ppe-RLP2 (b), Aru-RGP (c) and AruRLP2 (d). (D) Dose-dependent effects of Ppe-
496 RGP (a), Ppe-RLP2 (b), Aru-RGP (c) and AruRLP2 (d) in causing spawning of ovarian
497 fragment in either *P. pectinifera* or *A. rubens*. +++ denotes spawning occurred and most of
498 oocytes were matured, ++ denotes about 50% oocytes were matured, + denotes a few oocytes
499 were matured, and – denotes no spawning occurred. Symbols and bars represent the mean for
500 four separate assays using ovaries from four different animals and standard error of the mean
501 (SEM), respectively.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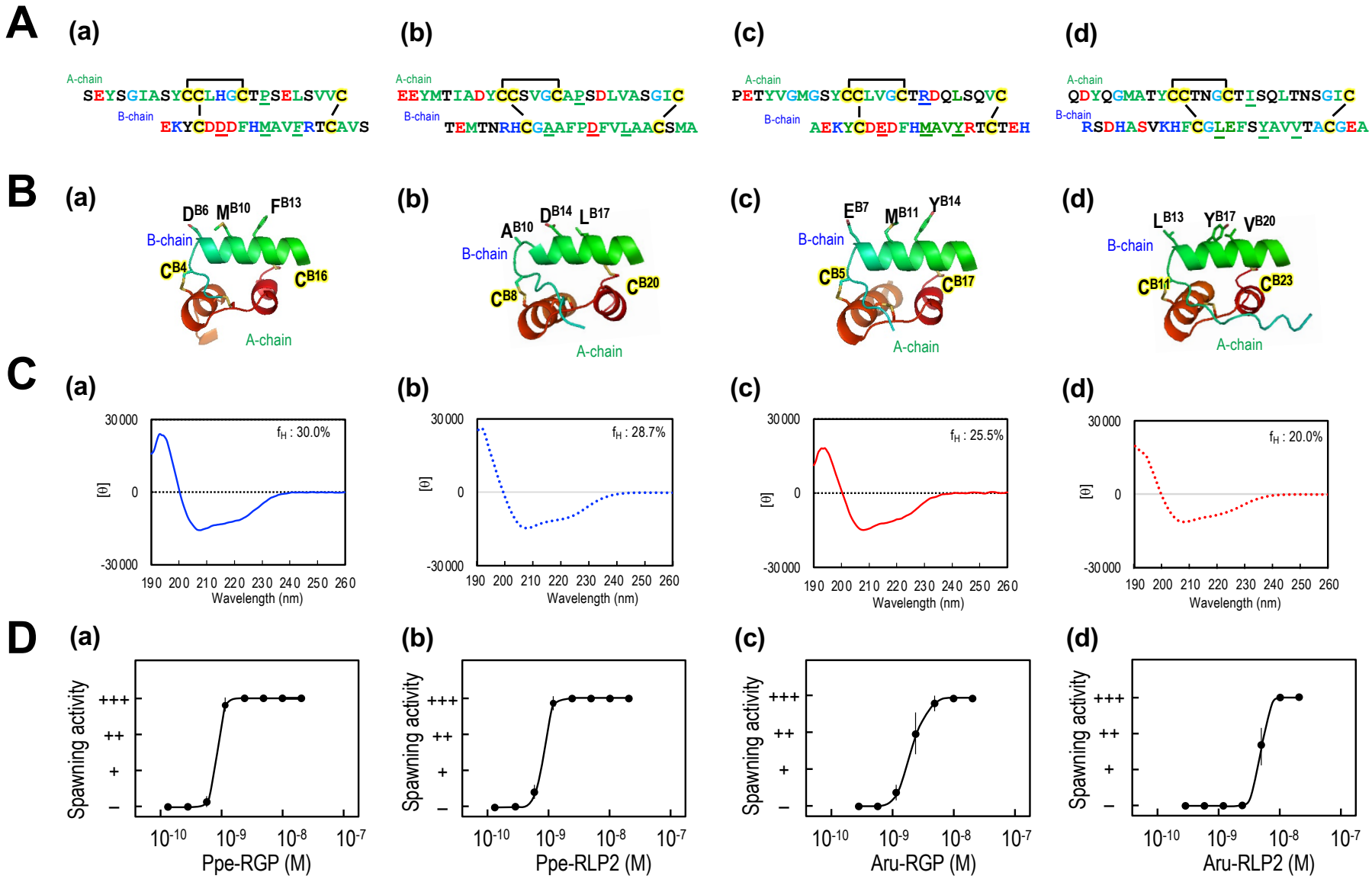
Signal peptide
B-chain
C-peptide
A-chain
Ppe-RLP2 1 MASQCRLILASISAVCLVIPSLMCLPAVQATEM---TNRHCGAAFPDFVLAACSMAKRS-IRSSPSLHDLLQAFKSDEYQANRYT--SPIHLRKREEYMTIADYCCSVGCAPSDLVASGIC 117
Pmi-RLP2 1 MASQCRLILASISAVCLVITSLMCLPAVQATET---TNRHCGAAFPDFVLAACSMAKRS-IRSSPSLHDLLQAFKSDEYQANRYT--SPIHLRKREEYMTIADYCCSVGCSPSDLVASGIC 117
Cse-RLP2 1 MTSKCRLILASASAVILVITSLMSLPTVQASEA---ANKYCGTAFPAAVWTACSMAKRS-IRSLPSFDEFLHAFKSKGQLDGRYD--TQIHLRKRQDYHGMANYCCSSGCTYDDLIASGIC 117
Aso-RLP2 1 MTSKYRLILASVPAVVFVIATLSLS--MVQADS---SSKHCGSAFPQFVWTACSMAKRS-NRSPRSLDDLLETFKSARHLDISYR--TPIRLSKRQDYDGMADYCCIIGCSTNELIASGIC 113
Asc-RLP2 1 MELHRHTGLALTPVVILLISFMVSIPMVDAGESK--AVRYCGTDFPAAVWSACAMAKRSSVRSPPTLFDMLSTNSRDGPMKYLYE--TQRRLRKRQDYEGIAYYCCTSGCSYEDLIASGIC 117
Adu-RLP2 1 MELHRHIGIASSPAVILLISFMVSVSIVQAGDSQ--ANRYCGTDFPAAVWSACAMAKRSSIRSPPTLYDLLASNSQDGLMKNLYE--TQRRLHKRQDYGGIASYCCTSGCSFEDLMASGIC 117
Aru-RLP2 1 MTSCSHQMLALLSAVYILIFFLGGLPAVHARSDHASVKHFCGLEFSYAVVTACGEAKRS-IRSAP-FFDMFPVFKSPERIPADFDDSSMIHVRKRQDYQGMATYCCTNGCTISQLTNSGIC 119
Aja-RLP2 1 MTSCRHRILALLSAVSMLIFFLGALPTVHASTD--QVKQYCGFEFSYAVVTACAEAKRS-IRSAP-FYELFPVFKSQERIPADFDDSSMIHVRKRQ--EGMATYCCTNGCSISQLTNSGIC 115
Mgl-RLP2 1 MTSCRQRIMALLAAVIFLIAFLGNLPTVHANND-SRVKQYCGLAFSYAVVTACAEAKRS-IRSAP-FYDLFPAFKS-ERIPDDFDDSTVFHVRKRQDYQGMATYCCSNGCSLSQLANSGIC 117
B
B-chain
Ppe-RLP2 TEMTNRHCGAAFPDFVLAACSMA
Pmi-RLP2 TETTNRHCGAAFPDFVLAACSMA
Cse-RLP2 SEAANKYCGTAFPAAVWTACSMA
Aso-RLP2 DSSSKHCGSAFPQFVWTACSMA
Asc-RLP2 GESKAVRYCGTDFPAAVWSACAMA
Adu-RLP2 GDSQANRYCGTDFPAAVWSACAMA
Aru-RLP2 RSDHASVKHFCGLEFSYAVVTACGEA
Aja-RLP2 STDQVKQYCGFEFSYAVVTACAEA
Mgl-RLP2 NNDSRVKQYCGLAFSYAVVTACAEA
A-chain
Ppe-RLP2 EEYMTIADYCCSVGCAPSDLVASGIC
Pmi-RLP2 EEYMTIADYCCSVGCSPSDLVASGIC
Cse-RLP2 QDYHGMANYCCSSGCTYDDLIASGIC
Aso-RLP2 QDYDGMADYCCIIGCSTNELIASGIC
Asc-RLP2 QDYEGIAYYCCTSGCSYEDLIASGIC
Adu-RLP2 QDYGGIASYCCTSGCSFEDLMASGIC
Aru-RLP2 QDYQGMATYCCTNGCTISQLTNSGIC
Aja-RLP2 Q--EGMATYCCTNGCSISQLTNSGIC
Mgl-RLP2 QDYQGMATYCCSNGCSLSQLANSGIC
C
Ppe-RGP
Mgl-RLP2 (FOR)
Aja-RLP2 (FOR)
Aru-RLP2 (FOR)
Adu-RLP2 (PAX)
Asc-RLP2 (PAX)
Cse-RLP2 (VAL)
Aso-RLP2 (VAL)
Pmi-RLP2 (VAL)
Ppe-RLP2 (VAL)
90
87
100
99
81
91
94
100
0.1

Figure 1.


A
(a)
A-chain
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B-chain
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(b)
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(c)
A-chain
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(d)
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B-chain
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B
(a)
DB6 MB10 FB13
B-chain
CB4
CB16
A-chain
(b)
AB10 DB14 LB17
B-chain
CB8
CB20
A-chain
(c)
EB7 MB11 YB14
B-chain
CB5
CB17
A-chain
(d)
LB13 YB17 VB20
B-chain
CB11
CB23
A-chain
C
(a)
30 000
0
-30 000
[θ]
fH : 30.0%
190 200 210 220 230 240 250 260
Wavelength (nm)
(b)
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0
-30 000
[θ]
fH : 28.7%
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Wavelength (nm)
(c)
30 000
0
-30 000
[θ]
fH : 25.5%
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Wavelength (nm)
(d)
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0
-30 000
[θ]
fH : 20.0%
190 200 210 220 230 240 250 260
Wavelength (nm)
D
(a)
Spawning activity
+++
++
+
–
10-10 10-9 10-8 10-7
Ppe-RGP (M)
(b)
Spawning activity
+++
++
+
–
10-10 10-9 10-8 10-7
Ppe-RLP2 (M)
(c)
Spawning activity
+++
++
+
–
10-10 10-9 10-8 10-7
Aru-RGP (M)
(d)
Spawning activity
+++
++
+
–
10-10 10-9 10-8 10-7
Aru-RLP2 (M)

Figure 2.