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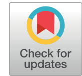
Japanese medaka as a model for studying the relaxin family genes involved in neuroendocrine regulation: Insights from the expression of fish-specific *rln3* and *insl5* and *rxfp3/4*-type receptor paralogues



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ABSTRACT

The goal of this paper is to establish Japanese medaka (*Oryzias latipes*) as a model for relaxin family peptide research, particularly for studying the functions of RLN3 and INSL5, hormones playing roles in neuroendocrine regulation. Medaka, like other teleosts, retained duplicate copies of rln3, insl5 and their rxfp3/4-type receptors following fish-specific whole genome duplication (WGD) and paralogous copies of these genes may have subfunctionalised providing an intuitive model for teasing apart the pleiotropic roles of the corresponding genes in mammals. To this end, we provide experimental evidence for the expression of the relaxin family genes in medaka that had previously only been identified *in-silico*, confirm the gene structure of five of the ligand genes, characterise gene expression across multiple tissues and during embryonic development, perform *in situ* hybridization with anti-sense *insl5a* on embryos and in adult brain and intestinal samples, and compare these results to the data available in zebrafish. We find broad similarities but also some differences in the expression of relaxin family genes in zebrafish versus medaka, and find support for the hypothesis that the *rln3a/rln3b* and *insl5a/insl5b* paralogues have been subfunctionalized. Given that medaka has a suite of relaxin family genes more similar to other teleosts, and has retained the gene for rxfp4 (which is lost in zebrafish), our results suggest that *O. latipes* may be a good model for delineating the ancestral function of the relaxin family genes involved in neuroendocrine regulation.

1. Introduction

Gene and genome duplications are important driving forces in evolution (Ohno, 1970), and both have contributed to the diversification of the relaxin peptide family and their receptors in vertebrates (Yegorov and Good, 2012). Two rounds (2R) of whole genome duplication (WGD) are now widely thought to have occurred early in vertebrate evolution and to have been instrumental in the evolution of vertebrate traits, while a third round (3R) of WGD occurred just prior to the origin of the teleostei (Fig. 1) and is thought to have contributed to the dramatic radiation of ray-finned fish (Ravi and Venkatesh, 2008; Kassahn et al., 2009; Canestro et al., 2013). Yegorov and Good (2012) demonstrated that the diversification of the relaxin family of genes was mostly driven by these WGD events (Fig. 2). They hypothesized that during 2R a singular pre-vertebrate *rln-/insl*-like gene duplicated into four relaxin family ligands (*rln*, *insl3*, *rln3* and *insl5*), which subsequently acquired distinct functions in the early vertebrate genome (i.e. neo-functionalization c.f. (Force et al., 1999)). During 3R, only the peptides *rln3* and *insl5* and several of their associated *rxfp3/4* type receptors retained duplicates, leading to a massive expansion and apparent sub-functionalization of the genes involved in neuroendocrine regulation in teleosts (Good et al., 2012; Yegorov and Good, 2012; Yegorov et al., 2014).

The duplicated genes that have sub-functionalised in fish genomes are increasingly used as a tool to tease apart the corresponding (non-duplicated) function of their orthologous genes in mammals (Ichimura et al., 2013; Seth et al., 2013). Both zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) have proven to be excellent models in which to employ both forward and reverse genetic approaches because of their high reproductive rate, extra-uterine development, rapid maturation,

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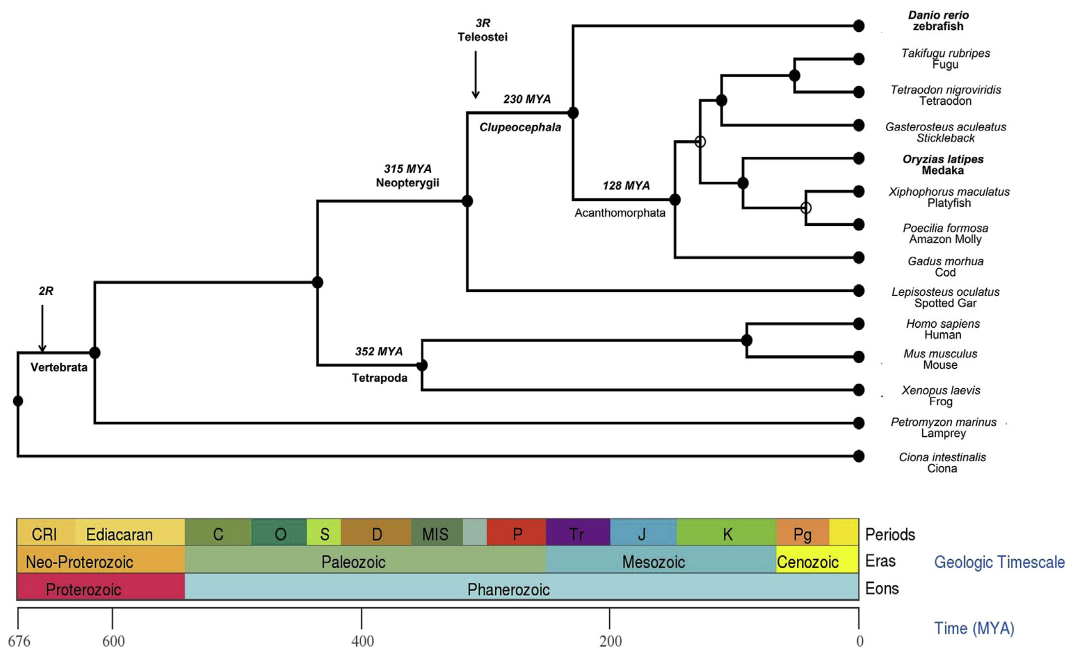

Fig. 1. Evolutionary history of vertebrate lineages relative to the geologic timescale (c.f. TimeTree.org). The approximate timing of 2R and 3R are indicated as well as the divergence dates of key vertebrate clades.

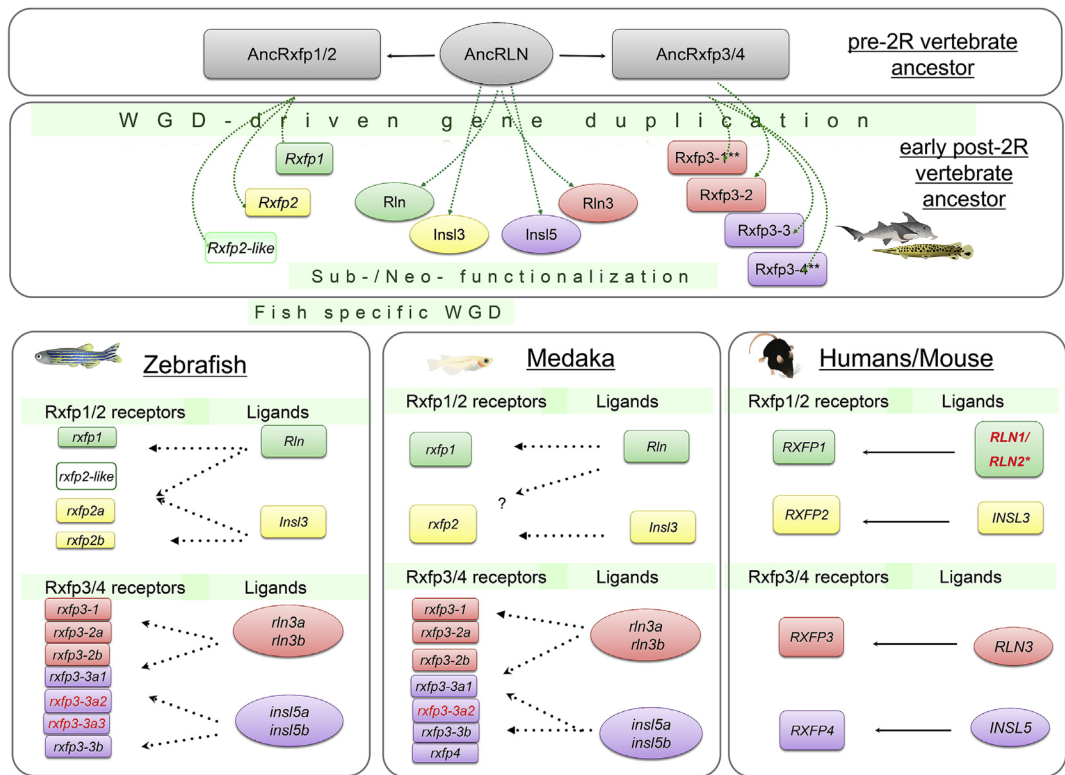

Fig. 2. Evolution of the relaxin family peptides and receptors in vertebrates (Adapted from Bathgate et al., 2018). A singular ancestral Rxfp1/2 like, Insl/Rln-like and Rxfp3/4-like genes present in the pre-2R vertebrate genome diversified into three Rxfp1/2, 4 Insl/Rln and 4 Rxfp3/4 genes in the post-2R genome. Teleosts harbour copies of most of these genes, and additionally retained post-3R duplicates of rln3 and insl5 as well as several of the rxfp3/4 type receptors through which they are thought to signal, while tetrapods, including human/mouse, lost many genes of these post-2R genes. All genes duplicated via whole genome duplication (WGD) events are given in black, while genes arising through small scale duplication (SSD) such as tandem duplications are shown in red. In addition to RLN1/RLN2 peptides in humans, there are two additional tandem duplications at the RLN locus in humans, INL4 and INSL6, which arose early during mammalian evolution but remain orphan ligands.

transparency of the eggs and embryos (enabling whole-mount *in situ* hybridization (ISH)), relatively low maintenance costs, as well as the tremendous genomic resource availability (Kirchmaier et al., 2015; Gut et al., 2017). Of particular relevance for the relaxin family, is the proven utility of medaka as a model for endocrine disorders in human especially metabolic diseases, including obesity, non-alcoholic fatty liver disease, and type 2 diabetes (Matsumoto et al., 2010; Ichimura et al., 2013 41; Seth et al., 2013; Gut et al., 2017; Fujisawa et al., 2018). Additionally, medaka is a good model for studying the effect of stress on behaviour, physiology and/or gene regulation since they are highly sensitive to environmental challenges, including novelty stress, exposure to predators, chemicals, and experimental other methods to induce stress.

The relaxin family ligands RLN3 and INSL5, and their receptors have been proposed to play roles in stress, satiety and metabolism. In both rat and mouse, the primary site of *RLN3* expression is the *nucleus incertus* (NI), as well as in the ventral and lateral periaqueductal gray (PAG) (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010); the teleost *RLN3* paralogs, *rln3a* and *rln3b*, are expressed in the homologous regions of the zebrafish brain (Donizetti et al., 2009). RXFP3, the receptor for RLN3, is expressed in areas of the brain involved in modulating stress and mood/anxiety via activation of corticotropin-releasing factor receptor-1 (CRF-1). Null mutation mouse models for *RLN3* and/or *RXFP3* showed evidence of reduced locomotion (Smith et al., 2009; Hosken et al., 2015), decreased anxiety (Watanabe et al., 2011; Hosken et al., 2015), decreased body weight (Sutton et al., 2009), and reduced sucrose consumption (Walker et al., 2015), while activation of RXFP3 has been found to reduce anxiety (Nakazawa et al., 2013; Ryan et al., 2013a; Zhang et al., 2015), modulate alcohol seeking behaviour (Ryan et al., 2013b), and leads to increased feeding and body weight, especially in females (McGowan et al. 2005, 2006; Sutton et al., 2009; Calvez et al. 2016, 2017). As a result of these studies, there is interest in the possible application of RLN3/RXFP3 signalling to neuropsychiatric disorders in humans (Kumar et al., 2017), and an *RLN3* polymorphism has been associated with obesity, hyper-cholesterolaemia and diabetes (Munro et al., 2012).

Although less research is available on the peptide INSL5 and its cognate receptor, RXFP4, in mammals, there is evidence that the signalling pair plays a role in metabolism, especially glucose metabolism and satiety. Unlike RLN3, the primary site of expression of INSL5 in mammals is the gut, with secondary expression in the hypothalamus (Conklin et al., 1999; Grosse et al., 2014). It is highly expressed in enteroendocrine cells (EECs) (L-cells) of the distal mammalian colon where it is hypothesized to affect behaviour and appetite by signalling via RXFP4 expression in EEC's as well as ganglia of the vagus nerve that innervate the gut (Burnicka-Turek et al., 2012; Grosse et al., 2014; Luo et al., 2015). In mice, Grosse and colleagues demonstrated that *Insl5* expression increased during chronic fasting, and proposed that it is an orexigenic hormone (Grosse et al., 2014), although other research argues that INSL5 affects energy balance by having both direct and indirect insulinotropic effects (Burnicka-Turek et al., 2012; Luo et al., 2015), or by influencing the gut microbiome and hepatic gluconeogenesis (Lee et al., 2016). In humans, Wagner and colleagues found differences in *INSL5* levels in obese and lean humans (Wagner et al., 2016), and *INSL5* and *RXFP4* have been proposed to be novel markers of colorectal neuroendocrine tumours (Thanasupawat et al., 2013). The available research in zebrafish suggests that it would be a good model for *insl5* research: the highest expression for both paralogs, *insl5a* and *insl5b,* using qPCR was intestine, with brain and gonad (for *insl5a*) as secondary sites of expression (Good-Avila et al., 2009; Good et al., 2012); *in situ* hybridization results suggest that the two paralogs are further spatially sub-functionalised in goblet cells (*insl5a*) and EEC (*insl5b*) (Venditti et al., 2018), while two of the receptors (*rxfp2-2a* and *rxfp3-3b*) were highly expressed in intestine, and all Rxfp3/4 type receptor genes were expressed in brain, although zebrafish, but not medaka is missing Rxfp4 (Good et al., 2012).

Given the potential roles of RLN3/RXFP3 and INSL5/RXFP4 in metabolism and for modulating the effect of stress on feeding behaviour, reproduction and growth, here we develop resources for studying the relaxin family genes in Japanese medaka, a fish that diverged from other teleosts later than zebrafish (Fig. 1), and which has retained a suite of relaxin family genes more similar to those in other teleosts (Yegorov et al., 2014), particularly an orthologue to human RXFP4 (Fig. 2). To this end, we designed primers, performed qPCR in embryonic stages and in multiple adult tissues, and then used these data as well as comparisons to data in zebrafish to propose sub-functions of paralogous copies of relaxin family genes in teleosts. The goal of this research is to facilitate the use of medaka as a model for studying the ancestral functions of relaxin family genes involved in neuroendocrine regulation.

2. Materials and methods

2.1. Primer design for quantitative analysis of gene expression

Using the protein coding sequence and chromosomal positions of the relaxin family of genes identified in previous work (Good-Avila et al., 2009; Yegorov and Good, 2012), the genomic regions housing the relaxin family genes were obtained from Ensembl (Version 91), and primers designed to span introns (where possible) and generate amplicons between 80 and 10bp using Primer 3 software (Untergasser et al., 2012) for the use of quantitative, real-time polymerase chain reaction (qRT-PCR). To confirm primer specificity and gene expression, RT-PCR was performed using cDNA (see protocol below) from select tissues using a PCR reaction volume of 25 µl, containing 2 µl cDNA, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 1 µl 5 mM dNTPs, 2.5 µl 10X buffer, 2 µl 25 mM Mg, 0.1 µl Taq DNA polymerase (Invitrogen). Unique amplicons were then sequenced at The Centre for Applied Genomics (http://www.tcag.ca/) and aligned to the genomic sequence to confirm sequence identity and C-domain regions for ligands.

Experimental animals: *O. latipes* were maintained in an Aquaneering, Inc. stand-alone aquarium system (San Diego, CA, USA) in the fish facility at the animal complex at the University of Winnipeg. Fish stock was maintained under an extended photoperiod of 14 h of light, and 10 h of dark. Fish were fed to satiety twice daily (10:00a.m./3:00p.m.) with flakes (O.S.I. Spirulina Flake Food, Cyclop-eeze 100 Gram Aquarium Food, and Zeigler Adult Zebrafish Diet)**.** The temperature in the aquarium tanks was ranged from 28 to 29 °C. Embryos were generated under conditions of natural cross-fertilization. Eggs/embryos were collected from the caudal end of the female medaka using a plastic dropper and then placed in a petri dish in culture medium consisting of 16 mL of 60X E3 medium (17.2 g NaCl, 0.76 g KCl, 4.9 g $MgSO_4 \cdot 7H_2O$, and 2.9 g $CaCl_2 \cdot 2H_2O$ in 1 L H_2O) and 3 mL of 0.01% Methylene blue in distilled water (1 L) and incubated at 29 °C. The culture medium was changed every day and the developmental stage of embryos assessed via microscopy (described in SuppFile1). To assess changes in gene expression at various developmental stages, embryos were collected at 7 distinct time points (0.5, 1, 2, 3, 4, 5 and 6 days post fertilization), and adult medaka were used to evaluate tissue-level expression.

Total RNA preparation and cDNA synthesis: The following tissues were collected from male and female medaka; brain, eyes, gills, heart, liver, spleen, intestine, kidney, gallbladder, ovary and testis (SuppFile1). To obtain 2 µg of RNA, 3 tissue samples per sex or 40 embryos (from 10 to 144 h post fertilization [hpf]), were pooled to generate pooled “biological” replicates. Total RNA from embryos and adult tissues were extracted using the miRNeasy plus Universal Mini Kit (QIAgen) containing QIAzol®, according to the manufacturer's protocol. To maximize RNA yields, QIAshredders® (QIAgen) were used to shred tissues prior to RNA extraction. The extracted RNA samples were converted to complementary DNA (cDNA) using iScript select cDNA

Synthesis Kits (Bio-Rad®) and random hexamers following the manufacturer's protocol. RT-qPCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), following the manufacturers protocol and run on a CFX Connect (Bio-Rad).

Real-Time, Quantitative PCR: Expression levels of *rpl7* and *β-actin* were used for gene normalization. For relative expression, an average of all genes in a given tissue or day of embryonic development was used as a calibrator for each gene. The relative expression levels and error bars were calculated by a standardization procedure that includes log transformation, mean centering and autoscaling as described by Willems et al. (2008). The analysis of adult tissues was performed on male and female medaka separately; differences in gene expression were assessed across tissues as well as between tissues, in the latter case normalized gene expression values were further calibrated to the tissue of highest expression.

In situ *hybridization.* Stellaris Probes: *In situ* hybridization was performed using insl5a and cholecystokinin a (*ccka*), the latter was employed as a positive control because it is known to be expressed from EEC's in the intestine of both mammals and fish, and was found to be expressed in high levels in medaka intestine and brain (Alnafea, 2017). The sequence for the coding region (exons) of *insl5a* and *ccka* including 300 base pairs of the 5'-UTR and 700 base pairs of the 3'-UTR were used to design anti-sense probes for *in situ* hybridization analyses. Anti-sense probes against the pre-mRNA sequences were designed using the Stellaris, Bioresearch software resulting in forty-one 18 bp anti-sense probes covering the UTR + exonic sequence of *insl5a*, and forty-eight 20 bp anti-sense probes for *ccka* (Supp File2, Tables 2 and 3). The mini-probes were tagged with Quasar 570 or Quasar 670 flouescent dyes for *insl5a* and *ccka* respectively and reconstituted in Tris-EDTA and aliquoted into smaller portions and stored at −20 °C until use. **Embryo Preparation:** Medaka embryos were collected and their chorions removed manually. Embryos were incubated overnight in 4% paraformaldehyde at 4 °C, then immersed in 70% ethanol prior to paraffin embedding. Paraffin blocks of medaka embryos were sectioned using a Leica microtome; tissues were cut into 5 μm thickness. Slides were stored at −80 °C prior to performing *in situ* hybridization. **Adult intestine and brain samples:** Adult tissues were dissected from adult medaka after euthanasia using MS222. Each tissue was subsequently embedded in cryomatrix embedding medium, immersed in liquid nitrogen and then cut into 5 μm sections. The cryostat microtome temperature was −26 °C for the chamber and blade, to enhance the quality of the tissue parts and sectioning and slides were stored at −80 °C prior to performing *in situ* hybridization. For paraffin-embedded tissues, tissues were incubated overnight in 4% paraformaldehyde at 4 °C, immersed in 70% ethanol prior to embedding in paraffin. Paraffin blocks were sectioned using a Leica microtome, and cut into 5 μm thick sections. Slides were stored at −80 °C prior to performing *in situ* hybridization. ***In situ* Hybridization and Imaging:** Medaka embryos were whole-mounted and prepared for hybridization following the manufacturer's protocol (Stellaris, Biosearch Technologies). To identify the localization of target genes within medaka tissues, medaka tissue sections were stained for DAPI, *insl5a* and *ccka*. Embryo and adult tissues were visualized using a LSM 710 Zeiss confocal microscope.

3. Results

Primer design and chromosome annotations: The first release of the *O. latipes* genome in 2008 was performed on strain HdrR. Nevertheless, some of the annotations are in disagreement with our predictions presented here; thus here we report annotations for five of the peptides obtained from aligning sequences generated from RT-PCR with primers flanking the B/C and C/A domains so as to capture the C-peptide and intron location. Three of the peptides were previously unannotated (*insl3, insl5a, insl5b*) and two of these did not have the C-domain specified (*rln3a, rln3b*) (SuppFile2, Table 3). Novel, gene-specific qRT-PCR primers that flanked the singular intron were designed for all genes except for *rln,* for which gene-specific primers were not identified (Table 1). A list of the updated accession numbers, and chromosomal locations of all relaxin family genes in Japanese medaka and four other teleost models is presented in SuppFile3.

Developmental expression of relaxin family genes in medaka: Analysis of the transcript abundance during embryonic stages revealed low expression of most peptide genes during embryonic developmental and relatively higher expression of the receptor genes. Transcript levels of *insl5b* were the highest of all relaxin family peptides in all stages of embryonic development, although *rln3a* had a relative expression > 1 on days 1 (stages 0–21) and 2 (stages 22–26), and *rln3b* and *insl3*, were expressed above the average of all genes on days 5 (stages 32–33) and 6 (stages 34–39) (Fig. 3).

On the other hand, most of the *rxfp* genes were more highly expressed, except for *rxfp3-3a1*, which consistently had very low expression, and is the only relaxin family peptide receptor gene in medaka that originated via small scale (local) duplication (Yegorov and Good, 2012, and SuppFile4). The receptor gene with the highest expression across all days was *rxfp3-3a2,* although *rxfp2* had similarly high expression in early stages of developments (days 1–3) and *rxfp3-3b* and *rxfp3-2b* exhibited high expression at later stages. Most of the receptors exhibited a similar fold change over time such that they increased in expression between days 1 and day 4 (stages 29–31), and then expression plateaued or decreased on days 5 and 6. The only exception to this pattern was *rxfp3-3b*, which decreased in expression between days 2 and day 3 (stages 27–28) and then increased during the later stages of embryo development (Fig. 3).

Gene expression in adult tissues via qRT-PCR. The relative abundance of the relaxin family gene mRNA in adult male and female Japanese medaka was measured in brain plus optic nerve (part of the central nervous system, CNS), gills, heart, spleen, kidney, liver, gallbladder, intestine, and gonads (testes or ovaries). Fold changes were calculated relative to the mean of all transcripts expressed in a tissue (Fig. 4), and relative to the tissue of highest expression for a given gene (SuppFile4).

Ligands: *rln3a* and *rln3b* were highly expressed in brain relative to other tissues (SuppFile4), but r*ln3a* exhibited slightly higher expression in female heart than brain, and *rln3b* also had strong expression in gonads and kidney and intestine (Fig. 4 and SuppFile4). As expected, *insl5a* and *insl5b* were most highly expressed in intestine in both sexes (SuppFile4), but *insl5b* exhibited a wider expression profile and was also highly expressed in brain, heart and gonad (SuppFile4). *Insl3* expression was very high in male testes (as expected) and in females exhibited low expression except in eye/optic nerve (Fig. 4).

Receptors: Of all *rxfp* genes, *rxfp1* and *rxfp2* exhibited the highest expression across tissues (Fig. 4). In males, both genes were the most highly expressed in kidney. In females, *rxfp1* and *rxfp2* exhibited broader expression, consistent with the wider expression of *insl3* in females (SuppFile4). In both sexes, *rxfp3-2a* was expressed in intestine and kidney, and additionally in brain and heart in females and in male testes, and *rxfp3-2b* was most highly expressed in female intestine, and in male brain and testes (SuppFile4). For all of the *rxfp3-3* and the *rxfp4* genes, the dominant tissue of expression was intestine, while *rxfp3-3a2* expression was higher in brain than intestine in both sexes, and *rxfp3-3a1* was also highly expressed in gonad (especially testes), and *rxfp3-3b* in testes.

***In situ* hybridization of *insl5a* in adult brain and intestine and in whole-mount embryos.** Hybridization of both *insl5a* and *ccka* was most evident in the intestinal epithelium; *ccka* is known to be secreted from I-cells in the EEC in mammals, while insl5a is secreted from mammalian L-cells; in medaka, the two peptide hormones were secreted from different but adjacent cells (Fig. 5), suggesting that *insl5a* in medaka is secreted by EEC cells, but a different cell-population than *ccka. Insl5a* expression was also observed in the adult brain, but the signal was diffuse in the mid- and hindbrain regions of adult medaka (not shown). Lastly, *insl5a* hybridization was observed in day 6 (stages

Table 1
Primers for relaxin family genes in Japanese medaka (*Oryzias latipes*).

Genes	Forward Primer 5′-3′	Reverse primer 5′-3′	Size (bp)	Efficiency
rln3a	CGAGCCGTCATCTTCACCT	ATGCTTGGGACTTTTCTGG	137	92%
rln3b	GGAGGTTCAAGATGGAAACG	ACGCTCCACCACAAAGTTCT	146	113%
insl3	GGACCAACACAAAACCCTCT	CCGGAAGGATCGAGAGAAC	137	102%
insl5a	CGCTGGAGGAGATTCTTGAC	CTGAAGCTGTCCAAGCTGTT	77	101%
insl5b	TTCTGAGGGCGTTGGTGT	GGTGCCGCTGTTCTCTTCT	118	109%
rxfp1	GGTCTTTGTGTGGGTCGTCT	ACATGGCGTGGAGTTTGTTT	101	97%
rxfp2	AGATCCTGCAAACCCAACAC	TGACAAGCAGATTGCCAAAG	121	96%
rxfp3-1	CCTGGGGTATCCTCATCAAA	TCAGACAGCTGTTGGAGTGG	114	100%
rxfp3-2a	TTCCCCCTGACTGTGTGTCT	CCCGGCTCTGTACTCTCTTC	87	99%
rxfp3-2b	GGCCTTTTGGGAAAGTTATG	TCTTCAAAGCGGAGGAGATG	126	110%
rxfp3-3a1	ACAAGCGCAGCATGAAAAC	CAGCACACGAAGAAGGACAA	91	118%
rxfp3-3a2	TGAGACAGGGTCGAAAGAGG	AAACGGCCAGCTAAAGTCAA	128	105%
rxfp3-3b	AGACGGCGCTGGATTTCAG	GCCCATGGCTGTGAGGAA	110	108%
rxfp4	TCTTTGGCCTTACCGTTTTG	GTTCAGCCCCGTGAGTAGAG	102	100%

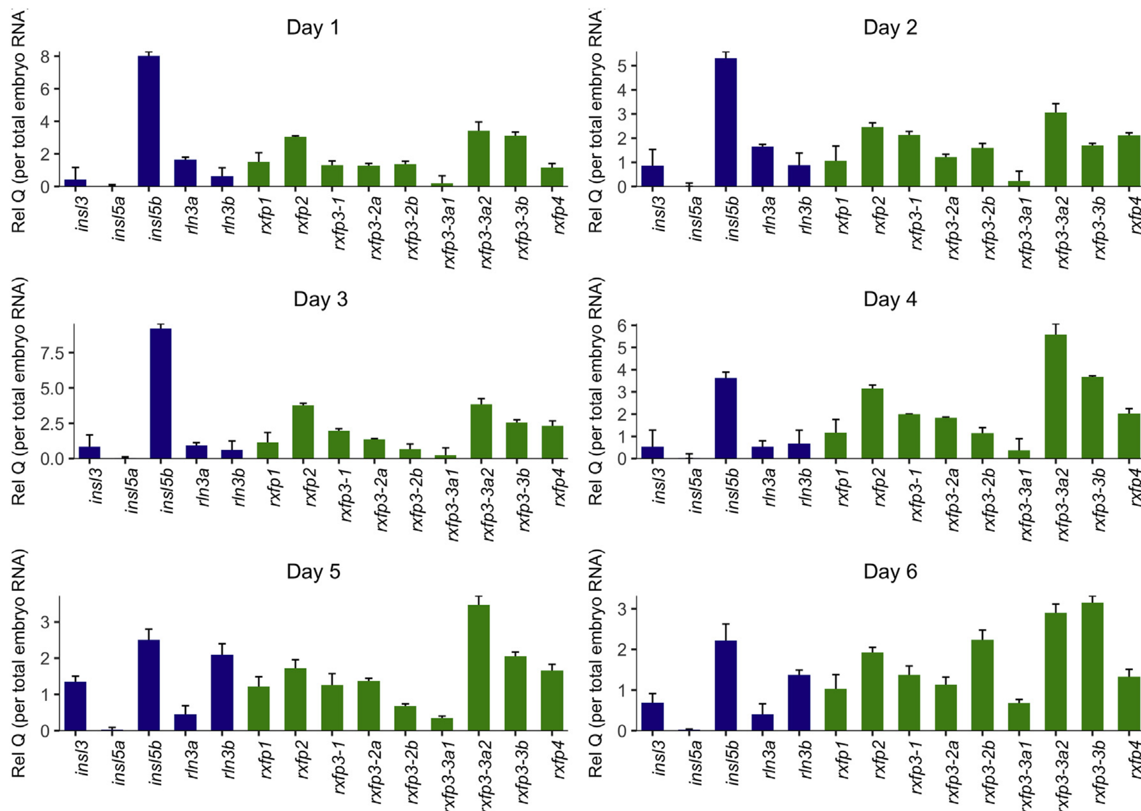

Fig. 3. Results of the qRT-PCR experiments describing the expression of relaxin family genes over six days of embryonic development (refer to SuppFile1for stages represented in each day). For the qRT-PCR analyses values greater than 1 indicate that the gene was expressed more than the average of all genes at that developmental stage.

34–39) whole mount embryos, with a hybridization signal in the intestine/pancreatic region (Fig. 6).

4. Discussion

We set out to inform on the use of Japanese medaka as another teleost model for investigating the function of relaxin family genes in vertebrates. In particular, we propose that medaka would be a good model to study the emerging roles of Rln3 and Insl5 peptides in metabolic and stress-related diseases in humans. Since the genes encoding these peptides and Rxfp3/4-type receptors, through which these peptides are thought to operate, underwent sub-functionalization in the post-3R teleost genome (Fig. 2), teleosts offer an intuitive model for teasing apart the pleiotropic roles of these genes in mammals.

The growing interest in medaka for endocrine disorder investigation (Untergasser et al., 2012; Ichimura et al., 2013; Kirchmaier et al., 2015; Fujisawa et al., 2018), suggests that it is a timely model for dissecting the role of relaxin family peptides involved in neuroendocrine regulation. To this end, we provide the first experimental evidence for the expression of the relaxin family genes that had previously only been identified *in-silico* in *O. latipes* (Good-Avila et al., 2009; Yegorov and Good, 2012), characterise gene expression across multiple medaka tissues, and compare these results to the data available in zebrafish (Donizetti et al. 2009, 2010; Good-Avila et al., 2009; Fiengo et al., 2012; Good et al., 2012; Venditti et al., 2018) to further delineate relaxin ligand-receptor relationships in vertebrates.

RLN3. In mammals, the physiological effects of RLN3 expression in the NI and PAG are mediated by paracrine stimulation of RXFP3-expressing neurons, which are proposed to act as a central signal linking nutritional status, reproductive function and stress (Tanaka et al., 2005;

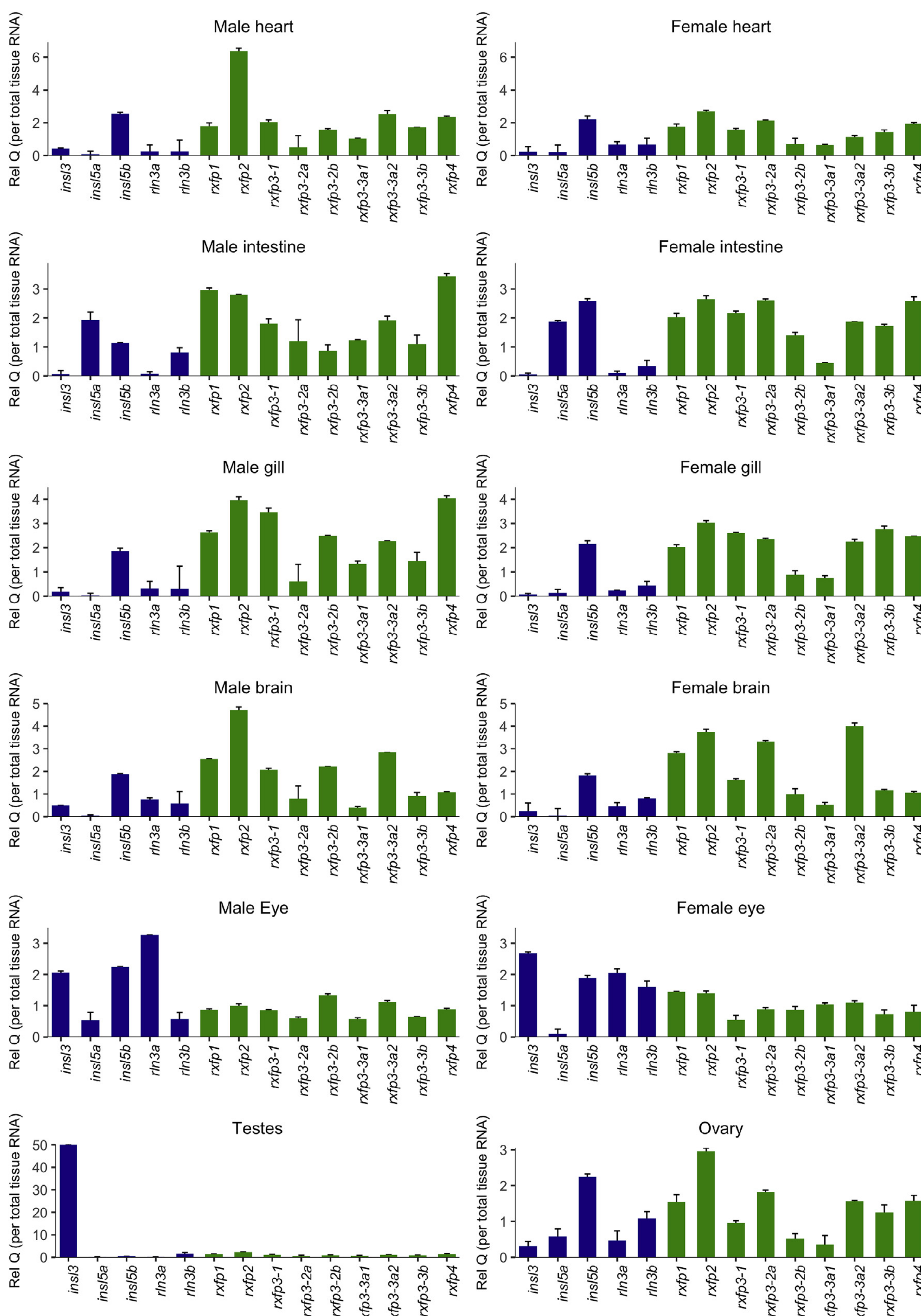

Fig. 4. Results of the qRT-PCR experiments describing the expression of relaxin family genes in seven organs in Japanese medaka. Relative expression of all ligand and receptor genes was quantified relative the mean expression of all relaxin family genes per organ in each sex.

McGowan et al., 2008; McGowan et al., 2014). Although the absolute level of *rln3a/rln3b* expression in medaka was low (perhaps due to the restricted subset of cells expressing them), both paralogs were more highly expressed in brain than any other tissue (SuppFile5), consistent with the results from all other teleosts examined (Hu et al., 2011; Good et al., 2012; Kusakabe et al., 2014) and with the high level of peptide conservation between teleost *rln3a/rln3b* and mammalian *RLN3* as well as between teleost *rxfp3-1* and mammalian *RXFP3* (Wilkinson and

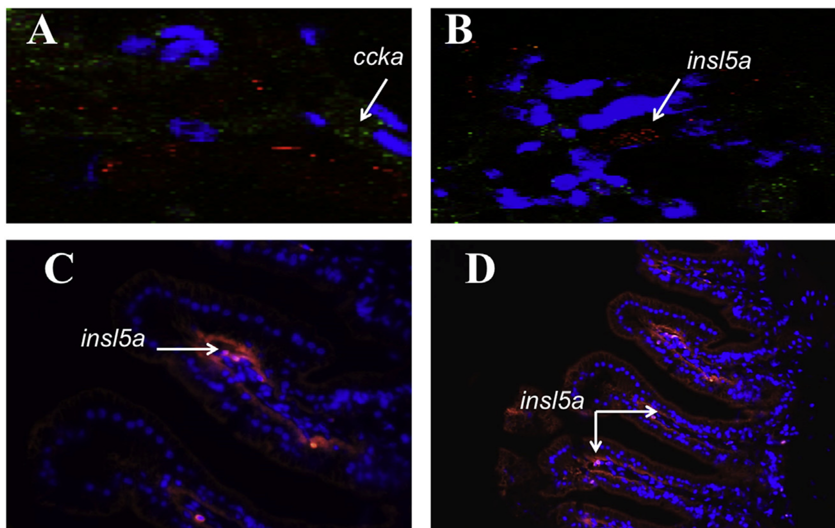

Fig. 5. *In situ* hybridization results in medaka. Cellular level of expression of *ccka* (positive control) (A) and *insl5* (B) in enteroendocrine cells (EECS) of the gut following sectioning of frozen tissue in 5 μm thick slices using a cryostat microtome. Cells were incubated with a nuclear fluorescent stain (DAPI), and then hybridized with *insl5a* (red) and *ccka* (green) probes (see methods). Paraffin embedded sections were also used to assess the spatial localization of *insl5a* expression (C, D). Paraffin blocks of medaka intestine were sectioned via microtome, and *in situ* hybridization performed using the *insl5a* antisense Stellaris probes and co-stained with a nuclear fluorescent stain (DAPI), revealing the location of *insl5a* probe in EECs.

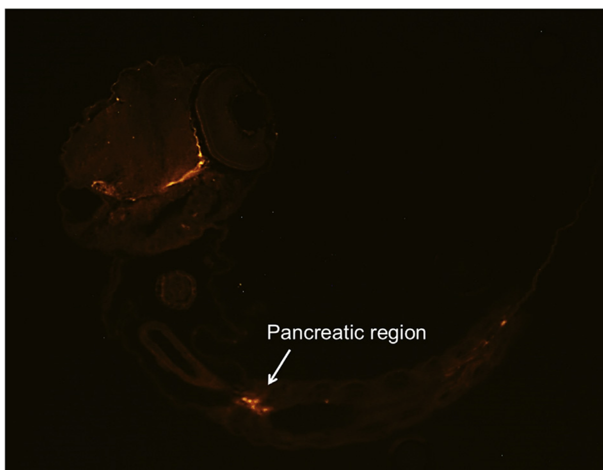

Fig. 6. Whole mount embryo *in situ* hybridization showing diffuse binding of insl5a sense probe in the brain, but cellular staining in the pancreatic/intestine.

Bathgate, 2007; Good et al., 2012; Bathgate et al., 2018). *In situ* hybridization experiments in zebrafish have shown that *rln3a* is expressed in the NI, whereas *rln3b* expression is localized to the PAG, which demonstrates sub-functionalization of teleost *rln3* (Donizetti et al., 2009).

The apparent sub-functionalization of *rln3a/rln3b* should be mirrored by sub-functionalization of their receptors. In medaka, we found high expression of *rxfp2, rxfp3-1, rxfp3-2a,* and *rxfp3-3a2* in adult brain; these results are similar to those found in zebrafish with the notable exception that in zebrafish *rxfp3-3b* was the receptor most highly expressed in brain while in medaka *rxfp3-3a2* had comparable expression but not *rxfp3-3b* (Good et al., 2012). *In situ* hybridization in zebrafish confirmed that *rxfp3-1* and *rxfp3-3b* were expressed in distinct subsections of the brain homologous to *rxfp3*-expressing regions, suggesting sub-functionalization (Donizetti et al., 2015). Since *rln3a* and *rxfp3-1* are pseudogenes in tetraodon, we propose that Rxfp3-1 and Rxfp3-2a are potential receptors for Rln3a, while Rxfp3-2b and possibly Rxfp3-3a2 (medaka) and Rxfp3-3b (zebrafish) are potential receptors for Rln3b and/or Insl5b (see below) in the brain.

INSL5. Our results provide evidence for spatial and temporal sub-functionalization of *insl5* paralogs in medaka during and post-embryogenesis that is largely consistent with data from zebrafish. A recent ISH study by Venditti et al. (2018) in zebrafish found that overall *insl5b* exhibited stronger expression during embryonic development, while *insl5a* was expressed in pancreas and intestine 72 hpf; both *insl5a* and *insl5b* were expressed in intestinal cells 96hpf. Consistent with Venditti et al., we demonstrate that *insl5b* was the most highly expressed peptide during embryonic development and that *insl5a* is expressed in the embryonic pancreatic region. There are at least four receptors that are hypothesized to signal by Insl5a/Insl5b stimulation in medaka: Rxfp3-3a1*,* Rxfp3-3a2*,* Rxfp3-3b and Rxfp4. Medaka *rxfp3-3a2* was the most highly expressed gene during development, whereas its small-scale genome duplication paralog*, rxfp3-3a1,* was expressed at low levels throughout development providing evidence of temporal sub-functionalization between these two paralogs and suggesting that Insl5b may bind to Rxfp3-3a2.

In adult tissues, we find both similarities and differences between zebrafish and medaka gene expression. For both species, *insl5a* and *insl5b* were highly expressed in intestine while ovary was the second highest site of *insl5a* production consistent with previous reports (Good-Avila et al., 2009; Good et al., 2012). On the other hand, medaka exhibited the highest transcript levels of *insl5b* detectable in brain, followed by intestine, heart, kidney and gonad; even though the absolute quantity of *insl5b* was lower than *insl5a* in all tissues except brain. The latter is unlike what is seen in zebrafish, where *insl5b* was expressed at very low levels in brain (Good-Avila et al., 2009; Good et al., 2012). The spatial sub-functionalization of insl5 paralogues is also different in the intestine in zebrafish and medaka. Using *in situ* hybridization, Venditti et al. (2018) found *insl5a* expression in goblet cells and *insl5b* expression in EEC's, while we found *insl5a* expression in EEC's in medaka. Goblet cells are responsible for making the mucous which lines the gut, and it is involved in both gut contractility and immune defense. EECs are cells underneath the goblet cells that release gut peptide hormones that control food intake, digestion and glucose hemostasis in both fish and mammals such as GLP-1 (Mojsov, 2000) and PYY (Volkoff, 2014) and they are also integral players in gut-immune interactions (Rooks and Garrett, 2016; Zietek and Rath, 2016). Many of the Rxfp3/4 type receptor genes that may bind insl5a/insl5b were highly expressed in medaka gut, whereas in zebrafish, their predominant tissue of expression was brain. For example, in medaka *rxfp3-2a, rxfp3-2b* (which may bind Rln3a/Rln3b), as well as *rxfp3-3a1, rxfp3-3b* and *rxfp4* were all highly expressed in intestine. In mammals, INSL5 is hypothesized to be involved in gut contractility, glucose homeostasis, satiety, and gut-microbiome interactions (Burnicka-Turek et al., 2012; Grosse et al., 2014; Luo et al., 2015; Ang et al., 2017; Lee et al., 2016), and was shown to disrupt the oestrus cycle in female mice (Burnicka-Turek et al., 2012). Given the above evidence of sub-functionalization of insl5 paralogs, including the higher expression of *insl5b* in brain, the high expression of both ligands in the gut, and the expression of *insl5a* in ovary, indicates that medaka may be a better model for teasing apart the functions of

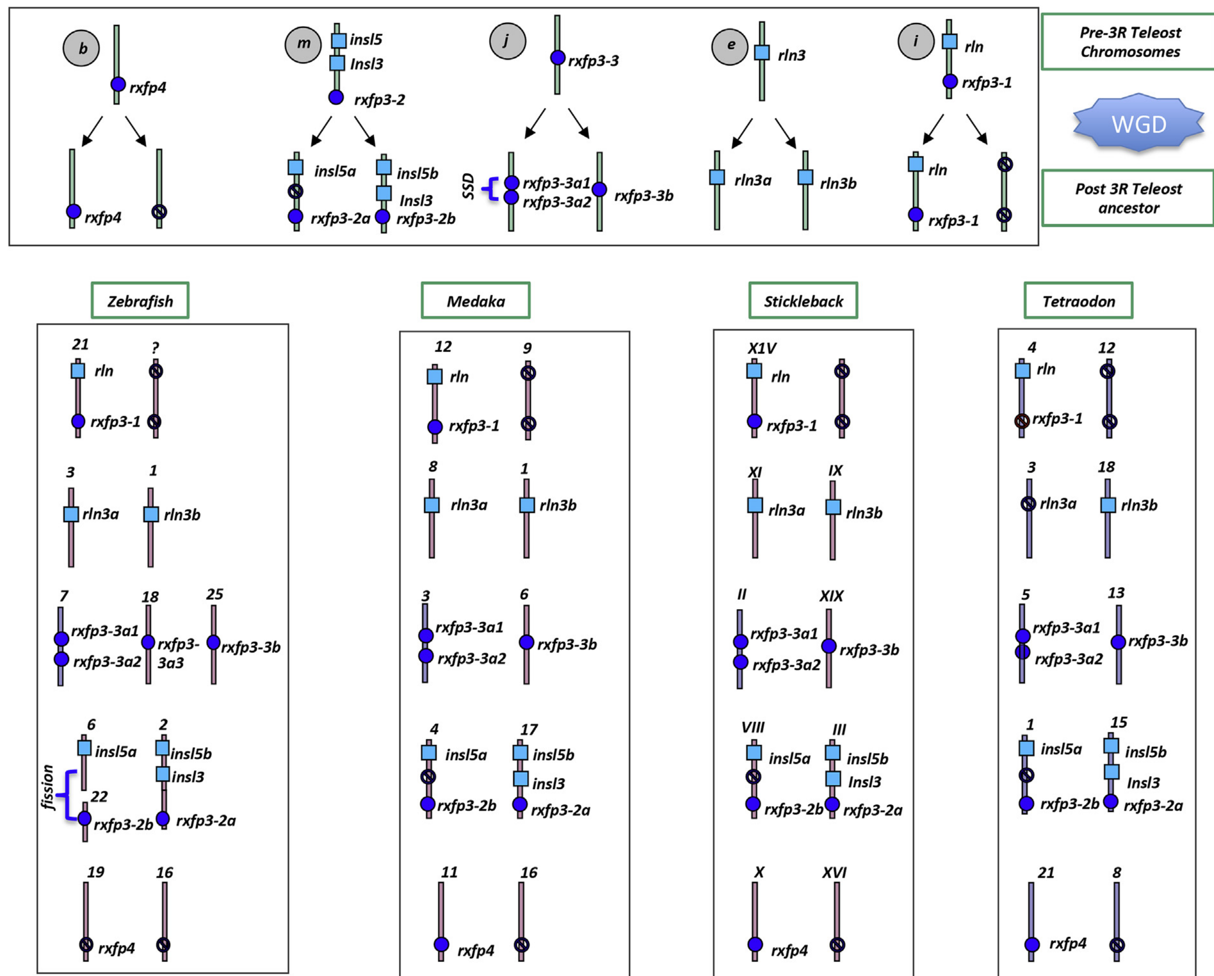

Fig. 7. Evolutionary history of the relaxin family peptides and rxfp3/4 receptors in the pre-3R and post-3R teleost genomes and the syntenic relationships and chromosomal location of the genes in four model teleost species: zebrafish, Japanese medaka, three-spine stickleback and tetraodon.

Insl5/Rxfp4 signalling in vertebrates.

4.1. Gene nomenclature and verification in teleosts

Although many of the relaxin family genes have recently been annotated at Ensembl, there are important exceptions. Firstly, following the nomenclature of Yegorov and Good (2012), the genes *rxfp3-2a* and *rxrp3-2b* are now largely annotated (i.e Ensembl ID and coordinates) but are usually unnamed. For precision, we depict the evolution of the genes in teleosts, and show how *insl3*, *insl5* and *rxfp3-2* genes are linked with *insl3*, *insl5a* and *rxfp3-2b* being on one chromosome in most teleosts while *insl5b* and *rxfp3-2a* are on a second chromosome (Fig. 7). A further issue that needs to be resolved is the nomenclature of the genes we called *rxfp3-3a1* and *rxfp3-3a2* (SuppFile3), but which Ensembl researchers have called *rxfp3-3a2* and *rxfp3-3a3*. By our analyses, *rfp3-3a1* and *rxfp3-3a2* are always *closely linked* on the same chromosome in all teleosts (Fig. 7), while *rxfp3-3a3* is present on a different chromosome and is only present in zebrafish and other early diverging teleosts. Despite these issues in need of resolution, the current inclusion of 49 teleosts on Ensembl, most of which have annotations for relaxin genes, means that it is an exciting time to study the relaxin gene family in fish.

4.2. Strengths and weaknesses of using teleosts as a model for relaxin family gene evolution

We propose the use of medaka and zebrafish as model species for the roles of Rln3 and Insl5 in metabolic and/or stress-related diseases because a) the extensive sub-functionalization of the genes in teleosts allows distinct functions to be separated while the early divergence (~450 mya) of teleosts from tetrapods (Fig. 1) allows elucidation of the ancestral function of these genes b) they permit low-cost large-scale *in vivo* investigations c) they possess the same organs and complex interplay required for transferrable investigation into mammalian metabolic syndromes. While fish would be good models to understand the ancestral function of relaxin family genes, they would not capture *de novo* changes of the roles of these genes in mammals, and as such would not be good models for studying e.g the function of mammalian relaxin that has neo-functionalised in placentals (Bathgate et al., 2018). This caveat may be more relevant for Insl5 than for Rln3, since the peptide sequence of Rln3 is very similar between mammals and fish, while that for Insl5 appears more divergent (Good-Avila et al., 2009).

An advantage of working with zebrafish and medaka is that there are now tremendous genomic resources available for both species. For zebrafish, the increased success of CRISPR-Cas system for targeted mutations has allowed researchers to generate mutants for many genes including *rln3a* (Chia, 2017). A variety of mutants are available through the Zebrafish Mutation Project whose aim is to create knockouts for all annotated zebrafish genes: there are currently knockouts available for ten relaxin family genes (See SuppFile3).

There are similarly rich resources for medaka. The four medaka genomes are accessible through a number of genome browsers including Ensembl and the Japanese genome browser (http://dolphin.nig.ac.jp/medaka/index.php). The NBRP genome browser also enables searches for SNPs in the HdrR, HNI, Nilan, HSOK, and Kaga strains

(http://medaka.lab.nig.ac.jp/cgi-bin/gb2/gbrowse/medaka/), and reference transcriptomes are now available for *Oryzias latipes* and *O. melastogma* (Lai et al., 2015). Given the almost complete annotation of relaxin family genes now on Ensembl (SuppFile3) and the wealth of resources available to study medaka, it already presents a good model with some advantages compared to zebrafish, such as a more similar suite of genes to human/mouse and other teleosts, apparent sub-functionalization of insl5 paralogs, while still having many of the practical benefits of working with zebrafish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.01.017.

References

Alnafea, H., 2017. Expression of Insulin superfamily genes in Japanese Medaka (Oryzias latipes) during development and under conditions of prolonged fasting. In: Biology. University of Winnipeg, Winnipeg, pp. 162.
Ang, S.Y., Hutchinson, D.S., Patil, N., Evans, B.A., Bathgate, R.A., et al., 2017. Signal transduction pathways activated by insulin-like peptide 5 at the relaxin family peptide RXFP4 receptor. Br. J. Pharmacol. 174 (10), 1077–1089.
Bathgate, R.A.D., Kocan, M., Scott, D.J., Hossain, M.A., Good, S.V., et al., 2018. The relaxin receptor as a therapeutic target - perspectives from evolution and drug targeting. Pharmacol. Ther. 187, 114–132.
Burnicka-Turek, O., Mohamed, B.A., Shirneshan, K., Thanasupawat, T., Hombach-Klonisch, S., et al., 2012. INSL5-deficient mice display an alteration in glucose homeostasis and an impaired fertility. Endocrinology 153, 4655–4665.
Calvez, J., de Avila, C., Matte, L.O., Guevremont, G., Gundlach, A.L., et al., 2016. Role of relaxin-3/RXFP3 system in stress-induced binge-like eating in female rats. Neuropharmacology 102, 207–215.
Calvez, J., de Avila, C., Timofeeva, E., 2017. Sex-specific effects of relaxin-3 on food intake and body weight gain. Br. J. Pharmacol. 174, 1049–1060.
Canestro, C., Albalat, R., Irimia, M., Garcia-Fernandez, J., 2013. Impact of gene gains, losses and duplication modes on the origin and diversification of vertebrates. Semin. Cell Dev. Biol. 24, 83–94.
Chia, S.-M., 2017. Behavioural Analysis of Zebrafish Relaxin 3a Mutant. National University of Singapore, pp. 138. ScholarBank@NUS.Repository.
Conklin, D., Lofton-Day, C.E., Haldeman, B.A., Ching, A., Whitmore, T.E., et al., 1999. Identification of INSL5, a new member of the insulin superfamily. Genomics 60, 50–56.
Donizetti, A., Fiengo, M., del Gaudio, R., Di Giaimo, R., Minucci, S., et al., 2010. Characterization and developmental expression pattern of the relaxin receptor rxfp1 gene in zebrafish. Dev. Growth Differ. 52, 799–806.
Donizetti, A., Fiengo, M., Iazzetti, G., del Gaudio, R., Di Giaimo, R., et al., 2015. Expression analysis of five zebrafish RXFP3 homologues reveals evolutionary conservation of gene expression pattern. J. Exp. Zool. B Mol. Dev. Evol. 324, 22–29.
Donizetti, A., Fiengo, M., Minucci, S., Aniello, F., 2009. Duplicated zebrafish relaxin-3 gene shows a different expression pattern from that of the co-orthologue gene. Dev. Growth Differ. 51, 715–722.
Fiengo, M., Donizetti, A., del Gaudio, R., Minucci, S., Aniello, F., 2012. Characterization, cDNA cloning and expression pattern of relaxin gene during embryogenesis of Danio rerio. Dev. Growth Differ. 54, 579–587.
Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., et al., 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151, 1531–1545.
Fujisawa, K., Takami, T., Fukui, Y., Nagatomo, T., Saeki, I., et al., 2018. Assessment of high fat diet-induced fatty liver in medaka. Biol Open 7https://doi.org/10.1242/bio.031534. bio031534.
Good, S., Yegorov, S., Martijn, J., Franck, J., Bogerd, J., 2012. New insights into ligand-receptor pairing and coevolution of relaxin family peptides and their receptors in teleosts. Int. J. Evol. Biol. 14 2012.
Good-Avila, S.V., Yegorov, S., Harron, S., Bogerd, J., Glen, P., et al., 2009. Relaxin gene family in teleosts: phylogeny, syntenic mapping, selective constraint, and expression analysis. BMC Evol. Biol. 9, 293.
Grosse, J., Heffron, H., Burling, K., Akhter Hossain, M., Habib, A.M., et al., 2014. Insulin-like peptide 5 is an orexigenic gastrointestinal hormone. Proc. Natl. Acad. Sci. U. S. A. 111, 11133–11138.
Gut, P., Reischauer, S., Stainier, D.Y.R., Arnaout, R., 2017. Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. Physiol. Rev. 97, 889–938.
Hosken, I.T., Sutton, S.W., Smith, C.M., Gundlach, A.L., 2015. Relaxin-3 receptor (Rxfp3) gene knockout mice display reduced running wheel activity: implications for role of relaxin-3/RXFP3 signalling in sustained arousal. Behav. Brain Res. 278, 167–175.
Hu, G.B., Kusakabe, M., Takei, Y., 2011. Localization of diversified relaxin gene transcripts in the brain of eels. Gen. Comp. Endocrinol. 172, 430–439.
Ichimura, K., Kawashima, Y., Nakamura, T., Powell, R., Hidoh, Y., et al., 2013. Medaka fish, Oryzias latipes, as a model for human obesity-related glomerulopathy. Biochem. Biophys. Res. Commun. 431, 712–717.
Kassahn, K.S., Dang, V.T., Wilkins, S.J., Perkins, A.C., Ragan, M.A., 2009. Evolution of gene function and regulatory control after whole-genome duplication: comparative analyses in vertebrates. Genome Res. 19, 1404–1418.
Kirchmaier, S., Naruse, K., Wittbrodt, J., Loosli, F., 2015. The genomic and genetic toolbox of the teleost medaka (Oryzias latipes). Genetics 199, 905–918.
Kumar, J.R., Rajkumar, R., Jayakody, T., Marwari, S., Hong, J.M., et al., 2017. Relaxin' the brain: a case for targeting the nucleus incertus network and relaxin-3/RXFP3 system in neuropsychiatric disorders. Br. J. Pharmacol. 174, 1061–1076.
Kusakabe, M., Ishikawa, A., Kitano, J., 2014. Relaxin-related gene expression differs between anadromous and stream-resident stickleback (Gasterosteus aculeatus) following seawater transfer. Gen. Comp. Endocrinol. 205, 197–206.
Lai, K.P., Li, J.W., Wang, S.Y., Chiu, J.M., Tse, A., et al., 2015. Tissue-specific transcriptome assemblies of the marine medaka Oryzias melastigma and comparative analysis with the freshwater medaka Oryzias latipes. BMC Genomics 16, 135.
Lee, Y.S., De Vadder, F., Tremaroli, V., Wichmann, A., Mithieux, G., et al., 2016. Insulin-like peptide 5 is a microbially regulated peptide that promotes hepatic glucose production. Mol Metab 5, 263–270.
Luo, X., Li, T., Zhu, Y., Dai, Y., Zhao, J., et al., 2015. The insulinotrophic effect of insulin-like peptide 5 in vitro and in vivo. Biochem. J. 466, 467–473.
Ma, S., Bonaventure, P., Ferraro, T., Shen, P.J., Burazin, T.C., et al., 2007. Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. Neuroscience 144, 165–190.
Matsumoto, T., Terai, S., Oishi, T., Kuwashiro, S., Fujisawa, K., et al., 2010. Medaka as a model for human nonalcoholic steatohepatitis. Dis Model Mech 3, 431–440.
McGowan, B.M., Minnion, J.S., Murphy, K.G., Roy, D., Stanley, S.A., et al., 2014. Relaxin-3 stimulates the neuro-endocrine stress axis via corticotrophin-releasing hormone. J. Endocrinol. 221, 337–346.
McGowan, B.M., Stanley, S.A., Donovan, J., Thompson, E.L., Patterson, M., et al., 2008. Relaxin-3 stimulates the hypothalamic-pituitary-gonadal axis. Am. J. Physiol. Endocrinol. Metab. 295, E278–E286.
McGowan, B.M., Stanley, S.A., Smith, K.L., Minnion, J.S., Donovan, J., et al., 2006. Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. Regul. Pept. 136, 72–77.
McGowan, B.M., Stanley, S.A., Smith, K.L., White, N.E., Connolly, M.M., et al., 2005. Central relaxin-3 administration causes hyperphagia in male Wistar rats. Endocrinology 146, 3295–3300.
Mojsov, S., 2000. Glucagon-like peptide-1 (GLP-1) and the control of glucose metabolism in mammals and teleost fish. Am. Zool. 40, 246–258.
Munro, J., Skrobot, O., Sanyoura, M., Kay, V., Susce, M.T., et al., 2012. Relaxin polymorphisms associated with metabolic disturbance in patients treated with antipsychotics. J. Psychopharmacol. 26, 374–379.
Nakazawa, C.M., Shikata, K., Uesugi, M., Katayama, H., Aoshima, K., et al., 2013. Prediction of relaxin-3-induced downstream pathway resulting in anxiolytic-like behaviors in rats based on a microarray and peptidome analysis. J. Recept. Signal Transduct. Res. 33, 224–233.
Ohno, S., 1970. Evolution by Gene Duplication. Springer-Verlag, Berlin, New York.
Ravi, V., Venkatesh, B., 2008. Rapidly evolving fish genomes and teleost diversity. Curr. Opin. Genet. Dev. 18, 544–550.
Rooks, M.G., Garrett, W.S., 2016. Gut microbiota, metabolites and host immunity. Nat. Rev. Immunol. 16, 341–352.
Ryan, P.J., Buchler, E., Shabanpoor, F., Hossain, M.A., Wade, J.D., et al., 2013a. Central relaxin-3 receptor (RXFP3) activation decreases anxiety- and depressive-like behaviours in the rat. Behav. Brain Res. 244, 142–151.
Ryan, P.J., Kastman, H.E., Krstew, E.V., Rosengren, K.J., Hossain, M.A., et al., 2013b. Relaxin-3/RXFP3 system regulates alcohol-seeking. Proc. Natl. Acad. Sci. U. S. A. 110, 20789–20794.
Seth, A., Stemple, D.L., Barroso, I., 2013. The emerging use of zebrafish to model metabolic disease. Dis Model Mech 6, 1080–1088.
Smith, C.M., Lawrence, A.J., Sutton, S.W., Gundlach, A.L., 2009. Behavioral phenotyping of mixed background (129S5:B6) relaxin-3 knockout mice. Ann. N. Y. Acad. Sci. 1160, 236–241.
Smith, C.M., Shen, P.J., Banerjee, A., Bonaventure, P., Ma, S., et al., 2010. Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. J. Comp. Neurol. 518 4016-1045.
Sutton, S.W., Shelton, J., Smith, C., Williams, J., Yun, S., et al., 2009. Metabolic and neuroendocrine responses to RXFP3 modulation in the central nervous system. Ann. N. Y. Acad. Sci. 1160, 242–249.
Tanaka, M., Iijima, N., Miyamoto, Y., Fukusumi, S., Itoh, Y., et al., 2005. Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. Eur. J. Neurosci. 21, 1659–1670.
Thanasupawat, T., Hammje, K., Adham, I., Ghia, J.E., Del Bigio, M.R., et al., 2013. INSL5 is a novel marker for human enteroendocrine cells of the large intestine and neuroendocrine tumours. Oncol. Rep. 29, 149–154.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., et al., 2012. Primer3–new capabilities and interfaces. Nucleic Acids Res. 40, e115.
Venditti, M., Donizetti, A., Fiengo, M., Fasano, C., Santillo, A., et al., 2018. Temporal and spatial expression of insulin-like peptide (insl5a and insl5b) paralog genes during the

embryogenesis of Danio rerio. J. Exp. Zool. B Mol. Dev. Evol. 330, 33–40.
Volkoff, H., 2014. Appetite regulating peptides in red-bellied piranha, *Pygocentrus nattereri:* cloning, tissue distribution and effect of fasting on mRNA expression. Peptides 56, 116–124.
Wagner, I.V., Flehmig, G., Scheuermann, K., Loffler, D., Korner, A., et al., 2016. Insulin-like peptide 5 interacts with sex hormones and metabolic parameters in a gender and adiposity dependent manner in humans. Horm. Metab. Res. 48, 589–594.
Walker, A.W., Smith, C.M., Gundlach, A.L., Lawrence, A.J., 2015. Relaxin-3 receptor (Rxfp3) gene deletion reduces operant sucrose- but not alcohol-responding in mice. Genes Brain Behav. 14, 625–634.
Watanabe, Y., Tsujimura, A., Takao, K., Nishi, K., Ito, Y., et al., 2011. Relaxin-3-deficient mice showed slight alteration in anxiety-related behavior. Front. Behav. Neurosci. 5, 50.
Wilkinson, T.N., Bathgate, R.A., 2007. The evolution of the relaxin peptide family and their receptors. Adv. Exp. Med. Biol. 612, 1–13.
Willems, E., Leyns, L., Vandesompele, J., 2008. Standardization of real-time PCR gene expression data from independent biological replicates. Anal. Biochem. 379, 127–129.
Yegorov, S., Bogerd, J., Good, S.V., 2014. The relaxin family peptide receptors and their ligands: new developments and paradigms in the evolution from jawless fish to mammals. Gen. Comp. Endocrinol. 209, 93–105.
Yegorov, S., Good, S., 2012. Using Paleogenomics to Study the Evolution of Gene Families: origin and Duplication History of the Relaxin Family Hormones and Their Receptors. PLoS One 7, e32923.
Zhang, C., Chua, B.E., Yang, A., Shabanpoor, F., Hossain, M.A., et al., 2015. Central relaxin-3 receptor (RXFP3) activation reduces elevated, but not basal, anxiety-like behaviour in C57BL/6J mice. Behav. Brain Res. 292, 125–132.
Zietek, T., Rath, E., 2016. Inflammation meets metabolic disease: gut feeling mediated by GLP-1. Front. Immunol. 7, 154.