# Physiological Studies on Relaxin Family Peptides in Japanese Quail (Coturnix japonica)

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# （ウズラのリラキシンファミリーペプチドに関する生理学的研究）

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**CONTENTS**

**Chapter I: General introduction** ..... 1

**1.1. Relaxin family peptide** ..... 2

**1.2. Relaxin family peptides receptor** ..... 4

**1.3. Source and Secretion of Relaxin.** ..... 5

**1.4. The Japanese quail (*Coturnix japonica*) as a pilot animal for studying RLN3 functions in birds.** ..... 8

**Chapter II: Expression of Relaxin 3 in the Ovarian Follicle of Japanese Quail** ..... 9

**2.1. Introduction** ..... 10

**2.2. Materials and methods** ..... 10

2.2.1. Animals ..... 10

2.2.2. Cell culture ..... 11

2.2.3. RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR) ..... 12

2.2.4. Quantitative RT-PCR ..... 13

2.2.5. Data analysis ..... 13

**2.3. Results** ..... 14

2.3.1. Tissue distribution of RLN3 ..... 14

2.3.2. Hormonal regulation of RLN3 ..... 14

2.3.3. Spatiotemporal expression of RLN3 in preovulatory follicles ..... 15

**2.4. Discussion** ..... 15

**Chapter III: Expression of Relaxin Family Peptide Receptor 1 and 3 in the Ovarian Follicle of Japanese Quail** ..... 26

**3.1. Introduction** ..... 27

**3.2. Materials and methods** ..... 27

3.2.1. Animals and tissue preparation ..... 27

3.2.2. RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR) ..... 28

3.2.3. Quantitative RT-PCR ..... 29

3.2.4. Zymography ..... 29

3.2.5. Data analysis ..... 30

**3.3. Results** ..... 31

3.3.1. Tissue distribution of RXFP1 and RXFP3 ..... 31

3.3.2. RXFP1 and RXFP3 mRNA expression in the ovarian theca layer before and after LH surge ..... 31

3.3.3. Proteolytic activity in the ovarian follicle ..... 32

**3.4. Discussion** ..... 32

**Chapter IV: General discussion** ..... 41

**Summary** ..... 48

**Acknowledgement** ..... 52

**References** ..... 53

# Chapter I

# General introduction

### 1.1. Relaxin family peptide

Relaxin was discovered in 1926 by Frederick Hisaw, who observed that an injection of serum from pregnant guinea pigs or rabbits into non-pregnant female guinea pigs caused relaxation of the interpubic ligament [1]. The following year, the same relaxing factor was found in the pig corpus luteum and rabbit placenta [2]. In 1930, the relaxing factor was formally named relaxin after it was extracted from pig corpus luteum [3]. For the next 15-20 years, numerous of studies demonstrated that the high levels of circulating relaxin in the blood during pregnancy in mammalian species particularly highlight important roles for relaxin in pregnancy such as expansion of the public ligament of mice [4] and growth of the cervix in rats [5]; and parturition such as relaxation of the uterine myometrium in guinea pigs [6] and cervical softening in cattle [7].

The primary structure of relaxin was identified in 1970 and then, this information was used to clone the first relaxin genes. Cloning of rat [8] and porcine [9] relaxin gene confirmed the peptide structures and showed that the peptides were produced as prohormones with a B-, C-, and A-chain structure like insulin. During processing, the signal peptide and C-domain are cleaved off to produce the mature A-B chains, which are linked together by three disulfide bonds, two between the chain and one within the A chain (Figure 1.1).

The first human gene (H1-RLN) was determined by screening a probe of the porcine relaxin cDNA sequence in human genomic library [10]. After that, the second human gene (H2-RLN) was identified by screening a cDNA library prepared from a human corpus luteum of pregnancy with radiolabeled relaxin-specific cDNA probes corresponding to the human RLN1 gene [11]. Because of the isolating from the corpus luteum, H2-RLN is a luteal cell product and the circulating form of relaxin in human.

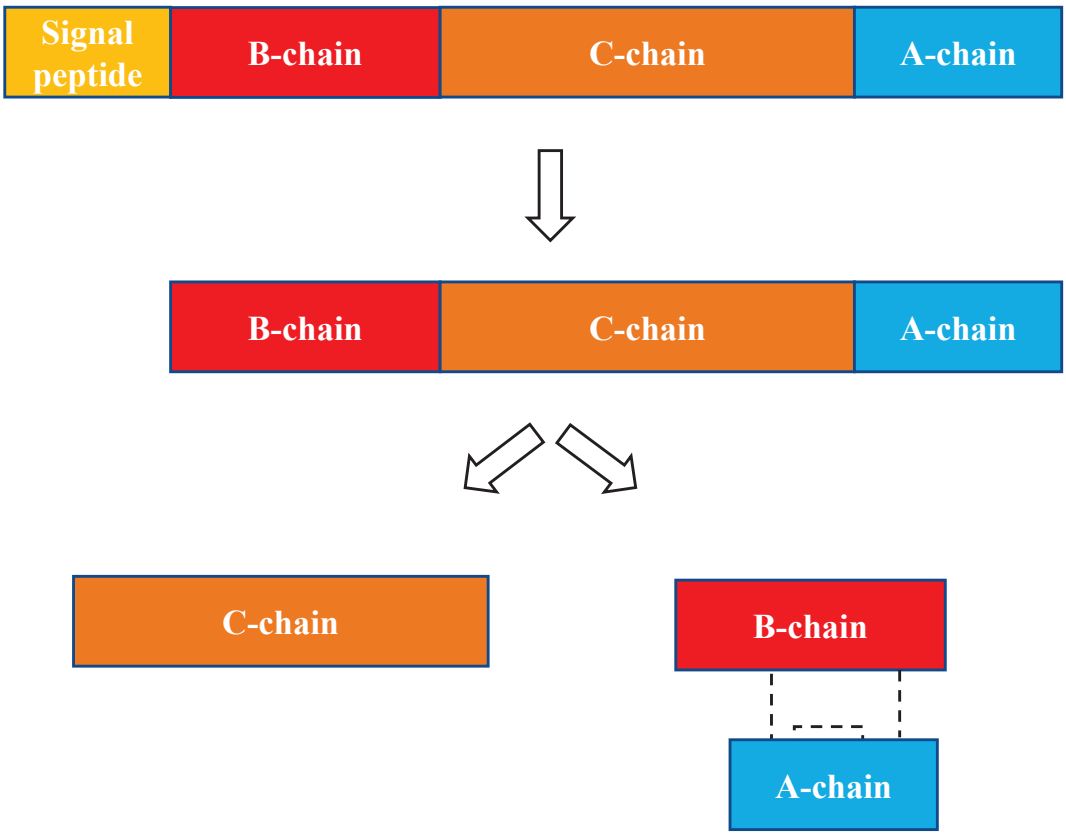


**Figure 1.1. Schematic illustration of the intracellular enzymatic processing of pre-pro-relaxin into its final product**. It was consisted of signal peptide, B-,C- and A-chain and two peptides chain are held together by three disulfide bonds, one within A chain and two between the chain. Ivell *et al*., [12]

Thus, it is the functional ortholog of rat and porcine relaxin. The most recent addition to the related peptides, RLN3 was discovered by screening the human genomic database [13]. The product of RLN3 is highly expressed in the brain and is unlikely to be circulating hormone [14]. Relaxin gene was subsequently clone from several species such as mouse [15] rhesus monkey [16] and chimpanzee [17], confirming the peptide structure and demonstrating that higher primates have three relaxin gene (H1, H2 and H3), while non-primates have two relaxin gene (RLN1, RLN3).

Furthermore, a variety of related peptides were discovered by using the cDNA cloning or the expressed sequenced tag (EST) database. These included insulin-like peptide 3 (INSL3) [18], INSL4 [19] by cDNA cloning, and INSL5 [20] and INSL6 [21] by EST database screening. Since the cloning of the first relaxin gene (RLN1), six additional human relaxin-like genes have been discovered, RLN2, RLN3, INSL3, INSL4, INSL5, and INSL6. Because the human and mouse genomes [22, 23] are now completely sequenced and searches of the databases have failed to identify additional members, it is likely that there are totally seven members of relaxin family peptide.

### 1.2. Relaxin family peptides receptor

Relaxin family peptides receptor 1 (RXFP1) is the cognate receptor for RLN1 or H1/H2-RLN [24]. RXFP2 shares close structural similarity with RXFP1 and is the cognate receptor for INSL3 [25]. Both receptors are characterized by a large extracellular domain consisting of a low-density lipoprotein class A (LDLa) module and ten leucine-rich repeat (LRR) motifs; seven transmembrane that are linked by extracellular and intracellular loops, and an intracellular domain [26]. These have two relaxin binding sites: a high affinity site, present in the LRR motifs, and a secondary low affinity binding site,

located in the transmembrane loops [27]. The presence of the unique LDLa module at the N-terminus of these receptors are essential for activation of the cAMP signaling pathway [28]. By contrast, RXFP3 and RXFP4 are different in structure from RXFP1 and RXFP2 because of the short extracellular domains [29, 30] (Figure 1.2). They are paired with their cognate ligands RLN3 and INSL5 [31, 32]. In addition, RLN1 from some species or H1/H2-RLN can bind to and active RXFP2 [33] and RLN3 will bind to and activate RXFP4 and RXFP1 [34]. However, INSL4 and INSL6 have no interactions with relaxin receptor, their primary receptors are unknown. The binding pair of relaxin and their receptor are summarized in Figure 1.3.

### 1.3. Source and Secretion of Relaxin.

In mammals, the source of RLN in reproductive tissues has been reported in the few past decades. The expression patterns and physiological roles of all receptors generally matches their respective ligands [35]. In pregnant animals, the corpus luteum is the main source of RLN1/RXFP1 in pigs [36], rats [37], and mice [38], but in horse [39], rabbit [40], hamster [41], cat [42], and dog [43], it was found in the placenta. The guinea pig is different in that uterus is the main site of RLN1 production during pregnancy [44]. As for non-pregnant animals, RLN1 levels are relatively low and localized to the luteal cells of the ovary [45]. As for other RLN family peptides, RLN3 is mostly expressed in brain [46]. It could be suggested that RLN3 is a neuropeptide in mammals. More detailed evidence is showed RXFP3 expression in the brain, suggesting that this receptor may have therapeutic targets [47-51].

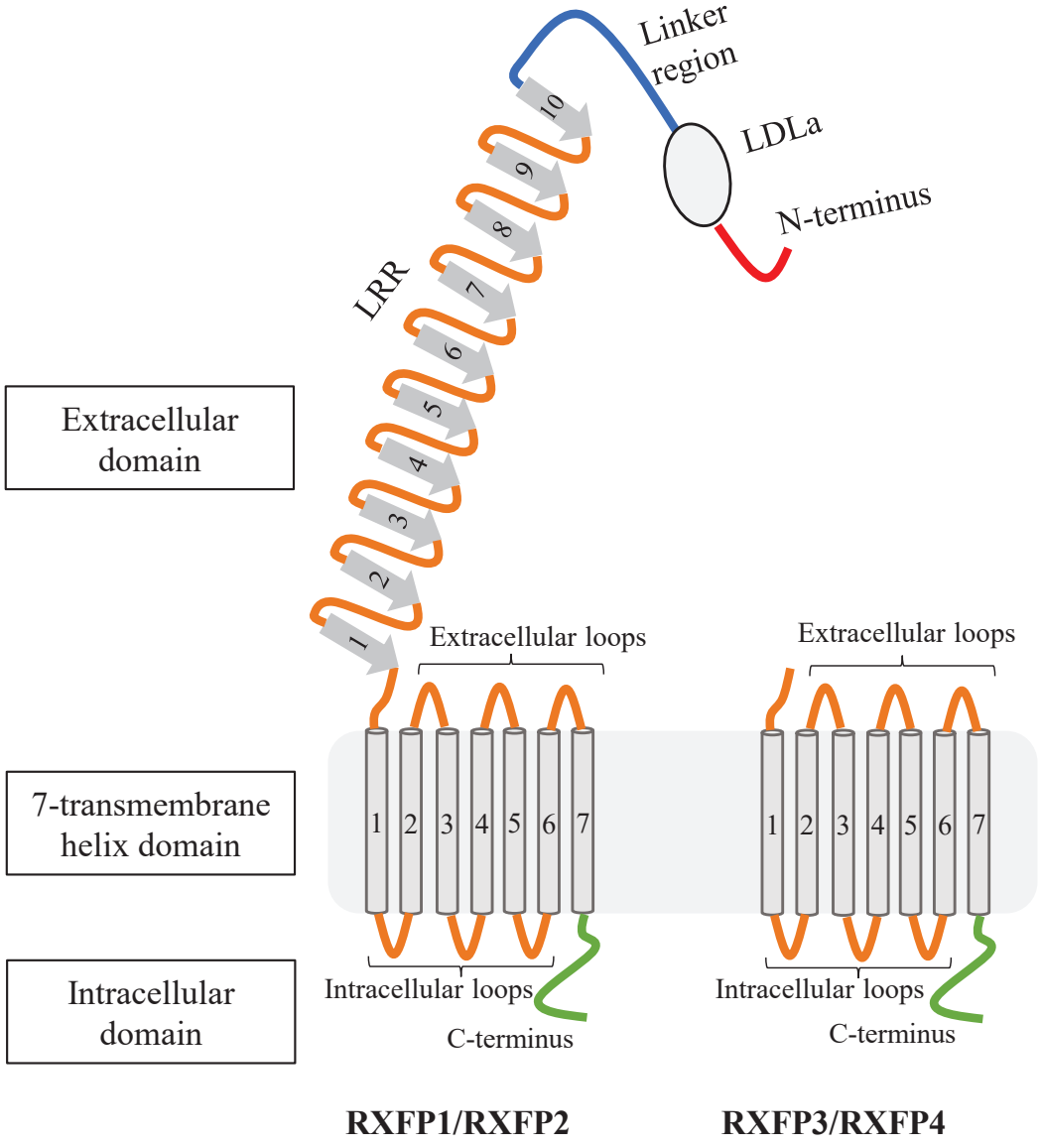


**Figure 1.2. Schematic illustration of structure of relaxin family peptide receptor**. RXFP1/RXFP2 molecule exposes a large extracellular domain that is constituted by the low-density lipoprotein class A (LDLa) domain and leucine-rich repeat (LRR) domain which are connected via a linker region. A membrane domain comprising of seven transmembrane that are linked by extracellular and intracellular loops, and an intracellular domain. RXFP3/RXFP4 molecule consisting only transmembrane domain and cytoplasmic domain. Speck *et al*., [52].

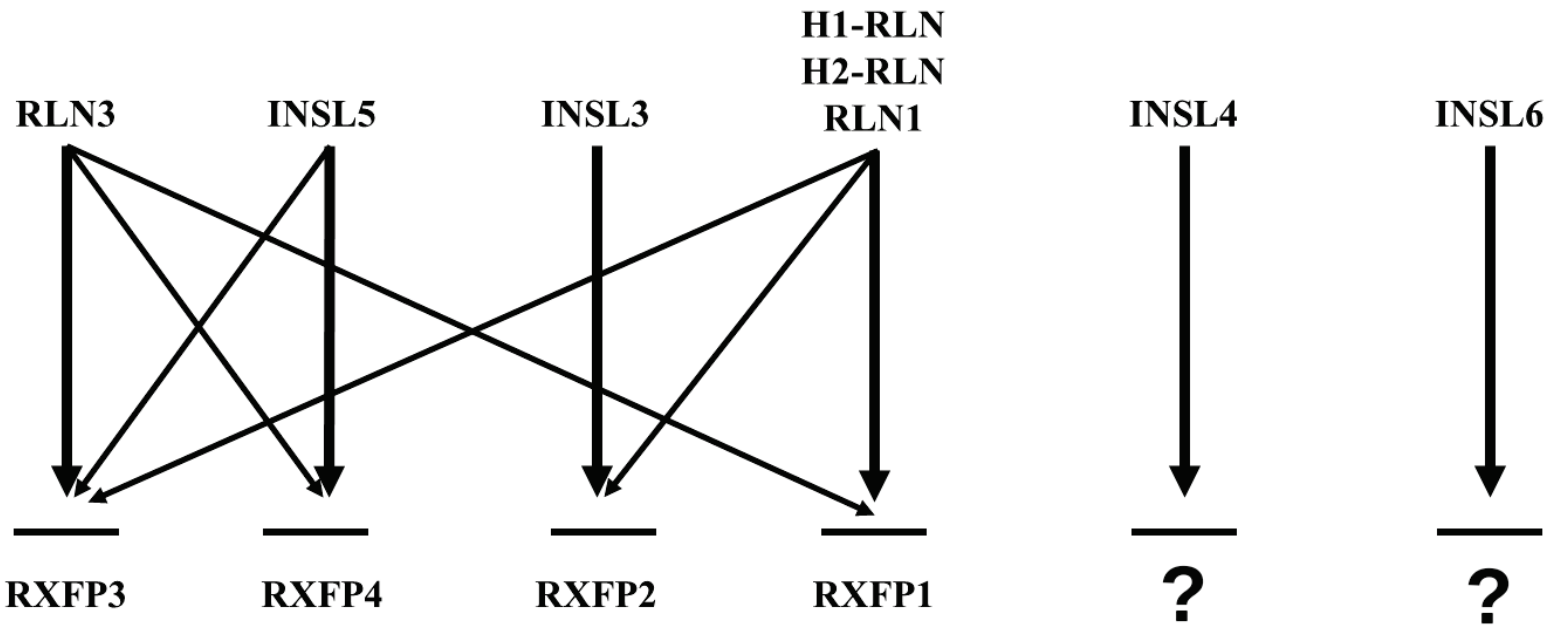


**Figure 1.3. Ligand-receptor relationships for the relaxin family peptides and their cognate receptors.** The thickness of the arrows reflects the affinity and specificity of the interaction. Ivell *et al*., [12].

### 1.4. The Japanese quail (*Coturnix japonica*) as a pilot animal for studying RLN3 functions in birds.

In birds, RLN-like activity has been reported in the ovaries [53, 54] and testis [55] of chickens. More recently, RLN3 expression was reported in the granulosa and theca layers of chicken ovaries [56]. However, the detail information on expression profile and function of RLN3 in birds is unknown. Japanese quail (*Coturnix japonica*) is an inbreeding subspecies of the genus *Coturnix* and belongs to the family *Phasianidae*, which is related to the chicken [57]. Japanese quail have serval advantages: small body, size, short generation interval, inexpensive rearing requirements, and high egg production [58]. To date, they have been widely employed as a useful model in embryology, endocrinology, genetics, nutrition, pathology, and physiology [59]. Thus, Japanese quail was provided as a valuable animal for avian research. They are extremely useful models for studying the physiological roles of RLN3 in bird reproductive tracts.

Therefore, a significant step towards evaluating the physiological roles of RLN3 in Japanese quail, would be determine whether the ovarian follicle acts as source and target tissue of RLN3. It will be also investigated the involvement of RLN3 during ovulation. In detail, this study aims at examining:

**1.** Expression of RLN3 in the ovarian follicle of Japanese quail

**2.** Expression of relaxin family peptide receptor 1 and 3 in the ovarian follicle of Japanese quail.

# Chapter II

# Expression of Relaxin 3 in the Ovarian Follicle of Japanese Quail

## 2.1. Introduction

The knowledge of RLN family peptides in avian species remains limited. More recently, it has been suggested that RLN3 secreted into the granulosa and theca layer of chicken ovary might function as a potential role in follicular development [56]. However, no studies have elucidated the hormonal regulation and physiological role of RLN3 in birds.

The objectives of this study were to investigate whether the ovary serves as a source of RLN3 in Japanese quail and characterized RLN3 expression in different-sized follicles. In addition, the granulosa layer was cultured with various steroid hormones to study the endocrine control of RLN3 expression. Moreover, the possible involvement of RLN3 was considered in the study during the ovulation process.

## 2.2. Materials and methods

### 2.2.1. Animals

Female Japanese quail (*Coturnix japonica*), six weeks of age, were obtained from Quail-Cosmos (Tashara, Japan). They were individually caged under a photoperiod of 14 h light: 10 h dark (lights on at 5:00) and provided with water and a commercial quail diet (Toyohashi-shiryo, Toyohashi, Japan) *ad libitum*. Almost all birds laid eggs regularly, ranging from 15:00 to 18:00 every day. The birds were monitored for time of oviposition which was recorded manually every 1 h between 15:00 and 18:00; the ovulation was assumed to occur 15–30 min after the time of oviposition. For gene expression analysis, various tissues were harvested from egg-laying quail at 15-30 weeks of age following sacrifice by cervical dislocation approximately 8–10 h before the expected time of

ovulation. The harvested tissues were frozen and stored at −80℃ until RNA extraction. For isolation of the granulosa layer, the ovarian follicles were removed from the birds 8 h before the expected time of ovulation and placed in phosphate-buffered saline (pH 7.4). The granulosa layer (granulosa cells, inner layer of the vitelline membrane, and basal lamina) was isolated from the theca layer according to the procedure reported by Gilbert *et al* [60]. Granulosa layers were obtained from the largest (F1), second largest (F2), and third largest (F3) follicles. To determine the detailed localization of RLN3 mRNA expression in the follicles, the F1 follicle was collected, and the theca and granulosa layers were separated as described above. The stigma (S), nonstigma (NS), and germinal disc (GD) regions of the granulosa layer were isolated as described by Jackson *et al* [61].

All experimental procedures for the use and care of animals in the present study were approved by the Animal Care Committee of the Faculty of Agriculture at Shizuoka University (approval number: 2018A-5).

### 2.2.2. Cell culture

The granulosa layers isolated from the three largest follicles (F1, F2, and F3) were placed into a six-well culture plate (Corning Incorporated – Life Sciences, Durham, NC, USA) filled with 3 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich Inc., St. Louis, MO, USA). The medium was supplemented with various steroid hormones, such as estradiol-17β, progesterone, and testosterone, to final concentrations of 0, 10, 100, and 1000 nM. These hormones were dissolved in 100% ethanol and added into the medium, its final concentration was never higher than 0.1% [62]. After incubation at 41℃ a humidified atmosphere of 5% $CO_2$ for the 6h, the granulosa layer was collected and stored at −80℃ for RNA extraction.

### 2.2.3. RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted from various tissues using the extraction reagent, RNAiso Plus (Takara Bio Inc., Shiga, Japan). After dissolving the resulting RNA pellet in RNase-free water, the concentration and purity were determined by absorption at 260 and 280 nm using a spectrophotometer (DS-11 Series Spectrophotometer, DeNovix Inc., Wilmington, DE, USA). Only RNA samples exhibiting an A260/280 ratio of 1.8 or greater were used for reverse transcription. Aliquots of 1 $\mu$ g of total RNA were used for cDNA synthesis in reaction mixtures (10 $\mu$ l) using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan).

Amplification of quail RLN3 (GenBank accession no. XM_015848927) was carried out using the specific primer set: 5′-GAAGCGGCTCTCCCTACTG-3′ (forward) and 5′-GGAGCAGATGCAGAGAAGCA-3′ (reverse). The specific primer set: 5′-GGCGCGGGTGATCATCGAGAA-3′ (forward) and 5′-GAGAGCGCCTCGTGGTGTTT-3′ (reverse) for the gene encoding quail S17 ribosomal protein (GenBank accession no. XM_015872709) was employed for normalization of the data. All PCR reactions were carried out in reaction mixtures (50 $\mu$ l) containing 5 mmol/ml $MgCl_2$, 0.4 mmol/ml dNTP, and 2.5 U Taq DNA polymerase (Takara Bio Inc.). The PCR protocol consisted of an initial denaturation for 2 min at 94℃, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 20 s, then a final extension step at 72℃ for 2 min. For the S17 primer pair, annealing at 53.4℃ was performed, and 25 cycles were employed for application. The PCR product was separated on 1% agarose gels containing ethidium bromide (1 $\mu$ g/ml) and visualized under UV transillumination. For quantitative evaluation, the intensity of the PCR product

bands was measured using ImageJ software (https://imagej.nih.gov/ij/index.html). The results were expressed as the RLN3 mRNA/S17 ribosomal protein mRNA ratio.

### 2.2.4. Quantitative RT-PCR

The primers and fluorescent probes (Table 2.1) for quail RLN3 and the S17 ribosomal protein gene were constructed to be within in the purified PCR product generated by RT-PCR, as described above. RLN3 mRNA quantitation of test samples were performed in conjunction with a standard curve (Figure 2.2) for each gene obtained by amplifying a 10-fold dilution series of purified PCR products. Quantification of gene expression by RT-PCR analysis was performed using a thermal cycler (DICE Real-Time System III; Takara Bio Inc.). PCR was carried out following reaction mixtures (50 $\mu$ l) containing template DNA, 400 nM TaqMan probes, 1X Probe qPCR Mix (Takara Bio Inc.), and 200 nM of each specific primer. Amplification and detection of the samples and standards were performed using a thermal profile setup: 5 min at 94℃ followed by 45 cycles of 10 s at 94℃, 10 s at 57℃, and 10 s at 72℃. The expression level of RLN3 was normalized to that of the S17 ribosomal protein gene.

### 2.2.5. Data analysis

All experiments were repeated 3-5 times. Normality and homoscedasticity of the data were confirmed using the Shapiro–Wilk test and Bartlett test, respectively, Differences between two groups were analyzed using the F-test followed by the Student's t-test. Differences between multiple groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ (student's t-test) was considered to reflect a statistically significant difference. Data are presented as the mean ± standard

error of the mean (SEM).

## 2.3. Results

### 2.3.1. Tissue distribution of RLN3

To investigate the tissue distribution of RLN3, various tissues from egg-laying birds was isolated and performed semi-quantitative RT-PCR analysis. As shown in Figure 2.1, RLN3 was mainly expressed in the granulosa and theca layers, with relatively weak expression being detected in a wide range of tissues except for any parts of the oviduct, or the vagina and pancreas. Next, the change in RLN3 mRNA expression during follicular development was investigated. As shown in Figure 2.3, higher expression was observed in the granulosa layer than in the theca layer in all follicles; in addition, the expression in the granulosa layer increased with increased follicle size.

### 2.3.2. Hormonal regulation of RLN3

To elucidate the hormonal regulation of RLN3 expression, granulosa layers obtained from F1, F2, and F3 were incubated for 6 h with 0, 10, 100, and 1000 nM of estradiol-17β, progesterone, or testosterone, and the expression of RLN3 was measured by quantitative RT-PCR analysis. As shown in Figure 2.4, the addition of 1000 nM estradiol-17β increased the RLN3 mRNA expression in all follicles whereas the addition of more than 10 nM progesterone suppressed RLN3 transcription. The addition of testosterone into the medium did not affect RLN3 expression in F2 and F3 but tended to decrease RLN3 expression at lower concentrations in F1. The expression of RLN in F1 was restored at higher testosterone concentrations.

### 2.3.3. Spatiotemporal expression of RLN3 in preovulatory follicles

To investigate the spatiotemporal expression of RLN3 mRNA, samples were separately isolated from granulosa and theca layers 8 h before the expected time of ovulation and immediately after oviposition, which were assumed to be before and after the LH surge, respectively. Furthermore, the expression in the granulosa layer around the S, NS, and GD regions of the granulosa layer were also quantitated. The expression of RLN3 mRNA in the granulosa and theca layers were shown to be significantly decreased after the LH surge compared with that before the LH surge (Figure 2.5). In addition, the highest expression was observed in the S region of the follicle, with this expression significantly decreasing after the LH surge. Relatively lower expression levels were observed in the NS and GD regions; these did not exhibit a similar reduction in expression (Figure 2.6).

## 2.4. Discussion

This present study demonstrates the profiles of RLN3 expression in mature female quail. It has discovered that 1) RLN3 expression was detected in the granulosa layer of the ovarian follicle and gene expression increased during follicular development; 2) RLN3 expression was enhanced by estradiol-17β whereas it was suppressed by progesterone; and 3) RLN3 expression was significantly higher in the S region than that in the other regions of the granulosa layer of the F1 follicle, with this expression decreasing following the LH surge.

Ghanem *et al* [56] reported that the expression of RLN3 mRNA in chicken ovaries was highest in the granulosa layer and significantly increased during follicular

development. Although these authors did not measure the expression levels in mature follicles (*i.e.,* F1, F2, or F3), these data suggesting that the main source of RLN3 in Japanese quail is the granulosa layer of mature follicles are consistent with the results from chicken. In pig, it was reported that the non-pregnant or immature pigs expressed a large amount of RLN protein and mRNA in the largest preovulatory follicles [45], which may be linked to the fact that the changes in RLN3 mRNA expression correspond with the developmental stage of the follicles.

In granulosa layer cultures, the data has shown that estradiol-17β stimulated RLN3 expression and that the effect of estradiol-17β was greater in the larger follicles than in smaller ones (F3<F2<F1). In female birds, estradiol-17β plays an important role in reproduction by stimulating follicular development [63]. Thus, the elevated effects of estradiol-17β on RLN3 expression during follicular development may reflect the role of estradiol-17β -stimulated RLN3 expression on the process of follicular growth. In contrast, it has displayed that the addition of testosterone in the culture medium showed no obvious effects on RLN3 expression in F3 and F2. Rather, testosterone suppressed RLN3 expression at lower doses (10 and 100 nM), whereas 100 nM testosterone resorted RLN3 expression at the highest concentration in F1 follicles. Although the cause of the distinction in F1 is unknown, it could be considered that testosterone may act as a negative regulator of RLN3 expression in avian species.

In female birds, testosterone is synthesized and secreted from the theca interna of the medium-sized follicles and is converted to estradiol-17β by aromatase in the theca externa [64, 65]. The increase in circulating testosterone that occurs approximately 6 h before ovulation is the result of secretion from the four largest follicles [63]; the follicular venous plasma of the fourth to second largest follicles contains a significantly higher

concentration of testosterone than that in peripheral plasma [66, 67]. Similarly, preovulatory follicle secretion of estradiol-17β is greatest from the third and fourth largest follicles 3–6 h prior to ovulation [63], with the concentrations of estradiol-17β in follicular venous plasma also being higher than those in peripheral plasma [66,67]. Although the effective concentrations of estradiol-17β and testosterone in granulosa cell culture were much higher than those of the circulating hormones, it is possible that the local concentrations of these hormones around the granulosa cells may also be much higher than the blood hormone concentrations.

In opposition to the stimulatory effects of estradiol-17β, RLN3 expression was suppressed by progesterone. In particular, RLN3 expression in the granulosa layers of all tested quail follicles was decreased by the addition of progesterone. In hens, 4–6 h before ovulation, the amount of progesterone produced by granulosa cells in the F1 follicle increased in response to LH, suggesting that LH stimulated progesterone production by the granulosa layer [63], concurrent with the decrease in RLN protein expression in an autocrine manner [45]. This assumption is also supported by the observation that the granulosa layer isolated from large follicles is particularly sensitive to LH because the number of receptors for LH increases as the follicles mature [68].

This study found that RLN3 mRNA expression significantly decreased after the LH surge compared with that before the LH surge. This result is consistent with culture experiments showing that the addition of progesterone suppressed RLN3 expression. Moreover, these data have shown that RLN3 expression in the stigma region of the granulosa layer of the F1 follicle significantly decreased after the LH surge. Jackson *et al* [61] reported that plasminogen activator, a proteolytic enzyme, plays an essential role in the extracellular matrix remodeling required for follicular development and ovulation.

Although this study did not assess either plasminogen activator or protease, the data has been showing the dramatic decline of RLN3 expression in the stigma region after the LH surge, which may relate to the process of ovulation. Notably, the expression of RLN family peptide receptor 1 (RXFP1), which is an authentic receptor for RLN3, was detected in the theca layer but not the granulosa layer in hen ovaries [56]. Therefore, it could be hypothesized that RLN3 expressed by the granulosa layer may stimulate the theca layer by binding with RXFP1, resulting in the alteration of protease activity and expression to promote ovulation.

In conclusion, this study provide evidence for the novel function of RLN gene family peptides in avian species. The results indicated that the granulosa layers in mature follicle express RLN gene, with the expression being highly localized in the stigma region. Although, the roles of RLN on protease expression or activity were not examined, it was hypothesized that RLN3 may be involved in ovulation tissue remodeling and ovulation because RLN expression dramatically declined after the LH surge.

**Table 2.1 List of primers and probes for Japanese quail target genes**

| Gene | Name | Sequence (5' to 3') | GenBank accession No. |
|---|---|---|---|
| RLN3 | Forward primer | GAATACGAGCCTGTGGCAGA | XM_015848927.2 |
| | Reverse primer | TAACTGCTGGGTCCTAGGGG | |
| | Probe | [FAM] AGTTCAAAGAACTTCTTCGCCAGGCAGAG [TAMRA] | |
| S17 | Forward primer | AACGAGAGCGCAGGGATAAC | XM_015872709.2 |
| | Reverse primer | CGTCACCTGAAGGTTGGACA | |
| | Probe | [FAM] CGTACCCGAGGTCTCTGCTCTTGATCAGGA[TAMRA] | |

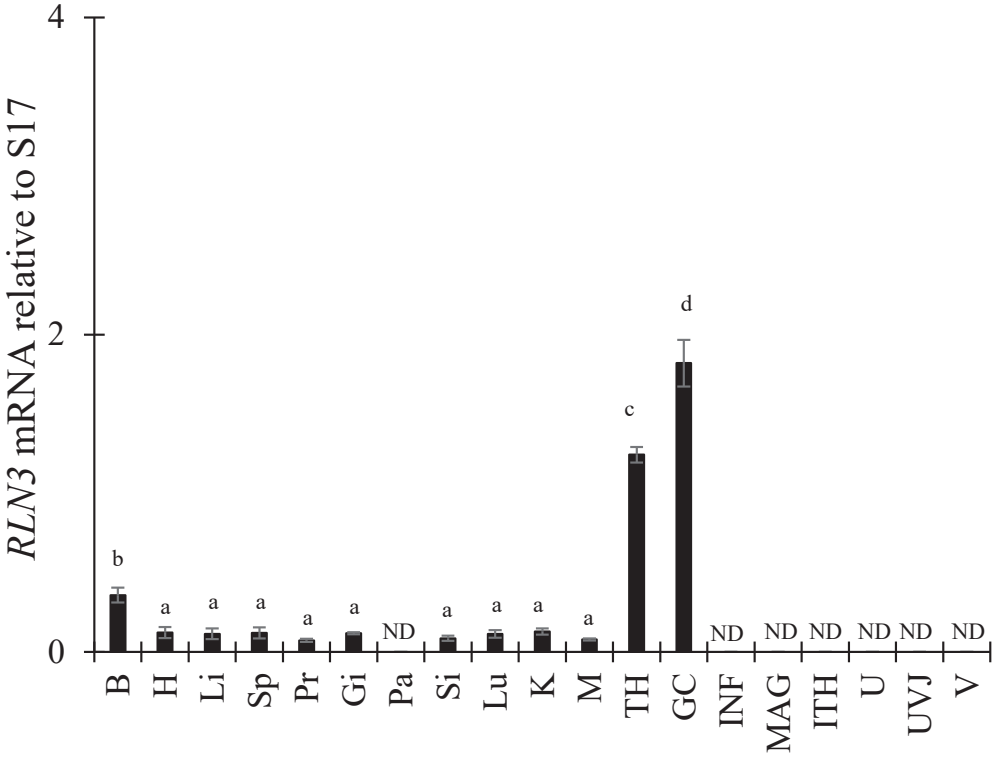


**Figure 2.1. Tissue distribution and RLN3 mRNA expression in Japanese quail**. Semi quantitative RT-PCR analysis was performed using total RNA from various tissue. The band intensities were normalized to those of the S17 ribosomal protein gene. Values represent the mean ± SEM from five animals for each tissue; value with different letters are significantly different ($P<0.05$). ND, not determined. B, brain; H, heart; Li, liver; Sp, spleen; Pr, proventriculus; Gi, gizzard; Pa, pancreas; Si, Small intestine; Lu, lung; K, kidney; M, muscles; TH, theca layer; GC, granulosa layer; INF, infundibulum; MAG, magnum; ITH, isthmus; U, uterus; UVJ, uterovaginal junction; V, vagina.

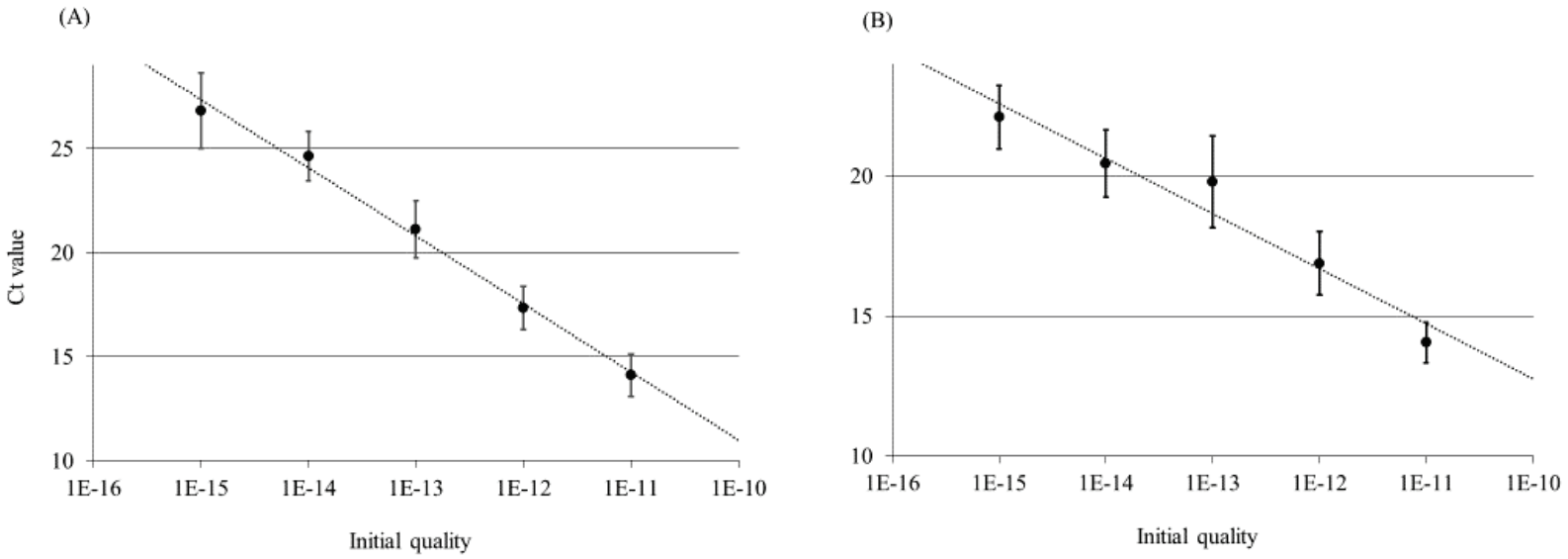


**Fig 2.2.** The standard curve of RLN3 (A) and S17 (B) ranging from $10^{-11}$ to $10^{-15}$ for real-time RT-PCR quantification. Each point represents the mean ± SD of 5 independent experiments.

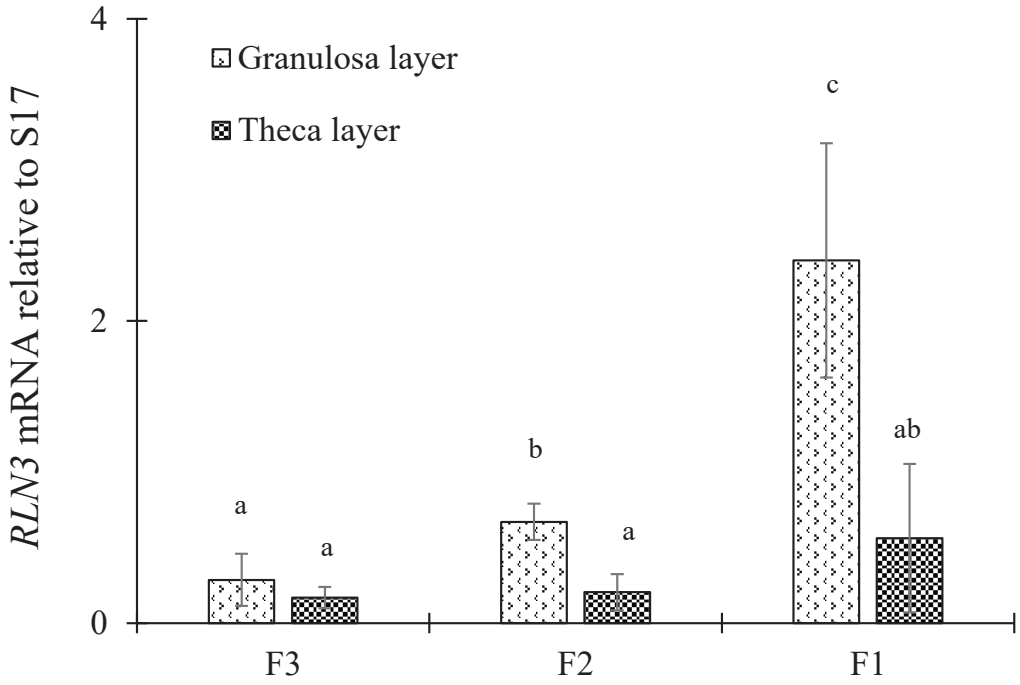


**Figure 2.3. Changes in RLN3 mRNA expression during follicular development**. Granulosa layers obtained from the largest (F1), second largest (F2), and third largest (F3) follicles were isolated 8-10 h before the expected time of ovulation. The tissues were extracted for RNA and expression was measured by quantitative RT-PCR. Values represent the mean ± SEM of triplicate experiments; value with different letters are significantly different ($P<0.05$).

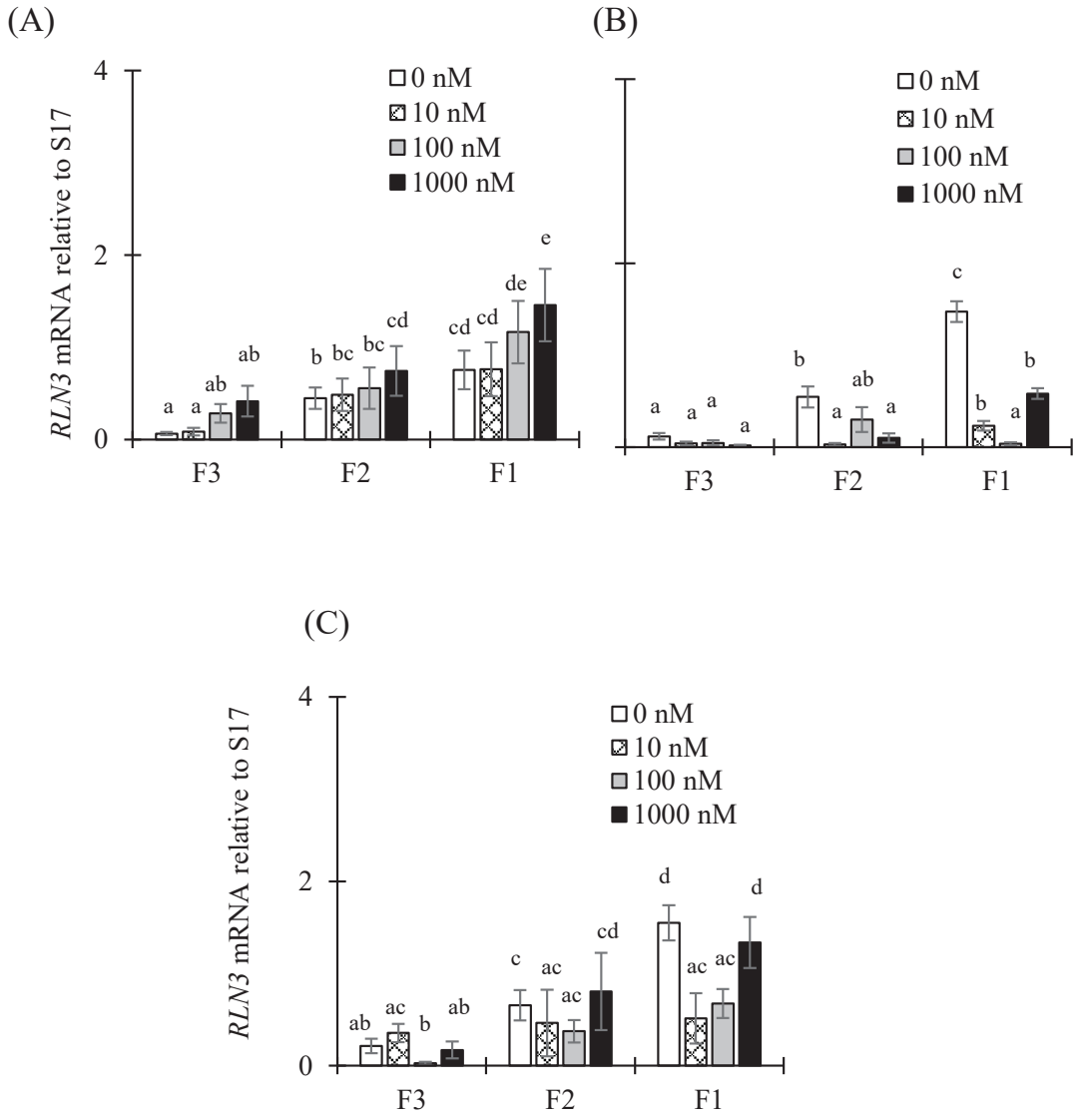


**Figure 2.4. Effect of various steroid hormones on RLN3 mRNA expression**. The granulosa layers of the largest (F1), second largest (F2), and third largest (F3) follicles were cultured with 0, 10, 100, and 1000 nM of (A) estradiol-17β ($E_2$), (B) progesterone ($P_4$), or (C) testosterone (T) for 6 h. After incubation, granulosa layers were collected to extract RNA, and expression was measured by quantitative RT-PCR.. Values represent the mean ± SEM of triplicate experiments; value with different letters are significantly different ($P< 0.05$).

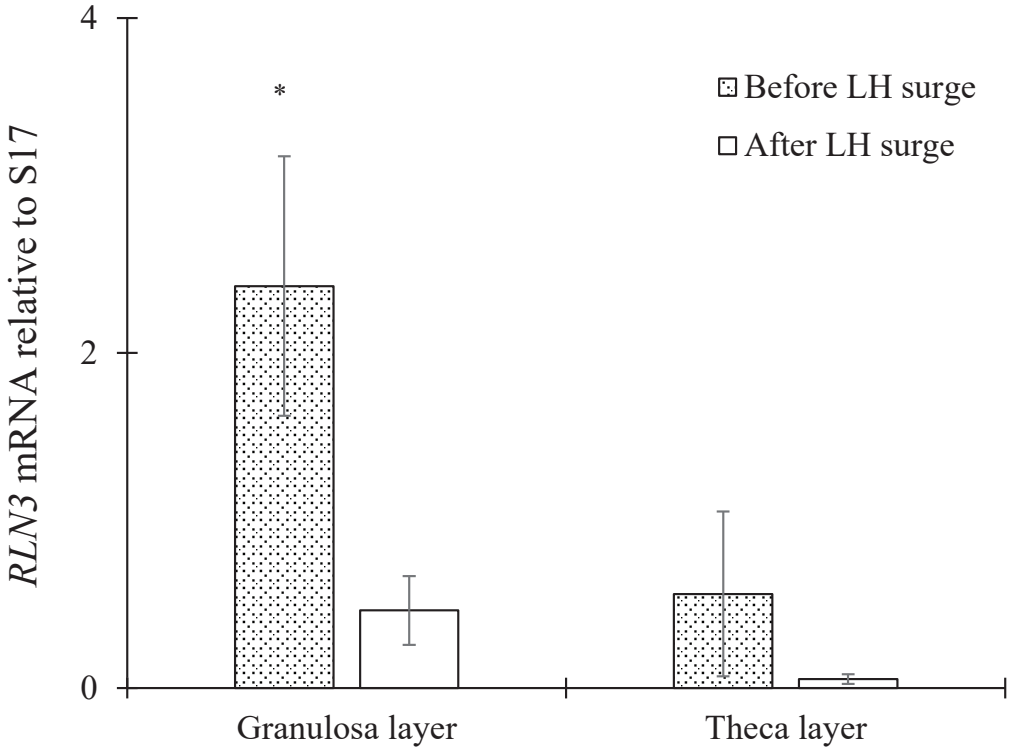


**Figure 2.5. Changes in RLN3 mRNA expression before and after the LH surge**. Granulosa layers and theca layers obtained from the largest (F1) follicle were isolated 8-10 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge). The tissues were extracted for RNA and expression was measured by quantitative RT-PCR. Values represent the mean ± SEM of triplicate experiments; value with different letters are significantly different ($P<0.05$). LH, luteinizing hormone.

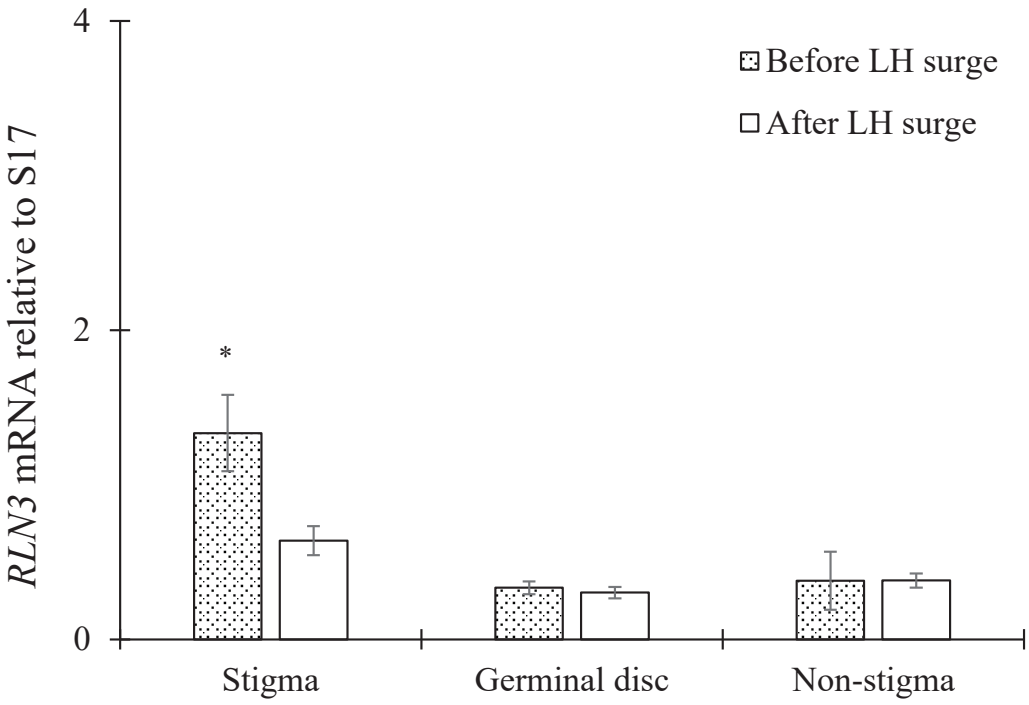


**Figure 2.6. RLN3 mRNA expression in the different regions of the granulosa layer before and after the LH surge**. The F1 follicle was collected 8-10 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge). The stigma, non-stigma, and germinal disc regions of the granulosa layer were separated. RNA was extracted from the tissues and the expression was measured by quantitative RT-PCR. Values represent the mean ± SEM of triplicate experiments; value with different letters are significantly different ($P<0.05$). LH, luteinizing hormone.

# Chapter III

# Expression of Relaxin Family Peptide Receptor 1 and 3 in the Ovarian Follicle of Japanese Quail

## 3.1. Introduction

In Chapter II, it was investigated the expression profile of RLN3 in Japanese quail and demonstrated that mRNA expression increases in granulosa cells during follicular development, especially in the stigma region prior to an LH surge. Thus, RLN3 may be involved in the processes of the follicular maturation and ovulation in Japanese quail. Recently, from quantitative RT-PCR analysis, RXFP1 and RXFP3 mRNA expression were detected in the theca layer and ovarian stroma, respectively [56]. Although RXFP1 and RXFP3 are known to be expressed in the chicken ovary, there is a lack of knowledge regarding the relaxin and its receptor. Therefore, this chapter focuses on the examining the expression of relaxin receptors in Japanese quail.

## 3.2. Materials and methods

### 3.2.1. Animals and tissue preparation

Female Japanese quail (Coturnix japonica), 15-30 weeks of age (Quail-Cosmos, Tahara, Japan), were individually caged under a photoperiod of 14 h light: 10 h dark (lights on at 5:00) and provided with water and a commercial quail diet (Toyohashi-shiryo, Toyohashi, Japan) *ad labium*. Almost all birds laid eggs regularly at the same time each day (15:00 to 18:00). The birds were monitored for the time of oviposition, which was recorded manually every 1 h between 15:00 and 18:00; ovulation was assumed to occur 15-30 min after the time of oviposition. For gene expression analysis, approximately 8-10 h before the expected time of ovulation, quail were killed by cervical dislocation. Then, the tissues were harvested and stored at -80℃ until RNA extraction. The three largest follicles (F1, F2, and F3) were also removed and placed in phosphate-buffered saline (pH

7.4). The granulosa and theca layers were cleanly separated by using the procedure of Gilbert *et al* [60]. All experimental procedures for the use and card of animals in the present study were approved by the Animal Care Committee of the Faculty of Agriculture at Shizuoka University (approval number: 2018A-5).

### 3.2.2. RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted by using the extraction reagent, RNAsio Plus (Takara Bio Inc., Shiga, Japan). The concentration and purity of the RNA were determined by absorption at 260 and 280 nm using a spectrophotometer (DS-11 Series Spectrophotometer, DeNovix Inc., Wilmington, DE, USA). Only RNA samples exhibiting an A260/280 ratio of 1.8 or greater were used for reverse transcription. Aliquots (1 $\mu$ g) of total RNA were used for first-strand cDNA synthesis in reaction mixtures (10 $\mu$ l) using oligo(dT) primer with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan).

The specific primer set: 5′- TGCGCAGCTGTAAGCCTAAT -3′ (forward) and 5′- TGGCGTCTGTTTCCCTCTTC -3′ (reverse) for quail RXFP1 (GenBank accession no. XM_015860873) was employed. Amplification of quail RXFP3 (GenBank accession no. XM_015848444) was conducted using the specific primer set: 5′-TCCCCCACTGAGAATGGGAT-3′ (forward) and 5′-TTTCAACTGGCTCTTCGGCA-3′ (reverse). The forward primer set of 5′-GGCGCGGGTGATCATCGAGAA-3′ and reverse primer set of 5′-GAGAGCGCCTCGTGGTGTTT-3′ were designed according to quail S17 ribosomal protein, which deposited under GenBank accession number XM_015872709. PCR amplification of RXFP1, RXFP3 and S17 transcripts was performed using 1 $\mu$ l of cDNA and separate reactions with a different number of cycles,

in a final reaction volume of 50 $\mu$ l in the presence of 5 mmol/ml $MgCl_2$, 0.4 mmol/ml dNTP, 2.5 U Taq DNA polymerase (Takara Bio Inc.), and the relevant specific primers. After initial denaturation for 2 min at 94℃, a variable number of amplification cycles were performed at 94, 60 and 72℃ (30 s each) for RXFP1; 94 and 58 ℃ (30 s each), followed by 72℃ (45 s) for RXFP3; and 94 and 53.4℃ (30 s each), followed by 72℃ (20 s) for S17. The amplifying RXFP1, RXFP3 and S17 were employed by using 35, 40 and 25 cycles, respectively. Amplification was followed by a final extension step at 72℃ for 2 min. Then, the PCR product was electrophoresed on 1% agarose gels containing ethidium bromide (1 $\mu$ g/ml) and visualized under UV transillumination. For quantitative evaluation, the intensity of the PCR product bands was measured using ImageJ software (https://imagej.nih.gov/ij/index.html). The results were expressed as the RXFP1 and RXFP3 mRNA /S17 ribosomal protein mRNA ratio.

### 3.2.3. Quantitative RT-PCR

For the quantification of gene expression by quantitative PCR analysis sets of primers and probes (Table 3.1) were constructed to be within the purified PCR product generated by RT-PCR, as described above. Quantification of the RXFP1 and RXFP3 mRNA test samples was performed in conjunction with a standard curve for each gene by amplifying a 10-fold dilution series of purified PCR products. Amplification and detection of the samples and standards were performed as previously described in Chapter II.

### 3.2.4. Zymography

Theca layers were isolated from the F1 follicle 8 h before the expected time of ovulation (before LH surge) and 2 h before the expected time of ovulation (after LH

surge), and then homogenized in ice-cold PBS supplemented with 0.5 $\mu$ g/ml leupeptin, 10 $\mu$ g/ml soybean trypsin inhibitor (SBTI), 1 mM phenyl-methyl-sulfonyl fluoride (PMSF), and 1 mM EDTA [69, 70]. The homogenates were centrifuged at 20,000 x *g* for 10 min to the remove cellular debris, and the supernatant, referred to as theca layer lysates, was divided into aliquots and stored at -80℃ until use. The concentration of the protein in the extracts was determined using the Bradford Protein Assay kit (Bio-Rad) as described by the manufacturer.

For gel electrophoresis, the theca layer lysates (16 $\mu$ g protein) were separated using an 8% SDS-PAGE gel containing 0.1% gelatin according to the procedure described in the previous report [70]. After the electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min. Then, the gels strips were excised along the lane casting, and each gel was incubated with 0.1 M glycine (pH 8.0) buffer containing 0.5 $\mu$-g/ml leupeptin, 50 $\mu$ g/ml SBTI, 1 mM PMSF, 1 mM EDTA and 10 $\mu$ M MG132 at 4℃ for 1 h with gentle agitation. After the incubation, the gel strips were incubated overnight at 37℃ to promote the enzyme reaction. After the reaction, gel strips were stained with Coomassie Brilliant Blue and the halo formation in the gels was observed.

### 3.2.5. Data analysis

All experiments were repeated 3 times. The normality of the gene expression data was evaluated using the Shapiro-Wilk test, and subsequently, the homoscedasticity was examined using the F-test (comparisons between two groups) or Bartlett test (multiple comparisons). The expression levels of RXFP1 and RXFP3 in each tissue and at each follicular stage were analyzed using the Steel-Dwass test as a non-parametric multiple comparison method. The RXFP1 and RXFP3 levels before and after the LH surge were

compared using Student's t-test because of their normality and homoscedasticity. $P<0.05$ was considered to represent a significant difference. Data are presented as the mean ± standard error of the mean (SEM).

## 3.3. Results

### 3.3.1. Tissue distribution of RXFP1 and RXFP3

The expression of RXFP1 and RXFP3 mRNA were investigated by semi-quantitative RT-PCR analysis of total RNA extracted from the various tissues of mature female quail. As shown in Figure 3.1A, RXFP1 mRNA ubiquitously expressed, and the highest expression was seen in the theca layer. Conversely, the expression of RXFP3 mRNA was relatively low in all tissues, and a heightened expression was not detected in the ovary (Figure 3.1B). Next, the change in RXFP1 and RXFP3 mRNA expression changed in theca layer during follicular development were examined. As shown in Figure 3.3, a lower expression of RXFP1 mRNA was observed in the F3. The gene transcription was dramatically increased in F2 and the higher expression was maintained in the largest follicle (Figure 3.3A). Further, the expression of RXFP3 mRNA in the theca layer was remained low throughout the follicular development (Figure 3.3B).

### 3.3.2. RXFP1 and RXFP3 mRNA expression in the ovarian theca layer before and after LH surge

This experiment was compared the expression levels of RXFP1 and RXFP3 in F1 follicle of theca layer 8-10 h before the expected time of ovulation and immediately after oviposition, which were assumed to be before and after the LH surge, respectively. Figure

3.4 shows that RXFP1 mRNA expression in theca layers significantly decreased after the LH surge compared with that before the LH surge, whereas no such reduction in the expression of RXFP3 was observed.

### 3.3.3. Proteolytic activity in the ovarian follicle

The protease activity in the theca layer lysates was detected by using zymography and the banding pattern and the protease activity was compared before and after LH surge. As shown in Figure 4A, high molecular weight protease was detected in the sample derived from before LH surge. This band was also detected in the sample isolated after LH surge. In addition to the high molecular weight band, a clear protease band migrating around 60 kDa in molecular weight was observed after LH surge. The activity of the 60 kDa protease was inhibited in the presence of SBTI, PMSF and leupeptin, whereas no such inhibitory effects were found when the gel strip was incubated with EDTA and MG132. Moreover, all the protease inhibitors we tested failed to inhibit the protease activity of the high molecular weight band.

## 3.4. Discussion

The present study investigated the expression profiles of relaxin receptor such as RXFP1 and 3, in mature female quails. The results suggested that RXFP1 expression is higher in the ovarian theca layer, but no such ovarian-specific expression was seen for RXFP3, indicating that RXFP1 mediates relaxin signaling in the quail ovary. This expression pattern is different from that of mammalian species such as mice, rats, macaques, and humans, which are expressed mainly in the brain [13, 22, 48, 51]. In addition, mammalian RLN3 bind with high affinity to RXFP3 and a lower affinity to

RXFP1 [12, 71]. Although there is no report regarding RLN3 binding to its receptors in birds, mammalian RXFP1 is reported to couple with Gs protein, suggesting its stimulatory effects through a cAMP-mediated signaling pathway [27, 72, 73] whereas RXFP3 is linked to Gi/Go protein, resulting in the inhibition of cAMP production [34]. As described in Chapter II, it was suggested that the main source of RLN3 in Japanese quail is the granulosa layer of mature follicles. Thus, it was hypothesized that RLN3 derived from the granulosa cells bind RXFP1 in a paracrine manner, and this binding may stimulate the cAMP-dependent pathway that leads to the activation of some biological responses in the theca layers. Regarding the expression of RXFP3 in chicken, Ghanem *et al* [56] reported that a higher expression was detected in the stroma of the chicken ovary. Although the expression of the receptors in the ovarian stroma in Japanese quail was not examined, another ligand besides RLN3 may be responsible for the binding to stromal RXFP3 because no expression of RLN3 was seen in the stroma [56]. By observing the expression patterns of receptor during follicular development, the expression significantly increased in the largest and second largest follicles, indicating that the RXFP1 mRNA expression fundamentally corresponds to the developmental status in the follicles. In addition, it has been found that the expression of RXFP1 significantly decreased when ovulation is imminent, and this expression pattern is quite similar to that of RLN3 in granulosa cells. These results suggested that RLN3 signaling mediated via RXFP1 is maintained during follicular development, and it attenuates dramatically after the LH surge.

Notably, the zymography data suggested that the 60 kDa protease in the theca layer was strongly activated after the LH surge. The direct link of RLN3 signaling with 60 kDa protease was not investigated, but RLN3 signaling through the binding with

RXFP1 may disturbs the activity of 60 kDa protease during follicular development. The dramatic decline of RLN3 signaling may potentiate the protease activity, and thus it may be important for the ovulation process. As indicated in Chapter II, it was demonstrated that RLN3 expression was high in the stigma region, where follicular rupture occurred at the time of ovulation, and it significantly declined when ovulation closed. Therefore, the protease activation may occur more efficiently in the stigma region. Because of its molecular size [74], it could be hypothesized that the 60 kDa protease is plasmin. This is also supported by the observation that the activity of 60 kDa protease was inhibited by serine protease inhibitors such as PMSF and leupeptin [75, 76]. Although the involvement of matrix metalloproteinase in the process of ovulation was also reported in Medaka fish [77, 78], the 60 kDa protease is probably not matrix metalloproteinase because the protease activity was not inhibited in the presence of EDTA [79, 80].

In conclusion, this study discovered that RXFP1 may be a primary receptor for RLN3 in Japanese quail because of its abundant expression in the ovarian theca layer. Furthermore, the finding of the present study suggests that the possible involvement of RLN3 signaling in the ovulation process because the dramatic decline of the RLN3 and RXFP1 expression matches with the activation of 60 kDa protease. These findings help clarify the ligand-receptor binding and physiological role of RLN3 and its receptor in birds.

**Table 3.1. List of primers and probes for Japanese quail target genes**

| Gene | Name | Sequence (5' to 3') | GenBank accession No. |
|---|---|---|---|
| RXFP1 | Forward primer | TGGTGGGATCGCTGGCTATT | XM_015860873.2 |
| | Reverse primer | GTGTTTGAAGCCCAGAAAATGCA | |
| | Probe | [FAM] TGTCTCAGAGGTGTCAGTCTTACTGTTGA | |
| RXFP3 | Forward primer | CAAGCAAGGCTGGAGGAAGTC | XM_015848444.2 |
| | Reverse primer | TCTGGGCTGTGGAGAATGCA | |
| | Probe | [FAM] TCCATCAACCTCTTTGTGACCAGCCTGGCT | |
| S17 | Forward primer | AACGAGAGCGCAGGGATAAC | XM_015872709.2 |
| | Reverse primer | CGTCACCTGAAGGTTGGACA | |
| | Probe | [FAM] CGTACCCGAGGTCTCTGCTCTTGATCAGGA[TAMRA] | |

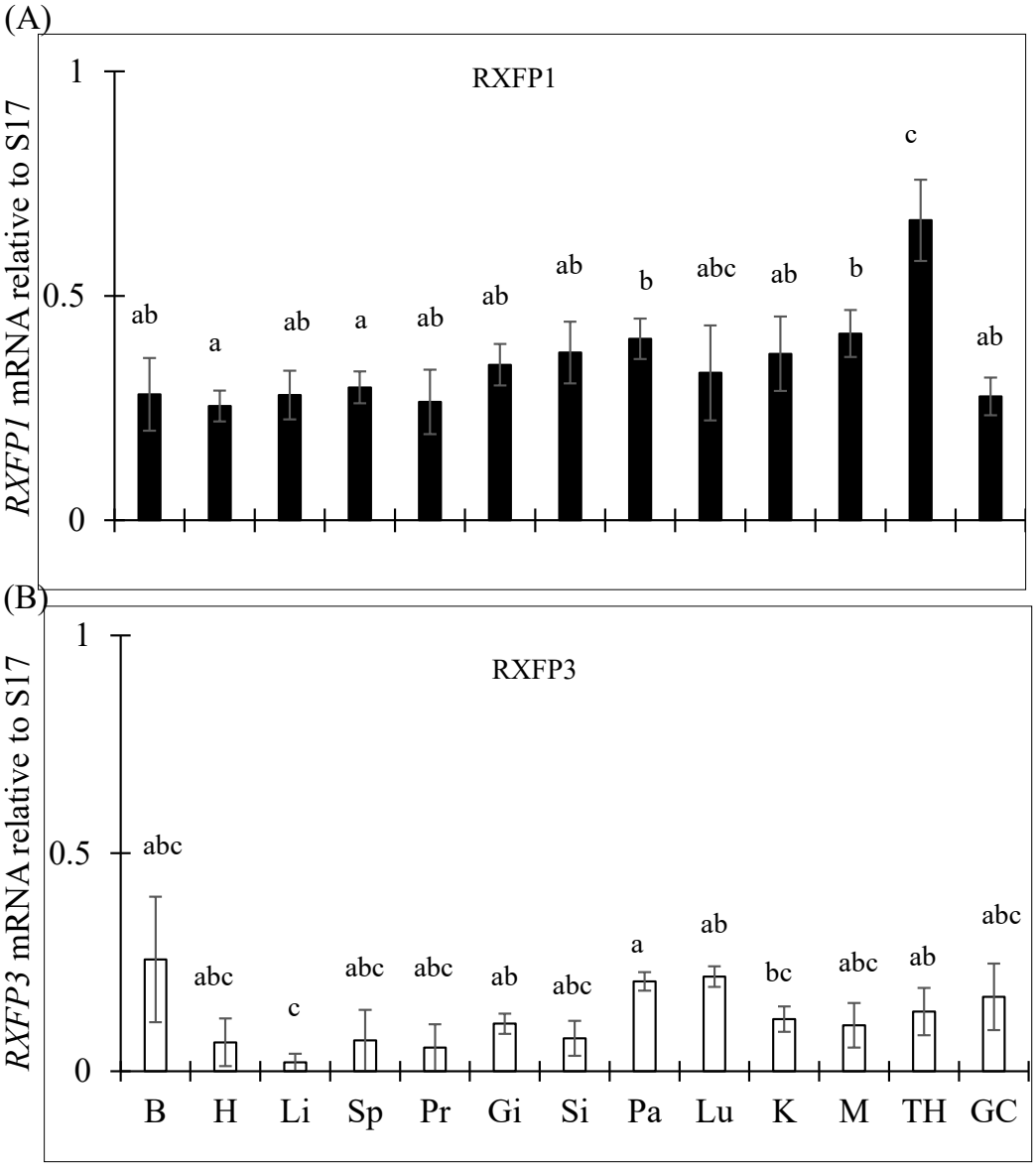


**Fig 3.1. Tissue distribution of RXFP1 and RXFP3 in Japanese quail**. Expression of (A) RXFP1 and (B) RXFP3 mRNA were measured by semi-quantitative PCR in various mature Japanese quail tissues. Each data point represents the mean ± SEM from three animal for each tissue; values with different letters are significantly different ($P<0.05$). B, Brain; H, Heart; Li, Liver; Sp, Spleen; Pr, Proventriculus; Gi, Gizzard; Si, Small intestine; Pa, Pancreas; Lu, Lung; K, Kidney; M, Muscles; TH, theca layer; GC, granulosa layer.

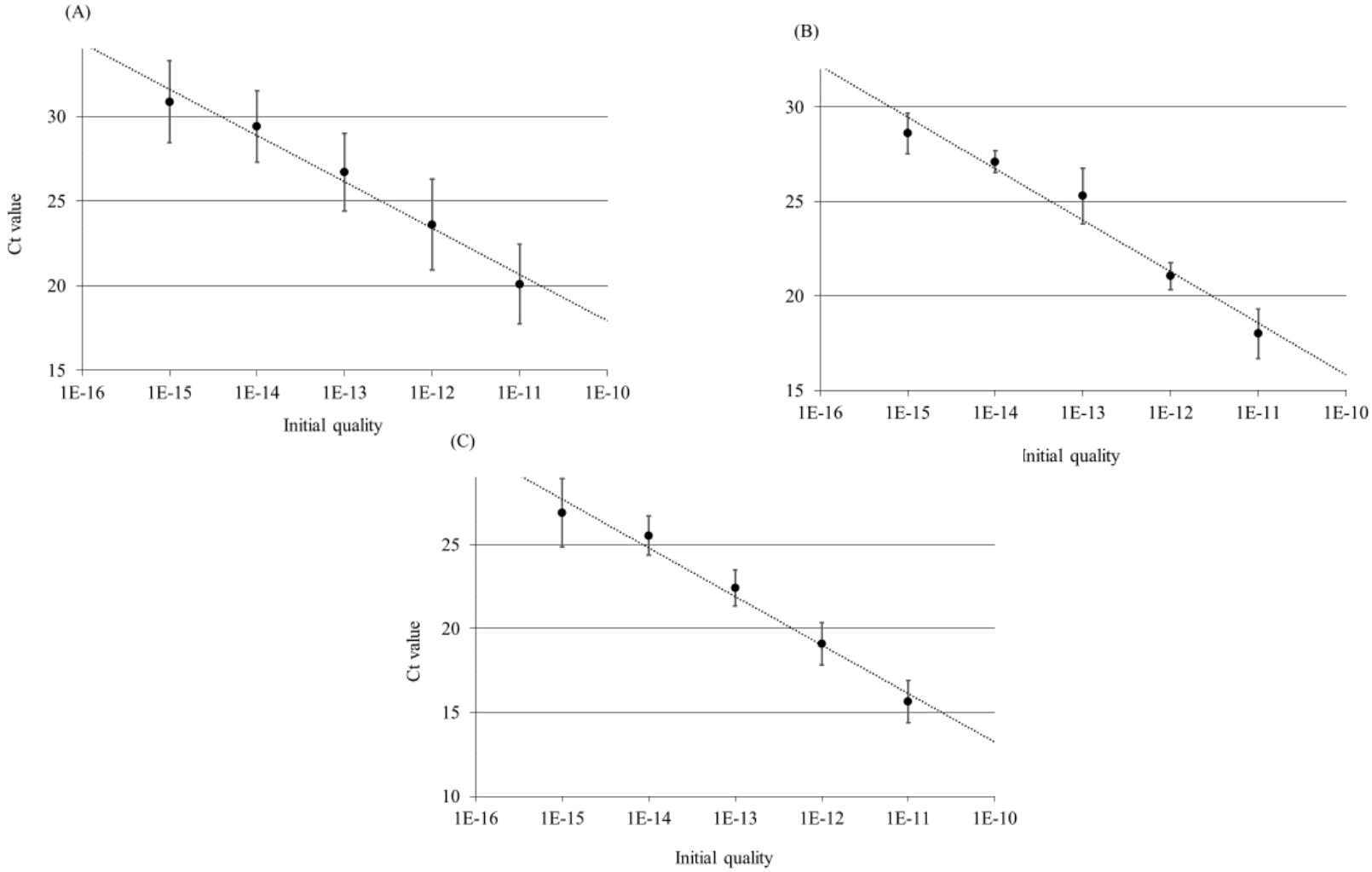


**Fig 3.2**. The standard curve of RXFP1 (A), RXFP3 (B), and S17 (C) ranging from $10^{-11}$ to $10^{-15}$ for real-time RT-PCR quantification. Each point represents the mean ± SD of 5 independent experiments.

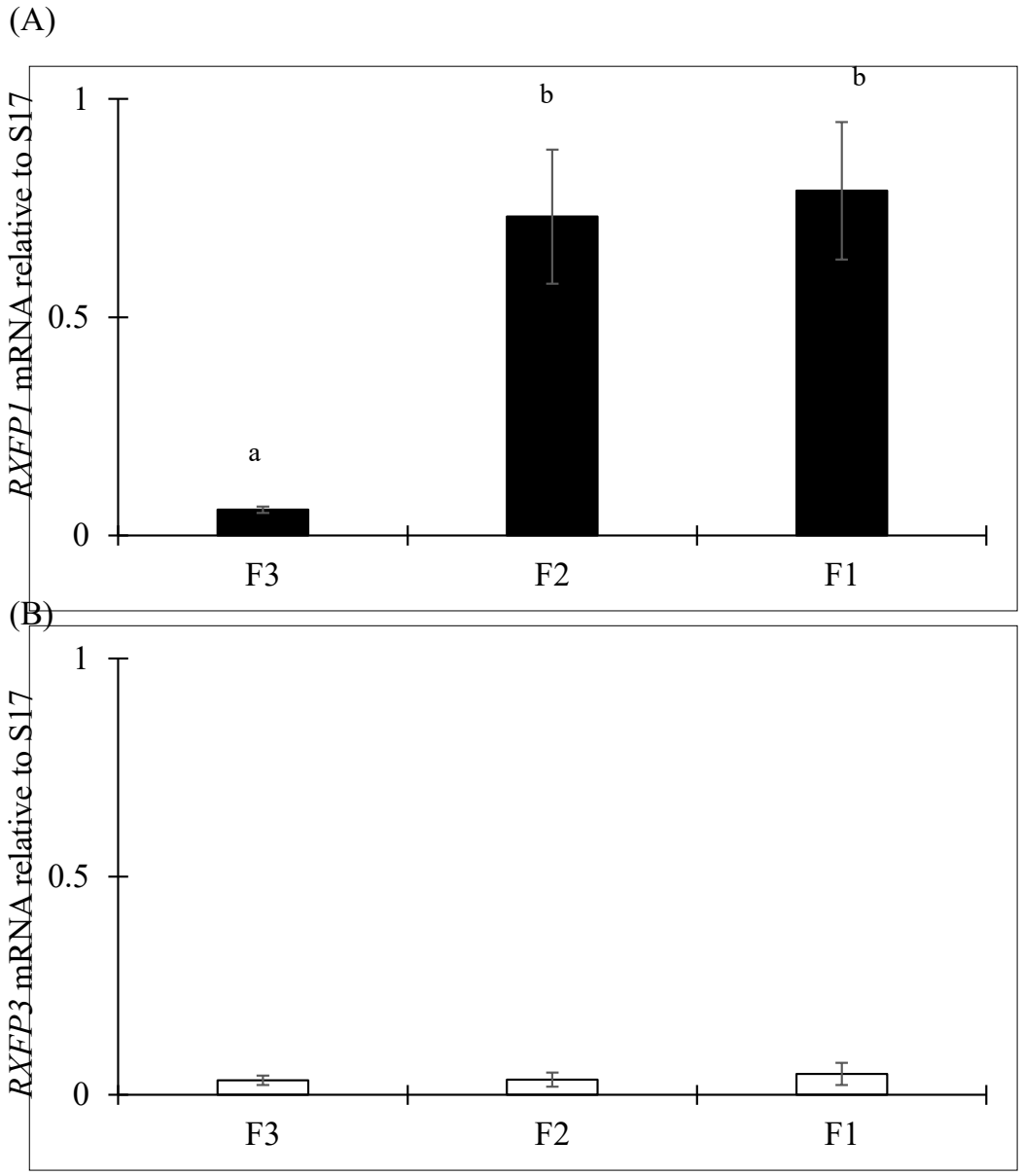


**Fig 3.3. Changes in *RXFP1* and *RXFP3* mRNA expression during follicular development**. The largest (F1), second largest (F2), and third largest (F3) follicles of theca layers were isolated 8-10 h before the expected time of ovulation. The tissues were extracted for RNA and the expression of RXFP1 (A) and RXFP3 (B) was measured by quantitative RT-PCR. Values represent the mean ± SEM of triplicate experiments; values with different letters are significantly different ($P < 0.05$).

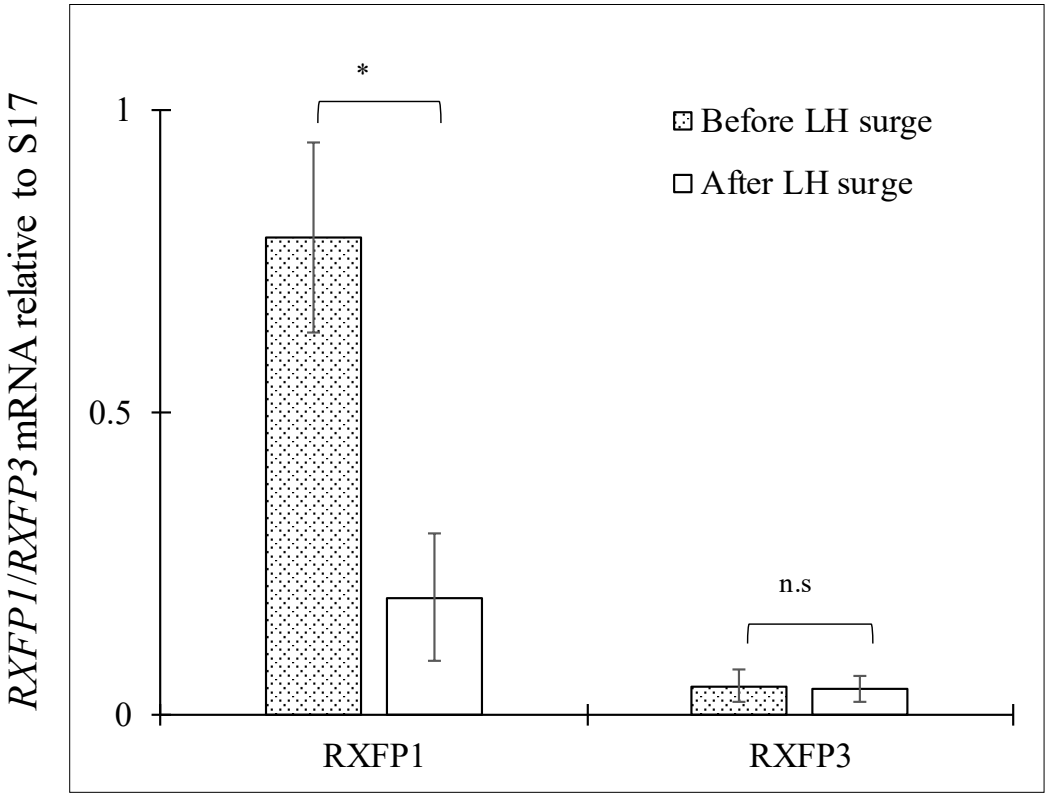


**Fig 3.4. Change in *RXFP1* and *RXFP3* mRNA expression before and after the LH surge**. Theca layers obtained from the largest (F1) follicle were isolated approximately 8-10 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge). The tissues were extracted for RNA and expression was measured by quantitative RT-PCR. Values represent the mean ± SEM of triplicate experiments; values with (*) are significantly different ($P<0.05$) and values labeled with n.s. are not significantly different ($P>0.05$). LH, luteinizing hormone.

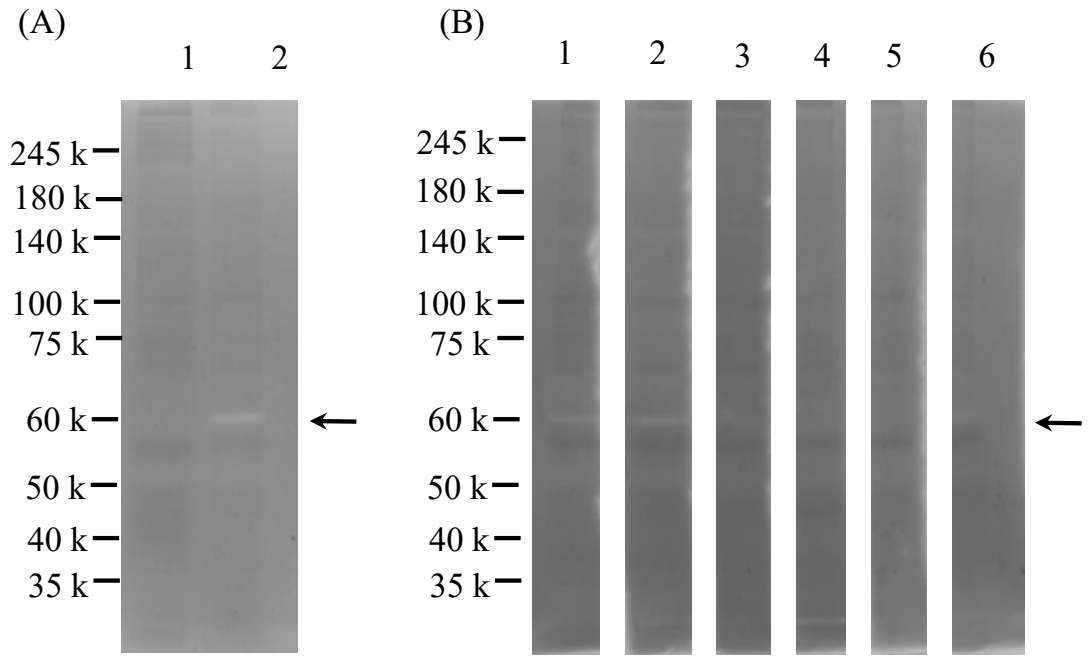


**Fig. 3.5. Detection of protease activity in theca layer**. (A) Zymography of the theca layer lysate before (lane 1) and after LH surge (lane 2). The samples (16 $\mu$ g of protein) were separated using an SDS-PAGE gel-containing gelatin, and the gel was incubated with 0.1 M glycine buffer (pH 8.0). (B) Zymography of theca cell lysates (after LH surge). The samples (16 $\mu$ g of protein) were separated using an SDS-PAGE gel-containing gelatin, and the gel strips were cut along the lane casting. Each gel strip was incubated in glycine buffer (lane 1) in the presence or absence of 1 mM EDTA (lane 2), 50 μg/ml SBTI (lane 3), 1 mM PMSF (lane 4), 0.5 μg/ml leupeptin (lane 5), or 10 μM MG132 (lane 6). The position of the 60 kDa is marked using an arrow. Representative results of repeated experiments are shown.

# Chapter VI

# General discussion

The present study is the first to demonstrate the possible physiological role of RLN3 in Japanese quail. Although, expression pattern of relaxin in nonmammalian species such as fishes and birds have been reported, the knowledge of the function remained unknown. In fishes, the expression of INSL3 was detected in the testis of zebra fish [81, 82], Japanese medaka [83], dogfish [84] and sand tiger sharks [85], while INSL5 was relatively higher in ovary. For other relaxin family peptide, they were found to broadly express in wide range of the tissues. In birds, it was discovered that the purified relaxin from ovary of the hen, which exhibited immunoreactivity to anti-porcine RLN antibodies, was bioactive as this material inhibited the spontaneous contractions of the estrogen-primed mouse uterus [54]. More recently, quantitative polymerase chain reaction (PCR) analysis revealed that RLN3 expression is highest in the granulosa layer and shows increased expression as follicles mature, but its receptors, RXFP1 and RXFP3, were highest in theca layer and ovarian stroma, respectively [56]. Although these are known to be expressed in the chicken ovary, there is a lack of knowledge regarding the RLN3 and its receptors RXFP1 and RXFP3. Therefore, the first experiment of the present study characterized the expression profiles of RLN3 and its receptor RXFP1 and RXFP3 in mature female quail.

In the first experiment, the results showed that RLN3 expression was upregulated in the granulosa layers during follicular development, and its expression was highest in the stigma region of the large follicle (F1) of granulosa layer but significantly decreased as the time of the expected LH surge approached. Regarding to the relaxin family peptide receptor, the results suggested that RXFP1 may be the primary receptor for RLN3 because of the higher expression of RXFP1 in the ovarian theca layer. The present study and Ghanem *et al*., [56] had investigated RLN3 and its receptor RXFP1 expression by

different size of follicles. These authors collected the immature follicles, meanwhile, this study focused on the three largest follicles (*i.e.*, F1, F2 and F3) for the expression profile. Although these authors did not measure the expression levels in mature follicles, it could be suggested that the granulosa layer is the main source of RLN3 while RXFP1 is produced in theca layer in ovary of Japanese quail, which is consistent with the results from chicken [56, 86]. However, these finding contrasts with that of mammalian species. In mammals, RLN3 and its receptor RXFP3 are highly expressed in the brain with this pairing being considered an important neuropeptide receptor system [87]. In avian species, the completed chicken genome allowed the identification of relaxin like genes. The sequence similarity and phylogenetic analysis showed the finding of two RLN3-like sequences but the other relaxin peptide family weren't identified [88]. It was hypothesized that in the chicken one of these RLN3 genes evolved a reproductive function, whereas mammalian RLN3 genes remained specific to the brain. Until now, phylogenetic analysis suggests that one of the chickens RLN3-like sequence is INSL5 homologs [89]. However, the function of INSL5 is still unknown. On the other hand, Lv *et al*., [86] isolated the various brain regions (telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus) and pituitary for gene expression analysis. It has showed that RLN3 secreted form the chicken pituitary cell and its receptor (RXFP1 and RXFP3) are differentially expressed in a wide range of chicken tissues. Because of the different way of sample preparation, it may have led to different results of RLN3 expression in Japanese quail. Thus, it could be suggested that in birds, RLN3 may be functional in the brain as neuropeptide like mammalian species or involved in various physiological functions.

In the second experiment, the most important finding of the present study revealed that RLN3 mRNA expression was enhanced by estradiol-17β and suppressed by

progesterone *in vitro*. It could be due to the increasing the number of LH receptors in the process of mature follicles [68]. In avian species, LH plays a major role in triggering ovulation by 4-6 h. Once LH peaks, the largest preovulatory follicle (F1) produces a significantly greater amount of progesterone compared to less mature follicles [63]. Additionally, the investigation of a stimulatory relationship between LH and progesterone *in vivo* indicates that the completely potentiated LH surge does not occur in the absence of progesterone secretion [90]. Therefore, it is possible that LH stimulates progesterone production by the granulosa layer concurrent with the decrease in RLN3 expression. This information will be supported the result of a significant decreased in the expression of RLN3, when ovulation approached. Although RXFP1 expression wasn't measured in the cell culture experiment, it could be believed that its expression is quite similar to that of RLN3 in granulosa cells.

In the third experiment, the result showed that the 60 kDa protease in the theca layer was strongly activated after the LH surge. In avian species, there is remodeling involving proteases such as plasminogen activator during follicular development. LH decreases both secreted and cell-associated plasminogen activator (PA), in the F1 follicle of granulosa cells in chickens [91]. These are similar to the report in chicken according to the Jackson *et al*., [92] showing the PA activity in the stigma region of the theca layer in the F1 follicle was very low at 8 h before ovulation (before the LH surge) and increased dramatically at 2 h before ovulation (after the LH surge). In this study, the zymography data together with the observation of the molecular size [74], it could be hypothesized that the 60 kDa protease is the plasmin. Thus, it could be assumed that the plasmin is involved in the ovulation process in birds.

Several studies have implicated the PA/plasmin in follicle rupture in rat, but

plasmin does not appear to be required for follicular rupture in mice [93]. Thus, it is thought that the PA/plasmin system would be immaterial for ovulation. In fishes, Ogiwara *et al*., [94] reported that the follicle rupture involving two different proteolytic enzyme activity, serine protease and MMPs, in Japanese medaka ovulation. In birds, the ovulation process involves a series of biochemical and morphological changes that occur in parallel within the preovulatory F1 follicle, leading to its rupture along the stigma and release of the mature oocyte. Because of the degradation of the extracellular matrix (ECM) by a cascade of proteolytic events mediated by different proteases, the follicle stigma region gets progressively thinner [95]. Recently, it has been reported that MMPs participate in the complex remodeling of extracellular matrix required for follicle development, ovulation, and atresia in the chicken ovary [96]. This pattern is different from that for the Japanese quail; however, it may be hypothesized that, like other species, the potential roles of MMPs and plasmin that are involved in different aspects of ovulation process in birds.

In mammals, an important finding was the observation that relaxin participates in ECM remodeling in many reproductive organs, including the ovary, by regulating proteolytic enzyme activity [97]. The Chapter II expected that the stigma region might have a greater amount of RLN3 production by the granulosa layers because it is the site of follicular rupture and RLN3 may be involved in the ovulatory process. Gathering data from these chapters, it proposed that RXFP1 is produced in theca cell that receives signal from its ligand RLN3 in granulosa cells. Then, this signaling begins several processes required for the follicular development and activation of PA/plasmin system leading to mature follicle rupture along the stigma and releases the mature oocyte.

In conclusion, the findings of this study represent a significant step towards

elucidating the physiological roles of RLN3 in birds and its possibility as a factor in the follicular growth and maturation. It also speculated that RLN3 expression in the stigma region of F1 follicle interacts with RXFP1 in theca layers as a paracrine factor that RLN3 may have a role in the ovulation process in the Japanese quail. Furthermore, the current study is the first to explore the information of the indispensable role of protease activity of 60 kDa in the ovulation of the avian species.

To deeply understand the functions of RLN3 in female avian species, the following future studies are necessary:

1. To clarify the signaling pathways activated by RLN family peptide and their receptor associate proteolytic activity during the dynamic remodeling process that takes places in the ovary prior to ovulation.
2. To develop the avian RLN3 immunoassay.
3. To measure cAMP serves as a useful indicator of receptor activity

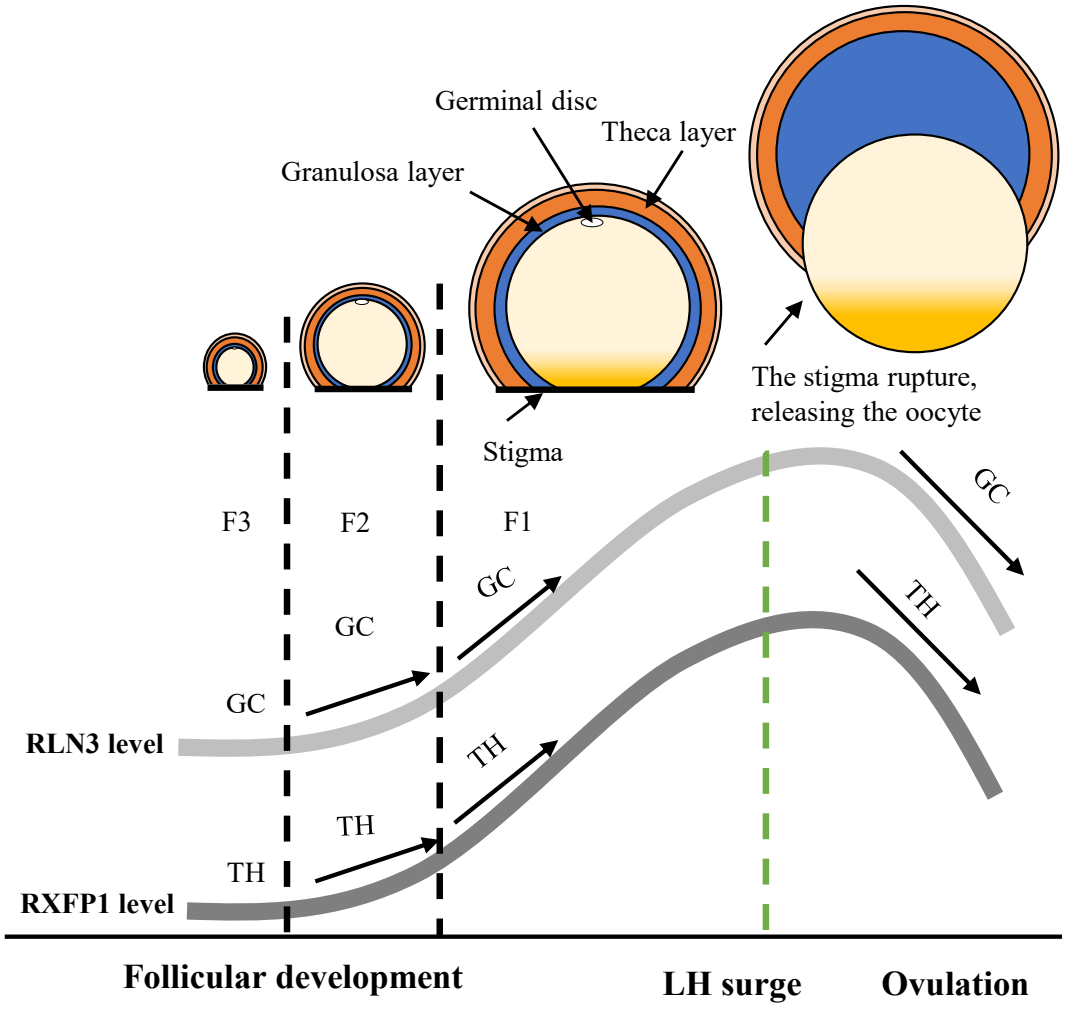


**Fig 4.1. Summary of cellular distribution of RLN3 and RXFP1 mRNA in the ovarian follicle before and after LH surge**. RLN3 mRNA was mainly expressed in the granulosa layer and its receptor RXFP1 mRNA was produced by theca layer. The RLN3 and RXFP1 mRNA expression was increased with follicular development and its expression was significantly decreased after LH surge. GC, granulosa layer; TH, theca layer.

# Summary

The relaxin-like peptides family is a group of peptide hormones, including relaxin 1-3 (RLN 1 – 3) and the insulin like peptides 3-6 (INSL3 - 6). The primary structure of the relaxin peptide is included an A chain and a B chain, which confer two inter-chains and one intra-chain.

Genomic library screening resulted in the cloning of human RLN1 (H1-RLN) and human RLN2 (H2-RLN). The circulating form of relaxin in humans is H2-RLN, which is considered to be the functional equivalent of all non-primate RLN1. RLN3, which has been identified in many species, acts as a neuropeptide in the brain. In the reproductive tissues in females, RLN1 has been found in the corpus luteum, placenta, and uterus in non-primates such as pigs, rats, mice, and guinea pigs. In avian species, it was recently reported that ovarian granulosa and theca cell is the source of RLN3 and their receptor (RXFP1 and RXFP3) in chicken. However, no studies have elucidated the expression profile and function of relaxin in birds. Therefore, a significant step towards evaluating the physiological roles of RLN3 in Japanese quail, would be determine whether the ovarian follicle acts as source and target tissue of RLN3 and their receptor (RXFP1 or RXFP3) and their changes in the follicular development. It will be also interesting to investigate whether RLN3 and their receptor could be a possible basis for screening ovulation process.

In the first step, by using RT-PCR analysis, RLN3 mRNA was found to mainly express in GC and theca cells of the ovary. The expression levels in the GC increased with the stage of follicular development. From the GC culture experiments, the RLN3 mRNA expression increased with the addition of estradiol-17β, whereas the addition of progesterone suppressed its gene transcription. More detailed analysis indicated that the RLN expression was highest in the stigma region of the follicle and its expression

significantly decreased when the time of expected LH surge approaching. It was demonstrated that the RLN3 expression is in the ovary of the Japanese quail. Because the RLN3 expression was highest in the stigma region, the result suggested that RLN3 could be related to the ovulation process in birds.

In the next step, as for the relaxin receptor, it was found that both RXFP1 and RXFP3 were ubiquitously expressed by performing RT-PCR analysis. Although the expression levels were relatively low, considerable expression of both receptors was observed in the ovary. The expression levels of RXFP1 were higher than that of RXFP3, indicating that the primary receptor for RLN3 in the ovary could be RXFP1. During follicular development, the theca RXFP1 expression increased, but it declined after the LH surge. More detailed analysis indicated that the RXFP1 expression was highest in the stigma region of the follicle, but no such reduction was observed after LH surge. Because of the higher expression of RXFP1 in the ovary, RLN3 may exert its biological activities through the binding to RXFP1. It could be suggested that RLN3 acts as the factor for the ovulation and maintaining follicular development may be mediated via RXFP1.

At the last step, the zymography data suggested that 60 kDa protease in the theca layer was strongly activated after the LH surge. The direct link of RLN3 signaling with 60 kDa protease was not investigated, but RLN3 signaling through the binding with RXFP1 may disturb the activity of 60 kDa protease during follicular development. The dramatic decline of RLN3 signaling may potentiate the protease activity, and thus it may be important for the ovulation process. As indicated above, RLN3 expression was high in the stigma region, where follicular rupture occurred at the time of ovulation, and it significantly declined when ovulation closed. Therefore, the protease activation may occur more efficiently in the stigma region. Because of its molecular size, we hypothesize

that the 60 kDa protease is plasmin. This is also supported by the observation that the activity of 60 kDa protease was inhibited by serine protease inhibitors such as PMSF and leupeptin.

The overall findings suggested that ovarian granulosa cell is the main source of RLN3 in Japanese quail and RXFP1 may be a primary receptor for RLN3. Furthermore, RLN3 signaling was involved in the ovulation process because the significant decline of the RLN3 and RXFP1 expression corresponds with the activation of 60 kDa protease. These findings help clarify the ligand-receptor binding and physiological role of RLN3 and its receptor in birds.

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