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Evidence for the Existence of Two Prolactin Isoforms in the Developing Pituitary Gland of the Goose (*Anser cygnoides*)

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Original article

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Compared to *Galliformes* such as chicken and turkey, very little is known about the existence and expression of isoforms of prolactin (PRL) in the pituitary glands of *Anseriformes*. In this study, by generating a rabbit-anti-goose (*Anser cygnoides*) PRL polyclonal antibody, we analysed the expression patterns of goose PRL isoforms in the embryonic and post-hatch development of the pituitary gland. Our results showed that two immunoreactive bands with molecular weights of about 23 and 26 kDa were detected using the Western blot technique, corresponding to the non-glycosylated (NG-) and the glycosylated (G-) isoform of PRL, respectively. The protein levels of the total PRL in a goose increased gradually from the embryonic day (ED) 22 to the post-hatch day (PD) 28, with a non-significant decrease on PD6. Furthermore, the percentage of G-PRL in the pituitary gland of the goose fluctuated from about 30.3% to 54.7% throughout the embryonic and post-hatch development. At the mRNA level, the expression of *PRL* increased steadily during the development and reached the highest levels on PD12, but later showed a non-significant decrease on PD28. The inconsistent expression patterns between the *PRL* mRNA and protein during the stages from PD6 to PD28 indicated that the *PRL* gene expression involves both transcriptional and post-translational regulation. Taken together, our data unequivocally demonstrated the existence of NG- and G-PRL in the pituitary gland of a goose and that the expression of the total PRL as well as the percentage of G-PRL significantly changed during embryonic and post-hatch development, indicating that the versatile biological functions of PRL during the ontogenesis of a goose could be closely related to changes in both its total expression and the degree of glycosylation in the pituitary gland.

Key words: isoform, glycosylation, ontogenesis, protein expression, mRNA expression, polyclonal antibody, poultry.

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The pituitary gland is usually referred to as the body's 'master gland' because it controls the activities and functions of other hormone-secreting glands, thereby playing an essential role in regulating growth and development, energy metabolism, reproduction and behaviour. The gland is structurally divided into the adenohypophysis and neurohypophysis. The tubercle and the distal part of the adenohypophysis are collectively called the anterior pituitary gland, which is mainly composed of eosinophilic cells, basophilic cells and chromophobe cells. These cells will gradually proliferate and differentiate during ontogenesis, and are eventually able to secrete different hormones that regulate a wide range of physiological functions (SCANES 2015). PRL is recognised as a pleiotropic polypeptide hormone with more than 300 physiological functions that is primarily synthesised and secreted by the lactotrophs of the anterior pituitary gland (BOLE-FEYSOT *et al.* 1998). In *Columbiformes* and *Galliformes*, changes in the plasma PRL concen-

tration have previously been reported to be strongly associated with tissue and organ development, as well as reproduction and incubation behaviour (UBUKA & BENTLEY 2011). In support of this finding, the mRNA and protein contents of *PRL* in the pituitary gland, pancreas, liver, kidney and gonads were observed to vary significantly during both embryogenesis and in the different reproductive stages of chickens and turkeys (ISHIDA *et al.* 1991; BÉDÉCARRATS *et al.* 1999c; HIYAMA *et al.* 2009; BU *et al.* 2015). In addition, it is well known that PRL exerts its pleiotropic actions mainly through interactions with its receptor (PRLR) (BOLE-FEYSOT *et al.* 1998), and there is evidence that the *PRLR* mRNA is expressed in a wide range of tissues and organs, including in the pituitary gland, adrenal gland, pancreas, gonads, skeletal muscles, heart, lungs, thymus, spleen, liver and kidneys (ZHOU *et al.* 1996; YAMAMOTO *et al.* 2003; LECLERC *et al.* 2007; BU *et al.* 2013).

In addition to the widespread expression of PRL and its receptor, the versatile functions of vertebrate PRL also depend on its structural diversity, which results from alternative splicing, proteolytic cleavage and post-translational modifications such as glycosylation, phosphorylation, polymerisation, sulfation and deamidation (CORCORAN & PROUDMAN 1991; FREEMAN *et al.* 2000; ZADWORNY *et al.* 2002; KANSAKU *et al.* 2008; HIYAMA *et al.* 2009; BU *et al.* 2013). Multiple isoforms of PRL, including glycosylated (G-) and phosphorylated ones, have been identified in the anterior pituitary glands of chickens using a one- and two-dimensional Western blot analysis after a glycosidase and neuraminidase treatment (HIYAMA *et al.* 2009), and different post-translational modifications have been evidenced to differently affect the structure of PRL, and thus the biological activities in the target tissues and cells of humans and of several mammals (FREEMAN *et al.* 2000). As the major post-translationally modified isoform, the G-PRL synthesised by the pituitary gland has been demonstrated to be able to be secreted into the blood and thus to modulate the responsiveness of extra-pituitary tissues to the circulating PRL, and the proportion of G- to non-glycosylated (NG-) PRL has been shown to change during different physiological states in chickens and turkeys (BÉDÉCARRATS *et al.* 1999a; BÉDÉCARRATS *et al.* 1999b; ZADWORNY *et al.* 2002; KANSAKU *et al.* 2008; HIYAMA *et al.* 2009). By contrast, very little is known about the existence and expression of G-PRL in the pituitary glands of *Anseriformes* during development, although several lines of evidence have shown that multiple PRL isoforms with different molecular weights were present in waterfowl (KANSAKU *et al.* 2005; LIU *et al.* 2008). Since the glycosylation of PRL could result in a very different topology and thus affect the receptor binding affinity, G-PRL may exert different biological actions than NG-PRL and changes in the ratio of G- to NG-PRL may partition the biological effects of PRL in the target tissues during development (ZADWORNY *et al.* 2002). Compared to chickens and ducks, domesticated geese (*Anser cygnoides*) exhibit a different egg incubation time, as well as a different post-hatch growth rate, stronger incubation behaviour and much lower egg production, but whether G-PRL exists in the pituitary gland of a goose and how its expression and proportions change during embryonic and post-hatch development remain unclear. Therefore, it is of particular importance to verify the existence of G-PRL in domesticated geese and to explore its physiological relevance during ontogenesis.

To achieve this goal, we first constructed the recombinant prokaryotic expression plasmid – namely pET-32a-goose *PRL* (g*PRL*) – and subsequently induced the *Escherichia coli* (*E. coli*) harbouring this construct to efficiently express the recombinant gPRL protein. Then, large amounts of recombinant gPRL protein were purified using His-Ni affinity chromatography and were used as the antigen to generate the rabbit-anti-gPRL polyclonal antibody. Finally, we quantified the contents of the total *PRL* mRNA and protein, as well as the percentage of G-PRL in the pituitary glands of geese during embryonic and post-hatch development using a quantitative real-time polymerase chain reaction (qRT-PCR) and the Western blot technique. This data was expected to lay a foundation for the further elucidation of the physiological actions of PRL isoforms during ontogenesis in *Anseriformes* such as geese.

Materials and Methods

Ethics Statement

All the experimental procedures involving the manipulation of birds were conducted in concordance with the 'Guidelines for Experimental Animals' of the Ministry of Science and Technology (Beijing, China). This study has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No.: 20180034).

Birds and Tissue Collection

All the experimental geese were provided by the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University (Ya'an Campus, Sichuan, China). A total of 49 female Sichuan White geese were used for the tissue collection. Regarding the generation of the rabbit-anti-gPRL antibody, one female Sichuan White goose was euthanised by carbon dioxide inhalation during the egg-laying period and the pituitary gland was immediately isolated for amplifying the cDNA encoding the mature peptide of gPRL. As for the investigations of the expression profiles of

PRL mRNA (n=3 individuals per developmental stage) and protein (n=3 individuals per developmental stage) during the embryo-to-hatching transition period, which is crucial for preparing the embryos for the abrupt switch from chorioallantoic to pulmonary respiration, six healthy female Sichuan White geese were euthanised by carbon dioxide inhalation on the embryonic day (ED) 22, ED26 and ED28, as well as on the post-hatch day (PD) 1, PD2, PD6, PD12 and PD28, respectively, for the purpose of collecting the pituitary glands. After sampling, all the tissues were snap-frozen in liquid nitrogen and were finally stored at -80°C until a further analysis.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each sample using the Trizol reagent following the manufacturer's instruction. The purity and concentration of the RNA were determined using a NanoDrop spectrophotometer. The cDNA was then synthesised from 1 μg RNA, using the Prime Script™ RT reagent kit with a gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions.

Construction and Characterisation of the Recombinant *gPRL* Prokaryotic Expression Plasmid

To construct the recombinant *gPRL* prokaryotic expression plasmid, based on the nucleotide sequence of the Sichuan White goose *PRL* gene (GenBank accession number: GQ202542.1), a pair of primers (Forward primer: 5'-ggGGTACCTTGCCTATCTGCCCCAATGGATCTG-3'; Reverse primer: 5'-ccgGAATTCTTAGCAATTGCTATCATGTATTAGG-3') containing the lowercased protective bases and the underlined Kpn I and EcoR I sites were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) to amplify the 600 bp cDNA encoding a 199 amino acid mature peptide of gPRL. The reverse transcription (RT)-polymerase chain reaction (PCR) was performed in a Bio-Rad thermal cycler (Bio-Rad, USA) under the following conditions: pre-denaturation at 95°C for 5 min, followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, an extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were evaluated via electrophoresis on 1.5% agarose gels and were purified using a gel extraction kit (Omega, USA). The purified PCR products were then cloned into the pMD19-T vector (Takara Biotechnology Co., Ltd., Dalian, China), and the resultant pMD19-T-*gPRL* was transformed into *E. coli* DH5α competent cells. The positive clones were screened by RT-PCR and were sent to the Tsingke Biotechnology Co., Ltd. (Chengdu, China) for sequencing. After the verification by sequencing, the plasmid expressing recombinant pMD19-T-*gPRL* was extracted using the Endo-free Plasmid Mini Kit II (Omega, USA). Then, both the pMD19-T-*gPRL* plasmid and the selected His-tagged prokaryotic expression vector pET-32a (Takara Biotechnology Co., Ltd., Dalian, China) were digested with the Kpn I and EcoR I restriction enzymes, and the respective fragment of interest was ligated to generate the recombinant plasmid expressing *gPRL* – namely, pET-32a-*gPRL* – and was transformed into DH5α competent cells. Finally, the positive clones were screened by RT-PCR and sent to the Tsingke Biotechnology Co., Ltd. (Chengdu, China) for sequencing.

Efficient Expression, Purification and Characterisation of the Recombinant gPRL Protein in BL21

After the verification by sequencing, the successfully constructed recombinant plasmid pET-32a-*gPRL* was transformed into the *E. coli* BL21. The *E. coli* harbouring this plasmid were further induced by IPTG to express the soluble recombinant gPRL protein, and the induction conditions for gPRL expression were optimised by testing the production yield under different concentrations of IPTG, as well as different induction temperatures and times. The optimal induction conditions were finally set at 1 mM IPTG at 25°C for 12 h. After the induction, the *E. coli* expressing gPRL were centrifuged at 10,000 × g for 10 min to remove the supernatants and resuspended in the pellet thoroughly in phosphate buffer saline (PBS). Next, the resuspended bacteria cell walls were broken by ultrasonic waves, and the resultant supernatants were retained via centrifugation and filtered through PES membranes. Then, the recombinant gPRL protein was purified by His-Ni affinity chromatography and was concentrated via ultrafiltration. Finally, the purified recombinant gPRL protein was characterised by both Coomassie brilliant blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels and a quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS) analysis, and its concentration was determined using a bicinchoninic acid protein assay kit (BCA; Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Preparation of the Polyclonal Antibody against the Recombinant gPRL Protein

Using the purified recombinant gPRL protein as the antigen, a total of four rabbits were immunised by a subcutaneous injection every two weeks for a total of four times. Before the immunisation, 2 ml blood was collected from each rabbit for separating sera as the negative control. At the first immunisation, 0.6 mg of gPRL emulsified with Freund's complete adjuvant (FCA) was subcutaneously injected into each rabbit, while at the second, third and fourth immunisation, 0.4 mg of gPRL emulsified with Freund's incomplete

adjuvant (FIA) was subcutaneously injected into each rabbit. After the fourth immunisation, all the immunised rabbits were euthanised with intravenous pentobarbital sodium, and an enzyme-linked immunosorbent assay (ELISA) coated with the recombinant gPRL protein was employed to determine the antiserum titer. Thereafter, the polyclonal antibodies specifically against gPRL were purified from the high-titer sera using a self-made recombinant gPRL-coupled affinity chromatography. The specificity, immunoreactivity and concentration of the purified rabbit-anti-gPRL antibody were determined using SDS-PAGE, Western blot and a BCA protein assay, respectively.

qRT-PCR

The mRNA expression levels of the *gPRL* gene in the embryonic and post-hatch pituitary glands were measured using a qRT-PCR. According to the obtained *gPRL* gene sequence, a pair of primers (listed in Table 1) were designed to detect its relative mRNA expression. Meanwhile, the primers of two internal control genes, i.e., goose *GAPDH* (GenBank accession number: XM_013199522) and *β-ACTIN* genes (GenBank accession number: M26111), were also designed (listed in Table 1). All the primers were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), and the qRT-PCR reactions were performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) using the SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd., Dalian, China). The reactions were conducted under the following conditions: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at the corresponding temperature of each primer pair for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all the qRT-PCR runs. The target specificity for each primer set was validated by a melting curve analysis, and the identity of all amplicons was verified by sequencing. All the samples were amplified in triplicate and the relative expression levels of *gPRL* mRNA were normalised to the reference genes, *GAPDH* and *β-ACTIN*, using the comparative CT method ($\Delta\Delta C_T$) (SCHMITTGEN & LIVAK 2008).

Protein Extraction and Western Blot

The pituitary glands from female Sichuan White geese during the embryonic and post-hatch developmental stages were homogenised individually in an NP-40 based extraction solution. Next, the supernatant was obtained by centrifugation at 10,000 × g for 10 min, and the protein concentration was determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, Nanjing, China). Then, equal amounts of protein lysates (~25 μg) mixed with a 5 × SDS-PAGE loading buffer were boiled at 95°C for 5 min and were then resolved by the 12% SDS-PAGE under reducing conditions. After being transferred to the polyvinylidene fluoride (PVDF) membranes, the membranes were blocked for non-specific binding for 1 h in the blocking buffer and were then incubated overnight with the rabbit-anti-gPRL polyclonal antibody at a dilution of 1:1000. After being washed five times with TBST (5 min each time), the membranes were further incubated with the HRP-linked goat-anti-rabbit lgG antibody at a dilution of 1:10000 at room temperature for 1 h. Thereafter, the membranes were washed five times with TBST (5 min each time), and the immunoblotted proteins were finally visualised by the enhanced chemiluminescence (ECL) solution (Beyotime Institute of Biotechnology, Nanjing, China), according to the manufacturer's instructions. A densitometric analysis was performed using Image Lab software (Version 4.1, Bio-Rad).

Statistical Analysis

All of the quantitative data was expressed as the mean ± standard deviation (SD). The *gPRL* mRNA and protein expression levels between the different developmental stages (n=3 individuals per developmental stage) were analysed by a one-way analysis of variance (ANOVA) after examining the homogeneity of variance using the Levene's test, followed by the Duncan's multiple range test. All statistical analyses were performed using the SAS v9.4 software (SAS Institute Inc., Cary, NC, USA), and a p-value less than 0.05 was considered to be statistically significant.

Table 1

The qRT-PCR primer pairs used in this study

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon/bp	Tm/°C
PRL	TAGTTGGGCGGGTTCATTCT	TTAGGCGGCACTTCAAAACC	183	60
GAPDH	GCTGATGCTCCCATGTTCGTGAT	GTGGTGCAAGAGGCATTGCTGAC	86	60
β-ACTIN	CAACGAGCGGTTCAGGTGT	TGGAGTTGAAGGTGGTCTCGT	92	60

Results

Construction of the Recombinant Prokaryotic Expression Plasmid pET-32a-*gPRL*

The cDNA encoding the mature peptide of gPRL, with a length of approximately 600 bp, was successfully obtained by RT-PCR and was validated by sequencing (Figure 1a). After ligating the fragments of both the pMD-19-T-*gPRL* and pET-32a digested by restriction enzymes (Figure 1b), the recombinant prokaryotic expression plasmid pET-32a-*gPRL* was successfully constructed and was validated by restriction enzymes digestion in combination with PCR amplification, as is shown in Figures 1c and d.

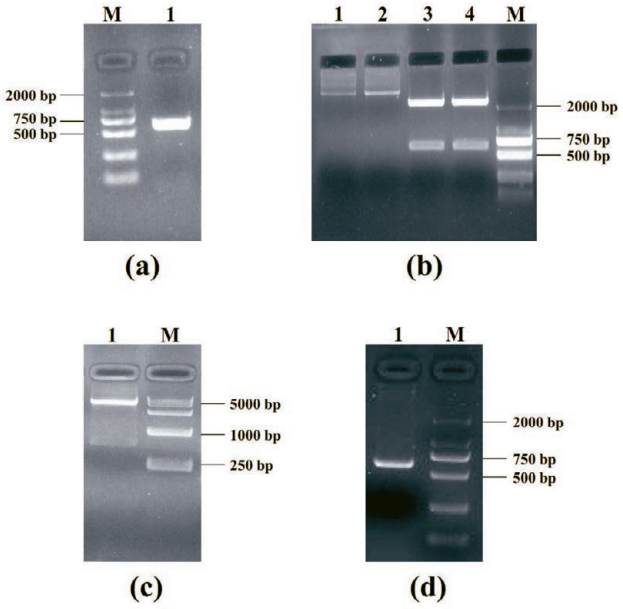

Fig. 1. Construction and characterisation of the recombinant prokaryotic expression plasmid pET-32a-*gPRL*. a – Agarose gel electrophoresis of RT-PCR products of the cDNA encoding the gPRL mature peptide. 1, RT-PCR products with a molecular weight of about 600 bp. M: DL 2000 DNA marker. b – Characterisation of the prokaryotic expression vector pET-32a and the recombinant plasmid pMD-19-T-*gPRL* by digestion with the Kpn I and EcoR I restriction enzymes. 1-2, agarose gel electrophoresis of the double digestion products of pET-32a; 3-4, agarose gel electrophoresis of the double digestion products of pMD-19-T-*gPRL*; M: DL2000 DNA marker. c – Characterisation of the recombinant prokaryotic expression plasmid pET-32a-*gPRL* by digestion with the Kpn I and EcoR I restriction enzymes. 1, agarose gel electrophoresis of the double digestion products of pET-32a-gPRL; M: DL5000 DNA marker. d – Characterisation of the plasmid pET-32a-*gPRL* by RT-PCR with the primer pairs amplifying the cDNA encoding the gPRL mature peptide. 1, agarose gel electrophoresis of the RT-PCR products with a molecular weight of about 600 bp; M: DL2000 DNA marker.

Prokaryotic Expression, Purification and Characterisation of the Recombinant gPRL Protein

The SDS-PAGE results of the supernatants obtained through the centrifugation of the pET-32a-*gPRL* expressed BL21 broken by ultrasonic waves are shown in Figure 2a. The high expression of the recombinant gPRL protein in the supernatants indicated that this recombinant protein is soluble. A His-Ni affinity chromatography was then used to purify the recombinant gPRL protein with different elution buffers (Figure 2b). The SAS-PAGE results of the protein solutions purified with different elution buffers showed that after an elution with 143 mM imidazole, one thick band with an expected size of about 39 kDa was observed (Figure 2c). After a verification by Q-TOF-MS and subsequent sequencing analyses, this purified protein was confirmed to be the recombinant gPRL protein (Figures 2d and e). These results suggested that the recombinant gPRL protein was successfully obtained in this study.

Purification and Characterisation of the Rabbit-anti-gPRL Polyclonal Antibody

After collecting the sera from the rabbits that were immunised with the purified recombinant gPRL protein, the rabbit-anti-gPRL antibodies were further purified using the self-made recombinant gPRL protein-coupled affinity chromatography. As is shown in Figure 3a, the dilutions of the rabbit-anti-gPRL sera exhibited multiple bands, with a thick band between 50- and 75 kDa; in contrast, the purified rabbit-anti-gPRL antibodies using the Tris-HCl elution buffer exhibited almost a single band at around 50 kDa. The molecular weight of the purified rabbit-anti-gPRL antibody was similar to that of the heavy chain of the IgG antibody (approximately 50 kDa), indicating that this generated rabbit-anti-gPRL antibody was reliable and was highly pure. Furthermore, we used the total protein extracted from the pituitary gland of the goose and the recombinant gPRL protein as the antigens to test the immunoreactivity and specificity of the rabbit-anti-gPRL antibody by a Western blot analysis, respectively. The results showed that the rabbit-anti-gPRL antibody specifically recognised not only two PRL isoforms with molecular weights of around 23- and 26 kDa (i.e. NG- and G-PRL) in the goose pituitary gland (Figure 3b) but also the recombinant gPRL protein with a molecular weight of around 39 kDa (Figure 3c), suggesting that this generated rabbit-anti-gPRL antibody was immunoreactive and had a high specificity against the gPRL protein

Changes in the mRNA Levels of *gPRL* in the Embryonic and Post-Hatch Development of the Pituitary Gland

Changes in the mRNA levels of *gPRL* in the embryonic and post-hatch development of the pituitary

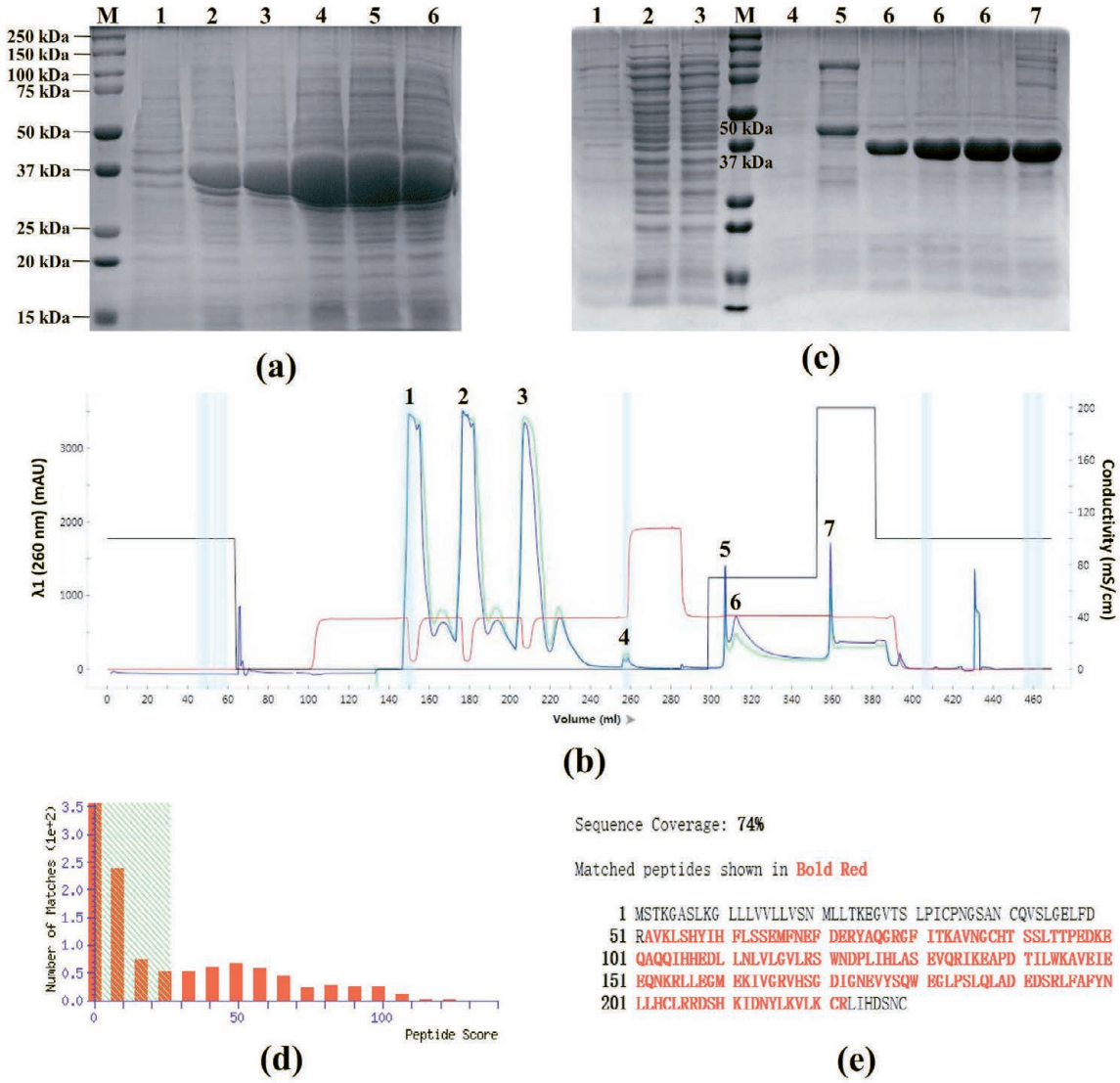

Fig. 2. Purification and characterisation of the recombinant gPRL protein. a – SAS-PAGE results of the supernatants obtained through centrifugation of the pET-32a-*gPRL* expressed BL21 broken by ultrasonic waves. 1-6, different concentrations of the supernatants from the ultrasonic disrupted pET-32a-*gPRL*-BL21; M, the pre-stained protein marker. b – Diagram of the elution peaks of the protein solutions purified from the supernatants from the ultrasonic disrupted pET-32a-*gPRL*-BL21 with different elution buffers using His-Ni affinity chromatography. Peak 1-3, the protein solutions eluted by the binding buffer consisting of 0.05 M NaH_2PO_4, 0.3 M NaCl and 0.005 M imidazole; Peak 4, the protein solutions eluted by 1 M NaCl; Peak 5-6, the first and second peaks of the protein solutions eluted by 143 mM imidazole, respectively; Peak 7, the protein solutions eluted by 400 mM imidazole. c – SAS-PAGE results of the protein solutions eluted with different elution buffers. The lane number corresponds to the peak number in (b); M, the pre-stained protein marker. d – Histogram of the scores of the peptides originated from the recombinant gPRL protein of about 39 kDa and characterised by quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS). Peptides having a score greater than 26 (outside the shaded area) are reliable. e – Sequence coverage of the peptides characterised by Q-TOF-MS.

glands of the geese are shown in Figure 4. The levels of *gPRL* mRNA remained low from ED22 to PD2. Thereafter, these levels significantly increased until PD12 ($p<0.05$) but then slightly decreased on PD28 ($p>0.05$).

Changes in the Protein Levels of gPRL Isoforms in the Embryonic and Post-Hatch Development of the Pituitary Gland

As is shown in Figure 5a, the Western blot analysis demonstrated the presence of two immunoreactive

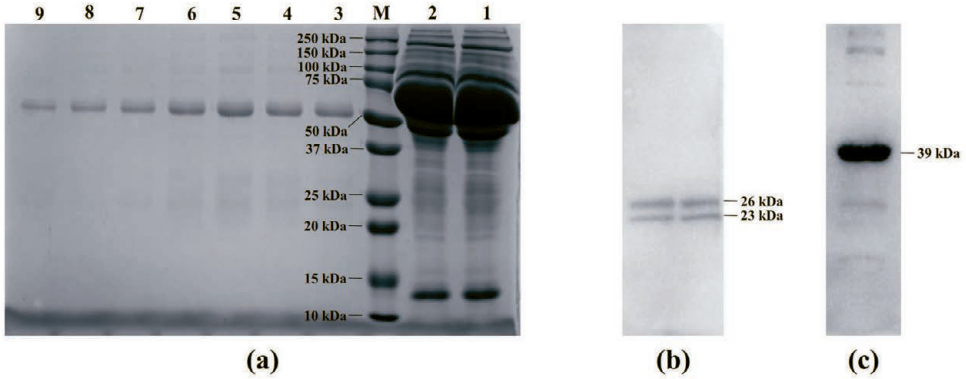

Fig. 3. Purification and specificity identification of the rabbit-anti-gPRL antibody. a – SDS-PAGE results of the rabbit-anti-gPRL sera before and after being purified using recombinant gPRL protein-coupled affinity chromatography. 1-2: the diluted rabbit-anti-gPRL sera; 3-9: the solutions purified from the diluted rabbit-anti-gPRL sera using the Tris-HCl elution buffer; M: the pre-stained protein marker. b – Western blot results of the total protein extracted from the pituitary gland of a female goose during the egg-laying period using the purified rabbit anti-gPRL antibody. c – Western blot results of the recombinant gPRL protein using the purified rabbit anti-gPRL antibody.

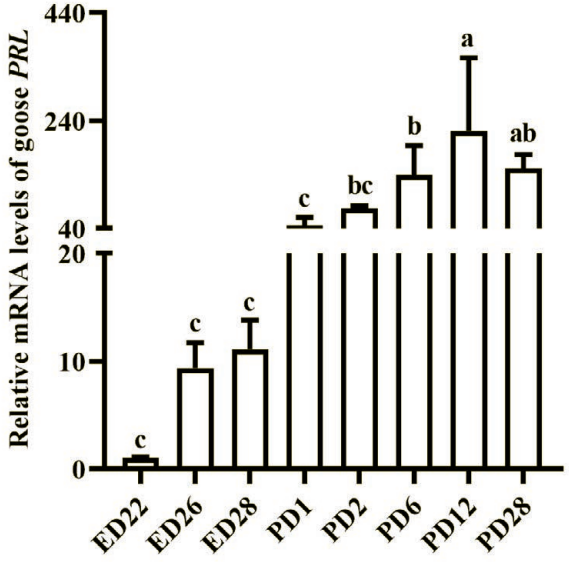

Fig. 4. Expression patterns of *PRL* mRNA in the pituitary glands of geese during the embryonic and post-hatch developmental stages. ED – embryonic day; PD – post-hatch day. Three individuals (n=3) at each developmental stage were used for the qPCR analysis and each sample was amplified in triplicate. Relative mRNA levels of *gPRL* were normalised to the reference genes *GAPDH* and *β-ACTIN* using the comparative C_T method. Data is expressed as the mean ±SD and was analysed using one-way ANOVA, followed by the Duncan's multiple range test. Different lowercase letters over the bar indicate significant differences between different developmental stages at $p<0.05$.

bands with molecular weights of about 23- and 26 kDa in the pituitary gland throughout the development stages of the geese, which corresponded to the non-glycosylated and glycosylated isoforms of gPRL, respectively. As is shown in Figure 5b, the protein contents of total PRL gradually increased from ED22 to PD2 followed by a non-significant decrease on PD6, and then increased again to reach the highest levels on PD28 ($p<0.05$). As is shown in Figure 5c, the relative proportion of G-PRL in the pituitary glands of the geese fluctuated from about 30.3% to 54.7% during the embryonic and post-hatch developmental stages, showing a significantly higher percentage on both ED22 and PD12 ($p<0.05$) while having the lowest percentage on ED26 ($p<0.05$).

Discussion

In addition to its critical roles in regulating incubation behaviour and reproductive performance (ZADWORNY *et al.* 2002; KANSAKU *et al.* 2008), PRL also plays an important role during ontogenesis in birds (DONEEN & SMITH 1982; BÉDÉCARRATS *et al.* 1999c; YAMAMOTO *et al.* 2003). To verify the existence of the glycosylated form of PRL and to explore its physiological relevance during ontogenesis in domesticated geese, in the present study we efficiently generated a rabbit-anti-gPRL polyclonal antibody. Through a Western blot analysis using this antibody, we showed the presence of two PRL isoforms, i.e. the NG- (~23 kDa) and G-PRL (~26 kDa), in the pituitary glands of developing geese. Furthermore, we revealed changes in the levels of total *PRL* mRNA and protein, as well as in the percentage of G-PRL in the pituitary gland of a goose during the embryonic and early post-hatch developmental stages.

In turkey hens, both the expression levels and stability of *PRL* mRNA change during different physiological states (TONG *et al.* 1997). Similarly, in the

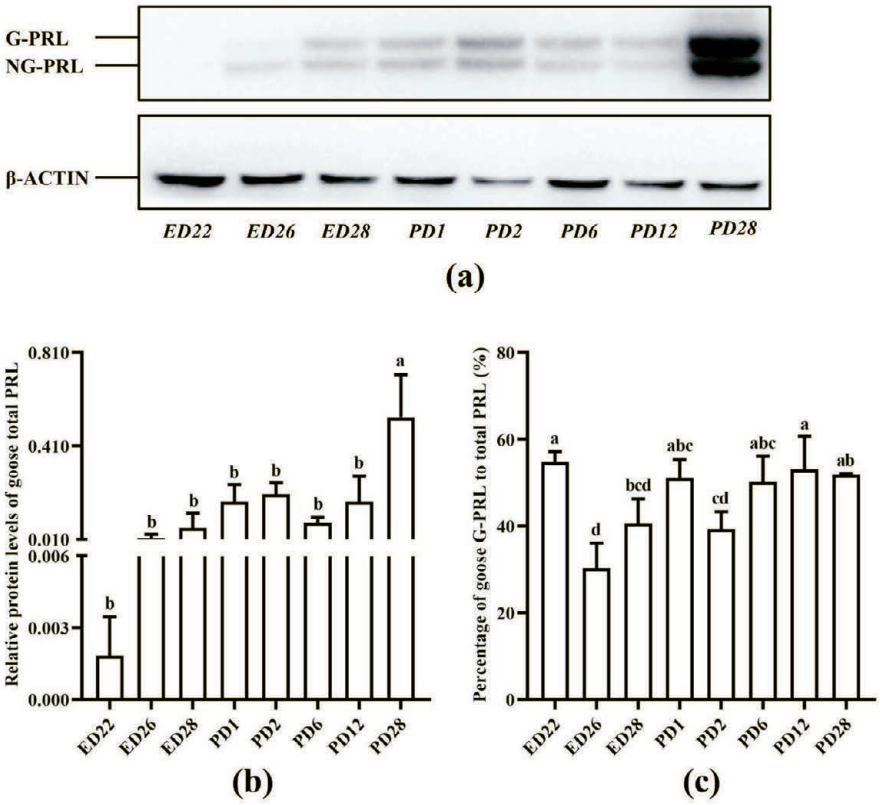

Fig. 5. The protein expression patterns of two PRL isoforms in the pituitary glands of geese during the embryonic and post-hatch developmental stages. a – Representative Western blot results of two PRL isoforms in the geese developing pituitary glands. b – Densitometric measurements of the relative protein levels of total PRL in the geese developing pituitary glands. c – Changes in the percentage of G-PRL in the geese developing pituitary glands. ED, embryonic day; PD, post-hatch day. Three individuals (n=3) at each developmental stage were used for the Western blot analysis. Data is expressed as the mean ±SD and was analysed using one-way ANOVA, followed by the Duncan's multiple range test. Different lowercase letters over the bar indicate significant differences between different developmental stages at $p<0.05$.

present study, we observed that the mRNA levels of *gPRL* in the pituitary gland progressively increased from ED22 to PD12, showing significant changes on PD6 when compared to the developmental stages before PD2 and reaching the highest levels on PD12. However, in chicken hens, it has previously been reported that the expression of *PRL* mRNA in the pituitary gland was initially maintained at a relatively low level until ED19, then increased gradually and reached the highest level on the day of hatching, before decreasing later on (ISHIDA *et al.* 1991). In turkey hens, although the *PRL* mRNA expression remained low during embryonic and early post-hatch development until 5 days before hatching, it increased significantly around the day of hatching and continued to increase during the first two post-hatch weeks (BÉDÉCARRATS *et al.* 1999c). Thus, it has been proposed that changes in the contents of *PRL* mRNA in the pituitary gland could be involved in the regulation of the physiological changes taking place during the embryo-to-hatchling transition in poultry, and that differences in the developmental patterns of *PRL* mRNA among chickens, turkeys and geese suggest that there are species-specific regulatory mechanisms controlling the PRL transcription during early ontogenesis. In support of this view, WONG *et al.* (1991) reported that the varying expression levels of *PRL* mRNA in the pituitary gland were related to changes in the PRL-releasing activity throughout the reproductive cycle of the domestic turkey hen. Furthermore, it has been shown that the varying levels of *PRLR* mRNA during the embryonic and early post-hatch developmental stages were involved in the hormonal control of osmoregulation (YAMAMOTO *et al.* 2003; LECLERC *et al.* 2007). Of particular note, although the mRNA and protein expression profiles of *gPRL* in the pituitary gland were almost similar from ED22 to PD2, they differed during the stages from PD6 to PD28, implying that the *gPRL* gene expression in the pituitary gland involves both transcriptional and post-translational regulation during ontogenesis.

In domestic birds, glycosylation is considered as the major post-translational modification of PRL, and both the non-glycosylated (~24 kDa) and glycosylated (~27 kDa) isoforms of PRL have been detected in the pituitary glands of chickens, turkeys and ducks (ZADWORNY *et al.* 2002; KANSAKU *et al.* 2008). In addition, multiple PRL isoforms with different molecular weights have been observed in the pituitary gland of the Magang goose (LIU *et al.* 2008). In this study, our results unequivocally demonstrated the ubiquitous presence of both NG- (~23 kDa) and G-PRL (~26 kDa) in the pituitary gland of the Sichuan White goose during its embryonic and early post-hatch developmental stages. Furthermore, it was observed that the percentage of G-PRL relative to total PRL fluctuated throughout the development cycle. Although the biological actions of G-PRL in the pituitary gland of poultry remain unclear, it is widely recognised that the glycosylation of PRL could alter its topological structure and thus affect its ability to bind to its receptors, which may result in secondary changes in its downstream signal transduction pathways that could eventually change its biological activities and functions (BOLE-FEYSOT *et al.* 1998; FREEMAN *et al.* 2000; ZADWORNY *et al.* 2002; CAPONE *et al.* 2015). Previous studies in humans and mammals have shown that glycosylation alters the biological activities of PRL (LEWIS *et al.* 1985; PANKOV & BUTNEV 1986; MARKOFF *et al.* 1988; SINHA 1995). In chicken hens, there is also evidence that compared to NG-PRL, G-PRL shows different effects on basal and gonadotrophin-stimulated steroidogenesis in *in vitro* cultured ovarian follicular cells, which is related to their differential actions on the expression of PRLR (HU & ZADWORNY 2017; HU *et al.* 2017).

In conclusion, the present study has demonstrated the existence of two PRL isoforms (i.e. G- and NG-PRL) in the pituitary gland of the goose and that the levels of total *PRL* mRNA and protein, as well as the percentages of the two PRL isoforms, change significantly during the embryonic and early post-hatch developmental stages, indicating that the versatile biological functions of PRL during the ontogenesis of the goose could be closely related to changes in both its total expression and the degree of glycosylation in the pituitary gland.

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Author Contributions

Research concept and design: S.H., J.W.; Collection and/or assembly of data: S.H. L.L., X.R., D.D., Lg.L.; Data analysis and interpretation: S.H., L.L., X.R., E.Q., H.H., J.W.; Writing the article: S.H., L.L.; Critical revision of the article:, J.W.; Final approval of article: S.H., L.L., X.R., E.Q., D.D., H.H., Lg.L., J.W.

Conflict of Interest

The authors declare no conflict of interest.

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