***animal* - The International Journal of Animal Biosciences**

**Dietary calcium deficiency suppresses follicle selection in laying ducks through mechanism involving cyclic adenosine monophosphate-mediated signaling pathway**

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**Supplementary Material S1**

*Plasma hormone assays*

Plasma concentrations of LH and FSH were assayed by radioimmunoassay (**RIA**).

FSH was measured by homologous RIA, following instructions provided with the kit from the same company. The human FSH RIA kit provided by the Beijing North Institute of Biological Technology (Beijing, China) included a highly purified preparation of human FSH, used as a standard, and a polyclonal rabbit antiserum. A highly purified preparation of human FSH was used as a standard, along with a polyclonal rabbit antiserum and FSH was iodinated by the chloramine-T procedure. The iodinated protein was separated on a PD-10 column followed by further purification on Sephadex G-75. The RIA was carried out according to the instructions that came with the RIA kit. Separation of bound and free radioactivity was achieved by incubation with a second antibody-coated cellulose suspension, centrifugation, and removal of the supernatant by aspiration. It is noted that the duck FSH amino acid sequence exhibited 96% homology to that of human. The lowest detectable level for FSH assay was 10 μ IU/mL. The antiserum used showed no cross-reaction with different purified preparations of LH (< 2%). Samples of a pool of plasma from ducks were included in each assay for a check on the reliability of each assay. The quality control assay were conducted for recovery rate based on the standard purified FSH, and the recovery rate was 82%. The intra- and interassay coefficients of variation were 11% and 20%, respectively.

LH was measured using a homologous RIA. Like FSH assay, reagents, including human LH was radioiodinated by the chloramine-T procedure according to the commercial recommendation procedure (Beijing North Institute of Biological Technology, Beijing, China). The iodinated protein was separated by gel filtration on a PD-10 column, and the assay procedure was performed according to the recommendations supplied with the RIA kit. It is noted that the duck FSH amino acid sequence exhibited 96% homology to that of human.The lowest detectable level of LH was 10 μ IU/mL. Cross-reactivity of the antibody used with LH was < 4% for FSH. The recovery rate for LH was 84%. The intra- and interassay coefficients of variation were 1.1% and 25%, respectively.

**Supplementary Tables**

**Table S1** *Composition and calculated analysis of experimental diets in laying ducks (%, as fed)*

|  |  |  |  |
| --- | --- | --- | --- |
| Variable | Dietary calcium content, % | | |
| 3.6 | 1.8 | 0.38 |
| Ingredients |  |  |  |
| Corn | 55.94 | 51.64 | 47.507 |
| Wheat bran | 10.30 | 16.83 | 16.795 |
| Soybean meal | 22.48 | 19.07 | 17.13 |
| Defatted rice bran | 0 | 5.85 | 15.83 |
| *DL*-Methionine | 0.15 | 0.15 | 0.15 |
| *L*-Lysine HCl | 0 | 0.05 | 0.06 |
| Tryptophan | 0 | 0 | 0.048 |
| Limestone | 8.46 | 3.85 | 0 |
| Dicalcium Phosphate | 1.37 | 1.26 | 1.18 |
| Salt | 0.30 | 0.30 | 0.30 |
| Premix1 | 1.00 | 1.00 | 1.00 |
| Total | 100 | 100 | 100 |
| Nutrient composition |  |  |  |
| CP | 17.05 | 17.05 | 17.05 |
| AME2 (Mcal/kg) | 2.52 | 2.52 | 2.54 |
| Ca | 3.60 | 1.83 | 0.38 |
| Available P | 0.36 | 0.36 | 0.36 |
| Methionine | 0.41 | 0.41 | 0.41 |
| Lysine | 0.85 | 0.85 | 0.85 |
| Cysteine + Methionine | 0.72 | 0.71 | 0.72 |
| Arginine | 1.15 | 1.14 | 1.16 |
| Tryptophan | 0.26 | 0.26 | 0.26 |
| Threonine | 0.64 | 0.63 | 0.64 |

1The premix provided the following per kilogram of diet: Vitamin A 12 000 IU, cholecalciferol D3 1 800 IU, Vitamin E 8.2 mg, riboflavin 9.6 mg, niacinamide 114 mg, D-pantothenic acid 28.5 mg, choline chloride 500 mg, cobalamin 30 μg, menadione 0.96 mg, DL-α-tocopheryl acetate 6 mg, Fe 52 mg, Cu 10.4 mg, Zn 91 mg, Mn 91 mg, I 0.52 mg, Co 0.26 mg, and Se 0.40 mg.

2AME, apparent metabolizable energy.

**Table S2** *Oligonucleotide primers for polymerase chain reactions of genes in laying ducks*1

|  |  |  |  |
| --- | --- | --- | --- |
| Gene1 | Accession Number | Primer pairs | Product length, bp |
| *ADCY1* | XM\_021268899.1 | F:5’CGCCACCTCCGTCAGCACC3’  R:5’TCCAGCCTCAGCCTGTCCTCTA3’ | 174 |
| *ADCY3* | XM\_021275394.1 | F:5’CTCGGTCCTCTGGGTCCTTATTTTG3’  R: 5’CGATGAGCACGATGGGCG3’ | 161 |
| *ADCY5* | XM\_021270949.1 | F:5’GAGGACCCCAAGGACAAGAA3’  R:5’GAGGAAGACTAAGGAGGCACAA3’ | 225 |
| *ADCY8* | XM\_021277472.1 | F:5’CGTCATGCAGCTGGTTATCC3’  R:5’TGTCTCAAGCCTCAGTCTGG3’ | 184 |
| *ADCY10* | XM\_013110146.2 | F:5’TCATCAGTCCTCACAAGGGCGA3’  R:5’ACACTCACTCAAGATTCCTCAACGA3’ | 180 |
| *PDE1A* | XM\_021270398.1 | F:5’ATGCTGTACAGGCTGGGATT3’  R:5’AGGGATCTTGAAACGGCTCA3’ | 221 |
| *PDE1C* | XM\_021268522.1 | F:5’GTTCCCAATGTGACCTTCTGCCTA3’  R:5’CTCTTTGTATTCCAGCCTCCTTCTT3’ | 164 |
| *ER1* | XM\_021275842.1 | F:5’TGCCAAGCCTGCCGACTA3’  R:5'GCTGGACTGTTCTTCTTGTTATGTT3' | 212 |
| *ER2* | XM\_021274556.1 | F:5’CATCTGCCCAGCTACCAAT3’  R:5’TGAGCCACATTTCATCATTCC3’ | 109 |
| *FSHR* | XM\_021267215.1 | F:5’GATAACTCTGGAAAGGTGGCATACT3’  R: 5’CACCGTGAAAGCAAACATCCA3’ | 115 |
| *LHR* | XM\_021267245.1 | F:5’GCTCTGTGATAACTTGCGTATG3’  R:5’ATTAGTTGATTCAGCTTGGTCCC3’ | 147 |
| *GJA1* | XM\_005017369.3 | F:5’CTCAAGGTGGTCCAGAAT3’  R:5’CTCGCTCACAGGTGTAGAT3’ | 232 |
| *GJA4* | XM\_026104659.1 | F:5’TACGACAAAGCCTTCCCCAT3’  R:5’GACAGATAAACGACGTGGCC3’ | 104 |
| *GJB3* | XM\_013104893.2 | F:5’CCCAATACCGTGGACTGCTACAT3’  R:5’TTCACCCTCTGCACGACCC3’ | 146 |
| *GJC1* | XM\_005018428.3 | F:5’AGCAAAACATGATGGCAGGCGA3’  R:5’CTATCTTATGCGGGCACGGCTT3’ | 179 |
| *GJD2* | XM\_005009786.3 | F:5’CCCAGCCTGTGCTTCATA3’  R:5’CACGCTTCATTGACTCCTG3’ | 112 |
| *VLDLR* | NM\_001310401.1 | F:5’CAGTGGTTCAGTAGGACACACCT3’  R:5’CAAACCTGTTTATCATCCCCAT3’ | 132 |
| *β-actin* | NM\_001310421.1 | F:5’GCTATGTCGCCCTGGATTT3’  R:5’GGATGCCACAGGACTCCATAC3’ | 174 |

1*ADCY1* to *10* = *adenylyl cyclase* *1* to *10; PDE1A* = *phosphodiesterases 1A*; *PDE1C* = *phosphodiesterase 1C*; *LHR* = *luteinizing hormone receptor*; *FSHR* = *follicle stimulating hormone receptor*; *ER1* or *2* = *estradiol receptor 1 or 2*; *GJA1* or *4* = *gap junction A1* or *A4*; *GJB3* = *gap junction B3*; *GJC1* = *gap junction C1*; *GJD2* = *gap junction D2*; *VLDLR* = *very low density lipoprotein receptor*.