# The Differential Expression of Hepatic Genes Between Prelaying and Laying Geese[1]

S. T. Ding,*[2] C. F. Yen,* P. H. Wang,* H. W. Lin,* J. C. Hsu,† and T. F. Shen*

*Department of Animal Science and Technology, National Taiwan University, Taipei 106; and †Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan

**ABSTRACT** Suppression subtractive hybridization was used to detect differential expression of genes in the livers of laying and prelaying geese. Liver tissues from prelaying and laying geese were dissected for mRNA extraction. The cDNA, reverse transcribed from liver mRNA of prelaying geese, was subtracted from the cDNA generated from the laying geese (forward subtraction). Five hundred seventy-six clones with possible differentially expressed gene fragments were observed by forward subtraction hybridization. After differential screening using the reverse and forward subtraction cDNA, 164 clones were subjected to gene sequence determination and further analysis. Using Northern analysis, 5 known and 8 unknown genes were shown to be highly expressed in the livers of laying geese compared with prelaying geese. Vitellogenin I, apoVLDL-II, ethanolamine kinase, G-protein gamma-5 subunit, and leucyl-tRNA synthase were highly expressed in the livers of laying geese compared with that from the prelaying geese ($P < 0.05$). The expression of these known genes suggests that their function in the liver of laying geese is primarily involved in lipid and lipoprotein metabolism. Several of these differentially expressed genes were found to be responsive to estrogen stimulation, confirming the involvement of these genes in the egg-laying function of the goose.

**Key words**: apoVLDL-II, laying goose, liver, vitellogenin I

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## INTRODUCTION

The liver in laying birds synthesizes lipids and packages them into lipoprotein particles for transport and deposition into developing oocytes (Perry et al., 1978). Yolk lipids, especially triacylglycerol, are the primary source of energy for developing embryos (Kuksis, 1992). Thus, the requirement for adequate lipid deposition to ensure proper embryonic development is important.

The deposition of lipids into the developing follicle (yolk) requires hepatic synthesis of small, yolk-specific very low density lipoproteins (**VLDL**) that can pass through the granulosa basal membrane. There is also the need for a lipoprotein lipase (**LPL**) inhibitor so that fatty acids are not cleaved from circulating lipoprotein triacylglycerol by peripheral tissue LPL (Walzem et al., 1999). The end result is that most of the triacylglycerol is deposited into the egg. The LPL inhibitor secreted by laying birds has been reported to be apoVLDL-II (Wiskocil et al., 1981; Schneider et al., 1990; Wiskocil et al., 1995). Therefore, there needs to be coordinated expression of several genes for the synthesis and deposition of egg yolk lipids.

From a reproductive standpoint, a short photoperiod has positive effects on sexual maturation in geese (Wang et al., 2005). Whereas there is considerable information on gene expression in the chicken (Carre et al., 2006), the goose genome has not been well studied. Understanding gene expression in the liver of sexually mature geese is a first step toward improving the reproductive efficiency of laying geese. Therefore, this study was conducted to determine the differential expression of genes in the liver of immature (prelaying) and sexually mature (laying) geese using suppression subtractive hybridization (**SSH**). A second objective was to study the effect of estrogen on the expression of some genes found in laying geese.

## MATERIALS AND METHODS

### *Experiment 1. Birds and Diets*

The animal protocol used in the present experiment was approved by the Animal Care and Use Committee of the National Chung Hsing University. All geese were purchased from a commercial goose farm and were raised according to the standard program used at the farm as described by Yen et al. (2006). Geese become sexually mature at approximately 15 mo of age, so goslings purchased in the fall of a given year will become sexually


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[2]Corresponding author: sding@ntu.edu.tw

mature during the winter of the following year. In the current study, the young, prelay geese were reared under a natural photoperiod (November 9 to April 8) and killed at 5 mo of age (average BW = 4.2 ± 0.6 kg). The laying geese started production at 15 mo and were killed at the age of 17 mo (average BW = 4.2 ± 0.4 kg). The daily photoperiod during production was 9L:15D. Geese were killed by electrical stunning followed by exsanguination. Liver samples were collected from individual geese (n = 6 per group) and stored at −70°C until extraction of RNA.

### Suppression Subtractive Hybridization

Total RNA was extracted from individual liver samples by the guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). The SSH procedure utilized the PCR-Select cDNA Subtraction Kit from Clontech (Palo Alto, CA) as previously described by Chang et al. (2007). In brief, 3 μg of mRNA of pooled sample from all 6 birds for each of the 2 periods (laying and prelaying) was reverse transcribed and double strand cDNA synthesized from the mRNA. The initial subtraction procedure detailed by Yen et al. (2006) was followed by a second hybridization to reduce false positive results (Luo and Lai, 2001). The differentially expressed gene fragments were then cloned into a pGEM-T Easy TA cloning vector (Promega, Madison, WI). We randomly picked 576 clones for further differential screening, sequencing, and Northern analysis to confirm the differential expression of genes between livers of laying geese and livers of prelaying geese.

### Differential Screening/Sequencing

The differential screening procedure followed the PCR-Select Differential Screening Kit User manual (Clontech). Details for the screening procedure were also described by Wang et al. (2006). This procedure was used to eliminate false positive clones. The sequences of 164 clones were determined by an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and confirmed by differential screening. The clones were selected because they were found to be differentially expressed in the livers of prelaying and laying geese. The sequences were then compared against the nonredundant nucleotide database in National Center for Biotechnology Information and the gene indices database in the Institute for Genomic Research using blastn to determine known and unknown genes. Thirteen genes were selected for transcript analysis based on known genes associated with lipid and lipoprotein metabolism and on adequate levels of mRNA expression to allow analysis by Northern blot procedures.

### Experiment 2

To study the effect of estrogen on gene expression in prelay geese, 10 female geese were randomly divided into 2 treatment groups (control, estrogen-treated) at 3 mo of age (BW = 3.92 ± 0.26 kg). The estrogen-treated geese were injected in the leg muscle with estradiol benzoate (0.325 mg/kg of BW; China Chemical & Pharmaceutical Co., Taipei, Taiwan) once a day for 2 consecutive days. The treatment dose followed the one previously used by Stake et al. (1981) in treating laying hens. The control geese were injected with the same concentration of corn oil (Sigma, St. Louis, MO). Geese were killed by electrical stunning followed by exsanguinations, and liver samples were collected from individual geese (n = 5 per group) and stored at −70°C until extraction of RNA. All the 10 livers were analyzed individually for the relative mRNA concentration of 8 of the 13 genes confirmed to be differentially expressed in the prelaying and laying geese in experiment 1. The procedures for RNA extraction and mRNA quantification were the same as described in experiment 1.

### Transcript Analysis

Electrophoresis and Northern blotting were performed using 20 μg of total RNA from each goose liver. The mRNA concentration of 5 known genes, including apoVLDL-II, vitellogenin I, ethanolamine kinase, G-protein gamma-5 subunit, leucyl-tRNA synthase, and 8 unknown genes was quantified by Northern blot analysis (Ding et al., 2004). The concentration of 18S rRNA, a housekeeping gene, was also quantified.

Radiolabeled probes synthesized by PCR were used for hybridization. The nested primer set from the PCR-Select cDNA Subtraction Kit (Clontech), forward = 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ and reverse = 5′-AGCGTGGTCGCGGCCGAGGT-3′ was used for all 13 genes cloned into pGEM-T Easy TA cloning vector (Promega). The source of the probes for 18S rRNA is indicated by Liu et al. (2005). The relative mRNA concentration was determined by phosphor-image analysis (Typhoon-9200, General Electric Company, Fairfield, CT) with ImageQuant software as previously described (Yen et al., 2005). The density value for each sample was normalized to the density value for 18S rRNA in the same sample to correct for extraction, sampling, gel loading, and membrane transfer variation.

### Statistical Analysis

All data from experiments 1 and 2 were analyzed by Student's *t*-test using the procedures of the SAS software (SAS Institute, 2001).

## RESULTS AND DISCUSSION

Five hundred seventy-six clones of gene fragments resulting from SSH assay were subjected to differential screening to reduce false positive clones. There were 164 clones confirmed as differentially expressed in the prelay and laying geese (Table 1). Sequences of these gene fragments showed that 26 genes were differentially expressed in the livers of laying geese compared with the prelay geese. Thirteen genes, including 5 known genes chosen

**Table 1.** Genes expressed in the liver of laying geese at greater levels than that in the prelaying geese

| Function | Gene | Accession no. | Clones | Length[1] (bp) |
|---|---|---|---|---|
| Known gene | | | | |
| | Vitellogenin I* | DQ980211 | 84 | 325 |
| | ApoVLDL II* | DQ980212 | 39 | 503 |
| | Ferritin H chain | DQ980213 | 13 | 348 |
| | Retinoblastoma-associated protein 140 | DQ980214 | 4 | 163 |
| | Fibrinogen gamma chain | DQ980215 | 3 | 281 |
| | Ethanolamine kinase* | DQ980218 | 1 | 319 |
| | G-protein gamma-5 subunit | DQ980219 | 1 | 152 |
| | Leucyl-tRNA synthase* | DQ980220 | 1 | 168 |
| | Vitamin D binding protein precursor | DQ980216 | 1 | 134 |
| | Fibronectin | DQ980217 | 1 | 256 |
| | Niemann-Pick disease, type C2 | DQ980221 | 1 | 221 |
| Unknown gene | | | | |
| | LEUG 1[2]* | DQ980222 | 1 | 131 |
| | LEUG 2* | DQ980223 | 1 | 763 |
| | LEUG 3* | DQ980224 | 1 | 302 |
| | LEUG 4* | DQ980225 | 1 | 266 |
| | LEUG 5* | DQ980226 | 1 | 223 |
| | LEUG 6* | DQ980227 | 1 | 201 |
| | LEUG 7* | DQ980228 | 1 | 174 |
| | LEUG 8* | DQ980229 | 1 | 242 |
| | LEUG 9 | DQ980230 | 1 | 157 |
| | LEUG 10 | DQ980231 | 1 | 270 |
| | LEUG 11 | DQ980232 | 1 | 221 |
| | LEUG 12 | DQ980233 | 1 | 707 |
| | LEUG 13 | DQ980234 | 1 | 195 |
| | LEUG 14 | DQ980235 | 1 | 157 |
| | LEUG 15 | DQ980236 | 1 | 217 |
| Total | 26 genes | | 164 | |

[1]Length of the longest sequence of a single clone or multiple clones of the same gene was indicated.

[2]LEUG = liver expressed unknown genes.

*Differentially expressed genes confirmed by Northern analysis.

on the basis of their metabolic importance and 8 unknown genes were confirmed by Northern analysis (Figure 1). The other unknown genes were not confirmed due to their relatively low mRNA concentrations in both groups of geese.

The ApoVLDL-II mRNA was more than 2-fold higher in laying geese compared with the prelaying geese (Figure 1). In this regard, the goose is similar to other poultry species in which apoVLDL-II is highly expressed in the liver of the laying birds (Wiskocil et al., 1981, 1995). The amino acid sequence for chicken apoVLDL-II was determined by Jackson et al. (1977). In avian species, apoVLDL-II is involved in the formation of small VLDL particles for deposition in the yolk (Walzem et al., 1999) and for the prevention of lipolytic activity by peripheral LPL (Schneider et al., 1990). In the second experiment, we also found that the expression of goose apoVLDL-II was induced by estrogen treatment (Figure 2), suggesting that this gene is responsive to estrogen. Similar data in chickens were reported (Kudzma et al., 1979), and Elbrecht et al. (1981) reported that embryonic hepatocytes in culture can be induced by estrogen to express apoVLDL-II.

Vitellogenin I was also highly expressed in the livers of laying geese compared with prelaying geese (Figure 1). Vitellogenin is a precursor of one of the major proteins in the avian egg yolk. Evans et al. (1988) cloned chicken vitellogenin I and III cDNA and demonstrated that they were induced by estrogen treatment. Estrogen secretion is greatly increased around the onset of egg-laying in the chicken (Senior, 1974). The expression of vitellogenin I is responsive to estrogen in the chicken (Wetekam et al., 1975; Bakker et al., 1988; Evans et al., 1998). In the present study, we demonstrated that goose vitellogenin I was highly expressed in the livers of laying geese but not in prelaying geese, suggesting that in the goose liver, this gene is also responsive to estrogen. Indeed, when estrogen was injected into prelay geese, the expression of vitellogenin mRNA was induced (Figure 2), confirming the responsiveness of this gene to estrogen. Similar data were reported in chickens (Wetekam et al., 1975; Deeley et al., 1977; Evans et al., 1988).

The G-protein subunit $\gamma 5$ was found to be highly expressed in the livers of laying geese compared with prelay geese (Figure 1). This protein is one of the G-protein complex families of proteins involved in extracellular signaling through an adenylate cyclase mediated pathway (Freissmuth et al., 1989; Gautam et al., 1990; Tang and Gilman, 1991) and is also involved in the activation of phospholipase C (Camps et al., 1992). Phospholipase C is involved in the cleavage of inositol phospholipids to generate diacylglycerol required for activation of protein kinase C (Nishizuka, 1995), whereas adenylate cyclase is involved in lipolysis and other physiological mechanisms involved in lipid metabolism (Holm et al., 1997). Fisher and Aronson (1992) cloned the $\gamma 5$ subunit for G protein from the liver of the cattle and rats, but goose G-protein $\gamma 5$

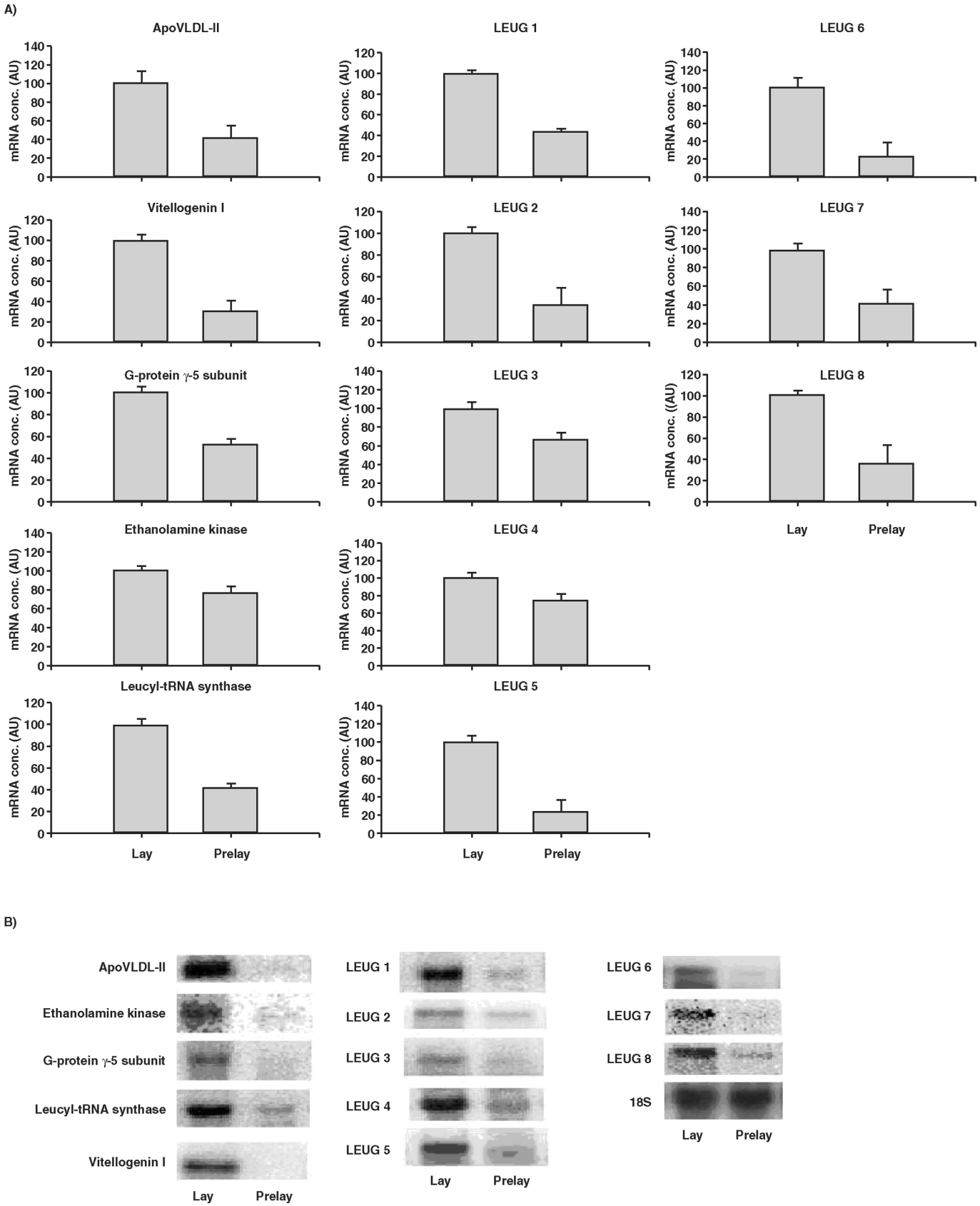


**Figure 1.** The differential expression of genes in the liver in laying geese (lay) compared with prelaying geese (prelay). On the day of sampling, 6 geese from each group were killed, and livers were dissected for RNA extraction. The relative mRNA concentrations of apoVLDL-II, ethanolamine kinase, G-protein γ-5 subunit, leucyl-tRNA synthase, vitellogenin I, and 8 liver expressed unknown genes (LEUG1 to 8) were determined by Northern analysis. The mRNA values were normalized to 18S ribosomal RNA (18S) content. Bars in Figure 1(A) are means with SE. For each gene, the relative mRNA concentration of the laying geese was significantly greater than that of the prelaying geese ($P \leq 0.05$). Conc. = concentration; AU = arbitrary unit.

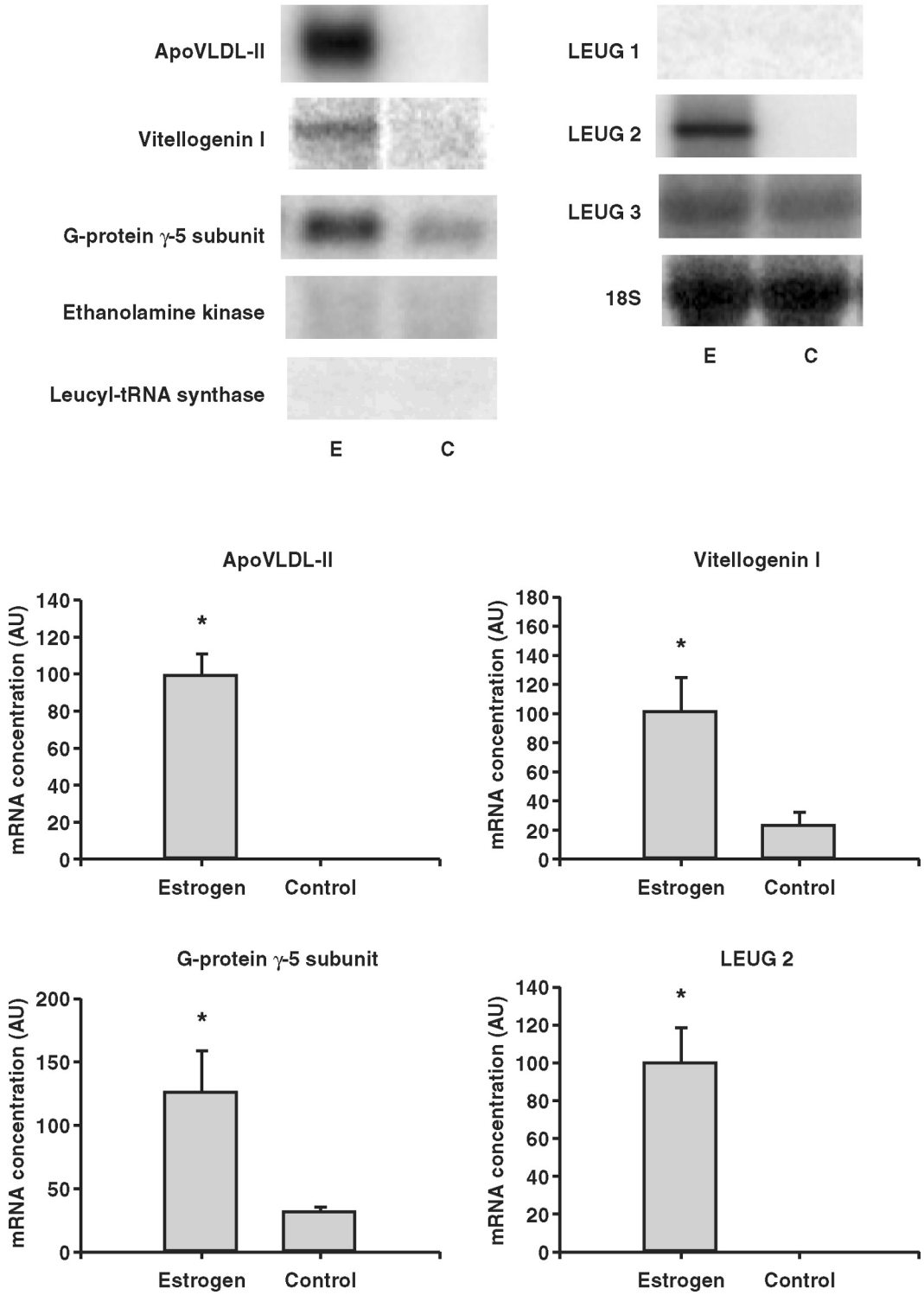


**Figure 2.** The effect of estrogen on the gene expression in prelaying geese. Ten female geese at the age of 12 wk were randomly divided into 2 treatment groups (BW = 3.92 ± 0.26 kg), control group (C) and estrogen-treated group (E). The geese from the E group were injected in the leg muscle with estradiol benzoate (China Chemical & Pharmaceutical Co., Taipei, Taiwan) at the level of 0.325 mg/kg of BW once a day during the 2-d experimental period. The geese in the C group were injected with the same amount of corn oil (Sigma, St. Louis, MO) in the leg muscle. The liver tissues were taken to analyze the mRNA concentrations for 8 of the 13 genes confirmed to be differentially expressed between prelaying and the laying goose. *Indicates significant difference between treatments. 18S = 18S ribosomal RNA; LEUG = liver expressed unknown gene.

subunit has not been reported in the literature. Whereas the mRNA was highly expressed in the laying goose compared with the prelaying goose, the relationship of the greater mRNA level to increased function remains to be demonstrated. In experiment 2, the estrogen injection also resulted in increased gene expression (Figure 2), confirm-

ing that it is responding to estrogen secretion in laying geese.

Ethanolamine kinase mRNA was highly expressed in the livers of laying geese compared with prelaying geese (Figure 1). There are 3 isoforms of ethanolamine kinase reported in different tissues of mammals (Aoyama et al., 2002). Ethanolamine kinase is the rate-limiting enzyme for phosphatidylethanolamine biosynthesis (Porter and Kent, 1990; Kent, 1995; Lykidis et al., 2001). This is the first report of ethanolamine kinase expressed in the goose liver. The greater mRNA level of this gene in laying geese compared with prelay geese suggests that in laying geese there is increased phospholipids metabolism. The expression of ethanolamine kinase mRNA was not induced by estrogen injections (Figure 2), suggesting that this gene is not estrogen-responsive. Because we observed that there was detectable level of this gene in older prelaying geese (5 mo of age; Figure 1) and no detectable level was found in the younger geese (3 mo of age), we speculate that the expression of this gene is age-related. There are no comparable data available in any avian species.

Leucyl-tRNA synthase was highly expressed in the livers of laying geese compared with prelay geese (Figure 1). This enzyme is a component of the translation machinery (Dang et al., 1985; Lindqvist et al., 1989; Rodovicius et al., 2004), and increased enzyme activity may represent an overall increase in protein synthesis needed for hepatic production of vitellogenin and apoVLDL-II. In the current study, we also found that the expression of Leucyl-tRNA synthase mRNA was also not induced by estrogen injection (Figure 2), again suggesting that this gene is not estrogen-responsive. Because there was detectable level of Leucyl-tRNA synthase mRNA in older prelaying geese (5 mo of age; Figure 1) and no detectable level was found in the younger geese (3 mo of age), we speculate that the expression of this gene is age-related. To the best of our knowledge, this is the first report to observe such a phenomenon in any avian species.

When some of the sequences from the current subtractive library were compared by Blast analysis with the sequences found in GenBank, there was no significant homology with known genes, so they were defined as unknown genes. The functions of these liver expressed unknown genes (**LEUG** 1 to 8) are not known, but they were highly expressed in the liver of the laying goose, suggesting their possible involvement in laying goose hepatic functions. In the current study, we also found that the expression of LEUG 1 and 3 mRNA was not induced by estrogen injections (Figure 2), indicating that these genes are not estrogen-responsive. The expression of LEUG 2, however, in the liver of the prelaying geese was increased by the estrogen injection, suggesting that this gene is responsive to estrogen stimulation. Further investigation is needed to identify specific functions of the unknown genes discovered in the current study, especially the LEUG 2 due to its responsiveness to estrogen treatment.

In conclusion, we have shown that there are a number of genes specifically expressed in the liver of the laying goose but not in prelay geese. To the best of our knowledge, this is the first report that demonstrates the expression of goose apoVLDL-II, vitellogenin I, ethanolamine kinase, G protein, and 8 novel genes in the laying goose liver. We also showed that several genes are responsive to estrogen stimulation, suggesting that these genes are important to egg laying in geese. Further studies on the functions of the unknown genes discovered in the current study will also enhance our understanding of the reproductive physiology of the goose.

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