BREEDING AND GENETICS

Validation of a Modified PCR-Based Method for Identifying Mutant Restricted Ovulator Chickens: Substantiation of Genotypic Classification by Phenotypic Traits

R. G. Elkin,*,[1,2] Y. Zhong,*,[3] R. E. Porter, Jr.,†,[4] and R. L. Walzem‡,[5]

*Departments of *Animal Sciences and †Veterinary Pathobiology, Purdue University, West Lafayette, Indiana 47907; and ‡Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616*

ABSTRACT Upon photostimulation, restricted ovulator (RO) female chickens exhibit endogenous hyperlipidemia, develop atherosclerotic lesions, and generally fail to lay eggs. This phenotype results from a point mutation in the gene specifying the very low density lipoprotein receptor (VLDLR), whose protein product normally mediates the massive oocytic uptake of egg yolk precursors from the circulation. Taking advantage of the single base change in the mutant VLDLR allele, a PCR-based method for the rapid identification of RO chickens was developed at the Biocenter and University of Vienna, Austria. However, this procedure was incompletely validated because phenotypic data were not obtained and conventional progeny testing of sons and grandsons was not performed. Here, the assay validation was completed by providing plasma lipid concentrations, plasma very low density lipoprotein particle sizes, or egg production records of PCR-genotyped females and their brothers and sires to demonstrate that each bird's phenotypic traits substantiated their genotypic classification. Moreover, several methodological modifications resulted in improved chemical safety, speed, and cost of preparing and analyzing genomic DNA from chicken erythrocytes. Because the ovaries of mutant RO females generally contain numerous vitellogenic follicles in the absence of a functional oocyte plasma membrane VLDLR, the existence of an alternate system for the oocytic uptake of plasma very low density lipoprotein and vitellogenin is suggested, whereas a physiological explanation as to why some, but not all, mutant RO hens are able to ovulate and lay eggs is lacking.

(*Key words*: hyperlipidemia, PCR, point mutation, restricted ovulator chicken, very low density lipoprotein receptor)

2003 Poultry Science 82:517–525

INTRODUCTION

A unique subpopulation of nonlaying White Leghorn chickens with hereditable hyperlipidemia and aortic atherosclerosis was first described by Ho et al. (1974). Because these defects were transmitted from a rooster to half of his female offspring, these researchers suspected that the condition was hereditable. Subsequent matings confirmed that the rooster, which was phenotypically normal in terms of its blood lipids, was only a carrier of the unidentified abnormal gene; hence, afflicted males were subsequently termed "carrier roosters" (Jones et al., 1975). Jones et al. (1975) also coined the term "restricted ovulator" (RO) to describe females with the mutant gene *ro*.

In comparison to wild-type (WT) hens, the blood plasma of sexually mature RO females is extremely lipemic and resembles egg yolk in color, and the ovaries of RO females contain numerous small yellow follicles and approximately 20 to 30 vitellogenic (yolk-filled) follicles ranging in size from approximately 3 mm to 20 mm (Figure 1). Moreover, a typical follicular hierarchy (Etches and Pettite, 1990) is absent, whereas abnormal follicles varying in both shape (some contain multiple nodules and are thus raspberry-like in appearance) and color (from pale yellow to green to chocolate brown) are evident (Figure 1) (Ho et al., 1974; Jones et al., 1975; Grau et al., 1979; Mitchell et al., 1979; Nimpf et al., 1989). Despite these striking changes in ovar-

Received for publication August 27, 2002.
Accepted for publication November 22, 2002.

[1]Present address: Department of Poultry Science, The Pennsylvania State University, University Park, PA 16802.
[2]To whom correspondence should be addressed: relkin@psu.edu.
[3]Present address: Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591.
[4]Present address: Wisconsin Veterinary Diagnostic Laboratory, 6101 Mineral Point Road, Madison, WI 53705.
[5]Present address: Department of Poultry Science, Texas A&M University, College Station, TX 77843.

Abbreviation Key: LRP = low density lipoprotein receptor-related protein; nt = nucleotide; OD = optical density; RO = restricted ovulator; RO_{sc} = screwy RO; *Taq* = *Thermus aquaticus*; VLDL = very low density lipoprotein; VLDLR = very low density lipoprotein receptor; WT = wild-type

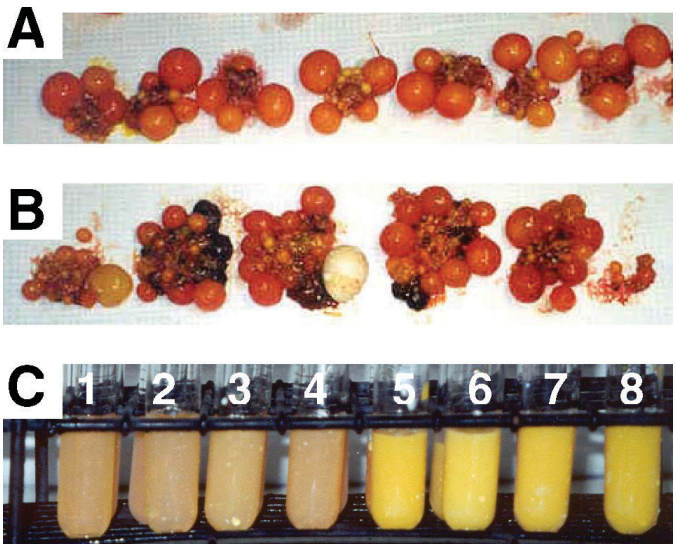

FIGURE 1. Representative ovaries and blood plasma from wild-type (WT) and restricted ovulator (RO) hens. In comparison to the ovaries of WT hens (panel A), the ovaries of RO females (panel B) contain numerous small yellow follicles and approximately 20 to 30 vitellogenic (yolk-filled) follicles ranging in size from approximately 3 mm to 20 mm. Moreover, a typical follicular hierarchy is absent, and abnormal follicles varying in both shape and color are evident. A hard-shelled egg found in the oviduct of one of the hens was removed and placed beside the respective ovary. As compared to blood plasma from sexually mature WT hens (panel C, tubes 1 to 4), blood plasma from sexually mature RO females (panel C, tubes 5 to 8) is extremely lipemic and resembles egg yolk in color. Samples were obtained from 85-wk-old second-cycle hens.

ian morphology, RO hens are able to lay eggs, although they are generally very few in number, small (<42 g; Grau et al., 1979), and tend to have darker colored yolks than those from WT hens (Ho et al., 1974; Birrenkott and McGibbon, 1975; Jones et al., 1975; Schjeide et al., 1976; Grau et al., 1979; Mitchell et al., 1979; Cho, 1981). In addition, the yolk ring structure of laid RO eggs has been reported to be completely disrupted, although rings have been observed in follicular yolk (Grau et al., 1979).

McGibbon (1977) was the first to provide evidence that the sterile condition associated with spontaneous follicular involution was sex-linked and resulted from a single gene defect at a locus (*ro*) on the Z chromosome. Nevertheless, the biochemical nature of the RO mutation remained unknown for more than a decade until Nimpf et al. (1989) reported that RO hens fail to express a functional 95-kDa member of the low density lipoprotein receptor supergene family, namely the very low density lipoprotein (VLDL) receptor (VLDLR). A study from the same laboratory (Stifani et al., 1990) then demonstrated that a single plasma membrane protein was responsible for the oocytic uptake from the maternal circulation of both VLDL and vitellogenin, the main yolk precursor macromolecules, and that ovarian membranes from RO hens failed to exhibit the presence of the 95-kDa VLDLR band when incubated with either radiolabeled VLDL or vitellogenin. Bujo et al. (1995) subsequently demonstrated that a single nucleotide substitution (G→C) in the VLDLR gene was responsible for this abnormal phenotype. As a result, a cysteine residue is converted into a serine, resulting in an unpaired cysteine and greatly reduced expression of the mutant VLDLR on the oocyte surface.

Taking advantage of the single base change in the mutant VLDLR allele, Bujo et al. (1996) then developed a PCR-based method for rapid identification of mutant RO chickens. Although this procedure confirmed the presence of the mutant gene in both a known RO carrier male and some of his daughters, as well and the absence of the mutant gene in some unrelated WT females, the method was incompletely validated because phenotypic data were not obtained, and conventional progeny testing of sons and grandsons was not performed. Therefore, the main objective of the present work was to completely validate the PCR method of Bujo et al. (1996) by confirming that each bird's phenotypic traits substantiated their genotypic classification, that only RO carrier roosters sired RO chicks, and that WT roosters never sired RO chicks. Moreover, several procedural modifications imparted advantages over the original methodology in terms of simplicity, speed, safety, and cost.

MATERIALS AND METHODS

Animals, Management, and Samples

Three hundred one eggs, resulting from the natural matings of three known RO carrier roosters with WT White Leghorn females, were obtained from J. J. Bitgood, University of Wisconsin. The eggs were incubated according to the conditions described in Elkin and Yan (1999), and 50, 41, and 19 chicks hatched from 100, 101, and 100 eggs set per sire, respectively. Following hatching, 108 of the chicks were transferred to battery brooders in an isolation facility at the Purdue University School of Veterinary Medicine. Marek's disease-infectious bursal disease vaccine was administered to the chicks via subcutaneous injection at 20 d of age.

At 32 d of age, 102 chicks were transferred to finishing cages and bled via the brachial vein using heparinized, 25-ga needles and 3-cc syringes. The blood samples were kept on ice until subsequent centrifugation at 6,000 × *g* for 10 min at 4°C. The plasma was decanted and submitted to the Purdue Animal Disease Diagnostic Laboratory for serological testing (*Mycoplasma synoviae*, *Mycoplasma gallisepticum*, and *Salmonella pullorum-typhoid*), and the erythrocytes were stored at −80°C prior to genomic DNA isolation and analysis by PCR (see below). Following negative serological test results, the birds were transferred to colony cages in an environmentally controlled room at the Grower Research Unit of the Purdue Animal Sciences Education and Research Center at 51 d of age. At 130 d of age, a second blood sample was obtained from each of the 98 surviving birds as described above and submitted for follow-up serological testing. Upon receipt of the test results, which were negative, 34 roosters and 64 hens were transferred to individual cages in an environmentally controlled room at the Layer Research Unit at 158 d of age. Individual egg production records were kept for the hens for 294 d, at which

time the flock was molted. Housing, lighting, and feeding regimes from 1 to 158 d of age (growing phase) and from 158 to 452 d of age (laying phase) are described in McDaniel et al. (1993), with the exception that the birds were not moved to the grower unit and layer unit until d 51 and 158, respectively, as compared to d 1 and 119, respectively. The relatively late transfer of the birds to the layer unit was due to the adherence to biosecurity protocols that precluded moving the birds until the serological test results were completed (and negative).

One additional blood sample was obtained via the brachial vein from 26 males and 52 females at 360 d of age. Blood was collected using 3-cc syringes, equipped with 25-ga needles, that were preloaded with 68 μL of an anticoagulant cocktail containing disodium EDTA (147 m*M*), penicillin/streptomycin/neomycin (150 U/0.15 mg/0.30 mg),[6] aprotinin (0.02 trypsin inhibitor U),[6] and sodium azide (68 m*M*). The blood samples were kept on ice until subsequent centrifugation at 6,000 × *g* for 10 min at 4°C. The plasma was decanted and shipped on ice to one of the authors (RLW), who was unaware of the identity of the samples, for subsequent analysis of plasma lipids and plasma VLDL particle size distributions (Walzem et al., 1994).

Propagation of the RO Colony and Assessment of the F_1 Population

On d 200, the vent areas of each of 29 PCR-genotyped roosters (11 RO and 18 WT) were feather-trimmed, and the birds were trained with regard to semen collection (Lake, 1957). Beginning on d 222, two White Leghorn WT hens each per rooster were artificially inseminated once weekly using separate, 0.05-mL aliquots of fresh, undiluted semen. The hens (derived from a Canadian randombred line, Strain 3N; W. M. Muir, 1997, Purdue University, personal communication) were 37 wk old at the onset of the breeding period and had documented rates of lay of at least 80% (hen-day production). Eggs were collected daily for 8 wk, but only those laid during the latter 4 wk were incubated as described in Elkin and Yan (1999). The chicks obtained were initially housed in colony cages in the grower unit and were subsequently transferred to individual cages in the layer unit at 125 d of age. Although the primary objective of this breeding protocol was to select and genotype a subpopulation of progeny in order to further validate the PCR method of Bujo et al. (1996), an additional, important goal was to assess the reproductive function of RO carrier roosters, the results of which are reported elsewhere (Elkin and Zhong, 2002).

Brachial vein blood samples were thus obtained from 54 female progeny of the RO carrier roosters (five daughters each from 10 roosters and four daughters from the eleventh) and 54 randomly selected female progeny from the 18 WT sires. The erythrocytes were isolated and stored at −80°C until subsequent genomic DNA isolation and PCR analysis was performed (see below). As was the case with the previous generation of birds, first-cycle individual egg production records were kept for all PCR-genotyped females from 125 to 456 d of age, at which time the flock was molted.

Genomic DNA Isolation From Erythrocytes

A GenomicPrep Blood DNA Isolation Kit[7] was used to isolate genomic DNA from thawed chicken erythrocytes. The kit is based on a modification of published procedures in which salt is substituted for toxic organic solvents in the deproteination step. Procedure B (*Extraction of DNA from Bird, Fish or Frog Nucleated Whole Blood*) was followed according to the manufacturer's instructions. The red cell lysate was also treated with RNase, which was considered to be an optional procedure according to the kit instructions.

The purity of the hydrated DNA was assessed by measuring absorbance (OD) at 260 and 280 nm. In general, samples having OD_{260}/OD_{280} values <1.60 or >2.00 were considered to be contaminated with protein or RNA, respectively, and the DNA extraction procedure was repeated on a new aliquot of erythrocytes. The amount of hydrated DNA was calculated by multiplying the sample OD_{260nm} value times 50, because an OD of 1.000 corresponds to approximately 50 ng/μL for double-stranded DNA (Sambrook et al., 1989). Sample concentrations varied from ~100 to 400 ng DNA/μL, which corresponded to an average yield of ~60 μg of DNA per 5 μL of erythrocytes originally extracted.

PCR Protocol

Three oligonucleotides, with the same nucleotide (nt) sequences as those employed by Bujo et al. (1996), were purchased[8] and used for PCR primers: P1, 5′-ACCCTAGTAAACAACCTCAATGATG-3′; P2, 5′-CCATTTACCATGTTCTCTTCAC-3′; and P3, 5′-CCATTTACCATGTTCTCTTCAG-3′. The nt sequences of the primers corresponded to nt 2,098 to 2,122 (sense), nt 2,177 to 2,198 (antisense; WT sequence), and nt 2,177 to 2,198 (antisense; mutant sequence), respectively, of the chicken VLDLR (Bujo et al., 1994).

A PCR reaction mixture (50 μL) was prepared in 0.2-mL MicroAmp thin-walled reaction tubes[9] and included the following constituents listed in the order added: 45 μL of PCR SuperMix,[8] 1 μL of primer P1, 1 μL of primer P2 or 1 μL of primer P3, and 3 μL of genomic DNA (150 ng). The PCR SuperMix is a 1.1× concentrated, ready-to-use mixture containing recombinant *Thermus aquaticus* (*Taq*) DNA polymerase (22 U/mL), 22 m*M* Tris-HCl (pH 8.4), 55 m*M* KCl, 1.65 m*M* $MgCl_2$, and deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP; 220 μM each). The reaction was run in a GeneAmp PCR system 9700[9] instrument for 30 cycles of 15 s at 94°C, 15 s at 65°C, and 30 s at 72°C. Pre- and post-PCR temperature

[6]Sigma Chemical Company, St. Louis, MO.
[7]Amersham Pharmacia Biotech, Inc., Piscataway, NJ.
[8]Life Technologies, Inc., Grand Island, NY.
[9]PE Applied Biosystems, Foster City, CA.

parameters included a pre-PCR hold for 10 min at 95°C and a post-PCR hold for 5 min at 72°C, immediately followed by ramp-down to 4°C and an infinite hold at that temperature.

Visualization of the PCR Amplification Products

Twenty-five microliters of the PCR-amplified products were mixed with 5 μL of a solution containing 25% Ficoll type 400[6] and 2.5% bromphenol blue and loaded on a 1.5% agarose[8] gel containing ethidium bromide. A 100-bp ladder solution[7] was diluted fivefold in 10 m*M* Tris-Cl/1 m*M* EDTA (pH 7.6) and mixed with the 25% Ficoll/2.5% bromphenol blue solution (62.5/37.5, vol/vol); 4 μL of the mixture was loaded in lanes 1 and 20 of a 20-well agarose gel. Gels were run on a Mini-Sub DNA electrophoresis cell[10] at 100 V for 45 min. The gels were then visualized under ultraviolet light and photographed using a DC-120 digital camera.[11]

Plasma Lipid and VLDL Particle Size Determinations

Plasma total cholesterol and triglycerides were measured using enzymatic assays as reported by Walzem et al. (1994). Plasma VLDL particle diameters and size distributions were determined optically by dynamic laser light scattering using a Microtrack Series 9200 ultrafine particle analyzer[12] (Walzem et al., 1994).

Protein Assay and Analysis of Egg Yolk Extracts by SDS-PAGE

The yolks from four freshly collected eggs (two each from one WT hen and one RO hen) were carefully separated from the albumens using an egg separator; any adhering albumen was removed by rolling the yolk on a moist paper towel. The yolk membrane was then punctured with a forceps, and the yolk was gently squeezed out into a graduated cylinder and mixed with five volumes of an ice-cold solution containing 20 m*M* Tris, 150 m*M* NaCl, 200 μ*M* EDTA, 1 m*M* phenylmethylsulfonylfluoride,[6] and 5 μ*M* leupeptin.[6] Yolk protein contents were measured in the presence of 12.8 m*M* SDS based on the method of Lowry et al. (1951) as modified by Elkin and Schneider (1994). Egg yolk extracts were subjected to SDS-PAGE on 4.5 to 18% gradient gels under reducing conditions (Elkin and Schneider, 1994). Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.[10]

RESULTS

Amplification Products from the Modified PCR-Genotyping Procedure

Initial work was aimed at using the modified genomic DNA isolation-PCR method to correctly genotype several known WT and mutant RO males and females from the University of Wisconsin parent flock. The PCR-amplified genomic DNA from known WT females, WT males, and RO males all resulted in the presence of a 413-bp product with the primer pair (P1/P2) specific for the wild-type gene (Figure 2, lanes 1, 5, and 7, respectively). The DNA from known RO females did not yield a PCR product with primer pair P1/P2 (Figure 2, lane 3) because in all Aves, females are the heterogametic sex. A product of 413 bp was also observed when genomic DNA from a known mutant RO female or RO male was amplified using primer pair P1/P3, which was specific for the mutant gene (Figure 2, lanes 4 and 8, respectively). In addition, the 413-bp product was not observed when the reaction mixture contained WT female or WT male genomic DNA and the primer pair P1/P3 (Figure 2, lanes 2 and 6, respectively).

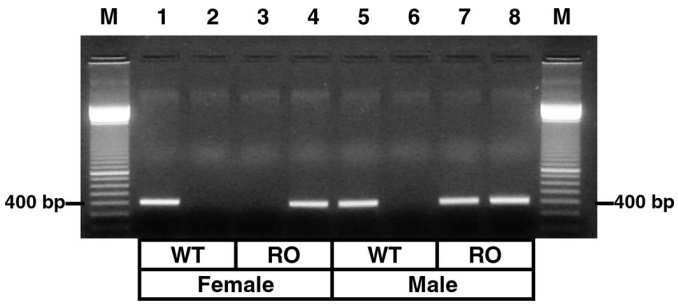

FIGURE 2. The PCR amplification of genomic DNA from wild-type (WT) and restricted ovulator (RO) chickens. The PCR-amplified fragments from 150 ng of genomic DNA from erythrocytes were obtained using primer pairs specific for the WT (lanes 1, 3, 5, and 7) or mutant very low density lipoprotein receptor genes (lanes 2, 4, 6, and 8), respectively. The PCR-amplified products were subjected to agarose gel electrophoresis as described by Bujo et al. (1996). Lanes 1 and 2: WT female; lanes 3 and 4: mutant RO female; lanes 5 and 6: WT male; and lanes 7 and 8: mutant RO carrier male. Lanes M contain as size markers the 100-bp ladder.

Egg Production and Plasma Lipids of White Leghorn Chickens Genotyped by the Modified PCR Procedure

Of the total of 102 chicks from which blood samples were obtained on d 32, four died prior to transfer to the layer unit (d 158) and 11 died during the 294-d laying period; thus, a total of 87 birds survived from hatch until the flock was molted at 452 d of age. The PCR genotyping of these surviving 29 males and 58 females revealed that 11 and 27, respectively, possessed the mutant VLDLR allele.

Overall hen-day egg production of the WT females averaged 70.4 % over the entire 294-d period, and only two birds (Figure 3, numbers 8 and 9) produced fewer than 172 eggs. In contrast, RO hens averaged 0.5% hen-day egg production, and no PCR-genotyped RO female laid more than five eggs during the entire 294-d period (Figure 3). The average total plasma cholesterol and triglyceride values in RO females were 635 mg/100 mL and 3,214 mg/100 mL, respectively, which were 3.8-fold and 2.8-fold higher than those of the WT females (166 mg/100 mL and 1,134 mg/100 mL, respectively; Figure 3). However, several outliers

[10]Bio-Rad Laboratories, Hercules, CA.
[11]Eastman Kodak Company, Rochester, NY.
[12]Leeds and Northrup, North Wales, PA.

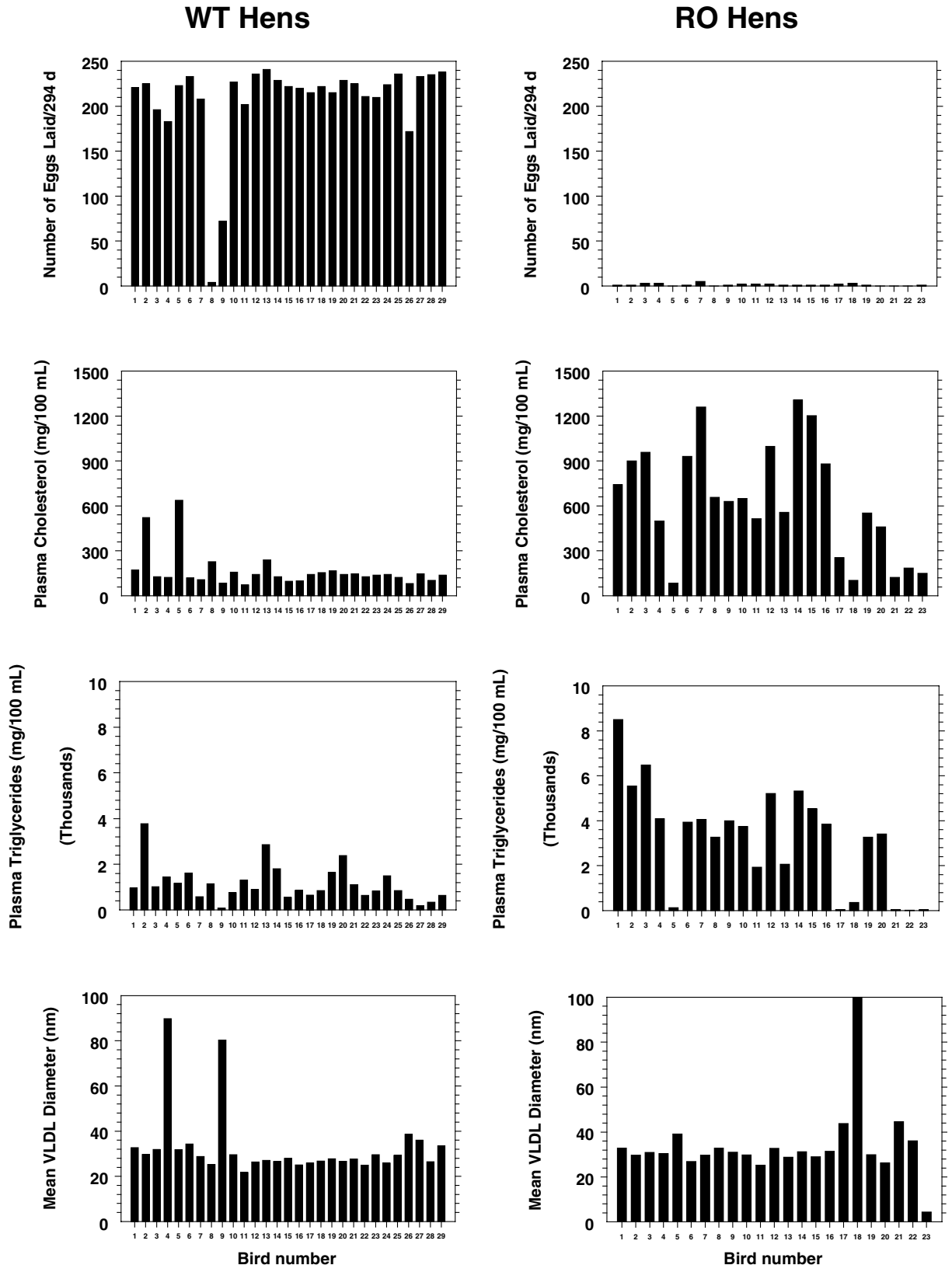

FIGURE 3. Egg production, plasma lipids, and mean plasma very low density lipoprotein (VLDL) particle diameters of PCR-genotyped, wild-type (WT) and restricted ovulator (RO) hens. Egg production was determined from 158 to 452 d of age for 29 and 23 WT and RO hens, respectively. Plasma total cholesterol and plasma total triglycerides concentrations and mean plasma VLDL particle diameters were measured in blood samples obtained when the birds were 360 d old. Values (average ± SD) for eggs laid/294 d, plasma total cholesterol, plasma total triglycerides, and mean plasma VLDL particle size were: 207 ± 50, 166 ± 121 mg/100 mL, 1,134 ± 797 mg/100 mL, and 33 ± 15 nm, respectively for WT hens and 1 ± 1, 635 ± 378 mg/100 mL, 3,214 ± 2,321 mg/100 mL, and 34 ± 17 nm, respectively, for RO hens.

were noted in both genotypes. For example, based upon her normal plasma total cholesterol and triglyceride values, it is possible that WT hen number 8 was an ovulating nonlayer, whereas the abnormally low plasma lipid values of WT hen number 9 were similar to those of a rooster (Figure 4) and suggested a lack of estrogenic stimulation of lipid synthesis. Moreover, six RO hens (Figure 3, numbers 5, 17, 18, 21, 22, and 23) had markedly reduced levels of plasma total cholesterol and triglycerides and, as such, may represent a sub-population of RO females that have both a mutation in the VLDLR and exhibit a lack of ovarian growth (e.g., as per the sixth ovary on the right in Figure 1B). In roosters, mean values for total plasma cholesterol [100 mg/100 mL (WT) vs. 83 mg/100 mL (RO)] and triglycerides [73 mg/100 mL (WT) vs. 74 mg/100 mL (RO)] were similar between genotypes (Figure 4).

Molar ratios of total plasma triglycerides to total plasma cholesterol differed between the female genotypes [3.3 (WT) vs. 1.9 (RO); data not shown] and were suggestive of dissimilar plasma VLDL compositions. In contrast, mean plasma VLDL particle diameters [32.7 nm (WT) vs. 33.9 nm (RO)] and size distributions [26.8% <23 nm and 11.3% >46 nm (WT) vs. 20.5% <23 nm and 14.5% >46 nm (RO); data not shown] were similar in WT and RO females, despite marked differences in egg production and plasma lipids (Figure 3).

In roosters, there appeared to be no influence of genotype on molar ratios of total plasma triglycerides to total plasma cholesterol [0.3 (WT) vs. 0.4 (RO); data not shown], plasma VLDL particle diameters [115.9 nm (WT) vs. 109.6 nm (RO); Figure 4], or plasma VLDL particle size distributions (both genotypes 0% <23 nm and essentially 100% >46 nm; data not shown).

Egg Production of White Leghorn F_1 Chickens Genotyped by the Modified PCR Procedure

Each one of the 11 PCR-genotyped, mutant carrier RO males produced at least one PCR-genotyped, mutant RO daughter (Figure 5). In addition, all of the WT daughters laid at least 104 eggs during the 331-d period, whereas mutant RO daughters generally laid less than 10 eggs, although there were four exceptions (one daughter from sire 2, one daughter from sire 4, and two daughters from sire 6 that laid 13, 46, 12, and 10 eggs, respectively; Figure 5). Genomic DNA was extracted from a second erythrocyte sample from the RO daughter (of sire 4) that laid 46 eggs; PCR testing once again confirmed the RO genotype.

Yolk Protein Profiles of Eggs from WT and RO Hens

The SDS-PAGE profiles of yolk proteins from eggs from one WT hen and an RO hen that laid 46 eggs/331 d (Figure 5) were fairly similar in terms of banding pattern, although in one of the two RO eggs, the proteolysis of apolipoprotein B and vitellogenin appeared to be somewhat incomplete (Figure 6, lane 3). This resulted in several high molecular

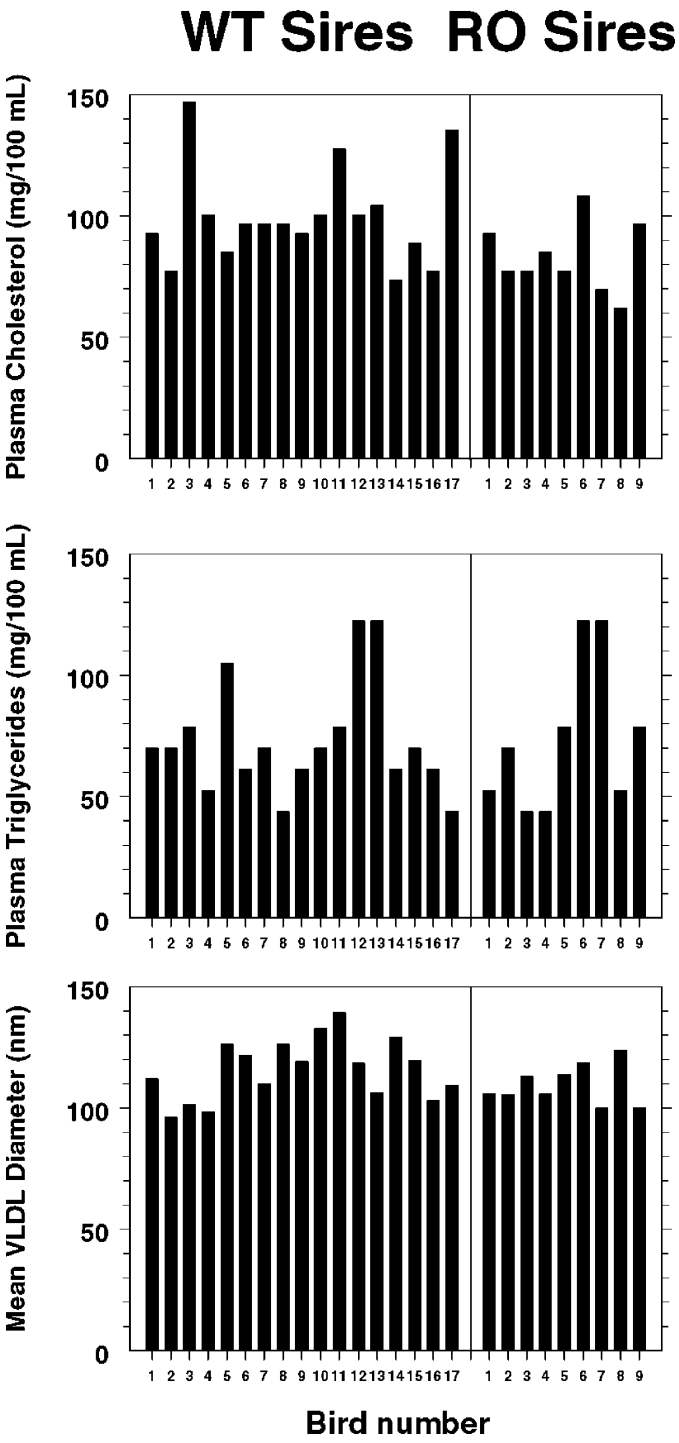

FIGURE 4. Plasma lipids and mean plasma very low density lipoprotein (VLDL) particle diameters of PCR-genotyped, wild-type (WT) and restricted ovulator (RO) roosters. Plasma total cholesterol and plasma total triglycerides concentrations and mean plasma VLDL particle diameters were measured in blood samples obtained at 360 d of age from 17 WT and 9 RO roosters. Values (average ± SD) for plasma total cholesterol, plasma total triglycerides, and mean plasma VLDL particle size were: 100 ± 20 mg/100 mL, 73 ± 23 mg/100 mL, and 116 ± 13 nm, respectively for WT roosters and 83 ± 14 mg/100 mL, 74 ± 31 mg/100 mL, and 110 ± 8 nm, respectively, for RO carrier roosters.

weight (>200 kDa) fragments that were not present in either the WT yolks (Figure 6, lanes 1 and 2) or the yolk from the other egg laid by the RO hen (Figure 6, lane 4). As a

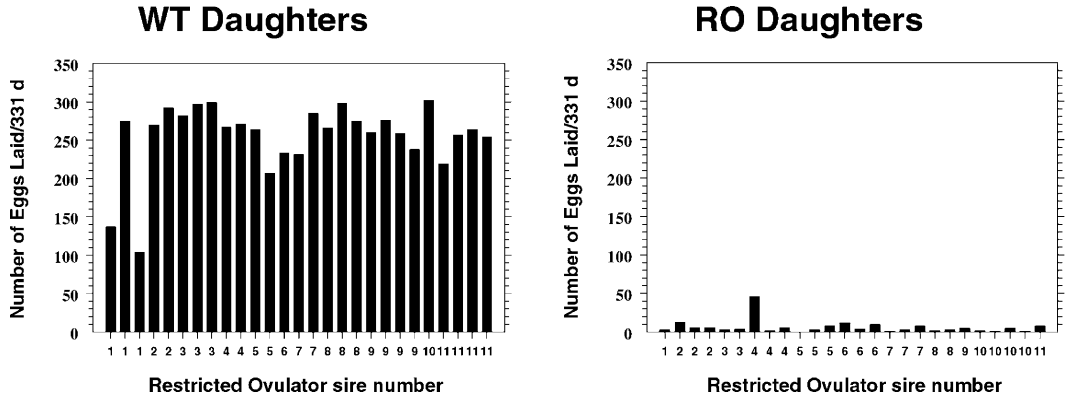

FIGURE 5. Egg production of the daughters of restricted ovulator (RO) sires. Individual egg production was monitored for either four or five PCR-genotyped daughters from each of 11 PCR-genotyped RO sires for 331 d. Fifty-four PCR-genotyped wild-type (WT) daughters from PCR-genotyped WT sires were also monitored for individual egg production. Values (average ± SD) for eggs laid/331 d were 255 ± 45 for WT daughters and 6 ± 9 for RO daughters.

result, less of the main apolipoprotein B fragments (~55 to 80 kDa) and vitellogenin proteolytic fragments (~37 to 45 kDa), which are generated by cathepsin D, were present. The other yolk protein bands represent additional apolipoprotein B fragments and vitellogenin fragments, as well as apovitellenin I, the unaltered yolk form of plasma apolipoprotein VLDL-II [6 kDa (monomer) and 14 kDa (dimer)]. The yolk protein profile from the other RO egg (lane 4) was virtually identical to that of the WT eggs (Figure 6, lanes 1 and 2).

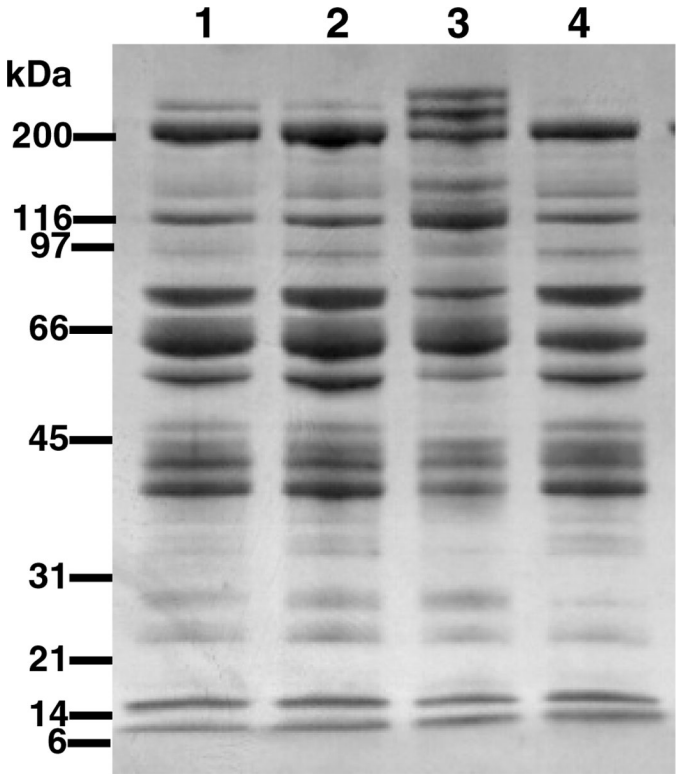

FIGURE 6. The SDS-PAGE (4.5 to 18% gradient gel) of whole yolk extracts from a wild-type (WT) and a restricted ovulator (RO) hen. Protein standard molecular weights (kDa) are indicated. Lanes 1 and 2 contained yolk extracts from two eggs from a WT hen; lanes 3 and 4 contained yolk extracts from two eggs from an RO hen that laid 46 eggs/331 d (a daughter of sire 4 in Figure 5). The weights of the eggs from which the yolks in lanes 1 to 4 were obtained were 67.04 g, 64.95 g, 36.17 g, and 52.62 g, respectively. Each lane contained 12 μg of protein. The gel was stained with Coomassie Brilliant Blue R-250. For a complete description of all bands present in whole egg yolk, see Elkin et al. (1995).

DISCUSSION

The mutant RO female chicken represents a model for an oocyte-specific receptor defect leading to familial hypercholesterolemia (Nimpf and Schneider, 2000). Moreover, the general presence of numerous vitellogenic follicles in the ovaries of mutant RO hens suggests the existence of a non-VLDLR-mediated mechanism of vitellogenesis.

Until recently, conventional progeny testing was the only available option with regard to identifying RO birds that possessed a mutant VLDLR allele. In this process, which took approximately 1 yr, sons of known carrier roosters had to be raised to sexual maturity and bred, and their daughters had to be evaluated for egg laying ability. Bujo et al. (1996) subsequently developed a PCR-based procedure for identifying RO chickens, which reduced the time required to identify mutant individuals from approximately 1 yr to several days. However, their method was incompletely validated because phenotypic data were not obtained, and conventional progeny testing of sons and grandsons was not performed.

In the present study, the RO genotyping procedure of Bujo et al. (1996) was modified through the employment of a different genomic DNA isolation method and an alternate PCR protocol. Because of these modifications, the initial goal of the present work was to demonstrate that the same 413-bp amplification products would be obtained with each respective primer pair when testing erythrocytes from several known WT and mutant RO males and females. Having confirmed this, our next goal was then to further validate the modified PCR procedure by 1) collecting long-term egg production records of genotyped females; 2) measuring select plasma lipids to assess whether all mutant RO females were hypercholesterolemic, hypertriglyceridemic, and were producing plasma VLDL particles associated with egg laying (Walzem, 1996); and 3) demonstrating that,

following breeding to WT hens, every PCR-genotyped carrier RO male produced at least one RO daughter and that every PCR-genotyped WT male produced only WT daughters. All of these objectives were accomplished. Moreover, the complete absence of false positives was noted, as the 413-bp amplification product never appeared when the mutant primer pair was used with WT genomic DNA and vice versa. Therefore, it was concluded that the modified PCR method was completely validated.

The current modified procedure also has several advantages over the original procedure of Bujo et al. (1996) in that 1) the use of a commercial kit, which has been available for over 5 yr, to isolate genomic DNA is both easier and comparatively safer for the analyst, since hazardous chemicals, such as phenol, are not employed; 2) the volume of reagents is scaled down by 50% (total volume of the reaction mixtures are 50 μL vs. 100 μL), thus imparting an economic advantage in light of the cost of *Taq* DNA polymerase; 3) the number of pipetting steps involved in assembling the PCR reaction mixture is reduced because one only has to add PCR SuperMix, primers, and template prior to placing the reaction tubes into the thermocycler; 4) the modified method is more rapid, since the use of thin-walled, 0.2-mL MicroAmp[9] tubes permits much shorter denaturation, annealing, and extension times for each cycle (15 s, 15 s, and 30 s, respectively, vs. 1 min, 1 min, and 1 min, respectively, for the original procedure); and 5) because oil-free PCR amplification is achieved when thin-walled, 0.2-mL MicroAmp tubes are used in the GeneAmp PCR system 9700 vs. the Perkin Elmer 480 DNA Thermal Cycler employed by Bujo et al. (1996), a potential source of contamination is eliminated.

Although egg production records and plasma lipid values generally supported the PCR-genotyping results, there was considerable individual variation within groups of females. Possible causes for nonlaying in WT birds was reviewed by Hutt et al. (1956), and egg production in mutant RO females has been reported by numerous investigators (Ho et al., 1974; Birrenkott and McGibbon, 1975; Jones et al., 1975; Schjeide et al., 1976; Grau et al., 1979; Mitchell et al., 1979; Cho, 1981). The observed differences in mean egg production between the two generations of RO females in the present report (1.4 eggs/294 d and 6.3 eggs/331 d) were fairly minor, with the exception of the RO hen from sire 4 that laid 46 eggs in 331 d. However, this value was far below the mean of 255 eggs/331 d observed in the WT hens.

The fact that the ovaries of mutant RO hens contain numerous vitellogenic follicles in the absence of a functional oocyte plasma membrane VLDLR is contrary to our present understanding of the vitellogenic process (Nimpf and Schneider, 2000) and suggests the presence of an alternate system for the oocytic uptake of plasma VLDL and vitellogenin. Moreover, an explanation as to why some, but not all, mutant RO hens are able to ovulate and lay eggs is lacking, although Grau et al. (1979) clearly recognized this phenomenon over two decades ago and stated the following: "Some daughters of certain matings, although clearly possessing the gene ro laid more eggs than typical ROs but fewer than normals. These, which have been classified as screwy ROs (RO_{sc}) were presumably less affected by the ro gene because of the presence of modifier genes. The yolks from RO_{sc} hens appeared less abnormal than those from RO hens."

One possible 'modifier gene' candidate is the low density lipoprotein receptor-related protein (LRP; Schneider and Nimpf, 1993), a 380-kDa low density lipoprotein receptor supergene family member expressed in ovarian follicles of both WT and RO females (Stifani et al., 1991). Since the oocyte LRP binds vitellogenin, and its ligand binding properties seem to overlap those of other low density lipoprotein receptor family members, it has been suggested that the 95-kDa VLDLR and the 380-kDa LRP may complement one another during the extremely rapid last growth phase of the oocyte (Stifani et al., 1991; Schneider, 1996). Therefore, although all mutant RO females have a nonfunctional oocyte VLDLR, it is possible that a compensatory increase in the expression of the oocyte LRP occurs in certain RO females (i.e., RO_{sc} individuals), which leads to enhanced vitellogenesis and the ability of to lay more eggs than typical RO hens. Moreover, the completely normal yolk protein profile in one of the two eggs from an RO hen that laid 46 eggs/331 d strongly suggested the existence of an alternate (i.e., non-VLDLR-mediated) vitellogenic process in which normal proportions of both plasma VLDL and vitellogenin were taken up by growing oocytes. Thus, in addition to determining why the apparent efficiency of yolk protein precursor proteolysis varied among follicles of the same RO_{sc} hen, it also would be of interest to examine oocyte LRP expression levels in an RO hen population.

Two of the hallmark phenotypic traits of mutant RO hens, namely extremely low egg production and an ovary containing a plethora of small vitellogenic follicles, are somewhat reminiscent of the overfed broiler breeder hen (Hocking et al., 1987; Renema et al., 1999). However, multiple hierarchies of large follicles are generally not noted in RO hens although, as in the case of overfed broiler breeders, the RO birds are both heavier and had more abdominal body fat than WT hens (R. G. Elkin, unpublished data).

Leszcynski et al. (1984) reported that sexually mature RO hens also exhibit a gross imbalance in their basal plasma circulating ratio of estrogen to progesterone (threefold higher and fourfold lower, respectively, than in WT hens). As a result, a progesterone surge is absent and ovulation does not generally occur in RO females. This finding is most likely due to an absence of large preovulatory follicles since, in chickens, only the granulosa cells of large preovulatory follicles possess receptors for luteinizing hormone, and luteinizing hormone is the major stimulator of progesterone secretion (Leszcynski et al., 1984). Thus, although vitellogenesis typically occurs in the ovaries of most RO hens to some extent, in many cases, oocyte growth apparently cannot be sustained at normal levels, and the vitellogenic follicles generally remain unresponsive to luteinizing hormone. This ultimately results in extreme hyperlipidemia, hyperestrogenemia, hypoprogesteronemia, a failure to ovulate, and an inability to lay eggs.

ACKNOWLEDGMENTS

The authors thank Ken Wolber and Tom Heim for their excellent supervision of the birds and their progeny. Appreciation is also extended to Cansu Agca, Ryan Meunier, Mickey Latour, and Stacey Neuman for their technical assistance and to J. James Bitgood, University of Wisconsin, for providing hatching eggs that enabled us to establish the RO colony and for his helpful suggestions. This work was funded in part by a grant from Progen Biotechnik GmbH (Heidelberg, Germany).

REFERENCES

Birrenkott, G. P., and W. H. McGibbon. 1975. Yolk development in restricted ovulators and their normal sibs. Poult. Sci. 54:1734 (Abstr.).

Bujo, H., R. G. Elkin, K. A. Lindstedt, J. Nimpf, J. J. Bitgood, and W. J. Schneider. 1996. A rapid, polymerase chain reaction-based procedure for identifying mutant restricted ovulator chickens. Poult. Sci. 75:1113–1117.

Bujo, H., M. Hermann, M. O. Kaderli, L. Jacobsen, S. Sugawara, J. Nimpf, T. Yamamoto, and W. J. Schneider. 1994. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. EMBO J. 13:5155–5175.

Bujo, H., T. Yamamoto, K. Hayashi, M. Hermann, J. Nimpf, and W. J. Schneider. 1995. Mutant oocytic low-density lipoprotein receptor gene family member causes atherosclerosis and female sterility. Proc. Natl. Acad. Sci. USA 92:9905–9909.

Cho, B. H. S. 1981. Endogenous hyperlipidemia and plasma fatty acid profiles of normal and hereditary nonlaying chickens. Comp. Biochem. Physiol. 68B:19–23.

Elkin, R. G., M. B. Freed, S. A. H. Danetz, and C. A. Bidwell. 1995. Proteolysis of Japanese quail and chicken plasma apolipoprotein B and vitellogenin by cathepsin D: Similarity of the resulting protein fragments with egg yolk polypeptides. Comp. Biochem. Physiol. 112B:191–196.

Elkin, R. G., and W. J. Schneider. 1994. Visualization of the chicken oocyte lipoprotein receptor by ligand blotting with biotinylated plasma and yolk very low density lipoproteins. Poult. Sci. 73:1127–1136.

Elkin, R. G., and Z. Yan. 1999. Association of mevalonate biosynthesis inhibition with reduced fertility in laying hens. J. Reprod. Fertil. 116:269–275.

Elkin, R. G., and Y. Zhong. 2002. Assessment of reproductive function in mutant restricted ovulator carrier roosters. Poult. Sci. 81:1280–1282.

Etches, R. J., and J. N. Pettite. 1990. Reptilian and avian follicular hierarchies: Models for the study of ovarian development. J. Exp. Zool. 4(Suppl. 1):112–122.

Grau, C. R., T. E. Roudybush, and W. H. McGibbon. 1979. Mineral composition of yolk fractions and whole yolk from eggs of restricted ovulator hens. Poult. Sci. 58:1143–1148.

Ho, K. J., W. D. Lawrence, L. A. Lewis, L. B. Liu, and C. B. Taylor. 1974. Hereditary hyperlipidemia in nonlaying chickens. Arch. Pathol. 98:161–172.

Hocking, P. M., A. B. Gilbert, M. Walker, and D. Waddington. 1987. Ovarian follicular structure of White Leghorns fed ad libitum and dwarf and normal broiler breeders fed ad libitum or restricted until point of lay. Br. Poult. Sci. 28:493–506.

Hutt, F. B., K. Goodwin, and W. D. Urban. 1956. Investigations of nonlaying hens. Cornell Vet. 46:257–273.

Jones, D. G., W. E. Briles, and O. A. Schjeide. 1975. A mutation restricting ovulation in chickens. Poult. Sci. 54:1780 (Abstr.).

Lake, P. E. 1957. Fowl semen as collected by the massage method. J. Agric. Sci. (Cambridge) 49:120–126.

Leszcynski, D. E., R. C. Hagan, S. E. Rowe, and F. A. Kummerow. 1984. Plasma sex hormone and lipid patterns in normal and restricted-ovulator chicken hens. Gen. Comp. Endocrinol. 55:280–288.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.

McDaniel, C. D., J. M. Balog, M. Freed, R. G. Elkin, R. H. Wellenreiter, and P. Y. Hester. 1993. Response of layer breeders to dietary acteylsalicylic acid. 1. Effects on hen performance and eggshell quality. Poult. Sci. 72:1084–1092.

McGibbon, W. H. 1977. Evidence that the restricted ovulator gene (ro) in the chicken is sex-linked. Genetics 86:S43–S44 (Abstr.).

Mitchell, A. D., S. E. Carlson, W. H. McGibbon, and S. Goldfarb. 1979. Hepatic HMG CoA reductase and cholesterol 7α-hydroxylase activities in normal and hyperlipidemic-restricted ovulator atherosclerosis-prone chickens before and after the commencement of egg laying. Atherosclerosis 32:11–21.

Nimpf, J., M. J. Radosavljevic, and W. J. Schneider. 1989. Oocytes from the mutant restricted ovulator hen lack receptor for very low density lipoprotein. J. Biol. Chem. 264:1393–1398.

Nimpf, J., and W. J. Schneider. 2000. From cholesterol transport to signal transduction: low density lipoprotein receptor, very low density lipoprotein receptor, and apolipoprotein E receptor-2. Biochim. Biophys. Acta 1529:287–298.

Renema, R. A., F. E. Robinson, J. A. Proudman, M. Newcombe, and R. I. McKay. 1999. Effects of body weight and feed allocation during sexual maturation in broiler breeder hens. 2. Ovarian morphology and plasma hormone profiles. Poult. Sci. 78:629–639.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Isolation of DNA from mammalian cells: Protocol I. Pages 9.16–9.19 in Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schjeide, O. A., W. E. Briles, S. Holshouser, and D. G. Jones. 1976. Effect of "restricted ovulation" gene on uptake of yolk-precursor protein. Cell Tiss. Res. 166:109–116.

Schneider, W. J. 1996. Vitellogenin receptors: Oocyte-specific members of the low-density lipoprotein receptor supergene family. Int. Rev. Cytol. 166:103–137.

Schneider, W. J., and J. Nimpf. 1993. Lipoprotein receptors: old relatives and new arrivals. Curr. Opin. Lipidol. 4:205–209.

Stifani, S., D. L. Barber, R. Aebersold, E. Steyrer, X. Shen, J. Nimpf, and W. J. Schneider. 1991. The laying hen expresses two different low density lipoprotein receptor-related proteins. J. Biol. Chem. 266:19079–19087.

Stifani, S., D. L. Barber, J. Nimpf, and W. J. Schneider. 1990. A single chicken oocyte plasma membrane protein mediates uptake of very low density lipoprotein and vitellogenin. Proc. Natl. Acad. Sci. USA 87:1955–1959.

Walzem, R. L. 1996. Lipoproteins and the laying hen: Form follows function. Poult. Avian Biol. Rev. 7:31–64.

Walzem, R. L., P. A. Davis, and R. J. Hansen. 1994. Overfeeding increases very low density lipoprotein diameter and causes the appearance of a unique lipoprotein particle in association with failed yolk deposition. J. Lipid Res. 35:1354–1366.