Overfeeding-Induced Ovarian Dysfunction in Broiler Breeder Hens Is Associated with Lipotoxicity[1,2]

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ABSTRACT In mammals, triacylglycerol (TAG) accumulation in nonadipose tissue, termed lipotoxicity, develops with obesity and can provoke insulin resistance, overt diabetes, and ovarian dysfunction. Leptin, an adipose tissue hormone, may mediate these effects. Feed-satiated broiler breeder hens manifest lipotoxicity-like symptoms. Changes in body and organ weights, hepatic and plasma TAG, nonesterified fatty acids (NEFA), ovarian morphology, and egg production in response to acute voluntary increases of feed intake were measured in 2 studies with Cobb 500 broiler breeder hens provided with either 145 or ≥290 g of feed/d per hen for 10 d. In both studies, no hen fed 145 g of feed/d exhibited ovarian abnormalities, whereas ~50% of feed-satiated hens did. Egg production in feed-satiated hens was reduced from 73.3 to 55.8% ($P = 0.001$). Morphology indicated that apoptosis-induced atresia occurred in the hierarchical follicles. Fractional weight of yolk increased from 29.3 to 30.6% ($P = 0.016$) and no longer correlated to egg weight. Body, liver, and abdominal adipose weights were significantly greater ($P < 0.05$) in feed-satiated hens, as were plasma concentrations of glucose, NEFA, TAG, insulin, and leptin ($P < 0.05$). Feed-satiated hens with abnormal ovaries had significantly more liver and abdominal fat, greater plasma leptin and TAG concentrations, and more saturated fatty acids in plasma NEFA than did feed-satiated hens with normal ovaries. Differences in severity of lipotoxic metabolic and hormonal responses among feed-satiated hens were closely linked to the incidence of ovarian abnormalities and granulosa cell susceptibility to apoptosis and necrosis.

Key words: broiler, feed intake, lipotoxicity, obesity, egg production

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INTRODUCTION

Genetic selection for rapid growth in modern broiler strains is collaterally associated with specific undesirable traits, including a higher incidence of skeletal deformities and metabolic diseases such as ascites, fatty liver, and kidney syndrome (Griffin and Goddard, 1994; Julian, 1998). In females, the capacity for rapid early growth coupled with free access to feed leads to enhanced adult fatness (Chambers et al., 1981; Havenstein et al., 2003a,b) and poor reproductive performance (Yu et al., 1992a,b; Robinson et al., 1993). These undesirable outcomes appear to arise from increases in feed intake that occurred concomitantly with genetic selection for rapid early growth (Barbato, 1994; Richards, 2003). In adulthood, persistence of the trait of increased voluntary feed intake results in actual food intakes in excess of the requirement for optimal adult health and performance as indicated by reduced livability and increased incidence of metabolic disease (Robinson et al., 1993; Griffin and Goddard, 1994). As a result, management of nutrition plays a large role in adult broiler breeder performance.

Restriction of feed intake to approximately 50 to 60% of ad libitum is an effective and practical management technique to reduce metabolic disease and improve egg production in broiler breeder hens. Broiler breeder hens fed ad libitum during the rearing period undergo sexual maturation and begin laying eggs earlier than their feed-restricted counterparts. However, hens fed ad libitum also exhibit a dramatic drop in egg production at an earlier age and consequently, produce fewer eggs overall (Bornstein et al., 1984; Yu et al., 1992a,b). These studies also show that hens fed ad libitum have heavier BW and increased body fat, with at least a 50% increase in fractional abdominal fat weight (relative to BW) at sexual maturity compared with feed-restricted hens. This degree of adiposity can be described as obesity.

Ovarian morphology, follicle sensitivities to hormonal factors, plasma gonadotropin and sex steroid patterns, hypothalamic and pituitary responsiveness, and sex steroid outputs from ovarian follicles from lean and obese

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broiler breeder hens have been compared to determine whether an endocrine mechanism could be identified as responsible for poor egg production (Hocking et al., 1987, 1989; Yu et al., 1992c; Bruggeman et al., 1998a,b; Onagbesan et al., 1999). Historically, adipose tissue has been considered a nonendocrine tissue. However, in the past decade, adipose tissue has been recognized as an active endocrine organ that mediates its effects through various hormones (Fruhbeck et al., 2001). The role of adipose tissue and the hormonal and metabolic signals it can elaborate are relatively unstudied in connection with reproductive function in poultry.

Leptin is the best understood of the adipose tissue hormones. Impaired leptin signaling is known to cause increased feed intake, massive obesity, and reproductive failure in mammals due to the absence of leptin-induced gonadotropin secretion (Zhang et al., 1994; Ahima and Flier, 2000). Obesity in otherwise normal mammals is variably associated with reduced insulin sensitivity, overt diabetes, hyperlipidemia, hyperleptinemia, hyperinsulinemia, and reproductive dysfunction (Ahima and Flier, 2000; Ben-Shlomo, 2002). Elevated nonesterified free fatty acids (**NEFA**) and glucose in fasted humans despite elevated insulin concentrations is thought to be diagnostic for insulin resistance and has been proposed as a causative agent for diabetic progression and complications (Chen et al., 1987; Golay et al., 1987; Byrne et al., 1994; Paolisso et al., 1995; Egan et al., 1996; Saloranta and Groop, 1996; Charles et al., 1997). In ZDF-fa/fa rats, a well-characterized model of nonfunctional leptin signaling, excessive accumulation of triacylglycerol (**TAG**) and fatty acids in nonadipose tissues as well as altered circulating and tissue lipid profiles (Wahle et al., 1991, 1994) are known to occur. This constellation of symptoms reflects "lipopenic" dysregulation that can cause lipid-related cytotoxicity. In humans and other mammalian models this condition has been termed "lipotoxicity" (Unger, 2002).

Based on these mammalian studies, we hypothesized that in feed-satiated broiler breeder hens metabolic homeostasis can be disrupted to create a lipotoxic condition that could drive the alterations in hormonal signals leading to ovarian dysfunction and reduced egg production. The objective of the present studies was to determine whether differences in adiposity, total hepatic fat content, plasma NEFA concentration and composition, as well as plasma concentrations of glucose, TAG, leptin, and insulin in feed-restricted and feed-satiated broiler breeder hens could be related to egg and ovarian indexes. If differences were found, a second objective was to determine whether ovarian granulosa cells from broiler breeder hens fed different amounts of food differed in their susceptibility to apoptosis or necrosis in vitro.

MATERIALS AND METHODS

Animal Management

Hens in both experiments were fed a soy and corn-based breeder layer mash (Table 1) that provided 2,750 kcal of ME/kg. Diet composition was calculated from published values for feed ingredients (NRC, 1994). Fatty acid composition of the diet was determined by the gas chromatography method described below. Feed was placed between 0830 to 0900 h within a 14L:10D photoperiod in which lights were turned on at 0500 h. Hens had free access to water throughout the experiment. Egg production and feed intake were recorded daily.

Table 1. Feed composition

Ingredient	
Protein	15.00
Fat	4.20
Calcium	3.40
Potassium	0.62
Sodium	0.16
Total ME, kcal/kg	2,750
Fatty acid composition, %, wt/wt dietary fat	
C16:0	10.8
C16:1 n7	0.2
C18:0	2.1
C18:1 n9	28.6
C18:2 n6	55.7
C18:3 n6	0.1
C18:3 n3	1.0
C20:0	0.4
C20:1 n9	0.2
C20:4 n6	0.3
C20:3 n3	0.3
C22:5 n3	0.3

Experiment 1. Thirty-four commercially reared Cobb 500 Fast Feathering broiler breeder hens (35-wk-old; peak egg production = 79.4% egg/hen per d at 33 wk) were used in the study. Hens were fed the breeder-recommended 145 g of feed/hen per d during a 5-d adaptation period, and during a subsequent 10-d period used to measure basal egg production. Blood samples were taken at the beginning and end of this 10-d period. Fifteen hens were sampled for tissues and ovarian evaluation on d 11. The remaining 19 hens were provided with 290 g of feed/hen per d for an additional 10 d. Blood and tissues were harvested from all hens at the end of this interval with ovarian evaluation occurring at necropsy. On alternate days, eggs were broken and yolk weight measured after gentle rolling on paper toweling to remove adherent white. Determined values were used to calculate fractional yolk weight as (yolk weight/egg weight) × 100%.

In this experimental design, each hen served as its own control for variables measured before necropsy such as egg production, fractional yolk weight, and plasma lipid parameters. A 10-d interval was chosen because a hierarchical follicle typically requires 7 to 10 d to mature to the point of ovulation. This interval allows plasma parameters measured at the start of a 10-d feeding period to be correlated to egg production during that same period. Flock subsampling on d 11 (n = 15) was used to create comparisons for tissue weights and values measured at necropsy on d 21 (n = 19).

Experiment 2. Sixty commercially reared Cobb 500 Fast Feathering broiler breeder hens (35-wk-old; peak egg production = 79.2% egg/hen per d at 33 wk) were fed

145 g of feed/hen per d during a 3-d adaptation period and a subsequent 10-d period used to measure basal egg production. On d 11, hens were separated into 3 groups of 20, such that hens of similar initial mean BW and egg production were formed. Groups were then randomly assigned to 1 of 3 groups: 145 g of feed/hen per d, 290 g of feed/hen per d, or free access to feed (ad libitum). Five hens from each group started the study protocol each week for 3 wk. Before initiation of the study protocol, hens were maintained on 145 g of feed/hen per d. Blood samples were taken at the beginning of the study period and again after the 10-d feed manipulation in conjunction with tissue harvest and ovarian evaluation. At necropsy, ovarian granulosa cells were collected according to the method of Gilbert et al. (1977). Cells were rinsed twice with PBS buffer and immediately dispersed in M199-HEPES medium containing 200 U/mL of type-2 collagenase and 0.3 mg/mL of trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min. Dispersed cells were evaluated for apoptotic morphology.

Necropsy and Tissue Collection

Hens were anesthetized with isoflurane before necropsy in accordance with an approved animal care protocol (Use and Care Committee, Texas A&M 2002-355AG). Liver, abdominal fat pad, and ovary were collected at necropsy. Weight of liver, ovary, and abdominal fat pad were divided by (BW/100) to estimate their fractional contribution. Liver steatosis and hemorrhage were judged using a 5-point scale (Walzem et al., 1993). Ovaries were weighed and follicles characterized in several ways. First, follicles were classified into 3 groups: hierarchical follicles (large yellow follicles, >8 mm), small yellow follicles (2 to 8 mm), and large white follicles (2 to 5 mm) according to the system devised by Gilbert et al. (1983). Ovarian morphology was generally characterized as normal or abnormal. Abnormal ovaries were defined as those with abnormal hierarchical follicles (i.e., atretic follicles or type 3 follicles); the occurrence of internal ovulation as judged by the presence of viscous and yolk-like fluid in the abdominal cavity; and ovarian involution as judged by an ovary weight less than 20 g. Shrunken follicles containing dark yellowish to brown yolk contents surrounded by loose connective tissue were classified as atretic. Type 3 follicles are a degenerated form of follicle that are >15 mm in diameter due to accumulation of nonyolk fluid that renders yolk material less viscous and light yellow to whitish in color (Liu et al., 2001).

Determination of Hepatic Lipid and Plasma NEFA

Internal standards [1,2-dipentadecanoyl-glycero-3-phosphocholine (C15-PC), triheptadecanoin (C17-TAG), and pentadecanoic acid (C15-NEFA); Nu-Chek Prep, Inc., Elysian, MN] were added to samples before lipid extraction to allow for accurate quantification of individual fatty acids.

Total lipids were extracted from liver homogenates and fasted plasma samples using chloroform/methanol (2:1; vol/vol). Thin-layer chromatography as described by Watkins et al. (2001) was used to separate and identify phospholipid and TAG fractions. After methylation, fatty acid methyl esters were extracted in hexane, and separated using a gas chromatograph (HP6890, Hewlett Packard, Wilmington, DE) equipped with a hydrogen flame-ionization detector and an HP capillary column (DB-23, 30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA) and HP ChemStation software (Hewlett Packard). A standard comprising 32 fatty acid esters was used to identify retention times of each fatty acid species. The degree of fatty acid saturation within a lipid class was calculated as $\Sigma m_i n_i/100$, where m_i is the percentage (mol/mol) and n_i is the number of C-C double bonds in fatty $acid_i$; the smaller the value, the more saturated the fatty acid content.

Morphometric Analysis for Granulosa Cell Apoptosis

Fluorescent staining using an Annexin-V-FLUOS/PI staining kit (Roche Diagnostics Corp., Indianapolis, IN) was used to identify morphological changes in granulosa cells that were indicative of apoptosis and necrosis. Apoptotic cells lose the ability to maintain phosphatidylserine within inner leaflets of membranes. As a result, phosphatidylserine is present on the outer leaflet of the plasma membrane. In this setting, Annexin V-FLUOS will bind to the cell surface with the intensity of green staining being proportional to phosphatidylserine on the cell surface. Due to loss of membrane integrity, necrotic cells are permeable to propidium iodide causing the nucleus to stain red, whereas apoptotic cells with membrane integrity will exclude propidium iodide and so nuclei will not stain red. Visualization of stained cells was conducted with an Olympus BX50 camera (Olympus America, Melville, NY) with a BX-FLA reflected light fluorescence attachment and Chroma 51006 filter set (Chroma Technology Corp., Rockingham, NY).

Plasma Glucose, TAG, Insulin, and Leptin Concentrations

Fasting plasma glucose concentrations were determined enzymatically using commercial kits from Wako (Richmond, VA). Plasma insulin and leptin concentrations were determined through a homologous double-antibody radioimmunoassay (McMurtry et al., 1983; Evock-Clover et al., 2002; McMurtry et al., 2003) using purified chicken insulin (Litron Laboratory, Rochester, NY) and recombinant-derived leptin (National Institutes of Health, Bethesda, MD) as standards.

The primary antisera against chicken insulin were raised in guinea pigs and the anti-guinea pig γ-immunoglobulin was raised from sheep. A rabbit anti-chicken leptin primary antibody and sheep anti-rabbit γ-immunoglobulin secondary antibody were used in the leptin

assay. The intraassay CV was 4.3% for insulin and 3.0% for leptin.

Statistics

Plasma values determined at the beginning and end of the basal egg production measurement period were found to be similar by paired *t*-tests; the values were treated as duplicates and averaged. Paired *t*-tests were used to evaluate differences in mean values for hens fed 290 g of feed/hen per d and for these same hens during measurement of basal egg production. Final data from hens fed 145 g of feed per d (n = 15) were compared with that from hens fed 290 g of feed per d (n = 19) using a GLM for unbalanced designs (SAS Institute, Inc., Cary, NC). Differences in mean values were tested using LSD and Tukey's procedures. A GLM in which egg production was the independent continuous variable and parameters such as organ weight and numbers of atretic follicles were the dependent variables was also used in data analysis. Data from Experiment 2 were analyzed by 2-way ANOVA to determine the effects of time and feeding level. Time was not significant and data were reanalyzed by 1-way ANOVA with feeding level as the classifying variable. Differences in mean values were tested using LSD and Duncan's procedures. There were no significant differences in outcomes for hens fed 290 g of feed/hen per d and those provided free access to food. For presentation in tables, data were pooled to form a ≥290 g of feed/hen per d or feed-satiated group. In both studies, comparisons between feed-satiated hens with normal and abnormal ovaries were tested using appropriate *t*-tests. Differences were considered significant at $P < 0.05$.

RESULTS

General

Results from Experiments 1 and 2 were consistent with one another; therefore, feed intake, organ, BW, and egg production data are presented for Experiment 1 only. Qualitative morphological results from studies with granulosa cells harvested in Experiment 2 are reported here.

Feed Intake, Organ Gain, and BW Gain

Hens fed 290 g of feed/hen per d finished the entire portion on d 1 and 2; thereafter, daily feed consumption declined gradually to reach approximately 250 g of feed/hen per d. As a result, the daily feed consumption of hens provided with 290 g of feed/hen per d averaged 266 g of feed/hen per d (Table 2). This rate of feed intake for 10 d resulted in 500 g of BW gain, increased absolute and fractional liver weight by 130 and 100%, respectively, and increased absolute and fractional abdominal fat weight by 36 and 18%, respectively ($P < 0.05$; Table 2). Increased feed intake also exacerbated liver steatosis and liver hemorrhagic scores ($P < 0.05$; Table 2). Overall, mean ovarian weight remained unchanged in response to higher levels of feed intake but its fraction of BW decreased ($P < 0.05$; Table 2). Separating feed-satiated hens into those with normal and abnormal ovaries (last 2 columns of Table 2) showed that ovaries were 18.9% ($P < 0.05$) heavier in feed-satiated hens with normal ovaries compared with those with ovarian abnormalities.

Approximately 52% (10/19) of the hens receiving 290 g of feed/hen per d exhibited ovarian abnormalities (Table 3). Of these, 6 hens had atretic hierarchical follicles, 1 had both atretic and type 3 follicles, 1 had atretic follicles and internal ovulation, 1 had type 3 follicles, and 1 experienced internal ovulation only. Regrouping data according to the absence (n = 9) or presence (n = 10) of ovarian abnormalities (the last 2 columns in Table 3) revealed that in hens with abnormal ovaries, the number of atretic follicles ranged from 1 to 4.

Similar BW gains and average daily feed intake levels between feed-satiated hens with or without ovarian abnormalities at necropsy ($P > 0.05$; Table 2) suggested that the presence of ovarian abnormalities was not associated with BW gain and daily feed intake level per se. However, ovarian abnormalities were associated with higher absolute and relative liver and abdominal fat weight, more pronounced liver steatosis, and lower absolute and relative ovary weights ($P < 0.05$; Table 2).

Egg Production, Fractional Yolk Weight, and Ovarian Morphology

Egg production was stable and averaged 73.3 ± 2.7% in hens fed 145 g of feed/hen per d (Table 3; Figure 1, panel A). Three days after the hens were provided with 290 g of feed/hen per d, egg production declined and ultimately reached 36% within 10 d. As a result, average egg production was lower (55.8 ± 2.8%, $P < 0.05$; Table 3). Interestingly, however, average yolk weight increased by nearly 1 g ($P < 0.05$; Table 3). Hens fed 290 g of feed/hen per d were out of lay an average of 1.6 d compared with the average (0.2 d) of hens fed 145 g of feed/hen per d ($P < 0.005$). There was no difference in the number of days out of lay for feed-satiated hens with normal or abnormal ovaries (Table 3). Egg production was 17.8% less in hens with abnormal ovaries compared with feed-satiated hens with normal ovaries ($P < 0.05$, Table 3) because 70%, rather than 56%, of hens failed to lay an egg on at least 1 day.

Among hens fed 145 g of feed/hen per d, fractional yolk weight was stable and amounted to 29.3 ± 0.07% (Figure 1, panel B, d 1 to 11) over the 10-d measurement period. Fractional yolk weight increased 4 d after provision of 290 g of feed/hen per d (Figure 1, panel B, d 11 to 21). The increase in fractional yolk weight was variable and of sufficient magnitude that the standard allometric relationship between yolk and egg weight was lost immediately after release from restriction as indicated by a loss in the significance of the correlation between these 2 measurements (Figure 1; panel B, open bars). During the last 6 d of this 10-d period, fractional yolk weight

Table 2. Feed intake, body and organ weights, egg production, and ovarian morphology of broiler breeder hens in Experiment 1

	Feed allowance		Ovarian morphology[1] of 290 g of feed/hen per d	
	145 g/hen per d (n = 15)	290 g/hen per d (n = 19)[1]	Normal (n = 9)	Abnormal (n = 10)
BW Start,[2] kg	3.6 ± 0.1[b]	3.7 ± 0.1[a]	3.7 ± 0.2	3.7 ± 0.1
BW End,[2] kg	3.6 ± 0.1[b]	4.2 ± 0.1[a]	4.2 ± 0.2	4.2 ± 0.1
Feed intake, g/hen per d	145[b]	266.0 ± 2.0[a]	268.6 ± 2.0	263.7 ± 3.0
Liver weight, g	64.5 ± 2.5[b]	151.4 ± 8.0[a]	134.3 ± 10.6[b]	166.8 ± 9.5[a]
Fractional liver weight, g/100 g of BW	1.80 ± 0.05[b]	3.59 ± 0.17[a]	3.18 ± 0.20[b]	3.97 ± 0.20[a]
Fatty liver color score[2]	2.0 ± 0.1[b]	3.3 ± 0.2[a]	3.0 ± 0.2[b]	3.6 ± 0.1[a]
Hemorrhage score[2]	1.1 ± 0.1[b]	2.0 ± 0.2[a]	1.7 ± 0.2	2.2 ± 0.3
Abdominal fat weight, g	80.4 ± 5.5[b]	109.6 ± 6.7[a]	96.5 ± 10.0[b]	121.4 ± 7.7[a]
Fractional abdominal fat weight, g/100 g of BW	2.21 ± 0.12[b]	2.59 ± 0.15[a]	2.25 ± 0.16[b]	2.90 ± 0.18[a]
Ovarian weight, g	65.4 ± 2.2	65.7 ± 3.0	71.7 ± 4.7[a]	60.4 ± 2.8[b]
Fractional ovarian weight, g/100 g of BW	1.82 ± 0.05[a]	1.58 ± 0.08[b]	1.73 ± 0.14[a]	1.44 ± 0.07[b]

[a,b]Values are means ± SEM. Means not sharing a common superscript letter within a row differ ($P < 0.05$) between 145 and 290 g/hen per d or between normal and abnormal ovary groups. Body weights within a column not sharing a common superscript number are heavier ($P < 0.05$) at the end than the start of feeding period ($P < 0.05$).

[1]5-point scale, with 1 = best condition and 5 = worst condition.

[2]Data from hens fed 290 g/hen per d were divided according to the presence of normal or abnormal ovarian morphology at necropsy using criteria described in Materials and Methods.

averaged 30.57 ± 0.20% ($P < 0.016$) compared with 29.3 ± 0.07%.

As shown in Table 3, hens consuming 290 g of feed/hen per d variably exhibited increased total numbers of hierarchical and atretic follicles ($P < 0.05$) in association with decreased numbers of small yellow follicles ($P < 0.05$). Hens receiving 145 g of feed/hen per d displayed no ovarian abnormalities (Table 3). Among hens with higher levels of feed intake, those exhibiting abnormal ovarian morphology possessed at least 1 atretic follicle, reduced weights in the F2 to F5 follicles, and had poorer egg production ($P < 0.05$; Table 3; Figure 1) compared with hens retaining normal ovarian morphology despite increased feed availability.

Plasma Glucose, TAG, NEFA, Insulin, and Leptin Concentrations

Plasma glucose, TAG, NEFA, insulin, and leptin concentrations were increased in feed-satiated hens in Experiments 1 and 2 ($P < 0.05$; Table 4). Hens with ovarian abnormalities displayed higher plasma TAG and leptin concentrations than their counterparts free of ovarian abnormalities ($P < 0.05$; Table 4). Plasma glucose, NEFA, and insulin concentrations did not show a similar divergence, and values for feed-satiated hens were similar regardless of ovarian status. Values for glucose, TAG, NEFA, insulin, and leptin in feed-satiated hens were lower and normal

Table 3. Ovarian morphology of broiler breeder hens in Experiment 1

	Feed allowance		Ovarian morphology of 290 g of feed/hen per d	
	145 g/hen per d (n = 15)	290 g/hen per d (n = 19)[1]	Normal (n = 9)	Abnormal (n = 10)
Egg/d per hen, %	73.3 ± 2.7[a]	55.8 ± 2.8[b]	62.2 ± 2.8[a]	51.1 ± 3.8[b]
Yolk weight, g	18.6 ± 0.3[b]	19.2 ± 0.2[a]	19.1 ± 0.2	19.3 ± 0.3
Hierarchical follicle weight,[2] g				
F1	18.1 ± 0.5	18.5 ± 0.3	18.9 ± 0.5	18.3 ± 0.4
F2	14.4 ± 0.6	14.2 ± 0.6	15.7 ± 0.8[a]	12.6 ± 0.5[b]
F3	9.9 ± 0.5	10.1 ± 0.7	11.4 ± 0.7[a]	8.9 ± 1.0[b]
F4	5.2 ± 0.5	5.8 ± 0.7	7.4 ± 1.0[a]	4.1 ± 0.6[b]
F5	2.9 ± 0.3	2.8 ± 0.5	3.9 ± 0.9[a]	1.8 ± 0.3[b]
Hierarchical follicles/ovary	6.4 ± 0.4[b]	7.7 ± 0.3[a]	7.4 ± 0.4	8.0 ± 0.4
Small yellow follicles/ovary	15.1 ± 1.4[a]	11.7 ± 0.7[b]	11.0 ± 1.0	12.3 ± 0.9
White follicles/ovary	12.5 ± 0.6	12.1 ± 0.7	12.2 ± 1.0	12.0 ± 0.9
Abnormal ovary frequency[1]	0/15	10/19	0/9	10/10
Atretic follicles/ovary	0[b]	0.8 ± 0.3[a]	0[b]	1.5 ± 0.4[a]
Average no. of days out of lay	0.2 ± 0.1[b]	1.6 ± 0.4[a]	1.4 ± 0.6	1.6 ± 0.6

[a,b]Values are means ± SEM. Means not sharing a common superscript letter within a row differ ($P < 0.05$) between 145 and 290 g of feed/hen per d or normal and abnormal ovary groups.

[1]Data from hens fed 290 g of feed/hen per d were divided according to the presence of normal or abnormal ovarian morphology at necropsy using criteria described in Materials and Methods.

[2]Nominal hierarchal ranking based on position within hierarchy rather than hours to ovulation.

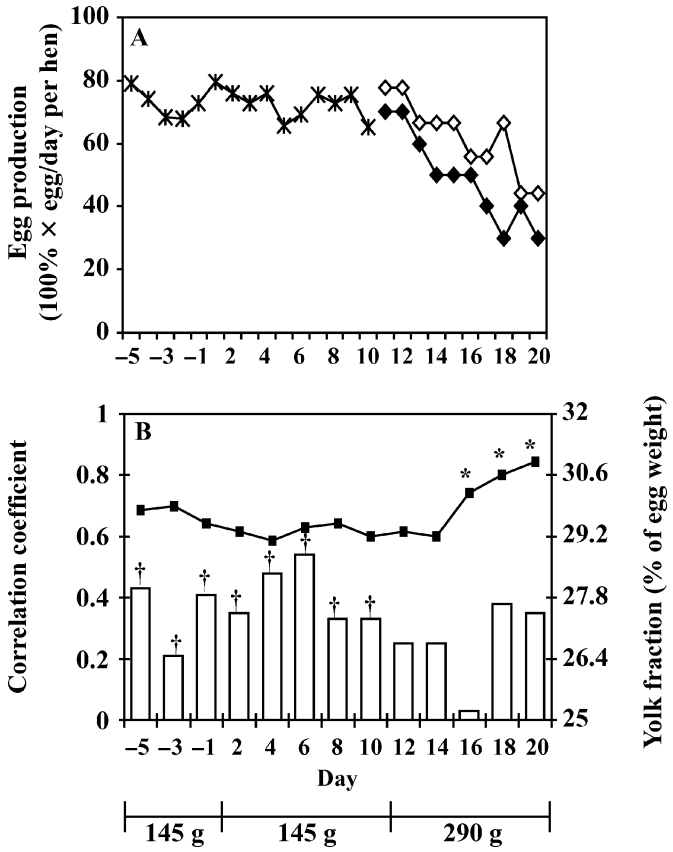

Figure 1. Daily egg production and relationship of yolk weight to egg weight. A) Egg/hen per d, flock size (n = 34) was reduced on d 10 (n = 15). Necropsy on d 20 (n = 19) established ovarian classification as abnormal (◆, n = 10) or normal (◇, n = 9). Feed intake from d –5 to d 10 = 145 g of feed/hen per d and from d 11 to d 20 = 290 g of feed/hen per d. B) Vertical bars represent correlation coefficients (left y-axis) between yolk and egg weight. Filled squares (right y-axis) represent fractional yolk weight. †Significant correlation between fractional yolk weight and egg weight. *Significant increase in fractional yolk weight relative to 145-g feed period.

during the period when their feed intake was 145 g of feed/hen per d (data not shown).

Hepatic Lipid Content and Fatty Acid Profile of Plasma NEFA

Hens fed 145 g of feed/hen per d had an average of 93.2 μmol of TAG/g of liver (Figure 2) whereas hens fed 290 g of feed/hen per d for 10 d had 275.8 μmol of TAG/g of liver ($P < 0.05$). This nearly 3-fold increase reflects a marked liver steatosis that was associated with increased liver hemorrhage (Table 2; $P < 0.02$). Hens that developed ovarian abnormalities had 23% more ($P < 0.05$) hepatic TAG than similarly fed hens gaining similar amounts of BW but with normal ovarian morphology (Figure 2; Table 2). Hepatic phospholipid concentration was not affected by feed intake ($P > 0.05$) or the presence of ovarian abnormalities (Figure 2). In plasma, provision of hens with 290 g of feed/hen per d increased plasma NEFA concentrations by 49% (Table 5; $P < 0.05$). This increase was not different in hens with normal and abnormal ovaries.

The fatty acids in plasma NEFA of hens fed 290 g of feed/hen per d exhibited increased proportions of C14:0 and C16:0 with reductions in the proportion of C14:1n7, C18:1 n9, C18:2 n6, C20:0, C20:1 n9, C20:4 n6, C20:5 n3, C22:0, C22:4 n6, C22:5 n3 ($P < 0.05$; Table 5). Analysis of NEFA concentration and fatty acid composition of the plasma samples taken from these hens before provision of 290 g of feed/hen per d were not different from those of hens fed 145 g of feed/hen per d before necropsy (data not shown). Plasma NEFA composition in hens exhibiting ovarian abnormalities showed relative increases in the amounts of C16:0 and C18:0, but lower relative amounts of C14:1 n7, C18:1 n9, C18:2 n6, and C22:0 when compared with feed-satiated hens that maintained normal ovaries ($P < 0.05$; Table 5). Fatty acid C16:1 n7 was notable in that feed-satiated hens that maintained normal ovaries exhibited a 34% increase whereas those with abnormal ovaries experienced a 42% decrease in the relative amount of this fatty acid in NEFA. As was seen in all other measurements, the differences caused by overfeeding per se, as well as the exaggerated responses of hens that developed ovarian abnormalities, were not apparent when hens were fed 145 g of feed/hen per d. Indeed, the degree of saturation for fatty acids in plasma NEFA in those hens were 0.29 ± 0.03% and 0.31 ± 0.03% during the time they were fed 145 g of feed/hen per d (individual fatty acid data not shown).

Apoptotic Morphology

After a 12-h incubation in serum-free conditions, granulosa cells collected from feed-satiated hens with or without abnormal ovarian morphology exhibited increased apoptosis as indicated by intense green staining with Annexin-V-FLUOS (Figure 3). Figure 3 shows membrane blebbing (indicated by white arrows) on the surface of granulosa cells of abnormal ovary. Necrosis also often occurred in granulosa cells from abnormal ovaries as indicated by appearance of red propidium iodide staining (Figure 3, 0-h incubation in panel A vs. 12-h incubation in panel B). Necrosis was rare and indications of apoptosis mild following a similar 12-h incubation of granulosa cells from hens fed 145 g of feed/hen per d.

Correlation Coefficients of Hens in Response to 290 g of Feed Per Hen Per Day

The egg production of hens fed 290 g of feed/hen per d (n = 19) was negatively correlated with atretic follicle number ($r^2 = 0.37$; $P < 0.01$), absolute and relative liver weights ($r^2 = 0.30$; $P < 0.05$ and $r^2 = 0.29$; $P < 0.05$, respectively), absolute abdominal fat weight and adiposity ($r^2 = 0.21$; $P < 0.05$ and $r^2 = 0.22$; $P < 0.05$, respectively), hepatic TAG content ($r^2 = 0.32$; $P < 0.05$), and plasma leptin concentration ($r^2 = 0.32$; $P < 0.05$). Egg production did not correlate to plasma insulin concentrations ($r^2 = 0.17$; $P < 0.10$).

Table 4. Effect of feeding level on plasma glucose, insulin, and leptin concentrations in Experiments 1 and 2

	Experiment 1			
	Feed allowance		Ovarian morphology of 290 g/d per hen	
	145 g/d per hen (n = 15)	290 g/d per hen[1] (n = 19)	Normal (n = 9)	Abnormal (n = 10)
Glucose, mg/mL	2.15 ± 0.05[b]	3.10 ± 0.05[a]	3.10 ± 0.06	3.12 ± 0.07
Nonesterified fatty acids, μmol/mL	0.29 ± 0.02[b]	0.42 ± 0.03[a]	0.42 ± 0.05	0.43 ± 0.04
Triacylglycerol, mg/mL	16.4 ± 1.4[b]	21.6 ± 1.0[a]	19.7 ± 1.5[a]	23.4 ± 1.1[b]
Insulin, ng/mL	2.41 ± 0.24[b]	4.83 ± 0.55[a]	4.48 ± 0.41	5.44 ± 0.94
Leptin, ng/mL	9.07 ± 0.29[b]	11.71 ± 1.08[a]	9.77 ± 0.89[b]	13.45 ± 1.47[a]
	Experiment 2			
	Feed allowance		Ovarian morphology of ≥290 g/d per hen	
	145 g/d per hen (n = 15)	≥290 g/d per hen[1,2] (n = 30)	Normal (n = 19)	Abnormal (n = 11)
Glucose, mg/mL	2.00 ± 0.04[b]	2.95 ± 0.04[a]	3.00 ± 0.06	2.90 ± 0.05
Nonesterified fatty acids, μmol/mL	0.40 ± 0.03[b]	0.55 ± 0.03[a]	0.55 ± 0.04	0.55 ± 0.05
Triacylglycerol, mg/mL	15.4 ± 1.4[b]	20.3 ± 0.5[a]	19.2 ± 0.6[b]	21.8 ± 0.7[a]
Insulin, ng/mL	2.18 ± 0.25[b]	3.30 ± 0.32[a]	3.25 ± 0.34	3.38 ± 0.68
Leptin, ng/mL	9.11 ± 0.46[b]	11.31 ± 0.50[a]	9.94 ± 0.36[b]	13.50 ± 1.57[a]

[a,b]Values are means ± SEM. Means not sharing a common superscript letter within a row differ ($P < 0.05$) between 145 and 290 g of feed/hen per d or between normal and abnormal ovary groups.

[1]Data from hens fed ≥ 290 g feed/hen per d were divided according to the presence of normal or abnormal ovarian morphology at necropsy using criteria described in Materials and Methods.

[2]Results were not different for hens fed 290 g of feed/hen per d and those fed ad libitum. Data were pooled as ≥290 g/d per hen.

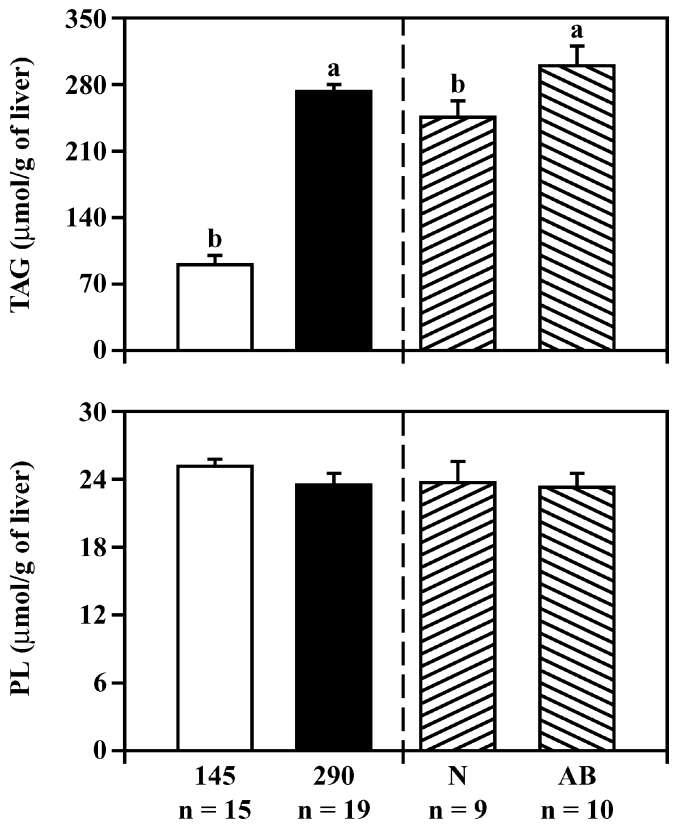

Figure 2. Hepatic triacylglycerol (TAG) and phospholipid (PL) concentrations of hens fed 145 or 290 g of feed/d. Hens fed 290 g of feed/d were further characterized as having normal (N) or abnormal (AB) ovaries. Bars not sharing a common superscript letter are significantly different for comparisons between groups fed 145 and 290 g of feed/d or between N and AB ovaries ($P < 0.05$).

In hens fed 290 g of feed/hen per d, plasma insulin concentration directly correlated with plasma leptin concentration ($r^2 = 0.62$; $P < 0.001$) and adiposity ($r^2 = 0.37$; $P < 0.01$). Plasma leptin concentration in these same hens correlated directly with adiposity ($r^2 = 0.37$; $P < 0.01$) and liver hemorrhage score ($r^2 = 0.20$; $P < 0.05$), and inversely with absolute ovary weight ($r^2 = 0.26$; $P < 0.05$), weight of the F1 ($r^2 = 0.21$; $P < 0.05$), F3 ($r^2 = 0.20$; $P < 0.05$), and F4 ($r^2 = 0.25$; $P < 0.05$) follicles. Numbers of atretic follicles correlated with liver hemorrhage score ($r^2 = 0.43$; $P < 0.01$). Other relationships included the positive correlation for the degree of saturation of plasma NEFA to that for hepatic TAG concentration ($r^2 = 0.26$; $P < 0.05$).

DISCUSSION

The results of these studies suggest an obesity-associated mechanism for impaired egg production commonly observed in feed-satiated broiler breeder hens. Broiler breeder hens rapidly became obese, gaining 500 g of primarily adipose tissue in 10 d when provided with 290 g of feed/hen per d. Moreover, persistent hyperglycemia despite significant increases in plasma insulin concentrations justifies classification of these hens as insulin-resistant. More than half of feed-satiated broiler breeder hens exhibited ovarian abnormalities such as expanded follicular hierarchies, atretic or type 3 follicles. Phenotypic separation of feed-satiated hens with normal and abnormal ovaries showed that significant differences in plasma leptin and TAG levels, hepatic TAG content, and adiposity rather than differences in insulin, glucose, or NEFA concentrations per se were most closely associated with ovarian abnormalities.

Table 5. Effect of feeding level on plasma nonesterified fatty acid profile of broiler breeder hens

	Feed allowance		Ovarian morphology of ≥290 g/d per hen	
Fatty acid, mol %	145 g/d per hen[1] (n = 15)	290 g/d per hen[1] (n = 19)	Normal (n = 9)	Abnormal (n = 10)
C14:0	2.8 ± 0.19[b]	4.4 ± 0.6[a]	4.2 ± 0.66	4.6 ± 0.54
C14:1 n7	1.72 ± 0.46[a]	0.96 ± 0.18[b]	1.23 ± 0.26[a]	0.72 ± 0.09[b]
C16:0	53.0 ± 2.2[b]	60.1 ± 2.48[a]	58.7 ± 1.55[b]	61.4 ± 0.44[a]
C16:1 n7	1.22 ± 0.16	1.15 ± 0.37	1.64 ± 0.38[a]	0.71 ± 0.12[b]
C18:0	21.3 ± 0.95[b]	25.4 ± 1.36[a]	23.5 ± 1.60[b]	27.1 ± 0.87[a]
C18:1 n9	9.6 ± 2.3[a]	3.0 ± 0.62[b]	4.6 ± 0.99[a]	1.5 ± 0.38[b]
C18:2 n6	2.8 ± 0.56[a]	1.41 ± 0.27[b]	1.93 ± 0.40[a]	0.94 ± 0.16[b]
C20:0	1.33 ± 0.28[a]	0.73 ± 0.06[b]	0.69 ± 0.12	0.76 ± 0.05
C20:1 n9	0.66 ± 0.18[a]	0.17 ± 0.09[b]	0.36 ± 0.21	Trace
C20:4 n6	0.88 ± 0.12[a]	0.50 ± 0.07[b]	0.48 ± 0.12	0.51 ± 0.10
C20:5 n3	1.57 ± 0.21[a]	0.94 ± 0.15[b]	1.19 ± 0.27	0.71 ± 0.11
C22:0	1.96 ± 0.25[a]	0.77 ± 0.09[b]	0.97 ± 0.17[a]	0.59 ± 0.07[b]
C22:4 n6	0.62 ± 0.15[a]	0.22 ± 0.08[b]	0.20 ± 0.15	0.23 ± 0.15
C22:5 n3	0.54 ± 0.11[a]	0.31 ± 0.08[b]	0.31 ± 0.11	0.31 ± 0.11
Degree of saturation,[2] %	0.35 ± 0.04[a]	0.17 ± 0.02[b]	0.22 ± 0.03[a]	0.13 ± 0.02[b]

[a,b]Values are means ± SEM. Means not sharing a common superscript letter within a row differ ($P < 0.05$) between 145 and 290 g/d per hen, or normal and abnormal ovary groups.

[1]Data from hens fed 290 g of feed/hen per d were divided according to the presence of normal or abnormal ovarian morphology at necropsy using criteria described in Materials and Methods.

[2]The degree of fatty acid saturation within a lipid class was calculated as $\Sigma m_i n_i/100$, where m_i is the percentage (mol/mol) and n_i is the number of C-C double bonds in fatty acid; therefore, the smaller the value, the more saturated the fatty acid content.

In mammals, leptin treatment attenuated reductions in plasma gonadotropins, blunted the alterations in the gonadal axis, and rescued the delay of ovulation caused by starvation (Nagatani et al., 1998; Ahima and Flier, 2000), whereas leptin excess was shown to have a deleterious effect on mammalian granulosa cell steroidogenesis and follicle maturation in vitro (Spicer and Francisco, 1997; Kikuchi et al., 2001). In rats, leptin administration significantly impaired in vivo and in vitro ovulation (Duggal et al., 2000). Similar to those mammalian studies, Paczoska-Eliasiewicz et al. (2003) showed that leptin treatment of Hy-Line Brown hens attenuated ovarian regression and reductions in ovarian progesterone and estradiol content induced by feed removal. The same study showed that prolonged leptin treatment during the refeeding period significantly hampered follicle entry into the hierarchy and follicle growth delaying ovarian restoration (Paczoska-Eliasiewicz et al., 2003).

Initial BW, subsequent feed intake, and BW gain were not different in feed-satiated broiler breeder hens that subsequently did or did not develop ovarian abnormalities. However, the composition of BW gain in hens that did develop ovarian abnormalities was different in that they gained more adipose and accumulated more hepatic TAG compared with feed-satiated hens with normal ovaries. As has been observed in mammals (Maffei et al., 1995), plasma leptin concentration was highly correlated with the degree of adiposity. In addition, elevated leptin correlated with liver hemorrhage, and liver hemorrhage was highly correlated to ovarian abnormalities. Leptin is a proinflammatory hormone with a number of systemic effects that are distinct from suppression of food intake (Vgontzas et al., 2003; Nakanishi et al., 2005). This suggests that hens with a higher capacity to direct metabolic fuels toward the liver for lipogenesis and to adipose tissue for storage are more susceptible to metabolic dysregulations that lead to reproductive dysfunction and a different plasma leptin response. In mammals, similar metabolic changes are termed lipotoxicity and are thought to underlie pancreatic β-cell failure and progression from insulin resistance to overt diabetes (Unger, 2002).

Elevated plasma NEFA with an increased fractional content of saturated fatty acids observed in feed-satiated broiler breeder hens may play a key role in the development of ovarian abnormalities. In cultured human granulosa cells, palmitic and stearic acids at physiological levels triggered cell death through down-regulation of an apoptosis inhibitor, Bcl-2, and up-regulation of an apoptosis effector, Bax (Mu et al., 2001). Oleate and linoleate induced progressively less granulosa cell apoptosis in parallel with the increase in the number of double bonds found in these 2 C18 fatty acids. That report underscores the critical role of actual fatty acid composition as well as concentration of plasma NEFA in saturated fatty acid-induced cell apoptosis. Granulosa cells from feed-satiated broiler breeder hens appear quite susceptible to apoptosis and may provide an in vivo model of saturated fatty acid-induced apoptosis.

The dichotomy of response to additional energy intake was also evident in studies with Leghorn hens (Walzem et al., 1993) where it was noted that force-feeding to 50% above usual caloric intake for 3 wk led to complete ovarian involution in 6 of 14 Leghorn layers. Dramatic increases in hepatic TAG content, liver hemorrhage, incidence of ovarian abnormalities, and impaired egg production indicate that feed-satiated broiler breeder hens typically experience fatty liver syndrome (Walzem et al., 1993, 1994). Broiler breeder hens in this study spontane-

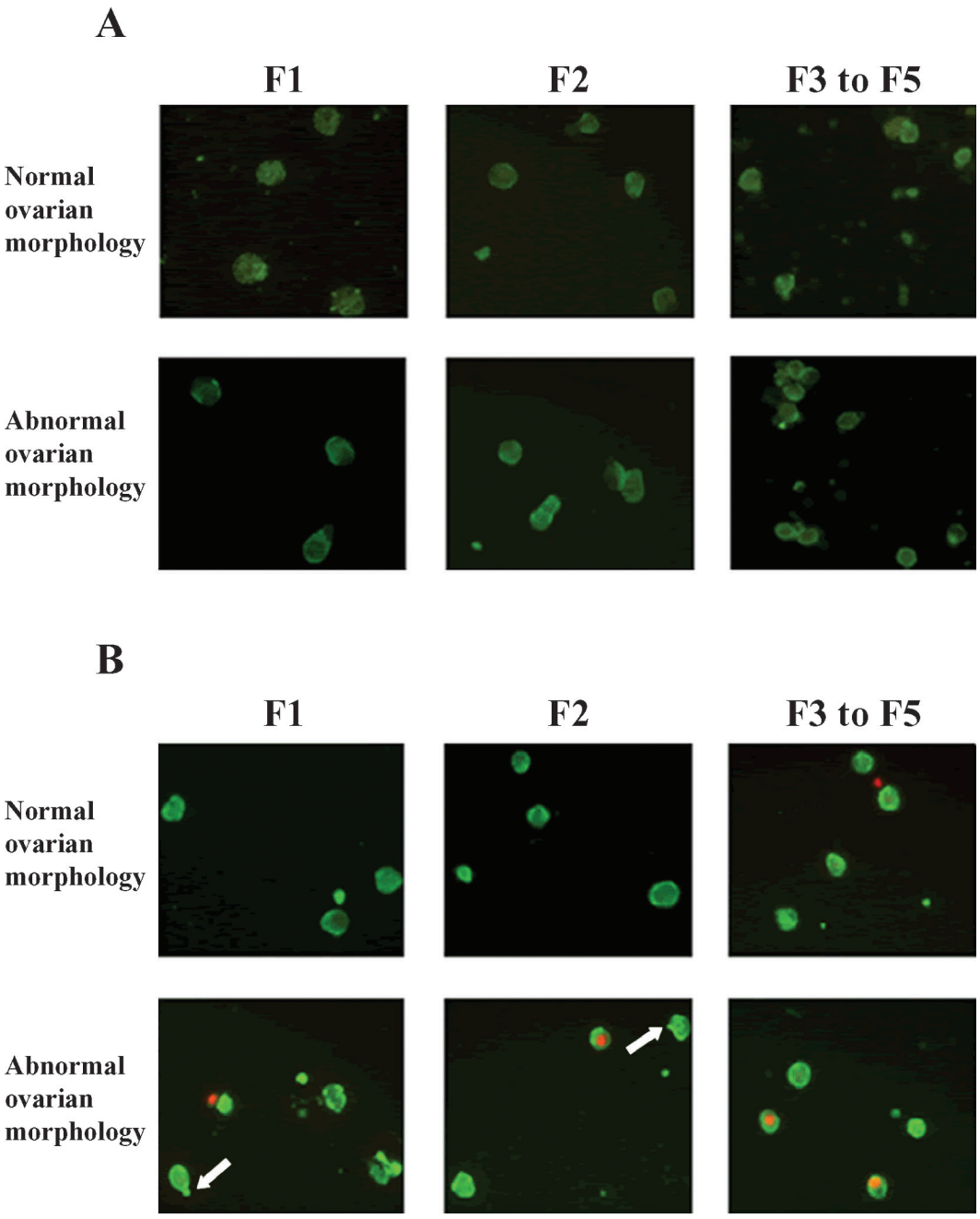

Figure 3. Hierarchical follicle (F1 to F5) granulosa cell morphology and staining with annexin V-FLUOS (green, brighter = apoptotic) and propidium iodide (red = necrosis) of hens fed 145 g of feed/d (normal) or ≥290 g of feed/d (abnormal) after 0 (panel A) or 12 h (panel B) of incubation in serum-free M199-HEPES medium at 37°C.

ously consumed 83% more than breeder-recommended feed intakes. Cassy et al. (2004) noted breed-specific differences in ovarian and hepatic leptin receptor amounts and distribution. Unlike the present study, the Hubbard strains used in that study showed no change in plasma leptin, insulin, or glucose concentrations. The Hubbard hens responded to excess feed with increased leptin receptor mRNA in liver F4, F3, and F1 follicle granulosa cells. In mammals, obesity and reproductive dysfunction are known to arise from defects in genes encoding either leptin or its receptor. A similar genetic diversity could also exist in poultry.

In the present study, follicular atresia within the hierarchy and impaired egg production of feed-satiated broiler breeder hens are consistent with earlier work by others (Hocking et al., 1987, 1989; Yu et al., 1992a,b). On average, hens consuming 145 g of feed/hen per d and feed-satiated hens had similar F1 follicle weights. However, feed-satiated hens did not maintain the expected significant relationship between yolk and egg weight. Loss of this latter relationship was introduced largely by a variable increase in the relative contribution of yolk to egg weight (Figure 1). An increase in actual (Table 1) and fractional yolk weight (Figure 1, panel B) coupled with declining egg

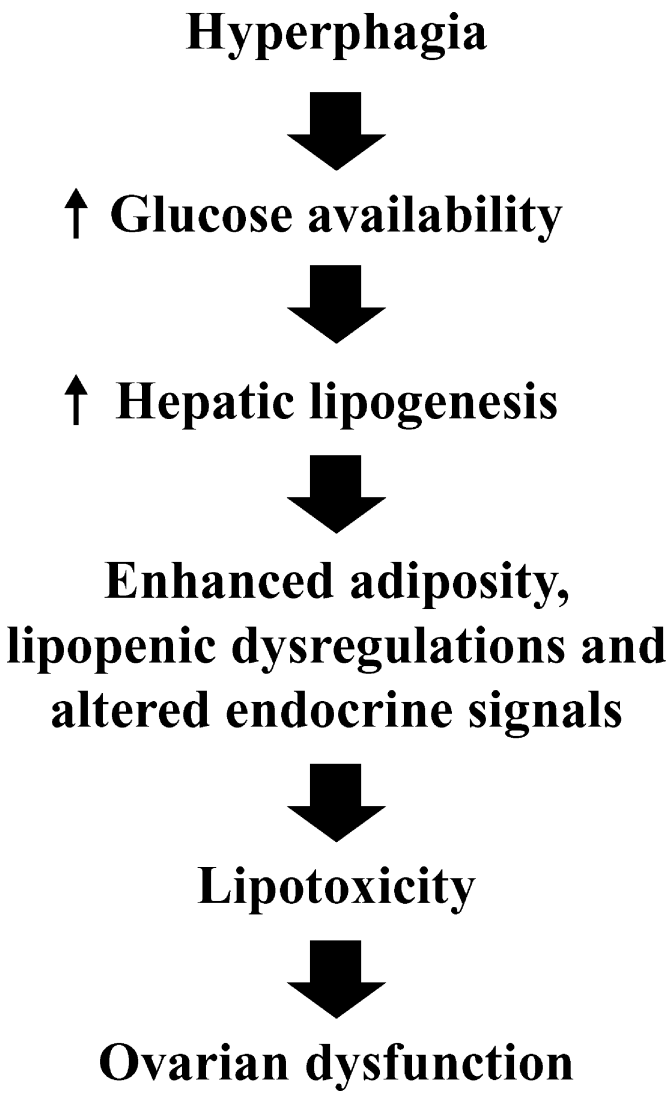

Figure 4. Hypothesized progression of events leading to lipotoxicity and reproductive dysfunction in broiler breeder hens.

production after 4 d could result from prolonged retention of follicles within the hierarchy of feed-satiated hens that allowed more plasma yolk-targeted very low density lipoprotein to be internalized. Although the time of oviposition was not determined in this study, prolonged retention of follicles within the hierarchy would increase exposure of all follicular cell types to altered metabolic and hormonal signals. Such a situation would increase the likelihood of granulosa cell dysfunction and induction of follicle atresia. Development of ovarian abnormalities in feed-satiated hens arose from differences in individual hen metabolism in response to the particular diet fed and environmental conditions. Such individual differences likely give rise to differences in the concentrations of critical metabolites that are the pivotal factors controlling the actual incidence of ovarian dysfunction within a flock.

Lipotoxicity is a theory that states that the accumulation of excess TAG and fatty acids in nonadipocytes results in altered intracellular signaling, cellular dysfunction, and cell death (Unger, 2002). Based on that theory, the enlarged yolk follicles, liver steatosis, enhanced adiposity, and reduced egg production observed in the feed-satiated hens studied here are the predicted outcomes to metabolic adaptation to inappropriately high feed intake. The proposed scenario is summarized in Figure 4. Lipotoxicity develops due to altered intracellular signaling arising from excessive fatty acid availability and altered cellular lipid (e.g., fatty acid) profile. Ovarian dysfunction as highlighted in Figure 4 is intended as prototypical target tissue, and organs such as the liver, adipose tissue, pancreas, and hypothalamic-pituitary axis, which contribute to normal ovarian function and have important independent functions, can also be affected.

Collectively, the results of the present study strongly indicate that unregulated feed intake and genetic divergence converge to influence reproductive function of broiler breeder hens. These effects appear to be mediated through lipopenic dysregulations and lipotoxic mechanisms similar to those documented for mammalian models of type 2 diabetes. The genetic divergence in the sensitivity of ovarian function in response to feed intake suggests a potential for further improvement in current broiler strains. Effective improvement of this complex trait may be best approached by first describing the metabolic basis for differences in lipid metabolism that are phenotypically associated with differences in female reproductive performance.

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