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DIETARY PROTEIN LEVELS AND THE RESPONSES OF BROILERS TO SINGLE OR REPEATED CYCLES OF FASTING AND REFEEDING

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ABSTRACT

The present study was designed to study short-term responses accompanying either chronic or acute fasting-refeeding cycles. Seven-day old Shaver broilers were fed diets containing either 120 or 300 g crude protein on either free choice basis or on 7 cycles consisting of 1 day of fasting followed by 2 days of feeding. In addition, birds fed free choice were subjected to the above regimen, but only for one cycle. Birds were bled and killed on day 1, 2 & 3 of the final cycle for each of these experiments. Measurements taken at these intervals included *in vitro* lipogenesis (IVL), growth and feed consumption, hepatic enzyme activities and plasma triiodothyronine (T_3), and thyroxine (T_4). Birds fed the lower level of crude protein free choice from 7 to 28 d ate less, were smaller and less efficient in growth. *De novo* lipogenesis and plasma T_3 were greater and T_4 was less in birds fed the lower protein diet. Birds subjected to repeated fasting-refeeding cycles exhibited striking changes on each day of the cycle. The lowest rate of IVL was noted following a 1 day fast and the greatest after 2 day of refeeding. This pattern was noted in birds fed diets containing either 120 or 300 g crude protein/kg although the responses were exaggerated in birds fed the lower level of protein. Chickens fed a low-protein diet in conjunction with a single fasting-refeeding cycle exhibited responses that were similar to chronic fasting-refeeding. The magnitudes of fasting-refeeding responses were magnified by repeated cycles of fasting-refeeding. Feeding a high level of protein modified some of the effects of a fasting-refeeding cycle.

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KEY WORDS: Chickens, Lipogenesis, Dietary protein, Feeding regimens

INTRODUCTION

There are generally two methods for altering lipogenesis in aves: 1) fasting-refeeding or 2) switching from high to low protein diets (1,2,3,4,5). Certain initial responses following feed replacement after fasting and a switch from a high to a low protein diet are similar (1). This observation indicates, initially, that metabolism might be similar in both regimens. A sequential examination of metabolic pathways following these changes reveals differences, however. For example, refeeding fasted birds results

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in high rates of lipogenesis that are sustained for three days (5). At this time, lipogenesis is approximately the same as that in controls fed free choice. In contrast, switching birds from a high to a low crude protein diet results in a sustained, high rate of lipogenesis (1,5).

Dietary crude protein is an unknown factor in defining the responses of broilers subjected to either chronic or acute fasting-refeeding events. The purpose of the experiments described in this presentation was to determine the role of dietary crude protein in modulating changes in intermediary metabolism during acute or chronic fasting-refeeding. An overall comparison between the effects of diet composition and feeding regimens reveals that feeding low-protein diets as a part of an alternating-high-low-protein feeding and refeeding fasted chickens results in similar responses (1). In both cases, *de novo* lipid synthesis increases. In contrast, high-protein diets and fasting decreased *de novo* lipogenesis (2). It is of interest if both these types of nutritional regimens can be used on a chronic basis to adapt intermediary metabolism in the broiler to further increase lipogenesis during the respective refeeding and low-protein phases of the two feeding regimens.

Table 1. Composition of the diets (g/kg diet)

Ingredient	Dietary crude protein (nitrogen x 6.25 (g/kg diet))	
	120	300
Isolated soy protein*		100
Soybean meal	112	400
Corn meal	767	400
Corn oil	17	40
Sand	15	
Dicalcium phosphate	40	40
Limestone	10	10
L-methionine		5
Selenium premix	1	1
Mineral premix	1	1
Vitamin premix	5	5
Cellulose	30	
Calculated Composition		
Metabolizable Energy (MJ/kg)	12.8	12.8
Fat (MJ/kg)	1.8	2.2
Carbohydrate (MJ/kg)	9.0	5.1
Protein (MJ/kg)	2.0	5.4
Lysine (g/kg)	6.0	17.3
Sulfur amino acids (g/kg)	10.3	10.3

* Soybean protein grade II (900 g/kg crude protein, 21726); Nutritional Biochemicals, PO Box 22400, Cleveland, Ohio 44122, USA; □ L-methionine (18915), US Biochemicals, PO Box 22400, Cleveland, Ohio 44122, USA; Selenium premix provided 0.2 mg Se/kg of diet; Mineral premix provided (mg/kg of diet): manganese 100, iron 100, copper 10, cobalt 1, iodine 1, zinc 100 and calcium 89; Vitamin premix provided (mg/kg of diet): retinol 3.6, cholecalciferol 0.075, biotin 1, vitamin E (d- α -tocopherol acetate) 10, riboflavin 10, pantothenic acid 20, choline 2 g, niacin 100, thiamine 10, vitamin B₆ 10, menadione sodium bisulfite 1.5, cyanocobalamin 0.1, folic acid 2 and ethoxyquin 150.

MATERIALS AND METHODS

Seven-day old, male, Shaver broiler chickens were assigned to one of four dietary treatment regimens with four pens per treatment (four birds per pen). These regimens were: 1) diets containing 120 or 300 g crude protein/kg fed on a free choice basis or 2) these same diets consisting of cycles of a one-day fast followed by a two day refeeding period. These birds were fed for seven cycles. All groups were fed until 28 d of age. On each day of the eighth cycle, one bird was selected from each pen and sacrificed. In addition, at this time, birds fed on a free choice basis were assigned to a single one-day fast followed by a two-day refeeding period. On each day of this cycle, one bird was selected from each pen and sacrificed. Diets are described in Table 1. This experiment was replicated twice.

In Vitro Metabolism - Lipogenesis

Livers were excised, washed in 155 mM-NaCl to remove blood and debris and then sliced (MacIlwain Tissue Chopper; 0.3 mm). Quadruplicate explants were incubated at 37 C for 2 h in Hanks' balanced salts (6,7) containing 10 mM-HEPES and 10 mM-sodium[2-¹⁴C]acetate (166 MBq/mol). All incubations were conducted in 3-ml volumes at 37 C for 2 hr under a 95% O₂-5% CO₂ atmosphere. At the end of the stated incubation periods, the explants were placed in 10 mL of 2:1 chloroform: methanol for 18 h and washed according to Folch *et al.* (8). The extracts were evaporated to dryness and dispersed in scintillation fluid. Radioactivity in the extracts was measured by liquid scintillation spectroscopy. *In vitro* lipogenesis (IVL) was expressed as μ moles of acetate incorporated into lipids per g of tissue.

In Vitro Metabolism - Enzyme Assays

Remaining liver tissues were homogenized (1:10, wt/vol.) in 100 mM-HEPES (pH 7.5)-3.3 mM- β -mercaptoethanol and centrifuged at 12,000 \times g for 30 min (9,10). The supernatant fractions were kept at -80 C until analyzed for the activities of malate: NADP⁺ oxidoreductase-[decarboxylating] (malic enzyme, EC 1.1.1.40), isocitrate: NADP⁺ oxidoreductase-[decarboxylating] (ICD-NADP, EC 1.1.1.42) and aspartate aminotransferase (AAT, EC 2.6.1.1). The activity of malic enzyme was monitored because of the enzyme's role in providing reducing equivalents (NADPH) for the synthesis of fatty acids. Isocitrate: NADP⁺ oxidoreductase-[decarboxylating] may function as both a residual source for the provision of NADPH and as a source of a coreactant for transamination. Aspartate aminotransferase aids in the removal of excess amine groups formed by feeding high-protein diets.

Malic enzyme activity (ME) was determined by a modification of the method of Hsu and Lardy (11). Reactions contained 50 mM HEPES (pH 7.5), 1 mM NADP, 10 mM-MgCl₂ and the substrate, 2.2 mM-L-malate (disodium salt) in a total volume of 1 mL. Portions (50 μ L) of the 12,000 \times g supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30 C.

Isocitrate: NADP⁺ oxidoreductase-[decarboxylating] activity (ICD) was determined by a modification of the method of Cleland *et al.* (12). Reactions contained 50 mM-HEPES (pH 7.5), 1 mM NADP, 10 mM MgCl₂ and the substrate, 4.4 mM DL-isocitrate in a total volume of 1 mL. Portions (50 μ L) of the 12,000 \times g supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30 C.

Aspartate aminotransferase activity (AAT) was determined by a modification of the method of Martin and Herbein (13). Reactions contained 50 mM-HEPES, 200 mM L-aspartate, 0.2 mM NADH,

1000 units per liter malate: NAD⁺ oxidoreductase (EC 1.1.1.37) and the substrate, 15 mM-2-oxoglutarate in a total volume of 1 mL. Portions (25 μ L) of the 12,000 \times g supernatants (diluted 1:10) were preincubated in the presence of the first four ingredients. Reactions were initiated by adding the substrate and following the rate of oxidation of NADH at 340 nm at 30 C. Enzyme activities are expressed as μ moles of product formed/min under the assay conditions (1).

Plasma Hormone Assays.

Both total T₃ and T₄ concentrations were estimated with a solid-phase single antibody procedure that is commercially available (ICN Biomedicals, Costa Mesa, CA, USA). These assays were validated for avian samples (10) by dispersing standards in charcoal-stripped chicken sera and by noting recovery of added T₃ and T₄ (94-97%). All assays were conducted as single batches to remove inter-assay variation.

Plasma Metabolites

Plasma uric acid and nonesterified free fatty acids were determined with commercially available kits (Sigma Chemical Bulletin No. 282, Sigma Chemical Co., St. Louis, MO and NEFA-C, Wako Pure Chemical Industries., LTD, Osaka).

Statistics

Data were analyzed by analyses of variance. Terms in the models were dietary protein and feeding regimens. Significance of mean comparisons was determined with the Bonferroni t-test (14). The model has been described by Remington and Schork (14).

RESULTS

Table 2 summarizes the effects of protein level and feeding regimens on growth and feed consumption. Although restricting birds to food on one day out of three decreased body weights, the birds consumed more food than did controls on the other two days of the cycle. Birds had access to food on 67% of time, but consumed 85% of the amount of food and were only 20% lighter in weight than were respective controls.

TABLE 2.

Dietary protein effects on the growth of broilers subjected to chronic or acute fasting-refeeding*

Protein	Regimen	Body wt (28 d)		Feed intake		Feed efficiency	
		(g)	300	(g)	300	(gain/feed)	300
120	Free choice	778	1142	1305	1491	0.49	0.67
120	Chronic fasting-refeeding	675	921	1116	1270	0.48	0.61
Pooled Standard Error		22.4		32.7		0.02	

* Broiler chickens growing from 7 to 28 days of age were fed diets containing either 120 or 300 g crude protein under one of two regimens (free choice or seven cycles consisting of 1 day of fasting followed by two days of refeeding.)

Neither dietary crude protein nor feeding regimens changed plasma glucose concentrations (Table 3). In contrast, feeding a greater level of crude protein increased plasma NEFA and Uric acid (P<0.05)

and decreased triglycerides ($P < 0.05$). Fasting for one day increased plasma NEFA and decreased triglycerides ($P < 0.05$) in birds subjected to acute fasting-refeeding. Only those birds given the lower level of crude protein exhibited responses during chronic fasting-refeeding.

TABLE 3
Dietary protein effects on plasma metabolites in broilers subjected to acute or chronic fasting-refeeding*

	Glucose†		NEFA‡		Uric Acid‡		Triglycerides‡	
	120	300	Protein Regimen (g crude protein/kg diet)		120	300	120	300
Free choice	15.1	14.2	170 ^a	334	355 ^a	600	2161 ^a	963 ^a
Acute Fasted 1 d	12.3	12.4	645 ^b	730 ^{ab}	333	309 ^b	825 ^{ab}	659 ^a
Refed 1 d	16.4	15.6	127 ^a	194 ^{ab}	346 ^a	723 ^a	1772 ^a	1220 ^a
Refed 2 d	14.8	14.7	268 ^a	184 ^{ab}	373 ^a	634 ^a	2840 ^{ab}	1137 ^a
Chronic Fasted 1 d	12.4	13.5	708 ^{ab}	337 ^a	144 ^{ab}	459 ^{ab}	786 ^a	1116 ^a
Refed 1 d	15.5	14.2	126 ^a	240 ^a	284 ^a	530 ^a	2083 ^a	1332 ^a
Refed 2 d	14.3	14.2	259 ^b	200 ^b	361 ^a	604 ^a	2653 ^a	1354 ^a
Standard Error	0.36	0.26	27.8	31.5	45.5	39.0	93.8	170.5

* Significant difference between protein levels at a time in a feeding regimen.

^b Significant difference between free choice for a protein level and a time increment in a feeding regimen.

* For a description of the dietary treatments, see Table 2; † 10^{-3} M; ‡ 10^{-6} M, NEFA, non-esterified free fatty acids.

TABLE 4
Dietary protein and *in vitro* metabolism in broilers subjected to acute or chronic fasting-refeeding*

	IVL†		AAT‡		ME‡		ICD‡	
	120	300	Protein Regimen (g crude protein/kg diet)		120	300	120	300
Free choice	696 ^a	144 ^a	751 ^a	1039 ^a	336 ^a	138 ^a	348 ^a	624 ^a
Acute Fasted 1 d	104 ^{ab}	87 ^b	305 ^b	1260	178 ^b	175 ^b	855 ^b	863 ^b
Refed 1 d	813 ^{ab}	336 ^{ab}	1271 ^b	1149	505 ^{ab}	182 ^{ab}	883 ^b	987 ^b
Refed 2 d	1563 ^{ab}	188 ^a	1571 ^b	1426 ^b	754 ^{ab}	118 ^a	1109 ^b	930 ^b
Chronic Fasted 1 d	200 ^{ab}	113	1135 ^{ab}	733 ^{ab}	414 ^a	144 ^a	544 ^{ab}	777 ^a
Refed 1 d	1601 ^{ab}	385 ^{ab}	1470 ^b	1584 ^b	838 ^{ab}	119 ^a	796 ^b	893 ^b
Refed 2 d	1856 ^{ab}	708 ^{ab}	1600 ^b	1803 ^b	1061 ^{ab}	199 ^a	921 ^b	1005 ^b
Standard Error	27.1	37.4	74.2	99.4	45.8	27.3	54.5	45.0

* Significant difference between protein levels at a time in a feeding regimen.

^b Significant difference between a free choice value for a protein level and the corresponding value for a time in a feeding regimen.

* For a description of the dietary treatments, see Table 2; † IVL = *in vitro* lipogenesis; values presented are μ moles [$2\text{-}^{14}\text{C}$]sodium acetate incorporated in hepatic lipids /kg body weight;

‡ AAT = aspartate aminotransferase, ME = malic enzyme, ICD = isocitrate dehydrogenase; values presented are units/ kg body weight.

Table 4 summarizes the effects of both dietary crude protein levels and feeding regimens on some measurements of *in vitro* metabolism. When fed on a free choice, the lower level of crude protein resulted in a greater rate of lipogenesis compared to the higher protein level. An acute fast followed by two days of refeeding first decreased ($P < 0.05$) and then increased ($P < 0.05$) lipogenesis compared to the free choice rate on both days of refeeding. This response was more noticeable ($P < 0.05$) in birds fed the diet containing 120 g crude protein/kg than in birds fed the diets containing the lower crude protein level. A comparison between chronic and acute treatment groups showed that the change in lipogenesis following refeeding was greater ($P < 0.05$) in the chronic than in the acute treatment group. Generally, ME activity paralleled that of IVL although responses following refeeding were not as great. These responses also included lower ME activities in birds fed the higher level of crude protein and in birds fasted 1 d. In contrast, feeding the higher level of crude protein and fasting for 1 d increased AAT and ICD activities.

The effects of feeding regimens and dietary protein levels on plasma thyroid hormone levels are presented in Table 5. Feeding the higher level of crude protein increased ($P < 0.05$) plasma T_4 and decreased ($P < 0.05$) plasma T_3 . Feeding regimens did not generally affect plasma T_3 levels. Exceptions were noted in birds fed the lower level of crude protein in conjunction with either fasting-refeeding regimen. Fasting decreased ($P < 0.05$) plasma T_3 compared to free choice.

TABLE 5
Dietary protein effects on plasma triiodothyronine (T_3) and thyroxine (T_4) in broilers subjected to chronic or acute fasting-refeeding*

	T_3		T_4	
	Protein Regimen (g crude protein/kg diet)			
	120	300	120	300
Free choice	4.7	2.7 ^a	10.8 ^a	23.8 ^a
Acute Fasted 1 d	2.6	2.4	26.9	24.8
Refed 1 d	3.4	3.0	10.3 ^a	18.9 ^a
Refed 2 d	4.3	3.2	12.2 ^a	24.9 ^a
Chronic Fasted 1 d	3.0 ^b	2.7	22.4 ^b	35.1 ^{ab}
Refed 1 d	4.4	2.7	8.6 ^a	22.1 ^a
Refed 2 d	5.2	3.7	9.7 ^a	22.1 ^a
Standard Error	0.22	0.21	1.28	1.93

^a Significant difference between protein levels at a time in a feeding regimen.

^b Significant difference between a free choice value for a protein level and the corresponding value for a time in a feeding regimen.

* For a description of the dietary treatments, see Table 2; † 10^{-9} M.

DISCUSSION

Few groups have offered any biochemical explanation for the decrease in lipogenesis coincident to the feeding of high-protein diets. An inverse relationship between the level of the dietary protein and the subsequent rate of IVL may indicate a decrease in the flow of substrates through glycolysis and an increase in the production of glucose from substrates that were formerly in the pathways leading to fat synthesis (18). The enzyme activities in the present study indicate that ICD functions in both lipid and

protein metabolism. The enzyme provides both a residual capacity for the production of reducing equivalents and a co-reactant for transamination (α -ketoglutarate). Competition exists between acetyl-CoA carboxylase and the aconitase-isocitrate dehydrogenase pathway for limited cytoplasmic citrate. The requirement for α -ketoglutarate as a co-reactant for transamination of excess amino acids depresses citrate levels and the subsequent activation of acetyl CoA carboxylase. Thus, citrate levels appear to control the avian enzyme more than the rat enzyme (19). Furthermore, Hillard *et al.* (20) reported that dietary carbohydrate was a potent regulator of avian lipogenesis, possibly though regulating the citrate supply.

Generally, lipogenic responses were somewhat magnified by repeated fasting-refeeding as opposed to a single cycle. In turn, dietary protein levels moderated responses. The higher protein diet increased plasma thyroxine and decreased triiodothyronine. In this respect, higher protein diets mimicked the effect of fasting on thyroid metabolism. Rats fed on an intermittent basis demonstrate many of the characteristics of meal-fed animals (15). Changing from a high to a low-protein diet and from a fasted to a fed state rapidly increased lipogenesis in a fashion similar to the meal-feeding response seen in rodents. The present study indicates that this increase is also greater when chickens are repeatedly fasted and refed or fed alternating-high-low protein diets. Indeed, these same responses have been noted in older chickens (16,17). The former study attributed this rapid change in lipogenesis to a decrease in fatty acid release from adipose tissue. This decrease increases CoA availability for the citrate cleavage and acetyl-CoA carboxylase (EC 6.4.1.2) reactions. In contrast, the latter study seems to indicate that the supply of reducing equivalents (NADPH) regulates *de novo* lipogenesis during rapid changes in lipogenesis. Based on a noted high correlation between ME activity and *de novo* lipogenesis, Yeh and Leveille (18) originally proposed that availability of NADPH regulates lipid metabolism in chickens fed on high-protein diets.

Oppenheimer *et al.* (21) found a positive correlation between T_3 level and hormone action at the cellular level. According to their hypothesis, a decrease in either tissue binding or circulating levels of T_3 (as is the case in the present study) would decrease enzyme activity. The data concerning the effects of low protein diets on plasma thyroid hormone levels agree with relatively recent results obtained with chickens (22,23,24,25), rats (26) and trout (27). Previously, Cree and Schalch (28) found that a reduction in the energy intake from fat calories decreased body weight and T_3 and T_4 in young rats. In contrast a later study from this same group (29) indicated although a reduction in carbohydrate energy also decreases body weight, there were no changes in either T_3 or T_4 . This study seemed to imply that in rats: 1) growth could be separated from thyroid function and 2) growth retardation was related to the quantity of the dietary energy rather than the quality of the energy sources in the diet. Analyses of data in these studies seem to indicate that thyroid hormone values may be of some usefulness in determining the protein nutritional status of chickens. It should be noted, however, that large differences among dietary treatments were required to meet significance because of the large variation in values in most previous studies.

An experiment was conducted to determine the interaction of dietary crude protein and feeding regimens (free choice, chronic or acute fasting-refeeding) on intermediary metabolism. Both fasting-refeeding regimens gave the familiar meal-feeding response, although repeated cycles fasting-refeeding gave responses greater than did a single cycle. The findings in the present study further reinforce our hypothesis that dietary protein alters lipid metabolism even during periods of fasting-refeeding. Although, high-protein diets result in a pattern of *in vitro* lipogenesis similar to fasting, certain levels hormone levels, particularly T_4 , are different from fasting values.

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