Effects of Substitution Between Fat and Protein on Feed Intake and Its Regulatory Mechanisms in Broiler Chickens: Endocrine Functioning and Intermediary Metabolism

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ABSTRACT The aim of this study was to investigate the influence of dietary macronutrient ratio on postprandial (5 or 48 h after refeeding) endocrine functioning and metabolites of the intermediary metabolism and to relate these parameters with dietary-induced alterations in energy, protein, and lipid metabolism. Male broilers were reared from 1 to 7 wk of age on isoenergetic diets with substitutions between fat and protein but similar carbohydrate contents [low protein (LP): 126 vs. 242 g protein/kg; low fat (LF): 43 vs. 106 g fat/kg]. The LP chickens had significantly increased postprandial plasma triglyceride levels. This was likely the result of stimulated hepatic lipogenesis, as corroborated by their significantly higher respiratory quotients. Plasma free fatty acid concentrations were higher in LP broilers, whereas glucose levels were unaffected by dietary composition, suggesting that these chickens preferred carbohydrates as an energy source over free fatty acids. Plasma uric acid levels were lower in LP compared with LF chickens, indicating a more efficient protein retention in the former group. LP birds that were fasted and refed at 48 h had higher plasma 3,5,3′-triiodothyronine (T_3) levels, corroborating their increased heat production. The postprandial T_3 increase was more pronounced in the LF chickens, possibly induced by their higher protein consumption.

In conclusion, diet-induced changes in heat production and energy partitioning are reflected in circulating levels of intermediary metabolites and hormones. Furthermore, nutritional studies should consider the ability of organisms to habituate to changed diet compositions and that alterations in feeding status follow higher-order responses.

(*Key words*: broiler chicken, macronutrient ratio, intermediary metabolism, endocrine functioning)

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INTRODUCTION

Energy deposition is the result of energy intake on the one hand and energy expenditure on the other hand and is controlled by a variety of mechanisms. Next to genetic factors, exogenous factors such as environmental conditions and nutritional factors (diet quantity and composition) interact strongly with the control and regulation of the energy flow. Regarding the dietary composition, it is well documented that the ratios between macronutrients (protein, lipid, and carbohydrate) have a major impact on zootechnical performance and on body composition of chickens (MacLeod, 1990, 1992; Buyse et al., 1992; Nieto et al., 1997; Collin et al., 2003). However, far less is known about the underlying causal mechanisms in terms of energy metabolism, intermediary metabolism, and endocrine functioning (see review by Buyse et al., 2001a). It must also be kept in mind that intentionally changing the concentration of one macronutrient in the feed has an effect on both of the other macronutrients, which makes it difficult to attribute the interpretation of observed effects to one particular macronutrient. Few studies have taken this point into consideration (Buyse et al., 2001a).

We have, therefore, initiated a series of studies with broiler chickens using isoenergetic diets in which the energetic substitution of one particular macronutrient was done by only one other macronutrient while keeping the concentration of the third macronutrient constant (Collin et al., 2003; Malheiros et al., 2003a,b, 2004; Swennen et al., 2004). These studies revealed that an isoenergetic substitution of fat energy for carbohydrate energy, with a constant crude protein level and therefore a constant me-

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Abbreviation Key: LF = low fat; LP = low protein; T_3 = 3,5,3′-triiodothyronine; T_4 = thyroxine.

tabolizable energy to crude protein ratio, has little effect on plasma concentrations of metabolites and hormones (Malheiros et al., 2003a, 2004). On the other hand, changing the dietary protein content did affect the protein and fat deposition in chickens as well as the intermediary metabolism and endocrine functioning (Collin et al., 2003).

The general aim of the presented experiment conducted with 2 isoenergetic diets with substitution between fat and protein contents was to investigate the influence of this isoenergetic substitution on endocrine functioning and key metabolites of the intermediary nutrient metabolism of broiler chickens and to relate these humoral parameters with measurements of the energy, protein, and lipid metabolism (such as apparent metabolizable energy intake, energy excretion, and energy retention as protein or fat; Swennen et al., 2004). Furthermore, possible effects of diet composition on these plasma parameters were not only evaluated during normal ad libitum feeding conditions but also during feed deprivation as well as after 5 h refeeding (postprandial changes). It is hypothesized that postprandial changes in these humoral factors may be involved in short-term control of feed intake.

MATERIALS AND METHODS

Experimental Design

Day-old male broiler chickens were purchased from a local hatchery.[2] The broilers were housed in an environmentally controlled poultry house with wood shavings as litter. The temperature was set at 35°C at 1 d of age and was decreased by 1°C every 2 d until a permanent temperature of 22°C was reached. The lighting schedule provided 23 h of light per day. Until 7 d of age, a commercial starter diet was provided ad libitum (for diet composition see Buyse et al., 2001b).

From 7 d of age, each group received one of the isoenergetic diets (Table 1). The diets contained the same ingredients, although some were in different quantities to create pairwise changes in protein and fat content and to keep gross energy content and carbohydrate concentrations more or less similar for both diets. The low protein diet (LP) contained 3,974 kcal of gross energy/kg, 126 g of protein/kg, 106 g of fat/kg, and 514 g of N-free extract/kg. The low fat diet (LF) contained 3,998 kcal of gross energy/kg, 242 g of protein /kg, 43 g of fat/kg, and 504 g of N-free extract/kg.

At 21 d of age, 12 broilers from each group were taken from the floor pens and housed in wire cages for adaptation to restraint housing conditions. The birds were provided assigned diets and water ad libitum. For the next 4 wk and repeated weekly with the other chickens, 3 LP and 3 LF chickens were each placed in 1 of the 6 respiratory cells for measuring the energy and protein metabolism. Chickens in the respiratory cells were provided continuous lighting and the same temperature schedule as used in the floor pens. After the adaptation period, the birds were fasted for 24 h, and then they were allowed to eat for 5 h so we could measure diet-induced thermogenesis. For the rest of the experimental period, the broilers were fed ad libitum to calculate total energy balance. The results for energy and protein metabolism have been reported by Swennen et al. (2004).

Blood samples were collected after 24 h of fasting, after 5 h of refeeding (postprandial stage), and after 48 h of ad libitum access to the diet. Blood was taken from a wing vein with a heparinized syringe and was collected into iced tubes. After centrifugation, plasma was stored frozen until analysis for hormone and metabolite contents. After euthanasia, liver and abdominal fat were excised and weighed. This research was approved by the Ethical Commission for Experimental Use of Animals of the Katholieke Universiteit Leuven.

TABLE 1. Experimental diets

Item	Diet: Low protein	Diet: Low fat
Ingredient (g/kg)		
Peas (<200 g of CP/kg)	31.369	31.369
Wheat	24.229	24.229
Maize starch	16.800	16.800
Soybean oil	9.507	1.961
Soybean meal	5.124	5.124
Monocalcium phosphate	2.365	2.365
Premix[1]	1.400	1.400
Chalk	1.307	1.307
L-Lysine	0.770	0.770
DL-Methionine	0.622	0.622
L-Threonine	0.393	0.393
Salt	0.337	0.337
L-Tryptophan	0.121	0.121
Soy protein	—	13.2
Celite	6.104	—
Energy and nutrient content		
Gross energy content (kcal/kg)	3,975	3,998
Protein (g/kg)	126.1	242.2
N-free extract (g/kg)	513.9	504.3
Fat (g/kg)	106.1	43.4
Ash (g/kg)	120.7	66.1
Fiber (g/kg)	23.0	22.4
Moisture (g/kg)	110.2	121.6
Starch (g/kg)	447.5	442.0
Sugars (g/kg)	21.7	21.7
Gross energy to protein ratio (kcal/g of protein)	31.52	16.51

[1]Premix supplied the following amount of vitamins and minerals per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 50 IU; vitamin K_3, 2.5 mg; vitamin B_1, 2.2 mg; vitamin B_2, 7.5 mg; vitamin B_3, 13 mg; vitamin B_6, 5.5 mg; vitamin B_{12}, 0.035 mg; niacin, 38 mg; folic acid, 1 mg; biotin, 0.2 mg; choline chloride, 650 mg; Fe, 45 mg; Cu, 25 mg; Mn, 60 mg; Co, 1 mg; Zn, 70 mg; I, 2 mg; Se, 0.4 mg; etoxyquin, 35 mg; butylated hydroxytoluene, 25 mg.

Plasma Metabolites and Hormones

Plasma 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4) concentrations were measured by radioimmunoassay as described by Darras et al. (1992). Intraassay coefficients of variation were 4.5 and 5.4% for T_3 and T_4, respectively.

[2]Avibel, Zoersel, Belgium.

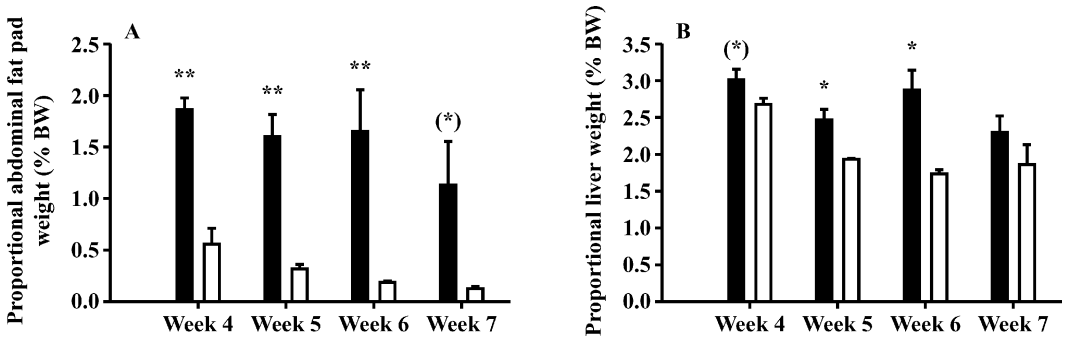

FIGURE 1. Proportional abdominal fat pad (A) and liver (B) weights expressed as a percentage of the body weight per dietary treatment: low protein (filled bars) and low fat (open bars) (n = 3 per week). Values are means ± SEM. Significant effect of the diets at the same treatment duration (*)$0.05 < P < 0.1$; *$P < 0.05$; **$P < 0.01$.

The plasma corticosterone concentration was measured using a specific radioimmunoassay kit[3] with a sensitivity of 0.39 ng/mL and low cross-reactions with aldosterone (0.20%), cortisol (0.40%), and deoxycorticosterone (3.30%). The intraassay variability was 3.6%.

Plasma glucose, triglycerides, and uric acid concentrations were measured spectrophotometrically with an automated apparatus.[4] Plasma free fatty acid concentrations were measured with a WAKO NEFA C[5] test kit, an enzymatic colorimetric test, modified for use in the Monarch Chemistry System.

Statistical Analysis

For each feeding condition [fasting, 5 h refeeding, ad libitum feeding (48 h), and postprandial changes defined as the change in plasma level between fasting and 5 h refeeding] separately, data were analyzed by ANOVA with diet composition and duration of the trial as classification variables.[6] In addition, within each feeding condition and treatment duration, differences between diets were analyzed by one-factor ANOVA. It is acknowledged that the age of the broilers increased with the duration of the trial. Hence both factors were intermingled and could not be analyzed separately. We, consequently, preferred to use the term 'duration of the trial' throughout the manuscript.

RESULTS

From 4 to 7 wk of age, absolute ($P < 0.0004$) and proportional ($P < 0.0001$) weights of the abdominal fat pad were significantly higher for broilers in the LP group. There was no effect of duration of the trial on this parameter (Figure 1). The proportional liver weight was significantly affected by the diet ($P < 0.0001$) and duration of the trial ($P < 0.01$). The LP broilers had a higher proportional liver weight, and this difference was significant at 5 and 6 wk of age (Figure 1). In both groups, proportional liver weights decreased with duration of the trial. There was no effect of diet or duration on the absolute liver weights in either group.

The outcome of the statistical analysis on plasma metabolite and hormone concentrations obtained after 24 h of fasting, 5 and 48 h of refeeding, and postprandial changes during the 5 h refeeding period is summarized in Table 2.

TABLE 2. Results of ANOVA according to diet composition (low protein and low fat) and duration of the experimental trial on plasma concentrations of metabolites and hormones

Parameter[1]	Diet	Duration	Diet × duration
After fasting			
Glucose	NS	NS	NS
Triglycerides	NS	0.0016	NS
Free fatty acids	0.0303	0.0003	NS
Uric acid	<0.0001	0.0325	0.0227
T_3	0.0014	NS	NS
T_4	NS	NS	0.0119
Corticosterone	NS	NS	NS
After 5 h refeeding			
Glucose	NS	NS	NS
Triglycerides	0.0012	<0.0001	NS
Free fatty acids	0.0022	<0.0001	NS
Uric acid	0.0001	0.0205	0.0486
T_3	NS	0.0006	0.006
T_4	NS	NS	NS
Corticosterone	NS	NS	NS
After 48 h refeeding			
Glucose	NS	NS	0.0138
Triglycerides	<0.0001	<0.0001	NS
Free fatty acids	0.0004	0.0005	NS
Uric acid	<0.0001	0.0325	NS
T_3	(0.06)	NS	NS
T_4	NS	NS	NS
Corticosterone	NS	NS	NS
Postprandial changes (after 5 h of refeeding) in			
Glucose	NS	NS	NS
Triglycerides	0.002	<0.0001	NS
Free fatty acids	(0.09)	NS	NS
Uric acid	0.0278	NS	NS
T_3	NS	0.0118	0.0105
T_4	0.0355	NS	NS
Corticosterone	NS	NS	NS

[1]T_3 = 3,5,3′-triiodothyronine; T_4 = thyroxine; $^{NS}P > 0.05$.

[3]IDS, Inc., Boldon, UK.
[4]Monarch Chemistry System, Instrumentation Laboratories, Zaventem, Belgium.
[5]Wako Chemicals GmbH, Neuss, Germany
[6]SAS for Windows, 1998, version 8 edition, SAS Institute Inc., Cary, NC.

In fasting conditions, there were no significant differences in plasma triglyceride concentrations between LF and LP chickens (Figure 2). Five hours of refeeding induced an increase in plasma triglyceride levels, and this elevation was more pronounced for LP chickens (diet effect: $P = 0.002$) and during the first part of the feeding trial (duration effect: $P < 0.0001$). Consequently, the LP chickens were characterized by significantly higher plasma triglyceride levels after 5 h ($P = 0.0012$) and 48 h ($P < 0.0001$) of refeeding. Regardless of the feeding conditions, plasma triglyceride levels decreased ($P < 0.0001$) with duration of the experiment, especially after the first week.

When fasted, free fatty acid levels averaged higher ($P = 0.03$) in the plasma of LP chickens compared with that of their LF counterparts (Figure 2). Five hours of refeeding reduced plasma free fatty acid levels. This effect tended ($P = 0.09$) to be more pronounced in the LF chickens and resulted in significantly ($P = 0.0022$) lower plasma free fatty acid concentrations for the chickens reared on the LF diet. Refeeding for 2 d induced an elevation in circulating free fatty acid levels. This increase was especially present in the LP chickens and resulted in significantly higher ($P = 0.004$) levels compared with those of LF chickens. Plasma free fatty acid concentrations decreased significantly with the duration of the trial, irrespective of the feeding status of the chickens.

Plasma uric acid concentrations were markedly affected by dietary composition (Figure 3). Indeed, irrespective of the feeding condition, LP chickens were characterized by significantly ($P < 0.0001$) lower levels of uric acid in their plasma compared with those obtained from LF chickens. Furthermore, 5 h of refeeding was also associated with more pronounced ($P = 0.028$) increases in uric acid levels for the LF chickens. For each feeding condition, plasma uric acid levels decreased significantly ($P < 0.05$) with the duration of the experiment until 6 wk of age. During the last week of the experiment, plasma uric acid concentrations increased again, although only for the LF chickens, resulting in a significant ($P < 0.05$) diet by duration interaction after fasting and 5 h of refeeding.

Fasted LP chickens were characterized by significantly ($P = 0.0014$) higher plasma T_3 levels compared with fasted LF chickens. Refeeding for 5 h increased circulating T_3 levels, although differentially according to the duration of the feeding period ($P = 0.0118$) and in interaction with diet composition ($P = 0.0105$). Indeed, the postprandial increases in plasma T_3 levels were more pronounced during the first week of the trial compared with later stages, especially for LF compared with LP chickens. As a consequence, LF chickens had initially the highest postprandial T_3 levels, but the difference between the diets was reversed with the prolongation of the feeding experiment (duration by diet interaction: $P = 0.006$). Plasma T_3 levels returned to intermediate values after 48 h of feeding and were on average ($P = 0.06$) higher for chickens fed the LP diet.

There was no overall effect of diet composition or duration of the trial on circulating T_4 concentrations for any

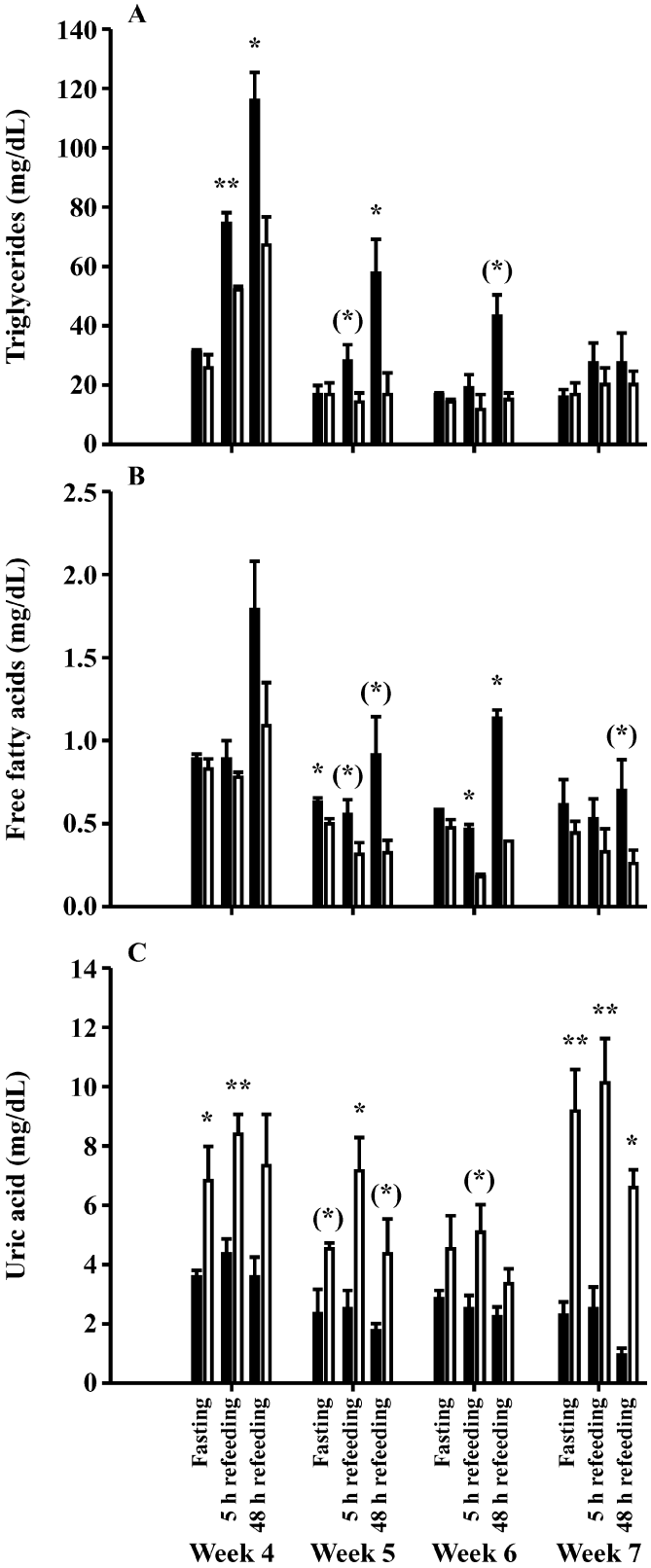

FIGURE 2. Plasma concentrations of triglycerides (A), free fatty acids (B), and uric acid (C) per dietary treatment (low protein: filled bars; low fat: open bars), per measuring point (after fasting, after 5 h of refeeding, and after 48 h of refeeding) and per week (n = 3). Values are means ± SEM. Significant effect of the diets at the same treatment duration and the same measuring point (*)$0.05 < P < 0.1$; *$P < 0.05$; **$P < 0.01$.

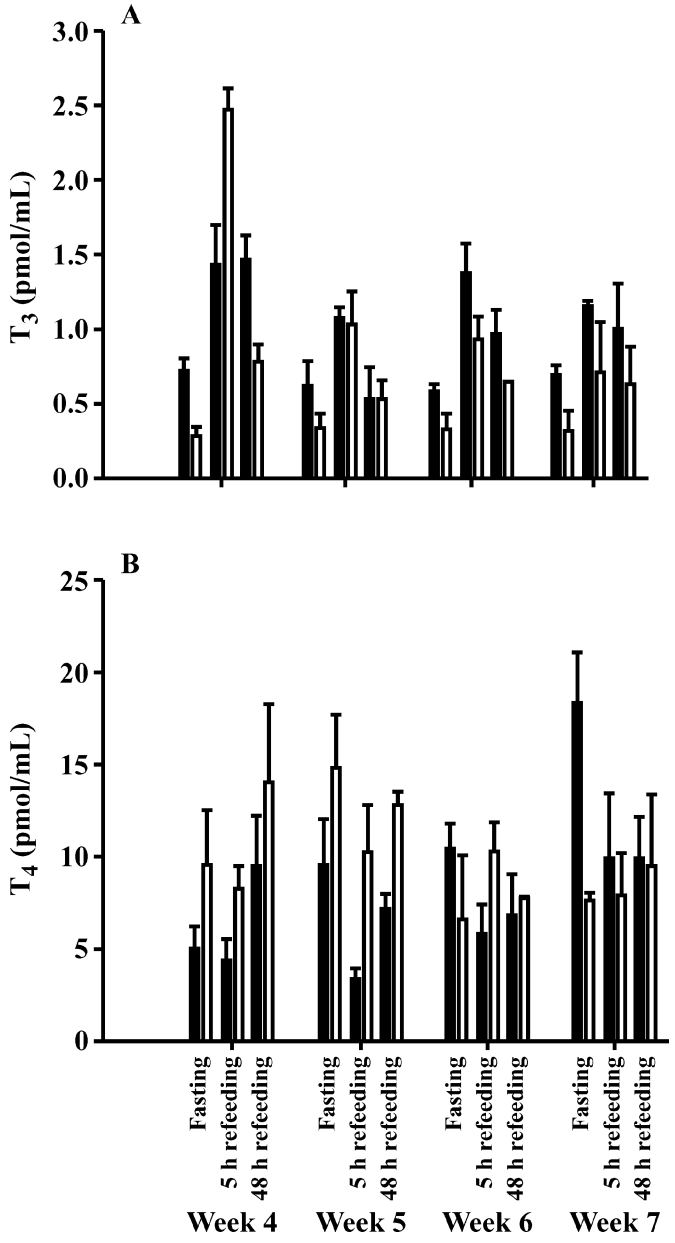

FIGURE 3. Plasma concentrations of 3,5,3′-triiodothyronine (T_3; A) and thyroxine (T_4; B) per dietary treatment (low protein: filled bars; low fat: open bars), per measuring point (after fasting, after 5 h of refeeding, and after 48 h of refeeding) and per week (n = 3). Values are means ± SEM.

of the feeding conditions. However, a significant (P = 0.0119) interaction between diet and trial duration was observed for fasting plasma T_4 concentrations. Indeed, during the first 2 wk of the trial, LP chickens were characterized by lower plasma T_4 levels than LF chickens, whereas the opposite was true for the last 2 wk. The postprandial decrease in plasma T_4 levels was more pronounced for the LP chickens (diet effect: P = 0.0355) compared with that of the LF chickens; the latter chickens showed even a slight postprandial increase in T_4 levels during the second part of the experimental period. In general, the changes in circulating T_4 levels induced by the feeding condition were opposite to what was observed for plasma T_3 levels.

Plasma glucose and corticosterone concentrations were not affected by diet or duration of the trial at any of the feeding conditions (data not shown).

DISCUSSION

Organ Weights and Plasma Metabolites

Besides higher fat intakes, the significantly increased plasma triglyceride levels of 5 and 48 h fed LP chickens were also very likely the result of stimulated hepatic lipogenesis. A high dietary energy to protein ratio enhances the de novo hepatic lipogenesis (Yeh and Leveille, 1969; Tanaka et al., 1983; Rosebrough and Steele, 1985) as well as hepatic malic enzyme activity (a key enzyme in the fat metabolism; Rosebrough et al., 2002). As the gross energy to protein ratio of the LP diet was almost twice as high as that of the LF diet (31.5 versus 16.5), a higher de novo lipogenic activity in the liver of LP chickens could be inferred. This enhanced lipogenic activity might explain the higher proportional liver weights due to lipid accumulation. Further evidence for an augmented de novo fatty acid synthesis of LP chickens is found in their significantly higher respiratory quotients (>1.0; Swennen et al., 2004).

The level of free fatty acids in the circulation is the net result of lipolysis on the one hand and cellular uptakes of free fatty acids for energy on the other hand. The LP chickens had higher free fatty acid levels compared with their LF counterparts, irrespective of the feeding status, which might have been the result of a reduction in free fatty acid uptake by peripheral tissues. Indeed, the plasma glucose levels were not affected by diet composition, despite 33% higher carbohydrate intake per metabolic body weight by the LP chickens (Swennen et al., 2004). This finding suggests that the LP chickens preferably used carbohydrates as an energy source rather than free fatty acids, resulting in higher circulating free fatty acid levels. In fasting conditions, however, the higher plasma free fatty acid levels of LP chickens may be the consequence of an enhanced lipolysis for energetic purposes. This hypothesis is based on the following observations 1) LP chickens have more body fat, and so more lipids are available for breakdown; 2) there was an absence of exogenous carbohydrates during fasting; 3) there was limited protein catabolism as reflected by low uric acid concentrations.

Uric acid is the end product of protein degradation in avian species. Regardless of feeding conditions, considerably lower uric acid levels were measured in the plasma of LP chickens compared with those of LF chickens, which confirms previous findings with ad libitum fed chickens (Rosebrough et al., 1996; Collin et al., 2003; Malheiros et al., 2003b). LP chickens consumed about 32% less protein per metabolic body weight compared with their LF counterparts, and as a reaction to this LP chickens improved their efficiency of protein retention (Swennen et al., 2004). Correlation analysis showed a negative relationship between plasma uric acid levels and efficiency of protein retention (r = –0.45; P < 0.05). It is, therefore, inferred

that reduced protein degradation/amino acid oxidation contributes to more efficient retention of dietary protein as a compensatory mechanism for a reduced protein intake. Differences in plasma uric acid levels between LP and LF chickens persisted in fasting conditions, which indicated a sparing effect on body proteins of these already growth-retarded chickens.

Thyroid Hormones

As observed previously (e.g., Harvey and Klandorf, 1983; Geris et al., 1999; Buyse et al., 2000, 2002), fasting reduced circulating T_3 levels. In this way, metabolic rate is reduced, which is a means to economize on body energy reserves. However, plasma T_3 levels of the fasted LP chickens were significantly higher compared with those of their fasted LF counterparts, which corroborates the higher fasting heat production values of LP compared with LF chickens (Swennen et al., 2004).

Refeeding induced a gradual increase in plasma T_3 levels as observed previously (Buyse et al., 2000, 2002). The postprandial T_3 increase differed between both diets, and the consumption of the LF diet elicited a greater response in T_3 compared with the LP diet. The magnitude of the postprandial increase in T_3 is positively related to the amount of feed ingested (Harvey and Klandorf, 1983). However, this argument does not explain the observed dietary effect because the amount of feed consumed during the 5-h refeeding period was similar for the LP and LF chickens (Swennen et al., 2004). Hence, the differential T_3 response must be due to unequal ratios of macronutrients (fat or protein) ingested. Because the LF chickens consumed a greater amount of protein compared with LP chickens, it is possible that this macronutrient is responsible for their more pronounced postprandial T_3 increase. Indeed, our previous study (Malheiros et al., 2003b) as well as those of others (Carew and Alster, 1997; Kita et al., 1998, 2002) indicated that the protein fraction of the diet has a greater impact on endocrine mechanisms than the fat content of the diet. However, an effect of the fat component on thyroid hormone metabolism including peripheral deiodination cannot be excluded. The postprandial T_3 response diminished when chickens were reared for longer on the experimental diets, which was especially the case for LF chickens. This habituation to new diets in terms of endocrine functioning and intermediary metabolism has also been observed by Collado and Tasaki (1981) and illustrates the drive of organisms toward homeostasis, even at another level (homeorhesis).

After 48 h of refeeding, plasma T_3 levels of LP chickens were, on average, higher compared with those of their LF counterparts. This finding is in agreement with several other studies (e.g., Rosebrough et al., 1987, 1996, 1999; Buyse et al., 1992; Carew and Alster, 1997) and confirms the higher heat production of fed LP chickens (Swennen et al., 2004).

Those studies also revealed that chickens fed on a LP diet are characterized by significantly lower plasma T_4 levels compared with chickens reared on a normal protein diet. Our findings with respect to the effect of diet composition on circulating T_4 levels are not so clear-cut, but this might be related to the duration of the experimental period. As an example, fasted LP chickens had, on average, the lowest T_4 levels during the first part of the experiment, whereas this was reversed during the last part (duration by diet interaction for fasting T_4 levels: $P = 0.0119$). Feed intake is associated with a decrease in plasma T_4 levels (with a concomitant increase in plasma T_3 levels), which was mainly due an increase in extrathyroidal deiodinase type-I enzyme activity and a decrease in deiodinase type-III enzyme activity (Van der Geyten et al., 1999; Buyse et al., 2000, 2002). The postprandial decrease in T_4 levels was more pronounced for the LP chickens and suggests a macronutrient-specific effect on deiodinase activity. A more profound discussion on the impact of dietary composition on thyroid hormone metabolism including hepatic and renal deiodinase activities is provided by Collin et al. (2003).

Response Dynamics

Feed deprivation affected most parameters compared with the normal status obtained after 48 h of refeeding. However, the upward or downward changes from fasting to ad libitum levels do not occur linearly but rather as a second or higher-order response. Indeed, plasma parameter levels observed after 5 h of refeeding are generally not intermediate between the fasting and ad libitum levels but are above the ad libitum level or below the fasting level. Such a higher-order response is not an uncommon biological phenomenon.

It is also noteworthy that differences between diets in circulating levels of some parameters, more in particular free fatty acids, uric acid, and T_3, were still present under fasting conditions. This carryover phenomenon illustrates the substantial effects of diet composition on bodily functions, even in the absence of the responsible factor, in casu macronutrients.

In conclusion, diet composition in terms of macronutrient ratios has profound effects on energy, protein, and lipid metabolism of broiler chickens. The diet-induced changes in body composition, heat production, and energy partitioning between protein and fat are also reflected in circulating levels of intermediary metabolites and hormones. Some of these easy-to-measure humoral factors can serve as bioindicators for more complex and more difficult-to-measure processes, such as heat production and amino acid degradation. Furthermore, nutritional studies should take into account the possibilities of organisms to habituate themselves to a changed diet composition as well as that alterations in feeding status follow a higher-order response.

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