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## Supplemental triiodothyronine, feeding regimens, and metabolic responses by the broiler chicken

R.W. Rosebrough\*, J.P. McMurtry

*Growth Biology Laboratory, Livestock and Poultry Science Institute, United States Department of Agriculture-Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA*

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### Abstract

There are conflicting results concerning the role of the thyroid hormones in lipid metabolism. The experiments in this report were designed to examine the role of  $T_3$  in modifying responses obtained by shifting birds from moderate to low protein diets. Birds were grown from 7 to 28 d on a diet containing 18% protein. At this time, birds were switched to a diet containing 12% protein  $\pm T_3$ . The switch was accomplished either immediately or after a 24 hr fast. Measurements taken included in vitro lipogenesis (IVL), hepatic enzyme activities and plasma metabolites and thyroid hormones. Simply switching to birds to the low protein diet increased IVL, but rates were similar for three days following the switch. Feeding  $T_3$  in this same regimen resulted in lower, but again, constant rates of IVL. In contrast, although switching protein levels after a 24 hr fast increased IVL, the rate after two days of refeeding was nearly double that following one day. This accentuated response was somewhat attenuated by including  $T_3$  in the diet. Neither fasting nor refeeding altered plasma  $T_3$  relative to ad libitum values. Supplemental dietary  $T_3$  increased plasma  $T_3$  and results were not affected by feeding regimens. Plasma  $T_4$  was greatest in birds fasted for 24 hr and least in birds fed  $T_3$ , suggesting that feeding regimens may regulate the conversion of  $T_4$  to  $T_3$ . It is suggested from this study that some of the effects of alterations in dietary feeding regimens can be modulated by  $T_3$ . © 2000 Elsevier Science Inc. All rights reserved.

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\* Corresponding author. Fax: +1-301-504-8623.

E-mail address: Rosebro@LPSI.BARC.USDA.GOV (R.W. Rosebrough).

## 1. Introduction

There are findings characterizing physiological responses to meal feeding and intermittent feeding in rodents. Both of these feeding regimens increase *de novo* lipogenesis, carcass and liver fat, and improve dietary energy utilization. An early report [1] showed that periodic feeding regimens increased *in vitro* lipid metabolism in chickens but did not improve energetic efficiency.

Fasting-refeeding is one of two methods for altering lipogenesis in birds. The other method involves switching from high to low protein diets [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 [6]. This observation indicates, initially, that metabolism might be similar in both regimens. A sequential examination of metabolic pathways following these changes revealed differences, however. For example, refeeding fasted birds resulted in a high rate of lipogenesis lasting for only three days following refeeding [6]. In contrast, switching birds from a high to a low protein diet resulted in a permanent increase in lipogenesis [1,4].

Although the thyroid gland partially controls avian growth, artificial changes in thyroid hormone levels do not always change lipid metabolism predictably. For example, Cogburn et al. [7] reported that dietary TRH increased plasma growth hormone (GH), thyroid hormone levels and body weight. On the other hand, long-term, dietary administration of thyroid hormones in another study decreased both growth and fat deposition,  $T_3$  being more effective than  $T_4$  [8]. Triiodothyronine ( $T_3$ ) was shown to increase lipogenic enzyme activities based on initial work [9,10], and fatty acid synthesis on later work [11]. Later studies indicated that  $T_3$  increased malic enzyme transcription, nuclear processing of transcript products or mRNA turnover [12]. More recent work demonstrated that  $T_3$  formed a nuclear hormone-receptor complex that enhanced transcription [13,14,15]. Therefore, it should follow that a relative increase in mRNA abundance results in an increase in enzyme protein synthesis and an increase in activity [16]. It is of interest to note that  $T_3$  inhibited lipogenesis in chickens [17].

The present study was conducted to further describe the roles of feeding regimens and thyroid hormone in the regulation of broiler lipid metabolism. The purpose of the experiment in this report was to determine if triiodothyronine would alter previously noted responses in lipogenic rates accompanying a change to a low protein diet. Furthermore, it was interest to note if feeding regimens would also affect the adaptation process.

## 2. Materials and methods

### 2.1. *Animals and diets*

The chickens were housed in an environmentally controlled room maintained at 20–23°C with a 12-hr light-dark cycle (0600–1800 hr light). All chickens were held under a quarantine that was certified by the station veterinarian. Chickens were observed daily for healthiness. One authorized animal caretaker was assigned to maintain chickens over the course of

the experiments. In addition, the research protocols were approved by the Beltsville Agricultural Research Animal Care Committee.

This experiment was replicated twice for a total sampling of 192 birds. At 7 d of age, male, Shaver broiler chickens were assigned to pens in a heated battery-brooder (4 birds per pen) and fed a diet containing 21% crude protein from 7 to 28 d. At 28 d, feed was withdrawn from one-half of the birds while the remaining birds were given a diet containing 12% crude protein + 0 or 1 mg T<sub>3</sub>/kg diet. At 29 d, the fasted birds were also subjected to one of these dietary treatments. One chicken was randomly selected from each pen replicate at 28, 29, 30, and 31 d of age (a total of four replicates for each treatment at every time increment). Birds were bled by cardiac puncture into combination syringe-collection tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Sarstedt Corp., Princeton NJ, USA). The chickens were then weighed and killed by decapitation-exsanguination. The livers were rapidly excised and placed in cold phosphate-buffered saline (PBS) (pH 7.0). Chickens were selected at 0900 to minimize diurnal variation. The blood samples were centrifuged at 600 × g and plasma samples were collected with individual Pasteur pipettes. Plasma samples were stored at –70°C for later analyses of hormones and metabolites.

## 2.2. *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 hr in Hanks' balanced salts [18,19] containing 10 mM-HEPES and 10 mM-sodium [2-<sup>14</sup>C]acetate (166 MBq/mol). All incubations were conducted in 3-ml volumes at 37°C for 2 hr under a 95% O<sub>2</sub>-5% CO<sub>2</sub> atmosphere. At the end of the stated incubation periods, the explants were placed in 10 mL of 2:1 chloroform: methanol for 18 hr according to Folch [20]. The extracts were evaporated to dryness and dispersed in scintillation fluid. Radioactivity in the extracts was measured by liquid scintillation spectroscopy. *In vitro* lipogenesis was expressed as μmoles of acetate incorporated into hepatic lipids per kg of body weight.

## 2.3. *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 × g for 30 min [6]. The supernatant fractions were kept at –80°C until analyzed for the activities of malate: NADP<sup>+</sup> oxidoreductase-[decarboxylating] (ME, EC 1.1.1.40), isocitrate: NADP<sup>+</sup> oxidoreductase-[decarboxylating] (ICD, EC 1.1.1.42) and aspartate aminotransferase (AAT, EC 2.6.1.1). The activity of ME was monitored because of the enzyme's role in providing reducing equivalents (NADPH) for the synthesis of fatty acids whereas ICD may function as both a residual source for the provision of NADPH and as a source of a coreactant for transamination.

Malic enzyme activity (ME) was determined by a modification of the method of Hsu and Lardy [21]. Reactions contained 50 mM-HEPES (pH 7.5), 1 mM-NADP, 10 mM-MgCl<sub>2</sub> and the substrate, 2.2 mM-L-malate (disodium salt) in a total volume of 1 mL. Portions (50 μl) of the 12,000 × 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 was determined by a modification of the method of Cleland et al. [22]. Reactions contained 50 mM-HEPES (pH 7.5), 1 mM-NADP, 10 mM-MgCl<sub>2</sub> and the substrate, 4.4 mM-DL-isocitrate in a total volume of 1 mL. Portions (50  $\mu$ 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 was determined by a modification of the method of Martin and Herbein [23]. Reactions contained 50 mM-HEPES, 200 mM-L-aspartate, 0.2 mM-NADH, 1000 units per liter malate: NAD<sup>+</sup> oxidoreductase (EC 1.1.1.37) and the substrate, 15 mM-2-oxoglutarate in a total volume of 1 mL. Portions (25  $\mu$ 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.

All enzyme activities are expressed as  $\mu$ moles of product formed/min under the assay conditions [6] and reported as  $\mu$ moles per kg of body weight.

#### 2.4. Plasma hormone and metabolite assays

Both T<sub>3</sub> and T<sub>4</sub> concentrations were estimated with solid-phase single antibody procedures that are commercially available (ImmuChem™ Triiodothyronine and ImmuChem™ Thyroxine, ICN Biomedicals, Irvine, CA). These assays were validated for avian samples [24] by dispersing standards in charcoal-stripped chicken serums and by noting recovery of added T<sub>3</sub> and T<sub>4</sub> (98%). Plasma IGF-I was estimated with a radio immunoassay as previously described [25]. Plasma triglycerides, uric acid and nonesterified free fatty acids were determined with commercially available kits (Sigma Chemical Bulletin No's. 334-UV and 292-UV, Sigma Chemical Co., St. Louis, MO; NEFA-C, Wako Pure Chemical Industries LTD, Osaka, Japan). Each hormone or metabolite was measured in a single assay to remove inter-assay variation.

#### 2.5. Statistical procedures

Data were then subjected to analyses of variance to test significance of the main treatment effects (feeding regimens, feeding times and T<sub>3</sub> supplementation). Procedures used are outlined in Remington and Shork [26].

### 3. Results

Fig. 1 summarizes the effects of triiodothyronine, feeding regimens and adaptation times on the responses of chickens to a low protein diet. It should be further noted that the following comparisons might be affected by the significant feeding regimen  $\times$  T<sub>3</sub> interaction noted for IVL, ME and ICD. As such, the only feasible comparisons were feeding regimens

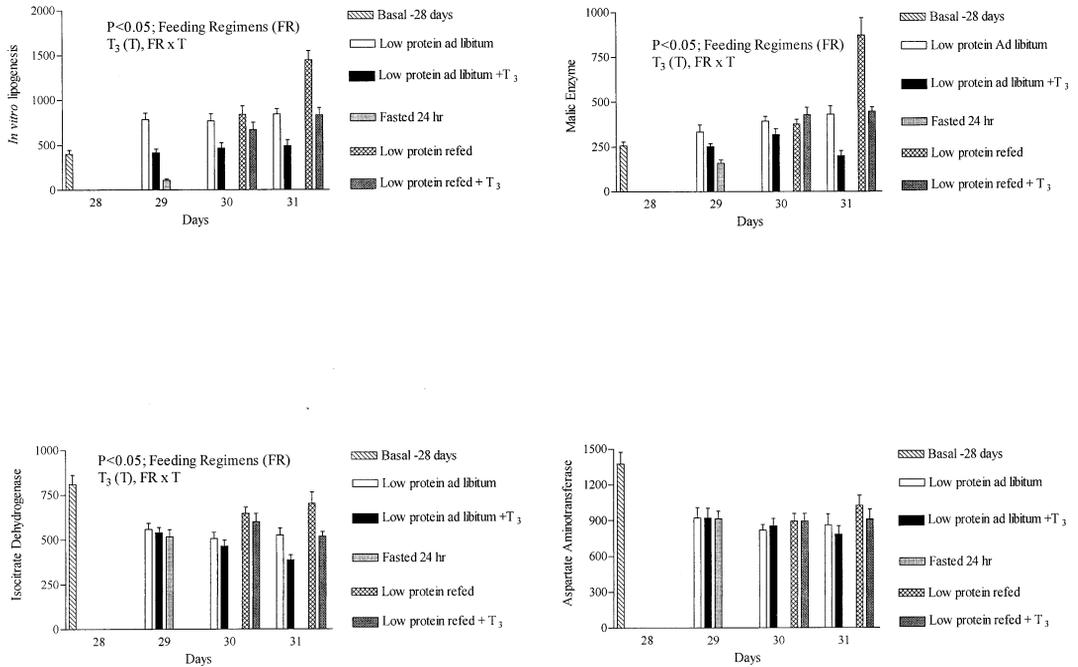


Fig. 1. Feeding regimens, time and triiodothyronine (T<sub>3</sub>) effects on in vitro metabolism in broilers. Broiler chickens were fed a standard starter diet from 7 to 28 d of age. At this time, a 24 hr fast was initiated for one-half of the birds. The other one-half was immediately switched to a low protein diet ± T<sub>3</sub>. The fasted birds were given this same dietary regimen at the end of the fast. Representative birds from each treatment were sampled on days one, two and three. Values are expressed per kg of body weight.

within each thyroid hormone treatment group and thyroid hormone treatments within each feeding regimen. Fasting decreased IVL ( $P < 0.01$ ) compared to the basal condition. In contrast, switching to a lower protein (ad libitum) diet increased ( $P < 0.01$ ) lipogenesis although values for this group were the same on days one, two and three of the experiment. In contrast, adding T<sub>3</sub> to the diet of the birds fed ad libitum attenuated ( $P < 0.01$ ) the effect of the switch to the lower protein but, again, values were the same for all three days of the experiment. Refeeding the low protein diet with out T<sub>3</sub> for 24 hr also increased ( $P < 0.05$ ) lipogenesis over all three days of the experimental period. This same effect was noted ( $P < 0.01$ ) in birds given T<sub>3</sub>, but the effect was not as notable. Changes in ME activity generally paralleled those noted for lipogenesis. Isocitrate dehydrogenase activity was only slightly affected by fasting-refeeding. Activity decreased ( $P < 0.05$ ) following a 24 hr fast but rebounded to the basal activity after 48 hr of refeeding. Neither feeding regimens nor supplemental T<sub>3</sub> had any effect on aspartate aminotransferase activity

Fig. 2 summarizes the effects of T<sub>3</sub> certain plasma hormone concentrations during adaptation to a low protein diet. Again, care must be used in interpreting this data because of significant feeding regimen x T<sub>3</sub> interactions as noted in the graphs. An immediate switch to a low protein decreased ( $P < 0.01$ ) plasma IGF-I as did a 24 hr fast. Refeeding the fasted birds with a low-protein diet further decreased ( $P < 0.01$ ) IGF-I. In contrast, adding T<sub>3</sub> to the low-protein diet attenuated ( $P < 0.01$ ) the effect of the low-protein diet on IGF-I.

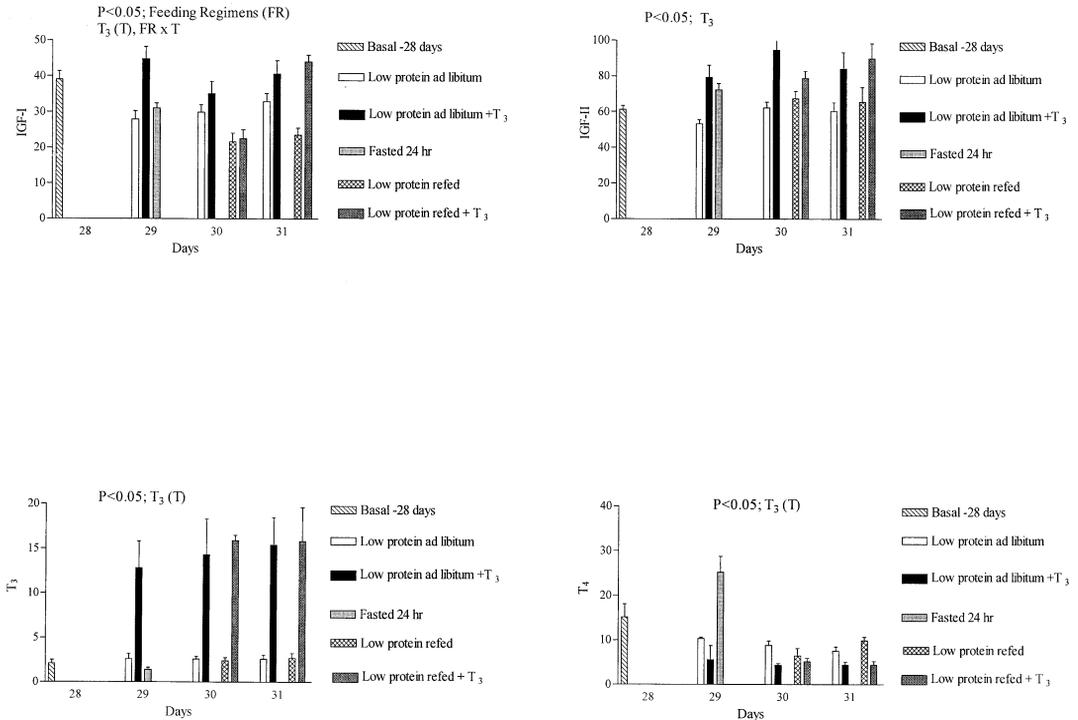


Fig. 2. Feeding regimen, time and triiodothyronine ( $T_3$ ) effects on plasma hormone concentrations. For an explanation of the experimental protocol, see Fig. 1. IGF-I = insulin-like growth factor, IGF-II = insulin-like growth factor II,  $T_3$  = triiodothyronine,  $T_4$  = thyroxine. All hormones are expressed as ng/ml.

Switching to a low-protein diet had no effect on plasma IGF-II, regardless of the feeding regimen used. In contrast, adding  $T_3$  to the low protein diet dramatically increased ( $P < 0.01$ ) IGF-II. This increase was noted for both feeding regimens. Neither fasting nor refeeding had any effect on plasma  $T_3$ . In contrast, fasting increased plasma  $T_4$ . As expected, adding  $T_3$  to the diet increased ( $P < 0.01$ ) plasma  $T_3$ , but decreased ( $P < 0.01$ ) plasma  $T_4$ .

Neither feeding regimens nor supplemental  $T_3$  affected plasma glucose (Fig. 3). Both fasting and supplemental  $T_3$  increased ( $P < 0.05$ ) plasma fatty acids (NEFA). Refeeding attenuated the effect of fasting on NEFA although the effect of dietary  $T_3$  was present on Days 2 and 3 (Days 1 and 2 of refeeding). Generally, supplemental  $T_3$  decreased ( $P < 0.05$ ) plasma uric acid. Switching to the lower protein diet increased ( $P < 0.05$ ) plasma triglycerides. It should be noted that supplemental  $T_3$  attenuated ( $P < 0.05$ ) this effect, somewhat.

#### 4. Discussion

The data in this study confirmed our long held hypotheses that changing birds to a lower protein diet would result in a rapid increase in de novo lipogenesis, and that the new rate would be sustained after the change. Indeed, lipogenic rates on Days 1, 2 and 3 were nearly

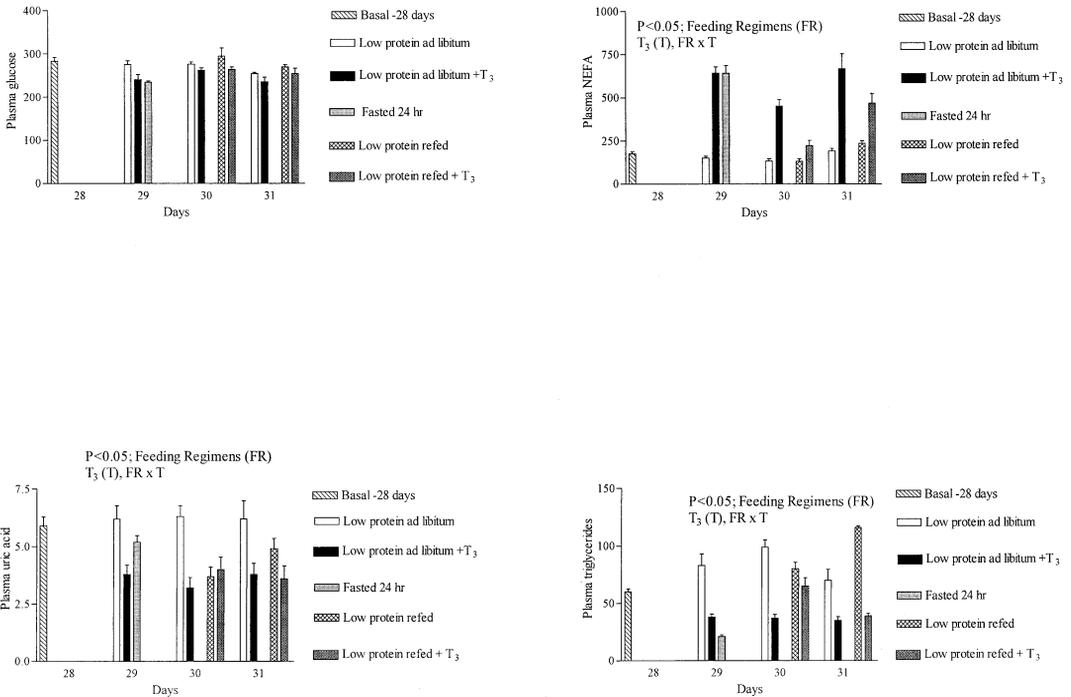


Fig. 3. Feeding regimens, time and triiodothyronine ( $T_3$ ) effects on plasma metabolite concentrations. For an explanation of the experimental protocol, see Fig. 1. Plasma glucose, uric acid and triglycerides are expressed as mg/100 ml while nonesterified fatty acids (NEFA) are expressed as  $\mu$ equivalents/liter.

identical when birds were merely changed to the lower protein diet. In contrast, those birds subjected to a bout of fasting-refeeding along with the change in dietary protein exhibited a much greater rate after 2 d of refeeding than did the birds which were just changed to a lower protein diet.

The present study demonstrated that fasting and then refeeding with a low-protein diet depressed plasma IGF-I compared to the prefasting state. Vasilatos-Younken and Scanes [27] have previously discussed the possibility that patterns of growth secretion may regulate plasma IGF-I in chickens. Kita et al. [28] reported that refeeding birds previously fasted for 24 hr restored plasma IGF-I values. The status of protein nutriture was not discussed in this study, however. We suggest that restoration of plasma IGF-I values also depends upon feeding a diet containing an optimal level of crude protein. In support of this hypothesis, we have reported that the chronic feeding of low-protein diets lowered plasma IGF-I values [17].

The changes in circulating IGF-I and IGF-II are not surprising because of their being bound to specific proteins and, thus, can be stable indicators of metabolic changes that both precede and regulate changes in circulating levels of these peptides. In a previous study concerning relative muscle size (RMS) in protein restricted chickens, we found a positive correlation between RMS and IGF-I [24]. Tomas et al. [29] found that IGF-I concentrations were positively related to protein accretion rates in several lines of chickens selected based on growth rates. Likewise, Scanes et al. [30] reported lower IGF-I concentrations in low

weight lines of birds compared to high weight lines. The findings of the present study complement those of Lauterio and Scanes [31] who described hormone development patterns in chickens severely altered by low protein diets (<100 g crude protein/kg). It should be noted that in the Lauterio and Scanes study, growth per se, protein nutriture and IGF-I values are interrelated.

In conclusion, the results of the present study, showing that fasting increases plasma IGF-II, verifies previous work from our group [32]. In contrast, refeeding did not decrease IGF-II. The present study does add to previous work by showing that switching from a higher to lower level of 'dietary crude protein has little effect on short term adaptations in plasma IGF-II.

The data from this experiment show conclusively that switching from a higher to a lower crude protein diet alters in vitro metabolism and certain plasma hormone concentrations. Furthermore, if the switch is conducted in conjunction with a fasting-refeeding regimen, effects are magnified. The latter observation points to the necessity of considering diet formulations before issuing blanket statements about the role of feeding regimens in regulating metabolic hormone profiles. This finding is especially important in assessing the effects of feeding regimens on the concentration of plasma IGF's. For example, up to this time, it has been accepted that refeeding fasted birds increases plasma IGF-I. We show that this statement may not be true if the refeeding regimen includes a low-protein diet.

## Acknowledgments

Mention of a trade name, proprietary product or vendor does not constitute a guarantee or warranty of the product by USDA or imply its approval to the exclusion of other suitable products or vendors.

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