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## The effect of the protein level in a pre-starter diet on the post-hatch performance and activation of ribosomal protein S6 kinase in muscle of neonatal broilers

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The cytoplasmic serine/threonine ribosomal protein S6 kinase (S6K1) plays a critical role in controlling protein translation. There is evidence that amino acids regulate S6K1 and protein synthesis in avian species, but the effect of dietary protein level on the activation of S6K1 in neonatal chicks is unknown. Therefore, the aim of the present experiment was to investigate the effect of different protein levels, supplied during the first 5 d post-hatch, on body growth, breast muscle development and on the activation of S6K1 and its downstream target, the S6, in neonatal chicks. Chicks were fed a pre-starter diet during the first 5 d post-hatch containing low (19.6% crude protein (CP); LP), medium (23.1% CP; MP) or high (26.7% CP) levels (HP) of protein. Weight gain of chicks fed the HP diet was higher ( $P < 0.05$ ) compared with those fed the LP diet during day (d)3–d5 and the numerical advantage of this group was maintained from d2 to d7. On d2 and d3, greater levels of S6K1 and S6 phosphorylation and/or activity were observed in chicks receiving the HP diet compared with LP and MP diets, without differences between results of the latter two dietary treatments. In conclusion, the present results suggest that early protein nutrition impacts the development of broiler chicks.

### Protein level: Broilers: Pre-starter diets: Ribosomal protein S6 kinase

The cytoplasmic serine/threonine ribosomal protein S6 kinase (S6K1) plays a critical role in controlling protein translation. Once S6K1 becomes phosphorylated on multiple serine and threonine residues, it becomes activated, and then phosphorylates several proteins involved in regulating the mRNA-binding step in translation initiation, including the S6, a component of the 40S ribosomal subunit<sup>(1)</sup>. In skeletal muscle, S6K1 is stimulated after refeeding in lower and higher vertebrates, such as fish<sup>(2)</sup>, chickens<sup>(3)</sup> and mammals<sup>(4)</sup>, partly through the post-prandial rise in plasma insulin level. The feeding-induced regulation of S6K1 also implies amino acids, whose plasma concentrations also increase after food intake, through a well-conserved mechanism involving the target of rapamycin/S6K1-signalling pathway<sup>(5)</sup>. In pigs, the activation by meal, insulin and amino acids of components related to translation initiation is developmentally regulated, with enhanced responses during the neonate period<sup>(6–10)</sup>. The decreased stimulation with age parallels the developmental decline in protein synthesis in skeletal muscle, which is due to lower efficiency of the translation process and the lower ribosome content<sup>(11)</sup>. Similar age-related changes might exist

in avian species since developmental declines in the synthesis capacity related to the number of ribosomes<sup>(12)</sup> and in the abundance of kinases involved in insulin signalling<sup>(13)</sup> have been reported in chick muscle.

Considering the dramatic effect of amino acids on proteins related to translation initiation in the neonatal period<sup>(14,15)</sup>, consumption of a high-protein (HP) diet could be of particular interest to improve protein synthesis and growth in that critical period. However, providing protein above animal requirement does not further enhance translation initiation factor activation or protein synthesis in neonate pigs<sup>(16,17)</sup>. Although an *in vitro* study indicates that amino acids (e.g. leucine and methionine) regulate S6K1 and protein synthesis in avian species<sup>(18)</sup>, the effect of dietary protein level on the activation of S6K1 in neonatal chicks is unknown. Moreover, chickens exhibit some differences concerning nutrition compared to mammals, since they consume feed continuously in little amounts<sup>(19)</sup> in contrast to meals consumed by mammals. The aim of the present experiment was to investigate the effect of three dietary protein levels, supplied during the first 5 d post-hatch, on the growth performance, breast muscle development and on

**Abbreviations:** BW, body weight; CP, crude protein; d, day; HP, high protein; LP, low protein; MP, medium protein; S6, ribosomal protein S6; S6K1, S6 kinase.

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the activation of S6K1 and its downstream target, the S6, in neonatal chicks. Chicks were fed a pre-starter diet during the first 5 d post-hatch containing protein levels (low- (LP), medium- (MP) or HP).

## Materials and methods

### Experimental design

Newly hatched broiler chicks ( $n$  360; Cobb-500) were obtained from a commercial hatchery (Belgabroed, Merksplas, Belgium), identified with a leg ring and weighed individually. The chicks were assigned randomly to twelve floor pens with wood shavings (thirty chicks/pen). The temperature was set at day old at 34°C and was decreased by 1°C every day until a final temperature of 29°C was reached. The light schedule provided 23 h of light per day. Three different experimental pre-starter diets, differing in protein content (Table 1; LP 19.6% crude protein (CP); MP 23.1% CP, recommended requirements; HP 26.7% CP), were given during the first 5 d to four pens each. The experimental diets were isoenergetic. Thereafter, a maize–wheat–soyabean-based starter diet was given (12.10 MJ metabolisable energy/kg, 220 g/kg CP) for the next 2 d. The study lasted 7 d. The present research was approved by the Ethical Commission for Experimental Use of Animals of the Katholieke Universiteit Leuven (P05100).

### Measurements

The body weight (BW) of each bird was recorded at days (d) 0, 1, 2, 3, 5 and 7. Feed consumption on a pen basis was recorded on these days. Feed conversion ratio on a pen basis was calculated.

BW gain, feed consumption and feed conversion ratio are calculated on a daily basis. At d0, d1, d2, d3, d5 and d7, five birds from each feeding group (LP, MP and HP; one to two chicks from each pen per feeding group) were euthanised by decapitation. Feed and water were provided until euthanasia. Retracted yolk sacs and breast muscle (pectoralis major) were dissected and weighed and are presented relative to BW. At d0, before starting the dietary treatments, five chicks were used to provide a baseline value. For samples taken during the first 3 d, breast muscles were quickly frozen in liquid nitrogen and stored at –80°C until further analysis of S6K1 activity and activation of S6K1 and S6 (only d3).

### Ribosomal protein S6 kinase and ribosomal protein S6 western blotting

For analysing the S6K1-signalling pathway, muscle lysates were prepared as previously described<sup>(20)</sup>. Tissue lysates (40 µg protein) were subjected to SDS-PAGE and western blotting using the appropriate antibody: anti-phospho-Thr389 S6K1; anti-phospho-Ser235/236 S6 or anti-S6 (Cell Signaling Technology, Beverly, MA, USA); anti-S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-vinculin (Sigma Chemical Company, St Louis, MO, USA). These commercial antibodies directed against mammalian proteins have been previously shown to cross react with chicken homologue

**Table 1.** Diet composition and calculated contents of the experimental diets in the pre-starter diet

	Diets		
	LP	MP	HP
<b>Ingredients (%)</b>			
Maize	60.0	60.0	60.0
Peas	5.3	5.1	5.0
Wheat	8.0	4.3	0.7
Full-fat soybeans	6.5	4.3	2
Soya protein	9.5	16	22.5
Soyabean meal	0.0	0.4	0.8
Potato protein	2.0	2.0	2.0
Vegetable oil	2.3	2.3	2.2
Monocalcium phosphate	2.8	2.3	1.7
Na <sub>2</sub> CO <sub>3</sub>	0.4	0.3	0.2
Premix*	1.0	1.0	1.0
Limestone	0.2	0.6	1.0
L-Lysine	0.6	0.5	0.4
DL-Methionine	0.3	0.2	0.0
L-Threonine	0.5	0.3	0.0
L-Tryptophan	0.1	0.2	0.3
Salt	0.2	0.2	0.3
Celite	0.2	0.1	0.0
<b>Calculated content</b>			
DM (g/kg)	893.7	896.4	899.2
Ash (g/kg)	50.9	50.6	50.4
Protein (g/kg)	196.4	231.9	267.4
Fat (g/kg)	74.1	71.3	68.5
Fibre (g/kg)	33.5	32.4	31.4
Carbohydrates (g/kg)	453.1	425.4	397.8
Metabolisable energy (MJ/kg)	12.5	12.5	12.4
Digestible lysine (g/kg)	12.2	14.6	16.0
Digestible methionine (g/kg)	7.2	7.3	7.3
Digestible methionine + cystine (g/kg)	9.4	9.9	10.4
Digestible leucine (g/kg)	14.9	17.9	20.9
Digestible threonine (g/kg)	11.2	10.0	8.8
Digestible tryptophan (g/kg)	2.8	4.0	5.2
Ca (g/kg)	9.5	9.4	9.3
Available P (g/kg)	7.0	5.7	4.4

LP, low protein; MP, medium protein; HP, high protein.

\*Premix supplied the following amount of vitamins and minerals per kilogram of diet: retinol, 3.6 mg; cholecalciferol, 0.075 mg;  $\alpha$ -tocopherol, 33.6 mg; menadione, 2.5 mg; thiamin, 2.2 mg; riboflavin, 7.5 mg; niacin, 13 mg; pyridoxine, 5.5 mg; cyanocobalamin, 0.035 mg; nicotinamide, 38 mg; folic acid, 1 mg; biotin, 0.2 mg; choline, 650 mg; Fe, 45 mg; Cu, 25 mg; Mn, 60 mg; Co, 1 mg; Zn, 70 mg; iodine, 2 mg; Se, 0.5 mg; ethoxyquin, 35 mg; butylated hydroxytoluene, 25 mg.

proteins (total proteins and phosphorylated forms) to a significant extent<sup>(20)</sup>. After washing, membranes were incubated with an Alexa Fluor secondary antibody (Molecular Probes, Interchim, Montluçon, France). Bands were visualised by IR Fluorescence by the Odyssey Imaging System (LI-COR, Inc. Biotechnology, Lincoln, NE, USA) and quantified by Odyssey IR imaging system software (Application Software, version 1.2).

### Ribosomal protein S6 kinase assay

Protein extractions and immunoprecipitation of muscle samples were performed as previously described<sup>(21)</sup>. Briefly, muscle lysates containing 500 µg protein were incubated overnight with an anti-S6K1 antibody (Santa Cruz Biotechnology). The immune complexes were precipitated on protein G sepharose (Amersham Biosciences, Uppsala, Sweden). After several washing steps, S6K1 activity was measured

by immune kinase assay according to the procedure in the S6K1 assay kit (Upstate Cell Signaling, Lake Placid, NY, USA) using [ $\gamma$ <sup>32</sup>P]ATP (Perkin Elmer Life Sciences, Boston, MA, USA).

### Statistical analysis

The data were processed using the statistical software package Statistical Analysis Systems software package version 8.2 (SAS Institute, Cary, NC, USA). The effects of age and protein level (LP, MP or HP) and interaction on relative feed intake, feed conversion ratio (on a pen basis), relative breast muscle weight and relative yolk weight were analysed by a general linear model (on individual chick basis). If the effect of protein level or interaction with age was significant, the means between the experimental groups were further compared per day by Tukey's test. For the S6K1 activity, due to the heterogeneity of variance, the non-parametric Kruskal–Wallis test was used to test differences between treatments. The expression and phosphorylation state of S6K1 and S6 were analysed by Tukey's test. Daily BW gain was analysed by the use of repeated measurements in a linear mixed-effects model containing age, protein level and interaction as variables. Leg ring numbers were used to identify the chicks during weighing. For all statistical analyses, significance was based on  $P < 0.05$ . In tables, all values are expressed as mean with pooled SEM.

## Results

### Growth performance and breast muscle development

Daily BW gain, daily feed intake and daily feed conversion ratio are presented in Table 2. During the first day, chicks

**Table 2.** Daily body weight (BW) gain (g/bird), daily feed intake (g/bird) and daily feed conversion ratio of chicks fed the low- (LP), medium- (MP) or high-protein (HP) pre-starter diet (Mean values with their pooled standard errors)

	Diet			Pooled SEM
	LP	MP	HP	
Daily BW gain (g/bird)				
d0–d1	3.66 <sup>a</sup>	1.18 <sup>b</sup>	1.55 <sup>b</sup>	0.14
d1–d2	9.32	9.60	10.30	0.42
d2–d3	12.46	13.17	14.44	0.36
d3–d5	13.48 <sup>b</sup>	14.54 <sup>a,b</sup>	16.18 <sup>a</sup>	0.59
d5–d7	18.61	19.88	20.97	0.60
Daily feed intake (g/bird)				
d0–d1	3.00	2.29	2.00	0.28
d1–d2	7.33	6.83	6.67	0.28
d2–d3	12.00	9.42	12.63	0.79
d3–d5	32.86	30.56	27.58	1.07
d5–d7	44.10	47.39	48.04	0.89
Daily feed conversion ratio				
d0–d1	0.82	1.94	1.29	0.27
d1–d2	0.59	0.67	0.61	0.03
d2–d3	1.04	0.93	1.00	0.06
d3–d5	1.27	1.09	1.09	0.05
d5–d7	1.26	1.28	1.23	0.02

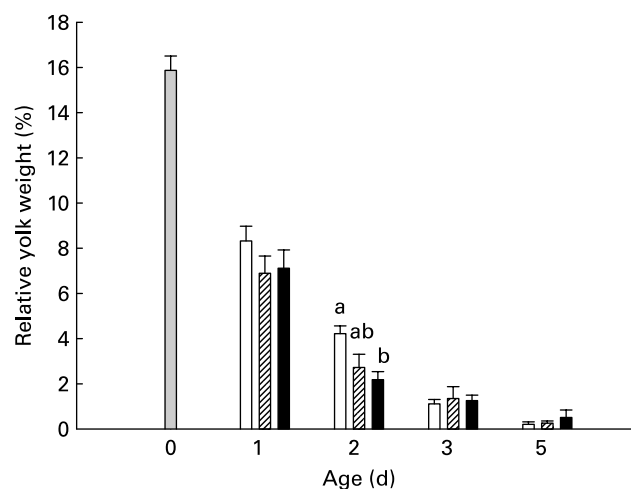
d, day.

<sup>a,b</sup> Mean values with unlike superscript letters in the same age group are significantly different ( $P < 0.05$ ).

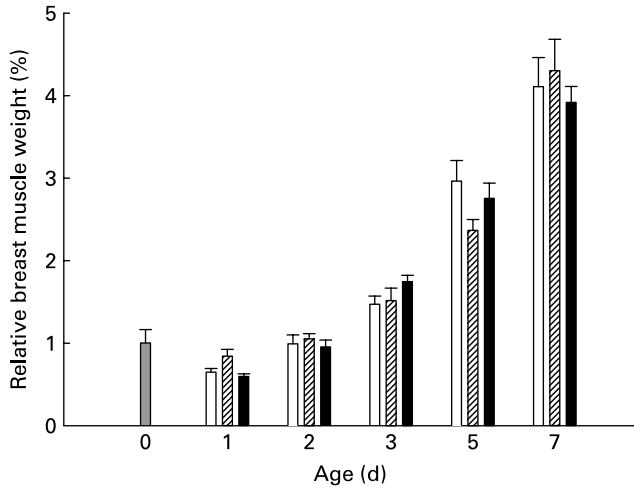
receiving the LP diet grew significantly faster than chicks receiving the MP or HP diet. From d2 to d3, chicks from the HP group tended to grow faster than chicks from the LP group ( $P = 0.09$ ). From d3 to d5, chicks from the HP group had significantly higher BW gains than chicks from the LP group. From d5 to d7, there was again a trend to a faster BW gain in the HP group compared with the LP group ( $P = 0.06$ ). Dietary protein level did not have a significant effect on daily feed intake or daily feed conversion ratio, irrespective of period. The general linear model on relative yolk weight showed a significant age effect ( $P < 0.0001$ ), whereas the effect of dietary protein level tended ( $P = 0.07$ ) to be significant with no significant diet  $\times$  age interaction (Fig. 1). However, on a daily comparison, relative yolk weight differed significantly between groups at d2 ( $P < 0.05$ ). Chicks from the HP group had a significantly lower relative yolk weight compared with chicks from the LP group ( $P < 0.05$ ), whereas the MP group did not differ from the HP ( $P > 0.05$ ) nor from the LP ( $P = 0.08$ ) group. Relative breast muscle weight increased with age ( $P < 0.0001$ ; Fig. 2). There was no effect of treatment ( $P > 0.05$ ) or interaction with age. However, from d2 to d3, the increase in breast muscle weight (absolute or relative weight) was numerically higher in the HP-fed chicks compared with the LP- and MP-fed chicks (absolute breast muscle gain from d2 to d3: HP 0.80 g; MP 0.54 g; LP 0.44 g; relative breast muscle gain from d2 to d3: HP 0.79 %; MP 0.46 %; LP 0.48 %).

### Activation of ribosomal protein S6 kinase and ribosomal protein S6 in the muscle

The activity of S6K1 in chick muscle is shown in Fig. 3 and was set at 1 at d0 (start of experiment). The S6K1 activity in the breast muscle slowly increased with age, the extent of the increase being greater in chicks from HP treatment compared with LP and MP groups. Consequently, at d2,



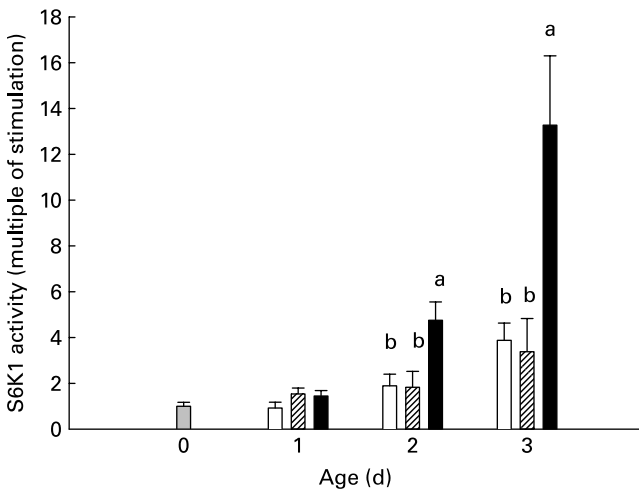
**Fig. 1.** Relative yolk weight (%) of chicks fed the low- (LP), medium- (MP) or high-protein (HP) pre-starter diet. <sup>a,b</sup> In the same age group, mean values sharing no common letter are different between groups ( $P < 0.05$ ). Values are shown as mean with their standard errors depicted by vertical bars ( $n = 5$  per day). Bar (■) at day 0 is baseline value, which was not included in the statistical analysis. □, LP; ▨, MP; ■, HP.



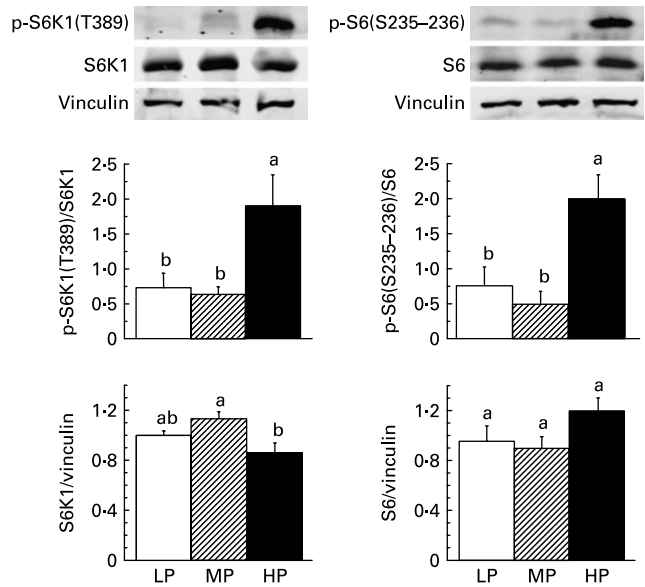
**Fig. 2.** Relative breast muscle weight (%) of chicks fed low- (LP), medium- (MP) or high-protein (HP) pre-starter diet. Values are shown as mean with their standard errors depicted by vertical bars (*n* 5 per day). Bar (■) at day 0 is baseline value, which was not included in the statistical analysis. □, LP; ▨, MP; ■, HP.

S6K1 activity in the HP group was about two times higher than the S6K1 activity in the other two groups. At d3, S6K1 activity in the HP chicks was about three times higher than that in the other groups.

Analysis of the multisite phosphorylation of S6K1 indicates that its activity is dependent on the phosphorylation of Thr389<sup>(22)</sup>. Therefore, the effects of protein level on the S6K1 phosphorylation on residue Thr389, which was normalised for total S6K1 content, were investigated (Fig. 4). It should be noted that in the MP chicks, the amount of



**Fig. 3.** Ribosomal protein S6 kinase (S6K1) activity in the breast muscle of chicks fed low- (LP), medium- (MP) or high-protein (HP) pre-starter diet. Before the pre-starter diet treatment (day 0), the S6K1 activity was measured in neonatal chicks and set at 1. Further activity measurements were expressed relative to day 0 (d0). <sup>a,b</sup>In the same age group, mean values sharing no common letter are different between groups but in the same age group (*P* < 0.05). Values are shown as mean with their standard errors depicted by vertical bars (*n* 5 per day). Bar (■) at d0 is baseline value, which was not included in the statistical analysis. □, LP; ▨, MP; ■, HP.



**Fig. 4.** Ribosomal protein S6 kinase (S6K1) and S6 activation in the breast muscle of 3-d-old chicks fed low- (LP), medium- (MP) or high-protein (HP) pre-starter diet. Representative western blots performed using antibodies raised against phospho-Thr389 S6K1, phospho-Ser235/236 S6, S6K1, S6 and vinculin as a protein-loading control. The signal obtained for S6K1 and S6 phosphorylation has been normalised by the level of the protein itself (S6K1 and S6, respectively) and that obtained for S6K1 and S6 protein content by the level of vinculin. <sup>a,b</sup>Mean values sharing no common letter are different between groups (*P* < 0.05). Values are shown as mean with their standard errors depicted by vertical bars (*n* 5).

muscle S6K1 was slightly higher than that in the HP chicks, while S6K1 content in the LP chicks did not differ from these in the two other groups. The S6K1 phosphorylation on Thr389 was about two- to threefold higher in the chicks from the HP diet compared with the Thr389 phosphorylation level determined in the chicks receiving the MP and LP diets.

To explore the consequence of the activation of S6K1, the phosphorylation of one of its downstream target, i.e. S6<sup>(20)</sup>, was examined (Fig. 4). Muscle S6 phosphorylation on Ser235/236 was higher in the chicks from the HP diet, with an about two- to threefold higher level compared with that of the chicks from the LP and MP diets. Muscle S6 content did not significantly differ between treatments.

**Discussion**

In the present study, we investigated the possible effects of the protein content in the first diet (5 d) of neonatal chicks on growth performance until 1 week of age. There were no diet-related differences in feed intake or feed conversion ratio. The HP-fed chicks had a numerically higher BW gain particularly compared with LP-fed chicks, but this enhanced growth only became significant between d3 and d5. It might be possible that the effect of the HP diet was delayed, which is in agreement with the results of Sklan & Noy<sup>(23)</sup>, who showed that HP intake enhanced BW at d7 but not earlier. Interestingly, Noy & Sklan<sup>(24)</sup> observed a decreased feed intake and improved feed efficiency by feeding increasing dietary CP (isoenergetic, equal fat content and lower carbohydrate content) to chicks during the first week

post-hatch, with relatively little effect on BW. It should be noted that in their experiment only carbohydrates decreased with increasing protein, while in the present experiment, the decrease in nutrients to maintain isoenergetic diets was divided over fat, fibre and starch. There was an increased yolk sac consumption in the HP-fed (26.7% CP) chicks at d2 compared with the LP-fed (19.6% CP) chicks. This contrasts the findings of Wiertelcki & Jamroz<sup>(25)</sup> who observed a lower growth rate and increased rate of yolk sac resorption in chicks fed a 18.5% CP diet compared with chicks fed an isoenergetic diet containing 22 or 20% CP. In addition, a higher yolk utilisation was found during the first 2 d post-hatch together with a decreased BW in chicks fed a LP diet, compared with chicks fed an isoenergetic diet low in fat or low in carbohydrate<sup>(26)</sup>. Taken together, these results give rise to two hypotheses for enhanced yolk consumption in relationship with post-hatch growth. On the one hand, it might be that the yolk can be used to compensate for a deficit in feed availability or imbalance in diet composition, reflected by a decreased BW as in the studies of Wiertelcki & Jamroz<sup>(25)</sup> and Swennen *et al.*<sup>(26)</sup>. On the other hand, rather than actually compensating for the lack of food/imbalance in diet composition, yolk consumption might be stimulated by feed availability<sup>(27)</sup> because of increased anti-peristaltic activity of the intestine<sup>(28,29)</sup> or diet composition, enhancing initial post-hatch growth<sup>(27)</sup>, as seen in the present study.

In the present study, the possible changes in S6K1 activation were investigated in the breast muscle of neonatal chicks fed diets differing in protein level. While Bigot *et al.*<sup>(21)</sup> recorded a 2.5-fold increase in S6K1 activity in chicks on the first day of feeding, no such fast increment was found in the present study. This lack of increment as soon as the chicks are fed might be due to line differences (Cobb-500 *v.* chickens stemming from fertile pedigree eggs provided by Hubbard-Isa), and/or differences in initial growth (approximately two- to threefold higher in the study of Bigot *et al.*<sup>(21)</sup>). Indeed, the responsiveness of S6K1 may be related to muscle growth rate as recently suggested<sup>(30)</sup>. Nevertheless, despite potential time-specific patterns, the present results show an increase in the activity of S6K1 during the first days' post-hatch, which is consistent with the activation of signalling components leading to translation initiation, *i.e.* S6K1<sup>(21)</sup> and protein kinase B (upstream of S6K1)<sup>(13)</sup>. It is noteworthy that this model of feeding in neonatal chicks is original since food is consumed throughout the day providing an almost continuous supply of nutrients in chickens<sup>(19)</sup>, in contrast to repeated cycles of post-absorptive and feeding states due to meals in mammals.

The chicks receiving the HP diet had a more rapidly augmented S6K1 activity compared with other groups, resulting in a higher activity compared with the MP and LP at d2 and d3. The greater increment in S6K1 activity in the breast muscle of neonatal chicks fed the HP diet indicates that S6K1 is sensitive to the level of protein in the diet. Similarly, there was an effect of dietary protein content on the phosphorylation of S6K1 at Thr389 (residue considered as critical in the activation and function of the kinase)<sup>(22,31)</sup>, which was found to be higher in the chicks receiving the HP diet compared with the two other groups. A similar pattern was observed for the phosphorylation of S6 (S6K1-downstream

target). Taken together, these findings show that dietary protein increases translation initiation factor activation in neonate chicks, in agreement<sup>(16)</sup> or disagreement<sup>(17)</sup> with contradictory data obtained in piglets. In fact, the presence or absence of a protein effect appears to depend on the diet composition of other macronutrients, such as lactose in neonatal pigs<sup>(16,17)</sup>.

In the present study, increasing protein level from 23.2% (MP, control standard diet) to 26.7% (HP, protein beyond the known requirement) increased the phosphorylation of S6K1, whereas in the studies of Frank *et al.*<sup>(16,17)</sup>, increasing protein to a level exceeding animal requirement did not further increase S6K1 phosphorylation in piglet muscle. These findings suggest some species-dependent differences, even though the activation of S6K1 in muscle of neonatal chicks is sensitive to dietary protein content as also observed in the muscle of piglets. Therefore, consumption of a HP diet clearly augmented the activity of S6K1 and activated S6K1 and S6 on d2–d3, potentially enhancing the translation of mRNA in breast muscle. Indeed, breast muscle weight gain between d2 and d3 was approximately 1.6-fold higher in the HP-fed chicks compared with the LP- and MP-fed chicks. Since a higher BW gain was observed in the chicks receiving the HP diet compared with the LP-fed chicks but not with the MP-fed chicks, it is probable that the protein level in the pre-starter diet affects also other tissues than breast muscle, particularly visceral tissues. Indeed, intestinal growth might be preferential in early chick development compared with muscle growth<sup>(27)</sup>.

In conclusion, we demonstrated greater levels of phosphorylation and activation of S6K1 and S6 in chicks that were fed high levels of dietary protein. These findings indicate that increasing protein above the recommended requirement further enhanced the activation of components related to translation initiation (*i.e.* proteins involved in the S6K1 pathway) in neonate chick muscle. Chicks fed the HP diet exhibited a slightly, numerically higher BW gain from d2 until d7, compared with the chicks fed the LP diet. Further studies are needed for better understanding the underlying mechanisms and integrating the short- and long-term consequences of early nutrition on protein metabolism, growth and tissue characteristics.

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