Effect of feed restriction timing on live performance, breast myopathy occurrence, and muscle fiber degeneration in 2 broiler chicken genetic lines

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ABSTRACT During recent years, research on meat quality in poultry has aimed to evaluate the presence and consequences of breast myopathies as well as the factors which can affect their occurrence by modifying the growth rate. A total of 900 broiler chickens were reared until slaughter (48 D) to evaluate the effect of 2 genetic lines (A vs. B) and feeding plans (ad libitum [AL], early restricted [ER], from 13 to 23 D of age, and late restricted [LR], from 27 to 37 D of age; restriction rate: 80%) on performance, meat quality, and breast muscle myopathies. Calsequestrin and vascular endothelial growth factor (VEGF) expressions, and muscle fiber degeneration (MFD) were recorded at 22, 36, and 48 D. Chickens in the AL treatment had greater final live ($P < 0.01$) and carcass weights and proportion of pectoralis major muscle ($P = 0.04$) compared to chickens in the LR treatment, whereas chickens in the ER treatment had intermediate final live (3,454 g) and carcass weights, and proportion of pectoralis major muscle (25.6%). Chickens of line A were heavier than chickens of line B ($P < 0.001$), and had a greater feed conversion rate. Chickens of line A also had a greater dressing out percentage ($P < 0.001$), but a lower proportion of pectoralis major muscle ($P = 0.04$), as well as a greater meat pH ($P < 0.001$), meat cooking losses ($P < 0.01$), and shear force of the pectoralis major muscle ($P = 0.03$). Calsequestrin and VEGF mRNA were significantly lower in ER and LR chickens compared to AL chickens after feed restriction and during refeeding ($P < 0.05$). MFD scores increased with chicken age ($P < 0.001$) and differed between genetic lines ($P < 0.001$). Neither feeding plan nor genetic line affected the occurrence of white striping or wooden breast condition.

Key words: meat quality, white striping, wooden breast, muscle fiber degeneration, calsequestrin, vascular endothelial growth factor

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INTRODUCTION

During recent years, research on meat quality in poultry has aimed to evaluate the presence and consequences of myopathies, especially white striping (**WS**) and wooden breast condition (**WBC**), in the pectoralis major muscle, as well as the factors affecting their occurrence. Both these myopathies have been classified as degenerative myopathies associated with regenerative changes of the breast muscle (Kuttapan et al., 2013a; Sihvo et al., 2014; Velleman and Clark, 2015; Radaelli et al., 2017); they affect the meat nutritional and technological properties (Mazzoni et al., 2015; Mudalal et al., 2015; Petracci et al., 2017). At commercial slaughter, WS may be detected at very high rates, including rates as high as 90% (Petracci et al., 2013b; Lorenzi et al., 2014; Kuttappan et al., 2017), whereas WBC has variable occurrence rates (Trocino et al., 2015; Bowker and Zhuang, 2016; Tijare et al., 2016; Kuttappan et al., 2017).

Several studies have shown a correlation between high growth rate and high breast yield and occurrence or degree of myopathies affecting the pectoralis major muscle and other muscles (Kuttapan et al., 2012, 2013b; Lorenzi et al., 2014; Trocino et al., 2015; Alnahhas et al., 2016). Recently, a strong genetic correlation of the WS condition in fast-growing chickens has been found ($h^2 = 0.65$; Alnahhas et al., 2016), although Bailey et al. (2015) found low genetic correlations between breast myopathies, body weight, and breast yield,

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and outlined the high contribution of environmental and/or management factors to the variance in the occurrence of WS (>65%) and WBC (>90%). In fact, rates and/or degrees of myopathies may be affected by ontogenetic factors, such as genetic line, slaughter weight, sex (Lorenzi et al., 2014; Trocino et al., 2015; Kuttappan et al., 2017; Papah et al., 2017), and/or management factors modifying the growth rate. As regards sex, Kuttappan et al. (2013a) reported no significant effect on WS occurrence, but they observed that females had a greater rate of normal breasts whereas males showed a greater rate of severely white-striped breasts. Moreover, Trocino et al. (2015) found that wooden breasts occurred more in males than in females. Among management factors modifying the growth rate, feeding strategies based on low-energy diets were first investigated (Kuttappan et al., 2012). Subsequently, quantitative (Trocino et al., 2015; Meloche et al., 2018a) or time-limited (Livingston et al., 2019) feed restrictions have been used to control growth and reduce the occurrence of myopathies. Recently, research has focused on the effect of decreasing dietary lysine (in particular) and amino acid density to control growth and reduce the rates and degrees of myopathies (Cruz et al., 2017; Bodle et al., 2018; Meloche et al., 2018b).

Some studies have also investigated the onset time of myopathies and muscle fiber degeneration (**MFD**) (Sihvo et al., 2017; Griffin et al., 2018) and interactions with feeding strategies (Radaelli et al., 2017). Early feed restriction (13 to 21 D of age) successfully reduced MFD during the period of feed restriction (Radaelli et al., 2017), but tended to increase white-striped breasts at commercial slaughter in comparison with ad libitum feeding (Trocino et al., 2015). This result was associated with the fast growth rate related to the compensatory growth during the refeeding period. Similarly, Meloche et al. (2018b) also highlighted that terms and timing for controlling curve growth by nutritional strategies (digestible lysine density) play a key role in final performance, final breast weight, and rates and degrees of myopathies. They found that the occurrence of severely affected breasts decreased when the highest reduction rate was used (i.e., 75% digestible lysine density from 18 to 26 D of age), but this had detrimental effects on breast yield at 42 D of age. On the other hand, the reduction of severely affected breasts without negative effects on breast yield was obtained by a lower reduction rate (85% digestible lysine) for a longer period (12 to 40 D) in broiler chickens slaughtered at 61 D of age (Meloche et al., 2018b).

Thus, the present study aimed to evaluate if feeding broiler chickens ad libitum or at a restricted rate during early (13 to 23 D of age) or late (27 to 37 D of age) growth may affect performance, slaughter and carcass traits, meat quality, and rates of myopathies in male chickens belonging to 2 different genetic lines. Moreover, MFD, mRNA expression of calsequestrin (**CS**), and vascular endothelial growth factor (**VEGF**) were recorded at different ages to evaluate interactions between timing of growth control by feeding strategies and muscle degeneration and regeneration.

MATERIALS AND METHODS

Experimental Facilities

The trial was performed at the poultry house of the Experimental Farm "Toniolo" of the University of Padova (Legnaro, Padova, Italy) during the period of May to June 2015, after a period (4 mo) during which the poultry house was not in use. The poultry house was equipped with a cooling system, forced ventilation, radiant heating, and controlled light systems. A total of 36 wire-net pens (125 cm wide × 260 cm long × 120 cm height) were used, and each pen was equipped with an automatic circular drinker (diameter: 39 cm) and a circular feeder (diameter: 37 cm) for manual distribution of feed. The pens had a concrete floor covered with wood shavings litter (depth 5 cm, 2.5 kg/m^2).

Twenty-four hours of light were provided during the first 2 D after the chickens arrived at the poultry house. After the first 2 D, hours of lights were progressively reduced until a 18L:6D light program was reached, which was maintained from the 12th day onwards.

Animals, Experimental Groups, and In Vivo Recordings

The study was approved by the Ethical Committee for Animal Experimentation of the University of Padova. All animals were handled according to the principles stated by the EC Directive 86/609/EEC about the protection of animals used for experimental and other scientific purposes.

A total of 900 male chickens belonging to 2 common commercial fast-growing genetic lines, line A (n = 450) and line B (n = 450), were used for the specific aims of the present study. Chickens were delivered by a commercial authorized means in compliance with Council Regulation (EC) n. 1/2005 on the protection of animals during transport to the experimental facilities of the University on the hatching day. All chicks had been vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease at the hatchery. On their arrival, 25 chicks were housed in each pen (36 pens in total), and chicks were randomly allocated to 6 experimental groups. The 6 experimental groups included 3 feeding plans (ad libitum—AL vs. early restricted—ER vs. late restricted—LR) × 2 genetic lines. Chicks were maintained in the experimental groups and pens from the day after their arrival until slaughtering at 48 D of age. Chicks were individually weighed the day after their arrival, identified by a leg mark, and weighed for live weight once a week until commercial slaughtering. Pen feed consumption was measured daily during the trial.

Diets and Feeding Plans

Three commercial diets in crumble form were administered during the trial, i.e., diet P1 (crude protein 20.1%, ether extract 5.05%, crude fiber 1.19%, ash 5.19%, lysine 1.29%) from 0 to 23 D, diet P2 (crude protein 18.9%, ether extract 4.93%, crude fiber 1.19%, ash 5.50%, lysine 1.20%) from 24 to 37 D, and diet P3 (crude protein 17.0%, ether extract 5.37%, crude fiber 1.34%, ash 5.26%, lysine 1.13%) from 38 D until slaughtering (on day 48). Diets were produced by a commercial feed mill (Martini, Budrio di Longiano, Italy). The following 3 feeding plans were adopted: chickens in 12 pens were fed ad libitum during the experimental trial, chickens in 12 other pens were restricted in the period from 13 to 23 D of age (early restricted), and the chickens in the remaining 12 pens were restricted from 27 to 37 D of age (late restricted). All feeding plans used the above described diets in 3 phases. The restricted chickens received 80% of the quantity consumed by the chickens fed ad libitum on the previous day. The restriction plan was calculated separately for the 2 genetic lines.

Sampling and Histological Analyses of Pectoralis Major Muscle

At 22 D, 36 chickens (1 chicken per pen, 1,105±147 g) were selected on treatment-wise average live weight and slaughtered to sample muscles for histological analyses. At 36 D, further 36 chickens (2,495±227 g) were selected and slaughtered with the same procedures. At 48 D of age, among chickens commercially slaughtered, 72 chickens (2 chickens per pen, 3,483±310 g) were selected on treatment-wise average live weight, and used to sample pectoralis major muscle tissues.

At slaughter, pectoralis major muscles were immediately sampled for histology. Samples were fixed in 10% buffered neutral formalin, stored at 4°C overnight, washed in phosphate-buffered saline (0.1 M, pH 7.4), dehydrated through a graded series of ethanol, and embedded in paraffin. Sections were cut at a thickness of 4 μm using a microtome (cryostat for frozen samples) and stained with hematoxylin and eosin to evaluate the general morphology of the tissues.

During microscopy examination (Olympus Vanox Photomicroscope, Japan), myopathic lesions, lipidosis, and fibrosis were assessed using a score ranging from 0 to 3 (0: normal; 1: mild; 2: moderate; 3: severe) as described by Radaelli et al. (2017).

CS and VEGF mRNA Expression Levels: RNA Preparation and Quantitative Real-Time PCR

Of the chickens slaughtered at 22, 36, and 48 D of age and used for histological analysis, a total of 36 chickens (i.e., 2 chickens × 6 experimental groups, from 3 feeding plans × 2 genetic lines × 3 slaughter ages) were used to sample pectoralis major muscles for mRNA expression of CS and VEGF.

At slaughter, pectoralis major muscles were immediately sampled and stored in RNA Later Reagent at –20°C. Subsequently, total RNA was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and trace genomic DNA contamination from total RNA was removed by DNase I treatment (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. The quantification and integrity of each RNA sample were estimated using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Two micrograms of total RNA from each sample was subjected to random hexamer primed first-strand cDNA synthesis in a volume of 20 μL using Superscript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green method with an Applied Biosystems 7500 Fast Real Time PCR System (Thermo Fisher Scientific), as described by Sacchetto et al. (2009).

All samples were run in triplicate. All values were normalized relative to the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using gene specific primers. Oligonucleotide primers used were as follows: CS 5′ AAGCAGTTCCAGATGACGGAGAT 3′ (sense), 5′ TTGGCATCCTTCTTGGAATCC 3′ (antisense); VEGF 5′ CAATTGAGACCCTGGTGGACAT 3′ (sense), 5′ GCAGCAACCCGCACATCT 3′ (antisense).

In the RT-PCR analyses, calculations were made using the Applied Biosystems software based upon threshold (**Ct**) values. Ct is the fractional cycle number at which the fluorescence passes the fixed threshold. Relative gene expression was quantified as follows: fold change = $2^{-(\mathrm{Ct})}$, where $\mathrm{Ct} = \mathrm{Ct}_{\mathrm{target}} - \mathrm{Ct}_{\mathrm{reference}}$ and $(\mathrm{Ct}) = \mathrm{Ct}_{\mathrm{sample}} - \mathrm{Ct}_{\mathrm{control}}$. All values were normalized relative to the expression of GAPDH.

Commercial Slaughtering, Carcass, and Meat Quality Recordings

At 48 D of age, all remaining chickens were slaughtered in a commercial slaughterhouse, after approximately 7 h of feed withdrawal and approximately 4 h of water withdrawal. Ready-to-cook carcasses were recovered after 2 h of refrigeration at 2°C, and individually weighed to measure slaughter dressing percentage.

A total of 216 carcasses (6 per pen), which had been previously selected on the basis of the slaughter live weight to be representative within a pen, were submitted to gross examination to evaluate the occurrence (presence or absence) and the degree (normal, moderate, severe) of WS on pectoralis major muscle and thighs (iliotibialis muscle) (Kuttappan et al., 2012, 2013b), and the occurrence (presence or absence) of WBC (Sihvo et al., 2014). Afterwards, out of the 216

carcasses, 108 (3 per pen) were further selected, as representative of the average live weight and variability of each pen, and transported to the Department (DAFNAE) laboratories to be stored at 2°C before meat quality analyses. Twenty-four hours after slaughter, carcasses were dissected for the main cuts (breast, wings, thighs, and drumsticks); pectoralis major muscles were separated from the breasts for meat quality analyses (Petracci and Baéza, 2011). The pH values of the pectoralis major muscles were measured in triplicate on their ventral side with a pH meter (Basic 20, Crison Instruments Sa, Carpi, Italy) equipped with a specific electrode (cat. 5232, Crison Instruments Sa, Carpi, Italy). The $L^*a^*b^*$ color indexes were measured in triplicate in the ventral side of the same muscles covered by a transparent plastic film, using a Minolta CM-508 C spectrophotometer (Minolta Corp., Ramsey, NJ) (Petracci and Baéza, 2011).

After measuring the pH and color indexes, one meat portion (8 cm × 4 cm × 3 cm) was separated from the cranial side of the pectoralis major muscle, parallel to the direction of the muscle fibers, and stored under a vacuum in plastic bags at –18°C until the meat analyses. Thawing and cooking losses were measured in this cut (Petracci and Baéza, 2011). After thawing, the meat portion was placed in a plastic bag and cooked in a water bath at 80°C until an internal temperature of 80°C was achieved. After 40 min of cooling, a meat portion (4 cm × 2 cm × 1 cm) was separated from the cooked cut to assess the maximum shear force with a LS5 dynamometer (Lloyd Instruments Ltd, Bognor Regis, UK) using the Allo-Kramer (10 blades) probe (load cell: 500 kg; distance between the blades: 5 mm; thickness: 2 mm; cutting speed: 250 mm/min) (Mudalal et al., 2015).

Statistical Analysis

Individual data of live weight and daily growth were analyzed by analysis of variance (ANOVA) with feeding plan, genetic line, and their interaction as main factors of variability, and with pen as a random effect, using the PROC MIXED procedure of SAS software (SAS Institute Inc., 2009). Pen data for feed intake and feed conversion were analyzed by ANOVA with feeding plan, genetic line, and their interactions as main factors of variability, and using the PROC GLM procedure (SAS Institute Inc., 2009). The frequency of myopathies at commercial slaughter was analyzed with the CATMOD PROC procedure (SAS Institute Inc., 2009) according to feeding plan, genetic line, and their interactions. Differences between the means with $P \leq 0.05$ were accepted as statistically significant.

The scores for MFD were analyzed using the PROC GLIMMIX procedure (SAS Institute Inc., 2009) with genetic line, feeding plan, age, and their interactions as fixed effects. The Bonferroni *t*-test was used for means comparison.

The expression levels of CS and VEGF transcripts were set to 1 for chickens fed ad libitum. Data were tested for normality using PROC UNIVARIATE procedures, and then submitted to statistical analysis for non-normal data using the PROC GLIMMIX procedure (SAS Institute Inc., 2009), with feeding plan as a fixed effect within slaughter age. Then the expression levels of CS and VEGF transcripts were set to 1 for chickens of genetic line A. The statistical procedure described above was used to test the genetic line as a fixed effect within slaughter age.

Differences between the means with $0.05 < P \leq 0.10$ were accepted as statistically significant.

RESULTS

Growth Performance

During the first growth period, the chickens submitted to early feed restriction exhibited lower daily weight gain (–15%; $P < 0.001$) and, thus, weighed less at 22 D of age ($P < 0.001$) than the chickens in the other 2 feeding plans, as a consequence of their lower feed intake (–16%; $P < 0.001$) (Table 1). During the second period, the early-restricted chickens exhibited greater daily weight gain than those always fed ad libitum (+5%) or submitted to late feed restriction (+9%) ($P < 0.001$), which is consistent with differences in feed intake (Table 1). On the other hand, during the same period, the late-restricted chickens had lower daily weight gain than those always fed ad libitum (–4%; $P < 0.001$). Thus, at the end of the trial, at 48 D of age, chickens always fed ad libitum had greater live weights compared to those submitted to late feed restriction (+2%; $P < 0.01$), whereas early-restricted chickens had intermediate live weights (Table 1). The feeding plan did not affect mortality rate.

The 2 genetic lines differed significantly in performance since the hatching day (Table 1). During the whole trial, chickens of line A exhibited greater daily weight gain (+6%) and feed intake (+10%), and, thus, were heavier (+6%) at 48 D of age than chickens of line B ($P < 0.001$) (Table 1). However, chickens of line A had greater feed conversion rates compared to chickens of line B (+4%) (Table 1). On the other hand, the mortality rate was significantly lower for chickens of line A than for chickens of line B ($P < 0.001$) (Table 1). A significant interaction between feeding plan and genetic line was recorded (Table 1): mortality was 9.33 and 17.6%, 12.7 and 14.4%, 6.00 and 22.2% in A and B chicks fed ad libitum, A and B chicks submitted to early restriction, and A and B chicks submitted to late restriction, respectively.

Carcass and Meat Quality

Changes in carcass weights were consistent with changes in final live weights: chickens always fed ad

Table 1. Live performance[1] (LS means) of broiler chickens until slaughtering.

Item	Feeding plan[2] (F)			Genetic line (G)		*P*-value			MSE
	Ad libitum	Early restricted	Late restricted	A	B	F	G	F × G	
Chickens (n)	238	242	238	378	340				
Live weight (g)									
On day 1	51.5	51.8	51.3	53.8^{A}	49.2^{B}	0.30	<0.001	0.99	3.95
On day 22	1,056^{B}	903^{A}	1,059^{B}	1,025^{A}	987^{B}	<0.001	<0.001	0.10	93
On day 48	3,482^{A}	3,454^{A,B}	3,399^{B}	3,548^{A}	3,342^{B}	<0.01	<0.001	0.65	278
First period (1 to 22 D)									
Weight gain (g/d)	47.8^{A}	40.5^{B}	48.0^{A}	46.3^{A}	44.7^{B}	<0.001	<0.001	0.10	4.36
Feed intake (g/d)	61.0^{A}	50.8^{B}	60.2^{A}	58.2^{a}	56.5^{b}	<0.001	0.04	0.41	2.33
Feed conversion	1.27	1.25	1.25	1.25	1.26	0.13	0.49	0.73	0.03
Second period (23 to 48 D)									
Weight gain (g/d)	93.6^{B}	98.3^{A}	90.2^{C}	97.2^{A}	90.7^{B}	<0.001	<0.001	0.34	9.10
Feed intake (g/d)	177^{B}	182^{A}	171^{C}	186^{A}	168^{B}	<0.001	<0.001	0.56	3.78
Feed conversion	1.88	1.84	1.88	1.90^{A}	1.83^{B}	0.07	<0.001	0.09	0.04
Whole trial (1 to 48 D)									
Weight gain (g/d)	73.0	72.4	71.2	74.3^{A}	70.1^{B}	0.05	<0.001	0.65	5.90
Feed intake (g/d)	122^{A}	120^{A,B}	118^{B}	126^{A}	114^{B}	<0.01	<0.001	0.30	2.91
Feed conversion	1.66	1.65	1.64	1.69^{A}	1.62^{B}	0.58	<0.001	0.11	0.04
Mortality[3] (%)	13.8	12.3	13.8	8.70^{A}	17.9^{B}	0.87	<0.001	0.03	-

MSE, root mean square error; SEM is equal to MSE/$\sqrt{n}$.
^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).
^{A,B}Means within a row lacking a common superscript differ ($P < 0.001$).
[1]Individual data: live weight and daily growth rate. Pen data: feed intake and feed conversion.
[2]Ad libitum, chickens fed ad libitum during the whole trial; early restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 13 to 23 D of age; late restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 27 to 37 D of age.
[3]Interaction feeding plan × genetic line. Mortality: 9.33 and 17.6%, 12.7 and 14.4%, 6.00 and 22.2% in A and B chicks fed ad libitum, A and B chicks submitted to early restriction, and A and B chicks submitted to late restriction, respectively.

Table 2. Slaughter results and carcass traits (LS means) in chickens slaughtered at 48 D.

Item	Feeding plan[1] (F)			Genetic line (G)		*P*-value			MSE
	Ad libitum	Early restricted	Late restricted	A	B	F	G	F × G	
Chickens (n)	238	242	238	378	340				
Cold carcasses (CC) (g)	2,699	2,669	2,624	2,758^{A}	2,570^{B}	<0.01	<0.001	0.97	230
Dressing out percentage[2] (%)	77.6	77.4	77.3	77.9^{A}	77.0^{B}	0.42	<0.001	<0.001	2.13
Dissected chickens (n)	36	36	36	54	54				
Live weight (g)	3,541	3,509	3,445	3,401^{B}	3,596^{A}	0.27	<0.001	0.24	252
Breast yield (% CC)	39.9	39.6	39.3	39.2^{b}	40.0^{a}	0.26	0.02	0.97	1.64
Pectoralis major (% CC)	26.4^{a}	25.6^{a,b}	25.4^{b}	25.6^{b}	26.2^{a}	0.04	0.04	0.95	0.78
Wings (% CC)	10.0	10.1	9.99	10.0	10.0	0.53	0.96	0.52	0.57
Thighs (%CC)	15.2	14.8	15.2	15.1	15.0	0.08	0.80	0.46	0.99
Drumsticks (% CC)	13.0	13.3	13.4	13.2	13.3	0.08	0.48	0.29	0.71
Hind legs (% CC)	28.2	28.0	28.6	28.2	28.3	0.25	0.86	0.77	1.46

MSE, root mean square error; SEM is equal to MSE/$\sqrt{n}$.
^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).
^{A,B}Means within a row lacking a common superscript differ ($P < 0.001$).
[1]Ad libitum, chickens fed ad libitum during the whole trial; early restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 13 to 23 D of age; late restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 27 to 37 D of age.
[2]Interaction feeding plan × genetic line. Dressing out percentage: 78.3 and 76.8%, 77.6 and 77.2%, 77.6 and 77.1% in A and B chicks fed ad libitum, A and B chicks submitted to early restriction, and A and B chicks submitted to late restriction, respectively.

libitum had greater carcass weights (+3%; $P < 0.01$) and greater proportions of pectoralis major muscle on the carcass (+4%; $P < 0.05$) compared to late-restricted chickens, whereas early-restricted chickens had intermediate values (Table 2). Regarding meat quality, lightness index was significantly lower in the pectoralis major muscles of chickens always fed ad libitum than in those submitted to early or late feed restriction ($P < 0.01$). Redness index was greater in the meat of early restricted chickens compared to late restricted chickens ($P < 0.01$) (Table 3).

The chickens of line A had heavier carcasses and greater dressing out percentages ($P < 0.001$), but lower breast yield and proportion of pectoralis major muscle ($P < 0.05$) in comparison with chickens of line B (Table 2). Chickens of line A also had a greater meat pH ($P < 0.001$), meat cooking losses ($P < 0.01$), and shear force ($P = 0.01$), and lower lightness index ($P = 0.01$) at the

Table 3. Meat quality traits (LS means) of pectoralis major muscle in chickens slaughtered at 48 D of age.

Item	Feeding plan (F)			Genetic line (G)		*P*-value			MSE
	Ad libitum	Early restricted	Late restricted	A	B	F	G	F × G	
Carcasses (n)	36	36	36	54	54				
pH	5.97	5.91	5.93	5.98^{A}	5.89^{B}	0.22	<0.001	0.55	0.13
L*	42.2^{B}	43.7^{B}	43.9^{A}	42.7^{B}	43.8^{A}	<0.01	0.01	0.53	2.20
a*	$0.64^{A,B}$	0.79^{A}	0.32^{B}	0.60	0.57	<0.01	0.80	0.76	0.58
b*	16.2	16.8	16.0	16.1	16.6	0.09	0.10	0.84	1.71
Thawing losses (%)	8.61	9.08	8.40	8.57	8.82	0.51	0.60	0.23	2.52
Cooking losses (%)	25.7	25.8	24.6	26.3^{A}	24.5^{B}	0.17	<0.01	0.78	3.01
Shear force (kg/g)	2.39	2.26	2.11	2.39^{a}	2.12^{b}	0.18	0.03	0.06	0.62

MSE, root mean square error; SEM is equal to MSE/$\sqrt{n}$.
[a,b]Means within a row lacking a common superscript differ ($P < 0.05$).
[A,B]Means within a row lacking a common superscript differ ($P < 0.01$).
Ad libitum, chickens fed ad libitum during the whole trial; early restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 13 to 23 D of age; late restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 27 to 37 D of age.

pectoralis major muscle, in comparison with chickens of line B (Table 3).

A significant interaction was recorded between the feeding plan and the genetic line ($P < 0.01$): chickens of line A showed the highest dressing out percentage when fed ad libitum compared to restricted feeding plans (78.3% vs. 77.6%), whereas differences among chickens of line B were less pronounced (76.8, 77.2, 77.1 in the case of ad libitum, early restriction, and late restriction feeding plan) (Table 2).

Rate of Myopathies at Commercial Slaughter

At gross examination, the rate of WS in breast meat (Figure 1a) and hind leg meat (Figure 2a) tended to be the lowest in chickens submitted to early feed restriction ($P = 0.10$ for breast meat and $P = 0.07$ for hind leg). Regarding genetic lines, the occurrence of severely white-striped breast meat was significantly greater in chickens of line A than in chickens of line B (25.9% vs. 7.41%; $P < 0.001$) (Figure 1b), whereas WS rates of hind leg meat were similar (Figure 2b). Finally, the occurrence of WBC was not significantly affected by the feeding plan nor the genetic line (Figure 3).

Histological Analyses of Pectoralis Major Muscle

At histological analyses, sections of pectoralis major muscles scored as normal (score: 0) had an organized skeletal muscle consisting of single muscle fibers covered by fibrous connective tissue, the endomysium, which insulated each fiber (Figure 4a); inside each muscle fiber, numerous longitudinally arrayed myofibrils were visible; nuclei were located peripherally, just beneath the sarcolemma, which was covered by the endomysium, and tended to move towards the fiber center only occasionally. Adipose tissue between muscle fibers was absent or very low, whereas inflammatory cells (lymphocytes and macrophages) or necrotic fibers were completely absent.

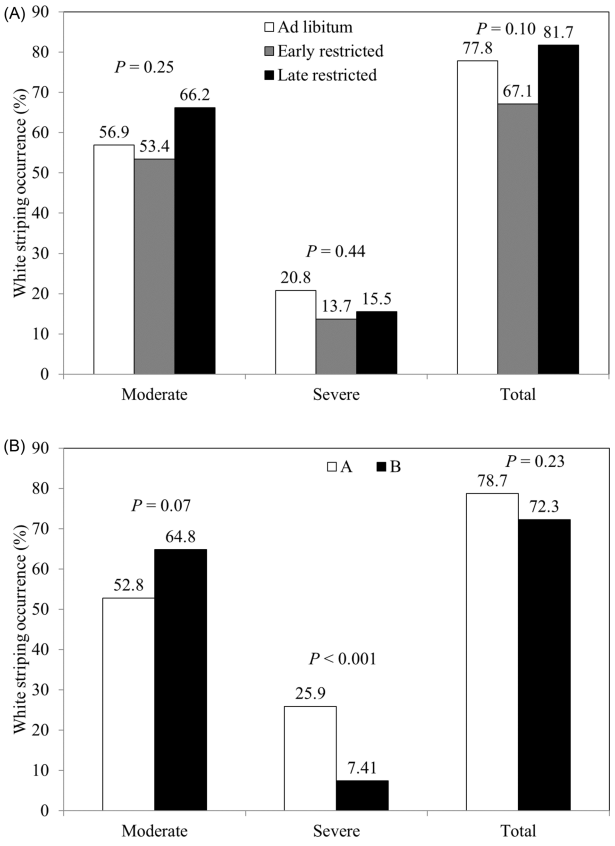

Figure 1. Occurrence (%) of chickens showing white striping (moderate, severe, total) of pectoralis major at gross observation performed at commercial slaughter (48 D of age): effect of feeding plan (ad libitum vs. early restricted vs. late restricted) (a) and genetic line (A vs. B) (b).

In sections exhibiting mild MFD (score: 1), most of the muscle parenchyma had a normal structure; however, numerous fibers appeared hypereosinophilic with loss of cross striations and in most cases internalization of nuclei (Figure 4b). Adjacent muscle fibers were separated by a greater quantity of connective tissue than the control, whereas inflammatory cells and adipose tissue were absent. In sections that were moderately degenerated (score: 2), muscle fiber structure was altered; hypereosinophilic fibers were detected which exhibited

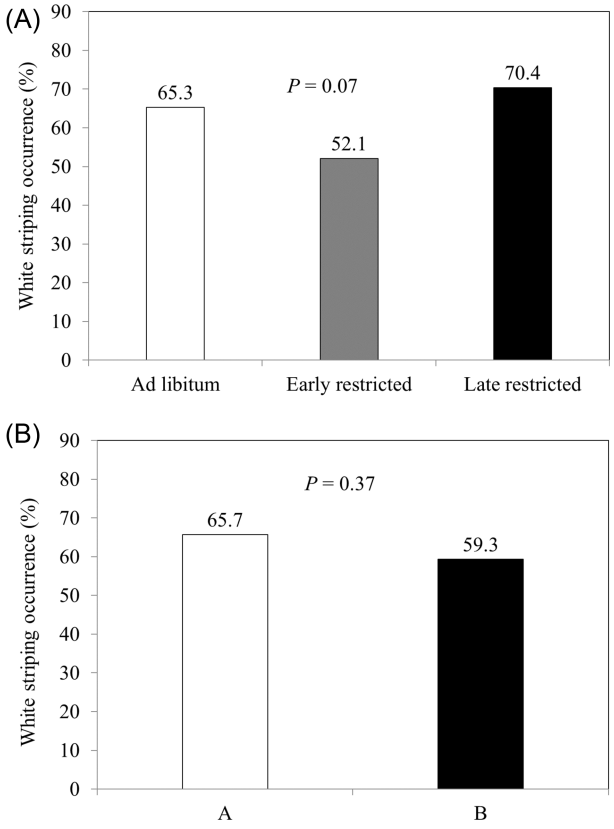

Figure 2. Occurrence (%) of chickens showing white striping of thighs at gross observation performed at commercial slaughter (48 D of age): effect of feeding plan (ad libitum vs. early restricted vs. late restricted) (a) and genetic line (A vs. B) (b).

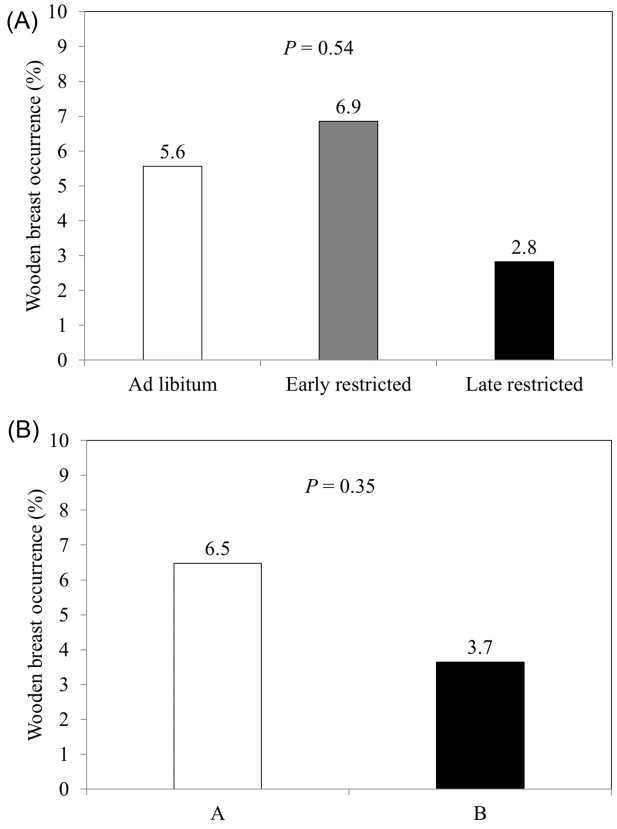

Figure 3. Occurrence (%) of chickens showing wooden breast of pectoralis major at gross observation performed at commercial slaughter (48 D of age): effect of feeding plan (ad libitum vs. early restricted vs. late restricted) (a) and genetic line (A vs. B) (b).

a vacuolar degeneration and rare fragmented fibers undergoing a phagocytic process, and appeared to be surrounded by inflammatory cells. The muscle parenchyma showed an increasing percentage of degenerating muscle fibers when compared to the previous stage (Figure 4c). Moreover, the interstitium among muscle fibers appeared infiltrated by inflammatory cells, such as lymphocytes and macrophages. Finally, in sections with severe MFD (score: 3), most of the fibers lost the typical cross striations and exhibited a massive necrotic process (Figure 4d and e). Fibers were scattered in an abundant collagen-rich connective tissue and exhibited a high variability in size (degenerating and regenerating fibers).

The MFD score significantly increased with the age of the chickens from 1.25 to 1.88 and 2.42 from 22 D to 36 and 48 D of age, respectively ($P < 0.001$) (data not reported in tables). Taking the average of the 3 ages, the MFD score was not affected by the feeding plan (1.93, 1.81, and 1.81 in ad libitum, early-restricted, and late-restricted chickens, respectively; $P > 0.10$), but differed between the 2 genetic lines (2.03 vs. 1.67 in A vs. B; $P < 0.001$) (data not reported). Nevertheless, the probability of the interactions between the main factors of variability (feeding plan or genetic line) with age approached statistical significance ($P = 0.07$ and $P = 0.06$, respectively). Regarding the interaction between the feeding plan and the age, the lowest MFD score (1.06) was recorded at 23 D of age in early-restricted chickens; the highest MFD scores were recorded in chickens always fed ad libitum at 36 D of age (2.14) and in all feeding plans at 48 D. Moreover, at 36 D of age, MFD scores were significantly lower in early- and late-restricted chickens compared to chickens fed ad libitum (1.92 and 1.58 vs. 2.14) (Bonferroni comparisons, $P < 0.05$) (Figure 5a). Regarding the interaction between the genetic line and the age, at 36 D of age, the MFD scores differed between the 2 lines (2.22 in line A chickens vs. 1.54 in line B chickens) (Bonferroni comparisons, $P < 0.05$) (Figure 5b).

CS and VEGF mRNA Expression Levels

At 22 D of age, CS mRNA expression levels were significantly lower in chickens that had been submitted to feed restriction compared to chickens fed ad libitum (Table 4). However, at 36 D of age, the reduction of mRNA in restricted animals compared to chickens fed ad libitum was not significant, whereas at 48 D of age, CS mRNA expression levels were significantly lower in chickens previously submitted to early or late-feed restriction compared to chickens always fed ad libitum ($P < 0.05$).

The VEGF gene transcriptional activity was always significantly lower in chickens immediately after feed restriction and during refeeding. Nevertheless, at 48 D,

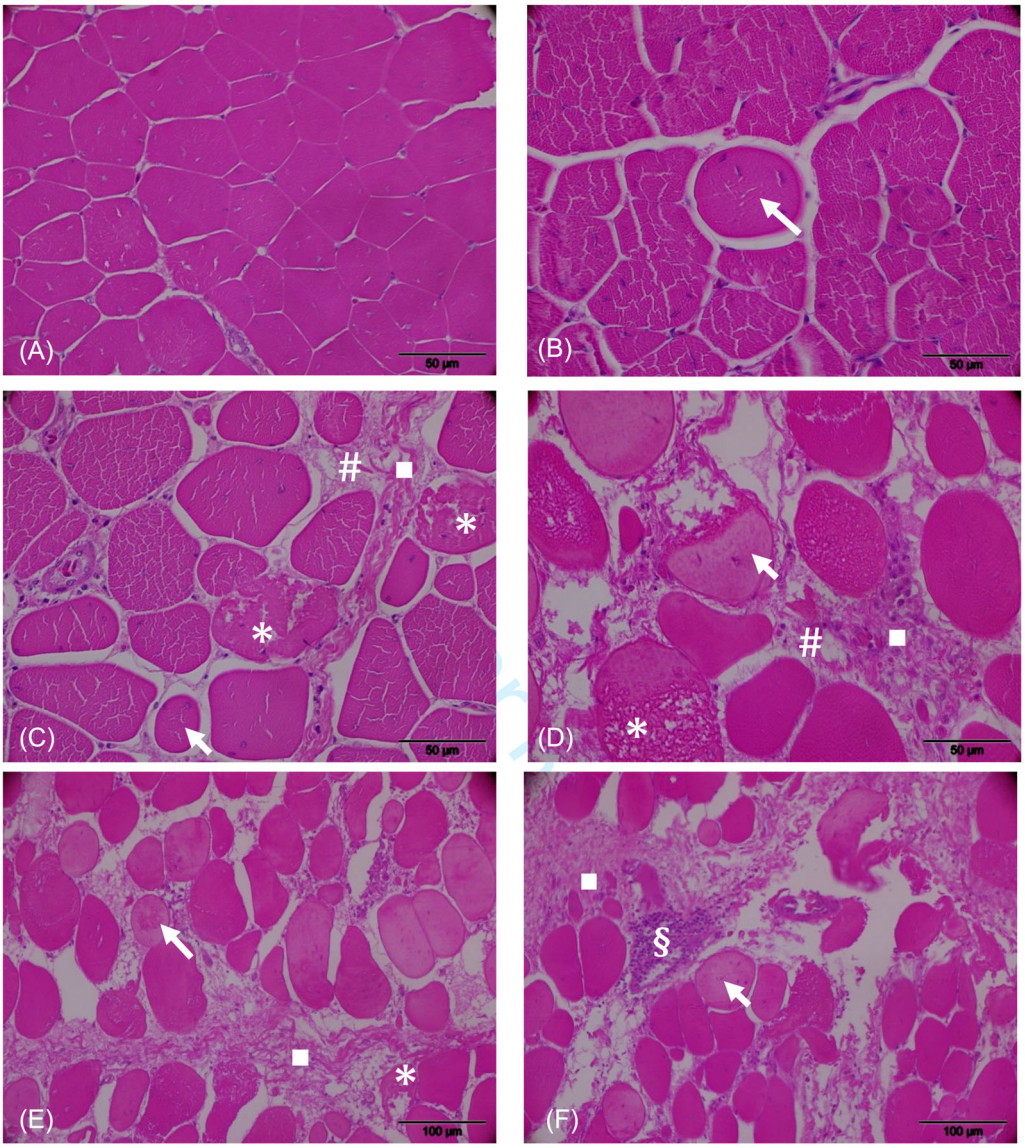

Figure 4. Sections of pectoralis major scored as normal (a), mild (b), moderately (c), and severely degenerated (d,e). All panels are stained with hematoxylin and eosin. Sections a and b were sampled at 22 D of age; sections c and e were sampled at 48 D of age (➞ iper-eosinophil fibers; ■ connective tissue,∗ necrotic fibers, # adipocytes, § lymphocytic infiltration).

the transcriptional activity of the VEGF gene was the lowest in chickens previously submitted to early restriction compared to those submitted to late restriction and those always fed ad libitum ($P < 0.001$).

The CS and the VEGF gene transcriptional activities did not differ according to the genetic line (data not reported).

DISCUSSION

In broiler chickens, feed restriction can be used to control growth curves and occurrence of some metabolic disorders and diseases associated with the high growth rates of selected genetic lines (De Jong et al., 2012; Sahraei, 2012; Butzen et al., 2013). Nevertheless, feed restriction plans should guarantee the same productive performance and carcass quality as ad libitum feeding.

Clearly, feed restriction affects growth rate as long as it is used: continuous time-limited feed restriction from 7 to 42 D (Livingston et al., 2019) or quantitative feed restriction (Meloche et al., 2018a) decreased the final body weight of chickens, whereas it improved the global feed conversion ratio. In the present study, feed restriction impaired the growth rate of early-restricted chickens during the first period (0 to 22 D; restriction from 13 to 22 D) and that of late-restricted chickens during the second period (23 to 47 D; restriction from 23 to 37 D of age). In the case of early-restricted chickens, the refeeding period was sufficiently long for the chickens to fully express their compensatory growth, so that their final body weight and overall performance (1 to 48 D) did not differ from those of chickens fed ad libitum. These results are consistent with previous studies adopting different feed restriction plans and systems (Urdaneta-Rincon and Leeson, 2002; Zhan

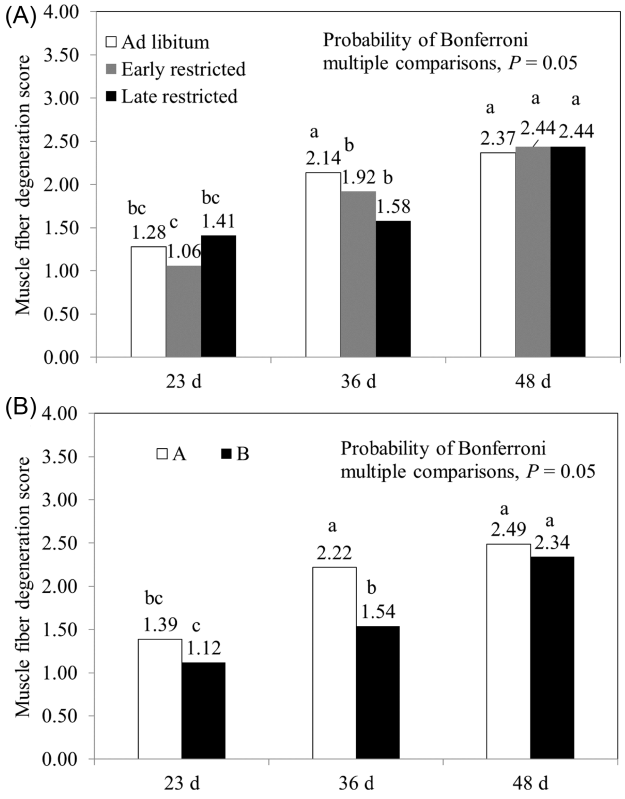

Figure 5. Muscle fiber degeneration score measured at pectoralis major at 23, 36, and 48 D of age: effect of feeding plan (ad libitum vs. early restricted vs. late restricted) (a) and genetic line (A vs. B) (b). Bars with different letters statistically differ (Bonferroni multiple comparisons $P < 0.05$).

et al., 2007; Butzen et al., 2013). However, this was not the case for late-restricted chickens in the present study, which had lower final body weight and overall performance (1 to 48 D) than chickens fed ad libitum. Indeed, in a previous trial, even early-restricted male and female chickens (13 to 21 D of age) slaughtered at 46 D of age approached, but did not completely recover, the same final live weight as chickens fed ad libitum (Trocino et al., 2015). In the present study, moreover, the overall feed conversion ratio was not affected by the feeding plan. Likely, the duration of the refeeding period was so long that it diluted the positive effect of compensatory growth in early-restricted chickens, and so short that it did not allow its full expression in late-restricted chickens (25 D in the former and 11 D in the latter chickens).

The effects of feed restriction on slaughtering yields and carcass traits are usually consistent with results of growth performance and final live weight and, accordingly, are affected by rate, duration, and timing of feed restriction and refeeding. Thus, Livingston et al. (2019) reported lower dressing percentage and breast muscle yield in continuously restricted chickens compared to ad libitum fed ones. Under our experimental conditions, the lighter late-restricted chickens had the lowest proportions of pectoralis major muscle on the carcass, despite the fact that there was no difference in dressing percentage. Gratta et al. (2017) also recorded a lower dressing percentage and breast yield in lighter early-restricted chickens compared to ad libitum fed chickens, which is consistent with previous results (Zhan et al., 2007; Butzen et al., 2013).

Regarding myopathies, the growth control by feed restriction was expected to have an effect on the reduction of rates and degrees of myopathies, as well as MFD at a histological level. In fact, myopathies have been associated with an increased muscle hypertrophy of fast-growing chickens, which brings about reduced capillary density adjacent to the myofibers (Hoving-Bolink et al., 2000; Joiner et al., 2014), thus affecting regeneration, degeneration, and necrosis of skeletal muscles (Velleman, 2015). Based on gene expression and pathways by RNA sequencing, localized hypoxia, oxidative stress, increased intracellular calcium, and the presence of muscle fiber-type switching have been proposed as responsible for WBC and WS (Mutryn et al., 2015; Soglia et al., 2016b; Marchesi et al., 2019).

Indeed, Kuttapan et al. (2012) reduced the occurrence of WS in broilers slaughtered at 54 D of age (from 75 to 53%; $P < 0.05$) by lowering the energy value of diets. Consistently, Livingston et al. (2019) reduced the rate of WS (from 90 to 41%) and WBC (95 to 86%), as well as the degree of these myopathies (2.87 to 1.64

Table 4. mRNA gene expression (LS means) of calsequestrin and vascular endothelial growth factor in pectoralis major muscle of chickens at different ages (22, 36, and 48 D).

	Feeding plan[1] (F)				
Item	Ad libitum	Early restricted	Late restricted	*P*-value	MSE
Calsequestrin (CS)					
22 D	1.05	0.69	-	0.03	0.09
36 D	1.05	0.90	0.83	0.36	0.11
48 D	1.03	0.68	0.68	0.04	0.10
Vascular endothelial growth factor (VEGF)					
22 D	1.01	0.49	-	<0.001	0.03
36 D	1.04	0.63	0.65	0.01	0.10
48 D	1.06	0.45	0.86	<0.001	0.07

MSE, root mean square error; SEM is equal to MSE/$\sqrt{n}$.
[1]Ad libitum, chickens fed ad libitum during the whole trial; early restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 13 to 23 D of age; late restricted, chickens received 80% of the quantity consumed by the chickens fed ad libitum from 27 to 37 D of age.

for WS and 2.89 to 2.14 for WBC) in feed-restricted male chickens at 42 D of age. On the other hand, Meloche et al. (2018a) successfully reduced the severity of WS and WBC at different ages (33, 43, and 50 D) according to feed restriction rate (from 95 to 85% of ad libitum). On the other hand, and differently from the results of the present study, when early feed restriction was used to prevent myopathies, the occurrence of white-striped breasts at commercial slaughter (46 D) tended to increase (Trocino et al., 2015), whereas at histological examination 97% of breasts showed MFD without significant differences between early restriction and ad libitum feeding (Radaelli et al., 2017). These results were ascribed to the fast (muscle) growth rate of early-restricted chickens related to their compensatory growth during the refeeding period, compared with chickens always fed ad libitum.

Nevertheless, myopathies and associated MFD degeneration occur early, and increase with age (14 D of age in Radaelli et al., 2017; 16 D in Griffin et al., 2018; 18 D in Sihvo et al., 2017), as observed in the present study. Radaelli et al. (2017) also demonstrated that MFD frequency and degree are under control as long as chickens are submitted to feed restriction, but the positive effects of feed restriction are soon lost after 1 wk of refeeding, and disappear completely after 2 wk.

In the present study, effects of the different feeding plans on skeletal muscle at different ages were also evaluated by studying CS and VEGF mRNA expression. Calsequestrin is a specific molecular marker of the junctional sarcoplasmic reticulum (SR) of skeletal muscle fibers, involved in calcium homeostasis. The primary function of the SR membrane system is to store and release calcium ions and, consequently, to control muscle fiber contractions. The SR is composed of 2 distinct portions: the junctional SR associated with transverse tubules and the longitudinal or non-junctional SR. Calsequestrin is the main calcium binding protein, and is located in the lumen of junctional SR.

In mammals, postnatal rearrangement of SR membranes is characterized by the proliferation of the junctional SR, which precedes the morphogenesis of non-junctional SR membranes (Franzini-Armtrong and Jorgensen, 1994). In chicken pectoralis major muscle, proliferation and rearrangement of the junctional SR occur during early postnatal development (between 18 and 28 D), and are associated with upregulation of CS and of ryanodine receptor/calcium release channels (another specific protein marker of junctional terminal cisternae involved in calcium homeostasis) (Damiani et al., 1992).

As CS mRNA and protein expression occur in parallel with post-natal morphological development of the junctional SR, in the present study, CS mRNA gene expression was used as a specific probe to study the effects of feed restriction and refeeding on the differentiation of the junctional SR in pectoralis major muscle at different ages. Our results demonstrate a reduction in CS mRNA content in chickens submitted to feed restriction compared to those fed ad libitum at 22 D of age, when proliferation and rearrangement of the junctional SR occur (Damiani et al., 1992). At 36 D, the absence of differences between feeding plans may be associated with the completion of proliferation and rearrangement of the junctional SR in existing fibers. In contrast, at 48 D, when the highest MFD was found for all chickens, a lower CS mRNA content was measured in the pectoralis major muscle of chickens previously submitted to feed restriction compared to chickens fed ad libitum. This result suggests that feed restriction might interfere not only with the capacity to sequester calcium into SR membranes, as already reported (Sporer et al., 2012), but also with the morphological development of SR in regenerating fibers.

In parallel with CS, we investigated the mRNA expression level of the VEGF gene. VEGF is a potent inducer of neovascularization in adult life, due to its activity as specific mitogen for vascular endothelial cells. The VEGF gene is among the hypoxia-inducible genes associated with vascular development (Acosta and Hernández, 2014). Moreover, VEGF exerts strong effects in stimulating proliferation and differentiation of many types of cells, including muscle fiber, following injury and damage (Arsic et al., 2004).

In this study, VEGF transcriptional activity was lower both in early- and late-restricted chickens (at 22 and 36 D of age, respectively) immediately after feed restriction, but also after refeeding at 36 and 48 D. Nevertheless, at 48 D, early-restricted chickens showed the lowest activity of VEGF gene transcriptional activity, which suggests that early feed restriction is damaging for VEGF gene expression. Consistently, Velleman et al. (2010) reported that early feed restriction affected the expression of genes involved in muscle cell proliferation and differentiation.

During feed restriction, the reduced nutrient intake decreased muscle fiber accretion, which is mainly associated with hypertrophy (Velleman, 2015). However, proliferation of the junctional SR and neovascularization were also likely depressed, which corresponded to a lower CS and VEGF expression. During refeeding, we could hypothesize that the expression of these genes remained lower in previously restricted chickens, compared to those always fed ad libitum, because their fast compensatory growth was especially associated with hypertrophy of muscle fibers, which decreased space for vascularization and, thus, induced hypoxia and MFD (Hoving-Bolink et al., 2000; Clark and Velleman, 2017).

Thus, feed restriction is effective in controlling MFD during both early (13 to 23 D) and late restriction (27 to 37 D), but also upregulates the expression of genes associated with regeneration and proliferation, as well as vascularization during both restriction and refeeding. Moreover, neither early nor late restriction had any strong positive residual effect on the rate of myopathies or MFD at commercial slaughter, whereas late restriction had negative effects on performance and carcass traits.

Consistently with our results, when the reduction of dietary lysine during different periods (Meloche et al., 2018b) or the reduction of amino acids density from 13 to 24 D (Bodle et al., 2018) was used to target breast growth to control the rate of occurrence of myopathies, the total rate of white-striped and wooden breasts was not affected. Nevertheless, lower WBC scores were obtained in male chickens at 45 D (Bodle et al., 2018).

The genetic lines tested in our study differed from each other from the first day until the end of the trial, with chickens of line A growing more than chickens of line B, even if the differences were greater during the second growth period. Comparison between broiler genetic lines within the same trial and with data from the literature is difficult because results may be affected by several factors, such as hatchery, reproductive stock age and condition, genetic line nutritional requirements, and feeding plans. Nevertheless, key information may be obtained about the prevalence of myopathies and interactions with growth rates (Kuttappan et al., 2012; Petracci et al., 2013b; Trocino et al., 2015). Some authors outlined differences between genetic lines along the growth period: during the first period high-breast yield genetic lines showed greater performance compared to standard breast-yield chickens, whereas during the second period an opposite trend was recorded (Petracci et al., 2013b; Trocino et al., 2015).

Regarding meat quality, few differences would have been expected between the 2 genetic lines included in this study, because both were high-growth selected hybrids. Nevertheless, meat from chickens of line A had greater pH, lower L^* index, and greater cooking losses and shear force than meat from chickens of line B, which could be related to the greater rate of severely white-striped breasts ($P < 0.001$; Figure 1) as well as wooden breasts ($P > 0.10$; Figure 3) in the former compared to the latter chickens. In fact, the correlation between these changes in meat quality and occurrence of myopathies has been widely demonstrated in several studies (Mudalal et al., 2015; Soglia et al., 2016a, b; Kuttappan et al., 2017).

Trocino et al. (2015) found a greater rate of severely white-striped breasts in high-breast yield than in standard-breast yield genetic lines, without differences in the total rate of abnormal meat. Petracci et al. (2013a) and Lorenzi et al. (2014) also found that standard genetic line chickens were less affected than high-breast yield genetic line chickens. Indeed, in the present study, the MFD degeneration scores had greater values in chickens of line A than in chickens of line B at 36 D of age, when differences in growth rates between genetic lines were larger, even if by slaughter age MFD scores were similar in the 2 genetic lines.

Thus, regardless of the feeding or nutritional strategies tested, or the genetic line used, to our knowledge, rates of myopathies are scarcely controlled, unless growth production is greatly impaired. In fact, MFD occurs early, and slows down when growth is reduced, but immediately restarts as soon as the growth rate increases, without any clear and strong beneficial residual effect at slaughter time on myopathy occurrence. At the present time, feeding strategies for growth control based on the reduction of nutrient intakes (different rates, duration, and timing) have been proven to exert a partial and not always consistent control only on the degree of myopathy.

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