Effect of age on the occurrence of muscle fiber degeneration associated with myopathies in broiler chickens submitted to feed restriction

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ABSTRACT To evaluate muscle fiber degeneration (MFD) associated with white striping and wooden breast, pectoralis major of 192 broilers differing for genotype (standard vs. high breast yield), gender, and feeding regime (ad libitum vs. restricted rate 80% from 13 to 21 d of age) were sampled at 14, 21, 28, 35, and 46 d of age for histological analyses by hematoxylin and eosin (H&E) staining to evaluate tissue morphology, Masson's trichrome to identify collagen presence, and Oil red and Nile blue for lipid presence. Microvessels (diameter $\leq$15 μm), nuclei positive to anti-cleaved lamin A and monoclonal proliferating cell nuclear antigen (PCNA) antisera were counted to assess apoptotic and regenerative processes, respectively. Significant differences were found according to feeding system, age, and their interactions. The frequency of chickens with MFD was higher with ad libitum than restricted feeding (75.0% vs. 62.5%; $P = 0.01$) and increased with age (18.8%, 28.1%, 75.1%, 96.9%, and 96.9% at 14, 21, 28, 35, and 46 d). However, at 14 d a similar frequency (18.8%) was found in all broilers; at 21 d, MFD occurred more in broilers fed ad libitum than in those under restriction (50.0% vs. 6.3%; $P < 0.01$); at 28 d differences were reduced (87.5% vs. 62.5%; $P = 0.10$) to disappear by 35 (100% and 93.8%) and 46 d (96.9% and 96.9%). The number of microvessels decreased with age (20.7 to 9.46; $P < 0.001$) and the number of nuclei positive to the anti-cleaved lamin A antibody increased. At histology, MFD at 46 d corresponded to loss of typical cross striations, massive necrotic process, degenerating fibers surrounded by inflammatory cells, scattered fibers in an abundant collagen-rich connective tissue, numerous adipose cells; necrotic fibers showed a high percentage of apoptotic nuclei, and regenerating fibers appeared positive to anti-PCNA antibody. In conclusion, MFD soon occurred after 2 wk of growth and increased dramatically within 28 d. Early feed restriction reduced MFD as long as animals were restricted, but no residual effect was recorded after re-alimentation.

Key words: Myopathies, gender, genotype, histology, apoptosis

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INTRODUCTION

The high productivity of poultry is the result of an intense genetic selection for fast growth rate and high breast yield, which has been continuously carried out during the last decades (Petracci et al., 2015), and is associated with the appearance of morphological abnormalities in skeletal muscles, large fiber diameters, as well as high proportion of glycolytic fibers (Dransfield and Sosnicki, 1999). Firstly, genetic selection for growth was associated with muscular problems, such as leg weakness and edema, focal myopathy, deep pectoral myopathy, and muscular dystrophy in broilers and turkeys (Siller, 1985). More recently, high growth rate and high breast yield of most common genetic lines have been associated with the occurrence of other myopathies affecting pectoralis major and other muscles, i.e., white striping and wooden breast (Kuttappan et al., 2012, 2013a; Lorenzi et al., 2014). In presence of white striping, pectoralis major exhibits a degenerative myopathy at fiber level (Kuttappan et al., 2013b), higher final pH, some minor differences in color indexes (lightness, red, and yellow), and higher cooking losses compared to not affected muscles (Mudalal et al., 2014; Mazzoni et al., 2015; Trocino et al., 2015). In case of wooden breast, pectoralis major also shows polyphasic myodegeneration with regeneration and accumulation of interstitial connective tissue or fibrosis at histology (Sihvo et al., 2014; Velleman and Clark, 2015), besides

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higher water losses during cooking and shear force compared to normal breasts (Mudalal et al., 2014; Trocino et al., 2015).

Indeed, low and similar patterns of heritability and genetic correlations for breast meat abnormalities (white striping, wooden breast and deep pectoral myopathy) have been measured in 2 genetic lines with different breast yields (Bailey et al., 2015). In addition, literature reports a wide variability at slaughter in myopathy occurrence (12% to > 70% at gross examination) which has been partially attributed to differences in bird live weight (Kuttappan et al., 2013a; Petracci et al., 2013; Lorenzi et al., 2014; Trocino et al., 2015). Accordingly, all factors affecting growth rate of broilers could play a role in modifying the occurrence of myopathies, e.g. feeding regime (Kuttappan et al., 2012; Trocino et al., 2015) or gender (Trocino et al., 2015). To our knowledge, no information is available on the onset of myopathies according to age and/or live weight.

For this reason, the present study aimed at evaluating the histological and immunohistochemical changes at different ages (14, 21, 28, 35, and 46 d) associated with the occurrence of myopathies in the pectoralis major muscle fibers of chickens belonging to 2 genetic lines (selected for standard or high breast yield), of both genders, fed ad libitum or at a restricted rate during the first growth period (from 13 to 21 d of age).

MATERIALS AND METHODS

Animals and Experimental Groups

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

A total of 192 chickens were used to sample muscles for histological and immunohistochemical analyses at different ages, out of 896 chicks used for controlling also growth performance, carcass and meat quality, and the occurrence of white striping and wooden breast at gross examination (Trocino et al., 2015).

All chicks were delivered by authorized transport means at the experimental facilities of the University of Padova on the hatching day. Half of the chicks were high breast yield genotype, the other half were standard breast yield type and both were sexed. All chicks had been vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease at the hatchery.

At their arrival, 28 chicks per pen were housed in 32 pens, randomly allocated to 8 experimental groups, i.e., 2 genotypes × 2 genders × 2 feeding plans (ad libitum vs. restricted), and controlled from the day after their arrival until commercial slaughtering at 46 d of age. During the experimental trial, half pens were fed ad libitum, the remaining half was restricted in the period 13 to 21 d of age. The restricted birds received the 80% of the quantity consumed by the chickens fed ad libitum on the previous day. The restriction program was calculated separately on the 4 groups obtained by the combination of 2 genotypes × 2 genders. Further details about the experimental facilities, the diets, the management of the chickens, and the procedures of commercial slaughter are given by Trocino et al. (2015).

At 14, 21, 28, and 35 d of age and at live weight equal to 547 ± 50 g, 1,013 ± 97 g, 1,770 ± 192 g, and 2,380 ± 258 g, respectively, 32 chickens (one chicken per pen) were slaughtered by cervical dislocation to sample muscles for histological analyses. At 46 d of age, among chickens submitted to commercial slaughter, 64 animals (2 birds per pen) (3,197 ± 342 g) were selected as representative in terms of average live weight and variability of the corresponding pens and used to sample pectoralis major.

Sampling, Histological and Immunohistochemical Analyses

At each slaughter, pectoralis major muscles were immediately sampled for histology. Samples were fixed in 10% buffered neutral formalin at 4°C overnight, washed in phosphate-buffered saline (**PBS**, 0.1 M, pH 7.4), dehydrated through a graded series of ethanol, and embedded in paraffin. Samples to be submitted to histochemistry were frozen in isopentane cooled by liquid nitrogen.

Sections were cut at a thickness of 4 μm using a microtome (cryostat for frozen samples) and stained with: 1) hematoxylin and eosin (**H&E**), 2) Masson's trichrome, 3) Oil red, and 4) Nile blue. H&E staining was employed to evaluate the general morphology of the tissues, Masson's trichrome (Bancroft and Stevens, 1975) was used to qualitatively assess the amount of collagen, whereas Oil red and Nile blue (Bancroft and Stevens, 1975) were used to identify the presence of lipids.

At 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). In details, the score (0) was attributed to samples presenting no necrotic fibers, no infiltration of connective tissue, and with normal or central nuclei; the score (1) was used when samples showed central nuclei, some fibers with hyaline cytoplasm, scarce necrotic fibers, absence of connective tissue infiltration; the score (2) was given when samples diffusely presented necrotic fibers, thickening of interstitial connective tissue, presence of inflammatory cells, and appearance of adipose tissue aggregates; finally, the highest score (3) was attributed to samples exhibiting a great amount of interstitial connective tissue and inflammatory cells, as well as of necrotic fibers and lobules of adipose tissue.

In addition, microvessels (diameter ≤ 15 μm) were counted on histological sections (Olympus Vanox

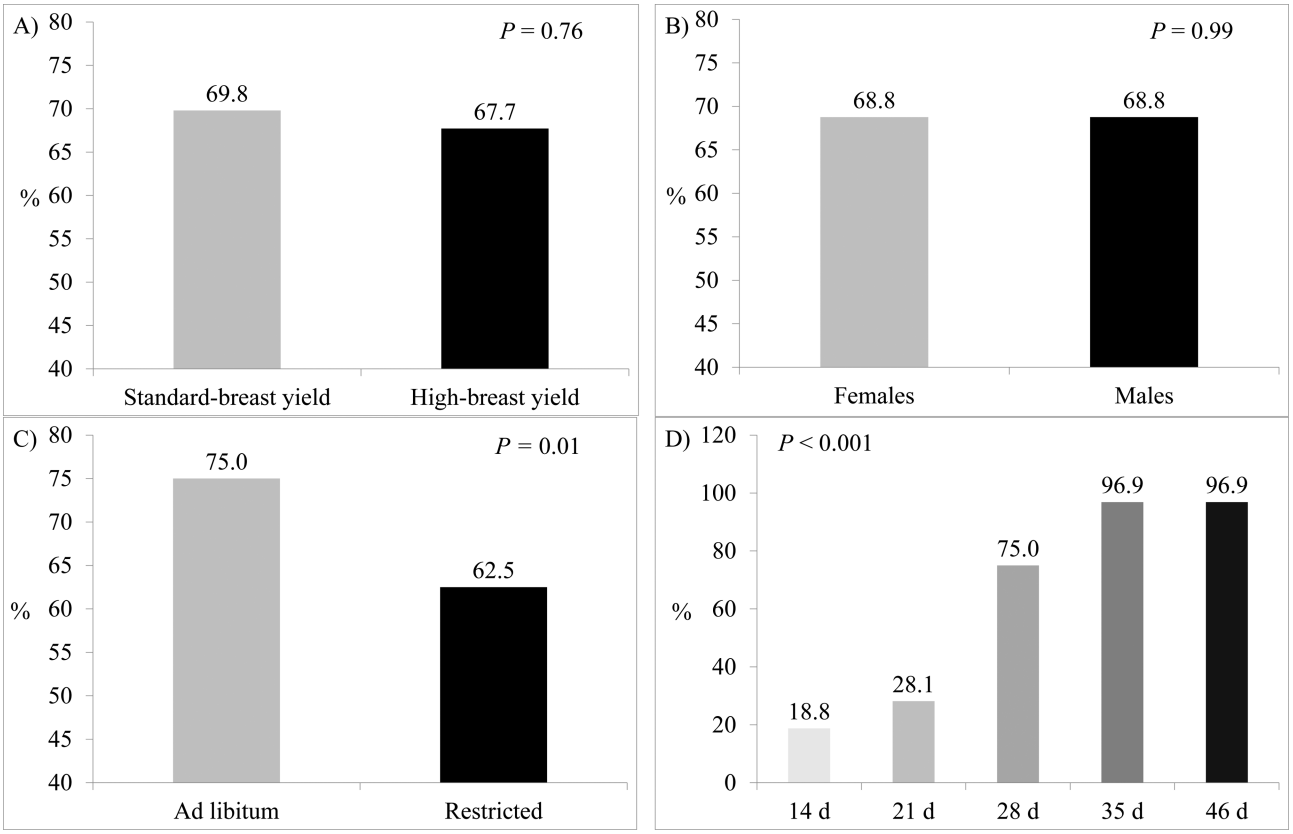

Figure 1. Percentage (%) of chickens showing muscle fiber degeneration at histological examination of pectoralis major according to (A) genotype (standard vs. high-breast yield), (B) gender (females vs. males), (C) feeding regime (ad libitum vs. restricted from 13 d to 21 d of age), and (D) age of chickens (14, 21, 28, 35, and 46 d).

photomicroscope, Japan) of pectoralis major muscles of all chickens slaughtered at 14 d and 46 d of age. Within each section, microvessels were counted independently in 5 areas (60,000 μm^2 each) by 2 trained observers.

Immunohistochemistry was carried by an automated immunostainer (BenchMark Ultra, F. Hoffmann-La Roche AG, Basel, Switzerland). Sections were deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Heat-induced antigen retrieval was performed using the BenchMark Ultra Cell Conditioning Solution CC2 (Ventana Medical Systems, Inc., Tucson, AZ) (pH = 6.0) at 91°C for 44 min. Sections were incubated for 32 min at room temperature with the primary polyclonal antibody anti-cleaved lamin A (cod. 2035, Cell Signaling Technology, Danvers, MA) diluted 1:100. Sections were then incubated with the detection system UltraView Universal DAB detection kit with HRP (horseradish peroxidase) enzyme directly conjugated to the secondary antibody (Hoffmann-La Roche AG, Basel, Switzerland). The quantitative assessment of anti-cleaved lamin A positive nuclei was made to assess the apoptotic processes by using a computerized image analyzer system (Olympus CellB, Japan) and as follows: (1) each haul was represented by 3 sections from each muscle sample; (2) 3 fields from each muscle section were analyzed; (3) within each field (60,000 μm^2), the number of positive nuclei was measured.

Three serial sections per each slaughtering were manually immunostained with a mouse monoclonal PCNA antiserum diluted 1:500 (Cell Signaling Technology, Danvers, MA) and incubated overnight at +4°C with the detection system EnVision FLEX/HRP and the EnVision FLEX Substrate Buffer EnVision FLEX DAB (Agilent Technologies, Dako Denmark A/S, Glostrup, Denmark). Then EnVision FLEX Substrate Buffer EnVision FLEX DAB was used as chromogen and all sections were counterstained with the EnVision FLEX Hematoxylin to assess the regenerative processes. The specificity of the immunostaining was verified by incubating sections with: (1) PBS instead of the specific primary antibodies; (2) preimmune sera instead of the primary antisera; (3) PBS instead of the secondary antibodies. The results of these controls were negative (i.e., staining was abolished). Moreover, skin and thymus of chickens were used as positive control tissues for both primary antibodies.

Statistical Analysis

The frequency of chickens showing any degree of muscle fiber degeneration (MFD) at pectoralis major was analyzed by PROC CATMOD (SAS Institute Inc., 2009) according to genotype, gender, feeding system, and age. Thereafter, differences according to the feeding system within age were assessed by the χ^2 test.

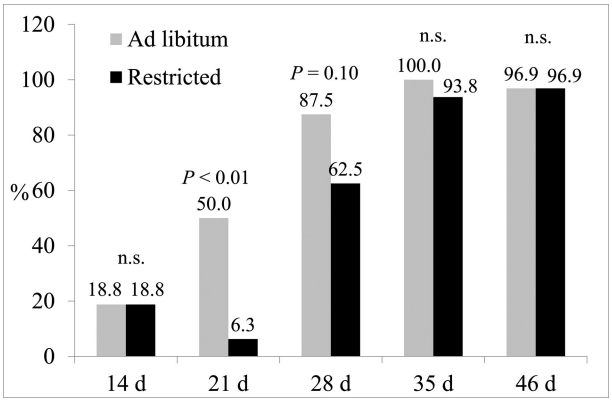

Figure 2. Percentage (%) of chickens showing muscle fiber degeneration at histological examination of pectoralis major between those fed ad libitum or submitted to feed restriction from 13 d to 21 d of age and at different ages (14, 21, 28, 35, and 46 d) (P-value of χ^2test within age).

The scores for muscle fiber degeneration, the number of apoptotic nuclei and the number of microvessels were analyzed by PROC GLIMMIX (SAS Institute Inc., 2009) with genotype, gender, feeding system, and age and their interactions as fixed effects. Differences between the means with $0.05 < P \leq 0.10$ were accepted as close to statistical significance.

RESULTS

The percentage of chickens showing any degree of MFD at histological examination was 68.8% on average (all slaughtering) and was not affected by the genotype or the gender (Figure 1A, B), whereas significant effects of the feeding system and the chicken age were measured (Figure 1C, D). The frequency of birds showing MFD was higher in birds always fed ad libitum compared to those submitted to early feed restriction (75.0% vs. 62.5%; $P = 0.01$) (Figure 1C). Moreover, as the age increased from 14 d to 46 d, the percentage of chickens showing MFD at pectoralis major significantly increased ($P < 0.001$) (Figure 1D). Indeed, when the effect of the feeding system was evaluated within age (Figure 2), at 14 d of age, a similar MFD occurrence was observed in all broilers; at 21 d (at the end of feed restriction), the MFD frequency was higher in chickens fed ad libitum compared to restricted birds (50.0% vs. 6.3%; $P < 0.01$); at 28 d, a similar trend was observed, but differences between the 2 groups were reduced (87.5% vs. 62.5%, $P = 0.10$) to disappear within 35 d and 46 d of age.

The MFD score was not significantly affected by the genotype, the gender or the feeding system, but it significantly increased with the age of the chick ($P < 0.001$) (Table 1). The MFD score also differed between chickens submitted to the 2 feeding systems depending on the age of observation at a level approaching statistical significance (probability of the interaction feeding system × age; $P = 0.10$): the score was higher in chickens fed ad libitum compared to those restricted

Table 1. Score for muscle fiber degeneration and number of apoptotic nuclei (LS means ± SE) in pectoralis major of broiler chickens differing for genotype, gender, feeding system and age.

Item	Breast yield (B)		Gender (G)		Feeding system (F)		Age (A)				P-value				
	Standard	High	Females	Males	ad libitum	Restricted	14 d	21 d	28 d	35 d	46 d	B	G	F	A	F × A
Score[a]	1.16 ± 0.08	1.19 ± 0.08	1.15 ± 0.08	1.21 ± 0.08	1.24 ± 0.08	1.12 ± 0.08	0.19 ± 0.14	0.38 ± 0.14	1.31 ± 0.14	2.09 ± 0.14	1.92 ± 0.10	0.79	0.64	0.32	<0.001	0.10
Apoptotic nuclei (n)	2.94 ± 0.49	3.39 ± 0.49	2.84 ± 0.49	3.49 ± 0.49	3.21 ± 0.49	3.12 ± 0.49	0.09 ± 0.82	0.44 ± 0.82	3.03 ± 0.82	3.81 ± 0.82	8.45 ± 0.58	0.53	0.36	0.89	<0.001	0.97

Score for muscle fiber degeneration: 0 (normal), no necrotic fibers, no infiltration of connective tissues, normal or central nuclei; 1 (mild) central nuclei, some fibers with hyaline cytoplasm, scarce necrotic fibers, absence of connective tissue infiltration; 2 (moderate), diffuse necrotic fibers, thickening of interstitial connective tissue, presence of inflammatory cells, and appearance of adipose tissue aggregates; 3 (severe), great amount of interstitial connective tissue and inflammatory cells, of necrotic fibers and lobules of adipose tissue.

at 21 d (0.69 vs. 0.06) and 28 d of age (1.50 vs. 1.13) (data not reported in tables). In contrast, the number of apoptotic nuclei increased only with the chicken age (from 0.09 at 14 d to 8.45 at 46 d of age; $P < 0.001$; Table 1).

The above-described evolution of MFD score with age corresponded to the following description at histological analyses: at 14 d of age, most sections of pectoralis major exhibited an organized skeletal muscle consisting of single muscle fibers covered by fibrous connective tissue, the endomysium, which insulated each fiber (Figure 3A); inside each muscle fiber, numerous longitudinally arrayed myofibrils were visible; nuclei were located peripherally just beneath the sarcolemma, which was covered by the endomysium. At 21 d, the histomorphology of muscle tissues showed an increasing range of myodegenerative lesions (Figure 3B-D; Figure 4A-D) from 1 (mild) to 3 (severe). Most of the muscle parenchyma exhibited a normal structure, although numerous fibers appeared hyper-eosinophilic, with loss of cross striations and internalization of nuclei (Figure 3B). Some hyper-eosinophilic fibers exhibited a vacuolar degeneration and rare fragmented fibers undergoing to a phagocytic process and appeared surrounded by inflammatory cells. At 28 d, the muscle parenchyma showed an increasing percentage of degenerating muscle fibers when compared to the previous stage (Figure 3C). Moreover, the interstitium among muscle fibers appeared infiltrated by inflammatory cells, such as lymphocytes and macrophages. At 35 d of age, muscle fibers appeared surrounded by an abundant collagen-rich connective tissue (Figure 3D). At 46 d, most of the fibers lost the typical cross striations and exhibited a massive necrotic process (Figure 4A–B) and degenerating fibers appeared surrounded by inflammatory cells (Figure 4B–C). At this stage, fibers were scattered in an abundant collagen-rich connective tissue and exhibited a high variability in size (degenerating and regenerating fibers) (Figure 4B–D). The connective tissue was rich in collagen fibers (Figure 5A–B) and numerous adipose cells (lipidosis) were detectable (Figure 5C–E).

Immunological analyses revealed the number of nuclei positive to the anti-cleaved lamin A antibody increased from 14 d to 46 d of age (Figure 6A–E). At 46 d, necrotic fibers showed a higher percentage of apoptotic nuclei (Figure 6D–E) and regenerating fibers appeared positive to the anti-PCNA antibody (Figure 6F–G). Both genotypes exhibited a similar pattern at immunohistochemistry.

The number of microvessels was not affected ($P > 0.10$) by genotype (14.5 ± 0.75 and 15.7 ± 0.72 in standard and high breast yield chickens), gender (15.9 ± 0.71 and 14.3 ± 0.76 in females and males), or feeding system (15.5 ± 0.75 and 14.7 ± 0.72 in chickens fed ad libitum and in those restricted from 13 to 21 d) (data nor reported in tables). In contrast, the number of microvessels was significantly higher in chickens at 14 d than at 46 d of age (20.7 ± 0.75 vs. 9.46 ± 0.72; $P < 0.001$).

DISCUSSION

Macroscopically, myopathy occurrence at commercial slaughtering has been found to range from 9.8% of white-striped breast fillets in broilers slaughtered at 42 d of age and 3.2 kg live weight (Ferreira et al., 2014) to 12.0% in light birds slaughtered from 45 d to 54 d of age (average live weight: 2.75 kg) (Petracci et al., 2013) to 55.8% in chickens slaughtered later (59 d to 63 d) (Kuttappan et al., 2013a) or 60.3% in heavy male birds (live weight from 3.8 to 4.2 kg) (Lorenzi et al., 2014) up to 86.7% in chickens slaughtered at 46 d of age (Trocino et al., 2015).

The correlation between white striping occurrence and growth rate/final live weight and/or breast yield in current broiler genotypes under commercial intensive conditions has been proposed but not fully demonstrated. In fact, some authors have found that heavier birds with thicker breasts are most likely to show white striping compared to lighter birds (Kuttappan et al., 2012; Petracci et al., 2013; Lorenzi et al., 2014), but others did not find a significant direct correlation between white striping and bird weight or significant effects of different genotypes (Trocino et al., 2015). High growth rates and high breast yield have also been considered responsible for wooden breasts (Sihvo et al., 2014), but the only significant difference in wooden breast occurrence has been reported between heavy males and light females slaughtered at the same age (Trocino et al., 2015). Indeed, according to Bailey et al. (2015), environmental and/or management factors may contribute to more than 65% and 90% of the variance in the occurrence of white striping and wooden breast, respectively. In fact, these authors found that heritability was low both for wooden breast (<0.10) and white striping (<0.34) and genetic correlations between breast myopathies and body weight ranged from less than 0.132 to 0.248.

When the same group of birds was controlled during growth, as in the present study, white striping appeared soon at 14 d of age (9.38% of controlled animals at gross examination) and increased at 21 d, 28 d, and 35 d (31.3%, 78.1, and 74.5%, respectively; data not reported in tables), while wooden breasts were observed only in birds at the last slaughtering at 46 d (74.5% and 12.2% of white-striped and wooden breasts, respectively; Trocino et al., 2015). Moreover, at gross examination, the 2 myopathies were often concomitantly present (Trocino et al., 2015; Soglia et al., 2016).

Whether white striping and wooden breast are different expressions of the same myopathy or not is yet to be demonstrated, as stated by Velleman (2015), but the histological lesions described are very similar. In fact, in the same animals of the present study, muscles affected by white striping or wooden breast at 46 d did not differ at an histological level and a range of microscopic lesions (i.e., internalization of nuclei, loss of cross striation, vacuolar degeneration and necrosis of fibers, lymphocytes and macrophages infiltration,

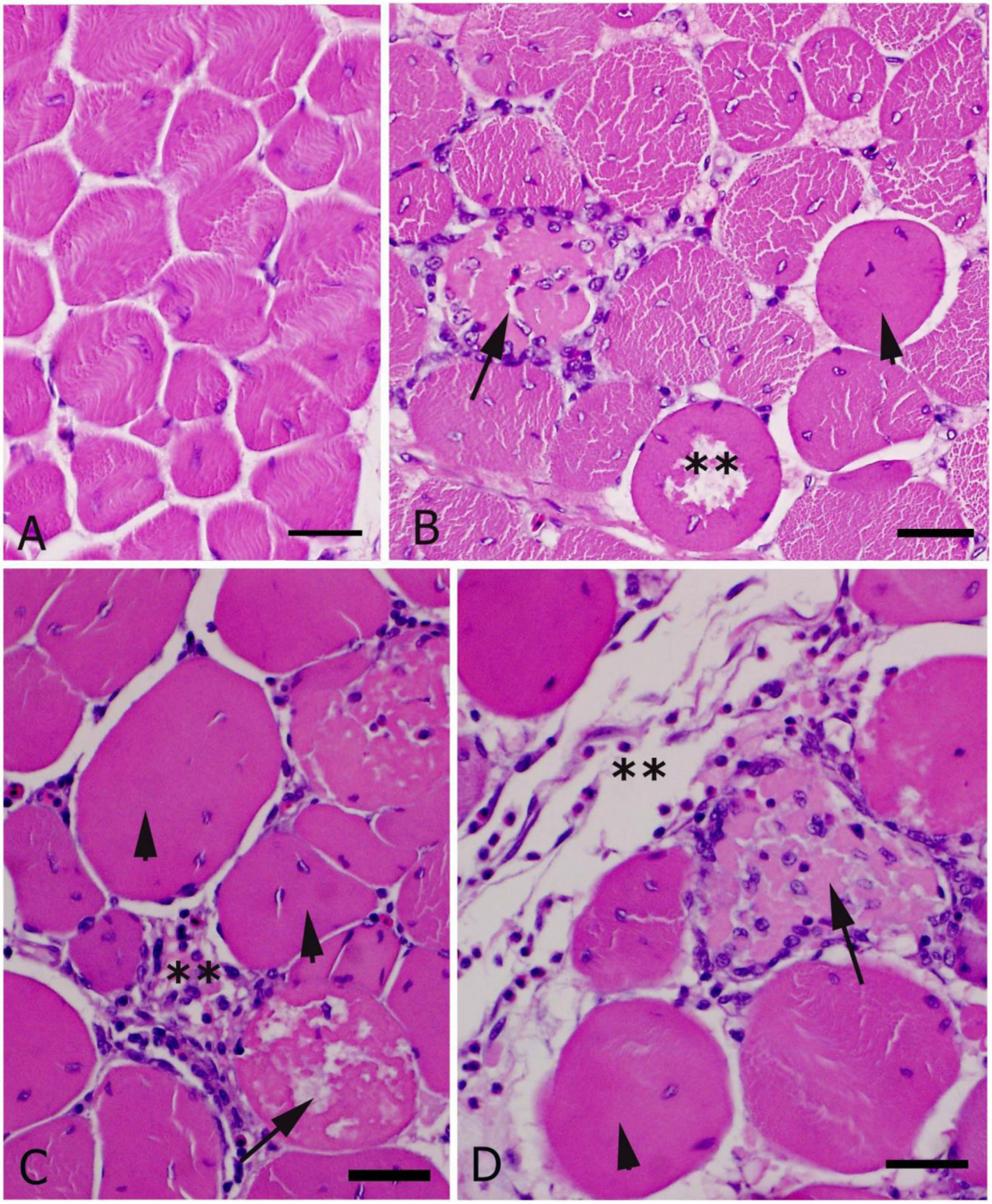

Figure 3. Histological evaluation of muscle fibers in chickens at 14 (A), 21 (B), 28 (C), and 35 (D) d of age. All panels are stained with hematoxylin and eosin. A) At 14 d, a normal histological aspect of muscle fibers is visible. In transverse section, the majority of muscle fibers exhibit nuclei arranged peripherally and consist of multi-nucleate cells with a striated pattern. Rare fibers show an internalization of nuclei. B) At 21 d, although most of the muscle parenchyma exhibits a normal structure, numerous muscle fibers appear hyper-eosinophilic (*arrowhead*), whereas others show a vacuolar degeneration (*asterisks*). Rare fibers undergoing phagocytosis appear fragmented and infiltrated by histiocytes, macrophages, and heterophils (*arrow*). C) At 28 d, hyper-eosinophilic fibers are abundant (*arrowheads*) and the interstitium is infiltrated by lymphocytes and macrophages (*asterisks*). D) At 35 d, degenerating fibers (*arrow*) are often surrounded by inflammatory cell infiltration and abundant collagen-rich connective tissue (*asterisks*). Arrowhead indicates a hyper-eosinophilic fiber. Scale bars: A, 20 μm; B, C, and D, 10 μm. Color version available in the online PDF.

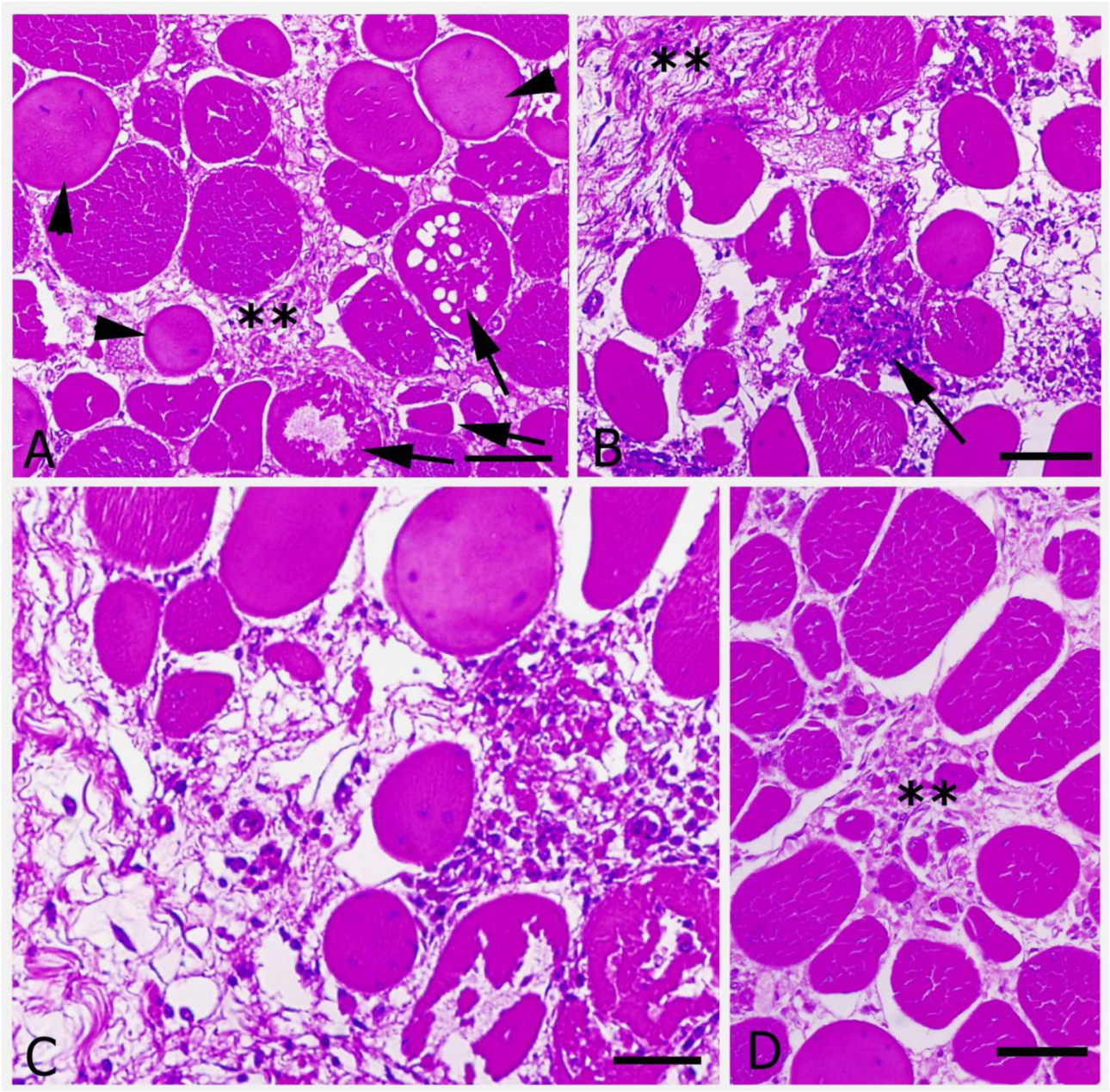

Figure 4. Histological evaluation of muscle fibers in chickens at 46 d of age. All panels are stained with hematoxylin and eosin. A) A gradual increase in degenerating fibers (*arrows*) scattered among hyper-eosinophilic fibers (*arrowheads*) is detectable. At this stage, the presence of collagen-rich connective tissue (*asterisks*) is higher than in previous stages. B) The number of muscle fibers, which show a variability in diameter size, is reduced when compared to that of previous stages. Fibers are replaced by connective tissue (*asterisks*). Arrow indicates a degenerating fiber infiltrated by inflammatory cells. C) An abundant connective tissue surrounds muscle fibers which exhibit a variability in diameter size, reflecting a regeneration process as evidenced in panel D (*asterisks*). Scale bars: A, B, D, 20 μm; C, 40 μm. Color version available in the online PDF.

degenerating and regenerating fibers of variable size, lipidosis and fibrosis) was observed (Trocino et al., 2015), as already reported by other authors (Kuttappan et al., 2013b; Sihvo et al., 2014; Velleman and Clark, 2015) in both white-striped and wooden breasts. In addition, in 2 different genotypes affected by wooden breasts, Velleman and Clark (2015) assessed different collagen distribution and arrangement of collagen fibrils, as well. In our study, at the last slaughtering either regenerating and degenerating fibers were observed, as previously reported by Sihvo et al. (2014). In fact, Velleman and Clark (2015) found that wooden breasts had increased expression of the myogenic transcriptional regulatory factors linked to satellite cell–mediated repair of muscle fiber damage, even if with some differences between genetic lines.

At 46 d, necrotic fibers showed the highest percentage of apoptotic nuclei and were surrounded by isolated regenerating fibers, which appeared positive to the anti-PCNA antibody in both wooden breast and white-striping myopathies. A regenerative process has been also described by Sihvo et al.

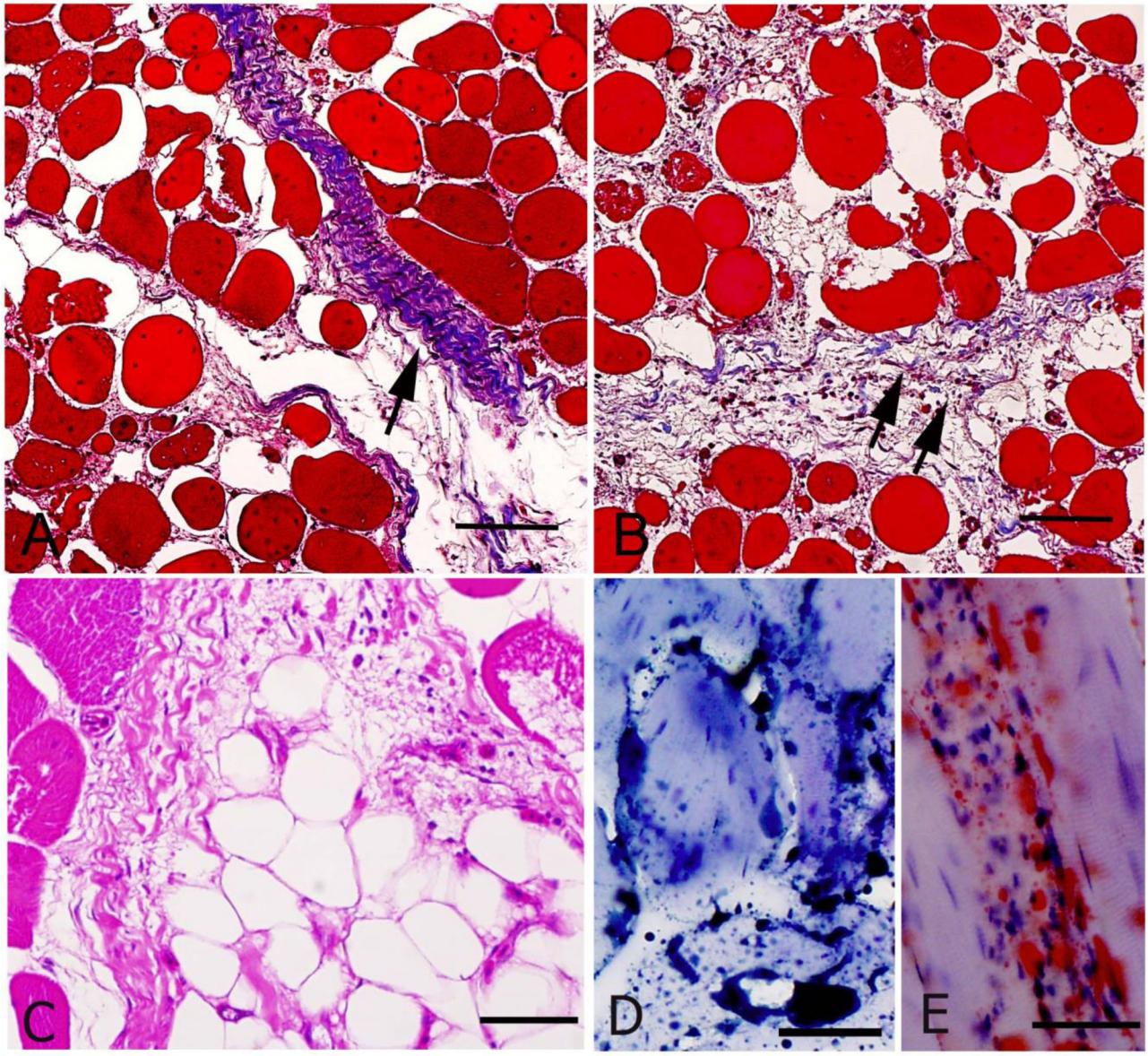

Figure 5. Histological evaluation of muscle fibers in chickens at 46 d of age. Panels A and B are stained with Masson's trichrome staining; panel C is stained with hematoxylin and eosin; panel D is stained with Nile blue; panel E is stained with Oil red. A, B) The numerous collagen fibers (*arrows*) distributed in the interstitium among muscle fibers are stained in blue by Masson's trichrome staining. C) Degenerated muscle fibers are also replaced by abundant adipose tissue. D, E) Adipose cells appear stained in blue and red by Nile blue and Oil red staining, respectively. Scale bars: A, B, 40 μm; C, D, and E, 20 μm. Color version available in the online PDF.

(2014) in wooden breast but not in white-striping myopathies.

Both myopathies have been associated with an increased muscle hypertrophy of fast-growing chickens, which brings about reduced capillary density adjacent to the myofiber (Hoving-Bolink et al., 2000; Joiner et al., 2014), thus affecting regeneration, degeneration, and necrosis of skeletal muscles (Velleman, 2015). The alteration of vascular support hinders the satellite cell-mediated repair mechanism, resulting in fibrosis (Siller, 1985). In addition, muscle multifocal degeneration and necrosis due to myopathies have been associated with altered Na and Ca metabolism since their levels increase, and fast-twitch skeletal muscle Ca-ATPase is overexpressed in wooden breast and white-striped pectoralis major (Soglia et al., 2016). The analysis of gene expression and pathway by RNA-sequencing reasonably support the hypothesis that localized hypoxia, oxidative stress, increased intracellular calcium, and the presence of muscle fiber-type switching may be responsible for wooden breast (Mutryn et al., 2015).

According to our results, the above-mentioned damaging mechanisms seem to occur early. In fact, at histological examination, we found that muscle fiber degeneration appeared soon after 14 d of age at an appreciable rate (18.8%) and affected half (50.0%) and nearly all (87.5%) the chickens fed ad libitum within 21 d and 28 d, respectively; at 35 d and 46 d, all breasts examined histologically showed muscle fiber degeneration (100% and 96.9%, respectively).

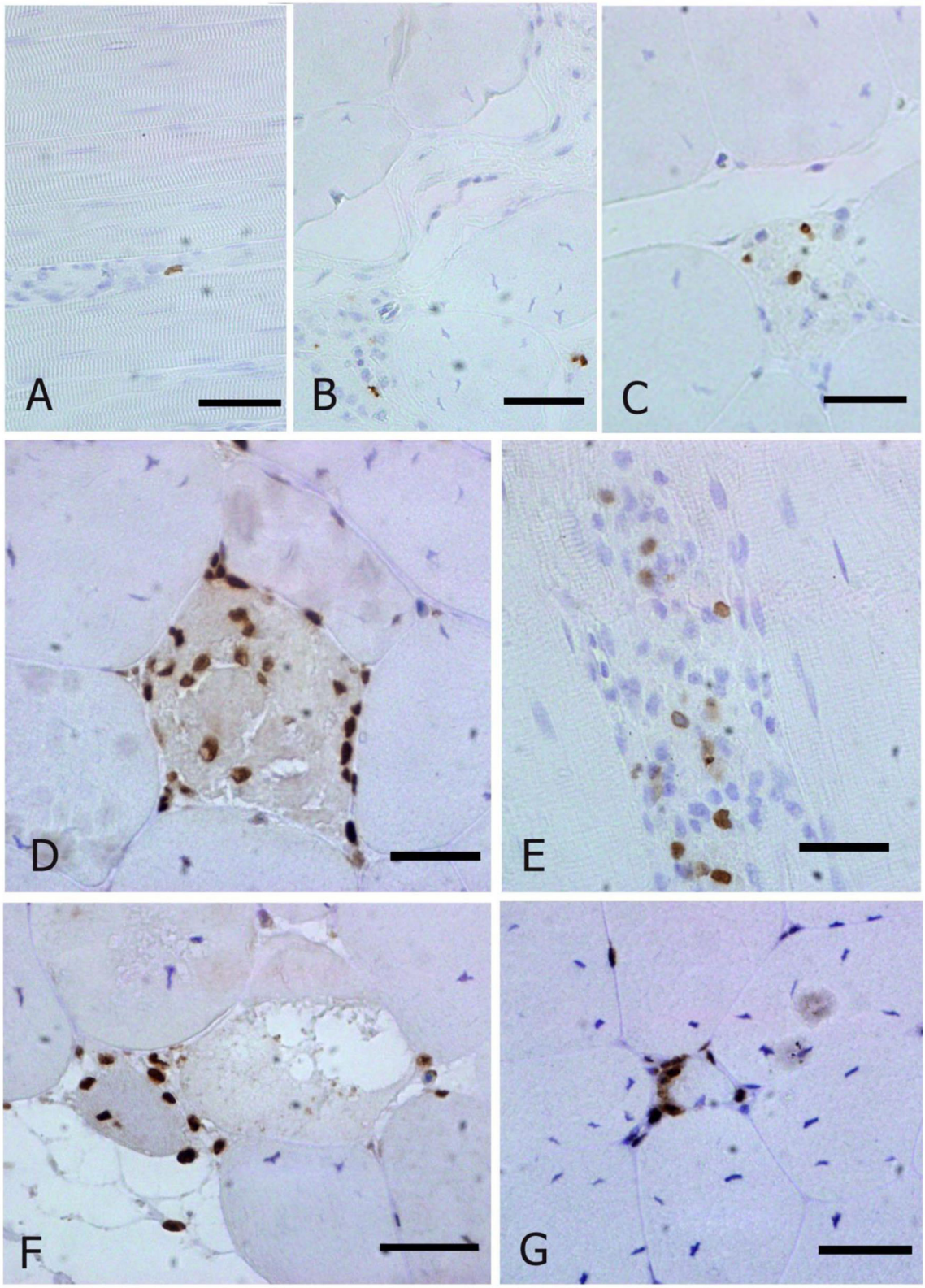

Figure 6. Immunohistochemical localization of anti-cleaved lamin A and PCNA in muscle fibers of chickens sampled at 21 (A), 28 (B), 35 (C), and 46 (D) d of age. Panels A-E are immunostained with the anti-cleaved lamin A antibody, whereas panels F and G are stained with the anti-PCNA antibody. A-D) The number of nuclei positive for anti-cleaved lamin A increased during chicken growth, reflecting the increase in the number of degenerating fibers. At 46 d, degenerating fibers (D, E) show abundant nuclei immunopositive for anti-cleaved lamin A, whereas regenerating muscle fibers (F, G) are immunostained with the anti-PCNA antibody. Both genotypes exhibited a similar pattern at immunohistochemistry. Scale bars: A, B, and C, 20 μm; D, E, F, and G, 10 μm. Color version available in the online PDF.

Moreover, the severity of lesions increased as the animal age increased and went together with a strong reduction of the number of small vessels (the most abundant ones). In contrast, Velleman et al. (2010) did not observe any muscle fiber degeneration or necrosis in the pectoralis major of chickens fed ad libitum and controlled, each 3 d posthatch to 42 d of age. Indeed, pectoralis major muscles selected at slaughter from heavy male broilers among the normal ones (i.e., without white striping and/or wooden breast at gross examination) showed myofibers with a normal profile, endo- and perimysial connective tissues without relevant alteration and few abnormal fibers (Soglia et al., 2016).

In our study, feed restriction was used to control bird growth and thus muscle fiber accretion from 13 to 21 d of age. Accordingly, during the same period, feed restriction successfully reduced the occurrence of muscle fiber degeneration compared to ad libitum feeding as measured histologically at 21 d of age. During the subsequent re-feeding period, however, restricted birds showed a compensatory growth that induced also an important damage at fiber level, as detected at 28 d of age. In addition, at gross examination of muscles at the last slaughtering (at 46 d of age), the occurrence of white-striped breasts increased by 10 percentage units in the restricted birds compared to those fed ad libitum (Trocino et al., 2015). Further insight would be necessary to assess whether a gradual re-alimentation program after feed restriction would maintain the benefits of feed restriction on myopathies occurrence. In fact, white-striping occurrence in broilers slaughtered at 54 d of age was reduced only when growth rate was controlled during the whole rearing period by the administration of low-energy diets (Kuttappan et al., 2012). On the other hand, a too early feed restriction (first 2 wk post-hatching) had negative effects on pectoralis major structure, in terms of poor organization, increased necrosis, and fat deposition, which were still fully evident at 42 d of age (Velleman et al., 2010). Finally, other strategies based on the dietary administration of antioxidants could play a role in reducing the progressive inflammation and oxidative stress and, thus, muscle fibers degeneration. However, previous results on the dietary supplementation of vitamin E proved a positive effect on the reduction of the average number of degenerated fibers per field in the pectoralis major only at early observations (28 d) which disappeared by 42 d and 49 d of age (Guetchom et al., 2012).

In conclusion, muscle fiber degeneration occurred early in broilers after 2 wk of growth, increased dramatically within 28 d of age, and was detected in almost all animals at histology within the end of the trial (46 d of age) regardless of genotype and gender. Feed restriction from 13 to 21 d of age was effective in controlling and reducing muscle fiber degeneration occurrence only as long as birds were under restriction and did not fully express their growth capability, but no residual positive effect was recorded after the re-alimentation period. This information about the time and factors affecting myopathies occurrence may contribute to arrange a suitable control of production factors in view of reducing the presence and the degree of abnormalities.

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