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The use of hematoxylin and eosin muscle staining and ImageJ as tools to assess the incidence and severity of white striping in chicken breasts^{1,2}

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ABSTRACT

The myopathy known as white striping (WS) increases deposits of fatty tissue in breasts (*Pectoralis major*) of high yielding broiler chickens. This condition threatens the poultry industry as it decreases consumers' willingness to purchase. To compare macroscopic (visual scoring) and microscopic (histological staining) methods as tools to assess WS, samples

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were collected from a trial evaluating the effects of growth rate (fast or slow) and L-carnitine supplementation (0 or 100 mg/kg) on performance parameters of broilers. Chicken breasts (*Pectoralis major*; n=144) were biopsied on the left cranial ventral region. Histological slides were prepared and stained (hematoxylin-eosin; H&E), photographed, and analyzed using ImageJ software. Increased incidence and severity of WS was visually observed in fast growing birds ($P<0.0001$) and those supplemented with 100 mg/kg of L-carnitine ($P=0.0348$). Fast growth rates increased average cell area ($P=0.0315$) and percentage of adipose tissue ($P=0.0007$), while cell count was higher in slow growing birds ($P=0.0171$). A significant correlation ($r=0.2375$; $P=0.0043$) was found between visual assessment of adipose tissue and percentage determined microscopically. Although it was possible to determine presence and severity of WS by using H&E staining, this technique is labor intensive and costly relative to subjective visual assessment, which is comparatively more resource efficient.

Key words: white striping, L-carnitine, growth rate, hematoxylin and eosin, ImageJ

RESUMEN

Uso de la tinción muscular hematoxilina y eosina e *ImageJ* como herramientas para evaluar la incidencia y severidad del *white striping* en pechugas de pollo

La miopatía conocida como 'white striping' (WS) aumenta la deposición de grasa en el músculo de la pechuga (*Pectoralis major*) en pollos parrilleros de alto rendimiento, reduciendo su aceptación por parte del público consumidor. Para comparar métodos macroscópicos (evaluación visual) y microscópicos (tinción muscular) como herramientas para estudiar la condición, se recolectaron muestras de un estudio que evaluaba el desempeño productivo de pollos parrilleros con diferentes razones de crecimiento (rápido y lento) y suplementación de L-carnitina (0 y 100 mg/kg). Se tomaron biopsias del lado izquierdo de la región ventral craneal de las pechugas (*Pectoralis major*; n=144). Se tiñeron (hematoxilina y eosina; H&E) laminillas histológicas, se fotografiaron y analizaron utilizando el programado 'ImageJ'. La incidencia y severidad del WS fue mayor en aves con crecimiento rápido ($P<0.0001$) y en aquellas suplementadas con 100 mg/kg de L-carnitina ($P=0.0348$), determinado mediante evaluación visual. El tejido de aves con tasas de crecimiento rápido presentó mayor área celular promedio ($P=0.0315$) y mayor porcentaje de tejido adiposo ($P=0.0007$), mientras las aves de crecimiento lento mostraron un mayor número de células ($P=0.0171$). Las muestras que visualmente mostraban mayor incidencia y severidad de la condición tuvieron mayor porcentaje de tejido adiposo (determinado microscópicamente; $r=0.2375$; $P=0.0043$). A pesar de que la tinción H&E demostró ser un método objetivo útil para determinar la incidencia y severidad del WS, el mismo requiere mayor tiempo y recursos, por lo que se favorece la determinación subjetiva que utiliza una escala hedónica y es más eficiente en términos de utilización de recursos.

Palabras clave: miopatía 'white striping', L-carnitina, tasa de crecimiento, hematoxilina y eosina, ImageJ

INTRODUCTION

Poultry production fulfills consumer demand for less expensive, low caloric and easy to cook meat (FAO, 2010). As an archipelago in the

Caribbean, Puerto Rico is not an exception, as poultry meat has been a staple of diet for decades. Yearly reports from the Department of Agriculture of Puerto Rico have shown a substantial increase in per capita consumption of poultry meat from 34.24 kg in 1991 to 47.97 kg in 2017 (Department of Agriculture of Puerto Rico, 2019). Like other marketable products, consumers indicate a willingness to pay for certain characteristics that define quality. Appearance is the most critical attribute in poultry products and can influence the acceptance or dismissal of a product (Fletcher, 2002). Therefore, detrimental changes in the appearance of poultry meat products can adversely affect marketability and subsequently, production.

Carvalho et al. (2021) defined white striping (WS) as the presence of white striations parallel to muscle fibers with distinct degrees of severity which are mostly present in the breast fillet. Although the etiology of WS is yet to be known, there is consensus that a connection exists between heavier birds and the presence of myopathy (Kuttappan et al., 2012a; Lorenzi et al., 2014; Russo et al., 2015). A study that measured consumer acceptance of three degrees of the myopathy concluded that as the severity of the condition increased, consumer acceptability decreased (Kuttappan et al., 2012b). In markets like Puerto Rico where poultry consumption is high, efficient and cost-effective assessment methods that determine the presence of the condition must be implemented to minimize negative consumer perception.

The objective of this study was to compare two assessment methods of detecting the presence and severity of WS in chicken breasts produced by broilers with different supplementation levels of L-carnitine (0 and 100 mg/kg), a compound used to increase fat metabolism and provide more energy (Corduk et al., 2007), as well as different growth rates (slow and fast). Visual assessment of WS was done using a hedonic scale (Bailey et al., 2015) to determine the severity of the condition on a macroscopic scale while histological image analysis was used to visualize the severity of the myopathy on a microscopic level.

MATERIALS AND METHODS

This study used a total of 144 *Pectoralis major* samples from broiler chickens from a previous study in which their diets were supplemented with two different levels of L-carnitine (0 or 100 mg/kg), and the broilers had different growth rates (slow or fast). Birds (Cornish Rock Cross) were harvested at six to nine weeks of age. Prior to collecting histological samples, visual severity assessment of WS was conducted using the hedonic scale established by Bailey et al. (2015), illustrated in Figure 1. Biopsy samples (6 mm in diameter; Integra®

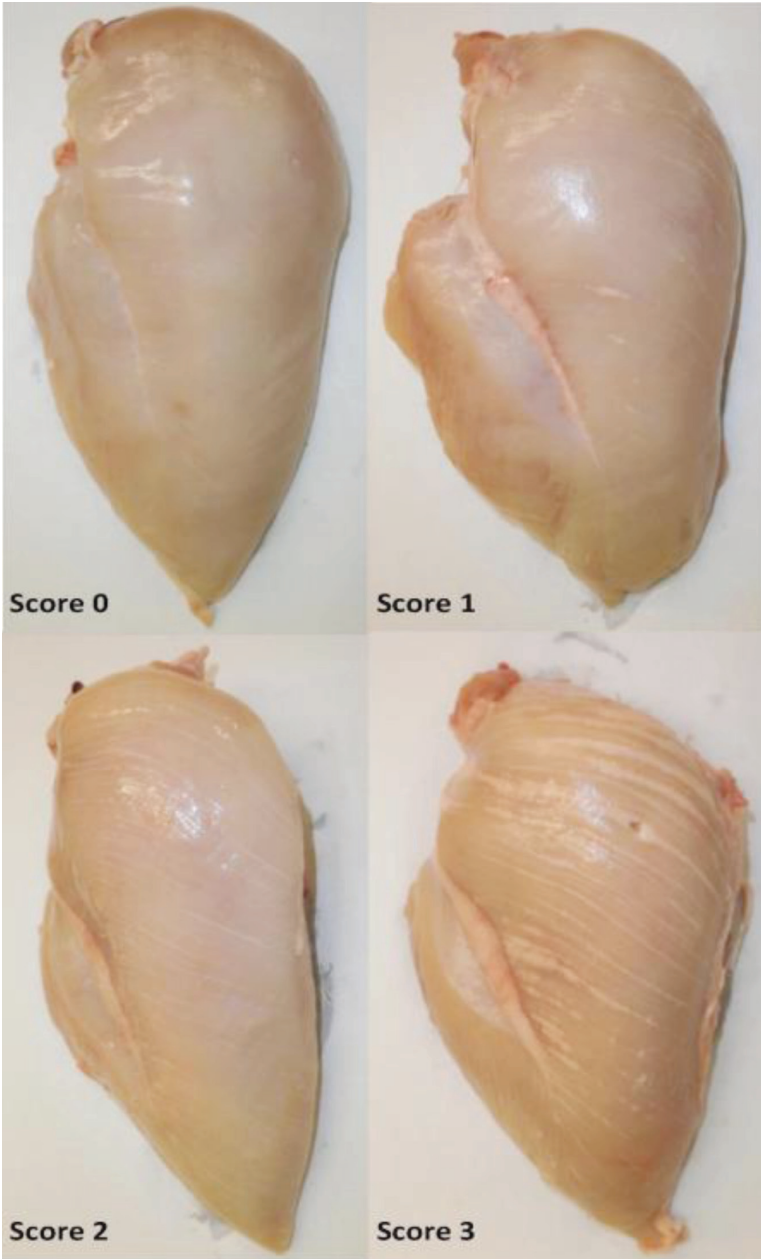


FIGURE 1. Visual assessment guide established by Bailey et al. (2015) where increasing values correspond to increasing severity of the white striping myopathy (0 = No WS; 1 = Mild WS; 2 = Moderate WS; 3 = Severe WS). Image was generously provided by Dr. Richard A. Bailey.

Miltex® biopsy punch needles)¹ were obtained from the superficial muscle fibers of the cranial region on the ventral left side from fresh (never frozen) breasts of each animal. Samples were placed in histology cassettes (Fisherbrand® TRUFLOW® tissue cassettes) and fixated in 10% formaldehyde. Tissue dehydration was performed by applying a series of consecutive and increasing concentrations of alcohol baths (70, 80, 95, 95, 100, 100, 100, and 100%) culminating in three consecutive baths of Xylene for dealcoholizing and ultimately clearing the tissue samples. Tissue embedding was performed using paraffin wax and subsequent sectioning was executed using a slice thickness of 7 µm and a 4° clearance angle. Slices produced were then floated in a distilled water bath at 43° C and placed in single, double or triple configuration, depending on the width of the slice print itself, on microscope slides prepared with an albumin drop prior to placement. Slides were then set on a warm plate at 37° C for 24 h to embed the paraffin wax. After the allotted time, a hematoxylin-eosin (H&E) staining protocol was performed (Kuttappan et al., 2013). Once the stained slides were completely dry, mounting medium was added and a coverslip slide was carefully placed to avoid the formation of air bubbles.

Triplicate crosscut images were then captured using a Nikon camera (Nikon Digital Sight DS-U3) connected to a Nikon microscope (Nikon Eclipse TS100) set at 10x magnification. The image scale was set at 100 µm using the NIS Elements D Software (Nikon, Melville, NY). Image analysis was carried out utilizing ImageJ software (v. 1.31) from which data regarding cell count, total cell area, average cell area, and percentage of muscle area relative to adipose tissue was obtained (Figure 2). The latter was used to calculate the percentage of adipose tissue (100 - % muscle area).

Data were analyzed with the Proc CORR of SAS (2012) to evaluate the relationship between the subjective visual assessment method and objective histological measures. Both methods were used to determine the presence and severity of WS. The main effects of supplementation, growth rate, and their interaction were also tested using the Proc GLIMMIX of SAS for all the response variables determined with the imaging analysis software. The TUKEY adjustment was used, and differences were determined with a *P-value* ≤ 0.05.

¹Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

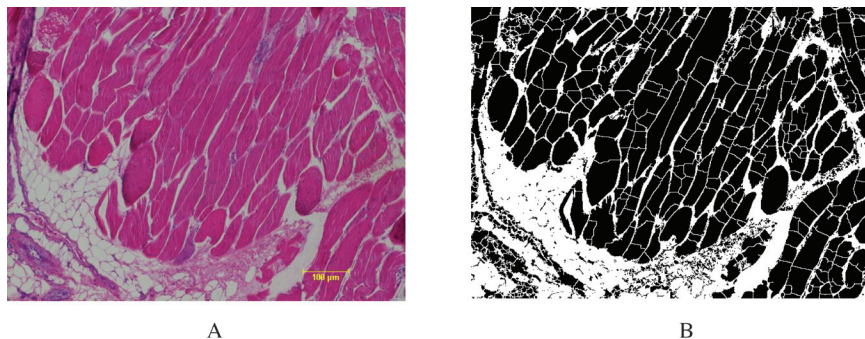


FIGURE 2. Stained (H&E)¹ histological image of poultry breast (*Pectoralis major*) tissue captured using a Nikon digital camera connected to a Nikon microscope (10x), where (A) is the original image and (B) is the same image analyzed using ImageJ software (v. 1.31). Black characterizes muscle tissue and white accounts for connective and adipose tissue. According to the visual guide established by Bailey et al. (2015) this sample displayed Moderate WS (Score = 2).

¹See color figure in digital version in <http://revistas.upr.edu/index.php/jaupr/>

RESULTS AND DISCUSSION

Main effects of L-carnitine supplementation, growth rate and their interaction are summarized in Table 1. No meaningful interaction was detected for any of the variables of interest ($P > 0.05$). However, growth rate had a significant effect on visual assessment of WS ($P < 0.0001$), cell count ($P = 0.0171$), average cell area ($P = 0.0315$) and percentage of adipose tissue relative to muscle cells ($P = 0.0007$). Slow growing birds had higher muscle cell count, while fast growing birds had more prevalent and severe WS visual evaluation scores, greater average cell area and a greater percentage of adipose tissue relative to muscle cells. Birds supplemented with 100 mg/kg of body weight of L-carnitine also displayed increased incidence and severity of WS when visually assessed ($P = 0.0348$). A significant correlation ($P = 0.0043$; $r = 0.2375$) was found between WS visual evaluation and the percentage of adipose tissue relative to muscle cells. An example of this relationship is demonstrated in Figure 3.

In a 2015 study, Bailey et al. were able to determine that WS has a genetic component but can be greatly influenced by non-genetic environmental factors as well. Therefore, studies designed to gain a better understanding of the WS condition tend to evaluate multiple factors as in the current study where dietary effects (supplementation with L-carnitine) and genetics (slow vs. fast growing bird strains) were evaluated. L-carnitine aids long chain fatty acids in penetrating the mitochondrial inner membrane and consequently producing ATP (Corduk et al., 2007; Rabie et al., 1997). As stated by Rabie and Szilágyi (1998), supplemen-

TABLE 1.—Means (±SE), main effects of growth rate, L-Carnitine supplementation, and their interaction on subjective and objective evaluation methods assessing white striping in poultry breast tissue (n=144; 36 birds per treatment group).

SUBJECTIVE MEASURE					
	Growth Rate		L-Carnitine, mg/kg BW	P-Values	Growth Rate * L-Carnitine
	Slow	Fast			
Visual Evaluation ¹	0.04±0.02 b	0.99±0.08 a	0.42±0.07 B	0.60±0.09 A	<0.0001 0.0348 0.0781
OBJECTIVE MEASURE					
	Growth Rate		L-Carnitine, mg/kg BW	P-Values	Growth Rate * L-Carnitine
	Slow	Fast			
Cell Count	1,001.01±33.22 a	890.5±32.30 b	913.99±35.29	977.53±30.96	0.0171 0.1672
Total Area (µm ²)	346,973±5,983.12	339,343±4,304.94	341,589±4,662.58	344,727±5,738.57	0.3038 0.6718
Average Cell Area (µm ²)	384.77±14.70 b	434.95±18.16 a	429.01±18.66	390.72±14.33	0.0315 0.0996
Fat Area (%) ²	36.4085±0.61 b	39.4347±0.63 a	38.5570±0.62	37.2862±0.66	0.0007 0.1500

¹Visual evaluation scale was adapted from Bailey et al. (2015) where, 0 = No WS; 1 = Mild WS; 2 = Moderate WS and 3 = Severe WS.

²Fat area (%) = Percentage of adipose tissue relative to muscle cells analyzed with ImageJ (v. 1.31).

a-b Lower case letters denote differences among growth rate within rows.

A-B Capital letters denote differences among L-carnitine within rows.

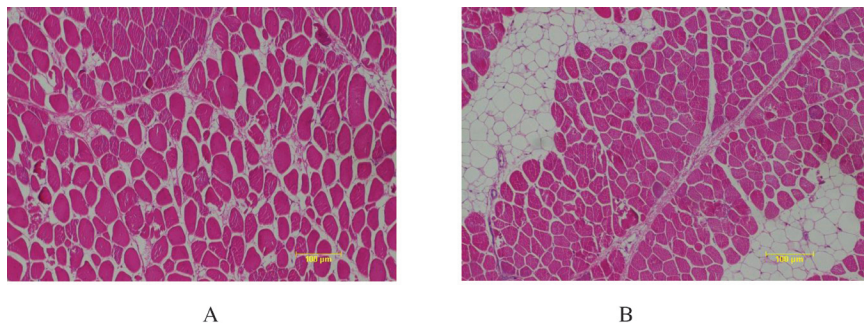


FIGURE 3. Stained (H&E)¹ histological images of poultry breast (*Pectoralis major*) tissue with (A) no indications of WS and (B) a sample with clear indications of WS. Based on the subjective scoring guide established by Bailey et al. (2015), sample A was scored as 0, indicative of No WS, and sample B was scored as 2, indicative of Moderate WS.

¹ See color figure in digital version in <http://revistas.upr.edu/index.php/jaupr/>

tation of exogenous L-carnitine could therefore metabolize dietary fat and promote growth through protein buildup. Thus, we hypothesized that by affecting the growth rate of our animals via supplementation of L-carnitine, identifiable and quantifiable amounts of infiltration and deposition of fat in the breast could be measured by objective and subjective methods. Even though no specific research was found relating supplementation of L-carnitine to the myopathy, copious published literature has highlighted its effects on growth and performance of meat birds with differing results. Although the current study was able to subjectively detect that birds supplemented with L-carnitine (100 mg/kg) had greater incidence and severity of WS ($P=0.0348$) compared with birds not being supplemented (0 mg/kg), meaningful differences were not identifiable after objectively evaluating histological poultry slides ($P=0.1500$). Perhaps higher concentrations of L-carnitine would have had more profound effects on muscle tissue and merited further investigation. For instance, some studies have evaluated performance attributes offering L-carnitine inclusion levels of up to 300 mg/kg (Parsaeimehr et al., 2014) and as high as 900 mg/kg (Murali et al., 2015). However, these studies focused on evaluating performance parameters and did not contemplate the micro and macroscopic effects of L-carnitine supplementation on WS development.

Our findings showed significant histological differences between slow and fast-growing poultry strains, where slow growing birds had greater muscle cell count and fast growing birds had greater cell size and greater amounts of adipose tissue relative to muscle tissue. In a histopathological study, WS was seen to produce chronic myopathic lesions, loss of cross striations, lipidosis, fibrosis, varying muscle fi-

ber size, among other histological anomalies when fillets were identified with a moderate or severe presence of the myopathy (Kuttappan et al., 2013). Russo et al. (2015) also histologically described WS and mentioned the presence of fibrosis, multifocal muscular degeneration, necrosis, and adipose tissue infiltration, especially in heavy broilers. Therefore, a greater number of muscle cells are expected to be present in birds with lesser incidence and severity of WS because the structural integrity of muscle would be retained. Also, due to factors like loss of cross striations and the apparent variation in muscle fiber size, it is expected that birds with a greater disposition for WS (fast growth rate) present greater muscle cell size as a result of losing cell composition. Likewise, we were able to detect greater amounts of adipose tissue in birds with fast growth rates, which concurs with work previously done on broiler weight and its relationship to WS (Kuttappan et al., 2012a; Kuttappan et al., 2012b; Lorenzi et al., 2014; Petracci et al., 2013). Therefore, our histological findings appear logical due to the nature of the myopathy and agree with previous studies.

CONCLUSIONS

Visual as well as histological differences were evident between fast and slow growing birds which concur with previous findings that associate greater incidence and severity of WS with increased growth rates. Therefore, both subjective (visual evaluation) and objective (histological image analysis with H&E staining) methodologies proved effective in assessing the presence and severity of WS. In comparison, microscopic image analysis with H&E staining is labor intensive and costly relative to subjective visual assessment, making the latter more resource efficient. On the other hand, other histological staining protocols specific for adipose and connective tissue may be worth exploring to gain a deeper understanding of this condition, considering that WS alters adipose and connective tissue deposition within the muscle.

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