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Research Note: Prospects for early detection of breast muscle myopathies by automated image analysis

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ABSTRACT White Striping (**WS**), Wooden Breast (**WB**), and Spaghetti Meat (**SM**) are documented breast muscle myopathies (**BMM**) affecting broiler chickens' product quality, profitability and welfare. This study evaluated the efficacy of our newly developed deep learning-based automated image analysis tool for early detection of morphometric parameters related to BMM in broiler chickens. Male chicks were utilized, and muscle samples were collected on d 14 of rearing. Histological procedures, including microscopic scoring, blood vessel count, and collagen quantification, were conducted. A previous study demonstrated our automated image analysis as a reliable tool for evaluating myofiber size, conforming with manual histological measurements. A threshold for BMM detection was established by normalizing and consolidating myofiber diameter and area into a unified metric based on automated measurements, also termed as "relative myofiber size value." Results show that severe myopathy broilers consistently exhibited lower relative myofiber size values, effectively detecting myopathy severity. Our study, aimed as proof of concept, underscores the potential of our automated image analysis tool as an early detection method for BMM.

Key words: image analysis, automated myopathy detection, histology, breast muscle, broiler chicken

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INTRODUCTION

White Striping (**WS**), Wooden Breast (**WB**) and Spaghetti Meat (**SM**) belong to a group of myopathies that affect the integrity of the breast muscle (Pectoralis major) of modern broiler chickens. Overall, at the histological level, breast muscle myopathies (**BMM**) share a common microscopic phenotype, with altered muscular architecture, pronounced myodegeneration, fibrosis and lipid accretion (Soglia et al., 2021). Their high incidence rate triggered an increasing concern in the poultry industry and research community (Kuttappan et al., 2016; Petracci et al., 2019). Indeed, issues related to consumers' acceptance, meat quality and processability have imposed substantial input costs on producers, estimated annually at up to 1 billion dollars in North America alone (Che et al., 2022). Breast muscle myopathies also affects animal welfare, as severe signs of myopathy are associated with impaired gait scores, which can be linked to common walking abnormalities and subtle behavioral changes in broilers (Norring et al., 2019). All of the above highlight the need for diagnostic and research tools capable of early detection of BMM. Early age detection, may enable immediate management intervention strategies in examined flocks, mitigating BMM, reducing economical loss and improving animal welfare.

In this study, we evaluated the efficacy of our newly developed deep learning-based automated image analysis tool —reported in Dayan, et al., (2023)— for early detection of morphometric parameters related to BMM in broiler chickens. We examined the consistency between data of automated image analysis to data from histological muscle phenotyping (microscopic scoring, blood vessel counts and collagen percentage). Our study, aimed as proof of concept for automated myopathy detection, introducing a unified metric of myofibers, termed as "relative myofiber size value."

MATERIALS AND METHODS

The animal study was reviewed and approved by IACUC: AG-20-16298.

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Broilers

Fertile eggs (n = 40, mean weight = 62.46 ± 4.4g) were obtained from 33-wk-old broiler hens (Cobb 500) at a commercial breeder farm (Y. Brown and Sons Ltd., Hod Hasharon, Israel). The eggs underwent standard procedures and following standard incubation conditions (37.8°C and 56% relative humidity). After hatching, male chicks were moved to brooders and raised according to the breeder recommendations (Cobb-Vantress). Throughout the rearing phase, the chicks were provided with a standard commercial starter diet (formulated by Brown feed mill, Kaniel, Israel) with ad-libitum access to water and feed.

Muscle Sampling and Histological Procedures

The muscle sampling procedure for histology was conducted according to a previously described method by Halevy et al., (2004). On d 14 of rearing, 12 chicks were euthanized by cervical dislocation, body weight, breast muscle weight and percentage were recorded, no significant differences were found (Table 1). Breast muscle samples were extracted from the superficial region of the proximal half of the left pectoralis major muscle, each measuring approximately 0.5 cm × 0.5 cm × 1 cm. The muscle samples were fixed in 3.7% formaldehyde in phosphate-buffered saline (**PBS**) at pH 7.4 (Sigma-Aldrich, Rehovot, Israel) for 24 h. Following fixation, the samples underwent a series of steps, including dehydration, clearing, and paraffin embedding. Cross-sections of 4 to 6 μm thickness were cut, using a Leica RM2135 Microtome (Leica Biosystems Nussloch GmbH, Germany). Following deparaffinization and rehydration steps, the sections were stained with Picrosirius (Sirius) Red Fast Green (**SRFG**). Images comprised of 12 stitched fields of X60 magnification were generated using EVOS FL Auto-inverted microscope (Life Technologies).

Morphometric Analysis: Microscopic Scoring, Blood Vessel Count, and Collagen Quantification

Microscopic scoring was evaluated using a 4-level severity scale (1,2,3,4) according to Che et al., (2022). The examination was conducted by a single individual and is referring to parameters related to myodegeneration, fibrosis, and lipidosis, which serve as markers for early detection of breast muscle myopathies, as early as 2 wk of age (Papah et al., 2017; Soglia et al., 2021; Vanhatalo et al., 2021). Each broiler was represented by 7 images which were individually scored and received a final average myopathy score, calculated by summing all scores and dividing by the number of scanned images per broiler. For example, broiler number 9 received a score of 2 in 2 scanned images and a score of 3 in 5 scanned images, thus obtaining a combined score of 19. To calculate the final average score, this sum was divided by the number of scanned images (7), to a final score of 2.71. Finally, the extent of the myopathy for each broiler was classified into 2 degrees: 1) moderate, encompassing all broilers with an average myopathy score below 2, and 2) severe, including all broilers with an average myopathy score higher than or equal to 2. Representative images of moderate (< 2) and severe (≥ 2) myopathies are presented in Figure 1.

Blood vessel count was performed manually on SRFG stained images, by a single individual, using Fiji-ImageJ counter application.

The evaluation of collagen proportion in the breast muscle was performed on the SRFG stained images using a semi-automated measurement method of color-based segmentation in Fiji-ImageJ software, according to Turgeman et al., (2008). The percentage of collagen (stained in red) out of muscle area (stained in green) was calculated as red area divided by the total area (red + green). Blank areas were regarded as artefacts and were excluded.

Table 1. Histologic traits and weight data of 14-d old broilers with moderate and severe myopathies.

Trait	Moderate myopathy	Severe myopathy	*P*-value
Myofiber diameter (μm)	24.42 ± 0.041*	21.22 ± 0.049	$P < 0.0001$
Myofiber cross sectional area (μm^2)	766.4 ± 2.13*	568.05 ± 2.25	$P < 0.0001$
Relative myofiber size value (geometric mean)	1.09 ± 0.002*	0.87 ± 0.003	$P < 0.0001$
Number of myofibers per mm^2	1302.24 ± 30.902*	1808.75 ± 94.57	$P < 0.0001$
% Collagen (types 1+3)	6.51 ± 0.41*	8.805 ± 0.97	$P < 0.0363$
Number of blood vessels per mm^2	242.53 ± 8.8*	212.08 ± 11.37	$P < 0.0385$
Number of myofibers supplied by 1 blood vessel	5.74 ± 0.23*	9.08 ± 0.62	$P < 0.0001$
Body weight (BW)	589.76 ± 11.26	566.64 ± 14.06	NS ($P < 0.23$)
Breast muscle weight	94.17 ± 3.86	88.91 ± 4.56	NS ($P < 0.39$)
% Breast muscle of BW	15.96 ± 0.41	15.69 ± 0.67	NS ($P < 0.73$)

Myofiber diameter and area were analyzed using automated image analysis as described by Dayan et al., (2023).

The proportion of collagen content out of muscle area was analysed using a semiautomated workflow and calculated according to: $\%\ collagen = \frac{collagen\ area}{collagen\ area + total\ muscle\ area} \times 100$

The number of blood vessels were manually quantified and the proportion out of muscle area was calculated according to: $\frac{number\ of\ vessels}{total\ muscle\ tissue\ area} \times 1,000,000$, with the multiplication converting units from μm^2 to mm^2.

The number of myofibers supplied by one blood vessel was calculated as follows: $\frac{myofiber\ per\ mm^2}{blood\ per\ mm^2}$

The relative myofiber size value was calculated by performing a geometric mean for the relative diameter and area traits of each myofiber, resulting in the acquisition of a relative myofiber size value. The formula is as follows: $\sqrt[2]{relative\ diameter\ value\ \times relative\ area\ value}$

Asterisk denotes for values that are significantly different between groups, as derived from one-way ANOVA followed by student's *t*-test ($p \leq 0.05$; NS = not significant). Results are presented as mean ± standard error mean, n = 7 moderate-classified broilers and 5 severe-classified broilers.

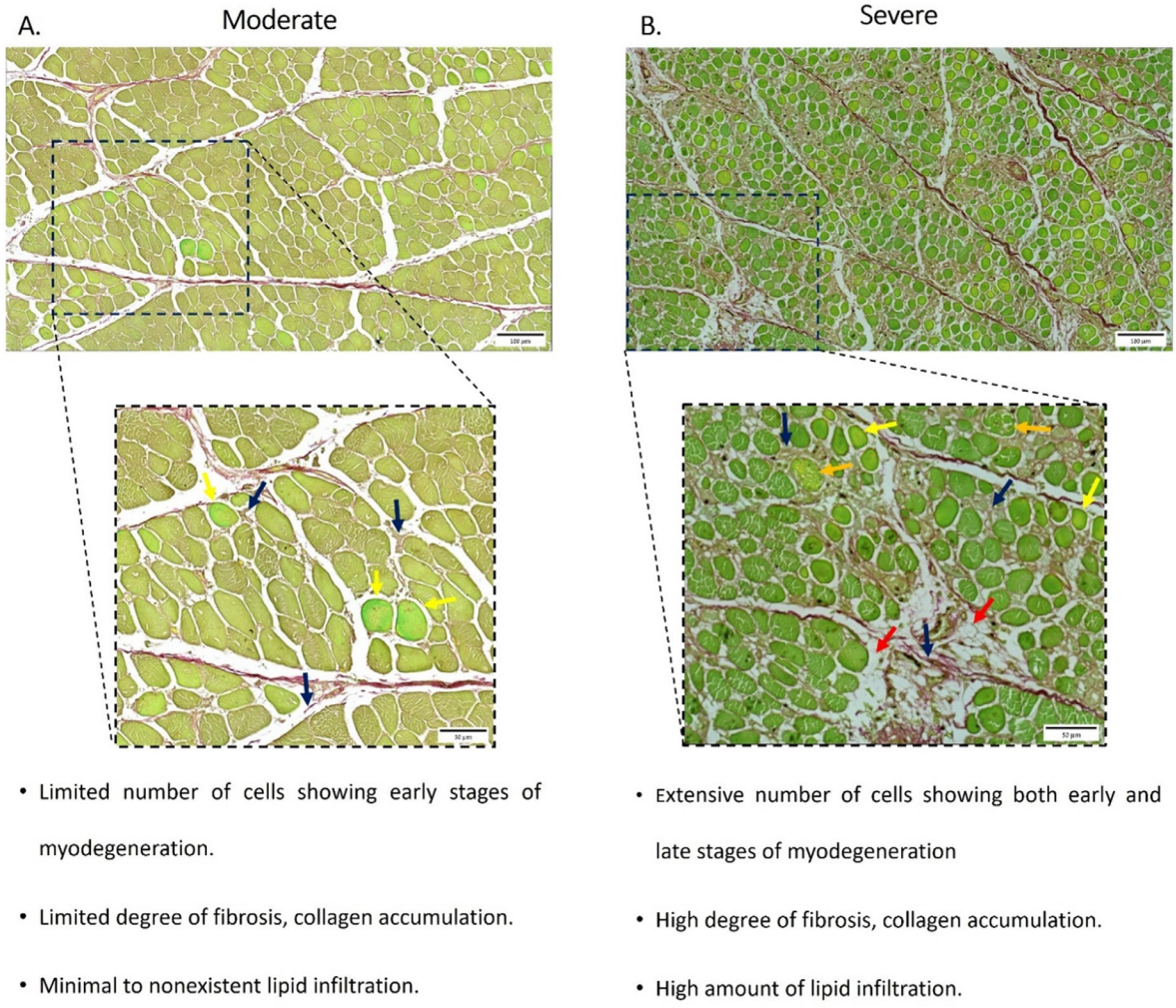

Figure 1. Histological scoring example of 14-day-old broilers' breast muscle, comparing severe vs. moderate myopathy: (A) represents moderate myopathy scoring (< 2), while (B) represents severe myopathy scoring (≥ 2). The yellow and orange arrows indicate early and late stages of muscular degeneration (myodegeneration), respectively. Red arrows represent adipose tissue infiltration and blue arrows show fibrosis and collagen accumulation in the cellular background. The histological images are stained with Sirius Red-Fast Green (SRFG) and consist of 12 stitched fields at X60 magnification, generated using EVOS FL Auto-inverted microscope.

Automated Image Analysis of Muscle Histology

The deep learning-based automated image analysis workflow was developed in our lab and reported in Dayan, et al., (2023). This workflow was shown as a reliable tool for rapid and precise evaluation of chicken breast muscle myofiber diameter and area. It maintains an accuracy rate of 99.91% compared to manual analysis, while demonstrating significantly enhanced speed and productivity with the ability to process 70 times more data sets in 38-fold less time (Dayan et al., 2023).

This workflow for Fiji (https://doi.org/10.5281/zenodo.7678528) is based on 1) PT-BIOP and Cellpose wrapper plugins, and 2) MorphoLibJ plugin (Dayan et al., 2023). The script and code are available as a zenodo repository (https://doi.org/10.5281/zenodo.7678527). The workflow includes image processing for the automated detection of myofibers and the morphological analysis with the extraction of myofiber metrics; the lesser diameter (μm) and cross-sectional area (μm^2). The last phase of the automated workflow includes exporting and saving of data to Excel files for further analysis and statistics.

Statistical Analysis

All data were distributed normally and subjected to one-way ANOVA followed by a student's t-test and considered significantly different with a P-value lower than or equal to 0.05 ($P \leq 0.05$). All values are presented as mean ± standard error mean (**SEM**). Statistical analyses were carried out using JMP-pro16 software (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Analysis of Muscle Histology

In order to determine the efficacy of our automated analysis method in early detection of breast muscle myopathies (**BMM**), we initially evaluated the myopathy score of each broiler, based on microscopic scoring (a detailed description appears in "M&M" section). As

shown in Figure 1, the broilers that were classified with moderate myopathy represented a lesser degree of myodegeneration, fibrosis, and lipid accretion (Figure 1A), while the severe-classified ones exhibited a significantly higher degree of these characteristics (Figure 1B). Next, we analyzed the numerical data of myofiber size obtained by our automated image analysis tool, along with additional semi-automated measurements of histological parameters related to myopathy. This analysis aimed to determine whether there is a consistency between the automated image analysis data and the myopathy ratings.

The average myofiber diameter (μm) and myofiber cross sectional area (μm^2), were significantly smaller in broilers classified with severe myopathy (15% and 35%, respectively, $P < 0.0001$) compared to those classified as moderate (Table 1). Furthermore, broilers classified with severe myopathy exhibited a higher number of myofibers per unit area (mm^2), with 39% more myofibers indicating an increase in their density, compared to moderate ones ($P < 0.0001$). In addition, severe myopathy broilers had 14% lower number of blood vessels per area (mm^2) compared to moderate ones ($P < 0.0385$). This difference is further highlighted when looking at the number of myofibers supplied by each blood vessel, where broilers classified as severe had almost double the amount of myofibers supplied by one vessel ($P < 0.0001$). To further assess the relationship between our histological score and parameters related to BMM, a collagen analysis was performed (Table 1). Results revealed a significantly higher collagen percentage (by 35%) in broilers classified with severe myopathy compared to those classified as moderate ($P < 0.0363$).

Together, our results of microscopic scoring equal to or above 2 (≥ 2) combined with higher collagen amount and decreased vasculature are in consistency with BMM phenotypic characteristics (Papah et al., 2017; Vanhatalo et al., 2021; Soglia et al., 2021; Che et al., 2022). Moreover, our results of increased myofiber density obtained by the automated image analysis are in accord with these publications. It can be concluded that a consistency exists between the data of automated image analysis and the manual and semi-automated morphometric data.

Determining a Threshold for Breast Muscle Myopathy Detection by "Relative Myofiber Size Value"

Given the observed quantitative differences between broilers classified with severe and moderate levels of BMM, we aimed to establish a one measurable value for detection. Such value would enable us to assess its likelihood based solely on the data obtained through our automated image analysis tool (i.e., diameter and cross-sectional area of myofibers). To address the discrepancy between diameter values (21−25 μm) and area values (570−765 μm^2), we normalized each trait relative to its overall average, reaching a relative value. Next, to consolidate the 2 normalized traits into a single final value, termed now as relative myofiber size, we calculated their geometric mean, as it considers the relative magnitudes of the numbers being averaged. The calculation for each myofiber involved multiplying the relative diameter value by the relative area value and then executing a square root of the calculation product (see formula in Table 1 legend).

The relative myofiber size, similar to other examined histological traits, was compared between broilers with moderate and severe myopathies (Table 1, Total score). The results revealed a dichotomous distribution: the moderate myopathy classified group received value higher than 1 and were found significantly different than the severe myopathy classified group, which received value lower than 1 ($P < 0.0001$).

Additionally, to assess whether the relative myofiber size value can indeed contribute in detection of myopathy, we analyzed the "relative myofiber size value" for each broiler (data not shown). Also, at the individual level, each of the moderate classified broilers received relative myofiber size value above 1 and each of the severe classified broilers received relative myofiber size value below 1 (with a 95% confidence level). In this context, relying solely on the objective measure of relative myofiber size value, we provide early detection of BMM, when 41.67% (n = 5) of examined broilers are associated with severe myopathy.

CONCLUSIONS

Our study successfully demonstrated the efficacy of an automated image analysis method for early detection of morphometric parameters related to BMM in broiler chickens. Through our automated measurements and also microscopic phenotyping, clear distinctions emerge between broilers classified as moderate and severe, revealing severe myopathy's pronounced myodegeneration, fibrosis, lipid accretion, lower myofiber size and increased density of myofibers. The introduced model of "relative myofiber size" provides a unified metric, showcasing a clear separation between moderate and severe myopathy broilers. This proof of concept, though with a limited sample size, highlights the potential of our automated image analysis tool for early detection and prediction of BMM. Early detection, facilitated by our approach, may contribute to poultry management, allowing for immediate application of preventive procedures such as adjustments of feeding programs, lighting regimes, etc. to reduce growth rates, a factor known to be associated with increased myopathy prevalence.

DISCLOSURES

The authors declare no conflict of interest.

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