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Spatiotemporal quantification of *Gallus gallus domesticus* myocardium components myocardium of *Gallus gallus domesticus*

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Abstract
This study aimed to investigate the morphological, functional, and structural components of the *Gallus gallus domesticus* myocardium. The evaluation was performed over a seven-week period to determine the remodeling of this organ in a lineage of broiler chicken. The study group consisted of twenty one animals aged between one and seven weeks old, separated in G1 to G7. The assessed parameters were: volume density of cardiomyocytes and interstitium; and densities of connective tissue, collagen fibers I and III, interstitium, and their correlations over the studied period, to determine body remodeling. The Vv(cardiomyocytes) values decreased from 73.89% to 47.78%. The Vv (interstitial) increased from 25.98% to 48.89%. The percentage of connective tissue increased from 3.29% to 13.75%. The type I collagen fiber increased from 0.06% to 0.5% in the last week. The type III collagen decreased 46% in the period, from 0.39% at baseline to 0.18% at the end.

Keywords: Heart, morphology, cardiomyocytes, collagen, broiler chicken

1. Introduction
Broilers have been subjected to intensive genetic selection over the years, which has led to a large increase of live weight. However, the weight of several organs, including the heart, has been reduced in relation to the animal live weight. The inversely proportional relationship between bird and organ weights, especially the heart, directly affects the physiological integrity of broilers (Rance *et al*., 2002; Nery *et al*., 2007) [16, 17]. Intensified effort on the part of the heart muscle, for example, especially in stressful situations, can lead to a remodeling of the organ and the development of ascites, a major cause of sudden death in poultry (Garcia-Neto & Campos, 2004; Abreu *et al*., 2010) [7, 1].
The cardiac muscle is supported by and inserted into a connective tissue skeleton composed mainly of collagen, especially type I and III fibers, in addition to elastic fibers (Burlew & Weber, 2000; López *et al*., 2001; Lombardi *et al*., 2003) [3, 20, 13, 12]. Together these structures act as myocardium support and filling, helping to maintain the alignment of cardiomyocytes.
The connective tissue can resist deformation to preserve its shape and thickness, preventing rupture while further contributing to the stiffening of the heart muscle. Specifically, types I and III collagen fibers are directly related to animal age; therefore, the fibers' arrangement and disposition change over time as the animals get older (Debessa *et al*., 2001; Martos *et al*., 2007) [6, 14].
To this extent, this study assessed the morphological, functional and structural myocardium components of the *G. gallus domesticus* broiler lineage. The parameters volume density of cardiomyocytes and interstitial tissue, and the densities of connective tissue, types I and III collagen fibers, and the correlations over the studied period, were evaluated to determine whether the heart underwent a remodeling process.

2. Materials and Methods
The twenty one hearts of male and female broilers (*G. gallus domesticus*) of the Master Gris Cou Plume lineage used originated from specialized commercial chicken farms.

The birds were fed commercial feed (Purina®). The birds' age ranged from one to seven weeks, and the hearts were divided into seven groups of three each according to age group (G1, G2, G3, G4, G5, G6, and G7). The study was approved by the Ethics Committee on Animal Use of the University of Brasilia (# 50607/2013).

Euthanasia was performed under deep anesthesia (Halothane, Cristália Laboratory). Subsequently, the hearts were collected and fixed in 10% formaldehyde for about 72 h. After that, four random 4-mm thick fragments, sectioned from each heart using a manual microtome, were submitted to conventional histological technique. The four slides prepared for each fragment were stained with picrosirius red to determine the volume density of cardiomyocytes (Vv[cardiomyocytes]), density of connective tissue and to characterize the types of collagen fibers. The picrosirius red staining was performed according to the technique described by Junqueira *et al.* (1979) [9]. The Sirius Red under polarized light stains the connective tissue pink. In addition, the collagen fibers were differentiated using polarized microscopy, in which type I fibers are stained red while type III fibers are stained greenish-yellow.

Three random fields for each heart were counted manually and on double-blind analysis, totalizing nine fields for each group. Total area was using the Delesse Principle, applied to the point test system: $A = P * Pt$ (μm^2), where *A* is the area occupied by the cardiomyocytes or interstitium, P is the number of points counted on the cardiomyocytes – the points that didn't coincide with this cells neither connective tissue were given as interstitium, and Pt is the total number of points of the test system..The result is expressed as volume density of cardiomyocytes (Vv[cardiomyocytes]) or interstitium (Vv[interstitium]), as percentual value.. The total number of points used in this study was thirty six on the software STEPanizer©.

The cardiomyocytes were quantified by image analysis using the STEPanizer© software 1.0. The density of the connective tissue and the collagen fibers was determined in the images via segmentation, using the Image ProPlus 6.0® software. A Leica optical microscope with polarized lenses and the ProRes CaputrePro 2.5® image analysis software were used.

The mean and standard deviation values of each parameter were obtained using the GraphPad Prism® 6 software, followed by the Kolmogorov-Smirnov normality test. Subsequently, the means were compared by one-way ANOVA. The Pearson correlation test was used to determine the correlation between time and Vv(cardiomyocytes), connective tissue density, collagen fibers I and III and interstitium, in addition to the correlations between the structures themselves. All tests were significant at $P<0.05$.

3. Results

The Vv(cardiomyocyte) values decreased from 73.89% to 47.78% ($P<0.0001$) between the first and last weeks, respectively (Figure 1A). On the other hand, Vv(interstitium) increased (p = 0.0002) between the first (25.98%) and seventh (48.89%) week (Figure 1B).

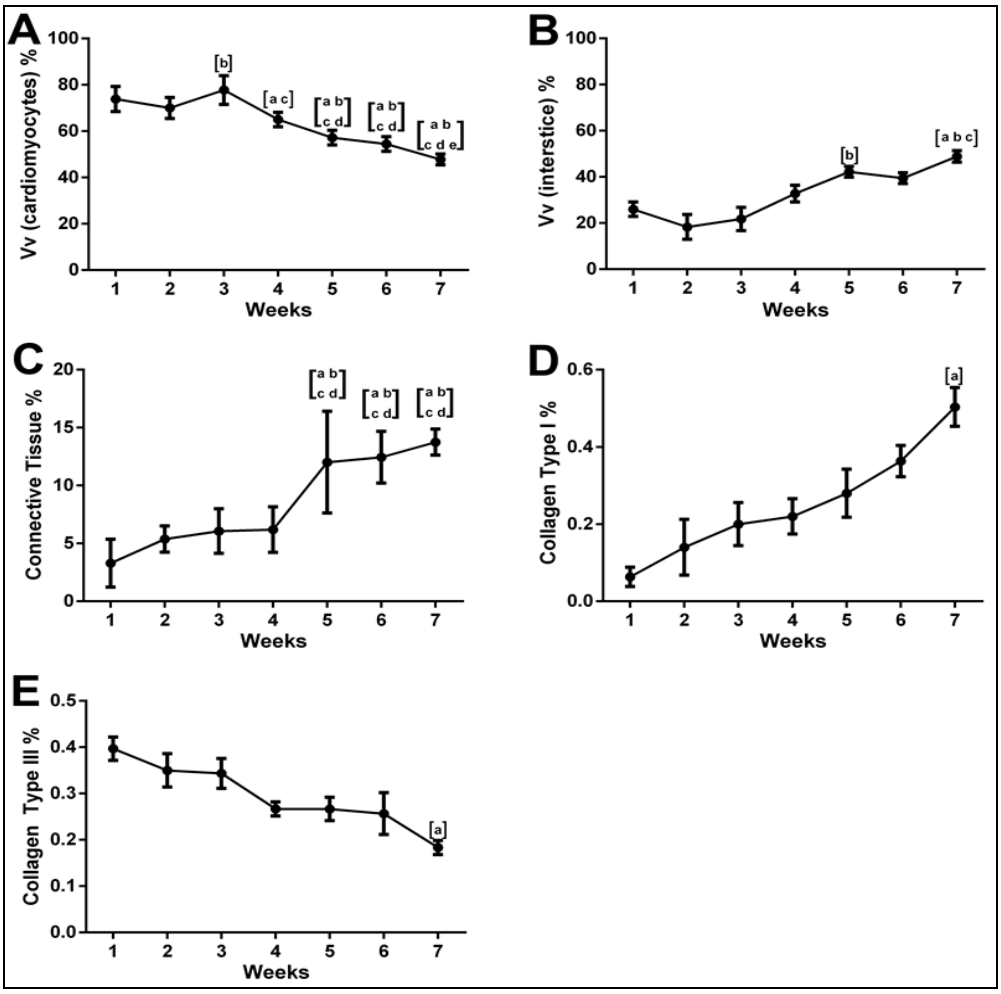

Fig 1: Means and standard deviations of volume densities of cardiomyocytes (A) and interstitium (B), and densities of connective tissue (C), type I collagen (D) and type III collagen (E) during the seven weeks of the study. The letters indicate significant statistical difference between weeks: [a] ≠ week 1; [b] ≠ week 2; [c] ≠ week 3; [d] ≠ week 4; [and] ≠ week 5.

Likewise, the connective tissue percentage, the main component of the interstitium, increased from 3.29% to 13.75% (P<0.0001) between the first and last week (Figure 1C). This result is consistent with increasing Vv(interstitium) and suggests that, unlike the cardiomyocytes, the connective tissue continued to expand. Type I collagen fiber also increased (p = 0.0103) from 0.06% in the first week to 0.5% in the last week (Figure 1D). In contrast, type III collagen fibers, like the cardiomyocytes, decreased (p = 0.0131) about 46%, from 0.39% in the beginning to 0.18% in the end (Figure 1E).

The Pearson correlation test showed statistically significant correlations between time (weeks) and Vv(cardiomyocytes) (r = -0.9946, P<0.0001), Vv(interstitium) (r = 0.9976; P<0.0001), connective tissue (r = 0.9554, p = 0.0008), type I collagen (r = 0.9747, p = 0.0002) and type III collagen (r = -0.9658; p = 0.0004). The correlations between time and Vv(cardiomyocytes) and collagen type III were strongly negative. On the other hand, the correlations between time and Vv(interstitial), connective tissue, and collagen type I were strongly positive. The correlations between Vv (cardiomyocytes) and Vv (interstitium) (r = -0.997; P<0.0001), connective tissue (r = -0.972; p = 0.0002), and type I collagen (r = -0.973; p = 0.0002) were strong and negative. The correlation between Vv(cardiomyocytes) and type III collagen (r = 0.962; p = 0.0005) was strongly positive. Unlikely, the correlations of Vv(interstitium) with connective tissue (r = 0.964, p = 0.0004) and type I collagen (r = 0.976, p = 0.0001) were strong and positive. Furthermore, the correlation between Vv(interstitium) and type III collagen (r = -0,968p = 0.0003) was strong and negative. The correlation of connective tissue with type I collagen (r = 0.930; p = 0.0024) was strong and positive, and contrary to the strong negative correlation observed with type III collagen (r = -0.884; p = 0.0082). Finally, the correlation between types I and III collagen fibers (r = -0.949; p = 0.0011) was strongly negative.

Table 1: Values of Pearson correlation (r) between time and volume density of cardiomyocytes and interstitium and density of connective tissue, type I collagen and type III collagen. All correlations were significant (P<0.05).

	Vv(cardiomyocytes)	Vv(interstitium)	Connective Tissue	Collagen I	Time
Vv(cardiomyocytes)	-	-0,99	-0,97	-0,97	-0,99
Vv(interstitium)	-0,99	-	0,96	0,97	0,99
Connective Tissue	-0,97	0,96	-	0,93	0,95
Collagen I	-0,97	0,97	0,93	-	0,97
Collagen III	0,96	-0,96	-0,88	-0,94	-0,96

4. Discussion

Cardiac remodeling is a complex combination of ischemia of the heart muscle and increased pressure in the myocardial wall, resulting in heart molecular, cellular, and interstitial changes (Weber, 2000) [3, 20]. Studies with rats have shown that the formation of cardiomyocytes reduces considerably in the first days after birth (Walsh *et al*., 2010) [20]. This fact could explain the possible cardiac remodeling observed in the birds of this study when the reduced formation of new cardiomyocytes was accompanied by deposition of connective tissue. This result seems to suggest that the rapid growth of broilers might favor an inversely proportional relationship between Vv(cardiomyocytes) and Vv(interstitium), which is mainly composed of connective tissue, as expressed in the results.

The broiler farms are known to feed high-protein and high caloric diet to favor rapid weight gain (Silva *et al.*, 2003; (Nery *et al.*, 2007) [19, 16]. Recently, it has been shown that obese mice underwent cardiac remodeling following a high caloric diet and the number of cardiomyocytes decreased in relation to other components of the myocardium (Schipke *et al.*, 2014) [18]. Schipke *et al.* (2014) [18] suggested that the significant reduction of Vv(cardiomyocytes) resulted from a high-calorie diet, despite the fact that the author did not pre-defined the calorie value of the diet that resulted in heart remodeling. Based on the previous results, it can be suggested that the significant reduction of the Vv(cardiomyocytes) of the birds in this study might result from the hypercaloric diet. Furthermore, it is noteworthy that the genetic model may also have been expressed in the present model, thus ensuring the cardiac remodeling as opposed to the evaluation over time of the birds.

Several physiological factors that suppress the proliferation of cardiomyocytes in mammalian models have been identified, among which the most important is perhaps the triiodothyronine (T3) hormone (Chattergoon *et al.*, 2012a; Chattergoon *et al.*, 2012b; Kinugawa *et al.*, 2005) [4-5, 10]. However, Holm *et al.* (2014) [8] reported that this hormone does not affect cardiomyocytes' maturation nor size in chickens. Thus, the proliferation of these functional myocardial cells is not terminated after birth as in other species, remaining at least until the sixth week after hatching (Li *et al.*, 1997) [11]. On the other hand, the inversely proportional relationship between Vv(cardiomyocytes) and connective tissue density over time shows that, although the cardiomyocytes maturation had not been completed, the proliferation of connective tissue overlapped the proliferation of these cells.

Quantitative evaluation of connective tissue in the cardiac muscle of dogs corroborate the results of this study. The positive correlation of time (age) with connective tissue density but negative with Vv(cardiomyocytes) reveals possible mechanisms that could potentially interfere with the increasing proportion of this tissue, such as advancing age (Benedicto & Bombonato, 2003; Martos *et al.*, 2007) [2, 14]. However, this possibility has not been fully elucidated. Furthermore, Martos *et al.* (2007) [14] stated that the evidence indicated possible metabolic changes of the connective tissue due to aging.

Regarding the collagen fibers present in the myocardium, Debessa *et al.* (2001) [6] stated that the type I collagen is more dense in the myocardium of older people while collagen type III tended to be more evident in younger individuals. The densities of both collagen fibers in the myocardium of birds were inversely correlated in this study. Also, the temporal correlation with the density of type I collagen fibers only was directly proportional, thus corroborating the results of Debessa *et al.* (2001) [6]. These results ensure that the structural pattern of these fibers can form the fibrous skeleton of broilers' heart.

It is known that the fibrous tissue deposition on the myocardium can have interstitial character, i.e., collagen fiber

deposition on the myocardial interstitium, especially the collagen type I. This process is apparently associated with secondary myocardial fibrosis due to hemodynamic overload with various consequences for the myocardial functioning. Initially, there may be resistance to blood flow due to vascular compliance, aggravating the myocardial hypoxia in cases of high O_2 consumption rate and hypertension. Furthermore, there are difficulties in the transmission of mechanical stress to the heart muscle due to the increasing rigidity of the ventricular chamber, which impairs the filling up of the chamber (Mill & Vassallo, 2001) [15].

5. Conclusion

In *G. gallus domesticus*, the age probably influenced the drop in the volume density of cardiomyocytes, thus causing their replacement by the component tissues of the heart and increasing the volume of the total tissue. Otherwise, the significant drop in the density of type III collagen fibers suggests a replacement by type I collagen fibers. This whole replacement process results from the fact that these are structural tissues, which are subject to changes during life.

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