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Original Article

Influence of Ammonia Gas on Histomorphometry and Histopathology of Broiler Chicken Tracheas

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ABSTRACT

The aim of the present study was to analyze the histomorphology and histopathology of broiler chicken tracheas submitted to different levels of ammonia gas. The experiment was conducted during different seasons in 2019 in the city of Garanhuns, Brazil. Three sheds with rice hull bedding were used: Shed 1, new bedding; Shed 2, reused bedding; Shed 3, reused bedding with an increase in moisture. Twenty-eight birds were housed per shed for a 42-day production cycle. On days D0 (control group), D7, D21, D35 and D42, seven birds per shed were euthanized. Fragments of the trachea were collected and placed into plastic containers with 10% buffered formalin solution (pH 7) for 24 h, subsequently undergoing routine histological processing. The thickness of the tracheal mucosa was measured using digital images, considering ten equidistant points as being separated by 100 µm. Histopathological lesions were analyzed considering distribution and intensity. The birds in Shed 2 (reused bedding) had greater quantity and severity of histopathological lesions, but the differences did not reach statistical significance. Regarding histomorphometry, birds reared in the higher temperature period had thicker tracheal mucosa at the end of the production cycle (D35 and D42) compared to those raised in the cooler temperature period. In conclusion, the difference in bedding did not significantly alter the tracheal mucosa of the birds, whereas temperature exerted an influence on the thickness of the trachea at the end of the production cycle.

INTRODUCTION

Brazil is the second largest producer of poultry meat in the world, with a total of 14.524 million metric tons in 2022 (ABPA, 2023). The Northeast region occupies the fourth place in terms of national chicken slaughter, with the state of Pernambuco accounting for approximately 46% of total slaughtered chickens in the region (ABPA, 2023).

Rosa *et al.* (2019) state that the profit margin in poultry farming is narrow and constant investment is needed. An important part of this investment is the bedding, as its quality directly affects production indices (Stojcic *et al.*, 2016). However, bedding can be used in several production cycles when adequate management is performed (Wang *et al.*, 2016). Bedding is expected to absorb the moisture from organic materials, create thermal isolation, and serve as physical protection to avoid foot lesions (Carvalho *et al.*, 2011; Campos *et al.*, 2018).

All organic litter, especially excrement from the birds, causes physicochemical changes in the litter, resulting in the production of ammonia gas (NH_3) (Davis & Morishita, 2005). NH_3 is considered the major atmospheric pollutant of poultry farming (YI *et al.*, 2016) and is capable of causing harm to humans (Schiffman, 1998; Iversen *et* *al.*, 2000; Davis *et al.*, 2015) and birds, especially in the respiratory system (Anderson *et al.*, 1964; Al-Mashhadani & Beck, 1985; Ansari *et al.*, 2016).

The main effects described are an increase in mucous in the trachea and swollen, inflamed lungs with hemorrhagic spots (Anderson *et al.*, 1964; Al-Mashhadani & Beck, 1985; Ansari *et al.*, 2016). However, there is little description of histological changes in the literature, with reports of loss of cilia, increase in the number of goblet cells, inflammation of the lamina propria, and submucosal edema (Oyetunde *et al.*, 1978; Al-Mashhadani & Beck, 1985; Zhou *et al.*, 2021). Moreover, there is a lack of studies involving a histomorphological analysis of the trachea.

Other factors also have a direct influence on bird wellbeing, such as thermal stress, which occurs when animals are unable to dissipate sufficient heat to the environment. This may happen for several reasons, such as an increase in ambient temperature (Rath *et al.,* 2015). Thermal stress is associated with gastrointestinal changes (Rath *et al.*, 2015), such as enteritis without morphological changes in the intestinal mucosa (Quinteiro-Filho *et al.,* 2010) and inflammation and necrosis in the liver, as reported by Tang *et al.* (2022). However, there are no reports in the literature associating high ambient temperature with damage to the respiratory system.

Therefore, the aim of the present study was to analyze histopathological and histomorphometric findings in the trachea of broiler chickens reared in different seasons and submitted to different levels of NH_3.

MATERIAL AND METHODS

Sampling

The experiment was conducted in the city of Garanhuns, state of Pernambuco, Brazil, which has reports of ammonia gas emitted from poultry litter causing harm to broiler chickens. The study was conducted following the Ethical Principles of Animal Experimentation issued by the National Animal Control and Experimentation Council (CONCEA) and received approval from the Animal Use Ethics Committee of *Universidade Federal Rural de Pernambuco* (UFRPE) (license number: 136/2018). According to the Köppen-Geiger classification, the climate in the region is classified as Aw (tropical with summer rains).

Birds were reared between February and March (season with higher temperatures, with averages between 33.6°C and 18.2°C and minimum absolute humidity of 30 to 31%) as well as between June and August, 2019 (season with cooler temperatures, with averages between 25.1°C and 14.5°C and minimum absolute humidity of 44 to 56%) (APAC, 2019abcde).

Three sheds measuring 2 x 2 m were used. All sheds were positioned in the east-west direction and were not climatized. Curtains were used to adjust the temperature, aiming at maximum resemblance to traditional production. The opening of the curtains was the same in all sheds, which were also close to one another and had the same wind direction.

The bedding of choice was rice hull, which is one of the most widely used types of bedding in the Northeast region of the country. New bedding was used in Shed 1. Reused bedding of fermented rice hull from the sheds of the ranch after one production cycle was used in Shed 1. The same bedding used in Shed 2 was used in Shed 3, being wet daily by the spraying of seven liters of water.

A total of 182 chicks (Ross breed) were used, with 91 birds for each experimental cycle divided among the three sheds (28 birds per shed). Each shed was considered one experimental group. In G1, G2 and G3, the birds were raised in the three sheds during the period of higher temperature. In G4, G5 and G6, the birds were raised during the period of cooler temperature. In each experiment, the control group was composed of seven zero-day birds, forming groups denominated Gx (production cycle from February to March) and Gy (production cycle from June to August). The maximum density was 11 birds/m^2 in all sheds, which is considered ideal for the breed according to the Ross Broiler Management Handbook (2014). The birds were reared in one 42-day production cycle. All groups received the same feed with similar feeders and drinkers, and the light program followed the Ross Broiler Management Handbook (2014).

All birds were weighed on a digital scale (SF 400, Global Home®) to track their development. Birds were euthanized in accordance with Resolution Nº 37/2018 (CONCEA Euthanasia Guidelines) on Days 0, 7, 21, 35 and 42 (D0, D7, D21, D35 and D42). Seven birds were euthanized on D0 to make up the control group. Twenty-one birds were euthanized on the other days (seven per group).

Histological and histomorphometric analyses

At all predetermined times, samples were collected from two regions of the trachea (upper and lower portions) and stored in plastic containers with 10%

buffered formalin solution (pH 7). After 24 h, the samples were transferred to 70% alcohol and sent for histological processing at the Animal Histology Lab of the Department of Animal Morphology and Physiology. All samples were processed using routine histology (Rocha *et al.*, 2016): dehydrated in increasing concentrations of alcohol, cleared in xylol, and embedded in paraffin. The blocks were cut into 3-µm slices, which were placed on slides and stained with hematoxylin and eosin, followed by analysis under a LEICA® DM500 trinocular optical microscope.

The histomorphometric analysis of the tracheal mucosa was performed using the protocol proposed by Nunes *et al.* (2002). Slides were examined and photographed with a 4x objective using a LEICA® DM500 optical microscope coupled to a LEICA ICC50 HD® digital camera. Images were captured using the Leica LAS EZ® software (Leica Microsystems, Buffalo Grove, IL, USA). Thickness of the tracheal mucosa was measured on digital images considering ten equidistant points as being separated by 100 µm using the Image J® software. The program was calibrated using a photomicrography with the same magnification (4 x) and a 0.01 mm LEICA micrometric ruler (50 mm – Reference 10310345), on which 69 pixels are equal to 100 micrometers.

The histopathological analysis of the trachea involved the determination of hyperemia, congestion, hemorrhage, squamous metaplasia, epithelial flattening, epithelial detachment, epithelial necrosis, hyperplasia and hypertrophy of the mucosal glands, deciliation, tracheitis, and fibroplasia. Lesions were classified in terms of distribution and intensity, using the protocol proposed by Sesti *et al.* (2013) with some adaptations (Table 1).

Ammonia gas levels inside the sheds were measured three times per day (6:00 h, 12:00 h and 18:00 h). An ammonia gas detector (Smart sensor, model: SSEYL AR8500) was used, which displays simultaneous readings of ammonia levels in ppm and ambient temperature in °C, as described by Bandeira *et al.* (2021).

Statistical analysis

All statistical analyses were performed with the aid of GraphPad Prism (version 7.0). The parametric ANOVA test was used to compare the thickness of the tracheal mucosa, both between upper and lower tracts and between groups. Histopathological lesions were compared using the nonparametric Kruskal-Wallis test, with the significance level set at 5%. The ANOVA normality test followed by Student's t-test were used for bird weight analysis, with the significance level set at 5%.

Table 1 – Scores used to asses the distribution and intensity of histopathological lesions.

Category	Score	Meaning
Distribution	0	Absent
	1	Focal
	2	Focally extensive
	3	Multifocal
	4	Multifocal to coalescent
	5	Diffuse
Intensity	0	Absent
	1	Discrete
	2	Moderate
	3	Accentuated

Source: Adapted from Sesti *et al.* (2003).

RESULTS AND DISCUSSION

The moisture of the bedding did not increase ammonia levels, as the values found in the groups reared with new bedding were very similar to those found in the groups reared with reused and wetted bedding (Figure 1). This was unexpected, as the release of ammonia gas depends on factors such as bedding moisture and ambient temperature, with greater release of this gas in wetter, colder locations (Aston *et al.*, 2019). Bandeira *et al.* (2021) demonstrated the cyclicity of NH_3, proving that ambient temperature exerts a strong influence on the emission of this gas in poultry farming.

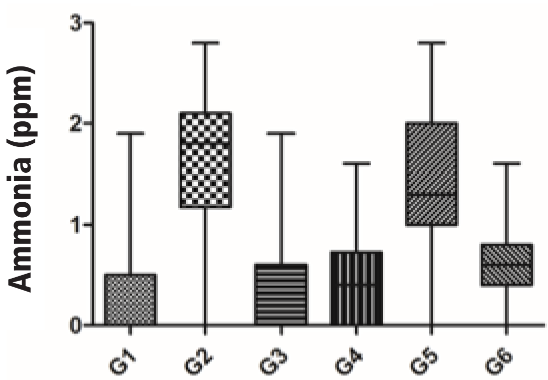

Figure 1 – Ammonia levels (ppm) measured with different bedding management in two seasons (higher temperature and cooler temperature). Each box represents an experimental group: G1 and G4 – birds reared in shed with new bedding; G2 and G5 – birds reared in shed with reused bedding; G3 and G6 – birds reared in shed with reused and wetted bedding. Groups G1, G2 and G3 were reared in the season with higher temperature; Groups G4, G5 and G6 were reared in the season with cooler temperature.

Fidan *et al.* (2020) also associated moisture and bedding thickness with an increase in NH_3. A bedding thickness of 1 cm was associated with poorer litter quality, as well as with an increase in moisture and NH_3. In the present investigation, the bedding had the same thickness in all treatments (approximately 15 cm). Fidan *et al.* (2020) state that bedding with this thickness leads to a reduction in atmospheric ammonia, which may have contributed to the low values found in the present study.

Eliotts & Collins (1982) found that an increase in bedding moisture led to a reduction in NH_3 in the first week of rearing broiler chickens, but the opposite occurred in the subsequent weeks. The outcome of the experiment differs from what was found in the present investigation, in which ammonia gas levels remained low. The authors attribute the reduction in ammonia gas to the greater dilution of NH_3, and point out that the production of this gas depends on the activity of aerobic bacteria, which are unable to perform their metabolism in a very moist environment, which causes a predominance of anaerobic bacteria. An environment favorable to anaerobic bacteria may have been present throughout the productive cycle in the present investigation.

Miles *et al.* (2011) created an equation capable of estimating the quantity of ammonia gas to be volatilized in a broiler chicken production. To arrive at this equation, the authors proved that the most influential factors were ambient temperature and moisture of the bedding, which, when high, can lead to an increase in NH_3 levels. However, there is a particular threshold of moisture and temperature at which production is maximized, with a tendency toward a reduction beyond this threshold (Miles *et al.*, 2011). This may explain the phenomenon seen in the present study, as groups G2 and G5 (birds reared with reused bedding) had higher NH_3 levels, whereas groups G3 and G6 (wetted bedding) had similar levels as those found in G1 and G4 (new bedding). This is in agreement with Liu *et al.* (2007), who concluded that, at a particular point, the increase in moisture reduced the emission of NH_3.

Previous studies have reported lesions associated with exposure to NH_3, such as metaplasia of the mucosal epithelium, mucosal hypertrophy (Oyetunde *et al.*, 1978), deciliation (Oyetunde *et al.*, 1978; Terzich *et al.*, 1998; Shi *et al.*, 2019), hypertrophy of mucosal glands (Terzich *et al.*, 1998; Shi *et al.*, 2019; Zhou *et al.*, 2020), necrosis, hemorrhage and tracheitis (Terzich *et al.*, 1998; Wang *et al.*, 2020; Zhou *et al.*, 2020). In addition to these lesions, others were also found in the present study, such as flattening and detachment of the mucosal epithelium, fibroplasia, hemorrhage and hyperemia (associated with inflammatory processes), tracheitis (with predominance of lymphocytes, histiocytes and plasmacytes), congestion (not associated with inflammatory infiltrate), loss of cilia, and depletion and hypertrophy of mucous glands.

Such lesions occurred due to diverse mechanisms linked to the action of ammonia gas, which is capable of provoking instability in the microbiota of the trachea, resulting in inflammatory reactions and even mucosal necrosis (Zhou *et al.*, 2021). According to Han *et al.* (2020), ammonia causes oxidative stress in the cells of the tracheal epithelium. This stress leads to mitochondrial dysfunction, which triggers cell death by necroptosis, a mediated cell death that causes a considerable inflammatory reaction. This is compatible with tracheitis being the lesion with greater occurrence (46.52%). However, not many areas of cell death were found. The other most common lesions were hyperplasia of mucosal glands (38.92%), squamous metaplasia of the mucosal epithelium (10.44%), and deciliation (4.11%) (Figure 2). However, no statistically significant differences were found between the upper and lower portions of the trachea, whether among groups or between seasons.

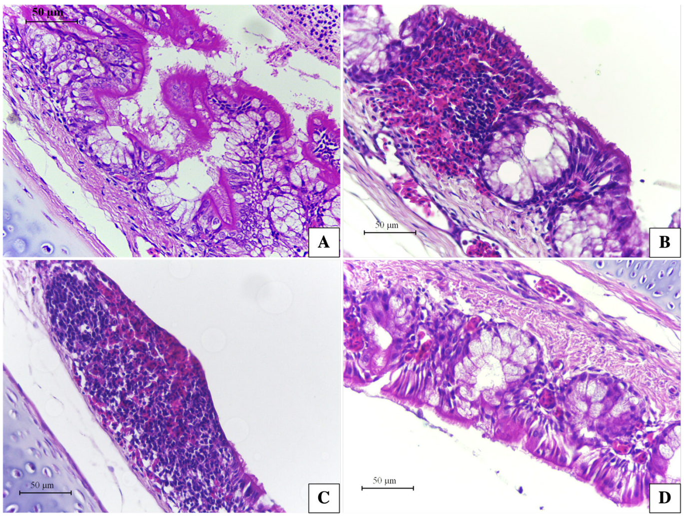

Figure 2 – Photomicrograph of the trachea of broilers raised at high temperatures, showing the main lesions found. A – Detachment of mucosal epithelium and areas of depletion and hypertrophy of mucous glands in chicken raised in a shed with new poultry litter, at 42 days, HE. B – Tracheitis with hemorrhage and hyperemia in the tracheal mucosa of a chicken raised in a shed with new poultry litter, at 35 days, HE. C – Tracheitis with hemorrhage and loss of cilia in the tracheal mucosa of a chicken raised in a shed with new poultry litter, at 35 days, HE. D – Congestion in the tracheal mucosa of a chicken raised in a shed with reused and moistened poultry litter, at 21 days, HE.

Nonetheless, the greater occurrence of lesions in this organ was in the period of cooler temperatures (186/316), which agrees with the data reported by Aston *et al.* (2019). The larger part of these lesions was found in the upper portion of the trachea, which was expected, as ammonia gas has good solubility in water (Al-Mashhadani & Beck, 1985; Xing *et al.*, 2016). Therefore, its concentration diminishes as it travels through the respiratory tract and blends with the mucous in the upper respiratory tract (Al-Mashhadani & Beck, 1985).

No statistically significant differences were found in the distribution or intensity scores (p>0.05) between the upper and lower portions of the trachea, whether among groups or between seasons. Most lesions had multifocal distribution and varied in terms of intensity. Areas of deciliation were always accentuated, while the other lesions ranged from mild to moderate.

With regards to histomorphometry, thickness of the trachea was similar in all groups on Day 7 (Figure 3-A). On Day 21, a slight increase was found in the groups in the cooler temperature period compared to those in the higher temperature period, but the difference was non-significant (p>0.05) (Figure 3-B). On Day 35 and 42, this situation was inverted, as the thickness of the trachea was greater in the groups in the higher temperature period compared to those in the cooler temperature period (Figure 3-C and D), and the difference was statistically significant (p<0.05).

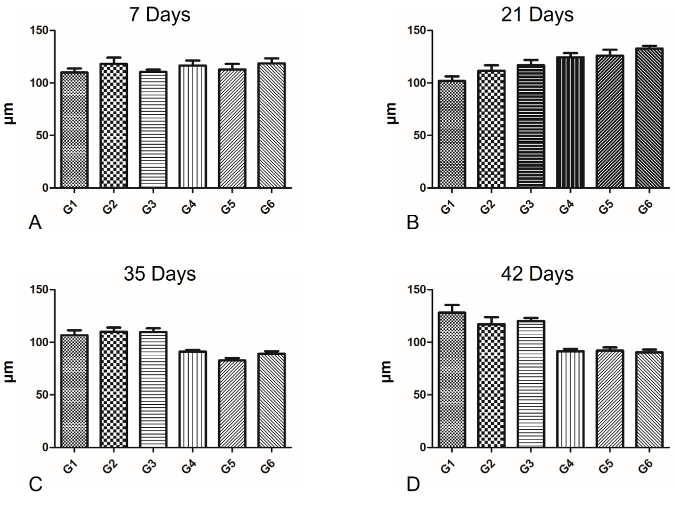

Figure 3 – Histomorphometric analysis of the tracheal mucosa of broiler chickens at 7, 21, 35 and 42 days of life reared with different bedding managements. Each box represents an experimental group: G1 and G4 – birds reared in shed with new bedding; G2 and G5 – birds reared in shed with reused bedding; G3 and G6 – birds reared in shed with reused and wetted bedding. Groups G1, G2 and G3 were reared in the season with higher temperature; Groups G4, G5 and G6 were reared in the season with cooler temperature. Differences in thickness of tracheal mucosa between seasons were only found on days 35 and 42 (p<0.05).

The fact that the thickness of the tracheal mucosa increased at the end of the production period in the higher temperature season was unexpected, as greater frequencies of epithelial metaplasia and tracheitis were found in the cooler temperature season. Both lesions promote an increase in the tracheal mucosa (Toro *et al.,* 2012; Eldemery *et al.*, 2017), as epithelial cell proliferation occurs in metaplasia and inflammatory cell infiltrate occurs in tracheitis.

In conclusion, despite different NH_3 levels and different environmental conditions, tracheal histopathological lesions were observed in all groups. With regards to histomorphometry, the tracheal mucosa was thicker in the period with higher temperatures. We believe that this experiment can serve as a tool for future studies, as well as directly support the poultry industry in improving the conditions of broiler chickens.

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