Single fluff-spray application of mother hen uropygial secretion analogue positively influences bursa of Fabricius development and the heterophil-to-lymphocyte ratio in ROSS 308 chicks

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ABSTRACT Stress is an important cause of illness and mortality in chick production. Stressors such as manipulation, absence of maternal care, transport, and housing can lead to welfare issues, immunodepression, and decreased productivity. The mother hen uropygial secretion analogue (**MHUSA**), a synthetic analog of a maternal semiochemical secretion, has been proven to protect chicks and broilers against stress, significantly reducing the heterophil-to-lymphocyte ratio. The aim of the present study was to test the effects of the MHUSA on chicks' stress when single-sprayed on their fluff at the age of 1 d. Two-hundred eighty ROSS 308 chicks were included in the study. At day 1, each chick received a spray of 200 μL of a 2% MHUSA aqueous solution (140 chicks) or the same amount of the excipient (control group, 140 chicks), and then chicks were housed in 2 separate rooms. To assess the persistence of the MHUSA after this single application, fluff was sampled from 10 chicks every day for 7 d and at day 13 and 19, weighed, placed in dichloromethane, and analyzed by gas chromatography. Blood smears and the bursa of Fabricius were collected every 3 d from 10 chicks of each group for 36 d to assess the heterophil-to-lymphocyte ratio and the bursa weight–to–BW ratio, respectively. Gas chromatography analysis showed that the MHUSA was present on chick fluff until day 5. The statistical analysis revealed that the heterophil-to-lymphocyte ratio was lower in the MHUSA group at day 4, 7, and 9 ($P < 0.0001$ for day 4 and 7; $P = 0.0377$ for day 9). The bursa weight–to–BW ratio was significantly higher in the MHUSA group than in the control group from day 4 until day 29. These results confirm the beneficial effects of the MHUSA on chicks' adaptation to the new environment and on bursa of Fabricius development, suggesting its potential role in improving chicks' immune response.

Key words: bursa of Fabricius, chick, MHUSA, pheromone, stress

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INTRODUCTION

Broiler chicks are exposed to stressful conditions during all phases of their life, such as transportation (Jacobs et al., 2016; Li et al., 2017), frequent handling (Wein et al., 2016), and high-density housing (Dennis et al., 2004; EFSA, 2010). In broiler chicks, as in other vertebrates, stress activates the hypothalamic-pituitary-adrenocortical (HPA) axis, with the consequent release of corticosterone by the adrenal glands (Mormède et al., 2007; Goessling et al., 2015). This glucocorticoid hormone exerts several effects that strongly reduce chickens' life quality, leading to decreased growth, increased fear behaviors and immunodepression, and a decrease in the gastrointestinal function (Shini et al., 2010; Scanes, 2016). For these reasons, stress is responsible for decreased productivity and meat quality and increased morbidity and mortality (Madec et al., 2006; Scanes, 2016; Wein et al., 2016).

In the last decade, the synthetic analog of a maternal semiochemical secretion named the mother hen uropygial

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secretion analogue (MHUSA) has been proven to play a role in the protection of chicks and broilers from the effects of stress (Madec et al., 2008a). This semiochemical is an analog of the secretion naturally produced by the uropygial gland of mother hens from 4 d before hatching until separation, and its efficacy in reducing stress and improving welfare in broilers has been verified by a decrease in the heterophil-to-lymphocyte ratio (**HLR**) and blood corticosterone levels (Madec et al., 2008b). Thanks to its effects on stress control, MHUSA application has also been associated with increased growth and meat quality in broilers (Madec et al., 2006, 2008c, 2009).

As mentioned before, farm chickens' lives are characterized by stressful conditions, such as transportation of day-old chicks (Jacobs et al., 2016), high-density housing (Dennis et al., 2004), and frequent manipulations (Wein et al., 2016). The aim of the present study was to evaluate if a single application of the MHUSA sprayed on the fluff of 1-day-old chicks could have an influence on some stress biological markers, such as the HLR (Goessling et al., 2015) and the bursa weight–to–BW ratio (BBR) (Cazaban et al., 2015).

MATERIALS AND METHODS

Animals and Husbandry

Chickens were reared according to current European Union regulations and to guidelines provided for the ROSS 308 strain. This study was conceived and performed in strict accordance with the French (2013-118) and European law (2010/63/EU) on the protection of animals used for scientific purposes. The experiment was approved by Ethics committee of Research Institute in Semiochemistry and Applied Ethology (IRSEA) (C2EA125).

Two-hundred eighty ROSS 308 chicks were purchased from a single commercial hatchery (Couvoir Grelier-Hendrix–La bohardière, St Laurent de la Plaine, France). After transportation to the module of Semiochemistry and Ethology for vertebrate species of IRSEA, they were randomly housed in 2 separate and independent experimental rooms, 140 chicks in each room, with relative humidity, daylight cycles, and temperature that were set according to recommendations for ROSS chickens in a commercial setting. Commercial feed was provided ad libitum, depending on the age of the chickens and according to recommendations. In addition, water was provided ad libitum.

Treatment Administration

At day 1, as 1-day-old chicks arrived at IRSEA's module, they immediately received the treatments. Half of the chicks (n = 140, the "MHUSA group" in the text) were individually sprayed on the fluff with 200 μL of a 2% MHUSA aqueous solution and housed in a room. The MHUSA solution was composed of the 4 compounds described by Madec et al: methyl laurate, methyl palmitate, methyl oleate, and methyl palmitate (2008c). Treatment concentration was selected according to previous studies (Madec et al., 2006), whereas the volume was chosen as it allowed a complete application on the chicks' back fluff. The remaining 140 chicks (the "control group" in the text) were sprayed with 200 μL of the excipient (a solution containing tween 80, water, methyl paraben, propyl paraben, 2-phenoxyethanol, Tinogard TT) and housed in the second room. These experimental rooms were completely independent, separated by a controlled pressure chamber and ventilated by 2 independent systems without any kind of communication between them. The stocking density at the beginning of the study was 11.7 chicks/m^2.

Fluff Sampling and MHUSA Persistence Analysis

Fluff was sampled every day from day 1 to day 7 and at day 13 and 19, cutting it with a pair of surgical scissors (one set of scissors per group, accurately washed between one chick and the following chick). For each sampling day, 10 chicks per group were sampled.

Fluffs were then individually weighed and analyzed by gas chromatography (GC-FID, PerkinElmer, Waltham). A sample of 5 mg of fluff was placed in 5 mL of dichloromethane, and the vials were placed for 30 min in an ultrasonic bath to extract the hydrophobic compounds of the MHUSA. After filtration, an aliquot of the solution was transferred into a 2-mL vial before gas chromatography mass spectrometry analysis. Gas chromatography mass spectrometry analyses were carried out using a GCMS QP2010 Plus system (Shimadzu Corporation, Kyoto, Japan) equipped with a Combi PAL AOC 5000 autosampler (PAL System, CTC Analytics). The capillary column was 30 m × 0.22 mm i.d. and had 0.22-μm film thickness SGE BP21 (nitroterephthalic acid modified polyethylene glycol). The GC oven temperature was initially held at 50°C for 3 min and was then ramped at 15°C min^{-1} to 240°C and held at this temperature for 10 min. The carrier gas was helium at 100 kPa. Analyses were performed in the splitless mode with a sample injection unit (250°C, 1 μL) or an OCI-PTV on-column/programmed temperature injection unit, depending on the analyte concentration.

The PTV injection conditions were as follows: 30-μL sample volume, splitless injection for 3 min, temperature initially held at 35°C for 1 min, then ramped at 300°C min^{-1} to 320°C, and held at this temperature for 15 min.

The identification of the analytes was performed on EI mass spectra in normal full scan mode from 40 to 350 m/z to avoid false positives because of matrix interferences. The transfer line and ionization source temperatures were set at 200°C. The scan speed and scan time were set at 5,000 amu/s and 0.08 s, respectively.

Blood Sampling for HLR Assessment

The HLR was evaluated at day 1, 4, 7, 10, 13, 16, 19, 22, 29, and 36 on 10 chickens per group. Animals were anesthetized with isoflurane by means of masks

adequate to their size and connected to an anesthetic machine (MSS International, Keighley, UK). When animals were deeply anesthetized, a small amount of blood was sampled by means of a 1-ml syringe connected to a 26 G needle. One drop of blood was used to obtain a blood smear for each chicken. Once air-dried, smears were stained using a May-Grunwald-Giemsa commercial kit (RAL555, RAL Diagnostic, Martignac, France) according to manufacturer's instructions for microscopic observation. A total of one hundred lymphocyte and heterophil cells were counted at 250x magnification, and the HLR was calculated by dividing the number of heterophils by the number of lymphocytes (Campbell, 2015).

Bursa of Fabricius Sampling and BBR Assessment

As for the HLR, the BBR was evaluated at day 1, 4, 7, 10, 13, 16, 19, 22, 29, and 36 on 10 chickens per group. After the blood sampling, chickens were humanely euthanized with an overdose of isoflurane and weighed. The bursa of Fabricius (**BF**) was collected during the necropsy and weighed with a precision balance, and its diameter was measured using a Vernier caliper. Then, the BF was placed in 10% neutral buffered formalin (pH 7.4) for histopathological analysis, according to previous literature (Cazaban et al., 2015). After 48 h, samples were submitted to routine tissue processing. They were paraffin embedded, and 4-µm-thick sections were then cut and stained with hematoxylin and eosin. The aim of the histological examination was to evaluate the presence of any possible BF alterations that could influence its weight.

The BBR was obtained according to the formula proposed by Cazaban et al. (2015): BBR = [BF weight (g)/ BW (g)] × 100.

Statistical Analysis

Statistical analysis was carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC). The parameters included in the statistical analysis were the following: the chick weight, BF weight, BF volume, BBR, and HLR. For each time point, the mean values of the MHUSA group (n = 10) were compared with those of the control group (n = 10). For each time point, assumptions of the normality and homogeneity of variances were verified, using the univariate procedure for normality and the t-test procedure for homogeneity of variances. If the conditions were verified, a Student's t-test was performed. If normality was not verified, the nonparametric alternative of the Wilcoxon test was preferred and computed using the npar1way procedure. If normality was verified but variances were heterogeneous, the Welch test was used, using the t-test procedure. The significance threshold was classically fixed at 5%.

RESULTS

The MHUSA was detected on the fluff of 10 of 10 chicks (100%) at all days until day 4, and in 8 of 10 chicks (80%) at day 5. The MHUSA was not detectable on chicks' fluff at day 6, 7, 13, and 19. The MHUSA presence on chicks' fluff and MHUSA's compounds concentration are reported in Table 1.

Regarding chicks' weight, a statistically significant difference was observed at day 1, with the MHUSA group having a higher weight than the control group ($P = 0.0326$, Student's t-test). No significant differences in weight were observed on the other sampling days.

The MHUSA group had a significantly lower HLR on day 4, 7, and 10 ($P < 0.05$), whereas no differences were observed on other days. The HLR data and related P-values are reported in Table 2.

The BF volume was greater in the MHUSA group from day 4 to the last sampling ($P < 0.01$). The MHUSA group also had heavier BFs and a higher BBR than the control group from day 4 to day 29 ($P < 0.05$). All the data concerning BF parameters are detailed in Table 3. The histological examination revealed that all the 200 BF (10 samples × 10 times × 2 groups) were well conformed, and no histopathological lesions were observed (Figure 1).

DISCUSSION

According to our results, the MHUSA seems to have positive effects on chicks' health when sprayed on their fluff at the first day of life. In fact, chicks that received this treatment had a reduced HLR until 10 d from its application and a more developed BF during all the period of study. Because these parameters are known as reliable indicators to measure the presence of stress in poultry (Cazaban et al., 2015; Goessling et al., 2015), we can assume that this single MHUSA application is able to help chicks to cope since their first day of life, with the stressful events that characterize the poultry production system. Because the native uropygial secretion is continuously secreted by the mother hen from 4 d before hatching until separation (Madec et al., 2008c), our study suggests that the application of the MHUSA from the first day of life can reproduce the natural effect of the native secretion in chicks that have never been in contact with the mother hen. Chicks that receive the native or the analog mother hen secretion are thus more able to cope with the farm environment than chicks deprived of this crucial element.

An HLR decrease was measured at the first sampling after the application day (day 4), and it was present until day 10 and disappeared at day 13. It is interesting to observe that MHUSA effects on the HLR persisted some days more than MHUSA persistence on chicks' fluff. In fact, the gas chromatography analysis revealed the presence of the MHUSA until day 5, although the HLR was significantly lower in the MHUSA group than in the control group until day 10. As is well documented, the HLR increases during stressful conditions by the activation of

Table 1. The MHUSA percentage of the presence on treated chicks' fluff, MHUSA's compound concentration in the fluffs' dichloromethane extract and concentration ranges from day 1 to day 19 (n = 10 per day of sampling).

	MHUSA	Methyl laureate			Methyl palmitate			Methyl oleate			Methyl linoleate		
Day	% Of positive chicks	Mean concentration (µg/L)	Range min-max	% Of positive chicks	Mean concentration (µg/L)	Range min-max	% Of positive chicks	Mean concentration (µg/L)	Range min-max	% Of positive chicks	Mean concentration (µg/L)	Range min-max	% Of positive chicks
1	100	19	4-90	100	285	9-2456	100	93	30-314	100	150	14-873	100
2	100	13	3-34	100	59	6-146	100	33	4-114	100	63	12-275	100
3	100	16	4-47	100	25	4-95	100	13	3-43	100	45	6-189	100
4	100	18	8-29	100	51	15-161	100	46	2-200	100	43	6-134	100
5	80	24	5-43	100	53	0-146	90	39	0-185	90	44	0-153	90
6	0	18	0-38	60	48	0-169	90	51	0-246	50	21	0-10	60
7	0	22	0-73	80	54	0-218	50	43	0-250	40	26	0-179	40
13	0	0	/	0	0	/	0	0	/	0	0	/	0
19	0	0	/	0	0	/	0	0	/	0	0	/	0

The MHUSA remanence was defined as the simultaneous presence of all the four compounds on the fluff of the single chick.
Abbreviation: MHUSA, mother hen uropygial secretion analogue.

Table 2. Heterophil-to-lymphocyte ratios in ROSS 308 chicks included in the study (n = 10 per group).

Parameter	Day 1	Day 4	Day 7	Day 10	Day 13	Day 16	Day 19	Day 22	Day 29	Day 36
HLR (SD)										
Control group	0.435 (0.087)	0.542 (0.070)	0.498 (0.071)	0.492 (0.181)	0.399 (0.170)	0.525 (0.118)	0.554 (0.107)	0.923 (0.107)	0.869 (0.105)	0.911 (0.045)
MHUSA group	0.439 (0.088)	0.245 (0.051)	0.277 (0.066)	0.349 (0.063)	0.349 (0.044)	0.521 (0.062)	0.531 (0.101)	0.914 (0.117)	0.860 (0.079)	0.866 (0.034)
P-value	0.9203	**<0.0001**	**<0.0001**	**0.0377**[1]	0.3874[1]	0.9353	0.6351	0.8504	0.8439	0.4257

Bold indicates statistically significant values.
Abbreviations: HLR, heterophil-to-lymphocyte ratio; MHUSA, mother hen uropygial secretion analogue.
[1]Welch's t-test; all the other comparisons were conducted using the Student's t-test.

Table 3. Bursa of Fabricius volume, weight, and bursa weight–to–BW ratios in ROSS 308 chicks included in the study (n = 10 per group).

Parameter	Day 1	Day 4	Day 7	Day 10	Day 13	Day 16	Day 19	Day 22	Day 29	Day 36
Bursa of Fabricius volume: mm^3 (SD)										
Control group	82.2 (26.3)	195.2 (66.6)	364.4 (160.2)	501.9 (194.3)	823.8 (156.5)	1762.2 (401.3)	1274.9 (459.9)	1938.3 (1102.4)	3566.2 (1050.3)	5504.3 (3282.9)
MHUSA group	105.3 (23.8)	344.5 (59.4)	678.8 (116.6)	1003.7 (242.3)	1479.8 (324.6)	2911.3 (861.9)	2991.1 (1174.8)	4191.6 (752.4)	6566.3 (1905.2)	9516.1 (3189.4)
P-value	0.0538	**<0.0001**	**0.0010**[1]	**<0.0001**	**<0.0001**[2]	**0.0022**[2]	**0.0011**[2]	**<0.0001**	**0.0004**	**0.0036**[1]
Bursa of Fabricius weight: g (SD)										
Control group	0.078 (0.022)	0.129 (0.033)	0.234 (0.056)	0.351 (0.139)	0.588 (0.111)	0.961 (0.213)	1.057 (0.349)	1.505 (0.595)	2.370 (0.515)	3.398 (1.393)
MHUSA group	0.087 (0.019)	0.162 (0.030)	0.314 (0.045)	0.514 (0.090)	0.812 (0.042)	1.381 (0.256)	1.889 (0.544)	2.396 (0.412)	3.143 (0.578)	3.766 (0.227)
P-value	0.3752	**0.0367**	**0.0024**	**0.0061**	**0.0007**	**0.0008**	**0.0007**	**0.0011**	**0.0055**	0.4688
BBR (SD)										
Control group	0.174 (0.054)	0.151 (0.029)	0.152 (0.024)	0.161 (0.054)	0.164 (0.026)	0.174 (0.036)	0.166 (0.061)	0.157 (0.045)	0.146 (0.035)	0.159 (0.056)
MHUSA group	0.174 (0.041)	0.192 (0.042)	0.195 (0.022)	0.222 (0.049)	0.216 (0.029)	0.255 (0.047)	0.269 (0.057)	0.237 (0.049)	0.196 (0.034)	0.164 (0.027)
P-value	0.9989	**0.021**	**0.0006**	**0.0167**	**0.0006**	**0.0004**	**0.0011**	**0.0015**	**0.0046**	0.8149

Bold indicates statistically significant values.
Abbreviations: BBR, bursa weight–to–BW ratio; MHUSA, mother hen uropygial secretion analogue.
[1]Wilcoxon two-samples test.
[2]Welch's t-test; all the other comparisons were conducted using the Student's t-test.

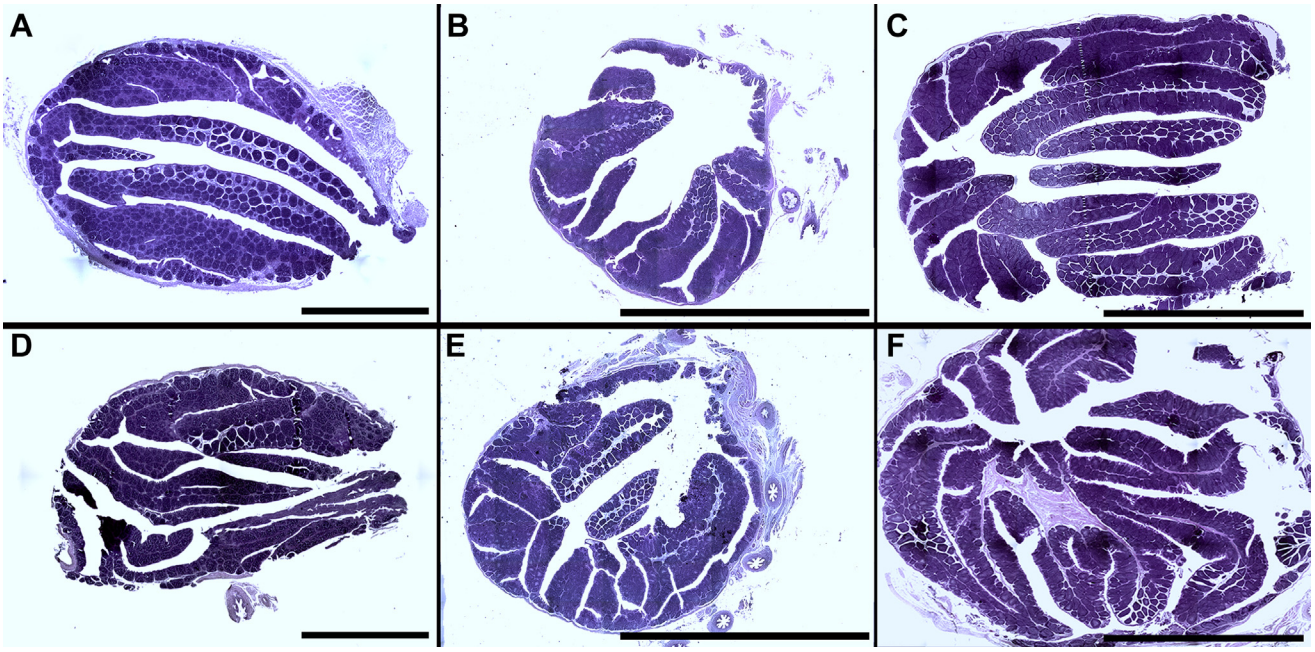

Figure 1. Sections of the bursa of Fabricius samples in control and MHUSA-treated chicks. (A, B, and C): The bursae of Fabricius belonging to chicks from the control group, sampled at day 1, 10, and 22, respectively. (D, E, and F): The BF belonging to chicks from the MHUSA group, sampled at day 1, 10, and 22, respectively. The bursae of Fabricius were submitted to the histological analysis to exclude the presence of any possible alteration that could influence its weight. Sections were stained with hematoxylin and eosin and observed at the light microscope. No alterations were observed in the 280 BFs. (hematoxylin and eosin stain, A, D: bar = 2 mm; B, E: bar = 1 cm; D, F: bar = 2 cm). Abbreviations: BF, bursa of Fabricius; MHUSA, mother hen uropygial secretion analogue.

the HPA axis, which leads to glucocorticoid production and release (Scanes, 2016). Glucocorticoids induce lymphocyte sequestration in other tissues, such as the spleen, lymph nodes, and bone marrow, decreasing their blood concentration and thus increasing the HLR (Davis et al., 2008). The MHUSA has been previously described to be able to protect chickens from this effect (Madec et al., 2008b), and the results of the present study further confirm this finding, also showing that this protection continues at least 5 d after the end of MHUSA persistence on the fluff. Because the HLR increase represents the consequence of a chronic growth of circulating glucocorticoids and of the associated progressive sequestration of lymphocytes in other tissues (Davis et al., 2008), this 5-d discrepancy could be the time needed before the expression of this mechanism. Consequently, after day 10, the HLR started increasing also in the MHUSA group, as chicks were less protected from rearing stressors by MHUSA effects.

Another interesting finding was that chicks that received the MHUSA had a more developed BF than control chicks in most phases of our study. The association between the MHUSA application and a higher BBR confirms the role of this semiochemical analog in protecting chicks from stressful conditions. Because this parameter represents the relative weight of the BF on the BW of the individual, it is a very accurate measure to evaluate BF development (Cazaban et al., 2015), and it is commonly used to investigate the efficacy of immunity, vaccines, dietary supplementation, and chickens' response to stress (Cazaban et al., 2015; Jeon et al., 2017; Zhang et al., 2017; Wu et al., 2018). Based on the literature, we chose to include this parameter in our study. The results showed that one single application of the MHUSA on chicks' fluff can improve the development of the BF from the first day of birth until 1 mo of life. The histological analysis revealed that all the BFs (from both the MHUSA and control groups) were normally conformed and did not have any kind of alteration. Thus, the difference in the BF weight (both as an absolute and relative value) should be due to a reduced proliferation of B cells in the BF of the chicks that did not receive MHUSA administration, as this organ is mainly composed of these lymphocytes (Tizard, 2012). To the best of our knowledge, this is the first report describing the effect of a maternal semiochemical analog on an immune organ in animals.

It seems logical that MHUSA effects on BF development are due to its capability to protect chickens from stressful conditions. Among its several effects, chronic stress induces the suppression of the immune system and the involution of immune organs (Shini et al., 2010). Moreover, Shini et al. (2008) clearly stated that the increase in corticosterone plasma levels leads to retardation in the BF development. Taking into account these findings, we can assume that the MHUSA may play a role in immunity regulation through its effects on glucocorticoid release, as revealed by the HLR and BF growth. Therefore, these results could open other interesting considerations about the influence of maternal semiochemical analogs on animal immunity.

On the other hand, one single MHUSA application had no effect on the chick BW, contrary to what was observed by Madec et al (2006; 2008c). In these 2

studies, the MHUSA was administered continuously from the beginning until the end of the study (during 4 wk or 80 d, respectively), using gelatin matrix blocks that diffused the semiochemical analog in farm air (Madec et al., 2006, 2008c), while in the present experiment, the treatment was available from day 1 to day 5, as shown by gas chromatography analysis. This difference may explain the results, as this dosage does not last long enough to improve the chickens' BW. Moreover, the HLR started increasing in the MHUSA group 5 d after the end of the treatment, showing that the protection against stress was becoming ineffective, presumably having a negative effect on the BW. Therefore, we can state that an MHUSA fluff application on day-old chicks protects them from stress during the first week, which represents a period rich in stressful conditions, such as transportation, adaptation to a new space, and frequent handling (Dennis et al., 2004; Wein et al., 2016; Jacobs et al., 2016), improving their welfare and preparing them to better cope with the following rearing phases. However, to continue obtaining effects such as stress reduction, increased growth, and meat quality (Madec et al., 2006, 2008c, 2009), it seems essential to continue the MHUSA diffusion from 1 wk after the first administration at day 1.

To conclude, this study demonstrated the potential use of the MHUSA to reduce chicks' stress during their first days of life, confirming its previously described effects and paving the way for its use immediately from the posthatching period.

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