PHYSIOLOGY AND REPRODUCTION

The effect of methionine and folic acid administered in ovo on the hematological parameters of chickens (*Gallus gallus domesticus*)

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ABSTRACT Methionine (**Met**), an essential amino acid in poultry diets, when overdosed may cause hyperhomocysteinemia, which is mainly a trigger for cardiovascular diseases in humans. Homocysteine is neutralized (remethylated) in the presence of folic acid (**FA**), which also plays an important role in hematopoiesis and participates in the synthesis of DNA, and its deficiencies may result in the development of neural tube defects. One of the basic tools in studying the impact of both xenobiotics and nutrients on the animal organism is hematological analysis. Thus, the aim of this study was to determine the effect of *in ovo* supplementation with Met and FA on the hematological parameters of broiler chickens. On the 17th day of incubation, embryonated eggs (Ross 308) were injected with 5 or 25 mg of Met per egg (M5 and M25), 3 and 15 mg of FA per egg (F3 and F15), or a mixture of these 2 compounds (M5/F3 and M25/F15). The broilers were reared in accordance with welfare regulations and fed with commercial diets *ad libitum*. Blood samples were collected on the first, seventh, and 35th day of rearing (D1, D7, and D35), and complete hematological analysis was performed. The observed changes in red blood cell parameters probably result from physiological changes occurring during bird growth. Mean erythrocyte volume decreased with the age of chickens in the control, M5, and M25 groups, but not in those supplied with FA. Among supplemented groups, the number of white blood cells on D1 was lower only in group M5 than in the sham (C) group. The analysis of leukograms showed no significant differences between the groups. Comparing D1 with D7 in the group injected with a higher dose of Met and FA (MF25/15), a statistically significant increase in the percentage of lymphocytes and a significant decrease in the percentage of heterophils were observed. In addition, in the group injected with a higher FA dose (F15), there was statistically significant reduction in the percentage of eosinophils and a significant increase in the percentage of monocytes at day 7 compared with day 1. It seems that Met supplementation led to temporary immunosuppression in the animals.

Key words: egg, amino acid, blood, toxicity

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INTRODUCTION

The avian (chick) embryo is a recognized model in biological and medical research (Stern, 2005, 2018); its advantage is that it provides the ability to observe individual stages of development using invasive (Schoenwolf, 2018) and noninvasive methods (Pawlak et al., 2011). This model is successfully applied in toxicological (Dżugan and Lis, 2016; Batoryna et al., 2018; Stark and Ross, 2019) and pharmacological studies (Lis et al., 2009; Pawlak et al., 2011; Bjørnstad et al., 2015). Moreover, the good manipulation tolerance of the egg structure of late-stage embryos allows parenteral immunization (*in ovo* vaccination) (Peebles 2018; Vandeputte et al., 2019) or, for stimulation of the chick's postembryonic development (Roto et al., 2016), supplementation with nutrients (*in ovo* feeding), for example, carbohydrates (Foye et al., 2006; Peebles, 2018; Retes et al., 2018) and amino acids (Ohta et al. 2004; Peebles, 2018), or others, for example, prebiotics (Stefaniak et al., 2019).

Methionine (**Met**) is an essential amino acid which is commonly used as a supplement in broiler diets to limit the use of other amino acids contained in feed

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(Jankowski et al., 2014; Albrecht et al., 2017). An increase in Met concentration stimulates the transport and absorption of lysine and arginine (Balnave et al., 1999; Bunchasak, 2009). Moreover, along with cysteine and glycine, it is the main constituent of the amino acid glutathione, which is an important link in the antioxidant system (Atmaca and Fry, 2005). In addition, this amino acid has a huge impact on the proper course of organogenesis in vertebrates and plays an important role in the body's production of tubulin, neurofilaments, and actin embryos (Coelho and Klein, 1990; Moephuli et al., 1997). Methionine deficiency can also handicap the formation of the circulatory and lymphatic systems of chick embryos, thus disturbing the division and differentiation of mesenchymal cells (Brosnan and Brosnan, 2006). In the light of this information, it seems that Met supplementation would have a positive effect on chick embryo development. On the other hand, it should be noted that Met undergoes methylation to homocysteine and may cause hyperhomocysteinemia when overdosed. In humans, hyperhomocysteinemia is mainly a trigger for cardiovascular diseases (atherosclerosis, congestive heart failure) and age-related macular degeneration, Alzheimer disease, and hearing loss (Osunkalu et al., 2010; Kim et al., 2018). Homocysteine is remethylated in the presence of folate (vitamin B9, folacin) (Scaglione and Panzavolta, 2014); therefore, providing it in a more stable form, namely, folic acid (**FA**), is medically recommended as beneficial for the circulatory system (Blom and Smulders 2011; Ganguly and Alam, 2015; Liu et al.,); it also acts against the formation of congenital and acquired heart defects (Lucock, 2004; Oakley et al., 2004; Cornel et al., 2005) and is recommended for pregnant women to reduce the risk of fetal malformations (Geisel, 2003). Folates also play an important role in hematopoietic processes and in the synthesis of DNA; their deficiencies lead to the formation of megaloblastic anemia, among others (Green and Data Mitra, 2017).

The aim of this study was to investigate whether *in ovo* supplementation with Met and FA influences the hematological parameters of chickens (*Gallus gallus domesticus*).

MATERIALS AND METHODS

Hatching Eggs

A total of 600 hatching eggs with the correct shape and weight (mean ± SD = 62 g ± 5.2 g) from the ROSS 308 line (Aviagen Group, Huntsville, AL) of 42-week-old parental broiler stock (Sławomir Domagała, Poultry Farm, Gołaczewy, Poland) were used in the experiment. The eggs were stored for about 72 h at a temperature (t) of 17°C ± 0.5°C and relative humidity (**RH**) of 70%; they were then gradually heated to 25°C for 12 h before the planned start of incubation and fumigated immediately before setting (Fasenko, 2007).

Incubation Technology

The eggs were set in Masalles 65 Digite laboratory incubators (Masalles Europe S.L., Barcelona, Spain) and incubated at 37.8°C ± 0.1°C and 50 ± 1% RH for 1 to 18 D of incubation (E1–E18) and at 37.2°C ± 0.1°C and 55 to 70% RH between E19 and E21. Until E18, the eggs were set on trays tilted at an angle of 45° and turned by 90° every h; they were then transferred to hatch baskets on E19. The candling for the elimination of eggs that were damaged, were unfertilized, or contained dead embryos was performed at E6 and E17 (immediately before *in ovo* injection).

Experimental Procedures

At E17, the embryonated eggs were selected and divided into 8 parallel groups (n = 70); the surface of every egg was disinfected with 70% ethanol. Next, a small hole in the shell (diameter about 2 mm) was made using an 18G needle (Nipro Medical Poland Ltd., Warszawa, Poland) at the big end of the egg. The eggs were injected with Met (L-Methionine; No. 64319, BioUltra, ≥99.5%; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a dose of 5 mg (M5) and 25 mg (M25) per egg, FA (No. F7876, ≥97%, Sigma-Aldrich, Merck KGaA) at a dose of 3 mg (F3) and 15 mg (F15), or a mixture of these 2 compounds (M5/F3 and M25/F15) (Table 1). The weight ratio of Met to FA (5:3) was similar to that in which both substances are present in the egg (Mine, 2008). The compounds were dissolved in 250 mikroL of 0.7% saline solution and injected into the amniotic sac according to the previously described method (Foye et al., 2006; Lis et al., 2009). The control group eggs were not injected (O) or injected only with 0.7% saline solution (C). After manipulation, the holes were sealed with hot paraffin, and incubation was continued. After hatching, 20 one-day-old chicks (10 females and 10 males) were decapitated for blood collection, 40 chicks

Table 1. Scheme of administering methionine and folic acid to chicken embryos.

Group	Eggs (number)	Methionine dose/egg	Folic acid dose/egg	Solution 0.7% NaCl/egg
O	70	–	–	–
C	70	–	–	250 µL
M5	70	5 mg	–	250 µL
M25	70	25 mg	–	250 µL
MF5/3	70	5 mg	3 mg	250 µL
MF25/15	70	25 mg	15 mg	250 µL
F3	70	-	3 mg	250 µL
F15	70	-	15 mg	250 µL
Total	560			

Table 2. Composition of the diet supplied to the broiler chickens of all the experimental groups.

Item	Starter (0–14 D)	Grower (15–28 D)	Finisher (29–35 D)
Wheat, %	60.80	65.60	69.30
Soybean meal, %	31.00	23.00	18.50
Soyabean oil, %	4.70	5.90	5.70
Rapeseed cake, %	0.00	2.5	4.00
Poultry mix broiler, %	3.50	3.00	2.50
Dry mass, %	88.57	88.92	88.82
Crude protein, %	21.74	20.57	19.45
Lipid, %	7.16	7.97	7.89
Fiber, %	2.75	2.74	2.85
Ash, %	4.72	4.58	4.03
ME, Kcal/kg	2,998	3,070	3,100
Lysine, %	1.25	1.16	1.08
Methionine, %	0.54	0.53	0.49
Methionine + cysteine, %	0.92	0.89	0.85
Threonine, %	0.85	0.81	0.77
Calcium, %	0.68	0.67	0.54
Phosphate, %	0.47	0.46	0.41
Sodium, %	0.16	0.16	0.16
Chlorine, %	0.22	0.22	0.26

Abbreviation: d, day of rearing.

were randomly selected in each group (20 females and 20 males), marked with individual wing marks, and reared in deep litter under production conditions in accordance with welfare regulations and instructions for rearing chickens in the Ross 308 line (Aviagen). Breeding of broiler chickens took place on deep litter for a period of 35 D. The broilers were fed with commercial pellet (De Heus-Polska, Łęczyca, Poland, Table 2) and water *ad libitum* according to their age. Feed conversion ratio was calculated as cumulative feed intake divided by weight gain on a group basis.

Collection of Blood Samples

At the first day of rearing (D1), 10 males and 10 females were randomly selected from each group; after decapitation, blood was collected from the jugular vein in EDTA-coated tubes. On the seventh day of rearing (D7) and after decapitation, blood from the jugular vein was collected from 20 chicks from each group (10 females and 10 males). On the 35th day of rearing (D35), blood was collected from 20 chicks from each group (10 females and 10 males) from the wing vein.

Hematological Analysis

Analysis of the number of red blood cells (**RBC**) (10^6/μL), hemoglobin (**Hb**) concentration in blood (g/dL), and hematocrit (**Ht**; %) was performed using the IDEXX LaserCyte hematology analyzer (IDEXX Europe B.V., Hoofddorp, The Netherlands). Moreover, the following were calculated according to standard formulas: mean corpuscular volume of red blood cell (**MCV**; fL) as Ht/RBC; mean corpuscular Hb (**MCH**; pg) as Hb/RBC; and MCH concentration (**MCHC**) as Hb × 100/Ht. The number of white blood cells (**WBC**) was determined by the manual method using Natt–Herrick's stain solution. The blood was diluted in this solution at a ratio of 1:200; subsequently, leukocytes and thrombocytes were counted in a Bürker chamber (BRAND GMBH + CO KG, Wertheim, Germany) (magnification: 400×). Smears were also made and stained using the MERCK Hemactolor staining kit from MERCK (Merck KGaA, Darmstadt, Germany).

Statistical Analysis

Hematological data were analyzed using the Kruskal–Wallis test followed by the multiple comparison (post

Table 3. Number of erythrocytes (10^6/μL) in the blood of chickens on the first, seventh, and 35th day of life (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	$1.70 \pm 0.618^{A,B}$	1.80 ± 0.112^{A}	2.72 ± 0.361^{B}
C	$1.80 \pm 0.407^{A,B}$	1.82 ± 0.172^{A}	2.77 ± 0.262^{B}
M5	$1.92 \pm 0.723^{A,B}$	1.85 ± 0.074^{A}	2.81 ± 0.203^{B}
M25	1.75 ± 0.622^{A}	1.82 ± 0.205^{A}	2.91 ± 0.266^{A}
MF5/3	1.88 ± 0.325^{A}	1.96 ± 0.212^{A}	2.76 ± 0.390^{A}
MF25/15	$2.07 \pm 0.187^{A,B}$	1.87 ± 0.172^{A}	2.91 ± 0.241^{B}
F3	$2.37 \pm 0.528^{A,B}$	1.94 ± 0.176^{A}	2.80 ± 0.176^{B}
F15	2.00 ± 0.299^{A}	1.89 ± 0.172^{A}	2.71 ± 0.226^{A}

$^{A–C}$Values marked with different capital letters in a given row differ statistically significantly ($P < 0.05$).

Table 4. Concentration of hemoglobin (g/dL) in whole blood on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	$10.35 \pm 1.559^{A,B}$	8.87 ± 0.585^{A}	12.48 ± 1.193^{B}
C	$10.37 \pm 1.167^{A,B}$	8.89 ± 0.564^{A}	12.49 ± 1.206^{B}
M5	$11.56 \pm 1.014^{A,B}$	8.75 ± 0.493^{A}	13.81 ± 0.867^{B}
M25	11.93 ± 0.984^{A}	9.08 ± 0.748^{A}	13.12 ± 1.131^{A}
MF5/3	10.59 ± 0.778^{A}	9.47 ± 0.792^{A}	12.62 ± 1.380^{A}
MF25/15	$10.32 \pm 0.502^{A,B}$	9.10 ± 0.600^{A}	13.12 ± 0.773^{B}
F3	$10.09 \pm 1.420^{A,B}$	9.26 ± 0.823^{A}	12.62 ± 0.623^{B}
F15	$10.62 \pm 1.961^{A,B}$	9.20 ± 0.758^{A}	12.39 ± 0.812^{B}

$^{A–C}$Values marked with different capital letters in a given row differ statistically significantly ($P < 0.05$).

Table 5. Hematocrit value [%] on the first, seventh, and 35th day of life of chickens (D1, D7 and D35).

Group	D1	D7	D35
	Mean ± SD	Mean ± SD	Mean ± SD
O	29.52 ± 3.476^{A}	27.79 ± 1.979^{A}	36.73 ± 5.297^{A}
C	30.44 ± 4.152^{A}	27.39 ± 2.294^{A}	36.89 ± 3.736^{A}
M5	32.61 ± 6.062^{A,B}	27.61 ± 1.407^{A}	38.39 ± 2.909^{B}
M25	31.63 ± 4.185^{A}	28.77 ± 2.787^{A}	39.71 ± 4.055^{A}
MF5/3	27.61 ± 3.185^{A}	30.07 ± 2.897^{A}	38.14 ± 5.930^{A}
MF25/15	29.46 ± 2.409^{A}	29.27 ± 2.566^{A,B}	39.71 ± 2.768^{B}
F3	27.71 ± 4.364^{A,B}	29.46 ± 3.106^{B}	38.43 ± 2.600^{C}
F15	29.55 ± 4.976^{A}	29.11 ± 2.924^{A}	36.77 ± 2.771^{A}

$^{A–C}$Values marked with different capital letters in a given row differ statistically significantly ($P < 0.05$).

hoc) test using Statistica 13.3 software (StatSoft Polska, Kraków, Poland). The level of significance was set at an α of 0.05.

RESULTS

There were no differences in the number of erythrocytes (RBC) in the blood microliter of chickens belonging to individual groups of the experiment, whereas a significant effect of age on this parameter was demonstrated (D7 vs D35) in the following groups: O ($P = 0.015$), C ($P = 0.047$), M5 ($P = 0.002$), MF25/15 ($P = 0.017$), and F3 ($P = 0.010$) (Table 3).

There were also no differences in the Hb concentration in the blood of chickens belonging to individual groups of the experiment, whereas as in the case of RBC, a significant effect of age (D7 vs D35) on the tested parameter was found in the following groups: O ($P = 0.005$), C ($P = 0.014$), M5 ($P = 0.001$), MF25/15 ($P = 0.001$), F3 ($P = 0.001$), and F15 ($P = 0.021$) (Table 4).

There was no effect of the injected substances on the Ht value, whereas the differences in time (age of the chicks) within particular groups were demonstrated. Statistically significant differences were recorded between day 7 and day 35 in groups M5 ($P = 0.001$) and F3 ($P = 0.001$) and between days 1 and 35 in groups MF25/15 ($P = 0.042$) and F3 ($P = 0.001$) (Table 5).

The MCV in day-old chicks injected with a lower dose of FA (group F3) was significantly lower than the mean recorded in the following groups: O ($P = 0.004$), C ($P = 0.000$), M5 ($P = 0.000$), and M25 ($P = 0.000$). A similar effect was not observed on the seventh or 35th day of the chick's life. In both control groups and groups injected with Met, a decrease in MCV was observed with age, and in the group injected with saline solution (C), the difference between the first and 35th day was statistically significant ($P = 0.001$). However, in groups supplemented with FA and a higher dose of a mixture of FA and Met, an increase in MCV was observed on the seventh day compared with the first day, and in the F3 group, it was statistically significant ($P = 0.000$; Table 6).

The MCH in erythrocytes in the first day of life of the hatchlings in group F3 was lower than in the case of other groups, and in relation to the group C and M25, the difference was statistically significant ($P = 0.023$ and 0.026, respectively). There was no influence of the age of the hatch (time) on the discussed parameter (Table 7).

There was no effect of injected substances and age of chicks (time) on MCHC (Table 8), platelet count in the blood microliter (Table 9), and leukocyte count (WBC) in the blood microliter (Table 10).

In the analysis of leukograms, no significant differences between the groups were observed. In contrast, the WBC image changed depending on the age of the chicks, and in the group injected with a higher dose of Met and FA (MF25/15), a statistically significant increase in the percentage of lymphocytes ($P = 0.003$), a significant decrease in the percentage of heterophils ($P = 0.002$), and a decrease in the heterophil-to-lymphocyte ratio ($P = 0.007$) was observed at day 7 compared with day 1. In addition, in the group injected with a higher dose of FA (F15), a statistically significant reduction in the percentage of eosinophils ($P = 0.014$) and a significant increase in the percentage of monocytes ($P = 0.016$) was observed at day 7 compared with day 1 (Table 11).

The final body weight range of 35-day-old chickens is as follows: for females, from 1,928 ± 167.9 g in the F15 group to 2,122 ± 231.3 g in the M5/F3 group; for males, from 2,160 ± 179.4 in the O group to 2,380.4 ± 278.8 g in the M5/F3 group; feed conversion ratio was similar in all groups (1.63–1.67).

Table 6. Mean corpuscular volume (fL) on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

Group	D1	D7	D35
	Mean ± SD	Mean ± SD	Mean ± SD
O	190.16 ± 59.100^{a,b,A}	154.89 ± 13.951^{A}	134.88 ± 3.283^{A}
C	171.97 ± 17.114^{a,b,A}	151.78 ± 18.231^{A,B}	133.20 ± 4.148^{B}
M5	186.55 ± 54.460^{a,b,A}	149.00 ± 8.003^{A}	136.84 ± 4.635^{A}
M25	196.08 ± 50.995^{a,b,A}	160.96 ± 28.407^{A}	136.42 ± 3.773^{A}
MF5/3	148.88 ± 19.089^{b,c,A}	155.06 ± 21.295^{A}	137.89 ± 4.590^{A}
MF25/15	142.67 ± 4.189^{b,c,A}	156.85 ± 15.331^{A}	136.42 ± 3.085^{A}
F3	118.46 ± 9.343^{c,A}	151.45 ± 4.088^{B}	137.08 ± 2.061^{A,B}
F15	147.96 ± 11.700^{b,c,A}	154.29 ± 2.371^{A}	135.63 ± 4.946^{A}

$^{A–C}$Values marked with different capital letters in a given row differ statistically significantly ($P < 0.05$).

$^{a–c}$Values marked with different lowercase letters in a given column differ statistically significantly ($P < 0.05$).

Table 7. Mean corpuscular hemoglobin (pg) on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	68.18 ± 22.900[b,c]	49.45 ± 4.478	46.13 ± 2.629
C	59.15 ± 9.957[a,b]	49.32 ± 5.876	45.15 ± 2.687
M5	71.31 ± 35.934[b,c]	47.23 ± 3.294	45.69 ± 1.736
M25	77.33 ± 29.991[a,b]	50.82 ± 8.813	45.12 ± 1.389
MF5/3	57.70 ± 11.181[b,c]	48.79 ± 6.046	45.86 ± 2.24
MF25/15	50.36 ± 5.636[b,c]	48.81 ± 4.434	45.12 ± 1.813
F3	43.46 ± 6.297[c]	47.74 ± 3.209	45.06 ± 1.467
F15	54.28 ± 14.555[b,c]	48.85 ± 1.667	45.73 ± 1.645

[a–c]Values marked with different lowercase letters in a given column differ statistically significantly ($P < 0.05$).

DISCUSSION

The ease of sample collection makes blood tests one of the basic diagnostic tools in both human and veterinary medicine (Talebi et al., 2005; Mitchell and Johns, 2008; Merska et al., 2013; Carisch et al., 2019). However, in practice, hematological tests are more rarely performed on avians than on mammals, mainly owing to the high labor intensity that is a result of the specificity of the blood cell structure and the absence of generally accepted reference values (Talebi et al., 2005; Keshavarz et al., 2019; Stefaniak et al., 2019).

An important element of hematological diagnostics is the assessment of RBC parameters, which often forms the basis of detecting malfunctioning of the circulatory system (Talebi et al., 2005). Methionine administered at higher doses may result in changes in the complete blood count (Toue et al., 2006). In our own studies, the analyzed RBC parameters, regardless of the age of birds and belonging to the experimental group, were generally in the range of reference values (Campbell, 2012). In chickens from groups injected with FA and a higher dose of a mixture of FA and Met, the number of RBC (Table 3) was statistically significantly higher in the first day of life than in the remaining groups. Increasing the RBC value may be indicative of transient hypoxia as a result of disturbances in the functioning of the cardiovascular system (Kalay et al., 2011). However, RBC count in all groups remained relatively stable at D7 and increased at D35, which could indicate the physiological nature of these changes and the fact that

Table 8. Mean corpuscular hemoglobin concentration (g/dL) on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	35.07 ± 3.294	31.94 ± 0.947	34.26 ± 2.861
C	34.27 ± 3.398	32.53 ± 1.380	33.90 ± 1.602
M5	36.50 ± 8.001	31.69 ± 1.066	33.40 ± 0.979
M25	38.38 ± 6.626	31.61 ± 0.796	33.07 ± 1.380
MF5/3	38.66 ± 4.294	31.54 ± 1.232	33.28 ± 1.710
MF25/15	35.28 ± 3.635	31.15 ± 1.064	33.07 ± 0.958
F3	36.72 ± 4.733	31.53 ± 2.039	32.88 ± 1.076
F15	36.45 ± 7.342	31.67 ± 1.348	33.75 ± 1.443

No statistically significant differences at $P < 0.05$.

Table 9. Number of platelets in the blood of chickens ($10^3/\mu L$) on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	23.33 ± 23.381	10.83 ± 5.845	5.83 ± 2.041
C	25.00 ± 17.889	6.67 ± 2.582	10.00 ± 7.746
M5	15.83 ± 10.685	13.33 ± 9.309	7.50 ± 4.183
M25	13.33 ± 6.831	8.33 ± 6.055	8.33 ± 6.055
MF5/3	15.83 ± 7.360	9.17 ± 5.845	28.00 ± 4.472
MF25/15	19.17 ± 15.303	11.67 ± 4.082	14.29 ± 8.864
F3	10.00 ± 7.746	13.75 ± 13.823	7.86 ± 5.669
F15	20.00 ± 8.944	10.00 ± 7.071	15.00 ± 5.774

No statistically significant differences at $P < 0.05$.

supplementation with FA at the stage of embryogenesis does not induce lasting changes in erythropoiesis. The observed effect of chick age (time effect) on RBC parameters is confirmed by the increased Hb level in all groups on day 35 (in comparison with day 7, these were often statistically significant differences) (Table 4). This is important due to the fact that chicken broilers are slaughtered at this age. The observed decrease of Hb concentration on D7 could be caused by the depletion of the yolk sac, the complete resorption of which occurs on the fourth to fifth day of life of the chick (Nangsuay et al., 2013). In chicks supplemented with FA (both separately and mixed with Met), the Ht value was stable or increased between D1 and D7, whereas it decreased in the other groups. (Table 5). In contrast, Ryu et al. (1995) did not find differences in Hb or Ht owing to Met or FA supplementation in the initial broiler diet. This phenomenon is in accordance with the observation that an excess of homocysteine formed as a result of Met methylation may reduce the Ht value (Osunkalu et al., 2010). Our result described previously seems to indicate that FA administered *in ovo* prevents such a decrease for some time.

On the other hand, a long-term increase in RBC parameters such as MCV, MCH, and MCHC is caused by a disorder of blood cell maturation. These symptoms are seen, among others, in megaloblastic and hemolytic anemia, both of which are caused by a deficiency of folates or disruption of their metabolic pathways (Ventura et al., 2004; Kingsley, 2010). However, a similar phenomenon was not observed in our research. Significantly lower values of MCV and MCH (Tables 6 and 7) were found only on D1 in group F3 in comparison with group C and group supplemented by Met (M), and this is difficult to explain. Whether alone or in combination with zinc, no effect of Met on hematological parameters in adult chickens was observed by Chen et al. (2018).

The normal number of leukocytes in the blood of adult chickens ranges from $12 \times 10^3/\mu L$ to $30 \times 10^3/\mu L$ (Talebi et al., 2005), or from $9 \times 10^3/\mu L$ to $32 \times 10^3/\mu L$ (Campbell, 2012), whereas in our research, WBC ranged from $17 \times 10^3/\mu L$ to $107 \times 10^3/\mu L$. In birds, immediately after hatching, the WBC value is usually higher owing to stress related to the hatching process,

Table 10. Number of leukocytes ($10^3/\mu L$) in the blood of chickens on the first, seventh, and 35th day of life of chickens (D1, D7, and D35).

	D1	D7	D35
Group	Mean ± SD	Mean ± SD	Mean ± SD
O	16.67 ± 12.517	84.17 ± 33.078	33.00 ± 13.038
C	33.33 ± 25.033	100.42 ± 43.830	38.75 ± 22.790
M5	20.83 ± 12.416	59.17 ± 14.634	37.50 ± 21.622
M25	39.17 ± 21.075	58.33 ± 29.098	29.17 ± 12.007
MF5/3	36.67 ± 46.440	106.67 ± 38.035	29.00 ± 21.622
MF25/15	40.00 ± 28.810	71.67 ± 27.508	24.29 ± 7.868
F3	55.83 ± 29.903	92.50 ± 40.089	27.68 ± 22.704
F15	35.00 ± 22.136	97.00 ± 44.525	39.29 ± 20.295

No statistically significant differences at $P < 0.05$.

and then, the number of WBC decreases with age (Latimer and Bienzle, 2010). In our own research, on D1, the number of WBC was lower only in group M5 than in group C (among the injected groups), whereas on D7, a noticeable but statistically insignificantly less number of WBC were observed in groups M5 and M25 (Table 10), which may indicate a decrease in the resistance of these chicks. Excessive Met supply and FA deficiency may be the cause of hyperhomocysteinemia, which according to Orzechowska-Pawiłojć et al. (2005) may be correlated with a decrease in the number of leukocytes. In addition, Adeyemo et al. (2010) showed the effect of Met on hematological parameters, in particular, on the number of WBC in the first 4 wk of rearing. On the other hand, Zang et al. (2017) did not notice any effect of Met supplementation of broiler diet on WBC differentiation count. In our study, the WBC differentiation count on D35 mostly followed the leukogram pattern of adult chickens provided by Campbell (2012), with the exception of monocytes (Table 11). Regardless of the group, on D35, the percentage of monocytes in complete WBC count was higher (11–20%) than the 0 to 7% reported by Campbell (2012). Moreover, on D35, in all the supplemented groups, the average percentage of this type of leukocyte was noticeably higher than in the controls (O and C groups). An increased percentage of monocytes in complete WBC count occurs, among others, in distressed birds (Nabity and Ramaiach, 2012) or in the case of necrotic changes in tissues (Campbell, 2012). Statistically significant differences were also found between D1 and D7 in group M25/F15 in terms of the percentage of lymphocytes (increase), heterophils (decrease), and the heterophil-to-lymphocyte ratio (decrease), and in group F15 in terms of eosinophils (decrease) and monocytes (increase). This may indicate a toxic effect of FA when used in excessively high doses.

CONCLUSION

The results of our own research indicate that changes in the blood picture caused by *in ovo* supplementation with FA and Met on the 17th day of embryogenesis do not cause permanent changes in the blood picture. Supplementation with Met alone led to temporary immunosuppression, whereas this effect was not seen in the groups supplemented with a mixture of Met and FA, which may affect the protective effect of FA. It seems, therefore, that it would be right to include this additive in bird feed supplemented with Met. At the same time, it should be noted that in our own studies, FA administered in high doses was toxic. Owing to the fact that the observed changes in the blood picture subsided by

Table 11. Differential leukocyte count (%) and heterophil-to-lymphocyte ratio (H:L) in chicken blood on the first, seventh, and 35th day of chickens' life (D1, D7, and D35).

		Lymphocytes	Eosinophils	Heterophils	Monocytes	Basophils	H:L
Day of life	Group	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
D1	O	46.08 ± 20.331	9.08 ± 2.929	37.50 ± 24.033	5.67 ± 3.357	1.67 ± 1.252	1.20 ± 1.068
	C	30.90 ± 12.577	11.20 ± 3.667	48.90 ± 2.437	6.80 ± 1.718	2.20 ± 1.037	2.13 ± 1.815
	M5	22.83 ± 5.811	7.00 ± 2.529	61.33 ± 10.519	5.00 ± 5.692	3.83 ± 2.994	2.90 ± 1.029
	M25	42.38 ± 3.473	11.63 ± 6.612	38.88 ± 7.962	4.50 ± 1.080	2.63 ± 1.701	0.93 ± 0.232
	MF5/3	38.80 ± 3.563	7.00 ± 2.449	39.80 ± 9.011	9.40 ± 3.209	5.00 ± 3.937	1.04 ± 0.297
	MF25/15	23.00 ± 13.466^{A}	11.00 ± 4.517	56.67 ± 16.488^{C}	7.33 ± 3.266	2.00 ± 0.894	3.39 ± 2.083^{E}
	F3	24.40 ± 9.456	8.60 ± 6.684	58.90 ± 12.310	6.60 ± 3.647	1.50 ± 1.000	2.83 ± 1.472
	F15	28.17 ± 3.971	11.33 ± 5.574^{B}	56.83 ± 5.456	3.00 ± 2.366^{D}	0.67 ± 0.516	2.04 ± 0.275
D7	O	74.67 ± 10.033	2.17 ± 1.169	10.50 ± 5.394	12.00 ± 5.621	0.67 ± 0.816	0.15 ± 0.102
	C	68.17 ± 10.943	2.00 ± 1.789	16.67 ± 11.021	13.00 ± 6.033	0.17 ± 0.408	0.27 ± 0.209
	M5	68.00 ± 12.712	3.00 ± 1.095	20.00 ± 10.488	8.50 ± 3.728	0.50 ± 0.836	0.33 ± 0.230
	M25	59.33 ± 17.095	3.83 ± 2.857	21.00 ± 10.449	14.83 ± 5.231	1.00 ± 1.095	0.43 ± 0.333
	MF5/3	80.17 ± 4.996	1.50 ± 1.224	10.00 ± 7.483	7.50 ± 4.324	0.83 ± 0.752	0.13 ± 0.106
	MF25/15	80.20 ± 11.691^{A}	1.20 ± 1.303	9.40 ± 9.128^{C}	8.80 ± 2.863	0.40 ± 0.547	0.14 ± 0.160^{E}
	F3	68.73 ± 15.148	1.33 ± 1.033	19.50 ± 14.223	9.83 ± 0.491	0.67 ± 0.816	0.34 ± 0.293
	F15	64.67 ± 5.854	1.00 ± 1.095^{B}	17.00 ± 5.657	16.50 ± 1.975^{D}	0.83 ± 0.753	0.27 ± 0.112
D35	O	62.17 ± 14.689	3.00 ± 3.033	23.11 ± 11.039	11.00 ± 4.690	0.50 ± 0.548	0.43 ± 0.280
	C	63.50 ± 9.935	2.83 ± 1.722	19.83 ± 7.468	13.83 ± 3.545	0.00 ± 0.000	0.33 ± 0.160
	M5	60.80 ± 9.731	1.20 ± 1.095	21.60 ± 5.941	15.80 ± 5.932	0.60 ± 0.894	0.37 ± 0.137
	M25	59.57 ± 6.528	3.14 ± 2.478	8.14 ± 6.309	17.57 ± 3.866	1.57 ± 1.397	0.31 ± 0.133
	MF5/3	67.17 ± 11.285	2.00 ± 0.894	15.17 ± 10.147	15.17 ± 3.545	0.50 ± 0.836	0.26 ± 0.222
	MF25/15	63.50 ± 13.404	1.50 ± 1.732	17.25 ± 11.087	17.25 ± 5.377	0.50 ± 0.577	0.32 ± 0.294
	F3	59.83 ± 5.345	3.00 ± 1.549	15.50 ± 6.442	20.50 ± 3.886	1.17 ± 1.169	0.27 ± 0.127
	F15	60.50 ± 13.925	3.33 ± 1.966	20.67 ± 15.174	14.17 ± 3.600	1.33 ± 1.211	0.42 ± 0.404

$^{A–E}$Values marked with the same capital letters in a given column differ statistically significantly ($P < 0.05$).

the 35th day of life (before slaughter of chicken broilers), it can be assumed that the possible enrichment of feed with Met and FA will not pose a threat to the consumer.

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