THESIS

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Master's in Animal Nutrition and Feed Safety Engineering

Kaposvar
2021

Hungarian University of Agriculture and Life Science

Kaposvar Campus

Master's in Animal Nutrition and Feed Safety Engineering

Effect of in ovo Methionine supplementation on the performance of broiler chicken

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Kaposvar
2023

Table of Contents

Tables

Figures

1. Introduction

According to the World Bank the rate of population increase is by 1 percent annually of which major contribution is by under-developed and developing countries. To meet the food demand, we need to increase the global food production with the same pace of its consumers. Poultry meat and eggs are the most affordable and accessible sources of animal protein. As per FAO 2020 report, poultry major contributor in the total meat production that is 40.6 percent which is around 337.3 million tons in total. According to Eurostat in 2020, the poultry meat production increased by 8.2 percent from the previous year in Hungary that is among the highest in the whole European Union while in whole EU the meat production is dropped by 3 percent. In 2012 revised report of FAO World agriculture towards 2030/2050", poultry production is expected to double in the world, particularly in the developing countries which will demand more intensified and ratified means of chicken feed production, quality of feed, ways of feeding and biosecurity measures. After the genetics improvement now, the major role is played by the nutrition. Nutrition in every livestock specie comprises most of the cost of the production so it is the most highlighted aspect for the animal production. In poultry, the 70 percent of the cost is of the feeding. The early feeding of the bird is very crucial as if the early feeding management is not proper the animal will not achieve its maximum potential later whatever we do. Thus, scientists around the world are striving to make it as perfect as possible so that maximum of a bird capabilities can be expressed. Apart from using the methods to make the early nutrition better by the brood hatching and patio hatchery technologies that provide nutrition after hatching the researchers tried to give the nutrients before hatching too. So, that the quality of chick being hatched can be improved by better structural development. The only way the pre-hatching nutrition can be provided to the chicken is by the *in ovo* technique. This *in ovo* technique was basically made for the injection of the vaccines into the chicken so that early passive immunity can be achieved by the bird earlier and strong immune competence so that the mortality can be decreased (SHARMA and BRUMSTER, 1982). Later researchers anticipated it as a major breakthrough in the early poultry nutrition to enhance the poultry liveability and productivity.

2. Literature Review:

2.1. Early Nutrition

In the past to increase the productivity of broiler the major focus of scientists was to get the optimum genetic potential of the birds by genetic selection process which increased the production performance by folds, but further increase was not proportionate with the growth of internal body organs with the body size which has deleterious effects on the health of the bird as in *Figure 1*. (SCHMIDT et al., 2009).

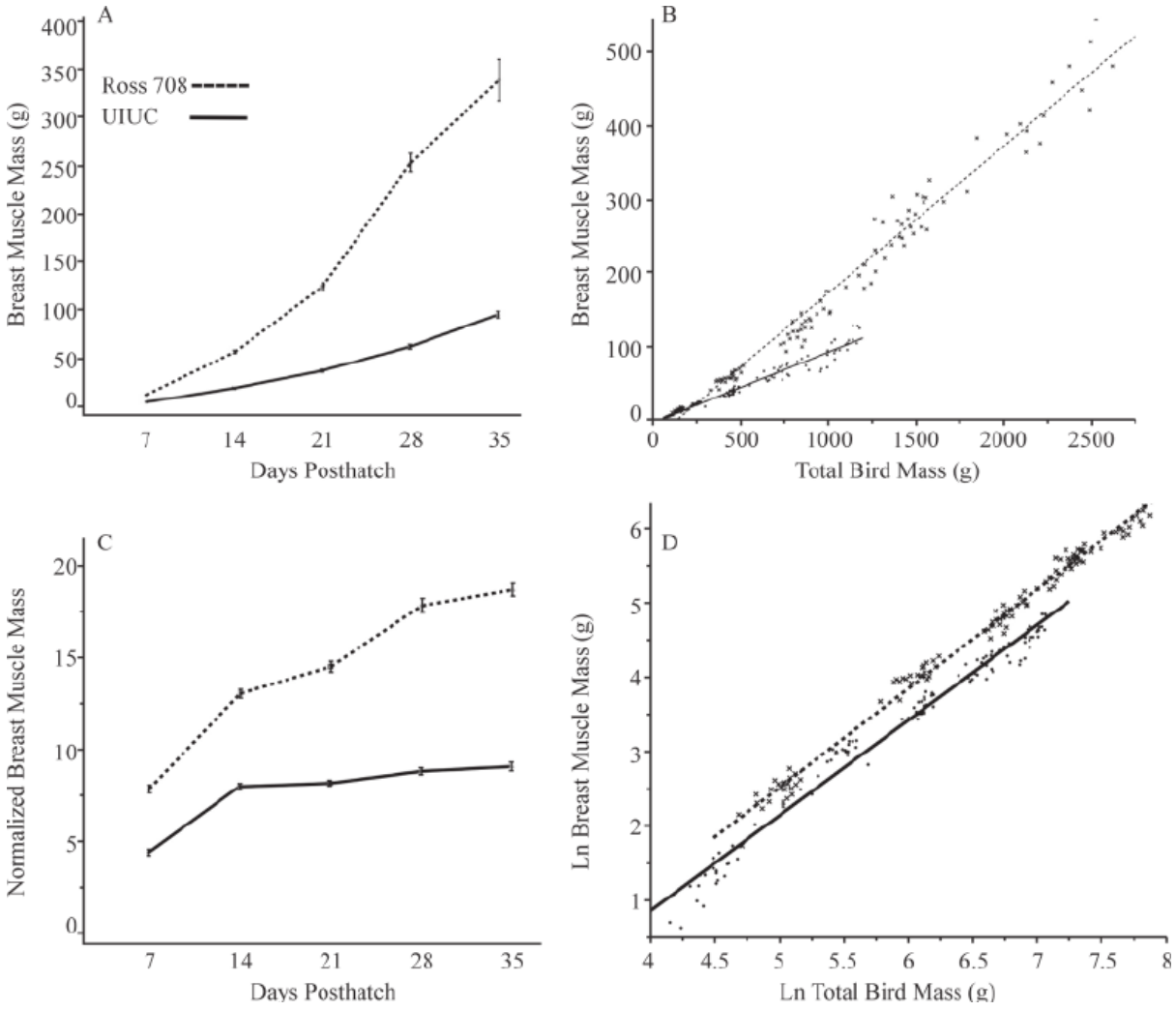


Figure 1: **Growth of breast muscle in heritage (UIUC) and Ross 708 broiler lines (SCHMIDT et al., 2009)**

A) Plot of breast muscle mass versus days post hatch. B) Plot of breast muscle mass versus total bird mass for both strains. C) Plot of normalized breast muscle mass (breast muscle mass divided by bird mass) × 100 versus day post hatch. D) Allometric plot: natural logarithm (ln) of breast muscle mass versus ln of total bird mass. Bars indicate SE of the measurements.

Achievement of better genetic potential goes along with the better feeding strategies and nutrition provided to the bird as feeding comprises the 70% of the cost of raising a bird (OAKLEY et al., 2013). So, nutrition plays a key role in realizing those genetic improvements as well. Chicks and eggs can be manipulated at different stages to get better feed efficacy. This alteration can be achieved during the last few days of hatching and early days post hatch as during that time the development of GIT is very fast (IJI et al., 2001) but in current scenario there is a delay of the first solid feed allowance. There is around 48-72 hours difference between hatch and placement in the farm which leads to stress that affects the development of the chicks' internal body systems (FERKET, 2001). It affects the productivity of the bird as it is reported that early rapid development of GIT and liver has positive correlation with the growth rate of the bird (LILJA, 1983). At the time of hatching the GIT is not fully developed so post hatch the development of the GIT is five times than the rest of the body organs (NITSAN et al., 1991) so for proper development and growth of chicken early feed is crucial to get the full potential of the bird. In a study conducted by Obun and his team (OBUN et al., 2013) it was revealed that delayed feeding from 12 hours to 72 hours that is a usual delayed time post hatch spent doing various activities like sexing, vaccination and transport. The production indicators like better body weight (BW), average daily body weight gain (ADG), feed intake (FI), decreased feed conversion ratio (FCR = feed intake/weight gain) and higher feeding efficiency (FE= weight gain/consumed feed) in the birds who were fed earlier than the rest. Significant difference can be observed in *Table 1*. Besides that, there was prominent decrease in the ileal digestibility coefficients of dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), nitrogen free extract (NFE) and Ash content as the post hatch delay time was increasing as in *Table 2* (OBUN et al., 2013). PINCHASOV et al., (1993) conducted an experiment on broiler chicks and turkey pullets with 24h and 48hrs of fasting. He recorded weight loss of around one tenth of the initial body weight of the chicks whose feeding was delayed for 48hrs. It affected the early growth of the birds adversely.

Table 1: Effect of delayed water and feed access to the broiler chicks on performance indicators of chicks (OBUN et al., 2013).

Parameters	Treatments (fasted hours)						SEM
	12	24	36	48	60	72	
Initial body weight (g/bird)	40.00	40.00	39.5	37.23	35.00	34.5.	0.06
Final body weight (g/bird)	573.00^{a}	572.22^{a}	557.00^{ab}	523.14^{b}	507.32^{c}	487.65^{c}	10.30
Body weight gain (g/bird)	533.00^{a}	532.22^{a}	517.50^{b}	485.91^{c}	472.32^{d}	453.15^{e}	9.25
Daily weight gain (g/bird)	19.04	19.00	18.48	17.35	16.87	16.18	0.35
Feed intake (g/birds)	1254.88^{a}	1256.21^{a}	1247.30^{ab}	1231.00^{b}	1223.87^{c}	1217^{c}	4.89
Daily feed intake (g/bird)	44.82	44.86	44.55	43.96	43.71	43.46	0.73
Feed conversion ratio	2.35^{c}	2.32^{c}	2.41^{b}	2.53^{ab}	2.59^{a}	2.69^{a}	0.46
Feed efficiency ratio	1.92^{a}	1.92^{a}	1.88^{a}	1.78^{b}	1.75^{b}	1.69^{b}	0.29
Mortality (%)	5	-	5	10	15	15	-

^{abcde} Mean with different superscripts are significantly different (P<0.05)

Table 2 Effect of delayed feeding on ileal digestibility coefficient of broiler chicks (OBUN et al., 2013).

Fasted period (Hours)	7 days old chicks						28 days old chicks					
	DM	CP	CF	EE	Ash	NFE	DM	CP	CF	EE	Ash	NFE
12	80.5^{a}	76.02^{a}	60.0^{a}	65.7^{a}	67.2^{a}	70.0^{a}	88.56^{a}	84.2^{a}	75.0^{a}	82.0	69.3	72.2
24	83.9^{a}	74.61^{a}	56.1^{a}	64.3^{a}	66.4^{a}	68.0^{a}	85.0^{a}	80.0^{a}	73.7^{a}	82.5	68.0	72.2
36	78.3^{a}	72.03^{a}	54.6^{a}	60.0^{a}	66.0^{a}	68.0^{a}	82.0^{a}	79.4^{a}	73.0^{a}	80.3	68.5	71.6
48	72.0^{b}	65.00^{b}	52.0^{b}	56.4^{b}	61.0^{b}	64.0^{b}	78.0^{b}	76.0^{b}	72.0^{a}	79.4	66.3	68.7
60	68.4^{b}	60.05^{b}	47.4^{c}	52.0^{b}	60.8^{b}	60.0^{bc}	77.0^{b}	72.0^{b}	68.4^{b}	76.7	66.0	68.4
72	62.0^{c}	54.64^{c}	42.2^{c}	45.5^{c}	56.0^{c}	58.0^{c}	77.0^{b}	70.0^{b}	66.6^{b}	75.1	66.4	68.0
SEM	0.06	0.10	0.04	0.11	0.06	0.11	0.08	1.10	0.01	0.06	0.05	0.4

^{abc} Mean with different superscripts are significantly different (P<0.05)

Therefore, providing the nutrients on the earliest bases became the prime focus that is resolved by the idea of Hatch Brood System and the Patio system. In my opinion, it cannot be economical for neither the hatcheries nor the farm owner even though it can resolve the early feed deprivation stress. Alternative option could be pre-hatch intervention or post hatch gel feeding in the hatching trays and the transport trays. AREAAER et al., (2020) conducted an

experiment by providing Hydrogel-95 to chick's post hatch for 2,4 and 6 hours and measured the growth parameters of chicks before they got the starter diet. It was observed that chicks who got hydrogel feed for six hours post hatch showed the best growth parameters as compared to control and the other treatment groups which got hydrogel feed for 2 and 4 hours.

2.2. What is *in ovo* technology and its history?

Word *in ovo* is a Latin word which means in the egg. In poultry, Marek's disease is the first disease against which vaccination is done right after the hatching. Reason of this *in ovo* technology initiative was even instead of giving post hatch vaccination shot the mortality rate was still high because chicks were being infected even before vaccination. So, Sharma and Brumster (SHARMA and BRUMSTER, 1982) float the idea that late-stage embryos have the capability to get immunity by the vaccination. For the vaccination of chicks Sharma and Burmester made the protective index as the efficacy measure between the conventional and the *in ovo* injection technology 3 days post hatch for the Marek's Disease HVT vaccine. They got the result that 93% protective index was achieved by *in ovo* injection on 18th day of incubation while only 21% protective index was achieved by the conventional method that is subcutaneous injection post-hatch on the back of the neck of the chick (SHARMA and BURMESTER, 1984). In 1985 a US based company EMBREX was licensed by USDA exclusive patent "Disease Control in Avian Species by Embryonal Vaccination". After EMBREX started working on *in ovo* injection machine in 10 years they were able to make a commercial prototype named INOVOJECT (GILDERSLEEVE et al., 1993) whose advanced version is now being used round the world. Thus, this *in ovo* injection technology is around 25 to 30 years old technology that was primarily used in the broiler chicken eggs for the vaccination programs against the Marek's disease (RICKS CA et al., 1999). Consequently, a lot of experimentations were carried out and later it started against all the early age viral diseases of the poultry like Infectious Bursal Disease, New Castle Disease, Infectious bronchitis and Avian Influenza so, now above 90% of the US hatcheries are using this *in ovo* Technology for vaccination purposes (E D Peebles, 2018).

2.2.1 Advantages and its consequences of *in ovo* technology

Before the *in ovo* technology, all the chicks were injected against the diseases after the hatching manually which was a very cumbersome process and takes a lot of personnel. Benefit of using *in ovo* injection technology in poultry industry is that it reduced the labour cost by automizing the process of the vaccination. In addition, the human error is also decreased in vaccination as

with the dual pressure technology the vaccination is done properly on the required site. On the other hand, large number of eggs can be vaccinated without any hassle. During this process all the chicks were man handled various times until they get vaccination, and it induces a lot of stress to the chicks which can lead to death by shock by using this incidence can be reduced noticeably. Along with that, manually vaccinating all the chicks one by one delays their early nutrition which is very important as the future growth of the chicks (OBUN et al., 2013). *In ovo* method greatly reduced this gap and the shifting of the chicks from the hatchery to the farm and growth of the chicks can be optimized eventually. All the chicks start the passive immunity earlier in their life than normal hatched chicks and higher mean antibody titre can be achieved in the whole flock as compared to conventional vaccination protocol chicks (SHARMA and BURMESTER, 1984). Good antibodies titre was achieved regardless of the day of vaccination, but the major fear was decreasing the hatchability of chicks but when chick embryos were vaccinated at the 18th day of incubation no significant difference in the hatchability was observed (SHARMA and BURMESTER, 1982). Although it is a complex process to carry out but with good quality egg, pro-active control of the environment in which this process is carried out and the bio secure injection machine make it germ free with effective disinfection.

2.2.2 Applications of *in ovo* technology

After exploring the area of *in ovo* vaccination, scientists used this idea to administer other supplements, nutrients, drugs and hormones with the idea to support the embryogenesis, hatchability, chicks' liveability and performance. Research has proved that by giving thyroxine hormone by *in ovo* feeding (IOF) increase the hatchability, higher chick weight, better quality chicks, improved productive performance of the bird and decreased mortality and second grade chicks (AFSARIAN et al., 2019).

JOHNSTON et al. in 1997 mentioned Elbrecht and Smith, 1992 research of „Aromatase enzyme activity and sex determination in chickens" in review "Applications in *in ovo* technology" that by the injection of aromatase inhibitor which block estrogen production when given to the eggs prior to the incubation at day 0, can produce 100% male phenotype as compared to nearly 50/50 male female ratio of non-injected eggs. It was also mentioned that administration of 800 U of chicken myelomonocytic growth factor (cMGF) can decrease the mortality of the young chicks caused by E-coli (JOHNSTON PA et al., 1997).

ICS et al. (2019) observed the effect of supplementing vitamin E at increasing rate on day 17.5 of incubation. They observed higher hatchability, better chick quality, body weight, better

chicks oxidative state, higher chick weight to egg weight ratio. Greater small intestinal developments were noted, and all of this added to better performance results of the bird. FARHAD et al. (2021) observed higher hatchability and oxidative state but no difference in carcass traits, immunity organ weights and immunity against New Castles Disease, when they administered Vit E and Vit C. According to BHANJA et al. 2012 *in ovo* administration of different vitamins have different functions pre and post hatch like Vitamin A and Vitamin C influence the embryonic development and Vitamin E and B1 are beneficial in early post hatch development.

SIWEK and his colleagues in 2018 deduced in their review article that by delivering the prebiotics and probiotics at day 12 in air sac we can achieve *in ovo* stimulation of the gut microflora. Stimulants are taken in by two ways. Water soluble prebiotics can pass through the chorioallantois membrane by the help of blood were visible in the embryonic feed duct at day 15. On the other hand, probiotics remain in the air sac were taken up by the chicken after day 18 when they start piping before hatch. In effect of prebiotics and probiotics better lifelong performance traits, GIT development, immune system evolution, yolk-sac absorption and abundance of microflora at the time of hatching were observed.

A Danish feed manufacturer Hamlet protein introduced a product named Hamlet Protein Avistart (HPA) for the early chick start for the day-old chicks which has better protein digestibility than normal soybean meal. In 2017, OMEDE et al. prepared an *in ovo* feeding (IOF) solution by suspending it in Milli Q water in different concentrations from 18.5 mg/ml to 150 mg/ml and extracted the supernatant after centrifugating the heated suspended solution of HPA which was later used as IOF solution. When this was administered in the eggs at day 18, it showed that this product had ability to give better hatching weights and gave better day 10 body weight gains thus improved the early post-hatch performance.

KALANTAR et al. (2019) trailed the 0.1ml and 0.2ml inoculation of Co enzyme Q10 also known as ubiquinone having the antioxidant role in the body at day 18 of incubation and it resulted in 6.54% higher hatchability and 4.74% body weight to egg weight ratio post-hatch in comparison to control non-injected groups. Moreover, higher immune organ weights, serum antibody titre against viral diseases was significant versus the controls.

2.2.3 Site and day of injection

There are five different sites in the egg which are used to administer the *in ovo* feeding solutions, named as embryo, amniotic fluid or amnion, allantois, air sac and the yolk as mentioned in the Figure 2 published in International hatchery practice article “Introducing a

new approach to *in ovo* vaccination for modern hatcheries" showing different compartments of eggs.

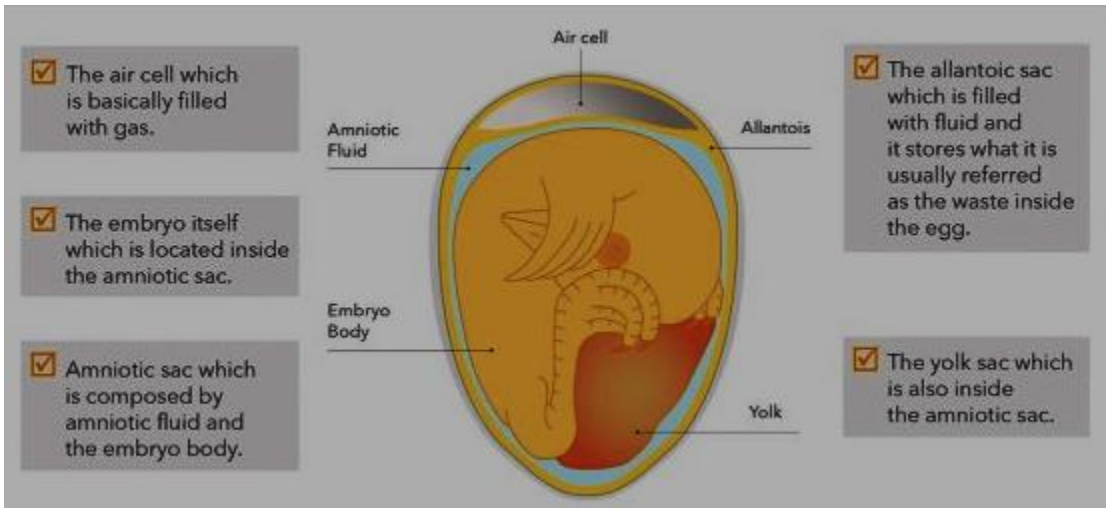


Figure 2: Different chicken embryo compartments and function (Introducing a new approach to *in ovo* vaccination for modern hatcheries).

Literature has sited different timelines for the administration of reagents into the egg for different purposes as in Table 3. Either the requirement is to change the embryonic development, embryonic microbiota or to feed the embryo for the post hatch starvation window. *In ovo* injection of aromatase inhibitor to produce males is given before the incubation so that male chick ratio could be increased (JOHNSTON et al., 1997) because there is no use of it giving in the later stage when the embryo gonads are already developed.

Another term used *in ovo* technology is *in ovo* stimulation which refers to stimulants used to vitalize the embryonic development for which the feeding is done in the midway of incubation. Prebiotics and symbiotics are given on the day 12 of incubation so that they can increase the desired native embryonic gut microflora when hatched. The injection site in *in ovo* stimulation differs from *in ovo* feeding. The injection site in this is air cell, which is easy, safe and do not disturb the embryogenesis. The reagents are taken up from there because during that time chorioallantois is highly vascularized (SIWEK at el., 2018).

As by GROFF-URAYAMA et al., 2019, did two experiments in which he did inoculation site and day of inoculation comparison. In treatment 1, they injected glucose in the allantoic space at day 16,17 and 18 and noted that hatchability was decreased regardless of day of inoculation. In treatment 2 they administered methionine and lysine on day 18 but this time in the air chamber. The hatchability was higher when injected in air sac than allantois. Thus, it was

concluded from this experiment that puncturing the air sac damage the embryo even accidentally the embryo in comparison to treatment 2. By noticing no difference in hatchability by day of inoculation, therefore we can say that by the 16th day embryos have the ability to take up the nutrients being injected in (GROFF-URAYAMA et al., 2019).

In 2010, LEITÃO et al. compared the efficacy of injecting at air sac versus allantois membrane by injecting the carbohydrates solution of maltose, sucrose and glucose. They noticed much lower hatching in the eggs where the injection needle erupted the air sac membrane and deduced that injecting at allantois can go further at the level of chorioallantois disturbing the oxygen and carbon dioxide exchange even embryo can be damaged by the needle.

For *in ovo* feeding, the optimum time to inject in the amniotic fluid is the late embryonic stage because this is the time when the amniotic fluids are absorbed by the embryo, so the injected nutrients are also taken in with the fluids which become the part of the enteric fluids (UNI Z et al., 2005). Furthermore, in late embryonic stage the air cell is totally dry and there is no vascularization in the chorioallantois. Thus, the gaseous exchange of the embryo is not disturbed and do not affect the viability of the embryo (SIWEK at el., 2018).

Table 3. *In ovo* methionine feeding table

Sr. no	Paper	Reagent	Conclusion	Site & Day	Author
1	The effect of feeding adequate or deficient vitamin B6 or folic acid to breeders on methionine metabolism in 18-day-old chick embryos	L-serine, L-betaine, and L-methionine	supplying folic acid and pyridoxine in broiler breeder diets is necessary for chick embryonic methionine metabolism	Amnion at 18^{th} day	LU et al., 2020
2	The effect of methionine and folic acid administered *in ovo* on the haematological parameters of chickens (Gallus domesticus)	Met 5 & 25mg. FA 3& 15 mg. Mixture M5/F3 and M25/F15 with 0.7% Saline	FA and Met on the 17th day of embryogenesis do not cause permanent changes in the blood picture	Amniotic sac at 17^{th} day	TOMBARKIEWICZ, BARBARA et al., 2020
3	Effects of *in ovo* injection of sulphur-containing amino acids on heat shock protein 70, corticosterone hormone, antioxidant indices, and lipid profile of newly hatched broiler chicks exposed to heat stress during incubation	Methionine + Cysteine 5.9mg & 3.4mg using 1ml saline 0.7% 1ml Saline	May be helpful to mitigate harmful effects of heat & oxidative stress	Amniotic fluid at 17.5^{th} day	ELNESR SS et al., 2019
4	Effects of *in ovo* Methionine-Cysteine Injection on Embryonic Development, Antioxidant Status, IGF-I and TLR4 Gene Expression, and Jejunum Histomorphometry in Newly Hatched Broiler Chicks Exposed to Heat Stress during Incubation	(Methionine + Cysteine) 5.9mg & 3.4mg 0.1ml of 0.75% saline	Improved embryonic development, IGF-I and TLR4 gene expression, antioxidant status and jejunum histomorphometry of newly hatched broiler chicks exposed to heat stress during incubation	Amnion at 17.5^{th} day	Elwan, Hamada A M et al.,2019
5	Effects of *in ovo* injection of lysine and methionine into fertile broiler (parent stock) eggs on hatchability, growth performance, caecum microbiota and ileum histomorphology	Met 2mg/0.2ml, Lys 2mg/0.2ml, Met + Lys 1+1mg/0.2ml in 0.2ml 0.5% saline	*In ovo* injection of lysine, methionine, and lysine + methionine did not affect relative chick weight, liveability, growth performance, caecum microbiota, and ileal villi length and thickness.	Air Sac at 16^{th} day	COSKUN ISA et al., 2018

6	Performance, intestinal morphometry, and incubation parameters of broiler chickens submitted to *in ovo* feeding with different techniques and amino acids	Methionine 20mg Methionine 30mg Lysine 20mg Lysine 30mg 0.5ml in 0.9% saline	The inoculation of methionine (20 and 30 mg) obtained data like the control group	Air chamber at 18^{th} day	GROFF-URAYAMA et al., 2019

2.2.4 Amount/ concentration of reagent to be used.

As stated by SIWEK at el. (2018) the amount of the reagent and characteristics of the nutrient to be injected also varies with the site we are going to inject. If the injection site is air sac in early embryonic stage the maximum volume could be injected is 0.2 ml as more than this will invade the outer and inner membrane of the egg and will lead to immediate death of the embryo. Secondly, the nutrient should be water soluble to be absorbed from here as they are taken up by the help of blood system. Moreover, if the nutrients are being inoculated at amnion level in the late embryonic stage the volume can be injected according to different literatures maximum ranges is 1.0-1.7 ml and it could be any type of the nutrient (protein, carbohydrates, amino acids and others) as they are taken up with the amniotic fluid into the enteric tract mentioned in *Table 3*.

The concentration of the nutrient can also put negative effects on the hatchability as it affects the equilibrium of the embryo and it was proposed that the osmolarity of the solution being injected should not surpass 650 millimole as more than this could lead to cellular oedema which ultimately kills the embryo (GROFF-URAYAMA et al., 2019).

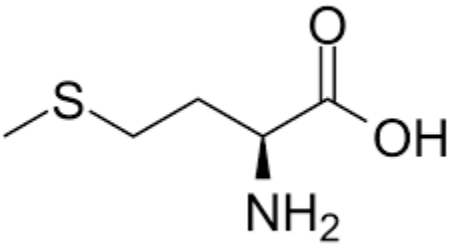
2.3. Role of Methionine

Chemical formula

Apart from the importance of providing proper crude protein in the diet now world has moved towards more precise nutrient supply by using the idea of Ideal Protein Concept or precision nutrition which gives nutritionist liberty to adjust the required amino acids separately (LEMME, 2003). In which amino acids are added in the feed with relation to the reference of Lysine in such proportion that added amino acids are totally used by the animal without adding to the excess N excretion by the amino acids. Protein in the diet is broken down into the amino acids in the body and then assimilated again to make the desired protein in the body. There are certain amino acids that cannot be produced by the body they are called the essential amino acids and methionine is one of the essential amino acids.

Methionine is a sulphur containing amino acid that is often the first limiting amino acid as well as functional amino acid in the poultry feed. Its chemical formula is $C_5H_{11}NO_2S$ and chemical structure shown in *Figure 3*.

Figure 3. Chemical structure of Methionine



It is involved in many metabolic pathways and its important role as methyl donor is in the methylation reactions, synthesis of feather follicles and muscle protein synthesis (FAGUNDES et al., 2020, VÁZQUEZ-AÑÓN et al., 2006). It also offers its methyl group for methylation reaction of DNA and a precursor of glutathione which reduce the oxygen reactive species (ROS) and prevent the cell from oxidative stress as well as it plays a vital role in the poultry in producing resistance against the heat stress (ELNESR et al., 2019). Being a sulphur containing amino acid it helps to produce other sulphur containing amino acids, increased growth performance in poultry (ZHAN et al., 2006) and enhanced immune system to the birds when added in the diet (ZHANG and GUO, 2008).

2.3.1 Sources of methionine

Now when the nutritionists are continuously working on decreasing the crude protein by adding the amino acids separately so that N-excretion could be reduced (LEMME, 2003). It created high demand for the synthetic methionine as a feed additive. Primarily, the major source of amino acids in the diet is the feed offered to the birds (e.g soybean, corn). The forms of available methionine in the market are DL-Methionine, methionine hydroxy analogue (MHA) and L-methionine. L-methionine is registered as a feed additive in 2014 by the EU Commission to be available commercially for the animal production (ULLRICH et al., 2019). The readily utilisable form of methionine which need no alteration to be absorbed in the small intestine is L-Methionine but other two available forms must be converted into L-methionine first by the help of enzymes in the liver and kidney (SHEN et al., 2015). It is assumed but not confirmed that L-methionine has better efficacy than other available forms, so it provides higher redox state, enhanced GIT development and growth performance in early growth stage of broiler birds (SHEN et al., 2015).

2.3.2 Role of methionine in embryonic development

Methionine plays a vital role in the differentiation of embryonic stem cells. CHEN et al., (2020) concluded that addition of methionine by *in ovo* technique help to enhance the follicles and feather growth in the chicks by activating the Wnt/β-catenin signalling pathway regardless of the type of methionine source. ELWAN et al., (2019) experimented by injecting methionine along with cysteine in amnion at day 17.5. It helped a better development of embryo and had positive effect on the Insulin like Growth Factor-1 (IGF-1). IGF-1 was effective in increasing the chick weight and on toll like receptor-4 (TLR-4). TLR-4 helps in acute heat shocks to the chicks as compared to the control also showed better antioxidant quality when exposed to heat. Furthermore, early Met supplementation did improve jejunum histomorphometry. They observed 29% increase in the villus area as well as increased villus depth and height in comparison to the control that is crucial for better digestibility and absorption of nutrients (ELWAN et al., 2019).

Deficiency of this amino acid can lead to many defects in the chick that can be fatal for the survival of the embryo or the chick. It has a significant role in the organogenesis, body's production of tubulin as well as formation of neurofilaments and its deficiency can hinder the formation of circulatory system and lymphatic system of the chick embryo (TOMBARKIEWICZ et al., 2020).

In the late embryonic stage when the glycogen reserves are near to depletion and to further support the development and provide energy for the piping and the hatching chick have to depend on the glucose generated by the gluconeogenesis which uses amino acids and if the situation further worsens muscle protein is then taken into use which hinders the further development and even could lead to late embryonic death (UNI Z et al., 2005). Thus, providing amino acids and nutrients in the late incubation stage stimulates the post-hatch GIT development and nutritional status of the chicken which help in attaining the optimum production potential of the animal (UNI Z et al., 2005).

3.Aim of the study

The aim of the study was to examine the effects of early nutrition strategies, specifically *in ovo* Met supplementation (0.5% DL-methionine solution injected to the amniotic fluid) in broiler chicken on growth performance and slaughter quality.

The effect of treatments were tested on Ross 308 eggs. *In ovo* intervention was performed on day 17 and eggs were assigned randomly to 5 groups.

4. Materials and methods

Hatching of 800 Ross 308 eggs divided into 5 treatment groups was carried out at MATE Kaposvar Campus Department of Farm Animal Nutrition. There were 5 treatment groups in the experiment as shown in Table 4. No intervention during incubation and early feeding administration (NI-0), in ovo saline group (IoS-0) with early feeding, no intervention during incubation with 48hrs delayed feeding (NI-48), in ovo saline with 48hrs delayed feeding (IoS-48), in ovo methionine with 48hrs delayed feeding (IoM-48).

Table 4: Experimental treatments

Treatment code	**Feed access**	**Early nutrition**
A (control1) -ve	Immediately after hatching	-
B (control2) +ve		*in ovo*, saline (NaCl)
C	Delayed (48 hrs)	-
D		*in ovo*, saline (NaCl)
E		*in ovo* AA Methionine

A=NI-0 No intervention during the incubation, immediate feeding

B=IoS-0 *In ovo* saline, immediate feeding

C=NI-48 No intervention during the incubation, 48h delayed feeding

D=IoS-48 *In ovo* saline, 48h delayed feeding

E=IoM-48 *in ovo* methionine, delayed feeding

4.1. Hatchery management

Hatching of 800 Ross 308 eggs (n=160/treatment) was carried out at the Department of Farm Animal Nutrition according to the Aviagen (2019) management guide. Before placing into the incubator, eggs were stored in cardboard boxes under 20^{o}C. Rotation and humidification were not neccessary due to the short storage time (6 days).

Trays of the incubator were signed according to the treatments.

4.2. Hatching timeline

Day -6 is the day when Ross 308 eggs came at the facility for incubation means 6 days before starting the experiment. At day 0 eggs were placed in the incubator at 37.9 degree celcius. At day 10 all the eggs were candled and fertile eggs were selected for further hactching process. Then on the day of *in ovo* injection all the previous fertile eggs were candled again and fertile and viable embroys were injected with Saline and Methionine containing solutions in designated egg groups. They were put again in the hatcher until the day 22 when eggs were hatched. Hatching protocol is mentioned in Table 6. Later, sexing of chicks were done on the basis of feathering.

Table 5: Hatching timeline

Day	Date	Tasks	Details
HATCHING			
-d6	2021.01.19	Arrival of 800 Ross 308 eggs	Storage until hatching
d0	2021.01.25	First day of incubation	Start, heating up the machine with the eggs
d10	2021.02.03	Candling	Selection of fertile/infertile/early dead embryos
d17	2021.02.10	Candling, *in ovo* intervention, placing into the incubator	Selection of dead embryos, *in ovo* intervention. Adjusting temperature and humidity according to the management guide.
d22	2021.02.15	Collecting and weighing chicks, ID numbers, sexing,	Fast feathering: pullet Slow feathering: rooster

4.3. *In ovo* intervention

The *in ovo* manipulation was carried out according to the protocol of Uni and Ferket (2003). Eggs were cleaned with a cotton wool dipped in iodine solution prior to *in ovo* intervention. Composition of the solutions used for *in ovo* supplementation were: 0.5 ml 0.9% NaCl solution for treatments B and D, 0.5 ml 0,5% methionine added to 0.9% NaCl solution. The eggs were carefully drilled to allow the needle to be inserted through the hole. Solutions were injected to the amniotic fluid (position of the embryos were checked to precise injection site) with a 21 G needle. A sterile, plastic tape was applied after the intervention to avoid the entry of pathogens all the process can be seen in Figure 4. After the process, eggs were placed into the incubator until day 22 of hatching.

Figure 4: In ovo intervention procedure

Table 6 Hatching management

Day of hatch			Temperature	Rotation	Ventillation %	CO_2 conc. tf%
Hatching						
	Date		**°C**			
1	01.25		37,9	Every 2 hours	0	0,60
2	01.26		37,9		0	0,60
3	01.27		37,9		0	0,60
4	01.28		37,9		0	0,60
5	01.29		37,9		0	0,60
6	01.30		37,9		0	0,60
7	01.31		37,8		0	0,60
8	02.01		37,8		0	0,60
9	02.02		37,6		0	0,60
10 Candling	02.03		37,6		0	0,60
11	02.04		37,5		5	0,35
12	02.05		37,5		5	0,35
13	02.06		37,4		10	0,35
14	02.07		37,3		10	0,35
15	02.08		37,3		15	0,35
16	02.09		37,2		20	0,35
17 Candling *In ovo* intervention, Placing into the incubator	02.10		37,1	Rotation stops, trays are horizontal	25	0,35
Incubation					**Incubation**	
18	02.11		37,0/36,7		30	0,35/0,60

19	02.12		36,7		30	0,60
20	02.13		36,5		40	0,60
21	02.14		36,2		50-100	0,60
22	02.15		36,2/35,8		50-100	0,35

4.4. Reception and housing

Right after haching each chick recieved a wing tag with ID number. Birds were sexed and weighed with gram precision. Two treatment groups were placed into pens immediately after hatching (NI-0, IoS-0). Feed intake of treatments NI-48, IoS-48, IoM-48 were delayed with 48 hours.

A total of 577 birds were placed in floor-pens (16 birds/pen, 8 pen/treatment). Birds were allocated to pens randomly. The installation has modern barn's environment technology complying with the EU regulations on the temperature, humidity, air movement, harmful gas, and dust concentration, as well as lighting hours and intensity requirements for livestock and the recommendation of Aviagen Ltd (2019).

4.5. Feeding management

In the trial, a 3-phase feeding program was used, starter phase between day 1-10; grower phase between day 11-21 and finisher phase between day 22-35. The feeds were formulated on a corn-soy bean meal basis and prepared in pelleted form. The birds were fed *ad libitum* from self-feeders during the trial. One feeder was presented per pen. Drinking water was available *ad libitum*.

Table 7: Composition and calculated nutrient content of the basal diet (g/kg) (g/kg)

Ingredients	**Starter (1-10)**	**Grower (11-21)**	**Finisher (22-35)**
Corn (grain)	482,40	510,65	532,15
Corn gluten (60%)	22,00	-	-
Soybean meal (44,2 %)	399,50	386,00	361,00
Sunflower meal	48,00	61,00	67,00
MCP	16,75	14,75	13,60
Limestone	15,60	14,95	14,25
NaCl	2,00	2,00	2,00
L-Lysin HCl	2,30	0,40	0,05
DL-Methionin	3,05	2,45	2,20
L-Treonin	0,65	0,05	-
Premix	5,00	5,00	5,00
Total	**1000,00**	**1000,00**	**1000,00**
Nutrient content (g/kg)			
AMEn (MJ/kg)	12,7	13,1	13,4
Crude protein	230,1	210,2	200,1
Crude fat	73,7	86,8	92,9
Crude fibre	29,1	28,6	27,8
Lysin	14,1	12,1	11,2
M+C	10,2	9,0	8,5
Threonin	9,4	8,2	7,8
Triptophan	2,8	2,7	2,5
Ca	10,0	9,0	8,5
$P_{avaliable}$	5,0	4,5	4,2
Na	1,6	1,6	1,6

Premix containes per kilogram feed: Zn: 22032 mg, Cu: 3200 mg, Fe: 16020 mg, Mn: 21948 mg, I: 300 mg, Se: 70 mg, Co: 20 mg, Vit. A: 3240000 IU, Vit. D3: 810000 IU, Vit. E: 20800 mg, Vit K3: 810 mg, Vit. B1: 810 mg, Vit. B2: 1890 mg, Vit. B3: 10800 mg, Vit. B5: 3240 mg, Vit. B6: 1350 mg, Vit B12: 6.8 mg, Folic acid: 270 mg, Biotin: 32 mg.

4.6. Data recording

4.6.1. Performance

Individual live weight was weighed with gram precision on days 0, 2, 10, 21 and 35 of the trial. Feed intake was recorded for the time intervals by measuring the offered and the left feed at the end of each phase. General health of the birds was monitored daily throughout the study. In case of dead birds, the weight of the dead body, the date and the suspected reason of mortality was recorded.

Table 8 : Schedule of events

Events			
d1	2021.02.15	Performance trial start	Taking NI-0, IoS-0 birds to pens, weighing individually
d3	2021.02.17	Start of groups with delayed feeding	Taking NI-48, IoS-48, IoM-48 birds to pens, weighing individually
d10	2021.02.24	Weighing	Weighing of animals and feed, switch to grower
d21	2021.03.08		Weighing of animals and feed, switch to finisher
d35	2021.03.22	Weighing	Weighing of animals and feed

4.6.2. Laboratory analyses

Nutritional content of the feed as dry matter, crude protein, fat, ash, and Ca and P was determined according to AOAC (1989)

4.6.3. Statistical analyses

Data was checked for outliers. The trial data was analyzed with two-way ANOVA for Live weight (LW) and average daily gain (ADG). One-way ANOVA was carried out for feed intake (FI) and feed conversion ratio (FCR) (SAS, 2004). The significance was considered at 5% ($P<0.05$) level, in case of significant treatment effect per comparison was performed with Tukey *pos hoc* test.

5. Results & Discussion

5.1. Live Weight (0-35 days)

The trial started with the incubation of eggs, the eggs were assigned into each of the 5 experimental groups. All the chicks were weighed by 0.1g precision after harvesting from the hatching machine at day 22. In this experiment, we measured the parameter of performance after getting immediate vs delayed feeding and supplemented with methionine. In Figure 5, there was significant difference of initial hatching weight among different treated groups that have no commom letter. Although statistically there should be no difference at hatch but IoS-48 and IoS-0 differed in their hatching weight.

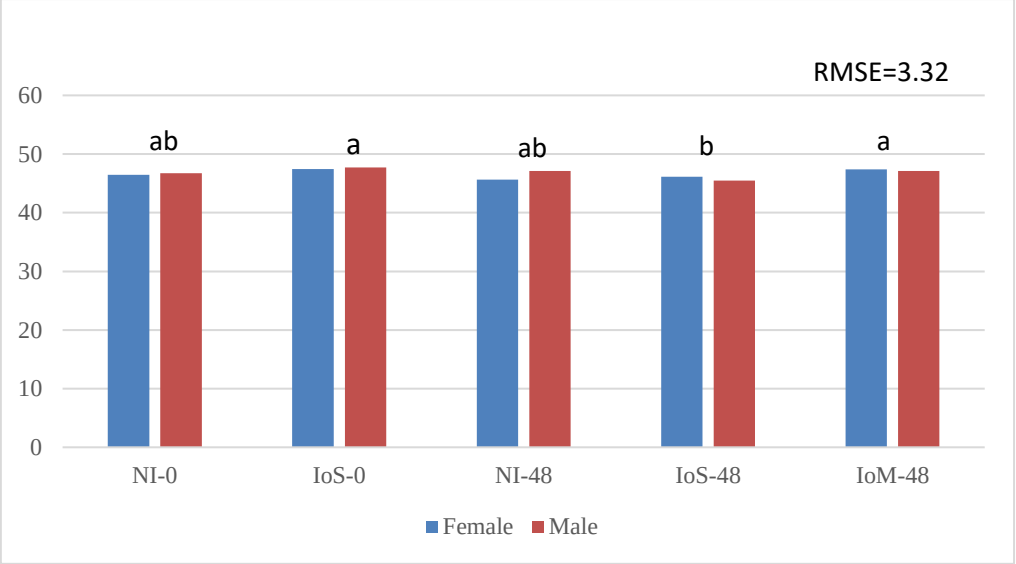


Figure 5. **Weight of newly hatched chicks in the treatment groups.**

NI-0 No intervention, immediate feeding;IoS-0 *In ovo* saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding; *significant difference between treatments (P=0.0021) and no difference between sexes (P=0.34)*

Methionine injection into amnion has no effect on the hatching weight of the chicks. No difference in the hatching weight of chicks is in line with the earlier findings by COSKUN ISA et al., (2018) and GROFF-URAYAMA et al., (2019) that methionine injection at day 16 or day 18 into air chamber or into amnion have no impact on the hatching weight of the chicks. In the study of GROFF-URAYAMA et al., (2019) there was also no difference between the male and female chick's weights as in our experiment that is in accordance with the breeding guide in our experiment.

At day 3, chicks were weighed again, we can see there is no significant difference between the sexes (P=0.47) but there was significant difference between the treatment groups (P<0.001).

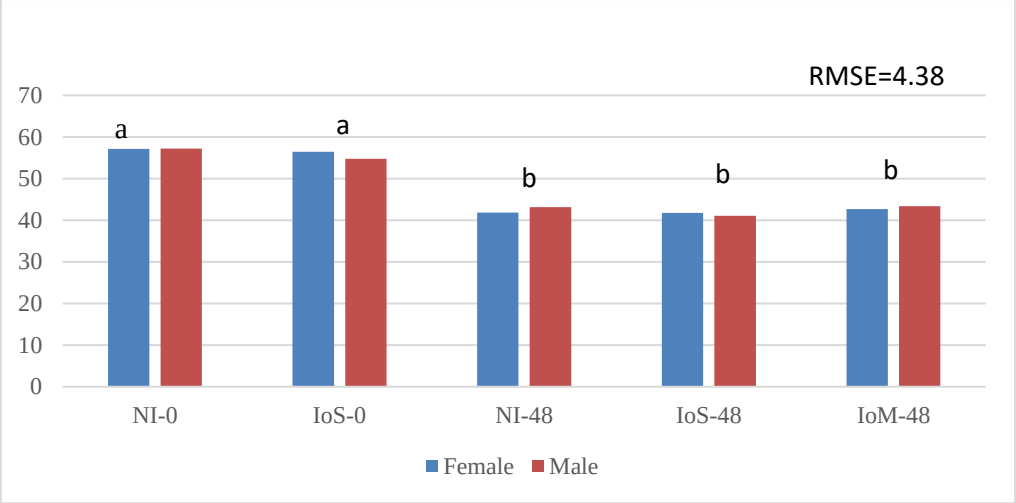


Figure 6. **Weight of 3-day old chicks in the treatment groups.**

NI-0 No intervention, immediate feeding;IoS-0 *In ovo* saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding; *"a" and "b" indicates treatment differences (P<0.001); no significant difference between sexes (P=0.47).*

The treatment difference can be explained by the early access to feed to NI-0 and IoS-0 as compared to the rest of three groups. Early access to feed 48hrs to the first 2 treatment groups will lead to higher body weight than the delayed fed group as reported previously by OBUN et al., (2020). 48hrs delayed feeding caused around 9% drop in weight as compared to their hatching weight. PINCHASOV et al., (1993) as concluded in a study of broiler chicks and turkey pullets that 48hrs of feed and water deprivation adversely affect the early growth of the bird. He also concluded that weight loss is about 1/10 of the hatching weight after 48hrs delayed feeding and we come by average of 9% weight drop.

Methionine can provide physiological changes like embryonic growth, resistance to heat stress and better GIT development. Based on this it was hypothesised that it might increase the performance of chickens as well but it does not. UNI et al., (2019) experimented that carbohydrates supplementation has positive impact on hatching weight and increase glycogen reserves post hatch which are useful in sustaining the delayed feeding post hatch. Because hatching birds require energy which was not possible to be provided by the methionine that why methionine have no effect on hatching weight and delayed feeding.

Figure 7 describes the weight gain of chicks at Day 10. Following graphs shows that there is significant difference between the treatment groups (P<0.001) but there is no significant difference between the sexes (P=0.48).

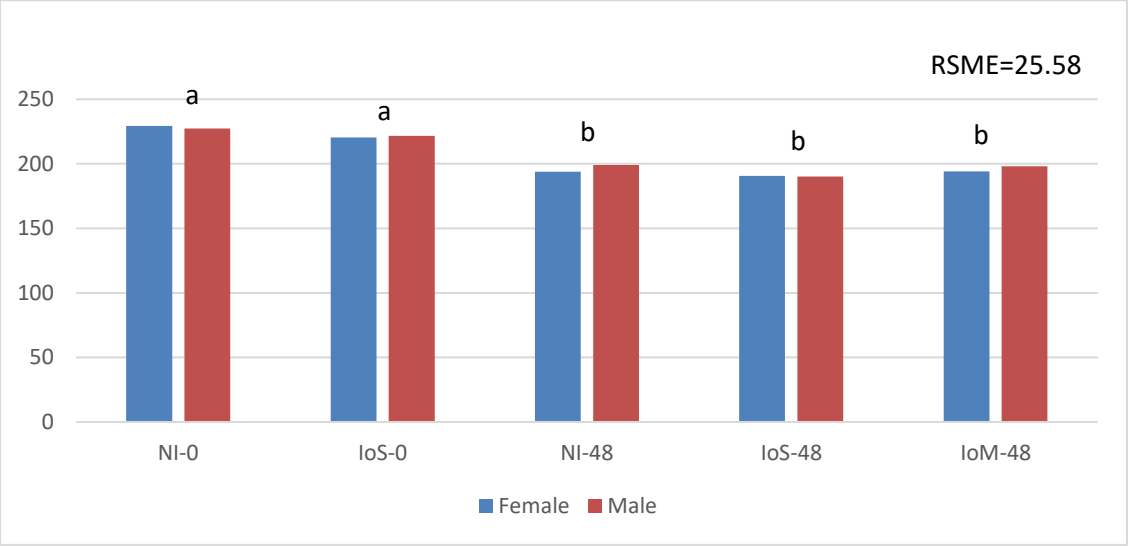


Figure 7. **Weight of 10-day old chicks in the treatment groups.**

NI-0 No intervention, immediate feeding;IoS-0 *In ovo* saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding. *"a" and "b" indicates treatment differences (P<0.001); no significant difference between sexes (P=0.48).*

At Day 21, chickens were weight and recorded weight shows in *Figure 8* that early access to feed groups NI-0 and IoS-0 and significantly higher body weight than other groups as P<0.001. While, there is no significant difference between the sexes as P=0.93

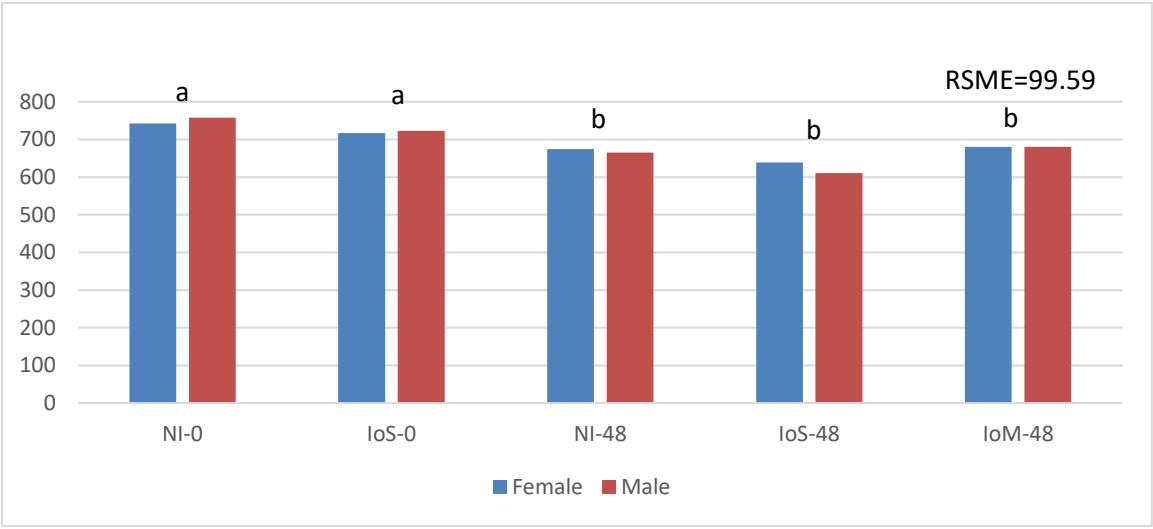


Figure 8. **Weight of 21-day old chicks in the treatment groups.**

NI-0 No intervention, immediate feeding;IoS-0 *In ovo* saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding. *"a" and "b" indicates treatment differences (P<0.001); no significant difference between sexes (P=0.93).*

Figure 9 demonstrates the weight of the chickens at the end of experiment day 35. Aquired data shows that there is tendencey of significance as sex P value is (P=0.1). Male chickens were heavier in four of the fives treatment groups except IoS-48. Furthermore, the difference among the treatments is significant P<0.001

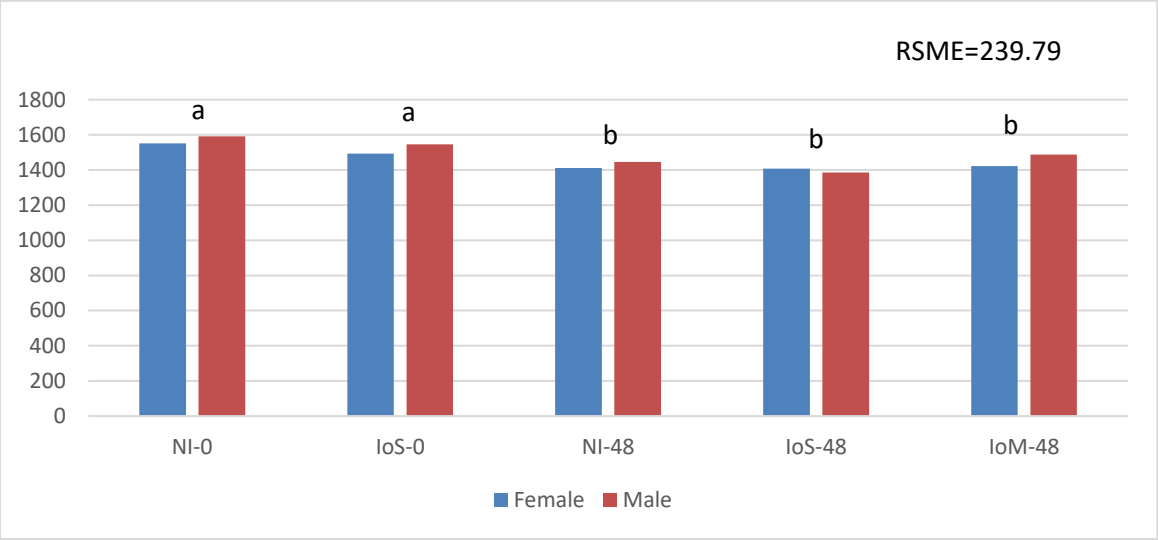


Figure 9 **.**Weight of 35-day old chicks in the treatment groups.

NIG- Non injected group; SIG-Saline injected group; DNIG-Delayed non injected group; DSIG-Delayed saline injected group; DMIG-Delayed methionine injected group. *"a" and "b" indicates treatment differences (P<0.001); Significant difference between sexes (P=0.1).*

Lower weight gain in the early fasted groups can be explain by late start of feeding and likely poorer development of digestive tract and limited ileal digestibility coefficients by the increasing fasting time as it was confirmed after 48hrs of fasting (OBUN et al., 2013).

During the whole experiment period no significant difference of weight between sexes was found.

48hrs or food deprivation gave rise to significantly low body weight until the end of the experiment in comparison to immediate fed groups and this is consistent with the study results of PINCHASOV et al., (1993)

It can be concluded that if the chick does not get early feed access the days to get the market weight will increase because it takes longer for the fasted chicks to get the desired weight (LI et al., 2022).

5.2. Average daily gain of chicks:

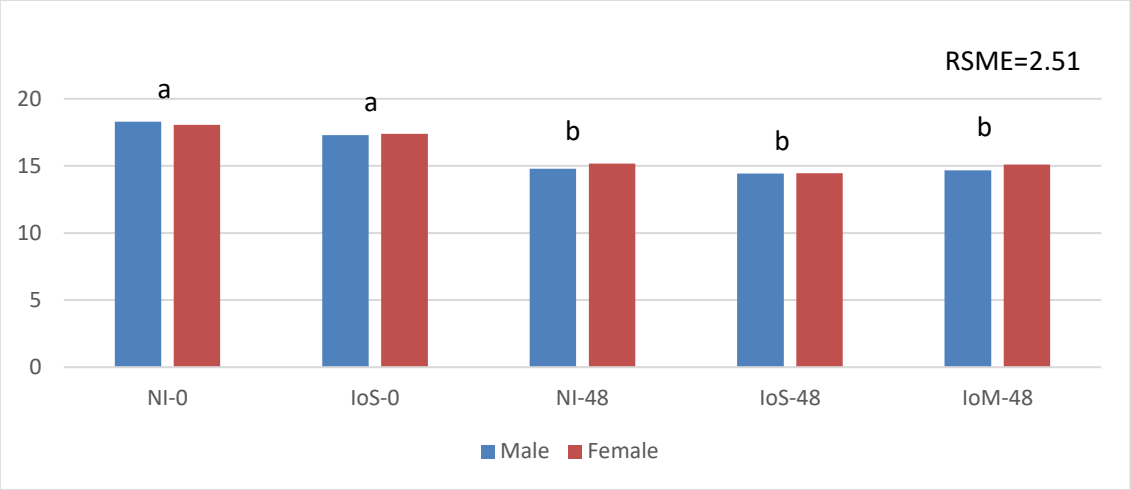


Figure 10. Average daily gain of the chicks (1-10 Days period).

NI-0 No intervention, immediate feeding; IoS-0 *In ovo* saline, immediate feeding; NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding; *"a" and "b" indicates treatment difference (P<0.001); no significant difference between sexes (P=0.52).*

During the first 10 days where chicks were fed with starter diet the average daily gain of the chicks who got feed immediately after hatch without delay have better average daily gain (ADG) than the other 3 treatment groups which got first feed after 48 hours delay. This again is due to early feed access to NI-0 and IoS-0.

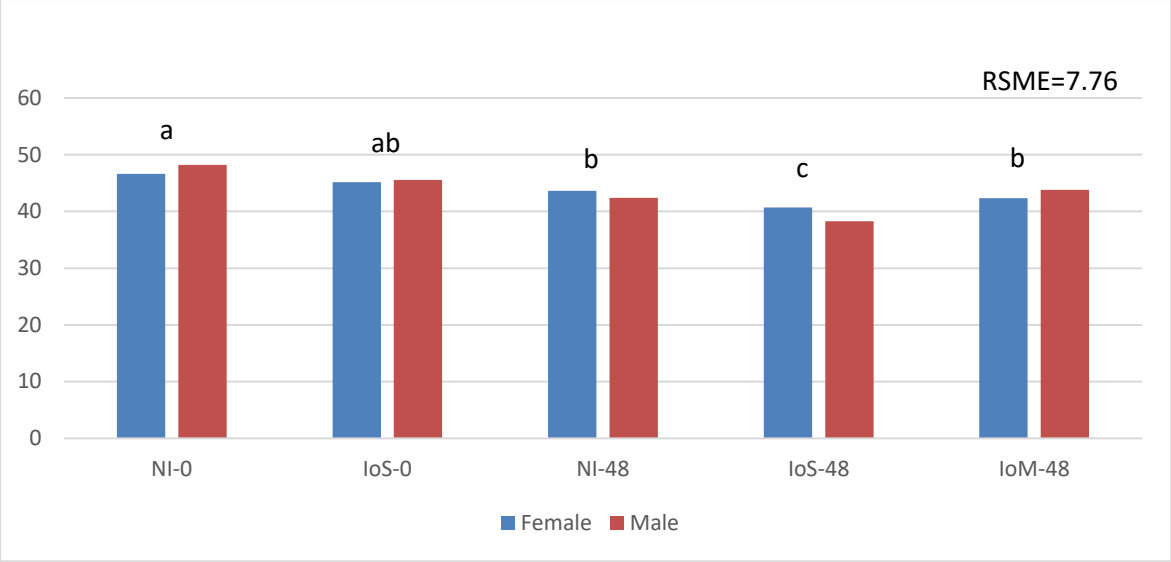


Figure 11. Average daily gain of the chicks (11-21 Days period).

NI-0 No intervention, immediate feeding; IoS-0 In ovo saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 in ovo saline, 48h delayed feeding;IoM-48 In ovo methionine, 48h delayed feeding; *"a" and "b" indicates treatment difference (P<0.001); no significant difference between sexes (P=0.9).*

Figure 11 demonstrates the ADG of the chicks from day 11 till 22 when chicks were getting the grower diet. The first 2 treatment groups still have better ADG than the delayed fed groups. There is also significant difference between the delayed fed groups where the IoS-48 shows less ADG than the rest of the 2 groups but by the end of experiment it cops up with the rest of the groups.

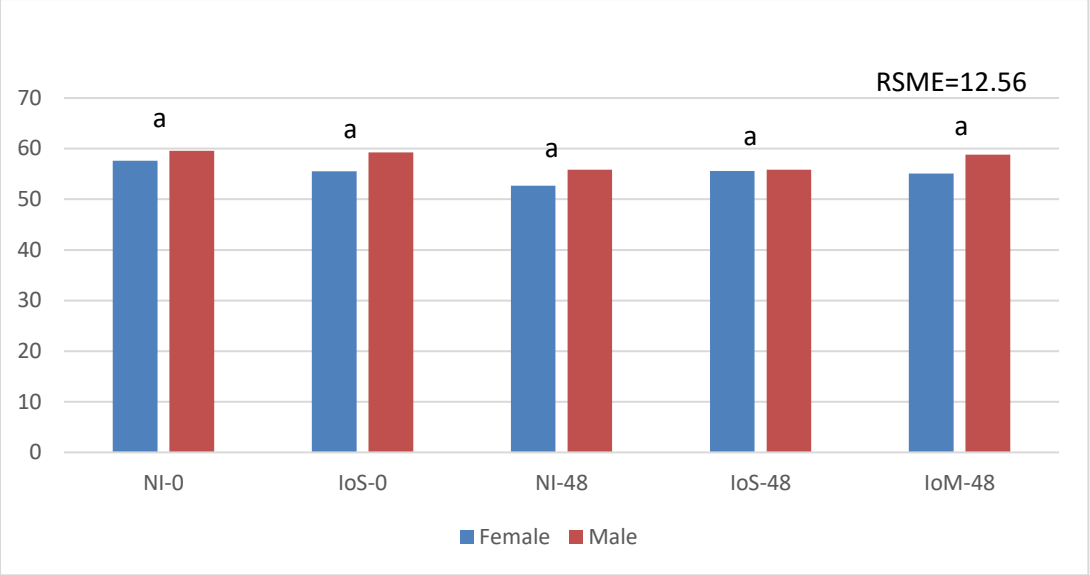


Figure 12. **Average daily gain of the chicks (22-35 Days period).**

NI-0 No intervention, immediate feeding;IoS-0 *In ovo* saline, immediate feeding;NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding;IoM-48 *In ovo* methionine, 48h delayed feeding; *"a" and "b" indicates treatment difference (P=0.12); significant difference between sexes (P=0.02).*

In the finisher diet period during day 22 till 35 that is the end of the experiment all the treatment groups have uniform ADG but there is significant difference between sexes where males showed better ADG by the end of the experiment than the females.

As the week passes, the weight gain getting equal in all groups is consistent with the experiment report by ALMEIDA et al., (2002) that after 1st week the weight gain become equal and showed no difference until the end of the experiment day 14. It is obvious that delayed fed groups of the compensatory gain later even though they could not match the weight gain as compared to the early fed groups (LI et al., 2022).

In ovo methionine injection also showed no significant difference in the weight gain in comparison to other treatment groups so methionine does not affect the weight gain of the chicken if given *in ovo.* The observed no difference in weight gain in our experiment is consistent with the experiment results of (GROFF-URAYAMA et al., 2019, COSKUN ISA et al., 2018)

5.3. Feed Intake & Feed conversion ratio

Referring to the feed intake (FI) which was recorded with the feeding phases between 1-10 days starter feed phase, 11-21 is grower feed phase and 22-35 is finisher diet phase, the following observations were made. During the first 10 days there was significant difference evident P<0.001. NI-0 showed the most feed intake as compared to IoS-0 and other delayed fed group. Delayed fed groups showed no significance difference among them, so Methionine do not have any effect on increasing the feed intake.

Table 9. **Feed intake of chicks for 35 days period in each group.**

NI-0 No intervention, immediate feeding; IoS-0 *In ovo* saline, immediate feeding; NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding; IoM-48 *In ovo* methionine, 48h delayed feeding

Trt	FI 1-10	FI 11-21	FI 22-35	FI total
NI-0	23.27a	70.65a	123.97	76.46a
IoS-0	22.03b	68.83ab	120.21	74.14ab
NI-48	18.14c	64.68ab	116.88	70.65ab
IoS-48	17.71c	62.77b	115.26	69.04b
IoM-48	18.22c	63.77ab	118.75	70.29ab
P-value	<0.001	0.0063	0.42	**0.02**
RMSE	0.78	4.41	9.25	4.61

abc Mean with different scripts are significantly different(P<0.05)

PINCHASOV et al., (1993) explained in his experiment that due to 48hrs fasting during the first week fasted chicks had less feed intake as compared to early fed chicks same as OBUN et al., (2013)

In the second phase, (11-21) feed intake of delayed fed groups got higher but still there was significant difference among the groups. Later, in the last time interval (22-35) there was no significant difference between any of the treatment groups (P=0.42). ALMEIDA et al., (2002), LI et al., (2022) experiments showed difference in feed intake in the earlier stage but by the end of experiment there was no difference in daily feed intake which is identical to our experiment results as well. OBUN et al., (2013) delayed feeding experiment showed no difference in daily weight gain between early and delayed feeding groups. While there will be difference in total feed intake among the early fed and fasting groups (P=0.02).

Table 10. **Feed conversion ratio of chicks for 35 days period of each treatment**

NI-0 No intervention, immediate feeding; IoS-0 *in ovo* saline, immediate feeding; NI-48 No intervention, 48h delayed feeding; IoS-48 *in ovo* saline, 48h delayed feeding; IoM-48 *in ovo* methionine, 48h delayed feeding

Trt	FCR 1-10	FCR 11-21	FCR 22-35	FCR total
NI-0	1.48a	1.71	2.42	2.04
IoS-0	1.47ab	1.74	2.34	2.02
NI-48	1.38c	1.72	2.39	2.04
IoS-48	1.38c	1.84	2.27	2.00
IoM-48	1.40bc	1.69	2.44	2.05
P-value	0.0002	0.64	0.56	0.91
RMSE	0.048	0.19	0.2	0.09

abc Mean with different scripts are significantly different($P<0.05$)

In the first 10 days there was significant difference among all the groups. In this period the fasted groups showed the lower feed conversion ratio (FCR) than the early fed groups but after this period there is no significant difference in FCR in any treatment group till the end of the experiment. Total FCR of the whole experiment period was also not significant ($P=0.91$).

Higher weight gain of chicks in the first period 1-10 negatively affects the feed conversion ratio (ALMEIDA et al., (2002). This negative effect of weight gain was only during the first 10 days later there was no difference. Overall, no difference in the feed intake and feed conversion ratio due to in ovo methionine injection observed in this experiment are in accordance with previous observations of (GROFF-URAYAMA et al., 2019, COSKUN ISA et al., 2018)

As per literature methionine is proved to improve the physiological changes in the bird like, heat stress, villus hight and width of small intestine (ELWAN et al., 2019). Based on supportive effect of methionine in physiology we devised it may help to improve growth of chickens with delayed feeding. But accounting the results of the experiment in ovo methionine alone cannot compensate for the negative effects of the post hatch delayed feeding. May be combination of energy source and methionine help to increase the performance of the bird. This can be explored further in future.

6. Conclusion

1. Early feeding is important for quick growth. Fasting of 48 hrs can have long-term negative impact on the final weight of the chick and can prolong the time required to get to market weight.
2. Methionine intervention into amnion at day 17 had no effect on the chick hatching weight, BW, ADG, FI and FCR of broiler chicks from day 1 to 35.

7. Acknowledgement:

The research was financed by GINOP-2.2.1-18-2020-00031 project.

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