Bioedukasi
Jurnal Biologi dan Pembelajarannya
Vol. 23 No 1, February 2025, page 110-115
e-ISSN: 2580-0094; p-ISSN:1693-3931
DOI: 10.19184/bioedu.v23i1.52812
Jurnal-dev.unej.ac.id/bioed/index.php/BIOED

The Effect of Increasing Testosterone and LPS Injection to the Chick of Domestic (*Gallus gallus domesticus*) Immunity and Growth

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Article Info
Article history:
Received January 14, 2025
Revised February 27, 2025
Accepted February 27, 2025

Keywords:
Body temperatur
Hormone testosterone
LPS *Escherichia coli*

ABSTRACT
In avian species, mothers have the ability to mitigate future challenges for their offspring by investing more or varied substances into their eggs. The primary goal of this strategy is to enhance the survival chances of their descendants. Since egg development occurs independently outside the mother's body, we manipulated the egg's contents by increasing androgen hormones in the albumin region. In this study, we introduced testosterone (T) and sesame oil injections during the early stages of embryo development by injecting the albumin position and observed the effects. After hatching, both groups of chicks were injected with LPS derived from *Escherichia coli* to assess their immune response and biometric growth. We measured the effects of T injections on body temperature, body mass, and the development of vital internal organs. The results revealed that chicks from the T-injected group exhibited three key indicators of improved condition: (1) a stronger response to LPS injection, as shown by a higher increase in body temperature compared to the control group, (2) similar body mass growth during early development, and (3) enhanced development of vital organs related to immunity. These findings suggest that embryos were able to utilize the increased testosterone during development to boost their immune responses against potential infections.

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1. INTRODUCTION

Maternal effects refer to the influence a mother's phenotype has on the characteristics of her offspring, independent of genetic factors. These effects enable mothers to adjust their children's traits to better align with the environments they will encounter, providing a level of flexibility beyond genetic inheritance. This flexibility is crucial for understanding processes like evolution, adaptation, and the outcomes of breeding programs. (Abruzzese et al., 2023; Groothuis et al., 2019). One way maternal effects manifest is through the exposure of embryos to hormones during development. Across various animal groups, from insects to humans, maternal hormones can shape the physiology, behavior, brain development, and physical traits of offspring.

Birds, in particular, offer valuable insights into these effects because their embryos develop outside the mother's body, So studies using bird models allow researchers to control and measure hormone exposure without affecting the parents. (Wang et al., 2023). Bird eggs contain considerable amounts of hormones that are deposited in the yolk by the mothers (Anderson & Navarra 2011). Among these hormones, androgens have been a primary focus of research. Studies in various bird species have shown systematic differences in androgen levels within and across clutches, suggesting that females may selectively distribute hormones like androstenedione (A4) and testosterone (T) in their eggs. This selective distribution can depend on factors such as the sex, quality, or laying order of the offspring, as well as environmental conditions like food availability and breeding density (Sapkota et al., 2020).

Several experimental studies have examined the effect of exposure to prenatal androgens on the offspring by injecting freshly laid eggs with T, A4, or both. In summary, increased levels of androgens may induce a variety of beneficial effects for the chicks, such as shorter incubation time (Eising & Groothuis, 2003) faster

post-hatching growth (Navara et al., 2006), increased competitive/aggressive behaviours (Riedstra et al., 2013). However, there is substantial variation in androgen deposition in eggs which suggests that the beneficial effects may be constrained by counter costs for the developing embryos. These costs may lie in the detrimental effects that androgens may have on the immune system (Müller et al., 2005), this may lead females to strategically adjust the allocation of these hormones in response to specific selective pressures.

The aim of this study was to test the hypothesis that chicks can overcome the negative effects of elevated androgen levels during embryonic development. Researchers injected eggs with a testosterone solution and sesame oil to mimic the natural increase in maternal androgens, then monitored embryonic development. They measured chick development by assessing body mass and recording temperature increases following LPS injection during early age. We had an experience in injecting LPS from *E coli* to stimulating immune system of the leghorn (Lelono et al., 2019) and domestic chicken using LPS from *S. tiphimurium* (Lelono & Surya, 2023) and in this study will use our own LPS extraction from *E coli* on domestic chicken (local Indonesian strain).

RESEARCH METHOD

Animal model

The eggs for the experiment were collected from the local chicken breeder in Sriwijaya Street, Sumbersari, Jember Regency. The Yudhistira local chicken (AKY) strain which characterize by their ability to produce both meat and eggs was use for this study. The adult chickens were raised in captivity in ratio 1 male and 5 females on the enclosure cages (2 * 3 * 3). In total there were 84 fertile eggs which lay less than two days to ensure their viability. All the eggs were cleaned, weighed, marked before starting the injection and then put in to the incubator. The temperature for the incubation is 30^{o}C and 20% of humidity, on day three and five each of the eggs was checked by candling method to indicate the development of the embryo. At day 5 there were 72 eggs ready for injection.

T and LPS solution preparation

Testosterone eggs were injected with a mixture of 100 µL vehicle (sterile sesame oil) and 50 ng of testosterone (46923, Sigma-Aldrich, DE), which constitutes the amount of ~2 sd of the naturally ocurring testosterone levels (mean = 127.2, sd = 23.3 ng) (Lelono et al., 2019). The LPS from *E. coli* bacterial culture was prepared by modified hot phenol-water extraction protocol (Rezania et al., 2011). In this protocol we were removed DNAse and RNAse, since the focus of the procedure to stimulate the lipopolisacradice on the wall membran of *E.coli*.

Testosterone injection

All of the 5 days incubation eggs were injected with testosterone (0.01 mL sesame oil + testosterone 50 ng /100 µl injection) as the treatment group and 0.01 sesame oil injections as the control group. Thirty-six of the eggs were in the T (testosterone hormone) group and the rest were in the S (Sesame oil) group. The solution injection was performed in the albumen region by making a small hole (~2 mm) drilled in the eggshell. The needle was inserted gently at a 45^{o}C angle into the egg albumen to ensure that the embryonic spot would not be interrupted. The hole was covered with melted paraffin (Lelono et al., 2019). All the process should be finished around 3 minutes to reduce the potential damage of the embryo. For the sterility of the injection we did all the preparation on the encase box.

Incubation eggs and raising chicks

The injected eggs both (T group and S group) were continuous their embryonic development on the incubator until hatch. All 17 day old chicks were raised on the aviary, the size of the metal cage is 75cm long * 75cm wide and 75 cm height. The temperatures in the cages were around 23 to 26^{o}C with 60% of humidity. Both group of the chicks were raised together, food and water were provided ad libitum. The size of the caged increase three times to provide the enough space for the movement and social interaction of the chicks

Injection of the LPS and body temperature measurement

Injection of the LPS solution was performed on 2-week-old chickens by intra-peritoneal injection method in a dose of 0.1 mL in the first injection 0.15 mL in the second injection and 0.2 mL in the third injection. The time difference between the first and second injection were 17 days after the first injection, as well as the third injection. The body temperature measurements were performed one hour before injection to collect the basal temperature and the second one was done one hour after injection. The third until thirteen of the temperature measurement were performed by inserting the probe of the anal digital thermometer for ten to twenty second until reach the constant grade. In order to minimize the discomfort we use the palm oil to lubricate the tip of the anal thermometer.

Organ collecting sample

The sampling organ and other biometrical data were done after the third injection. The section of the chicken body were focused on the data of total body mass, crude carcass, liver, heart, gizzard, duodenum, jejunum, bursa fabricius, thymus and speleen. Organ weight was tested using bivariate correlation to determine the relationship between organs. Prior to the bivariate correlation test, organ weight was first converted into relative weight with the formula:

Relative weight (%): $\frac{Organ\ weight}{Live\ weight}\ x\ 100\%$

Ethical clearance statement

All of the experiment protocol has been tested, analyzed and proved by the medical faculty ethical committee. The formal certificate number is 3135/UN25/1.10.2/KE/2024 declares at 26 July 2024. The entire researcher member has been trained for handling the animal's model and the head of the team possess the license to handle and perform an experiment with animal model from Groningen University.

Statistical Analysis

To start the statistical analysis all of the data were tested their normality and continue to the parametric test. The correlation analysis between body mass and other internal organ were used the bivariate correlated model with all data related to the relative weight as variable. We then make a list of the R and P value to see how strong the correlation between two organ especially those who related to the immune system.The temperatur differences due to the LPS injection To analize were analyzed by linear mixed model with body temperatur as depended variable; treatment group between Testosteron versus Sesame Oil injection and the temperatur with or without LPS injection as fixed factor, the chicks identity were used as random factor. For the posthoc test to analyze the different between T vs control group we use independent sample t test. The growth of the chick were analyzed by mixed model with the body mass, treatment group, sex of the chick, the age and the combination between treatment group with the sex and age as a fixed factor and ID as random factor.
All of the statistical analysis were used the SPSS version 22.

2. RESULT AND DISCUSSION

Result

The body mass of the chicks was not different between T and C groups (F=2,877; p=0,118), there is no difference between male and female chicks from day old chicks to 62 days old (F=0,162; p=0,695), however there is a significant different of the age along their development phase (F= 86,559; p=0,00). There is no difference in the interaction between treatment with sex (F=0,002; p=0,963), but there is significant different in the interaction between Treatment with age (F=2,964; p=0,008). The impact of the LPS injection to the increasing of temperature of the chicks in the treatment group (Testosterones and Sesame oil) were not significant (F=2,016; p=0,178), the different between LPS injection and non LPS were significant (F=284,083; p=0,00) while the interaction between treatments and LPS injection were significantly different (F=3,739; p=0,054). We than continue the indpendent T-test and found that the body temperature of the T were higher than C (t=2,14; p=0,33). The correlations between internal organs were displayed in the table 1.

Table 1. Comparison of mean organ index (g/kg body weight) (+SD) of domestic chickens in testosterone and sesame oil.

Organ	Control	Testosteron	*P result*
Lymph	0.26 ± 0.12	0.29 ± 0.04	0.538
Bursa fabricius	0.30 ± 0.10	0.36 ± 0.11	0.322
Duodenum	0.80 ± 0.07	0.83 ± 0.18	0.697
Jejenum	3.51 ± 0.41	4.19 ± 0.57	0.023
Gizzard	3.03 ± 0.39	3.27 ± 0.77	0.466
Liver	2.08 ± 0.27	1.93 ± 0.15	0.210
Thymus	0.47 ± 0.10	0.56 ± 0.27	0.450
Heart	0.43 ± 0.05	0.45 ± 0.09	0.554

Discussion

In this study, we examined whether chicks could overcome the negative impact of elevated androgen levels during embryonic development and how they would respond to an immune challenge after LPS injection during their early life. The initial indicator of immune response was their reaction to LPS injection (extracted from E. coli) as an immunological stimulus. We observed that the T group exhibited a significantly higher average temperature increase compared to the control group, suggesting a stronger ability to respond to the LPS injection (figure 1). This temperature rise reflects their physiological response to bacterial infection, a critical survival mechanism given the variety of potential infectious threats in their environment. The immune response to LPS in experimental animals typically results in fever, alterations in white blood cell counts, hypotension, intravascular coagulation, and shock, which can be fatal following injection with pure LPS or live/dead Gram-negative bacteria (Sampath, 2018). In most Gram-negative bacteria, the O antigen helps evade the immune system, while LPS, specifically lipid A, is responsible for the strong toxic effects, including mitogenicity, pyrogenicity, complement activation, and lethality. The immunogenic potential of lipid A varies by bacterial species and growth conditions (Mazgaeen & Gurung, 2020). In *E. coli*, lipid A triggers immune stimulation and induces strong proinflammatory responses in host cells (Mazgaeen & Gurung, 2020; Steimle et al., 2016).

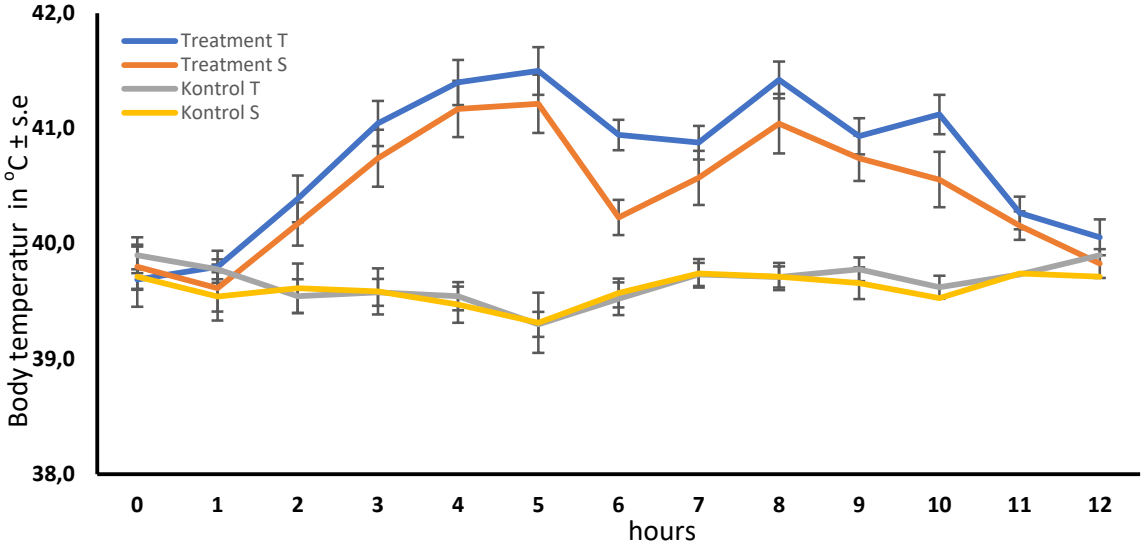

Figure 3. The pattern of body temperatur post-LPS injection in two different group of chicks which showed in blue line for the T group and orange line for the C group. And the other line showed the average of body temperature without LPS injection; the grey is belong to the T group and yellow is belong to the control group.

The first indication of hormone treatment was an increase in body mass (from day 0 to day 64). In this study, the chicks' development showed no significant differences between the treatment groups or between the sexes. There was a slight trend of male chicks in the treatment group outperforming females in weeks 3, 7, and 8, but this difference was not statistically significant. The findings suggest that testosterone supplementation significantly increased body weight during a specific period, particularly between days 15 and 18, but had no effect on body weight at days 24 and 27. Testosterone, a steroid hormone with anabolic and androgenic effects, promotes lean muscle mass growth (Bain, 2010). Testosterone stimulates an increase in Growth Hormone (GH) in the anterior pituitary, this growth hormone influences the body's physiology including protein, fat, and carbohydrate metabolism and bone growth (Reinecke et al., 2005).

Testosterone hormone injection in eggs is only administered in the albumen, while the accumulation of testosterone use during the embryonic period is in the yolk (Groothuis & Schwabl, 2008). The use of testosterone hormones may not be fully utilized by the embryo, but the results of body weight data show a difference in weight between the testosterone injection treatment group and the sesame oil injection group, this difference assumes that testosterone provides long-term effects that can be used in the future. The increasing T concentration via in vivo methods on chicken embryos before incubation, showed that plasma testosterone levels increased significantly in both sexes on days 12, 15, and 19 after testosterone injection with an increase of up to 3.9 times (Henry & Burke, 1999). Whereas, in this study 5 days incubation, so that the accumulated use of testosterone may not be used optimally.

Here, we compared the relative body mass of the chicks to the size of key organs, particularly those vital to the immune system. We found that in the treatment group have a higher average although the difference was not significant (Table 1). In contrast, the control group showed no such indication of a relationship between body mass and immune-related organs. The bursa of Fabricius is particularly important for immune function, as noted in previous studies (Adriaansen-Tennekes et al., 2009; Birhan, 2019; Parmentier et al., 2004). This increase may be due to the application of LPS extract, which is immunogenic and can induce immune stress in animal and damage immune organs.

This data suggests that the treatment group continued to increase the biomass of immune-related organs during early development. These findings align with other study, who identified the bursa of Fabricius, thymus, and spleen as the three primary organs involved in the avian immune system (Mehrzad et al., 2024). The thymus is especially crucial, as it provides the environment necessary for proper T cell differentiation. In the thymus, lymphoid progenitors or pre-T cells proliferate and develop into effector cells for cell-mediated immunity and regulatory cells that modulate immune responses (Hoffman et al., 2023). Our study shows that most of the immune-related organs in the treatment group developed well, contributing to the enhanced immune competence of the chicks.

Our hypothesis proposed that chicks would overcome the negative effects of elevated androgen levels during embryonic development. Our findings suggest that chicken embryos were able to withstand the elevated testosterone levels and even utilize them to enhance their immune competence during early development,

ultimately increasing their chances of resisting infectious diseases. This hypothesis can be explained by three possibilities. First, embryos may harness the testosterone hormone during critical developmental stages, incorporating it into their metabolic processes and overall embryonic development. This could explain the chicks' improved immune competence after hatching. This result offers an alternative explanation, as in our previous study (Lelono, 2019), we did not observe any effects when testosterone was administered before the incubation period. Second, elevated prenatal exposure to yolk testosterone may play a key role, as this substance is important during early embryonic development. For instance, it has been found that yolk testosterone increases metabolic rate (Tobler et al., 2007). Since metabolic rate governs nearly all biological activities (Brown et al., 2004), including components of the immune system (e.g., Kankova et al., 2018), it is possible that under natural conditions, where food is not always abundant, the negative effects may become more pronounced, either during the chick phase or at a later stage.

3. CONCLUSION

This study revealed three key findings: first, chicks in the T group exhibited a stronger response to LPS injection by showing a greater increase in body temperature compared to the C group. Second, these chicks also displayed similar body mass during early development. Third, the T group developed vital immune-related organs more effectively. Together, these results suggest that the embryos were able to utilize the elevated testosterone during embryonic development to enhance their immune response to potential infections. This study show that if the negative effect of testosterone addition can be solved. So, it is hoped that the results of this study can be a reference in the future to develop superior strains, especially in the immune system, growth, and development of chickens.

ACKNOWLEDGEMENT

This work was kindly supported the DRTPM Research Grant No. 0459/E5/PG.02.00/2024, who provided the finansial and other administrative support. We thank Efie Fajriah Eka Dewi, Ayu Diah Utami, Agatha Dei Gratia, Wardza Izza Wulandari for the supporting and their valuable contribution in providing laboratorium expense, animals care takers, statistical analysis and constructive discussion during the study.

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