Sex-Embryo Determination Using the Heart Rate as a Non-destructive Method in the Avian Species: Study on Japanese Quail (*Cortunix japonica*)

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Abstract. Sex determination of the embryo during the incubation phase is crucial in the poultry industry. This study focused on solving one of the crucial problems during the incubation phase in the laying poultry breeding industry, namely determining the sex of the embryos. The early stages of research will focus on collecting primary data on heart rate as a non-invasive method and folowed to the specific response to the embryonic development of the Japanese quail (*Cortunix japonica*) to determine the sex of the embryo during incubation period. As the animal model, we use the layer strain of the Japanes Quail (*Cortunix japonica*) since they are easy to handle and produce continuous eggs. The quail represents the small types of laying avian species that are economically important as egg producers in some Asian countries. We incubate the Japanese quail eggs and measure the embryo's heartbeat on day 7 in the 37 °C and variety of the temperature measurement. We found the significant correlation between heart rate to the environment temperature. This indicate that embryo has an ability to adjust their metabolic rate depend on the environmental condition. We also found the different patterns of the embryo's heartbeat based on their sex. The clear difference between male and female shows on day 10, and this difference continues until one day before hatching. The results of this study contribute to the information related to the embryonic response to changes in ambient temperature during the incubation period of small birds represented by quails and could be used as starting point to investigate the various species especially the avian layer strain such as leghorn.

Keywords: Embryo · Heartbeat · Japanese quail · Incubation · Avian

1 Introduction

In the productive laying poultry breeding industry, female chicks have a high commercial, meanwhile, the male chicks will be destroyed [1, 2] because they have no economic value [2, 3]. This condition is a waste of resources and has a negative impression of the animal welfare. Therefore, determining the sex of fertilized eggs especialy the female

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one is very important to increase the production efficiency of the laying poultry industry [4]. Several methods of determining embryonic sex have been developed, such as the differences in hormone estradiol (E2) levels in allantois fluid [5], the blood detection by a series of technique s, such as machine vision vascular yolk sac morphology, and orientation models [6], and fluorescence spectroscopy based on haemoglobin differences [7–9]. More importantly, poultry eggs of different sexes contain different volatile substances at incubation day 8 with 2-undecanone as the characteristic odour, a metabolite related to testosterone (T) [10]. Steroids play an essential role in the reproduction, development, and growth of chicken embryos, sex differences, and sex determination during the embryonic period. The incubate eggs contains the most powerful and prevalent endogenous steroid hormones such as Estrone (E1), E2, estriol (E3), T, androstenedione (A4), and dihydrotestosterone (DHT) [11, 12]. Sex steroids are mainly secreted in the gonads and then spread to the bloodstream, eventually acting on target cells [11]. Since blood hormone concentrations reflect physiologically available body hormones, sex differences in chicken egg embryos can be explored by detecting serum steroid hormones between males and females [6].

However, the crucial weakness of the sex determination methodology mentioned above is the sampling stage. Hormonal uptake of eggs requires an invasive method of extraction by perforating the shell of the egg. This stage carries the risk of increasing the potential for infection. Therefore, we need another method that can minimize risk but still be effective, namely the non-invasive method. We can use non-invasive methods to determine the foetus's embryonic development in eggs using a heart rate detector. This non-invasive method is seen as a very significant breakthrough because it preserves the embryo's life until it hatches. So far, the selection of heart rate as an indicator of embryonic development has been carried out by several researchers [13]. However, consistent results and standard procedures have not been found [14, 15]. Based on this background, this study is focused on a study to determine the sex of an egg embryo using a non-invasive method. This study hypothesizes that male embryos will show significant differences in heart rate since the incubation period.

2 Materials and Methods

2.1 Experiment Design

Javanese quail's eggs as research objects were obtained from breeders around Jember who specifically quail strain for egg production. Eggs come from a cage containing male and female parents with 10 females and 1 male. The ratio of 1′10 for quail is sufficient to ensure that each egg produced by the female contains an embryo. These fertile eggs ware then recorded by biometric data, namely weight, length, and width. For identification, each egg is marked by affixing white label paper to be easy to recognize.

The eggs that had been biometrically recorded and labelled were then placed in an incubator at 37 °C. Eggs are arranged in alternating grooves to allow fresh air to exchange freely. The eggs' position is placed in a horizontal position with a shift in position every 12 h to balance the yolk and albumin positions. After one week of incubation periods, the heart rate data were collected regularly at the same time every day using the "Digital Egg Monitor" monitor. All of the fertile eggs then continue to incubate until they hatch

at days 15 and 16. The chick then identified the individual differences using small sticky paper stickers on their legs. Those chicks then rose up to one month old until the sexes' differences were clear.

2.2 Animals' Model

Quail eggs as research objects were obtained from breeders who breed quail for egg and seed production in Maesan Bondowoso. Eggs come from a cage containing male and female parents with a composition of 20 females and 5 males. Generally, males weigh 140–143 gr and females 143–146 gr. Select types of quail lay eggs ranging from 250–300 eggs per year [16]. The age of quail as a source of eggs is 6 months and has been produced regularly for at least 4 months, marked by a pencil, egg size, and daily production. These fertile eggs are then recorded in the biometric data, namely weight, length, and width. For identification, each egg is marked by affixing white label paper so that it is easy to recognize.

2.3 Avian Embryo Heart Rate Detector

Several researchers have developed a heart rate detector, and this tool adopts a method of using a laser beam to detect changes that occur in eggs. The measured change is a rhythmic vibration or beat that is generally displayed by the heart organ. This tool will minimize harmful effects and even reduce the cessation of the embryonic metabolism process, leading to death. Although initially intended to replace the candling method, the information generated by this tool very accurately describes the biological activity of the embryo. In addition to life indicators (indicated by heart rate), it can also recap the embryo's movements, which can be translated as markers of life activities.

This tool, "Digital Egg Monitoring/Buddy," was developed by Avitronic - England Company to detect life markers in embryos that are difficult to see with ordinary candling methods. The heartbeat counting method's flexibility is in eggs that have more thickness and eggs with cryptic patterns. In patterned eggs, the embryo's presence becomes challenging to be exposed using the light bias which is the basis for the handling.

The eggs that had been biometrically recorded and labelled were then placed in an incubator at 37 °C. Eggs are arranged in alternating grooves to allow fresh air to exchange freely. The eggs' position is placed in a horizontal position with a shift in position every 12 h to balance the yolk and albumin positions. After one week of incubation periods, the heart rate data were collected regularly at the same time every day using the Digital Egg Monitor. Each eggs was recorded for two different data, first the heart beat and second the temperature of measurement. The electric laser thermometer was used to measurement of the eggs temperature in the digitals' egg monitor box. The eggs measurement continues until day 14 of incubation and all eggs then euthanized in the freezer to stop the incubation process for further analysis.

2.4 Statistical Analysis

The research data is in the form of the biometric distribution of quail eggs biometry, heartbeat and temperature measurement, which will be analysed for normality. The

mean of eggs size, temperature of measurement, and heartbeat, will be presented in the result section. The hatchability was counted the number of eggs show hear beat at day 14 divided by the total eggs. Firstly, the biometric distribution data of quail will be analysed for normality. The difference of embryo sex will be analysed using mixed models with each egg's identity as a random effect. Models included sex of the embryo, days of the incubation, and the interaction between the sex of the embryo and days of the incubation as a fixed effect and eggs mass as covariate. The second source of data analysis is the embryonic heart rate during the incubation period as the dependent factor and the temperature of incubated eggs measurement is an independent factor. To analyse the correlation between temperatures of measurement to the embryonic heartbeat we use bivariate analysis correlation, we use correlation at 0.01 levels (two tailed). All data were processed using IBM SPSS statistics 22 software.

3 Results and Discussion

3.1 Result

The general information of Japanese quail in this study is presented below: Mean of eggs $\pm$ SE = 11.08 $\pm$ 0.03, Mean of temperature $\pm$ SE = 33.42 $\pm$ 0.06, Mean of heart beat $\pm$ SE = 233.67 $\pm$ 1.67 and the hatchability is 64.24%. The Pearson correlation between temperature and heart beat is 0.767 (significant at 2-tailed 0.01 levels).

The heartbeat of male and female embryo at day 10 up to day 14 were not significantly different ($F(1:59.04) = 0.478$, $p = 0.478$). However, there was an effect of days to the different of incubation periods ($F(4:239.08) = 26.02$, $p = 0.000$) to the heart beat of the quail embryo where female ($N = 24$) higher than male ($N = 15$). Interestingly, there was an interaction effect of sex and days of incubation to the heat beat of the embryo ($F(239.08) = 3.655$, $p = 0.007$). But there was no effect of eggs mass to the heartbeat of the embryo ($F(1:59.52) = 1.168$, $p = 0.284$).

3.2 Discussion

We analyse the effect of the temperature measurement to the heartbeat Japanese quail embryo during incubation periods using Buddy digital egg monitor as a non-invasive method. We showed the significant correlation between temperatures of the measurement to the heart beat. The embryonic heartbeat detection was started in day 6 up to day 14 (one day before hatch). This result demonstrated that the heartbeat of the embryo will increase if the temperature of were higher as shown in the Fig. 1. This result in line with the study held by Sheldon [17] in the Zebra Finch (*Taeniopygia guttata*). They found the heart rate increased throughout embryonic development and was positively correlated with ambient temperature.

The heart beat method's concern to determine of the avian embryo development has been shown by Glahn [13]. But they encountered difficulties in managing the overlap between normal and hypothermia conditions during the incubation period. Over all, the advanced method of determining embryonic heartbeat has been done by Sheldon [17] using the Buddy digital egg monitor (Vetronic Services, Abbotskerswell, Devon, UK).

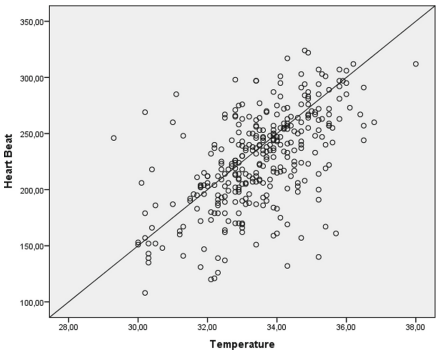

Fig. 1. The correlation between the temperature of measurement to the the Japanese quail embryonal heartbeat. The increase of temperature of the measurement will followed by the higher embryonic heartbeat.

Oviparous embryos show a range of behavioural and physiological responses that enable them to cope with external environmental challenges [18, 19]. For example, environmental conditions such as temperature can profoundly affect the rate and trajectory of embryonic development. Animals should be particularly vulnerable to sudden warming at the embryonic stage. Embryos, being relatively small and immobile, have limited capacities to thermoregulation. Studies of natural nests suggest that thermal fluctuations have little direct impact on embryonic performance [18, 20], however, these studies cannot reveal the impacts of temperatures that exceed the current range.

The embryo's hormonal content probably stimulates the metabolic rate which in turn to the embryonic. Wang [12] found the T hormone content of the embryo at day 10 up to 16 of incubation period where females significantly higher than males embryo. In the early stage of incubation, the maternal hormones were mainly concentrated on the egg yolk. The concentration of maternal hormone then shifted by the embryonic hormone production [21]. With the development of the embryo, endogenous hormones began to synthesize mainly in gonads. The higher T concentration in the female embryo could be stimulated by gonadal development where the female embryo already produces an oocyte [22]. The importance of androgen in the development of the embryo has been reviewed by Groothuis [23]. This group includes testosterone (T), Androstenedione (A4) and 5 alpha-dihydrotestosterone (DHT) that have many functions in an organism and can exert strong effects during development. In the beginning, mothers may use these androgens as a tool to influence the embryo's development in order to adjust its phenotype to the prevailing or future environmental conditions [24, 25].

In this study we also found no significant difference between male and female heartbeat embryos during the incubation period. However, we found the interaction between sex and the days of incubation to the embryo's heartbeat. The different patterns of the heartbeat between male and female embryo have been shown in Fig. 2. The differences of a heartbeat between male and female embryo start in day 12 up to day 14 (one day before hatch). This result demonstrated that the male and female embryo shows the difference in a heartbeat where females are significantly faster than males.

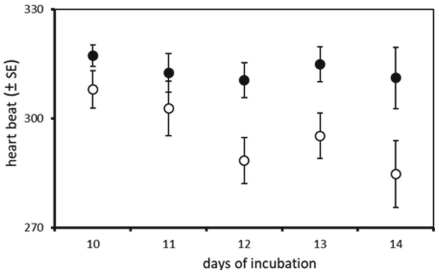

Fig. 2. The pattern of the Japanese quail embryonic heartbeat. The female embryo (filled circle) show higher heartbeat compared to the male embryo (open circle) from days10 up to day14.

The female's embryo heartbeat is higher than the male, similar to the adult phase of avian species such as domestic chicken. The heartbeat method's concern to determine the sex difference of avian embryo has been shown by Glahn [13]. They found that female embryos exhibited higher mean heart rates than males (2 to 4 beats/min) for Days 15 to 19 than the male chicks during incubation periods. However, they encountered difficulties in managing the overlap between normal and hypothermia conditions during the incubation period, and this implemented the method to become impractical for industrial scale.

Increased levels of yolk androgens may induce a variety of effects on the chicks' developmental time, growth, behavioral phenotype, immune functions, and physiology, some being positive and others, such as on immune function and metabolic rate, detrimental [25–30]. As the incubation continues, the sex-specific differences are sex-specific hormone metabolism by the embryo already in the first 3 days of incubation, depending on the differential transfer of other yolk components influenced by male quality. Indeed, avian embryos heavily metabolize maternal androgens very early in development [31–34]. Interestingly, since the differences of sex-hormone concentration already present at the early stage of development, the manifestation via heartbeat showed at day 10 up to 14 of the incubation period, warranting further study.

In conclusion, our results suggest that the correlation of the temperature ambient of the avian heartbeats has been shown since the embryonic phase. Another result showed that the avian heartbeat's sex-specific pattern has been shown since the embryonal phase. These results open new avenues for further studies on other avian species such as domestic chicken, especially in the poultry industry.

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Authors' Contributions. AL and BS designed the experiment, AL performed the experiments, AL and BS analysed the data, AL wrote the first draft of the manuscript and BS wrote with AL the final version. All authors read and approved the final manuscript.

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