Non-destructive Classification of Chick Embryos Based on Heartbeat, Body Motility and Growth Using Signal Processing of Near-infrared Light

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ABSTRACT

Poultry became the largest source of meat consumed in the world when it overtook pig production in 2017. In order to further promote sustainable poultry production systems to feed future generations, the monitoring and sorting of chick embryos rather than waiting to grade day-old chicks would enable higher productivity and more efficient use of resources. In this respect, non-destructive monitoring of chick embryos (hatching time, gender, cardiac abnormalities) during incubation could provide vital management insights for poultry farmers and other stakeholders. Non-destructive identification of hatch-window, gender and cardiac arrhythmias are also promising research areas with respect to promoting animal welfare in the industry. This is because late hatch chicks are a source of many complications in subsequent chick grading and rearing due to their delayed growth and retarded post-hatch performance. Hence, an understanding of the physiological factors leading to late chick hatching, along with early detection of these factors could avoid disadvantageous post-hatch culling.

Besides, there is a gender biased preference in dimorphic birds like chickens where the male is the preferable gender in broiler, and the female in layer strains. For broiler production female chicks are not economically viable due to their lower growth rates compared to male counterparts. Every year more than 7.0 billion day-old layer strain cockerels are culled globally by asphyxiation with carbon dioxide or maceration due to the noted gender biased production preference; raising serious ethical issues and resulting in significant economic losses. Therefore, non-intrusive, early chick embryo sexing before hatching is a crucial issue both from a commercial and ethical (animal welfare) point of view. This has driven increasing research effort and the desire of other stakeholders in the industry to find a solution. On the other hand, cardiac arrhythmia is considered an important cause of cardiovascular disorder in birds. Little is known, however, about arrhythmia during embryonic stages, chiefly because of their relative rarity and difficult of diagnosis. Unfortunately, the electrocardiogram (ECG) method cannot be used to study avian embryos due to the eggshell barrier. Therefore, an alternative method is an important tool if avian or reptilian embryo research in this area is to advance.

To remedy these issues, the study investigated the feasibility of using a near infrared (NIR) sensor together with signal processing to simultaneously monitor embryonic body motility, heart beats both in-terms of frequency and strength, as well as opacity (embryonic growth), towards the end of predicting hatch-window, and detecting gender and cardiac arrhythmia. Broiler chicken eggs (ROSS 308), selected based on size, weight, shell colour, were incubated for these purposes. Embryonic activity signals, based on NIR transmitted LED light and photodiode detection, of individual embryos were measured every 24 hours from day 6 to 19 of incubation. Peak

frequencies and the power spectrum from these signals were extracted using Fast Fourier Transform and numerical integration. The signals were also filtered using a low pass filter to visualize the characteristic signal patterns.

Two types of chick embryo body movements were found throughout the whole incubation period. During the early stage of incubation (day 6 to 8), the movement has a periodic pattern with a characteristic shape that differs among the embryos with a frequency ranging from 0.3-0.8 Hz. In the mid to late stages of incubation, movements were random, and had a lower frequency range from 0.2-0.6 Hz. Body movement strength was highly random, especially after day 10 of incubation. Heart rate during the incubation varied from 3.8 to 4.8 Hz in the period from day 8-19, while heartbeat strength sharply increased during incubation, peaking at day 14 to 16, and then subsequently subsiding. while heartbeat strength sharply increased during incubation, peaking at day 13 to 14, and then subsequently subsiding. While heartbeat was found to be related to hatch window, body motility and opacity were dependent on chick embryo gender during incubation. In terms of cardiac output, the higher the cardiac output the shorter the incubation period (an earlier hatch window). Heartbeat can be used to separate late hatch chicks with an accuracy of about 90%. On the other hand, male embryos during the second half of incubation were found to be more active and heavier than female embryos in-terms of body motility and opacity (growth), respectively. Chick embryo gender can be detected based on body motility strength and growth with the accuracy of 84% before hatching. In addition, we also investigated and interpreted embryo cardiac signals with naturally occurring bradycardia throughout the incubation. We found a normal heart rate (HR) during first half of incubation, but the HR had two frequency components in the mid incubation period and finally a much lower heart rate up until hatching. Early detection of potentially abnormal chicks with cardiovascular defects could significantly contribute to the humane treatment of these embryos and increase production efficiencies by identifying high quality embryos.

These results indicate that the NIR sensor, combined with signal processing, has the potential to non-invasively detect various hatch-groups, gender and cardiac arrhythmia. These techniques will enable poultry hatchery managers to ensure more precise monitoring and to enable sorting of embryos with the aim of obtaining uniform and high-quality chicks. Moreover, this could help to reduce costs by reducing the rearing time of unwanted embryos (space, energy and labor costs), as well as minimizing post-hatch handling for sorting of unwanted embryos. This research could also open many new dimensions of non-destructive research in precision poultry production and biomedical engineering fields such as embryo grading systems, indirect blood pressure measurement in avian and reptile models; cardiovascular drug design research; oxyhemoglobin level estimation in blood and in many other areas in the future.

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The author

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Chapter 1 General Introduction

1.1 Background

Poultry is the largest source of consumed meat in the world [1]. According to meat consumption statistics, poultry meat consumption is rapidly increasing compared to other sources of animal and fish protein, such as beef, pork, wild fish, cultured fish, and lamb, due to its easy access and lower price. Besides, the eggs from are a great source of protein for low income people worldwide and is a common item in daily food menus for all classes of people as well. Therefore, research on viable and healthy embryo detection, together with hatching time and gender prediction would enable more sustainable, precision, poultry production systems and potentially contribute to the immense challenge of feeding future generations.

1.2 Problem Statement

To ensure poultry production improves its sustainability for both meat and eggs, at all scales of operation, such production systems are critically dependent on the supply of uniform and high-quality day-old chicks from the hatcheries that supply the chicks at this stage. But to efficiently ensure uniform and high-quality day-old chicks, monitoring of embryos and early intervention during incubation will be a crucial step, as any small variation in embryo development at this stage can result in big differences in post-hatch performance. Some embryos show inferior growth (weakness, thin and noisy, soft and lethargic etc) performance, such as late-hatch, male in layer and female in broiler production, chicks with cardiovascular diseases (e.g. cardiac arrhythmias). These types of chicks are normally rejected from further rearing. In addition, Ernst et al. [2] reported that over 3% of embryos die within 3 days from the start of incubation, more than 0.3% of embryos before day 18 and 8% of embryos are dead at transfer to hatcher tray.

This is because late hatch chicks are a source of many complications in subsequent chick grading and rearing due to their delayed growth and retarded post-hatch performance. Hence, an understanding of the physiological factors leading to late chick hatching, along with early detection of these factors could avoid post-hatch culling. On the other hand, there is a gender biased preference in dimorphic birds like chickens where males are the preferred gender in broiler and females in layer strains. For broiler production female chicks are not economically viable due to lower growth rates compared to their male counterparts. Besides, every year more than 7.0 billion day-old layer strain cockerels are culled globally by asphyxiation with carbon dioxide or maceration due to this gender biased production preference; raising serious ethical issues and resulting in significant economic losses. Non-destructive methods to predict hatching window and gender is promising research areas in industrial and animal welfare from this perspective. Therefore, non-intrusive, early chick embryo sexing before hatching is a crucial issue both from a commercial and ethical (animal welfare) point

of view. This has driven increasing research given the desire of stakeholders in the industry to find a commercial solution.

Therefore, monitoring of embryos and early intervention during incubation is a very important issue in order to reduce loss of energy, excessive use of incubator space, increased time and handling costs. Future precision poultry production will be an approach that monitors birds closely in order to maximize the efficiency of the entire system through minimizing losses and unnecessary resource expenditure.

1.3 Literature Review

1.3.1 Chick embryonic developmental stages

Work by Byerly [3] on fundamental chick embryonic growth components, which destructively sampled large numbers of embryos, has established the mass of an embryo increases exponentially during incubation (Figure 1-1). The first half of incubation, day 1 to 10, is called the embryo formation phase, whereas the second half, from day 11 to until hatching, is called the growth phase.

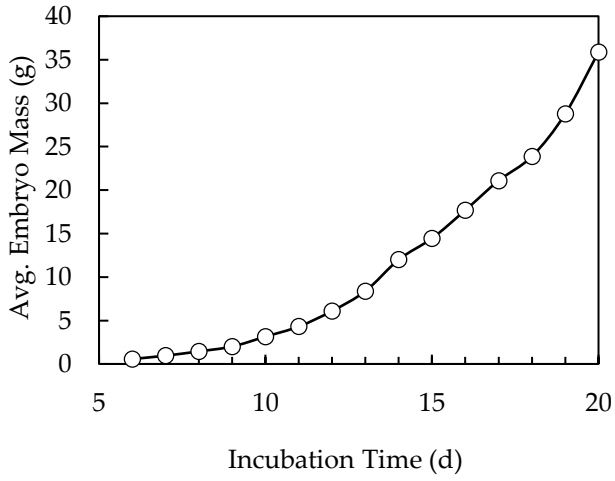


Figure 1-1 The generalize growth patterns of embryo[3]

The allantois appears on the fourth day of incubation and gains maximum weight from day nine to the eleven. The allantois is very turgid during this period and exudes liquid, thus losing weight rapidly on standing for even a short period.

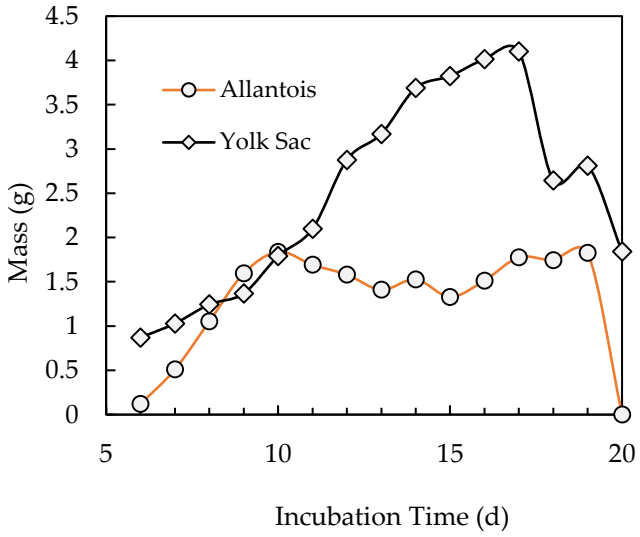


Figure 1-2 The generalized growth pattern of yolk sac and allantois[3]

The growth pattern of the yolk sac is shown graphically in Figure 1-2. From the sixth day on, the weight of a yolk sac in healthy egg steadily increases by the middle of the incubation period. The curves in Figure 1-2 show two ascending segments and a final descending segment. The first segment extends from the beginning of the incubation period to the ninth or tenth day. During this period the yolk sac grows peripherally until it almost completely surrounds the yolk. The yolk itself increases rapidly in size during the first four days of incubation due to absorption of water from the albumen. The yolk sac is of approximately constant thickness during this process of yolk enclosure. The second segment of the curves extends from the end of the first segment to the fifteenth or sixteenth day of incubation. The yolk decreases in size during this period due to the excretion of water into the allantois. Possibly this fact accounts for the initiation of a new mode of growth of the yolk sac: the formation of radiating lamellae which invade the yolk. These lamellae are much deeper in the peripheral portion of the yolk sac than in the central part. The third segment of the curve extends from the fifteenth or sixteenth day to hatching time. The size of both yolk and yolk sac decreases. The yolk becomes slightly more hydrated and finally both are drawn into the body[3]. Other physio-anatomical changes of the chick embryo over incubation time are summarized in Table 1-1 (adopted from Hamburger and Hamilton[4]).

Table 1-1 Embryonic development of domestic fowl [4]

Day	Age (h)	Developmental Event
1	6-7	Primitive streak begins
2	32-36	Bending and contraction of heart begin
	38-49	Haemoglobin synthesis begins, heart becoming S-shaped
3	65-72	Allantois now distinct, embryo surrounded by amnion
4	84	First active movements of head and neck occur
5	108-120	Trunk movements begin, four-chambered heart formed
6	120	Amnion begins to contract rhythmically
	144	Limbs now participate in whole body movements
		Active transport of amino acids by yolk sac membrane
7	150-156	Independent limb movements, sexual differentiation
8	180-192	Mineralization of bone begins
9	192-206	Chorio-allantois becomes fixed to shell
10	216-240	Whole body movements become jerky and random
	252	Amniotic contractions much reduced
12	264-288	Albumen absorption, calcium absorption from shell starts
13	288-312	Length and amount of activity phases reach a peak
14	312-336	Body begins to assume position along axis of egg
16	360-384	Embryo capable of respiratory movements
17	384-408	Co-ordinated and stereotyped movements begin
18	420	First behavioural response to light
19	432-456	Absorption of allantoic fluid completed,
		Yolk sac withdrawal begins
20	456-480	Begins to breathe and vocalize, shell pipped
		Withdrawal of yolk sac completed

1.3.2 Chick embryo body movement

Embryo movement represents a valuable epigenetic factor in vertebrate development [5], [6], as well as reflecting developmental establishment of neuromuscular interactions [7]. Moreover, correct tissue formation and anatomical structures associated with embryonic locomotion depend on the specific patterns of these movements[8]. The establishment of the locomotion system in vertebrates also determines the degree of wing and leg freedom in postnatal life [9], [10].

The avian embryo moves for almost the whole incubation period, and the greater part of this movement is active rather than passive[11]. The movements exerted by the embryo during incubation can be summarized as body movements including limb (leg and wing) movement and amniotic contraction, cardiovascular movement and respiratory movement. Previous researchers reported that the first active movement of the embryo head and neck start from day 4 (84 h of incubation) and the first trunk movement begins at day 5[4]. The first rhythmic movements begin at day 6, but this movement is gradually lost as the head and body become larger and heavier[12]. As the embryo transitions from the formation phase to growth phase, the movement pattern changes to a random and jerky one from day 10 with reduced amnion contraction reported by Hamburger and Hamilton[4]. They also reported that in the activity phase, both the length and the absolute amount of activity of the embryo peaks at day 13.

In-case of respiratory movement, although the embryo is capable of respiratory movement, the embryo only begins to breathe and vocalize at day 20[4]. Before this period, the allantois is fused with the shell membrane, normally near the air cell, which acts as a special respiratory organ for gas exchange and to excrete body waste.

1.3.3 Chick embryo heart beats

Although the heart of the chick embryo begins to form at the end of day 2, the movement of this tiny s-shaped heart is very small and is of a very low magnitude. Many avian biological scientists have reported cardiovascular movement in the range of 200 to 300 beats per minute throughout the incubation period based on destructive, semi-destructive and finally non-destructive methods.

Chicken eggs have been well studied for heart rate (HR) developmental patterns using Electrocardiography from day 15 to 21 by Laughlin et al. [13]; from day 6 to 20 by Tazawa and [14], Impedance Cardio Graphy from day 3 to 9 by Akiyama et al.[15]; from day 7 to 20 by Howe et al.[16], Ballistocardiography by Pawlak and Niedziolka [17], Acoustocardiography from day 12 to 20 by Akiyama et al. [15], and the Buddy Egg Monitor by Lierz et al[18].

In general, the embryonic HR steadily increases during the early stages of incubation during the conversion of the primordial tubular heart into a four-chamber configuration, followed by relatively

slow changes in HR during the last stages of incubation, though this passive movement does not follow any strict time dependant pattern. Embryonic HR tends to decrease toward the end of prenatal incubation with subsequent increase during the perinatal period, which is observed in many precocial birds[19]. The HR begins to become irregular from days 13 to 14 of incubation [15], [20]. Maximum HR of chick embryo has been reported to be on days 14-15 and then decreases to 250 bpm on day 18[15].

In the last few years, several researchers have shown considerable interest in heart rate (HR) measurements of chick embryo using invasive, semi invasive and non-invasive methods. Most of these techniques were destructive or semi-destructive except for the Ballistocardiograph, Acoustocardiograph and Buddy Egg Monitor methods.

1.3.3.1 Ballistocardiography

This non-invasive method measures the minute movements of the entire egg that result from cardiac contractions and blood ejection from the heart of the avian embryo (this ballistic movement is designated as BCG). The Ballisto-cardiograph measures vibrational capacitance as the ballistic movement of the eggshell. Body movement severely interferes with this device`s measurement.

1.3.3.2 Acoustocardiography

Cardiogenic pressure changes can be detected by a condenser microphone with a differential pressure transducer and is termed an Acoustocardiogram[21], [22]. Acoustocardiography is a non-invasive system and is relatively unaffected by embryonic activities compared with ECG, BCG. While it can be successfully used for HR measurements in chick embryos during the last half of incubation, the microphone often fails to detect ACG during the last stage of prenatal incubation and in the perinatal period [19].

1.3.3.3 Buddy egg monitor

The Buddy Egg Monitor was invented for hobbyists who wanted to monitor parrot eggs based on diffuse transmission. A limitation of this method is that it has a high probability of including body movements in heart rate measurements since it incorporates frequencies from 50 BPM. Moreover, it’s readings can be interrupted by body movements; a very common occurrence in avian embryos.

1.3.4 Chick hatch-window

Nowadays, the supply of high quality and uniform batches of chicks is considered one of the most important challenges to breeders and poultry farmers [23]. However, the homogeneity of the day-old chick cohort is frequently compromised by a wide-spread hatch window; a window that contains chicks with short to long incubation periods [24], [25]. This negatively affects that cohort’s post-hatch performance. It is well known that late hatch chicks show extensively inferior quality

(growth rate, mortality and disease susceptibility) in post-hatch performance [26]. Moreover, late hatch chicks cause many downstream complications in post-hatch chick sorting, feed and water supply, vaccination and maintenance of the rearing environment, which are correlated with hatching time differences (24-48 h) from those of the early hatch chicks.

Although the linkage between embryo growth, hatching time and day-old chick uniformity are known through studies of the effect of incubation temperature, it is less clear how physiological variability in embryo growth effects the timing of hatching [27], [28]. For instance, how the cardiac activity of chick embryos during incubation affects the hatch window has not yet been studied. However, cardiac changes are suspected to influence the growth of the embryo and the subsequent hatch window. According to Ar and Tazawa, avian embryos tend to stay in the egg for a fixed period, with all embryos having a more or less constant total number of heart beats during the incubation period [29]. Thus, the growth of the embryonic heart could be a good indicator of chick embryonic growth and stage of maturity. Hence, higher cardiac activity during incubation may shorten the hatching time of chick embryos. In oviparous organisms, the duration of incubation can be a critical life history variable [30]. Hence the embryonic development history of a chick embryo during the incubation period might have significant importance for precision prediction of hatching and post hatch chick performance. In these circumstances, a cardiac physiological study of various hatch groups, especially late hatch chick embryos, is extremely important for developmental physiology and precision poultry production system management. Early detection during incubation of potentially late hatch embryos could significantly contribute to the humane treatment of these chicks (late hatch chicks are discarded) and support production efficiencies (minimize labor, energy and space utilization).

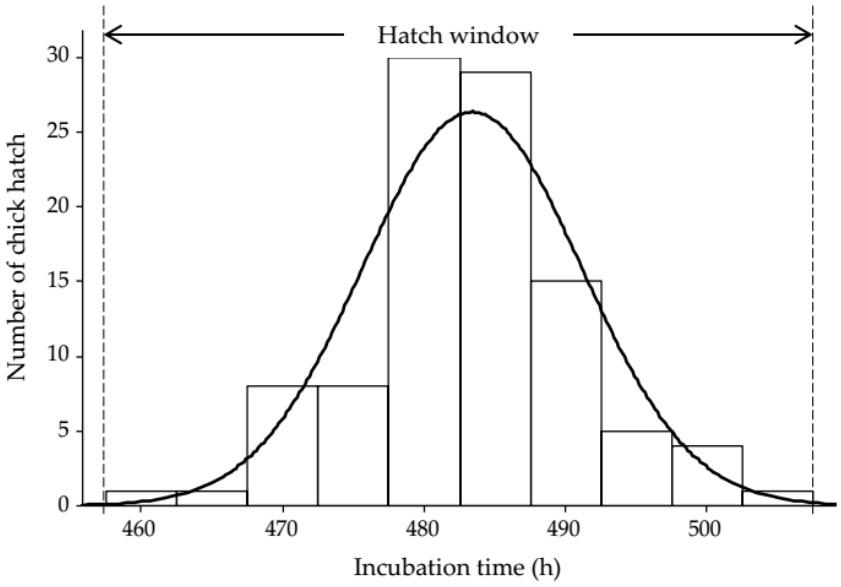


Figure 1-3 A typical hatch-window of chick embryos

1.3.5 Embryonic cardiac arrhythmia

Cardiac arrhythmia is considered important cause of cardiovascular disorder in birds. Little is known, however, about arrhythmia during embryonic stages, chiefly because of their relative rarity and the difficulty of diagnosis. The hearts of birds are morphologically similar to mammals, containing a full atrial and ventricular septum [31]. Although ECG, a non-invasive technique, is widely used in humans, such a method cannot be applied to avian or reptile embryos due to the presence of the eggshell. Therefore, a non-invasive method together with signal processing is keenly sought for advancing cardiovascular studies of avian or reptilian embryos. Some researchers have recently used the Buddy System and Embryonic Vital Scope for heart rate (HR) measurement of chicken or reptile embryos [18], [30], [32], [33]. A limitation of the Buddy egg monitor is that its readings can be obstructed by embryonic activity; a very common occurrence in avian embryos [18], [30]. For example, heartbeat signals can be obstructed by body motility during signal acquisition. Thus, the Buddy Digital Egg Monitor can't isolate heart beats from the mixed signal. This device can be considered a first generation, non-invasive HR measurement system.

Arrhythmia, which has its origin in the heart, can be classified into sinoatrial (SA) arrhythmia, atrioventricular (AV) arrhythmia and ventricular arrhythmia [34]. Based on the heart rate, it is also classified as bradycardia or bradyarrhythmia when the heart rate is below the normal range and tachycardia or tachyarrhythmia when the heart rate is above the normal range. If the heart rate is much higher it is called flutter, or cardiac fibrillation. When the heart rate is much lower, it is called severe bradycardia. Relatively little is known about these disorders in avian species, chiefly because of their relative rarity and difficulty of diagnosis [35], [36]. Very few cardiac arrhythmias studies (of those, mostly for tachycardia) have been carried out to date for avian embryos and all of them were invasive (e.g. ECG). Moreover, in all cases the arrhythmias were drug induced or under stress conditions, which does not speak to many naturally occurring incidences [37]–[40]. Therefore, it is imperative to develop a non-invasive diagnosis of chick embryo cardiac arrythmias at an earlier stage (during incubation) of their life (e.g. onset time, types, mechanism etc.) for a wide range of studies: precision poultry production systems (e.g. embryo grading systems); cardiovascular development; cardiovascular medicine, and for future reference. Here, we explored, a non-invasive method to diagnosis cardiac arrhythmia based on an optical sensor together with signal processing. In such a system, the intensity of transmitted light is mostly affected by pulsatile blood volumes and mechanical activity of the heart during blood pumping.

1.3.6 Chick embryo gender

There is a gender biased preference in dimorphic birds like chickens where males are the preferred gender in broiler and females in layer strains. For broiler production female chicks are not

economically viable due to lower growth rates compared to their male counterparts [41]. Extra feed costs are required for females to gain saleable weight. Moreover, female chickens in broiler farms result in complexities during shipping to market due to their lower body weight gain compared to males. In contrast, the male layer chick cannot be used for either egg production or meat production due to its lower growth rate and feed conversion ratio compared to a broiler chick. Besides, every year more than 7.0 billion day-old layer strain cockerels are culled globally by asphyxiation with carbon dioxide or maceration due to this gender biased production preference; raising serious ethical issues and resulting in significant economic losses [42].

Figure 1-4 Post-hatch culling of day-old chicks by maceration and asphyxiation

Therefore, non-intrusive, early chick embryo sexing before hatching is a crucial issue both from a commercial and ethical (animal welfare) point of view. This has driven increasing research effort and the desire of stakeholders in the industry to find a solution.

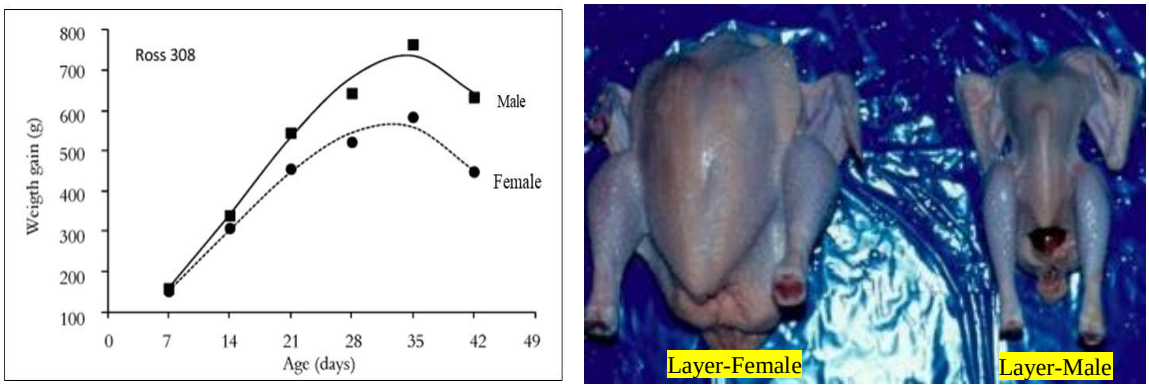


Figure 1-5 Growth curve of Ross 308 (broiler) and carcass size of female and male of layer chicken [43]

In the last few decades, many researchers have attempted to apply various strategies to detect embryo gender before hatching, or even better the gender of fertile eggs before incubation based on

differences in DNA content in blastoderm, hormonal difference (estrogen) in allantoic fluid and fluorescence properties of embryo blood [42], [44], [45]. Moreover, it is well established that sex differences exist in the heart rate, embryo weight in second half of incubation, egg content (maternal investment), egg odour, DNA content, blood fluorescence intensity and Raman scattering. But none of these methods has been used commercially since they are destructive methods. A commercially applicable method will need to be non-intrusive: not affecting the integrity of eggshells or the embryo within, such as having negative effects on embryonic development, hatchability or post-hatch development. In addition, it should be rapid enough to be applicable to large numbers of eggs; be economically feasible, and acceptable from an ethical point of view [46]. Here, we addressed, two non-invasive methods to detect gender of chick embryos based on embryo body motility and opacity value of the embryos during incubation.

1.4 Motivation of the Study

Like precision agriculture, precision livestock or more specifically precision poultry production can provide a new form of improved poultry production management. Precisely monitoring and grading chick embryos during incubation can greatly contribute to precision poultry production. This is because abnormal and weak embryos can have inferior post-hatch performance during rearing. Hence, it is much more beneficial to sort chick embryos before hatching rather grading of day-old chicks.

The uniformity of chicks means they are similar in size, weight, cardiovascular performances and have a similar growth pattern in incubation. Bad quality chicks refer to those chicks that have bad performance characteristics, such as late hatch chicks (e.g. weak, soft, lethargic), chicks with cardiovascular disturbances (e.g. arrhythmia), female chicks in broiler and male in layer breeds (lower growth rate). A common observation is that similar sized day-old chicks often show different growth patterns in their growth stages based on hatch window and gender and other abnormalities. Hence, growth, physical and cardiovascular performance patterns during incubation may be very important factors, besides genetic factors, that determine post hatch performance. Heart rate and some other features of cardiac movement may be valuable parameters to evaluate cardiovascular condition and performance of embryos.

Currently, a candling technique is widely used manually in the poultry industry to determine hatching egg fertility, as well as to remove dead embryos from the incubator. But this method is commercially very inefficient due to it being laborious, time consuming and has a low precision. Moreover, it is very difficult to monitor brown shell eggs and it can be used only for limited purposes. So in actuality, there is no non-invasive automatic method for monitoring chick embryonic movements. In a recent review on non-invasive studies of avian embryos, some researchers [17],

[18] have explored the use of devices like Buddy Egg Monitor, Ballistocardiograph and Acoustocardiograph for the purpose of heart rate counting in chicken embryos. The limitations of the methods are that they have a high probability of including body movements in heart rate measurements, since it allows for frequencies from 50 BPM. Moreover, it's readings can be obstructed by body movements; a very common occurrence in avian embryos.

Besides, non-destructive studies of hatching eggs or chick embryo in terms of hatch-window and gender have received little attention to date. Thus, non-destructive methods, which could predict hatching time and gender, could fuel innovations in the poultry industry from both a production and animal welfare perspective. This is because late hatch chicks are a source of many complications in subsequent chick grading and rearing due to their delayed growth and retarded post-hatch performance. Hence, an understanding of the physiological factors that lead to late chick hatching, along with early detection of these factors could avoid post-hatch culling. On the other hand, there is a gender biased preference in dimorphic birds like chicken where males are the preferred gender in broiler and females in layer strains. For broiler production female chicks are not economically viable due to lower growth rates compared to their male counterparts. Besides, every year more than 7.0 billion day-old layer strain cockerels are culled globally by asphyxiation with carbon dioxide or maceration due to this gender biased production preference; raising serious ethical issues and resulting in significant economic losses. Therefore, non-intrusive, early chick embryo sexing before hatching is another crucial issue both from a commercial and ethical (animal welfare) point of view. This has driven increasing research effort and the desire of stakeholders in the industry to find a solution.

To remedy these issues of hatch-window, gender and cardiac arrhythmia, we need new and improved sensing technologies for future precision poultry production. I would like to investigate the feasibility of using a near infrared (NIR) sensor together with signal processing to solve the above problems. This NIR LED transmitted light maybe such a technique to overcome the limitations of visible transmission spectroscopy as it has higher transmission for chicken eggs, is not influenced by eggshell colour, and uses a single wavelength that lowers the complexity of the data processing needed.

1.5 Research Undertaken

The overall objective of this research is to examine the feasibility of using NIR transmission for non-destructive monitoring of embryonic motility, heartbeat, cardiac arrhythmia, growth, hatch-window and gender differences during incubation. This research was divided into seven sub-sections:

1. **Experimental Methodology (Chapter 2):** a detail explanation about common experimental procedures, signal acquisition system, as well as relevant signal processing techniques for efficient data extraction from the signal.
2. **Characterization of chick embryo body and cardiac movements (Chapter 3):** investigation of the behavioural pattern of embryo body and cardiac movements in-terms of frequency and strength non-invasively from day 6 to 19 of incubation period using Near Infrared transmitted light together with signal processing.
3. **Cardiac Signal Behavior of Early and Late Hatch Chick Embryos (Chapter 4):** investigation of the pattern of embryonic cardiac activity in-terms of frequency and strength of various hatch-groups non-destructively from day 8 to 19 of incubation period using Near Infrared transmitted light combined with signal processing.
4. **Detection of Chick Embryo Gender Based on Body Motility (Chapter 5):** investigation of gender differences and development of sex classification based on embryo body motility strength using a NIR sensor.
5. **Broiler Chick Embryo Sexing based on Opacity of Incubated Eggs (Chapter 6):** investigation of gender differences and development of sex classification models based on opacity value of hatching eggs during incubation.
6. **Diagnosis of chick embryonic cardiac arrhythmia (Chapter 7):** development of a noble technique to monitor embryonic arrhythmia based cardiac activity signal in frequency domain.
7. **Conclusion and Perspectives (Chapter 8):** Summarizing the research findings and their impacts and future potential research scope in light of the current advancement.

1.6 Conclusions

Therefore, the present research was designed to investigate the behavioural pattern of embryonic body movements, heart beats, in-terms of frequency and strength, opacity (growth), with prediction or detection of hatch-window, gender and arrhythmia during the incubation period using near-infrared sensor.

Chapter 2 Experimental Setup, Signal Acquisition and Processing

2.1 Experimental Setup

2.1.1 Materials

A total of 51+155 light brown large type eggs laid by a 54-week and 45-week old parent flock (ROSS 308 strain, Japanese name "Chunky") from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto, Japan), were selected based on major diameter (59.5±3.0 mm), minor diameter (46±1.0 mm), mass (68±5.0g) and shell color (r ratio = 0.375±0.015) (Figure 2-2). An image based sorting of egg shell color was performed using a machine vision system [47] shown in Figure 2-1. The eggs were color sorted to eliminate any bias due to shell color in gender differences. A color image analysis method was performed to sort eggshell color using the RGB color space. Red of the RGB chromaticity is defined by eq. (1). The chemical pigment "protoporphyrin" released during eggshell formation is responsible for the brown color in the shell. The image acquisition system for egg sample selection was developed using a color camera (model: Imaging Source DFK21BU04, Sensor Type: CCD, Sensor Specification: Sony ICX098BQ) with C mount lens and ring LEDs as a lighting source.

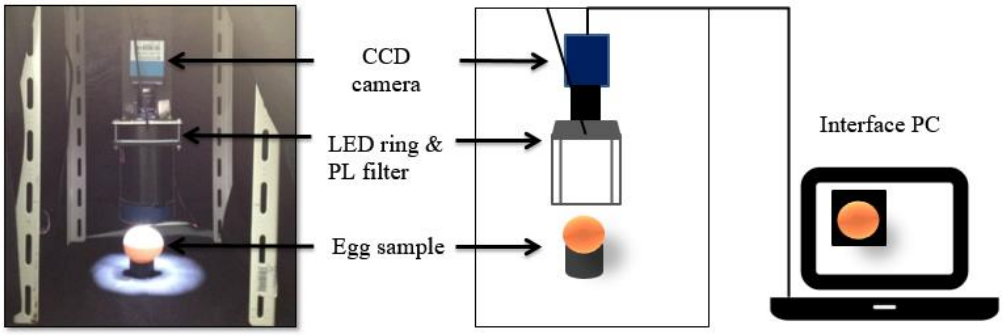


Figure 2-1 Color image based egg sorting system

$$r\ ratio = \frac{R}{R+G+B} \qquad \text{[eq.1]}$$

where, R, G and B are the red, green and blue components of the image respectively.

2.1.2 Pre-incubation tasks

The eggs were stored at 18.0°C for three days, including sorting time after collecting from farm. Prior to setting the eggs into the incubator, the eggs were preheated for 16 h (first 6 h at 28 °C and the remaining 10 h at 30°C).

2.1.3 Incubation

The eggs are then incubated at 37.8 °C and 55% RH in a commercial incubator (SSH-02, Showa Furanki, Saitama, Japan) as per Lourens et al.[27] with egg rotation at every 1 h interval. The eggs were transferred to hatcher trays at day 19 and the humidity of the incubator was increased to 60% from day 19 to until hatching [48].

Figure 2-2 Sorted eggs sample for incubation

2.1.4 Working principle of NIR sensor (Prototype-I)

An Embryonic Vital Scope (EVS) hereafter referred to as an NIR sensor, which consists of an LED light source (870 nm) with 16 series resistances that control the input current, and a photo diode which receives the light passing through the egg sample was used (Figure 2-3 and Figure 2-4). The light received is converted into current by the photo diode, which is further converted to a voltage signal using a trans-impedance amplifier. The average output voltage was maintained between 3-10 V. If the LED light is not strong enough to reach 3 V, the input current was increased by automatically adjusting the resistance (Table 2-1). This happens when the embryo inside the shell becomes larger, generally during the last half of incubation.

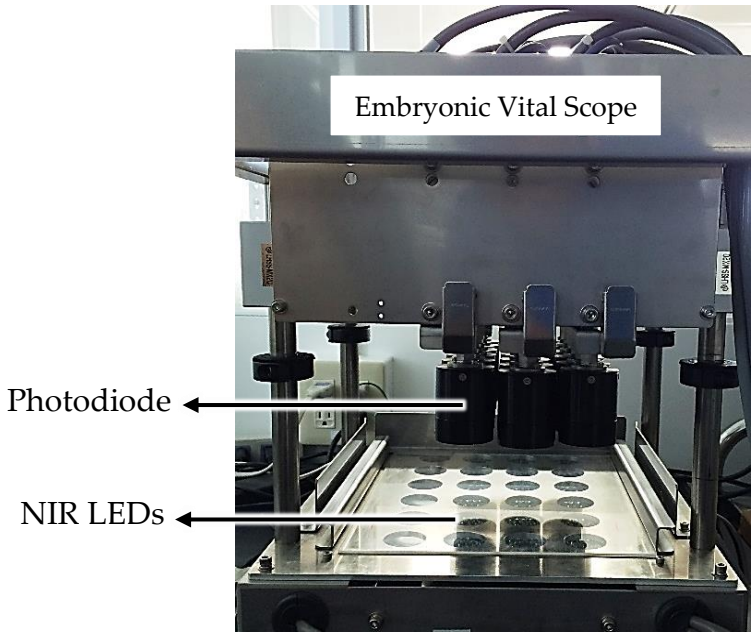


Figure 2-3 Actual image of near infrared sensor (Adopted from NABEL Co., Ltd)

Table 2-1 Series resistances of NIR sensor and corresponding LED input current

Resistance Level	LED current, mA	Resistance Level	LED Current, mA
1	0.73	9	17.52
2	0.75	10	19.33
3	2.41	11	20.95
4	2.65	12	24.35
5	6.21	13	30.86
6	8.08	14	50.68
7	12.76	15	69.44
8	14.34	16	96.15

As the sampling points of the signal was 33.3 per second, which is determined by the LED emission cycle of 30 ms, the Nyquist frequency of this sensor was 16.5 Hz which can be used for detecting any movements near 5.0 Hz.

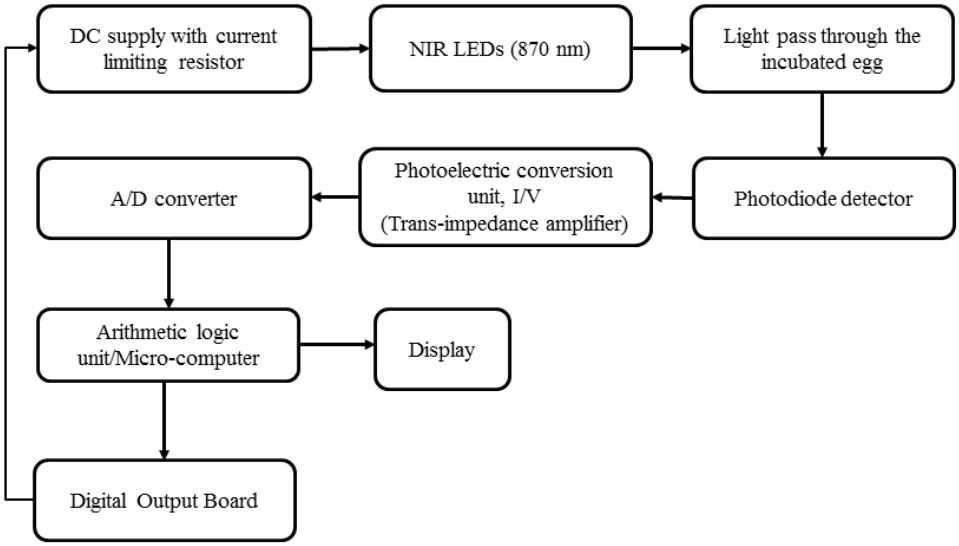


Figure 2-4 Flow diagram of working principle of near infrared sesnor

Pulse LED lighting was used with the NIR sensor. The lighting system consists of 6 LEDs, which remain on for 8 ms, and are turned off for another 22 ms (Figure 2-5). The sampling point of the photodiode was during LED ON condition after five times of time constant. The signal was also sampled during LED OFF mode for only reference purposes. This valued is not accounted for as this intensity was negligible in the Off mode.

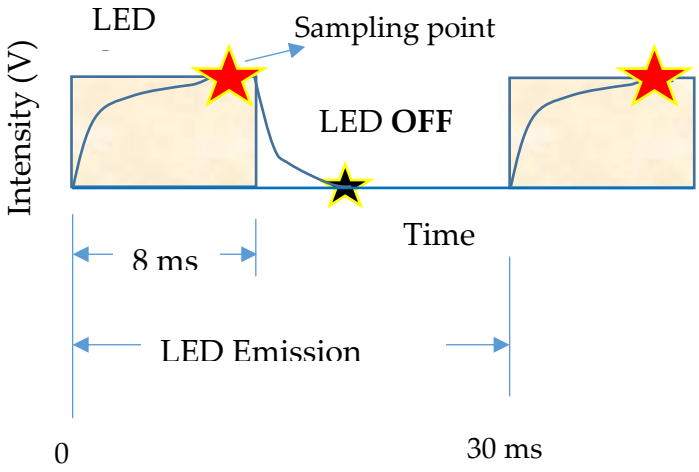


Figure 2-5 Sampling rate of near infrared sensor (Prototype I)

2.1.5 Working principle of NIR sensor (Prototype-II)

We updated and built a new device with finer signal resolution, a continuous input voltage control system, and, finally, reduced cost by using an oscilloscope. A different wavelength (810 nm) in the NIR region was used for this device, as well. This is because there are no differences in absorbance for oxyhemoglobin (HbO_2) and deoxyhemoglobin (Hb) blood at this wavelength. The rational for

selecting the NIR wavelength are: it has a relatively high transmission and higher penetration ability up to several centimeters in the NIR region than any other optical methods in biological tissues [49], [50].

This prototype sensor was designed and fabricated to acquire vital signals from chick embryos in a vertical optical configuration (Figure 2-6). The device consists of light emitting diodes (LEDs) as light sources and a pre-amplifier photodiode (Model: S9269, Hamamatsu, Japan). The dominant emitting wavelength of the LEDs (Model: L810-33AU, epitex, Japan) is 810 nm (spectral range: 800-820 nm). The light received is converted into current by the photodiode that is further converted into a voltage signal by amplification using an integrated amplifier (op-amp). During measurement, the average output voltage was maintained at a relatively constant voltage (V) for eggs on the same day using a vertical optical direction (Bottom to top) and a variable LED intensity controller (ranges was 1.7 -12.0 V). When the embryo inside the egg starts becoming larger in the latter half of incubation, the input current intensity needs to be increased to keep the output voltage similar. The amplified voltage signal was connected to an Oscilloscope (PC-interface type) through BNC cables. The Oscilloscope was then connected through a user interface to a PC with a USB connector. The user interface was developed based on the software development kit (SDK) in Microsoft excel using a Visual Basic for Application (VBA). The sampling rate per second of the signal was 1000 (sampling points per second). This high frequency signal is essential to observe the precise shape of the mechanical work been undertaken by the heart during pumping. The mechanical work done by the heart results in an electrical impulse generated by the heart in the SA node. The electric impulse is normally generates from the SA Node and propagated from the atrium to ventricle through the AV node, Bundle of His and finally Perkinje Fibers [34], [51], [52]. But the optical sensor works on the mechanical movement of the heart and the pulsatile blood flow that result from the electrical impulse. The oscillation due to embryonic movements and heart beats was 1-2 % of the average output voltage and centered around the mean value.

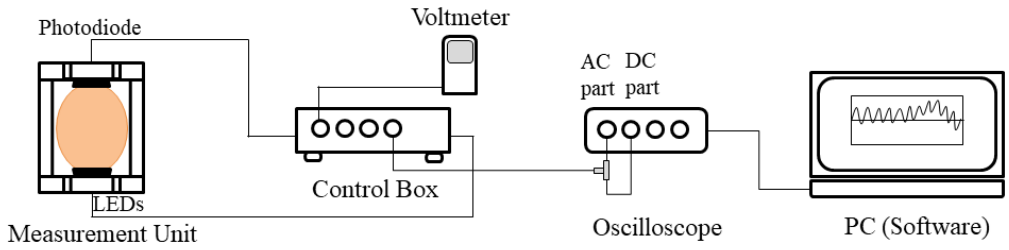


Figure 2-6 Schematic diagram of NIR sensor (Prototype II) during signal acquisition. The oscilloscope was connected to control box and PC with BNC cable and USB cable respectively

2.2 Signal Processing

2.2.1 Signal acquisition

From incubation day 6 to 19, eggs were also taken out from the incubator every 24 h for 9.003 seconds signal acquisition of each egg. To minimize the exposure time of the egg outside the incubator, eggs were immediately placed back into the incubator after the measurements.

The signal fluctuations due to embryonic movements are normally about 1.0% of the overage output voltage and appear around a mean value (Figure 2-7). Hence, the baseline is shifted by deducting the mean output voltage when considering only embryonic movements.

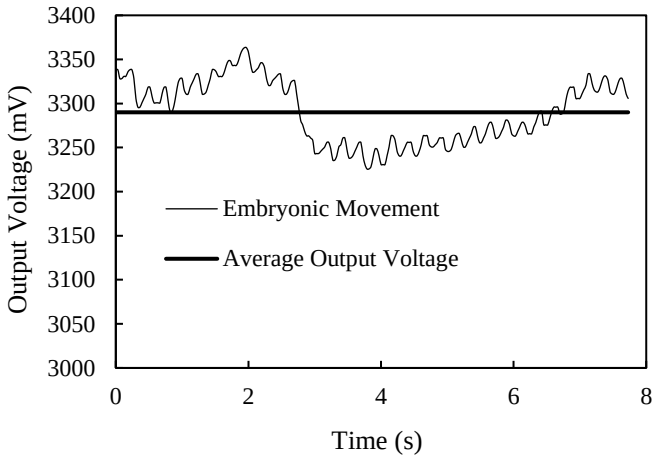


Figure 2-7 Overview of signal components of NIR sensor

2.2.1.1 Opacity calculation of incubated eggs

Opacity refers to the amount of light loss during transmission through the egg sample. The concept of using opacity in this dissertation is that as the embryo and its components grow gradually during incubation, the transmitted light intensity is gradually reduced over incubation time either by increasing absorbance due to the embryonic components or changes in reflection, which result in increasing amount of light loss over the incubation period. Therefore, the changes of both the amount of light loss and mass of embryonic components are in the same direction. The opacity values of all incubated eggs were calculated based on the following mathematical formulas.

$$Transmittance, T = \frac{Output\ light\ intensity}{Input\ light\ intensity} = \frac{Output\ current, I_{out}}{Input\ current, I_{in}} \quad \text{[eq.2]}$$

and

$$Absorbance, A = -\ln T = \ln T^{-1} = \ln \frac{I_{in}}{I_{out}} \quad \text{[eq.3]}$$

Since the near infrared sensor proposed here is not fully equivalent to near infrared spectroscopy, the opacity is mathematically defined by the following formula after modification from the absorbance equation.

$$Opacity = \frac{I_{input}}{I_{output}} = \frac{LED\ Current}{Output\ Voltage} = \frac{I_{LED}}{V_{out}} \quad \text{[eq.4]}$$

As we know, $I_{output} \propto V_{out}$ [eq.5]

Therefore, the opacity means the amount of input current necessary to get the unit output voltage after passing light through the egg sample. The average trans-impedance voltage, V_{avg} for individual eggs at incubation days 6 to 19 was determined by the following formulas:

$$Opacity = \frac{LED\ input\ current,\ \text{mA}}{Average\ Output\ Voltage,\ \text{V}} = \frac{I_{LED}}{V_{avg}} \quad \text{[eq.6]}$$

Where,

$$V_{avg} = \frac{1}{290}\sum_{n=10}^{300} V_{out} \quad \text{[eq.7]}$$

where *n* is the sampling counts and sampling frequency was 33.3 samples/s. The first 10 sampling points were used for adjusting resistance and input current for the LED to reach an output voltage between 3 to 10 V.

2.2.1.2 Calibration method for opacity values

The opacity value not only depends on transmission of egg, but also on the properties of the electrical device (such as egg seat number or position, photodiode sensitivity and LED luminous efficiency at 10 different egg seats). Therefore, calibration is necessary to eliminate the effects of device and position of egg seat (Figure 2-8).

$$Opacity\ (EggL, SeatN) = \frac{LED\ current}{Output\ voltage} = \frac{LED\ current}{Transmittance\ (EggL) \times PD\ sensitivity\ (SeatN) \times LED\ luminous\ efficiency} \quad \text{[eq.8]}$$

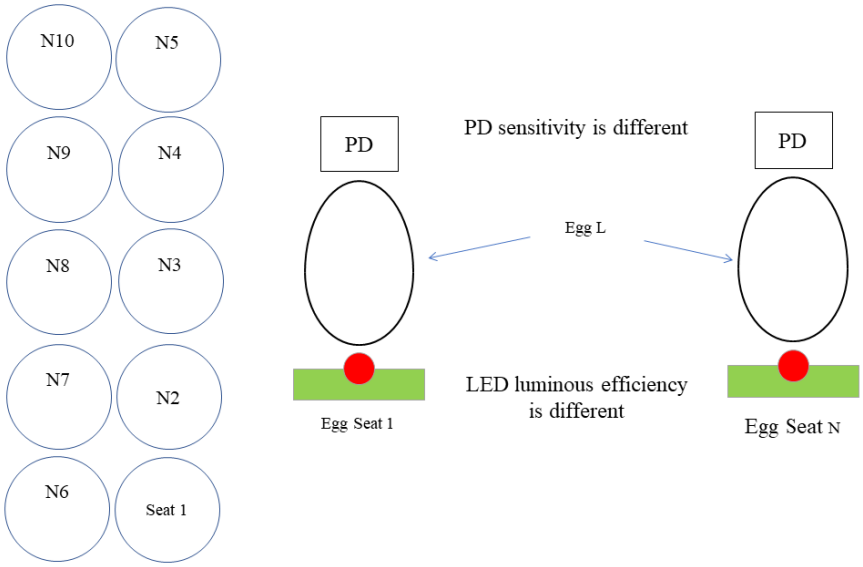


Figure 2-8 Dummy egg made of urethane and polyacetal and its dimensions

The opacity values of all egg samples were calibrated with a dummy egg made up of urethane and polyacetal, shown in Figure 2-9.

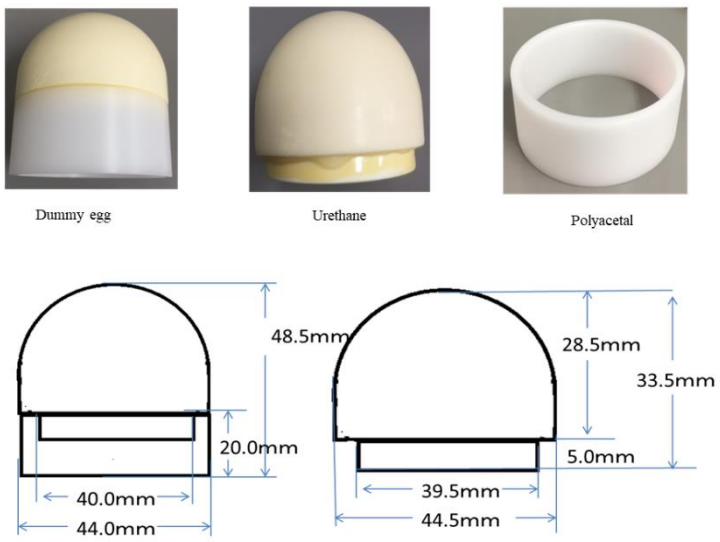


Figure 2-9 Different egg seats of NIR sensor (N represent seat other than seat 1)

The opacity of egg L at egg seat N can be converted to the opacity of egg L at egg seat 1 by using the following formula.

$$\frac{Opacity\ (EggL, SeatN)}{Opacity\ ratio\ (Seat\ N\ and\ 1)} = Opacity\ (EggL, Seat1) \qquad \text{[eq.9]}$$

where,

$$Opacity\ ratio\ (SeatN\ and\ Seat1) = \frac{Opacity\ (Dummy\ Egg, SeatN)}{Opacity\ (Dummy\ Egg, Seat1)}$$

$$= \frac{Transmittance(Dum.Egg)x\ PD\ sensitivity\ (Seat1)x\ Luminous\ efficiency\ (}{Transmittance\ (Dum.Egg)x\ PD\ sensitivity\ (SeatN)xLuminous\ efficiency\ (}$$

$$= \frac{PD\ sensitivity\ (Seat1) \times LED\ luminous\ efficiency\ (Seat1)}{PD\ sensitivity\ (SeatN) \times LED\ luminous\ efficiency\ (SeatN)} \quad \text{[eq.10]}$$

From eq.(8), eq.(9) and eq.(10)

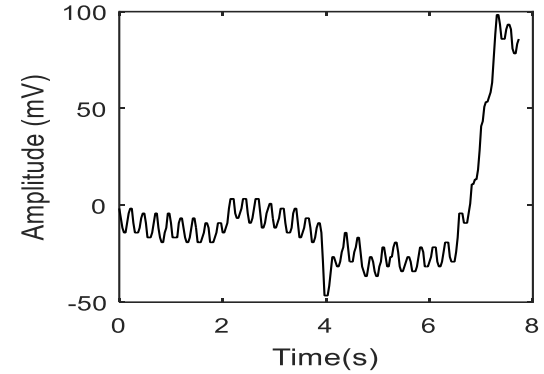
$$\frac{Opacity\ (EggL, SeatN)}{Opacity\ ratio\ (Seat\ N\ and\ 1)} \quad \text{[eq.11]}$$

$$= \frac{LED\ Current}{Transmittance\ (EggL) \times PD\ sensitivity\ (Seat1) \times LED\ luminous\ Efficiency\ (Seat\ 1)}$$

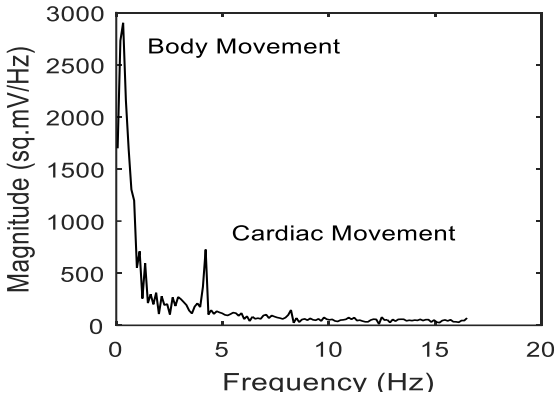
$$= Opacity\ (EggL, Seat1)$$

2.2.2 Fast Fourier Transform of signal

The voltage signals obtained by the NIR sensor were transformed into the frequency domain using Fast Fourier Transform (FFT) of 256 samples for peak frequencies that represent embryonic body and cardiac movement frequency (Figure 2-10).



A. Original signal after mean shifting



B. Signal after Fast Fourier Transformation

Figure 2-10 Original voltage signal and FFT signal (day 13)

2.2.3 Power spectrum estimation and signal de-trending

The area under respective peaks were also calculated for power, which represents movement strength or energy, using trapezoidal numerical integration after half mean de-trending of the FFT signal, as shown in eq.(3), Figure 2-11 and Figure 2-12.

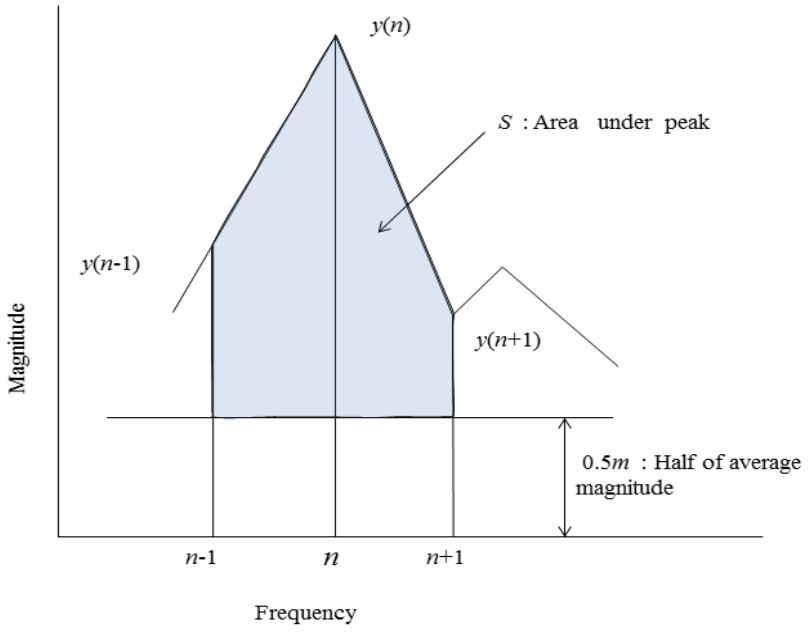


Figure 2-11 Calculation of area under curve after half mean de-trending of FFT signal representing signal strength or energy corresponded to peak

$$Area\ under\ peak = \int_a^b (f(x) - 0.5m)dx$$
$$\approx \frac{b-a}{2N}\sum_{n=1}^{N}(f(x_n) + f(x_{n+1}) - m) \qquad \text{[eq.12]}$$

where, *y=f(x)*, *a* is frequency value at *(x-1)* sampling position and *x* is sampling no. at peak position, *b* is frequency value at *x+1* sampling position, *N*=2 as 3 consecutive points is considered and spacing is *(b-a)/N*.

$$Average\ magnitude, m = \frac{1}{128}\sum_{n=1}^{128} y(n) \qquad \text{[eq.13]}$$

where, *y* is magnitude of FFT signal.

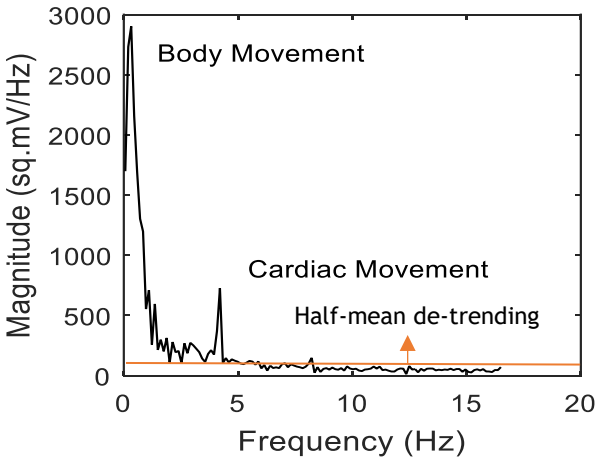


Figure 2-12 De-trending of FFT signal

Finally, movement signal strength for all egg samples for every day were compensated by dividing the value with output average voltage, as the output voltage for all samples were not same rather within the range of 3-10 V and the signal strength depends on this output voltage.

2.2.4 Digital filtering of signal

The signals were also filtered by a classical low pass digital Butterworth filter to visualize the signal pattern at an early stage of incubation (Day 7). This classical filter with 10 order was also effective at removing noise from the signal. All the signals were processed using MATLAB R2015a software. Finally, the values of heartbeat strength and movement strength of every embryo were compensated for by dividing with the average output voltage of the trans-impedance amplifier.

2.2.5 Data analysis

2.2.5.1 Descriptive statistical analysis

Mean values and standard deviation of data sets are a very common way to see data trends and dispersion. The co-efficient of variance (CV) is sometimes more useful than standard deviation when observing the degree of data variation among samples. Hence, the extracted features from the signal of all the eggs were used to calculate mean value (μ), standard deviation (σ) or co-efficient of variance (ε) with the following equations.

$$\mu = \frac{\sum_{n=1}^{k} x_n}{k} \qquad \text{[eq.14]}$$

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2} \qquad \text{[eq.15]}$$

$$\varepsilon = \frac{\sigma}{\mu} \quad \text{[eq.16]}$$

In statistics, the CV is also known as relative standard deviation (RSD) which is a measure of dispersion of a frequency distribution. It is often expressed as a percentage.

The significant difference was calculated based on an unpaired two sample t-test. The two-sample t-test is a parametric test that compares the location parameter of two independent data samples. The t-test statistic is shown in equation eq. (17)-(18).

$$t = \frac{\bar{x} - y}{\sqrt{\frac{s_x^2}{n} + \frac{s_y^2}{m}}} \quad \text{[eq.17]}$$

where $\bar{x}$ and y are the sample means, s_x and s_y are the sample standard deviations, and n and m are the sample sizes.

In the case where it is assumed that the two data samples are from populations with equal variances, the test statistic under the null hypothesis has a Student's t distribution with $n + m - 2$ degrees of freedom, and the sample standard deviations are replaced by the pooled standard deviation.

$$s = \sqrt{\frac{(n-1)s_x^2 + (m-1)s_y^2}{n + m - 2}} \quad \text{[eq.18]}$$

In the case where it is not assumed that the two data samples are from populations with equal variances, the test statistic under the null hypothesis has an approximate Student's t distribution with a number of degrees of freedom given by Satterthwaite's approximation. This test is sometimes called Welch's t-test.

2.2.5.2 Principle component analysis

Principal component analysis (PCA) is an effective data dimension reduction technique without discarding any useful information. PCA uses projections to reduce a large amount of variables into a new set of variables, which account for most of the variability between samples (Figure 2-13). It depends on the data matrix, transformation and its scaling. Each of the new variables (called principal components (PCs)) is a linear combination of the original measurements and therefore contains information from the entire variables. PCA fits new axes (variables) in the data space. The first axis is chosen in the direction of maximum variability. This way the amount of information in the first principal component (PC) is maximized. The second axis is chosen to be orthogonal to the first, so the second PC is uncorrelated with the first one. This operation is continued until a sufficient amount of variation is explained by the new variables. The score of the object is the value on the principal component axis, where the object is projected. The problem with this method is that it assumes a Gaussian distribution in the dataset.

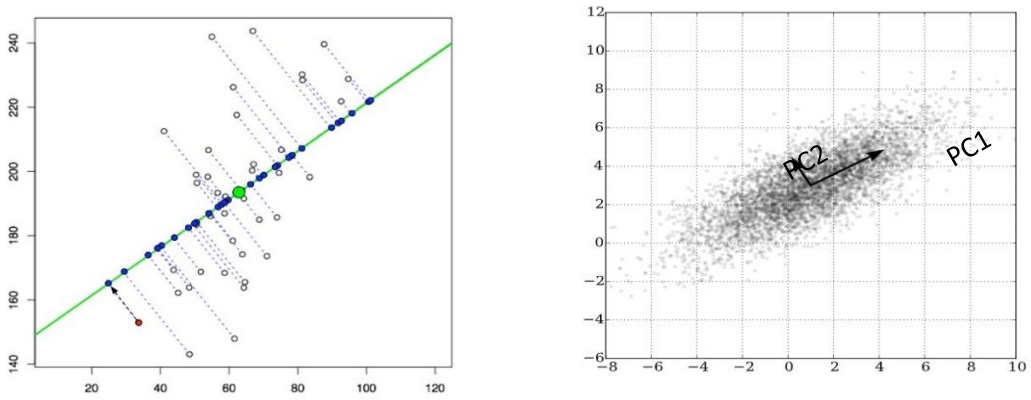
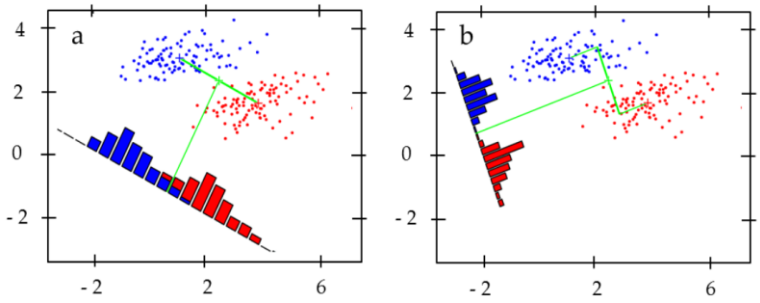


Figure 2-13 Illustration of PCA: (a) showed the projection in eigen space and (b) showed two principle components explaining variation in data matrix.

2.2.5.3 Classification Algorithms

Various classification methods (LDA, SVM and KNN) were used using the 2 PCs to discriminate between male and female embryos. Linear discriminant analysis (LDA), also known as Fisher's linear discriminant, is one of the most commonly used linear data classification techniques (Figure 2-14). LDA is used to find the linear combination of features which best separate two or more classes of objects or event. The resulting combinations may be used as a linear classifier. LDA easily handles the case where the within-class frequencies are unequal and their performance is well examined. This method maximizes the ratio of between-class variance to the within-class variance in any particular data set thereby guaranteeing maximal reparability [53].



(a) Samples from two classes (overlap) (b) Samples from two classes (improved separation)

Figure 2-14 Illustration of LDA technique: (a) samples from two classes along with the histograms resulting from projection onto the line joining the class means. (b) Corresponding projection based on the Fisher linear discriminant, showing the greatly improved class separation [54]

SVM is used for binary classification. It is works on optimal separating hyperplane between the two classes by maximizing the margin between the classes' closest points (support vectors) Figure 2-15. The performance of the SVM model is influenced by the selection of the kernel function (e.g. radial basis function, linear function, gaussian function etc.). The linearity and non-linearity are dependent

on the kernel function. We used SVM kernel functions with default parameter values in MATLAB. The gaussian is a non-linear kernel function which also can be defined as a Gaussian radial basis function kernel in MATLAB. This kernel function can have superior performance when the data structure looks circularly symmetric. In this case, though radial basis function kernel can separate the classes, the results can be over-trained.

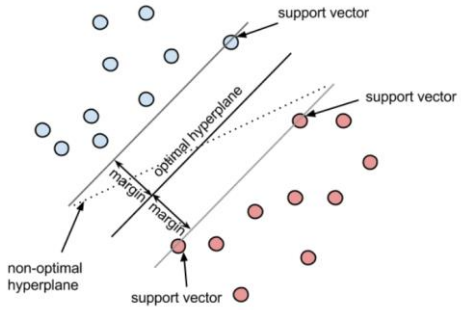


Figure 2-15 Illustration of SVM classifier [55].

KNN algorithm is a non-parametric method used for classification or regression. According to KNN rule, an unclassed sample is assigned to the class represented by most of its k-nearest neighbors in the training set. The k-NN classifier does not rely on any assumption concerning the statistical distribution of the data, but instead it relies on a positive integer k, a distance metric function (e.g. Cosine) between input patterns, and a labelled training set (Figure 2-16).

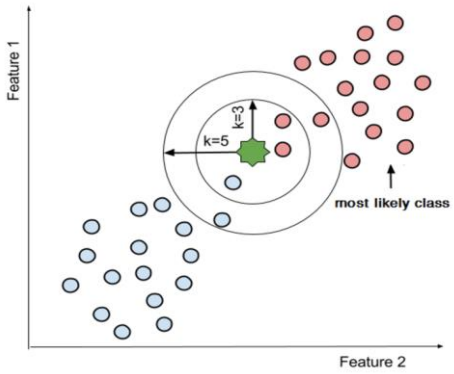


Figure 2-16 KNN classification for a two-class problem when the K parameter is set to "3" and "5". The green star represents a unseen sample point [55].

Performances of all classification models were evaluated using Recall (*R*), Precision (*P*), F-score (*F*) and overall accuracy values as shown in eq. (19)-(21).

$$R = \frac{T_P}{T_P + F_N} \quad \text{[eq.19]}$$

$$P = \frac{T_P}{T_P + F_P} \quad \text{[eq.20]}$$

$$F = \frac{2P \times R}{P + R} \quad \text{[eq.21]}$$

where T_P is true positive, F_P is false positive, T_N is true negative, and F_N is false negative.

Chapter 3

Characterization of Chick Embryo Body and Cardiac Movements

Abstract: Embryonic movements, body and cardiac activity, are important physiological phenomena for chick embryo development. Currently, there is no complete non-invasive method for simultaneously quantifying chick embryonic body motility and cardiac rhythm during incubation. This study investigates the use of a near infrared sensor to simultaneously monitor embryonic body and cardiac movements. Light brown chicken eggs (ROSS 308) were incubated for chick embryos activity signals measurement from day 6 to 19. Signal features (peak frequencies and signal energy) of chick embryo periodical activity were extracted to quantify both body and cardiac movements using Fast Fourier Transform and numerical integration. Two types of body movement were found throughout the whole incubation period. During the early stage of incubation, the movement was periodic; with the pattern differing between embryos. In the mid to late stages of incubation, movements were irregular and had a lower frequency compared to the periodic motion. Heart rates throughout the incubation period varied from 3.8 to 4.8 Hz, while heartbeat strength sharply increased during incubation, peaking at day 13 to 14, and then subsequently subsiding. These results indicate that near infrared sensing, combined with signal processing, has the potential to monitor embryo motility and cardiac rhythm that could be used in developmental physiology, cardiovascular medicine and precision poultry production systems.

Keywords: Chick embryo motility; cardiac rhythm; near infrared sensing

3.1 Introduction

3.1.1 Motivation

During early embryonic development, embryo movement represents a valuable epigenetic factor in vertebrate development [5], [6] and reflects the developmental establishment of neuromuscular interaction [7]. The establishment of the locomotion system in vertebrates also determines the degree of freedom in the wings and legs in postnatal life [9], [10]. Conversely, restricted early embryonic movements can result in positional abnormality, and reduced heart rate, blood flow, oxygen or nutrient delivery; resulting in decreased growth of the embryo [9], [10]. Moreover, light deviations from normal patterns of fetus activity can have long lasting functional disturbances in post hatch life [56]. Hence, it is important to monitor embryonic movement during incubation and be able to quantify this physiological activity.

From the early twentieth century many researchers have investigated avian embryo physiology and developmental biology during incubation using destructive and semi-destructive methods; but these invasive methods leave many questions unanswered (e.g. undesirable effects on hatchability and ethical issues) for scientists and other stakeholders. Moreover, physiological parameters (e.g. body motility, cardiac rhythm) are an important indicator of normal embryonic growth that could be used to detect weak, abnormal or dead embryos as well as infertile eggs, and thereby provide a means for detecting, removing and preventing contamination from these unproductive embryos, thus reducing production costs (energy and labor expenditure) and maximizing space utilization.

In a recent review of non-invasive studies of avian embryos it was noted that currently there are no techniques to quantify embryo movements frequency or/and amplitude except visual inspection using Candler [28]. Existing non-destructive devices, such as the buddy egg monitor [18], acoustocardiography [20], [21] and the ballistocardiograph [57], [58] have been utilized for the sole purpose of heart rate measurement of chick embryos. The buddy egg monitor was invented for hobbyists who want to monitor parrot eggs. A limitation of this device is that it has a high probability of including body movements in the heart rate measurements, since it allows for frequencies from 50 beats per minute (bpm) and can't separate out body movement. Moreover, it's readings can be interrupted by embryonic activities; a very common occurrence in avian embryos [18], [30]. For example, heartbeat signals can be interrupted by body motility during signal acquisition. Thus, the method used in this Buddy Digital Egg Monitor can't isolate the heartbeat from the mixed signal.

While equipment required for acoustocardiography is expensive, and a special audio-isolated room is necessary for measurement making it impractical for daily use [18]. Moreover, the measurement of heart rate by this method is not very accurate as the very small signal can be obscured by large artefacts caused during embryo movement [59]. As for ballistocardiograph, it measures vibrational capacitance as the ballistic movement of the eggshell. This device has also a low accuracy for heart

rate measurement due to its high sensitive to embryo body motility [18], [59]. Interestingly, none of the above methods can quantify the embryo body motility, which is considered an important physiological parameter of embryonic development (e.g. motor system) during incubation [60]. To date, the only way of counting embryonic movement frequency in the literature is candling, which is a visual inspection procedure that is not practical for industrial use [28].

When embryonic movement has been investigated, while body movement frequency or heart rate, an important parameter of health status like many other animals [18], have been measured, the strengths of both movements have not been examined. To characterize cardiac activity in terms of strength is also important, since cardiac signal amplitude is a measure of stroke forces or the energy of the pumping heart; a potential indicator of cardiac and embryonic growth, and their soundness. Hence, a robust method that can simultaneously characterize non-invasively both body (whole body, head and/or limbs) and cardiac movements (heart beats) in terms of both frequency and strength, could be used to monitor embryos in physiological studies and enhance the development of precision poultry production systems.

3.1.2 Objectives

Therefore, the present research set out to establish a standard methodology to investigate non-invasively the behavioral pattern of embryonic body and cardiac movements during the incubation period using near infrared light. Use of near infrared light on hatching eggs was singled out due to its high transmission rate compare to visible light ; a vital characteristic due to the high opaqueness of incubated eggs in the last half of incubation [61]. Transmission intensity decreases during incubation due to the formation and growth of the embryonic components (embryo, allantois and yolk sac).

3.2 Materials and Methods

3.2.1 Materials

This research was carried out in strict accordance with the animal experiment regulations of Kyoto University. A total of 100 light brown large type eggs laid by 54-week old parent flocks (ROSS 308) were collected from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto, Japan). Embryos from flocks younger than 30 weeks old may need several additional hours to complete development, compared to older flocks. Incubation time increases again when parent flocks are older than 60 weeks. Moreover, hatch rate (higher mortality) is reduced as the flock age increases. Eggs were selected for incubation based on major diameter (59.5±3.0 mm), minor diameter (46±1.0 mm), mass (68±5.0 g) and shell color (red ratio, r = 0.375±0.015). The purpose of this egg sorting was to minimize experimental variation among egg samples. Prior to incubation, all eggs were stored for 5 days at 18.0 (± 0.5) °C and 80 (± 5) % relative humidity (RH). A color image-based sorting of eggshell color was performed using a Machine Vision System.

2.1 Egg Incubation

Before setting the eggs into the incubator, eggs were preheated for 16 h (first six hours at 28°C and the remaining 10 hours at 30°C) to reduce thermal shock on blastoderm and to reduce early embryo mortality , thereby increasing hatchability [47], [62]. Prior to incubation at 37.8°C and 55% RH in a commercial incubator (SSH-02, Showa Furanki, Saitama, Japan), the transmission light signal of all eggs was measured using NIR sensor called Vital Scope, which is hereafter referred to as the signal at incubation day 0 (Figure 3-1). During incubation, eggs were turned automatically through an angle of 90° every hour. From incubation day 6 to 19, eggs were taken out of the incubator every 24 h to acquire a 9 second transmission signal for each egg. Eggs were then immediately placed back into the incubator after the measurements to minimize the exposure time of the egg outside the incubator.

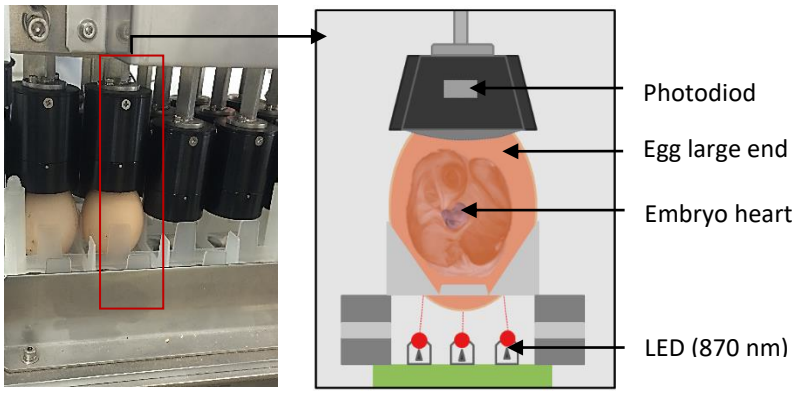


Figure 3-1Pictorial and schematic representation of experimental setup for signal acquisition by near infrared sensor

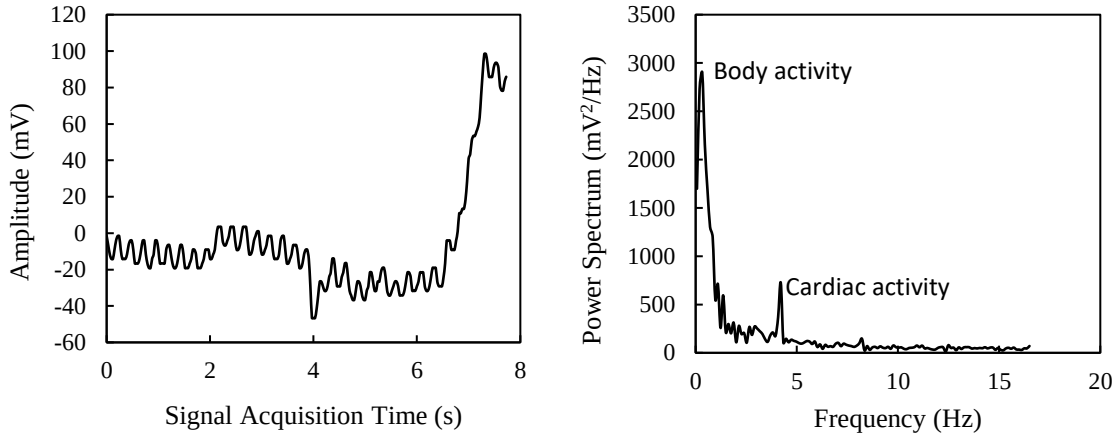
3.2.2 Signal Acquisition by Vital Scope

An Embryonic Vital Scope (EVS) was developed by NABEL Co., Ltd. for the sole purpose of detecting dead embryos the during last half of incubation based on chick embryo dynamic activity. It consists of six LEDs that emit light at 870 nm and a photodiode which receives the light passed through the egg. Any movements due to embryonic body movement and cardiac cycle lead to changes in the intensity of the transmitted light. The light received is converted into a current by the photodiode, which is then further converted to a voltage signal by amplification using a trans-impedance amplifier (Figure 3-1). Both the embryo size and the transparency of the egg vary widely during incubation. The average output voltage depends on the transparency of the egg, if we use a constant LED light intensity. The fluctuations in the transmission signal due to embryonic movements (including heartbeat) were 1.0 % of the average output voltage centered around the mean value. When the average output voltage is too small, the fluctuation due to embryonic movements becomes so small that observation of the heartbeat signal is difficult. Conversely, when the average output voltage is too large, its value is saturated, no fluctuation appears in the output voltage. The

average output voltage was maintained between 3-9.76V. When the transmission light intensity does not result in a 3V output then the input current to the LEDs is automatically increased by adjusting the resistance. Conversely, if the average output voltage is beyond 9.76V, the input current is decreased automatically. The signal sampling rate was 33.3 per second, which was determined by the LED emission cycle of 30 ms. As the Nyquist frequency of this sensor was 16.5 Hz, the sensor can be used for any movements less than or equal to 5 Hz.

3.2.3 Signal Processing and Data Analysis

The voltage signals obtained by the NIR sensor were transformed into the frequency domain using Fast Fourier Transform (FFT) with 256 samples equal to 7.72 seconds for peak frequencies and power spectrum. The high-frequency peak of power spectrum represents the cardiac pulses, whereas the low-frequency peak corresponded to embryo body movement (Figure 3-2). A similar method has also been used for extracting cardiac pulse by other researchers [63].



(a). Original signal after mean shifting (b). Signal after FFT

Figure 3-2 It shows original time domain chick embryo activity signal (a) and power spectrum of the chick embryo activity signal at day 13 (b). The left figure is showing heart beats (small fluctuations) and embryonic body movement (big fluctuations).

The area under the respective peaks was also calculated for the signal power using a trapezoidal numerical integration after half mean de-trending of the FFT signal, which represents movement strength. Such de-trending is usually used for baseline removal of low magnitude fluctuations of the FFT signal. The area under the Fourier transformed signal curve is proportional to the power of the signal, which can be calculated by summation or integration [64]. The physical meaning of the signal power typically indicates energy or strength of the signal, where amplitude for a particular frequency contributes a vital part. Finally, the values of heartbeat strength and movement strength of every embryo were normalized by dividing by the average output voltage of the trans-impedance amplifier. The signals were also filtered by a classical low pass digital Butterworth filter to visualize the signal pattern during the early stage of incubation. The frequency and impulse response of the filter are shown in Fig.6. All the signals were processed using MATLAB R2015a software.

The mean value and standard deviation of the data set are a very common way to visualize data trends and dispersion. The CV is sometimes more useful than standard deviation when observing the degree of data variation among samples. Hence, the extracted features from the signal of all eggs were used to calculate a mean value (μ), standard deviation (σ) and coefficient of variance ($\mathcal{E}$).

3.3 Results and Discussion

The scope of this paper is to establish a novel technique to measure the embryonic dynamic activity including the cardiogenic signal in terms of frequency and strength. The NIR sensor was successfully used to quantify chick embryo body activity from incubation day 6 to 19 and cardiac activity from day 8 to 19. The device was not sensitive enough to capture the cardiac activity of all embryos from incubation day 6 to 7, though it was possible for some embryos. Hence the data of day 6 and 7 for cardiac event are not presented in this paper.

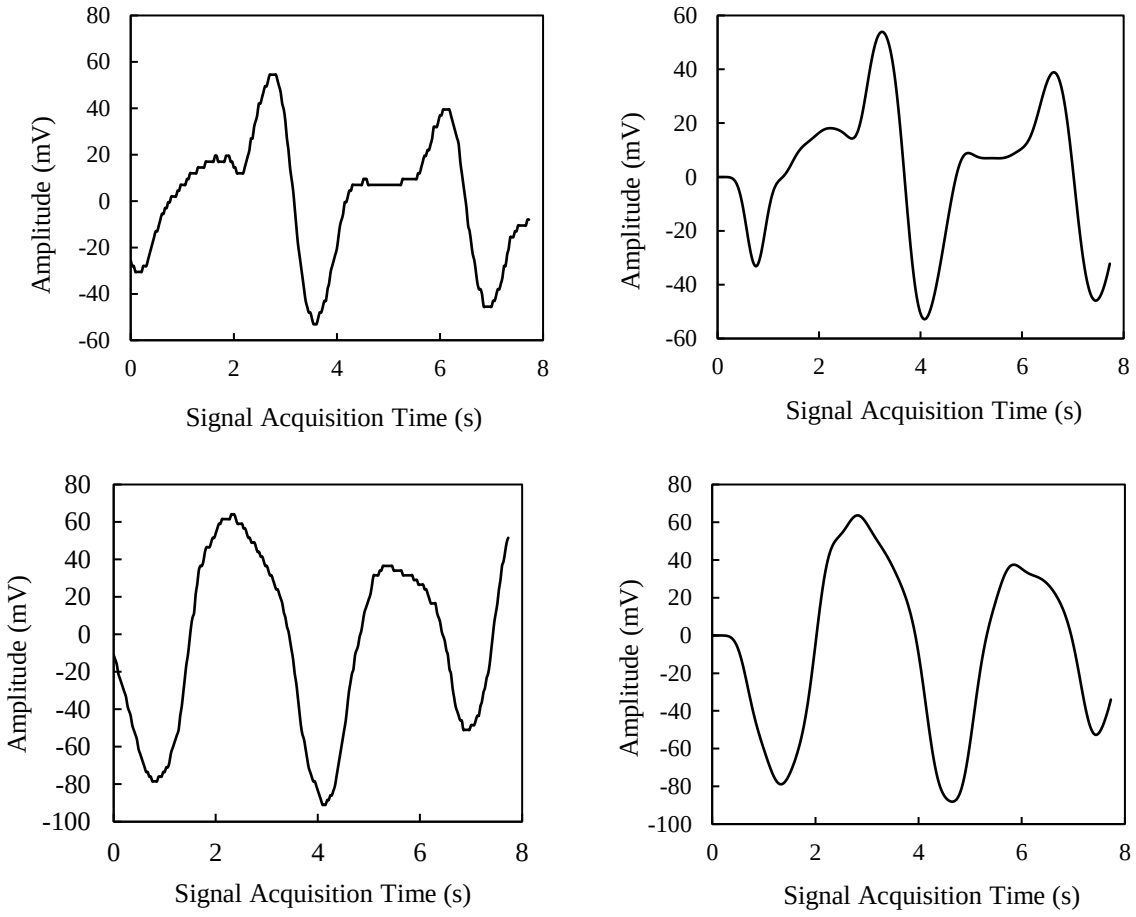
3.3.1 Chick Embryo Body Movement Pattern

The chick embryos were moving for almost the whole incubation period, but the pattern, rate and the nature of the movements were changed during the incubation period. Two types of embryonic activity, body and cardiac movements, were observed over the incubation period. In the early stages of incubation (day 6-8) body movements were characterized by a periodic signal with frequency a between 0.3 to 0.8 Hz, 19-50 beats per minute (bpm), the mid, and later stages of incubation by irregular movements at a lower frequency, 0.2 to 0.6 Hz (12-36 bpm) depending on the embryo (Figure 3-3 and Figure 3-5). First trunk movements begin at day 5 of incubation, whereas rhythmical amnion contraction begin at day 6 [4].Similar rhythmic movements at day 7-8 have also been observed using invasive methods [65] at similar frequencies [28]. Chick embryonic body activity can be characterized as periodic in nature, or quite sudden and jerky- irregular in nature [12], [65].

3.3.2 Early stage periodic movement

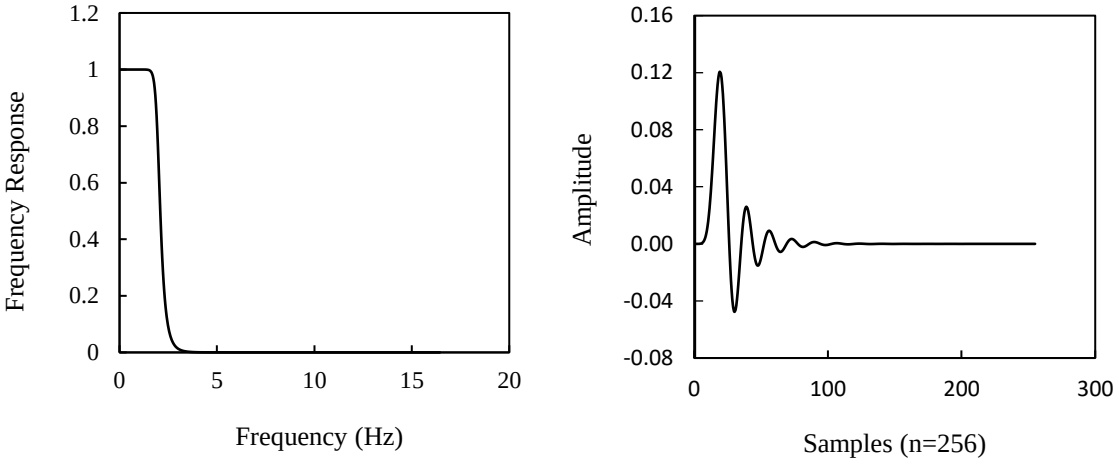
Rhythmic patterns were observed from around day 6 to 8, though the amplitude was lower on day 6 (Figure 3-3). The rate and amplitude became peaked at day 8 and rhythmic movements were disappeared by day 9 of incubation. This periodic pattern differs from embryo to embryo and may result from the individual vein network and orientation of the yolk sac, allantois and other internal structures, which are not identical for all the embryos. This rhythmic movement, though, disappears as soon as the head and other organs start becoming heavier [66] at about day 9-10 of incubation. These movements serve to prevent adhesions between the embryo and the surroundings membranes [67]. The increasing weight of the embryo, with the weakening of the amniotic contractions, contribute to a decline in rhythmic movements from the tenth day onwards [67]. In addition, embryo movements play a vital role in developmental establishment of neuromuscular interaction, formation of tissues and anatomical structures of the locomotion system [8], [68]. These early stage movements are also intricately linked to locomotion system development [69]. Another researcher described this

kind of movement as a swinging movement [12]. He observed that such movements are slow and irregular at first and then increase in frequency and amplitude until the end of the ninth day. He also stated that it becomes very rapid, regular, rhythmic and very extensive between day 6 to 9.



(a). Signal patterns before digital filtering (b). Signals after low pass filtering

Figure 3-3 The time domain activity signals of two characteristic rhythmic movement patterns of embryos at day 7. The cut off frequency was 1.9 Hz which means embryo body movement.



(a). Frequency response of digital low pass filter

(b). Impulse response of digital low pass filter

Figure 3-4 Frequency and impulse responses of classical digital Butterworth finite impulse response (FIR) low pass filter (order 10) applying a cut off frequency of 1.9 Hz.

3.3.3 Embryonic body movement frequency

Chick embryo body movement was found in the low-frequency region, from 12 to 50 bpm depending on embryo and developmental stages (Figure 3-6). The mean frequency though becomes higher from day 7 to 8 due to developmental rhythmic motions being replaced by irregular movements over the rest of the incubation period. Interestingly, these findings are similar to a statement of Nechaeva [60] who summed up the findings from many researchers [12], [67], [70], [71]: the rhythmic contractile activity of the chick embryo amnion can be continuous or packed; the duration of contractions is 2 to 7 s, and their frequency varies from 6 to 20 bpm; the frequency of contractions gradually increases reaching a maximum at day 7 to 9, then it decreases to a certain level by the 13–14th day of incubation and remains almost unchanged until day of 18–19. According to the statement of Kuo [12] the movement is most violent of all on the seventh and eighth days. He also stated that both frequency and extent are diminished in many cases after day 9 of incubation, as the body of the chick embryo becomes heavier. Wu et al. [67] reported that he observed bilaterally synchronized movements at around day 9 of incubation. The slight variations in the frequency range and peak position may be due to differences in pre-incubation, incubation conditions, breeds and flock ages. These findings also complied with candling and visual inspections with average body movements of 30 to 40 bpm from incubation day 7-20 [28].

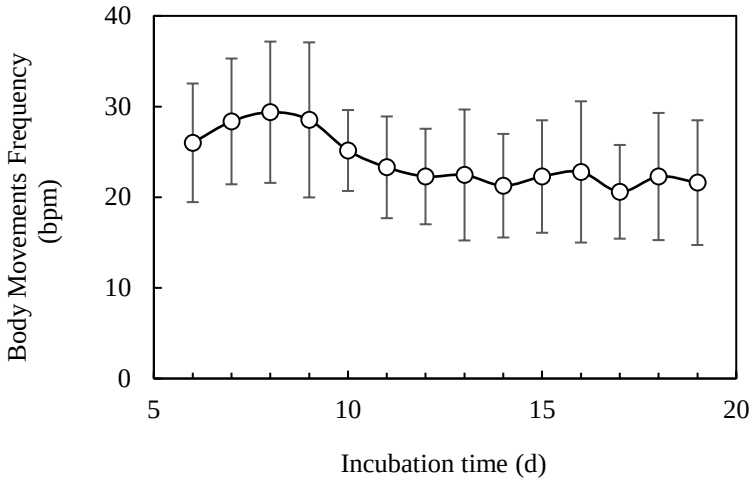


Figure 3-5 Average body movement frequency of 46 chick embryos with standard deviation during incubation (days 6 to 19)

3.3.4 Embryonic body movement strength

During the early stages (day 6-8) both body movement strength (BMS) and the coefficient of variance (CV) were relatively low (Figure 3-6) compared to that in the following stages, since the embryos are small and immature during these stages. The early segment of body movement strength had the first peak between day 7-8. Subsequently, strength reduced as the head started becoming heavier. The periodic movement changes to an irregular movement as the embryo transitions to the growth phase from the formation phase. Motility during days 9-13 change to a random and unintegrated one; when the embryo is active, different embryo parts, such as the head, wings, legs and beak move independently of each other in constantly changing combinations and appear to be unpredictable [72]. Average body motility strength peaked during the middle of the last half of incubation, on days 13 to 14, and then subsequently subsided. The amount of embryo activity (jerky and random movements) increased up to day 13 and then the amplitude reduced [11]. The higher CV indicates highly irregular and jerky movements which were observed throughout the second half of incubation. A different behavior of strength was observed from days 17-19, which is called the pipping period. During this time, the embryos generally right their position to start internal pipping and then external pipping, as noted by Bekoff [73], who reported that smooth, apparently coordinated movements move the chick into the hatching position at day 16-17. The typical and coordinated movement at day 17 or little earlier, is unlikely to result in a complex pre-hatching posture [74]. Moreover, during pipping period, the embryos become almost mature and the internal components begin being absorbed by the embryo abdomen and therefore, the movement strength decreases as it is difficult to move freely for large and mature embryos during the last few days before hatching. Another important reason for higher CV during the last half of incubation could be due to variations in the developmental stages, gender and hatch window dependency of the embryos.

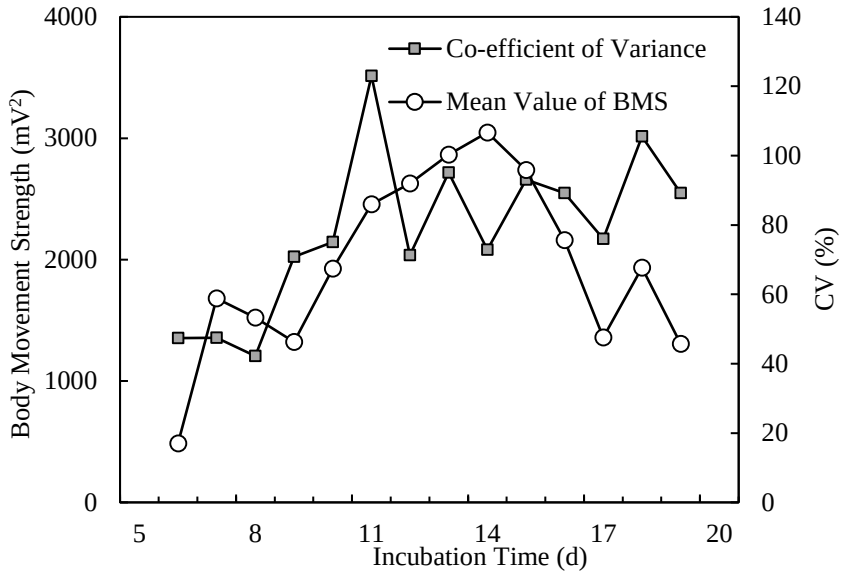


Figure 3-6 Average body movement strength (BMS) of 46 chick embryos with coefficient of variance (CV) during incubation (days 6-19)

3.3.5 Embryonic heart rate

The intensity of transmitted light is affected by both blood volumes released and vibrations of the heart during pumping (systole and diastole). This changing pattern in transmitted light intensity results in small fluctuations in the signal representing the heart rate. The heart rate varied between 3.8 to 4.8 Hz (228 to 290 bpm) for all embryos from days 8 to 19 (Figure 3-7); similar to the heart rate observed by many researchers [63], [75], [76] using destructive and semi-destructive methods. Youssef et al. [63] measured the heart rate of embryos using microscopic video measurement technique after windowing the egg shell and they found a frequency range of 4.4 ± 0.18 Hz at day 14. The heart rate of chick embryos at various stages of incubation using destructive methods have been extensively studied in the past and found to be within the range of 2.5 to 5 Hz. Heart rate measurements using the existing three non-destructive methods (e.g. Buddy Digital Egg Monitor, Ballistocardiography and Accoustocardiography) have been found to be from 170 to 300 bpm; 170-290 bpm for digital egg monitor, 200 to 250 bpm using Ballistorcardiography and 240 to 300 bpm for Accoustocardiography in last half of incubation [17], [18], [20]. The heart rate of the embryo in the last half of incubation varies little, dropping slightly from day 13 and rising again slightly until day 16. Although the actual mechanism for heart rate variation over the incubation period is still not clearly understood, the changes may be due to heart size, shape, the structure of cardiac muscle, and embryonic developmental stages along with the response to the external incubation environment. Besides, some researchers [20], [77] stated that the development of innervation and the embryonic growth associated with different somatic activities might be related to the changes in daily heart

rates. Heart rates are also dependent on the activity of the autonomic nervous system. According to Aubert et al. [75], vagus and sympathetic nerves control the heart rates by slowing and accelerating the rate respectively. Finally, the heart rate during the last few days decreases until the first half of day 19 [76], [78]. The differences in heart rate within the embryos has been associated variation in developmental stages (hatch window), growth potentiality, gender of embryos, egg contents, biological age of egg and embryo, genetic diversity within the breed, and also influenced to a degree by variations in the incubation and pre-incubation conditions. The average heart rate of female embryos is higher than that for male embryos [79]. The heart rate is also influenced by incubation temperature. Cooling from 34.7 to 31.1ºC causes a decrease in heart rate of up to 25%, a decrease in blood pressure and blood flow and vice versa [80]. Hypothermia is associated with bradycardia and a decrease in the peak velocity of blood flow during systole [81]. Thus, it would appear to be important to non-invasively observe the cardiac rhythm of individual embryos in order to undertake appropriate treatment if necessary. The heart rate also depends on egg mass which has an allometric relationship with mean heat rate [29].

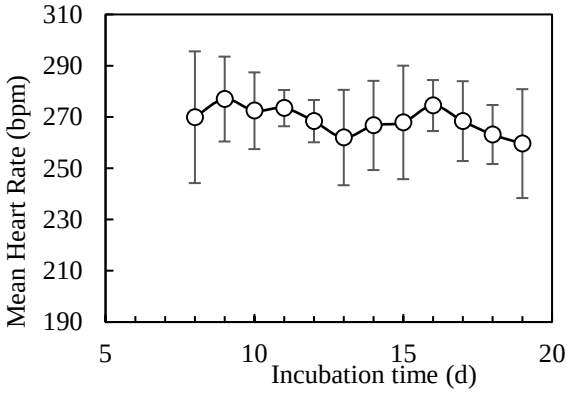


Figure 3-7 Mean heart rate of 46 chick embryos with standard deviation (days 8-19)

3.3.6 Embryonic heartbeat strength

The transmission based near infrared signal power (high-frequency component after FFT) is used to define cardiac movement strength where the amplitude and phase are the main components of signal power. The intensity changes in blood volume and vibration during diastole and systole contribute to changes in signal amplitude and phase of the cardiac signal. The heart beat strength sharply increased with the incubation time except for a slower rate around day 10, peaking at days 14-15 and then subsiding (Figure 3-8). The heart rate, heart stroke volume and oxygen (O_2) pulse are three important regulatory parameters for embryonic metabolic activity. According to the Fick principle, O_2 consumption is the product of heart rate and stroke volume that represent the amount of O_2 in the total blood volume. The average heart rate is stable over the mid-period of incubation and O_2 consumption increases, therefore, stock volume and other circulatory adjustments need to be attained

[82]. Thus, this might be one important factor for higher heartbeat strength during this incubation period. Although this is a new parameter to characterize cardiac rhythm, the physical meaning of strength (signal power) can be thought to represent the relative growth rate of the heart and embryo during the incubation period. This signal strength can be influenced by the coordinated position of the heart within the embryo affecting the transmitted light during the pumping of the heart. During the early days of incubation, the heart becomes more open and visible from outside of embryo. But as the embryo starts growing with the incubation time, the heart becomes less visible due to somatic tissue layers covering the heart which can dampen the magnitude of cardiac rhythm wave propagation, thus influencing transmitted light intensity late in the incubation period. Therefore, cardiac signal strength, as used in this paper represents the relative growth and maturity of the embryo and its heart.

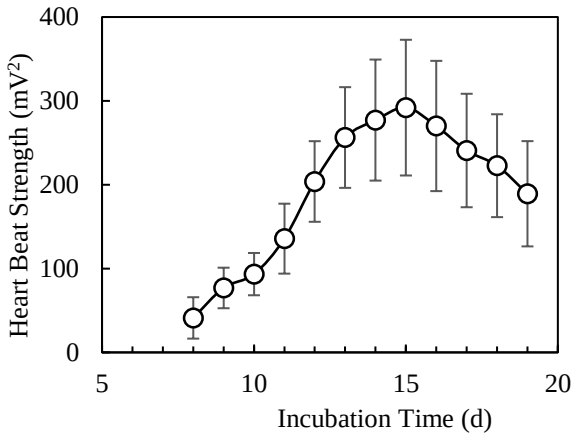


Figure 3-8 Mean heartbeat strength of 46 chick embryos with standard deviation (days 8-19)

3.4 Conclusions

This research implemented a novel technique for non-destructive monitoring embryonic body activity and cardiac rhythms of chick embryos simultaneously using near infrared sensing, combined with signal processing. During the early stages of embryonic development, body motion was found to be rhythmic, while these movements latter subsided into highly irregular activity. On the other hand, heart rates throughout the incubation period varied from 3.8 to 4.8 Hz, while heartbeat strength sharply increased during incubation, peaking at days 13 to 14, and then subsequently subsiding. This technique is more robust than the current techniques used as it not only can separate body activity from heart beats but also can quantify both of the movements in terms of frequency and strength. Moreover, heart rate measurement is not disturbed by embryo body motion, which is a very common problem with the previous non-invasive methods of heart rate measurement. These physiological parameters of chick activity could be used as a potential tool for the study of developmental biology of avian embryos and enhance of precision poultry production system in the future.

Chapter 4

Cardiac Signal Behaviour of Early and Late Hatch Chick Embryos

Abstract: Supply of uniform batches of chicks is considered as a big challenge to poultry production; a parameter intricately linked to hatch window. Late hatch chicks are a source of many complications in subsequent chick grading and rearing due to their delayed growth and retarded post-hatch performance. Hence, an understanding of the physiological factors to late chick hatching, along with early detection of these factors could avoid post-hatch culling. A total of 51 eggs (ROSS 308), sorted based on size, weight and shell colour, were incubated for the cardiac activity signal measurement during incubation using a near-infrared sensor. The voltage signals obtained from the sensor were transformed into the frequency domain to get heart rate and heartbeat strength. After hatching, the monitored chicks were then retrospectively allocated to hatch groups dependent on their actual hatching time. The heart rate data of all the eggs from day 10 to 13 were then used to develop classification model to separate late hatch chick embryos from regular hatch chick embryos using linear discriminate analysis (LDA) and support vector machine (SVM) algorithms. The late hatch chick embryos showed significantly (p-value < 0.05) weaker cardiac performances than the early hatch group. The LDA model can classify the late hatch chick embryos with an accuracy of 93%. Thus, the cardiac signals have the potential for early pre-hatch detection of weak chick embryos destined to be late-hatching and the application of this to precision poultry production systems.

Keywords: Heart beats, non-invasive, hatch-widow, animal welfare

4.1 Introduction

4.1.1 Motivation

Chicken became the largest source of global meat and a vital source of reasonably priced protein by overtaking pig meat production in 2017. To meet this growing demand, the poultry production must be efficiently scaled up. Thus, precision poultry farming, in particular high-quality chicks, will play an essential role.

Nowadays, the supply of high quality and uniform batches of chicks is considered as one of the most important challenge to breeders and poultry farmers [23]. However, the homogeneity of the day-old chick cohort is frequently compromised by a wide-spread hatch window; it contains chicks which have had short to long incubation periods [24], [25]. This negatively affects that cohort's post-hatch performance. It is well known that late hatch chicks show extensively inferior quality (growth rate, mortality and disease susceptibility) in post-hatch performance [26]. Moreover, late hatch chicks cause many downstream complications in post-hatch chick sorting, feed and water supply, vaccination and maintenance of the rearing environment relate to their hatching time differences (24-48 h) from those of the early hatch chicks.

Although the linkage between embryo growth, hatching time and day-old chick uniformity are known through studies of the effect of incubation temperature, it is less clear how physiological variability in embryo growth effects the timing of hatching [27], [28]. For instance, how the cardiac activity of chick embryos during incubation affects the hatch window has not yet been studied. The heart is a vital organ in chick embryos, which plays an important role in embryonic development during incubation. An embryo's heart undergoes complex changes throughout the developmental stages of incubation. These cardiac changes are suspected to influence the growth of the embryo and subsequent hatch window. According to Ar and Tazawa, avian embryos may stay in the egg for a fixed period and all embryos have a more or less constant total heart beat during their incubation span [29]. Thus, the growth of the embryonic heart could be a good indicator of chick embryonic growth and maturity. Hence, higher cardiac activity during incubation may shorten the hatching time of chick embryos.

In oviparous organisms, the duration of incubation can be a critical life history variable [30]. Hence the embryonic development history of chick embryo during the incubation period might have significant importance for precision hatching and post hatch chick performance. In these circumstances, a cardiac physiological study of various hatch groups especially late hatch chick embryos is highly important on the field of developmental physiology and precision poultry production system. Early detection during incubation of potentially late hatch embryos could significantly contribute to the humane treatment of chicks (late hatch chicks are discarded) and production efficiencies (minimize labor, energy and space utilization).

Visible transmission spectroscopic method cannot be used to monitor hatching eggs at the second half of incubation due to very low transmittance [61]. Besides, eggshell has high absorbance in the UV-Vis region. Therefore, near-infrared (NIR) could be a good option to study hatching egg and quantify dynamic activity of chick embryo as NIR has higher transmission in hatching egg even second half of incubation.

4.1.2 Objectives

Therefore, the present research was designed firstly to investigate the behavioral pattern of cardiac activity of various hatch groups non-invasively during incubation period using near infrared (NIR) sensor and secondly, to separate late chicks from regular hatch chicks based on their cardiac activity signal.

4.2 Materials and Methods

4.2.1 Materials

This research was carried out in strict accordance with the animal experiment regulations of Kyoto University (Animal experiment approval number: 28-59). A total of 51 light brown eggs laid by a 54-week old parent flock (ROSS 308 strain, Japanese name "Chunky") from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto, Japan), were selected based on major diameter (59.5±3.0 mm), minor diameter (46±1.0 mm), mass (68±5.0 g) and shell color (red ration, r = 0.375±0.015), for incubation studies. Uniformly graded fertile eggs were used to minimize heterogeneity among the embryos, hence to reduce the effects of zootechnical parameters on hatch window. Prior to incubation, all eggs were stored for 6 days at 18.0 (± 0.5) ℃ and 80 (± 5) % of relative humidity (RH). A color image analysis method was performed to sort eggshell color using RGB color space.

One incubator (SSH-02, Showa Furanki, Saitama, Japan) consist of three egg trays was used for incubation of fertile eggs (20+20+11=51) at 37.8 °C and 55% RH [27]. Prior to setting the eggs into the incubator, eggs were preheated for 16 h (first 6 h at 28 ℃ and for the remaining 10 h at 30℃) to reduce thermal shock on blastoderm and to reduce the early embryo mortality. After day 18, the eggs were transferred to hatcher three trays maintaining temperature at 37.8 °C and 60 % RH. The embryo cardiac signal of all incubated eggs was measured using the near-infrared sensor from day 8 to 19 of incubation.

4.2.2 Near Infrared Sensor

An Embryonic Vital Scope (EVS), hereafter referred as an NIR sensor consists of six light emitting diodes (LEDs) that emit light of 870 nm and a photodiode that receives the light passing through the egg. The light received is converted into current by the photodiode that is further converted into a voltage signal by amplification using a trans-impedance amplifier. The average output voltage was maintained between 3.00-9.76 volts (V). If the LED light was not enough to reach 3V, the input

current was increased by adjusting resistance automatically. This happens when the embryo inside the egg starts becoming larger at the latter half of the incubation. The sampling rate per second of the signal was 33.3 (sampling points per second), which was determined by the LED emission cycle (30 ms). As the Nyquist frequency of this sensor was 16.5 hertz (Hz), the sensor can be used for any movements less than or equal to 5 Hz. Fluctuations due to embryonic movements are normally 1.0 % of the average output voltage and center around the mean value (Figure 4-1). Therefore, the part of the signal assigned to embryonic movements was normalized by dividing by the average output voltage, as output voltage varies with the egg sample due to the variation in transmittance.

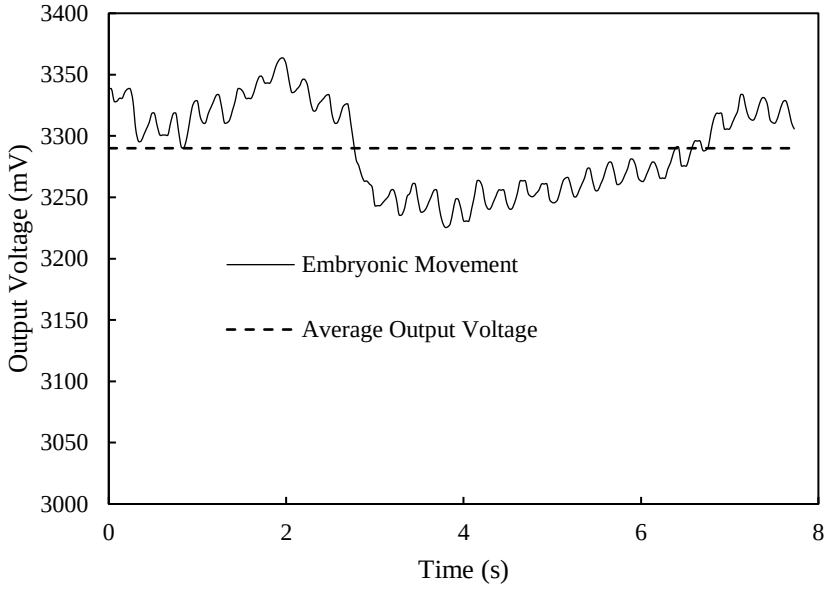


Figure 4-1 Diagram of time domain output signal of NIR sensor explaining embryonic movement as signal fluctuation around the average output voltage

4.2.3 Signal Acquisition

From incubation day 8 to 19, eggs were taken out from the incubator every 24 h for 9 seconds during which the signal (10 eggs in one tray) was acquired in vertical optical configuration (Figure 4-2). To minimize the exposure time of the egg outside the incubator, eggs were immediately placed back into the incubator after the measurements. However, the temperature of the sample eggs outside the incubation were carefully maintained properly by using warm electric blanket to avoid the influence of ambient temperature on activity signal of embryo.

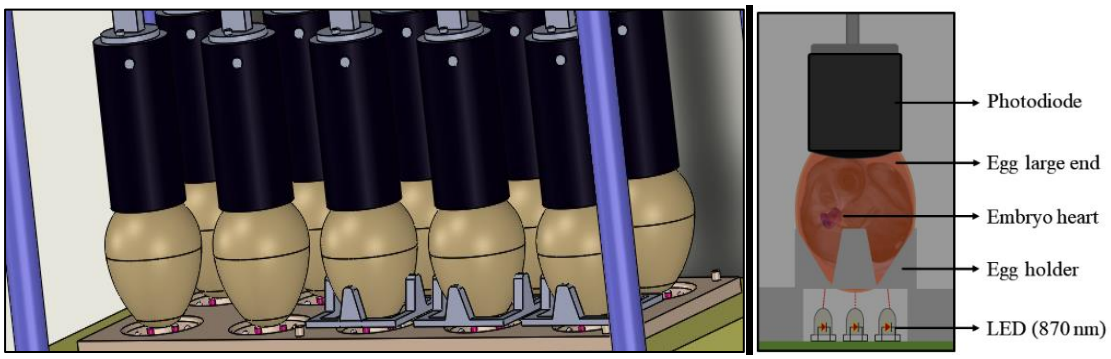


Figure 4-2 Schematic diagram of near infrared sensor during signal acquisition in vertical optical configuration. The measurement capacity of devise was 10 eggs at a time.

4.2.4 Signal Processing and Data Analysis

The voltage signals obtained by the NIR sensor were transformed into the frequency domain using a Fast Fourier Transform (FFT) of 256 sampling points equivalents to 7.72 seconds for peak frequencies and power spectrum. The high-frequency peak represents the cardiac movement of the chick embryo whereas the low-frequency peak represents embryo body movement. A similar approach has also been applied to extract cardiac pulse by other researchers [63], [83]. Body movement is out of the scope of this paper. The high frequency value in FFT signal represents heart rate in hertz (Hz), cycles/s which can be used as beats per min (bpm) after multiplying by 60.

The area under the respective peaks was also calculated as a measure signal power (energy), which represents heartbeat strength using trapezoidal numerical integration after half mean de-trending of the FFT signal. The de-trending is normally used for baseline removal of low magnitude fluctuations of the FFT signal. The area under the Fourier transformed signal curve is proportional to the power of the signal, which can be calculated by summation or integration [64]. The physical meaning of signal power is normally equated to the energy or strength of a signal, where the amplitude for a particular frequency contributes a vital part. All the signals were processed using MATLAB R2015a software. Finally, the values of heartbeat strength were normalized by dividing by the average output voltage of the trans-impedance amplifier.

The mean value and standard deviation of data set are a very common way to see the data trend and dispersion. Hence, the extracted features from signal of all eggs were used to calculate the mean value and standard deviation.

4.2.5 Actual hatching time identification

The hatching process of all embryos were recorded by an automatic image capturing algorithm using MATLAB 2015a software. From incubation day 19, eggs in the hatcher trays were monitored by two web cameras to record the time of hatching for each egg until the hatching process ended (Figure 4-3). During the monitoring period, images were captured at 5 min intervals and saved automatically

on the hard disk of a personal computer. The time of capture was also recorded simultaneously. Later, the period of incubation required for each egg to hatch was calculated as the duration between the beginning of incubation to the time of chick emergence [47].

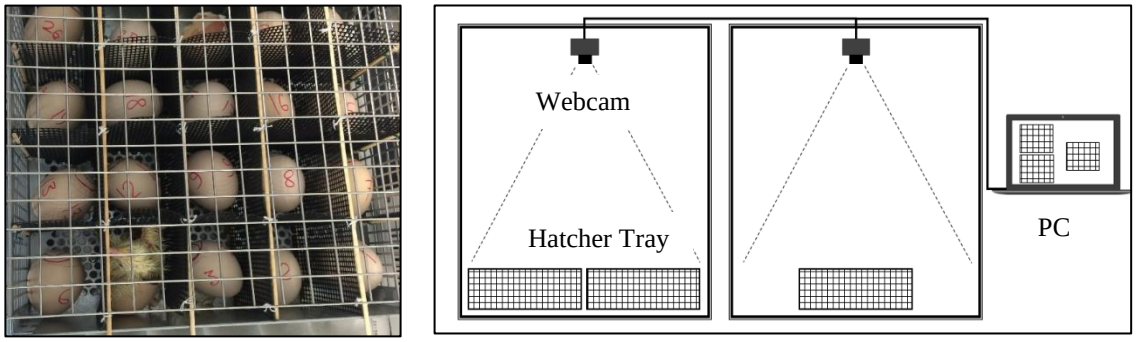


Figure 4-3 Monitoring of hatching process to obtain hatching time using automatic image capturing system

Embryos were retrospectively assigned to hatch or cohort groups based on incubation length [26]. The late hatch group was defined as those chicks that came out 26 h after the first chick hatched, whereas chick embryos that hatched with less than 482 h of incubation were considered as the early hatch group based on hatch window. Chicks normally come out after 24 h from the first hatch chick are considered as late hatch chick. Besides, some researchers also considered early and latch hatch groups before 480 h and after 493 h of incubation respectively [84].

4.2.6 Multivariate data analysis

Principle component analysis (PCA) was done to extract information from the heart rate data (day 10-13) and the multivariate classification methods such as linear discriminate analysis (LDA) and support vector machine (SVM) were then used for further classification of the late hatch chick embryos relative to regular hatch groups (early and middle hatch). Principal components (PCs) scores were used as the new data matrix in the classification step. During classification model development, all samples (38 regular and 7 late hatch chick embryos) were divided into two groups i.e. a training set (70%) and validation set (30%). Model performances were evaluated using Recall (R), Precision (P) and F-score (F) and overall accuracy values. All statistical analysis was performed using MATLAB R2015a software. Two sample t-test was used to see the statistically significant difference (p-value <0.05) between early and late hatch groups.

4.3 Results and Discussion

The scope of this paper is to establish a novel technique to measure cardiac activity signal of various hatch groups in terms of frequency and strength and application of this signal to classify late hatch chicks. The NIR sensor was successfully used to quantify cardiac activity from day 8 to 19. The device was not sensitive enough to capture the cardiac activity of all embryos from incubation day

6 to 7, though it was possible for some embryos. Hence the data of day 6 and 7 for cardiac event are not presented in this paper. Incubator with high precision was used in this experiment, hence slight variation in temperature, humidity and air flow inside incubation and its effects on cardiac activity of embryos were considered as negligible. Measuring chick embryo cardiogenic signals have at least two potentially important functions: observing the physiology or developmental biology of chick embryos of various hatch groups during incubation, and in applications for precision poultry production systems.

4.3.1 Hatch window of chicks

The spread of the chick's hatch window (ROSS 308) was found to be 39 h, where the first chick came out at 470 h and the last at 509 h. The average time of incubation for early, mid and late chicks was 478 h, 490 h and 498 h, respectively. The 80 % chicks were hatched (by 495 h of incubation) before 14 hours of pull. The average hatch window depends on breed, age of parent flock, pre-incubation conditions, the quality of egg, egg size, and incubation conditions [25]. The hatch window of a broiler breed, in this case, was found to be normally distributed, where eight chicks were considered as early hatch and seven chicks as late hatch and rest of the chicks as middle hatch based on the hatch window (Figure 4-4).

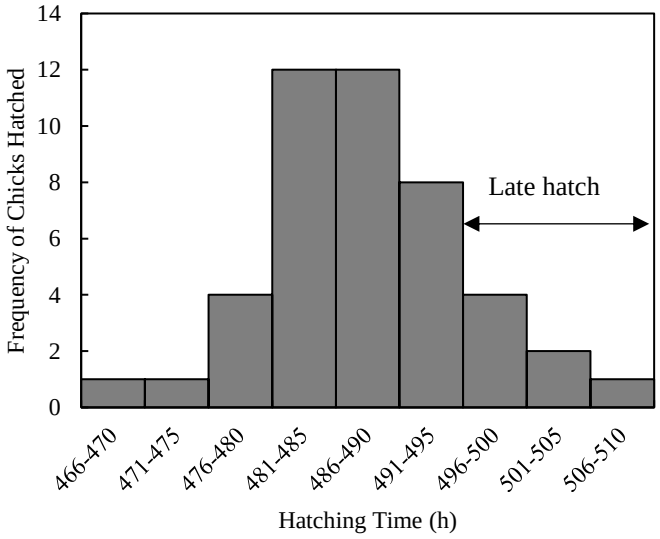


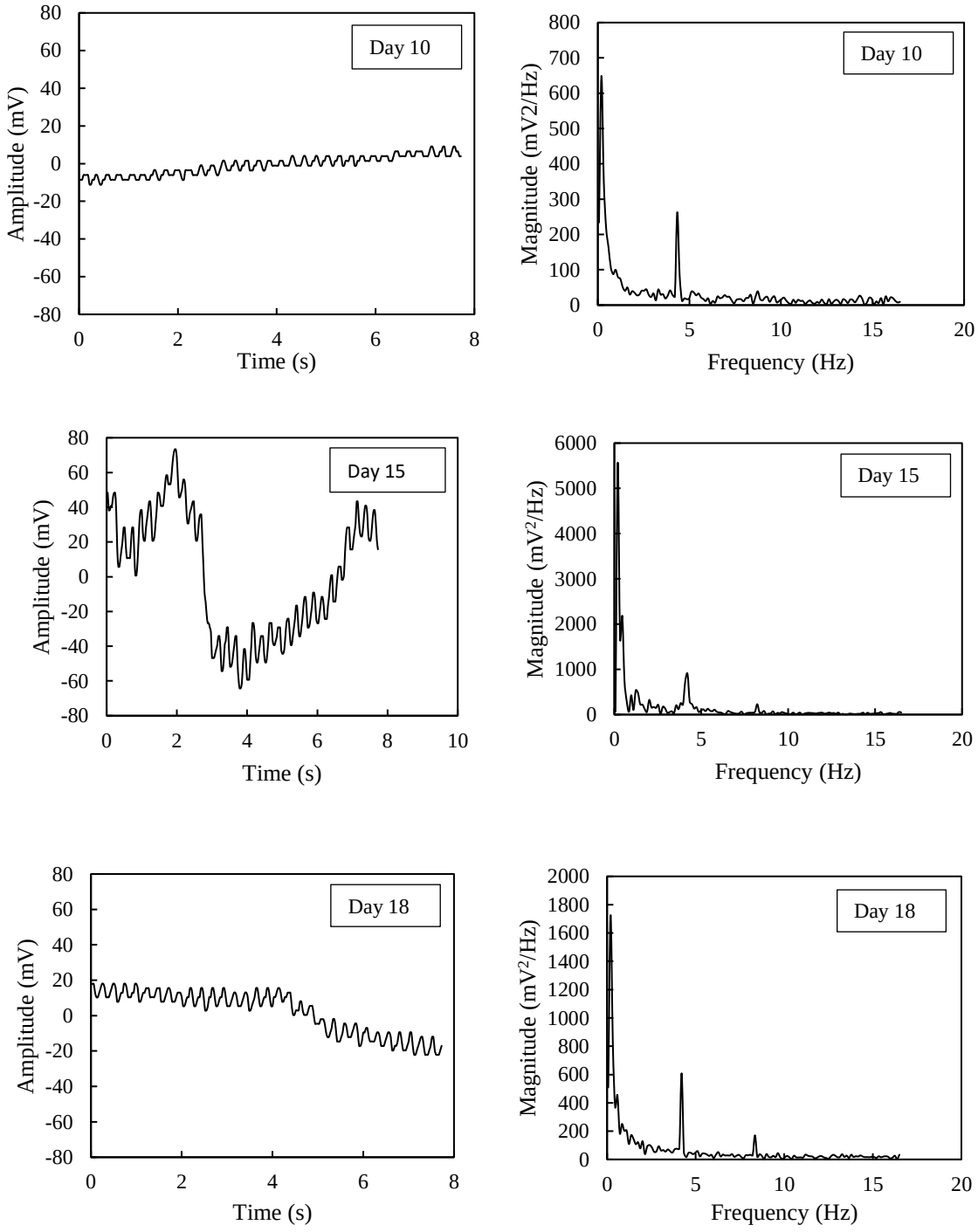
Figure 4-4 Hatch window of chicks (ROSS 308) spread over 39 hours

The hatch window of broiler breed in this experiment shifted to earlier depending on parent flock age (54 weeks old), egg storage time before incubation and preheating (16 h) before incubation. Embryos from flocks younger than 30 weeks may need additional 5-7 hours to complete development, compare to older flocks. Incubation time increases again when parent flocks are older than 60 weeks. Storage of the eggs also has a major influence on the length of the incubation period, probably because the internal components of fertile eggs undergo physiochemical changes during

this period. Besides, the pre-incubation heating lower than incubation temperature also shorten the incubation length of embryos, because blastoderm start cell division with increasing temperature. Pre-heating is a common practice to reduce early embryos mortality during incubation due to thermal shock on blastoderm.

4.3.2 Hatch group heart rate patterns

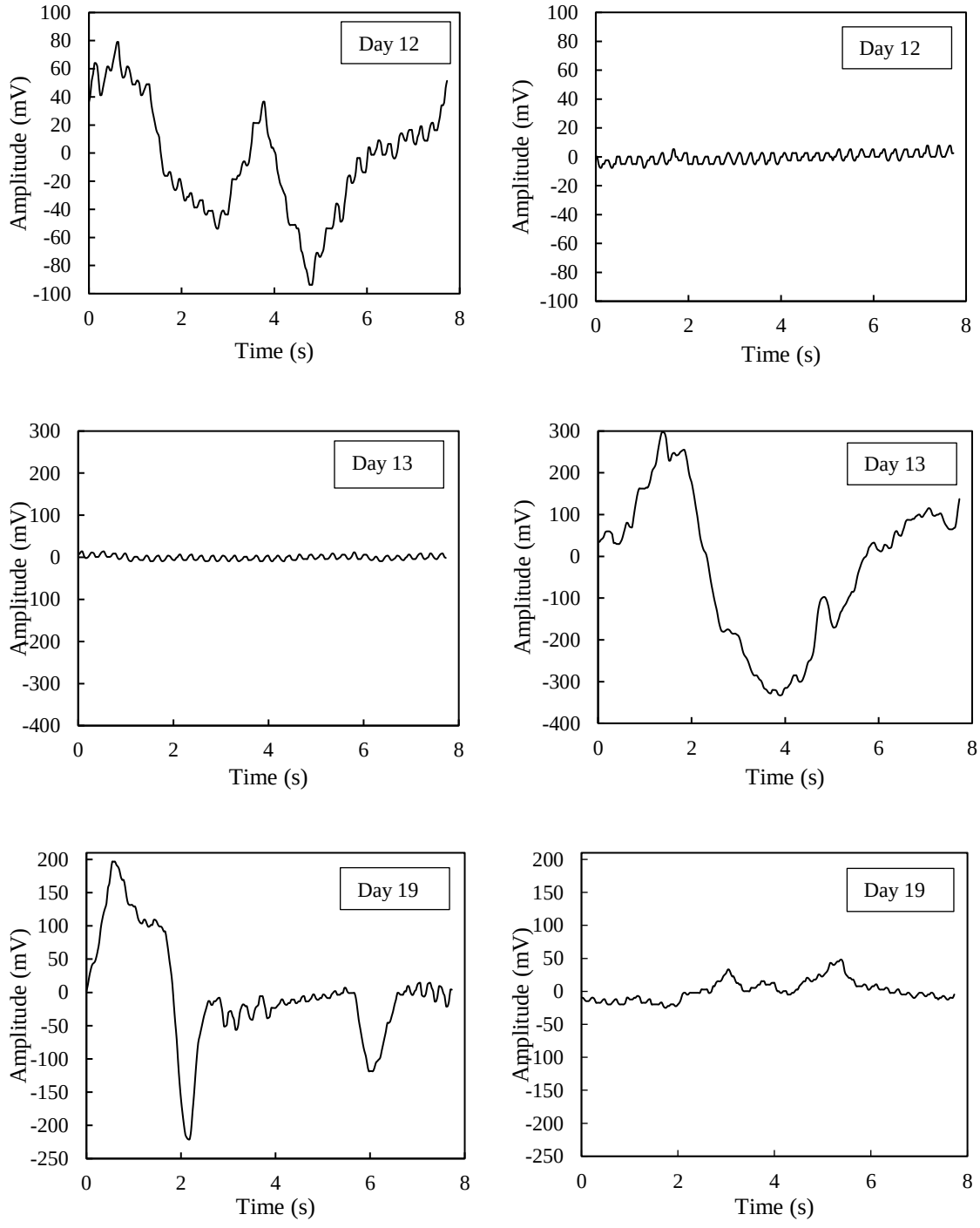
The intensity of transmitted light is affected by both blood volumes released into the system and vibrations of the heart during pumping (systole and diastole). This changing pattern in transmitted light intensity gives small fluctuations in the signal representing heart beats [83]. Based on frequency analysis, the heart rate was found to be between 3.8 to 4.8 Hz (228 to 290 bpm) for all embryos from day 8 to day 19 (Figure 4-5 and Figure 4-7); similar to heart rates observed by many other researchers [63], [75], [76] using destructive and semi-destructive methods. The heart beats per second were also visible in the time domain signal on different incubation days (Figure 4-6). The heart rate of the embryo over the incubation period varied little dropping slightly from day 13 and the again increasing slightly until day 16. The heart rate depends on activity of the autonomic nervous system. According to Aubert et al. [75], vagus and sympathetic nerves control the heart rates by slowing and accelerating the rates respectively. Finally, the heart rate on the last few days showed a decreasing trend until first half of day 19 [85] which is an important indicator to observe the developmental stages of the embryo.



a) Signal after mean output voltage shifting

b) Signal after FFT

Figure 4-5 Typical original signal and corresponding FFT signal of a chick embryo at incubation day 10, 15 and 18

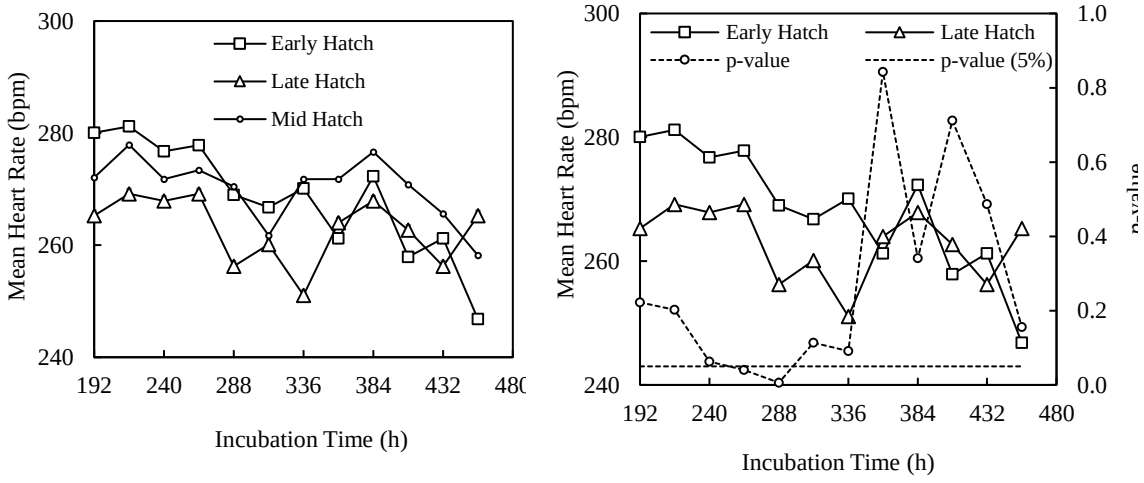


a) Early Hatch Embryo b) Late Hatch Embryo

Figure 4-6 Typical original signals of one early and one late hatch chick embryos at incubation day 12, 13 and 19

Late hatch chick embryos had the lowest average heart rate of all the hatch groups in the early stages of incubation until incubation day 13 (Table 4-1). On the other hand, the early hatch chick embryos had the highest heart rate among the three hatch groups before day 12. Interestingly, middle hatch chick embryos had heart rates between early and late hatch groups, but rising to be the highest during

the last few days before hatching (Figure 4-7). The heart rate behavior for the late hatch group in the last few days of incubation might be due to two reasons; firstly, they tried to recover their early heart rate trail and secondly, they may still at an earlier developmental stage compared to other groups. One interesting thing to notice is that early incubation heart rate reflects subsequent hatch window more than other periods; if you compare early to middle hatch groups. Besides, the decreasing trend of heart rate before hatching is a sign of chick embryo maturity which is absent in the case of late hatch chick embryos even at day 19 [78]. According to the findings of Ar and Tazawa, avian embryo stays in the egg for a fixed period of time, with all chick embryos having a more or less constant heart beats during the incubation period [29]. Moreover, cardiac activity is a vital physiological parameter of embryos that influences growth and maturity. Thus, the late hatch chicks, which by definition had a longer incubation period to complete their cardiac cycles during their entire embryonic developmental stages. It makes sense that the late hatch group should have a lower cardiac performance, since they are found to be lethargic and week after hatching and show inferior post hatch performance in terms of growth, mortality and disease susceptibility [84].



a) Average heart rate pattern various group

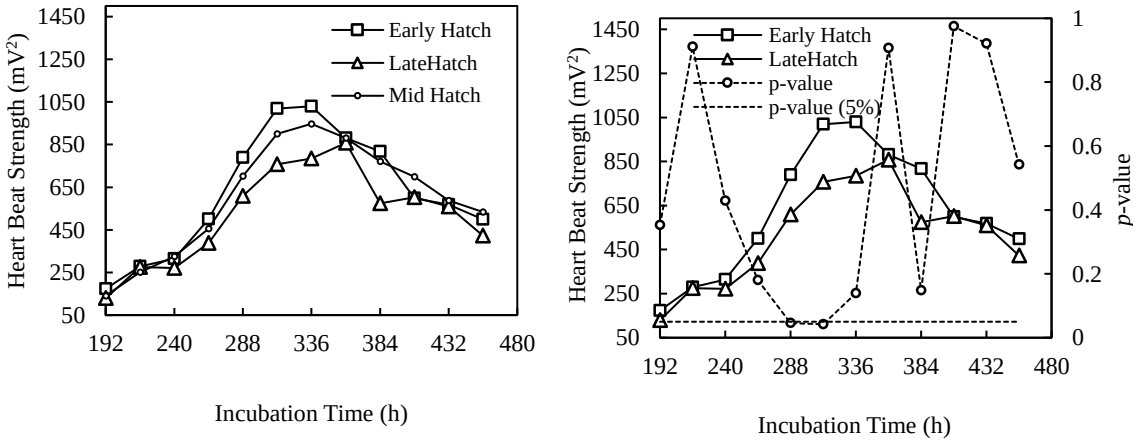
b) Differences between early and late group

Figure 4-7 Chick embryo average heart rate pattern for various hatch groups during incubation day 8-19 (192 h-456 h)

Although the early hatch chicks showed a higher heart rate until day 13 (312 h) than the late hatch chicks, statistically significant differences were found (p-value< 0.05) only at day 11 (264 h) (p-value=0.04) and 12 (288 h) (p-value=0.006). Some studies also found physiological differences between early and late hatchers, such as different thyroid hormone levels and differences in organ weight and maturity [84].

4.3.3 Hatch group heartbeats strength patterns

The transmission based near infrared signal power (high-frequency component after FFT) was used to define cardiac movement strength where amplitude and phase were the main components of signal power (energy). The heart beat strength for all hatch groups followed a similar pattern, sharply increasing from the beginning of incubation, slowing down around day 10, and then increasing again and peaking at day 14-15, before subsiding (Figure 3-8). Although this is a new parameter to characterize cardiac movement, the physical meaning of strength (signal power) can represent the relative growth of the heart and embryo during the incubation period [83]. This signal strength can be influenced by the coordinated position of the heart within the embryo influencing the transmissive light during the pumping of the heart. Early in the incubation, the embryonic heart becomes more open and visible. But as the embryo starts maturing the heart becomes less visible during this period of incubation due to somatic tissue layers covering the heart, which dampens the magnitude of the propagated cardiac rhythm wave, thus influencing the transmissive light late in the incubation period. Therefore, cardiac signal strength as defined in this paper represents the relative growth and maturity of the embryo. The late hatch chick embryos had a weaker cardiac strength over the incubation period than the other hatch groups, especially around day 11-14 (Table 4-2). Similar to heart rate pattern, the strength of heartbeat during early incubation was closely associated with the subsequent hatch window, although the middle hatch group tried to recover strength late in the incubation. For late hatch group, the peak position of heartbeat strength was at day 15; a delay in the developmental stage compared to the other hatch groups.



a) Average heart beat strength pattern of various hatch groups

b) Differences in strength between early and late hatch groups

Figure 4-8 Chick embryo average heart rate pattern for various hatch groups during incubation day 8-19 (192 h – 456 h)

Though early hatch chicks had a higher cardiac strength over the incubation period, there was only a significant difference between early and late hatch groups at day 12-13 (p-value = 0.04). Major changes in the behavior of the embryonic heart during incubation day 12-13 might be influencing

hatch window. Amplitude (vital part of signal strength) differences between early and late hatch groups at various incubation days were also visible in time domain signal which was similar to the power spectrum (signal strength) in the frequency domain analysis.

Table 4-1 Average heart rate (HR) of regular and late hatch chick groups

Cardiac Parameters	Incubation Day											
	8	9	10	11	12	13	14	15	16	17	18	19
HR^R (bpm)	270	278.30	273.20	274.42	270.54	263.59	269.51	268.29	275.64	269.52	264.4	258.48
SD	26	16.07	15.95	6.88	6.58	18.03	14.90	24.08	10.21	16.76	11.08	23.13
HR^L (bpm)	266	270.10	268.99	268.99	257.90	261.23	252.35	265.66	268.99	263.45	256.8	264.55
SD	27	19.90	9.43	6.99	8.64	6.99	24.55	9.73	6.99	6.11	13.34	6.11

^{R}Regular hatch; ^{L}Late hatch; SD: Standard deviation

Late hatch chick embryos had a significantly (p-value < 0.05) lower average heart rate at day 11 and 12, and average heart beat strength at day 12 and 13 than the early hatch embryos (Figure 4-7 *and* Figure 4-8). This relatively weak cardiac activity of the late hatch group might be one of the vital factors responsible for requiring a longer incubation period to complete their entire developmental stages. Finally, the large standard deviation values for the heartbeat strength maybe due to either to genetic variation, variation in the developmental stages and/ or incubation conditions.

Table 4-2 Mean heartbeat strength (HBS) of regular and late hatch chick groups

Cardiac Parameters	Incubation Day											
	8	9	10	11	12	13	14	15	16	17	18	19
HBS^R (mV^2)	142.7	261.09	323.90	457.71	707.39	911.18	961.43	877.54	770.20	683.07	577.5	532.64
SD	85.6	90.16	78.88	133.40	179.45	212.14	213.88	274.35	299.1	261.56	184.6	227.53
HBS^L (mV^2)	130	285.32	285.70	405.57	622.09	756.52	832.74	868.86	597.52	613.68	585.3	502.57
SD	60.2	48.36	120.60	139.38	127.94	225.66	199.35	238.67	202.75	106.86	152.8	239.22

4.3.4 Multivariate Classification

PCA was applied to the cardiac features of incubation day 8-19, as well as selective day of incubation, to obtain the best discrimination between late and regular (early and middle hatch groups) hatch embryos. The best observed PCA model was obtained with the heart rate of 10-13 incubation day to detect late hatch chick embryos. The first two PCs of this model explained 88% of the total variance in the dataset (Figure 4-9). Though geometrical exploration of the PC score plot gives the cluster trends, it cannot be used directly as a tool for discrimination of different hatch groups. Therefore, LDA and SVM classification methods were used using 4 PCs to discriminate between regular and late hatch chick embryos. A training sample set was used to construct a classifier and a validation sample set was used for evaluating the performance of the classification. These results indicate that maximum discrimination between regular and late hatch group could be achieved with a 93% accuracy by LDA method (Table 4-3). The late hatch group (15.2% of total

embryos) was considered as positive class due to a skewed type data set where recall was obtained 0.67. In this case, a higher recall value indicates good performance of the model to identify late hatch group. On the other hand, a higher precision value (close to 1.0) indicates the model to detect regular hatch chick embryos accurately.

Table 4-3 Classification result of the validation set (LDA with 4 PCs)

Model	Classification			
	Precision	Recall	Accuracy (%)	F-Score
LDA (4 PCs)	1.00	0.67	93	0.8
SVM (RBF)	0.6	1.0	86	0.75

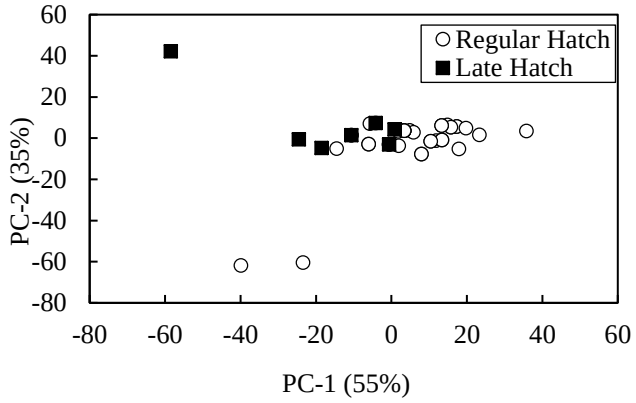


Figure 4-9 Scores plot of regular and late hatch chick embryos heart rate (first two PCs)

4.4 Conclusions

Firstly, this research revealed that cardiac signals (heart rate and heartbeat strength) are important physiological parameters, which are closely associated with embryonic development and maturation of chick embryos, and influence the subsequent hatch window. It has been reported that all the embryos complete incubation with more or less the same number of cardiac cycles. Hence, the early and mid-hatch embryos with their faster heart rate and stronger heartbeat strength complete the total cardiac activity of incubation earlier. Secondly, from a commercial production perspective, early detection of late hatch chick embryos during incubation is a big challenge for poultry farmers worldwide. From this perspective, cardiac signal features offer the potential to detect embryos destined to hatch late; thus, avoiding their post hatch killing. This early detection of potential late hatch chicks is also important from a humane treatment viewpoint, since these chicks are normally discarded by hatchery management personnel due to their inferior post-hatch performance. Moreover, this can reduce economic cost by reducing raring time of unwanted eggs/embryos which require space, energy and labor cost, minimizing post-hatch handling for sorting and unwanted embryos could be used for other purposes like vaccine production. Lastly, since cardiac activity effects the hatch window and hatching time influences post hatch chick performance, cardiac activity of chick embryos during incubation could be a valuable life history variable to study in the future in relation to post hatch chick performance.

Chapter 5

Detection of Chick Embryo Gender Based on Body Motility

Abstract: Non-destructive monitoring of chick embryos during incubation can provide vital management insights for poultry farmers and other stakeholders. Moreover, culling of huge numbers of male day-old chicks in layer production is raising a big ethical issue worldwide. Although sex differences in the activity of human fetuses is well established, any differences in avian embryo activity have yet to be resolved. Hence, this study investigated physiological differences between male and female embryos in terms of body dynamic activity and indirect differentiation of embryo gender based on this activity. Fertile eggs from broiler breed (ROSS 308) were used for this purpose. A near infrared (NIR) sensor consisting of light emitting diodes (LEDs) and a Si-photodiode was used to measure embryo motility non-invasively during incubation. Embryo body dynamic activity was separated from the activity signal in frequency domain using Fast Fourier Transform (FFT). The area under the low-frequency peak was then calculated for signal power using a trapezoidal numerical integration, defined as body movement strength. Principle component (PC) scores from embryo movement strength were used for gender classification by Support Vector Machine (SVM) and K-Nearest Neighbors (KNN) algorithms. Male chick embryos were significantly (p-value<0.05) more active during incubation. The formation of sex organ and hormonal differences from day 7-9 of incubation appear to be the reason for the sex differences in dynamic activity of embryos in the latter half of incubation. Understanding the embryo activity behaviour could contribute to resolving developmental biology, industrial and animal welfare issues.

Keywords: Near infrared sensing; signal processing; embryo motility; gender differences

5.1 Introduction

5.1.1 Motivation

To improve the efficiency of egg and meat production, the poultry industry would ideally raise only female chicks for layer strains, and for broiler strains only male due to their faster growth rate and greater food conversion efficiency [46]. However, every year a billion male day-old layer chicks are culled globally by maceration or gassing due to this gender biased preference; raising serious ethical issues and resulting in significant economic losses [86]. Male layer chicks can neither be used for egg production, nor meat production due to their lower growth rate compared to broiler chicks. In contrast, female broiler chicks are not economically viable due to their lower growth rate compared to male chicks [41]. Hence, the gender biased preference for female and male chicks in layer and broiler farms, respectively. Shipping female broiler to market is uneconomical due to lower body weight gain compared to males. It needs extra cost for feeding female chicks to achieve the same salable weight. Therefore, early chick embryo sexing before hatching is a crucial issue from both a commercial and ethical (animal welfare) point of view. A non-invasive method of gender identification before or during incubation would solve these problems; thereby maximizing productivity of the hatchery, while minimizing energy, labour, and feed consumption inputs, as well as environmental loads.

Currently, there is no non-invasive method for fertile egg sexing before hatching. The existing commercial methods are based on morphological markers, such as feather growth rate (covers and primary), anal vent (cloacal) and plumage color of day old chicks [46]. These widely used conventional methods are manual, time consuming, need skilled manpower and are not cost effective. In the last few decades, many researchers have attempted to apply various strategies (morphological, enzymatic, hormone and molecular assays) to detect embryo gender before hatching or even the gender of fertile eggs before incubation based on differences in DNA content in blastoderm, hormonal difference (estrogen) in allantoic fluid and fluorescence properties of embryo blood [42], [45], [87]. But none of these methods can be used commercially. A commercially applicable method needs to be non-intrusive: not affecting the integrity of eggshells and interior of eggs; and have no negative effects on embryonic development, hatchability or post-hatch development. In addition, it should be easily scaled to process large numbers of eggs; be economically feasible, be rapid and acceptable from an ethical point of view [46]. Since the various gender differences that have been reported to date require destructive measurements, a different approach is needed. Sex hormonal differences in chick embryo at incubation day 8-9 suggest there could be gender differences in embryonic motility and morphological behavior. Moreover, it is well established that sex differences exist in the activity level of human fetuses and chickens are relatively closely related to mammals in the phylogenetic tree. Besides, embryo movement represents a valuable epigenetic factor in vertebrate development and reflects the developmental establishment

of neuromuscular interaction [6], [7]. Hence, the embryonic activity during incubation may also carry valuable sex dependent information.

No conventional method has succeeded in monitoring embryonic movement non-invasively. Although visible transmission spectroscopic methods have the potential to being non-destructive, they cannot be used to monitor hatching eggs during the second half of incubation due to very low transmittance [61]. Besides, the eggshell has a high absorbance in the Ultra Violet to Visible (UV-Vis) region. However, NIR has the potential to quantify the dynamic activity of chick embryos, since NIR has higher transmission through hatching eggs.

5.1.2 Objectives

Therefore, the present research was designed to investigate, firstly, gender differences in chick embryo body motility, and then, secondly, to develop a classification of male and female chick embryos before hatching based on sex specific movement activity using an NIR sensor. Gender detection during incubation could significantly contribute to the humane treatment of chicks and improve productivity (meat and egg production) of hatcheries (minimize labor, energy and space consumption).

5.2 Materials and Methods

5.2.1 Materials

This research was carried out in strict accordance with the animal experiment regulations of Kyoto University, Japan (Approval number: 28-59). Fertile light brown eggs laid by a 54-week old parent flock (ROSS 308 broiler strain, Japanese name “Chunky”) from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto, Japan), were selected based on major diameter (59.5±3.0 mm), minor diameter (46±1.0 mm), mass (68±5.0 g) and shell color (red ration, r = 0.375±0.015), for incubation. Prior to incubation, all eggs were stored for 5 days at 18.0 (± 0.5) °C and 80 (± 5) % of relative humidity (RH). The eggs were color sorted to eliminate any bias due to shell color in gender differences. A color image analysis method was performed to sort eggshell color using the RGB color space. Red of RGB chromaticity is defined by eq. (1). The chemical pigment “protoporphyrin” released during eggshell formation is responsible for the brown color in the shell. The image acquisition system for egg sample selection was developed using a color camera (model: Imaging Source DFK21BU04, Sensor Type: CCD, Sensor Specification: Sony ICX098BQ) with C mount lens and ring LEDs as a lighting source (Figure 5-1).

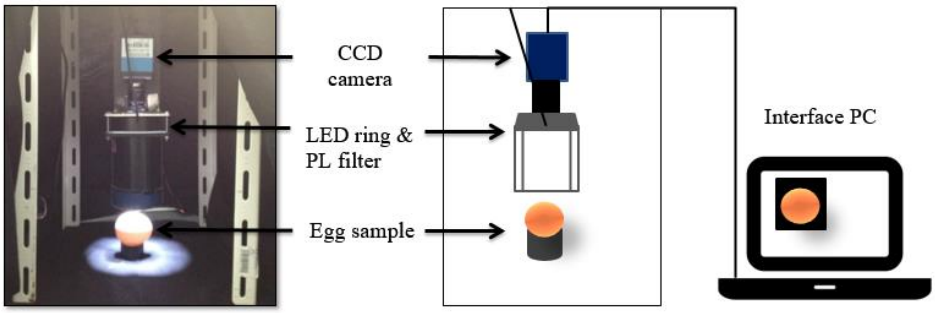


Figure 5-1 Actual and schematic diagram of egg image acquisition system. (Camera Dimension: H: 50.6 mm, W: 50.6 mm, L: 56 mm, height: 20 cm from object centre to the camera lens)

One incubator (SSH-02, Showa Furanki, Saitama, Japan) consist of three egg trays was used for incubation of fertile eggs (20+20+11=51) at 37.8 °C and 55% RH. Prior to setting the eggs into the incubator, eggs were preheated for 16 h (first 6 h at 28 °C and for the remaining 10 h at 30°C) to reduce thermal shock on blastoderm and to reduce the early embryo mortality [47]. After day 18, the eggs were transferred to hatcher three trays maintaining temperature at 37.8 °C and 60 % RH. The embryo motility signal of all incubated hatching eggs was measured using the near-infrared sensor from day 6 to 19 of incubation.

5.2.2 Near-infrared Sensor

An NIR sensor was used for embryonic activity signal acquisition during incubation (Figure 5-2). It consisted of six LEDs (Model: L870-04-35, Epitex, Japan) that emit light at 870 nm and a photodiode (Model: S9269, Hamamatsu Photonics, Japan) that receives the light passing through the egg, was used for this experiment. The spectral rage of the photodiode was 340 to 1100 nm with a peak sensitivity at 960 nm. The photo sensitivity at the peak wavelength was 0.62. In the case of LEDs, the spectral range was 860 to 880 nm with a peak wavelength at 870 nm. The purpose of the selection of NIR (870 nm) wavelength have several reasons such as less variation in transmittance of light through the egg in second half of incubation; relatively higher transmission, and penetration ability NIR region up to several centimeters than any other optical methods in biological tissue [50]. The most important point for choosing 870 nm was the minimal variation in transmission during incubation. Large variations in transmission are undesirable because of the very low intensity of transmission in the latter half of incubation. Ideally, we would like to use the NIR sensor over as wide range of the incubation period as possible, such as from Day 6 to 19. But transmittance of the incubated egg changed widely. Hence, control of LEDs intensity to keep the output voltage in a suitable range, not saturated or not too small, was necessary. In this case it was easier to control LED intensity, when the variance of transmittance was narrow. The light received is converted into current by the photodiode that is further converted into a voltage signal by amplification using a trans-impedance amplifier. The sampling rate per second of the signal was 33.33 (sampling points

per second), which was synchronized to the LED emission cycle (30 ms). Oscillation in the signal due to embryonic movements are around 1.0 % of average output voltage and center around the mean value [88]. Therefore, the part of the signal assigned to embryonic movements was normalized by dividing by the average output voltage, as output voltage varies with the egg sample due to the variation in transmittance.

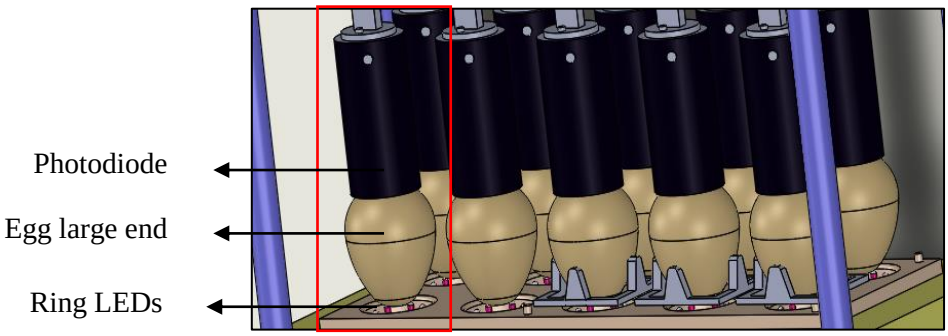


Figure 5-2 Schematic diagram of NIR sensor during signal acquisition of incubated eggs. (Photodiode Specification: Size: 10.1 x 8.9 x 40 mm, Active Area: 5.8 x 5.8 mm, Built-in Rf = 1 GΩ, Cf = 5 pF, Supply Voltage (op amp): ±20 V; Power dissipation: 500)

5.2.3 Egg incubation and signal acquisition

Before incubation at 37.8 °C and 55% RH in a commercial incubator, the signal of all eggs was measured using the NIR sensor at it is referred to as incubation day 0. During incubation, eggs were turned automatically through an angle of 90° every hour. From incubation day 6 to 19, eggs were taken out from the incubator once daily for signal acquisition with the NIR sensor. To minimize the exposure time of the egg outside of the incubator, eggs were immediately placed back into the incubator after the measurement. However, the temperature of the sample eggs while outside of the incubation were carefully maintained using a warm electric blanket to avoid the influence of ambient temperature on the activity of the embryo.

5.2.4 Embryonic activity signal processing

The voltage signal obtained by the sensor was transformed into the frequency domain using FFT of 256 sampling points equivalents to 7.68 seconds signal for peak frequencies and power spectrum. The data size ($2^n = 2^8$=256) was used for efficient conversion into frequency domain for FFT. We take 9 seconds signal with sampling interval of 30 ms. Therefore, 1 second is equivalent to 33.33 sampling points. Reversely, 256 sampling points is equal to 7.68 s (256/33.33=7.68 s). Hence, the frequency resolution in FFT signal was approx. 0.13 Hz. The resolution was designed based on the embryo movement frequency behavior. Potential use of research findings in the future is one of vital reasons for doing research. When we are supposed to use this technology in industrial purposes in large scale, it is not feasible to use signal more than 5-10 seconds. Longer time signal acquisition is only useful when signal would taken inside incubation without any interruption the eggs which could

increase the accuracy of scientific findings. But in this case, there would be a problem like the effect of longer duration of lighting on embryos and hatchability.

The high-frequency peak represents the cardiac movement of the chick embryo whereas the low-frequency peak represents embryo body movement [33], [88]. A similar approach (i.e. calculation of movement rate from frequency domain signal) has also been applied to extract frequency of movement by previous researchers [63]. The heart beats measurements (high-frequency part) are out of the scope of this paper.

The area under the low-frequency peak, *A*, was calculated to measure signal power (energy), which represents embryo body movement strength (BMS) using trapezoidal numerical integration after half mean de-trending of the FFT signal, as expressed by eq. (22). The de-trending is normally used for baseline removal of low magnitude fluctuations of the FFT signal. The physical meaning of signal power is normally equated to the energy or strength of a signal, where the amplitude for a particular frequency contributes a vital part.

$$A \approx \int_a^b (f(x) - 0.4m)dx\,,$$

$$= \frac{b-a}{2N}\sum_{n=1}^{N}(f(x_n) + f(x_{n+1}) - 0.8m) \qquad \text{[eq.22]}$$

Where x_n is the frequency at the low-frequency peak position n in the discrete frequency domain, $f(x_n)$ is the spectrum of x_n, a and b are the $(n\text{-}1)$th frequency $x_{(n-1)}$ and the $(n+1)$th frequency $x_{(n+1)}$ in the discrete frequency domain, N is 2 as three consecutive points is considered and spacing is $(b\text{-}a)/N$. The average magnitude (m) is calculated by eq. (13). All the signals were processed using MATLAB R2016a software. Finally, the values of body movement strength (BMS) were normalized by dividing by the average output voltage of the trans-impedance amplifier.

5.2.5 Gender identification of day-old chicks

The conventional sex determination of day-old chicks are feather sexing, vent or cloacal sexing and plumage color sexing. The feather sexing method is a widely used method in commercial poultry hatcheries due to its high accuracy and independency from breeds [46]. Hence, the feather sexing method was applied to determine the gender of day-old chick samples in this experiment. Primary wings feathers are significantly longer than the coverts in the case of females, where primary wing feathers for the male are shorter or about the same length (Figure 5-3).

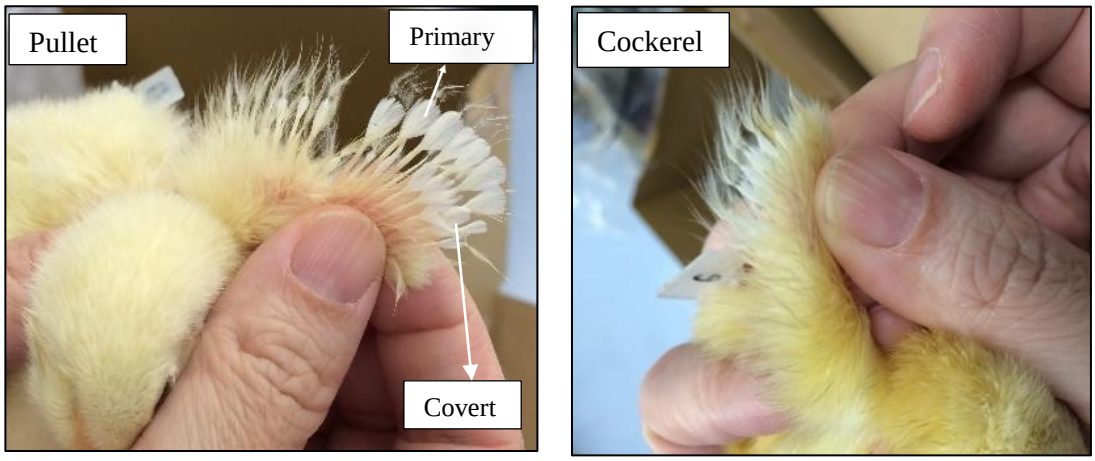


Figure 5-3 Gender identification of day-old chick by feather sexing method. Feather distribution pattern in left diagram for pullet and right diagram for cockerel. In pullet, primary is bigger than covert whereas primary is shorter or same in cockerel.

The eggshell thickness at three different sides such as large end (L.End), center and small end (S.End), of all egg samples and left and right shank length of neonatal chicks after hatching were also measure as supportive data using a micrometer and slide caliper, respectively.

5.2.6 Data analysis

After extracting BMS, the area under the peak of the frequency domain signal, we used BMS data from incubation days 10, 11, 16, 18 for PCA. Hence, the dimension of the feature vector was 4X46 where 4 means 4 incubation days and 46 is the egg (embryo) sample size. Those incubation days was chosen based on a *t*-test (p-value < 0.1). We used all sample to calculate eigen vectors of PCA. PC Scores of BMS were used as new input variables in classification models, SVM and KNN for classification of the male embryos. PCs scores were used as the new data matrix in the classification step. During classification model development, all samples (21 male and 25 female embryos) were used for 5-fold cross validation (CV) by taking 80% as a training set and 20% for testing set to check the models. For double checking the best model found in CV, data set was again divided into two groups i.e. a training set (70%) and validation set (30%) for testing of the best model. Model performances were evaluated using Recall (*R*), Precision (*P*), F-score (*F*) and overall accuracy values as shown in eq. (19)-(21). Two sample *t*-test was used to see the statistically significant difference (*p*-value <0.05) between male and female groups for various parameters.

5.3 Results and Discussion

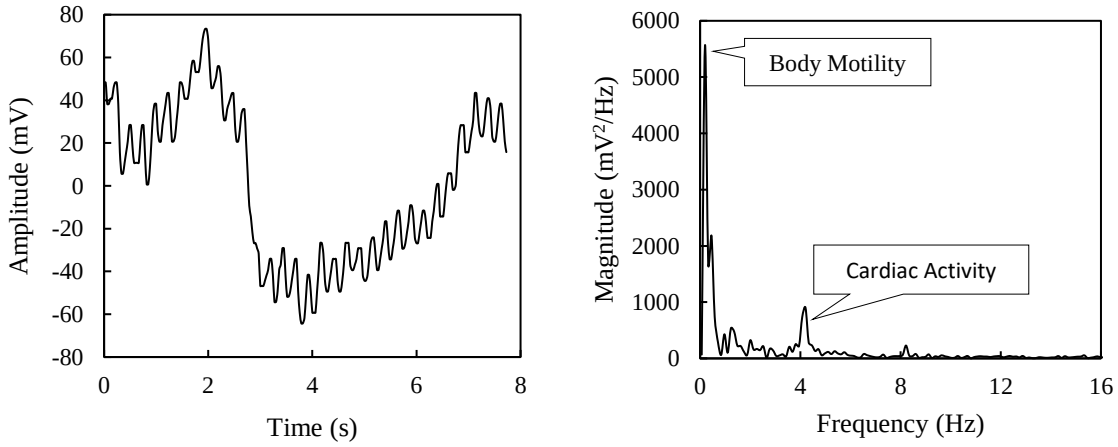
5.3.1 Gender specific sorted egg properties

After determining the actual gender of chicks by the feather sexing method, it was confirmed that there were no significant differences in male and female fertile eggs used in terms of egg mass (g), major and minor diameter of eggs (p-value < 0.05). Data showed that egg mass and shape parameters

were independent of gender for the broiler breed. Eggs were sorted based on size, shape and shell color to eliminate effects of these parameters on sex specific embryo body dynamic activity.

5.3.2 Gender differences in body movement patterns

The intensity of transmitted light is affected by both embryonic body movement and cardiac rhythm during blood pumping. This changing pattern in transmitted light intensity results in changes (low-frequency part) in the signal represent embryonic body movement where small waves (high-frequency part) in the signal representing heart beats (Figure 5-4). Based on frequency analysis, chick embryo body movement was found in the low-frequency region, from 12 to 50 beats per minute (bpm) (0.2-0.8 Hz). Before the onset of embryonic respiratory movement from day 18-20 of incubation, chick embryos have only two types of motility; body motility and heart beats. It is well established that body motility rate is 12-50 (<1.0 Hz) bpm whereas heart rate is 150-300 bpm (2.5-5.0 Hz) [33], [60].

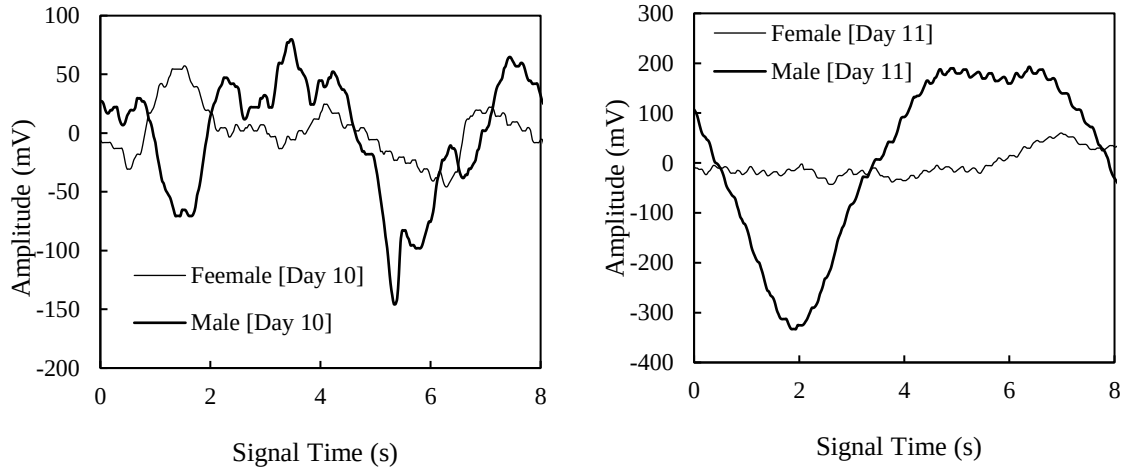


(a) Signal after mean (output voltage) shifting (b) Signal after Fast Fourier Transform (FFT)

Figure 5-4 Typical signal (a) and corresponding FFT signal (b) of an embryo at incubation day 15. The small, rapid waves (high-frequency component) in (a) represent heart beats and big, slow changes (low-frequency component) represent body movement.

The transmission based near-infrared signal energy (low-frequency component after FFT) was used to define embryo BMS. The strength of the signal depends on the amplitude and phase of the time domain motility signal. Amplitude (vital part of signal strength) differences between gender groups on various incubation days are visible in the time domain signal (Figure 5-5). The female embryos had a weaker BMS over the incubation period than the male groups, especially after day 9 (Figure 5-6). Though the male embryo group had a higher movement strength after day 9. A significant difference between male and female groups was only observed at day 10 (p-value < 0.05), day 11 (p-value <0.1) and day 16 (p-value <0.1). Gender differences were apparent every day after incubation day 9 once gender is fixed morphologically. Although this phenomenon significant differences prevailed after day 9 of incubation, differences in motility strength declined in the last

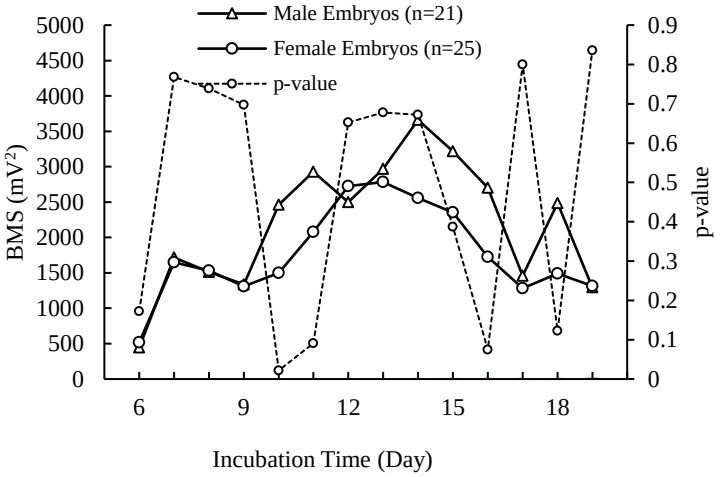
few days of incubation due to voluntary and irregular behavior movements. The embryonic motility signal was more regular and periodic in nature in the first half of incubation. In latter half, the signal was sometimes irregular and it's because of more than one component in the signal due to the independent movements of wings, legs and head. Prominent gender differences were found at days 10-11. This motility was a regular and periodic in this time. Gender detection by day 10-11 is desirable as the unwanted embryos could be used for other purposes like vaccine production.



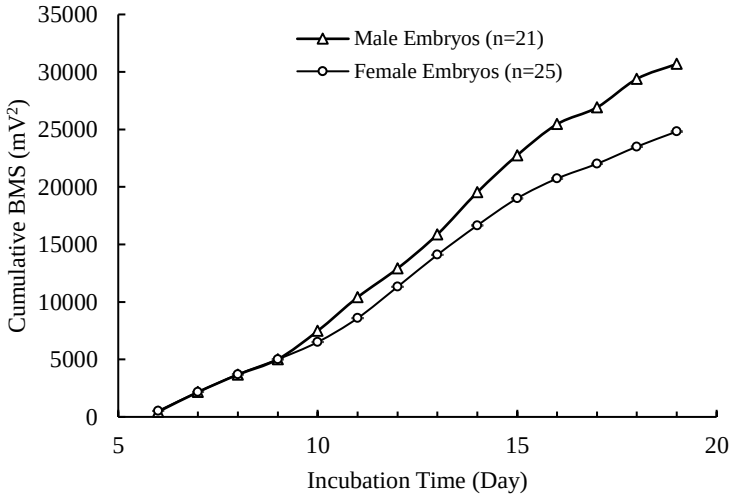
(a) Signal at day 10 (b) Signal at day 11

Figure 5-5 Typical signals of one male and one female embryos with similar features (egg mass, egg shape index, hatching time and chick weight) at day 10 and 11.

The movement pattern during incubation of both male and female groups followed a similar pattern, sharply increasing from the beginning of incubation, slowing down around day 9, and then increasing again and peaking at day 14-16, before subsiding, whereas Cumulative BMS gradually increased over incubation time. During the early stages (day 6-8) body movement strength was relatively low compared to that in the following stages, since the embryos are relatively small and immature during these stages. The early segment of the BMS gave the first peak between days 7-8 as a result of rhythmic embryonic movement during this stage. Subsequently, strength reduced at day 9 as the head started becoming heavier. The differences in behavior of strength was found at day 17-19 which is called the pipping period. During this time, the embryos generally come to an upright position to start internal pipping and then external pipping [73]. He reported that were smooth, apparently coordinated movements as the chick moved into the hatching position at day 16-17. Moreover, during the pipping period, the embryos become almost mature and the internal components started to absorb by the embryo abdomen and therefore, the movement strength decreases as it is difficult to move freely for the large and matured embryos during the last few days before hatching.



(a) BMS pattern of male and female embryos



(b) Cummulative BMS pattern of male and female embryos

Figure 5-6 Gender differences in average body movement strength (BMS) and cumulative body movement strength during incubation.

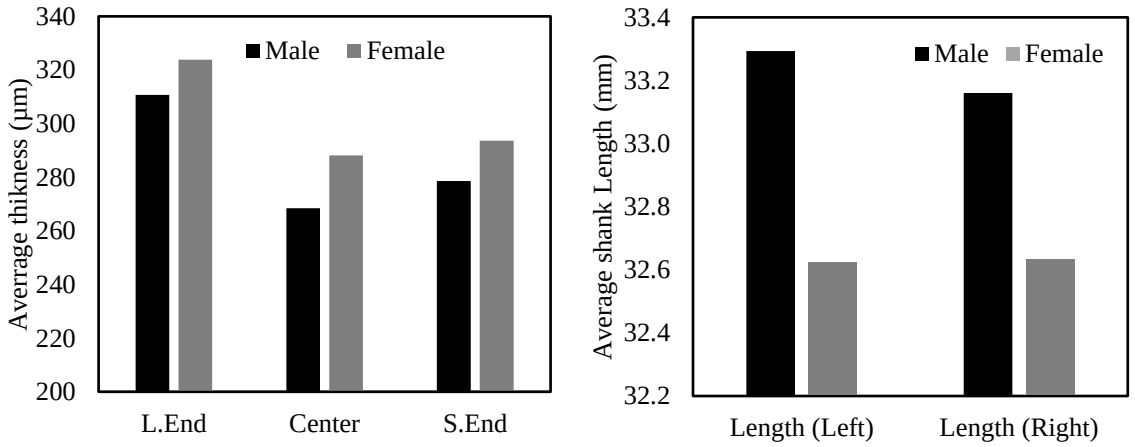
The sex hormonal differences in chick embryo at incubation day 8-9 support the gender differences in embryogenic activity and morphological behavior in the second half of incubation. Morphological sex differentiation is asymmetric in female and symmetrical in male birds in which two testes develop from the medulla. Increasing amounts of estrogens are produced by ovaries from 7.5 days onwards. Male gonads convert labeled precursors mainly into testosterone that can be identified at 7.5-10 days when dehydroepiandrosterone is used as a precursor, at 10 and 18 days with progesterone, and at 18 days also with pregnenolone as a precursor [89].

Morphological differentiation begins between day 5.5 and 6.5, with the ovary and testis formation completed by day 8.5 of incubation in chick embryos. The biosynthesis and secretion of gonadal hormones occurs after sexual differentiation of the gonads [90]. For this reason, the sex differences

in chick embryo dynamic activity after day 9.0 might be due to the effects of sex hormones (estrogen, testosterone and androgen) on embryo behavior and morphology. Interestingly, estrogen receptor alpha is expressed in the gonads of both sexes prior to sexual differentiation in the chick embryo [91]. This expression is seen primarily in the gonadal cortex. Expression is downregulated in males as development proceeds where as in females, expression is also turned off in the right gonad [92].

5.3.3 Gender differences in post-hatch shell thickness and chick shank length

As shown in Figure 5-7, sex specific differences were also found in post-hatch eggshell thickness and shank length of chicks just after hatching. Although significant differences were found in center (p-value < 0.05) and small end (p-value < 0.05) of male eggshell and female eggshell, there was no difference at the large end (p-value > 0.05). Significant differences were also found in the shank length of the male and female day-old chicks. The higher shank length of male chicks and lower post-hatch shell thickness at the center and small end indicated that male embryos absorb and utilize more calcium from shell compared to female embryos. It also suggested to assume that higher embryonic body motility might help the male embryos in dissolving and assimilation of calcium from the eggshell, especially from the lower part of the shell.



(a) Gender differences in shell thickness at three different sides of eggs

(b) Gender differences in left and right shank length of day old chick

Figure 5-7 Gender differences in post-hatch eggshell thickness and shank length of neonatal chick (♀=25, ♂=21). (L.End means Large End and S.End means Small End of egg).

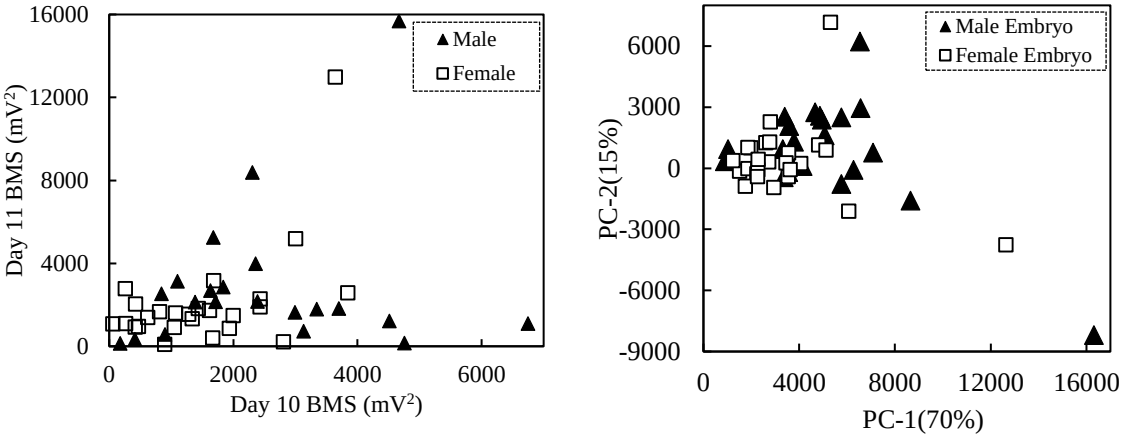
5.3.4 Gender classification

PCA was applied to embryonic movement strength of incubation at days 10-19, as well as other selective days of incubation, to obtain the best discrimination between male and female embryos. The best observed PCA model was obtained with motility strength on incubation days 10, 11, 16 and 18 to classify the gender of embryos. The first two PCs of this model explained 85% of the total variance in the dataset (Figure 5-8). The higher values in the PC score represent the bigger

movement (bigger amplitude move) during incubation by the embryos. The bigger movement might be from the several reasons such as bigger embryo, more active or strong embryos and/or taken lead in developmental stages which could be treated as explanatory variables. Because male embryos are more active, strong and higher growth compare to female growth. Various classification methods (SVM and KNN) were used using the 2 PCs to discriminate between male and female embryos. SVM is used for binary classification. It is work based on optimal separating hyperplane between the two classes by maximizing the margin between the classes' closest points (support vectors). The performance of the SVM model is influenced by the selection of the kernel function (e.g. radial basis function, linear function, gaussian function etc.). The linearity and non-linearity are dependent on the kernel function. We used SVM kernel functions with default parameter values in MATLAB. The gaussian is a non-linear kernel function which also can be defined as a Gaussian radial basis function kernel in MATLAB. This kernel function superior performance when the data structure looks circularly symmetric. In this case, though radial basis function kernel can separate the classes, the results can be over-trained.

KNN algorithm is a non-parametric method used for classification or regression. According to KNN rule, an unclassed sample is assigned to the class represented by most of its k-nearest neighbors in the training set. The k-NN classifier does not rely on any assumption concerning the statistical distribution of data but instead it relies on a positive integer k, a distance metric function (e.g. Cosine) between input patterns, and a labelled training set.

The results indicate that discrimination between male and female embryo groups could be achieved with an 84% accuracy using the SVM linear model (Table 5-1). The male group was considered as a positive class in the data set, with a recall obtained of 0.67. In this case, a higher recall value indicates good performance of the model to identify males. On the other hand, a higher precision value (close to 1.0) indicates the model is detecting female chick embryos accurately. The higher precision value here indicates a more similar behavior within female embryos, whereas a lower recall value indicates higher dispersion of male embryos which is clearly shown in PC bi-plot. This might incorporate some biological activity patterns of male and female embryos during incubation. The PC scores of most of the male embryo are appears in right and top corner indicating higher motility strength and vice versa for female embryos. Exceptional two male embryos PC scores appear in left side due to their non-motility states (inactive session) during signal acquisition.



(a) Scatter plot of male and female embryos' BMS at days 10-11

(b) Scores plot of male and female chick embryos' BMS

Figure 5-8 Scatter plot and scores plot of male and female chick embryos BMS. First two principal components covered 85% of variation in PCA eigen space. Two outlier male embryos PC scores are apparent on left side of the eigen space due to their non-motility stage.

The scope of this paper is to establish a novel technique to quantify the body motility signal for male and female embryos in terms of strength, and the application of this signal for indirect measurement of male and female embryos. Chick embryo activity signal measurement has at least two potentially important functions: observing the sex dependent embryonic physiology or developmental biology of chick embryos during incubation, and in applications for precision poultry production systems.

Table 5-1 Comparison of classification results of the cross validation and validation set

Model	Classification			
	Precision	Recall	Accuracy (%)	F-Score
SVM-Linear (5-Fold CV)	0.78	0.67	76.0	0.72
SVM-Gaussian (5-Fold CV)	0.67	0.67	70.0	0.67
KNN-Cosine (5-Fold CV)	0.74	0.67	74.0	0.70
SVM-Linear (Validation)	1.00	0.67	84.0	0.80

5.4 Conclusions

This research revealed that chick embryo body dynamic activity during incubation is a vital physiological parameter, which is closely associated with embryonic development, and is influenced by the subsequent gender of the embryo. The motility strength behavior for the male and female groups in the last half of incubation could attributed to sex hormone after complete gonadal differentiation at day 8.5 on motility behavior of embryos. From a commercial viewpoint, detection of male and female chick embryos before hatching is a crucial challenge for poultry farmers and other stakeholders worldwide. From this perspective, this feature offers the potential to detect embryo gender; thus, maximizing the productivity of hatcheries. This detection of chick embryo gender is also important from a humane treatment viewpoint, since male chicks are normally culled in layer breeds due to gender biased preferences. Moreover, this can facilitate a new era in sustainable and precision poultry production, maximizing meat production by rearing male and female chicks in different sheds; minimizing energy, space consumption and reducing biological waste production. Moreover, understanding dynamic activity behavior may also enhance the understanding the embryo development and to identify high quality embryo in precision chick production system in the future.

Chapter 6

Broiler Chick Embryo Sexing based on Opacity Value of Incubated Eggs

Abstract: In broiler production there is a gender biased preference for males, while for layer strains it is females. Thus, there is need for non-intrusive, early sex discrimination of chick embryos that is currently not being met. Hence, the motivation for the current study to investigate sex-based growth difference in terms of opacity in order to classify the sex of incubated eggs. As a model, eggs from a broiler strain (ROSS 308) were sorted based on size, mass and shell color, and then incubated. During incubation an Embryonic Vital Scope, which consists of LEDs and a Si-photodiode integrated with amplifire that receives the light that passes through the egg, was used to measure average output voltage for individual eggs. After hatching feather sexing was used to determine the actual gender of the day-old chicks. A two-sample t-test was used to see the significant differences between male and female groups and gender classification model were developed using various algorithms. As the embryo became larger during incubation, the amount of transmitted light was reduced. Consequently, the opacity, the ratio of input LED current over average output voltage, increased. Male chick embryos showed higher opacity than female embryos (p-value <0.05). This method allowed us to determine the embryo gender before hatching with an accuracy of 84%. Although this method is still not 100% accurate, it is an important step towards developing an early, accurate and non-invasive means for sexing chick embryos in an industrial setting in the near future.

Keywords: Embryonic growth; near-infrared sensing; signal processing; gender prediction

6.1 Introduction

6.1.1 Motivation

There is a gender biased preference in dimorphic birds like chicken where male is preferable gender in broiler and female in layer strains. For broiler production female chicks are not economically viable due to lower growth rates compared to their male counterparts [41]. Extra feed costs are required for females to gain salable weight. Moreover, female chickens in broiler farms cause complexities during shipping to market due to their lower body weight gain compared to males. In contrast, the male layer chick cannot be used for either egg production or meat production due to its lower growth rate and feed conversion ratio compared to a broiler chick.

Besides, every year more than 7.0 billion day-old layer strain cockerels are culled globally by asphyxiation with carbon dioxide or maceration due to this gender biased production preference; raising serious ethical issues and resulting in significant economic losses [87]. Therefore, non-intrusive, early chick embryo sexing before hatching is a crucial issue both from a commercial and ethical (animal welfare) point of view. This has driven increasing research effort and the desire of other stakeholders in the industry to find a solution.

In the last few decades, many researchers have attempted to apply various strategies to detect embryo gender before hatching, or even better the gender of fertile eggs before incubation based on differences in DNA content in blastoderm, hormonal difference (estrogen) in allantoic fluid and fluorescence properties of embryo blood [42], [45], [87]. Moreover, it is well established that sex differences exist in the heart rate, embryo weight in second half of incubation, egg content (maternal investment), egg odor, DNA content, blood fluorescence intensity and Raman scattering. But none of these methods has been used commercially since they are destructive methods.

A commercially applicable method will need to be non-intrusive: not affecting the integrity of eggshell or the embryo within, such as negative effects on embryonic development, hatchability and post-hatch development. In addition, it should be rapid enough to be applicable to a large numbers of eggs; be economically feasible, and acceptable from an ethical point of view [46]. Sex hormonal differences in chick embryo at incubation days 8-9 have been found, suggesting that if these gender differences in embryonic morphological development could be detected non-intrusively, they may form the means for an alternative means of early sexing. Our hypothesis, based on well establish phenomena of sexual dimorphism of broiler chicks where males are substantially heavier than females [41], is that the opaqueness of incubated male fertile eggs will absorb more light as it passes through the eggs than eggs with the smaller female embryo.

Optical methods have clear advantages in an industrial setting compared to other non-invasive methods. Moreover, the optical spectrum that is obtained contains information about the structure of the biological sample [87]. Because the eggshell and the embryo within have a high absorbance in the UV and visible region respectively, it is thought near-infrared (NIR) light is probably the

better option to study hatching eggs and quantify embryonic growth, since NIR has a higher transmission through hatching eggs, even during the second half of incubation [33]. In fact, NIR spectra (e.g. IR spectroscopic imaging, NIR Raman spectroscopy, NIR fluorescence signal of 820-1000 nm) has already been used *in ovo* sexing of chick embryos, though these methods were invasive.

6.1.2 Objectives

Therefore, the present research was designed to establish, firstly, a novel method to establish gender differences in chick embryonic growth in terms of opacity, and then, to classify male and female chick embryos before hatching based on opacity. Opacity could be defined as the amount of light lost when passing through the incubated fertile eggs. Gender detection during incubation of male embryos could significantly contribute to the humane treatment of chicks and improve productivity (meat and egg production) of hatcheries and to reduce environmental loads (minimize labor, energy and space consumption).

6.2 Materials and Methods

6.2.1 Materials

This research was carried out in strict accordance with the animal experiment regulations of Kyoto University, Japan (Approval number: 28-59). Fertile light brown eggs laid by a 54-week old parent flock (ROSS 308 broiler strain, Japanese name “Chunky”) were used in this experiment. Eggs were obtained from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto, Japan). Eggs were sorted based on major diameter (59.5±3.0 mm), minor diameter (46±1.0 mm), mass (68±5.0 g) and shell color (red ratio, r = 0.375±0.015), for incubation. Prior to incubation, all eggs were stored for 5 days at 18.0 (± 0.5) °C and 80 (± 5) % of relative humidity (RH). A color image analysis method was performed to sort eggshell color using the RGB color space.

2.2 Experimental Methods

The incubator (SSH-02, Showa Furanki, Saitama, Japan) consists of three egg trays was used for incubation of fertile eggs (20+20+11=51) at 37.8 °C and 55% RH [47]. Prior to setting the eggs into the incubator, eggs were preheated for 16 h (first 6 h at 28 °C and for the remaining 10 h at 30°C) to reduce thermal shock on blastoderm and hence to increase hatchability. After day 18, the eggs were transferred to a hatcher with three trays where the temperature and relative humidity were maintained at 37.8 °C and 60 %, respectively. The input LED current (I_{LED}) and average transimpedance voltage, V_{avg} were measured for all incubated eggs using the near-infrared sensor from days 6 to 19 of incubation. Before incubation in an incubator, the signal of all eggs was measured using the NIR sensor and it is referred to as incubation day 0. During incubation, eggs were turned automatically through an angle of 90° every hour. From incubation day 6 to 19, eggs were taken out from the incubator once daily for signal acquisition using a vertical optical

configuration. To minimize the exposure time of the egg outside of the incubator, eggs were immediately placed back into the incubator after the measurement. Moreover, the temperature of the sample eggs while outside of the incubation were carefully maintained using a warm electric blanket to avoid the influence of ambient temperature on the embryo.

6.2.2 Embryonic Vital Scope

This was an NIR sensor, which consisted of six LEDs (model: L810-33AU, epitex, Japan) that emit light at 870 nm and a Si photodiode with preamp integrated with feed-back resistance and capacitance (model: S9269, Hamamatsu Photonics, Japan) that receives the light passing through the egg, was used for this experiment. The light received is converted into current by the photodiode that is further converted into a voltage signal using an integrated operational amplifier (op amp). The sampling rate per second of the signal was 33.3 (sampling points per second), which was synchronized to the LED emission cycle (30 ms). The average output voltage (V_{avg}) was calculated from the 9 seconds output voltage (V_{out}) signal for each egg.

6.2.3 Opacity Calculation

The Opacity can be defined as the amount of light lost when passing through the hatching egg sample. As the embryonic components became larger during incubation, the amount of transmitted light was reduced, hence the photodiode current and subsequently the output voltage decreased. Consequently, the opacity, the ratio of input LED current over average output voltage, increased with the growth of embryonic components such as embryo, allantois, yolk sac and other structures. The higher opacity value means the higher input current, mA (i.e. higher input light intensity) is necessary to get the same output voltage, V. The opacity is proportional to the light absorbance of the egg sample. The opacity was calculated based on the eq. (6)-(7) (section 2.2.1.1).

6.2.4 Gender and hatching time identification of day-old chicks

The conventional sex determinations of day-old chicks are feather sexing, vent or cloacal sexing and plumage color sexing. The feather sexing method is a widely used method in commercial poultry hatcheries due to its high accuracy and independency from breeds [46]. Hence, the feather sexing method was applied for gender identification of day-old chicks. One-day-old male birds in the hatcheries are manually sorted out by feather or vent sexing, which provide accuracies of up to 98% [93]. Primary wings feathers are significantly longer than the female cohorts, where primary wing feathers for the male are shorter or about the same length.

The weight and shank length of neonatal chicks after hatching were measure as supportive data using a digital balance and a slide caliper. Hatching time was also calculated based on real time monitoring and image capturing during hatching process.

6.2.5 Data analysis

Opacity values (Days 16-18) were used as input variables in classification methods, such as discriminant analysis (DA), support vector machine (SVM), logistic regression and k-nearest neighbor (KNN) for classification of the male embryos. During classification model development, 5- fold cross validation method was used for all samples (21 male and 25 female embryos). Model performances were evaluated using contingency matrix and Recall (*R*), Precision (*P*), F-score (*F*) and overall accuracy values as shown in section 2.2.5.3 (eq. (19)-(21)). Male embryo was defined as a True Positive (T_p) class and female embryo as True Negative (T_n) class in the prediction models. Hence, False Positive (F_p) and False Negative (F_n) represented number of female embryos identified as male embryos and number of male embryos identified as female embryos respectively. In this case, the precision value was mostly influenced by F_p which indicated the misclassification of female embryos as male embryos. This means lower number of misclassifying female embryos will increase the precision value. On the other hand, as only male embryos are input variables for recall calculation, lower number of misclassifying male embryos will increase the recall value. Hence, recall value was independent of female embryos whether accurately classified or miss classified. Two sample *t*-test was used to see the statistically significant difference (*p*-value <0.05) between male and female groups for various parameters.

6.3 Results and Discussion

The scope of this paper is to establish a novel technique to monitor non-invasively embryonic growth in terms of opacity during incubation and to determine sex of hatching eggs during this incubation period. Although near-infrared sensor was used to monitor embryonic development from day 6 to 19, the prediction models was developed based on the opacity values of days 16-18. This research has several important functions: observing gender differences from the developmental biology of embryos, and the application of this to enhance precision poultry production and animal welfare.

6.3.1 Gender specific sorted egg properties

After determining the actual gender of chicks, it was confirmed that there were no significant differences in male and female fertile eggs used in this experiment in terms of egg mass (g), major and minor diameter of eggs at a p-value < 0.05. The data showed that egg mass and shape parameters were independent of gender for this broiler breed (Figure 6-1). Eggs were sorted based on size, shape and shell color to eliminate any effects of these parameters on sex specific embryonic development.

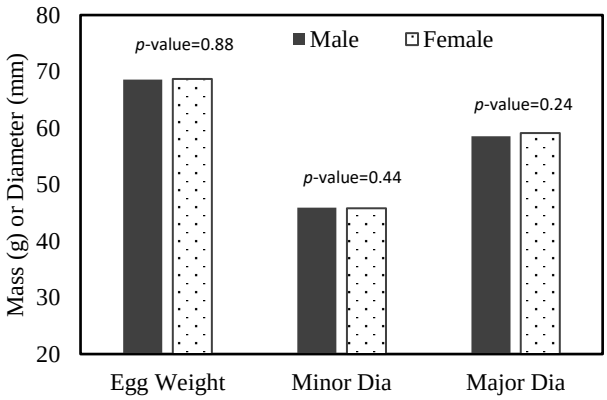


Figure 6-1 Relative values of male and female eggs mass and shape parameters (major and minor diameter) used for the experiment.

6.3.2 Gender differences in opacity of hatching Eggs

Near infrared signal transmission was used to determine the opacity of incubated fertile eggs. As the embryonic components became larger with incubation, the amount of transmitted light was reduced, hence the photodiode current and subsequently the output voltage decreased. Consequently, the opacity, ratio of input LED current over average output voltage, increased. But the change was not linear over this period as this parameter varied depending mostly on embryo size, yolk sac and allantois growth patterns.

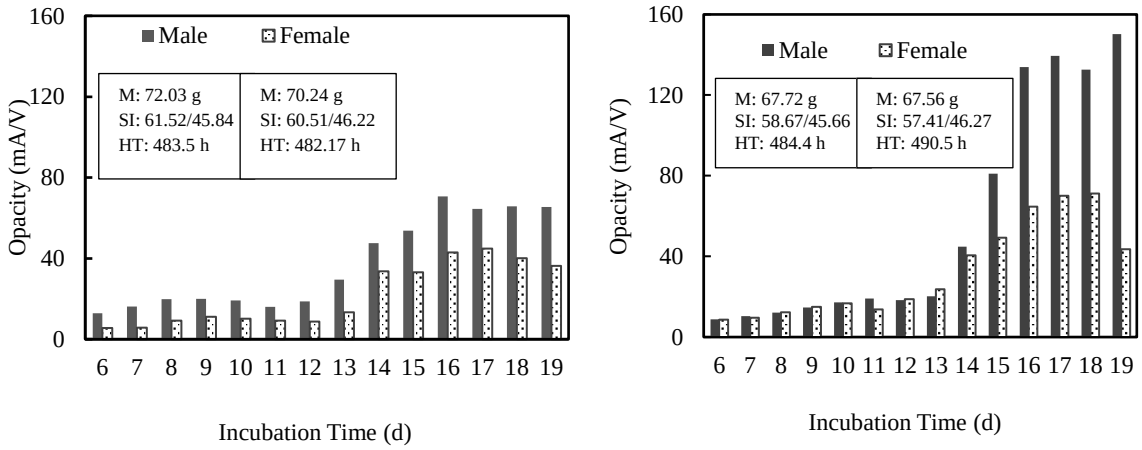


Figure 6-2 Opacity values of two set of male and female embryos with similar features (egg mass, egg shape index, and hatching time) from days 6- 19. M= Mass; SI= Shape Index= Major axis/ Minor Axis; HT= Hatching Time

The opacity pattern during incubation of both male and female groups followed a similar pattern, gradual increase from the beginning of incubation, slowing down around day 9, and then increasing again and peaking at day 16-18, before subsiding (Figure 6-2). During the first half of incubation (until day 10) opacity was relatively low compared to that in the second half, since the embryos are relatively small during these stages and it is called formation phase. During the pipping period (days

17 onward), the embryos have almost matured and the internal components start to be absorbed by the embryo abdomen and therefore, opacity decreases as absorbance by the allantois and yolk sac are minimal or not detectable for matured embryos during the last few days before hatching. Perhaps, another factor for decreasing of opacity is backward light scattering due to the skin and feathers of matured embryo. In first phase, day 6-12, opacity was influenced to a degree by peripheral growth of yolk sac and big changes in allantois size. At this formation phase the growth rate of the embryo is much lower than that during the last half of incubation, called the growth phase. Allantois gains its maximum weight at between day 9-11 [3]. From day 12-16, the biggest influences are the rapid growth of the embryo and formation of lamellae invading the yolk. Normally, the embryo enters into the exponential growth phase from day 11. A new mode of yolk sac growth begins during this phase as radiating lamellae forms and invades the yolk [3]. This rapid growth of the embryo and the invading of the yolk may be responsible for rapid changes seen in opacity between days 12-16 of incubation. From day 17 to hatching, opacity declines or remains static until hatching.

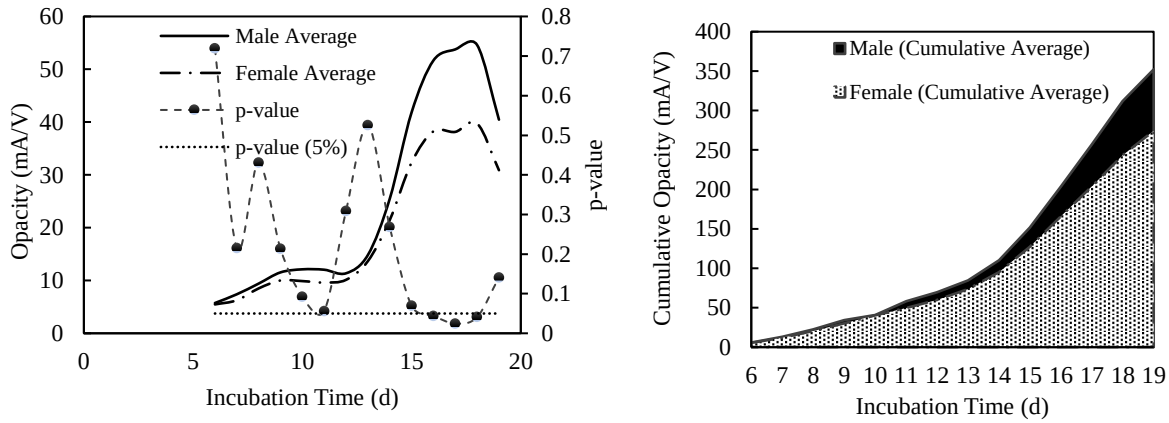


Figure 6-3 Gender differences in average opacity (left figure) and cumulative opacity (right figure) during incubation days 6-19

The female embryos had a lower average opacity over the incubation period than the males, especially after day 15 (Figure 6-3). Though the male embryos group had a higher value after day 10, a significant difference between male and female groups was only observed at days 11-12, 15 (p-value < 0.1) and days 16-18 (p-value <0.05).

Many avian species (such as chicken, duck, goose, turkey) are sexually dimorphic in body weight with the males being substantially heavier than females [41]. According to Burke and Sharp's findings, the mean wet body weight of male embryos is significantly greater than that of females at days 11, 13 and 18 of incubation. They also found that this difference also exists with twin embryos; the male having the heavier body weight. Body weight chick embryo gains in the second half of incubation. One can speculate that the sex determining gene (s) results in inherently different rates of cell multiplication and growth between the sexes. Alternatively, one could speculate that sex

differences in growth-regulating hormonal agents are responsible for differences in growth. Growth hormones, such as prolactin, thyroid hormones, and androgens have been shown to increase growth of chickens [41].

The sex related hormonal differences between chick embryos at incubation days 8-9 agree with the gender differences observed in embryogenic morphology in the second half of incubation. Male gonads convert labeled precursors mainly into testosterone that can be identified at days 7.5-10 when dehydroepiandrosterone is used as a precursor, at days 10 and 18 with progesterone, and also at day 18 with pregnenolone as a precursor [89]. Morphological differentiation begins between days 5.5 and 6.5, with the ovary and testis formation completed by day 8.5 of incubation in chick embryos. The biosynthesis and secretion of gonadal hormones occurs after sexual differentiation of the gonads [90]. For this reason, the sex differences in chick embryo accelerate after the first half of incubation, which might be associated with the effects of sex hormones on embryo growth, together with internal structural morphology.

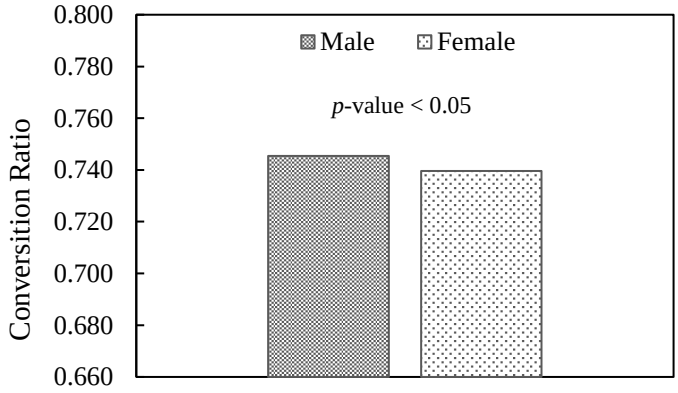


Figure 6-4 Conversion ratio (chick weight/egg weight) of broiler strain (♀=25, ♂=21).

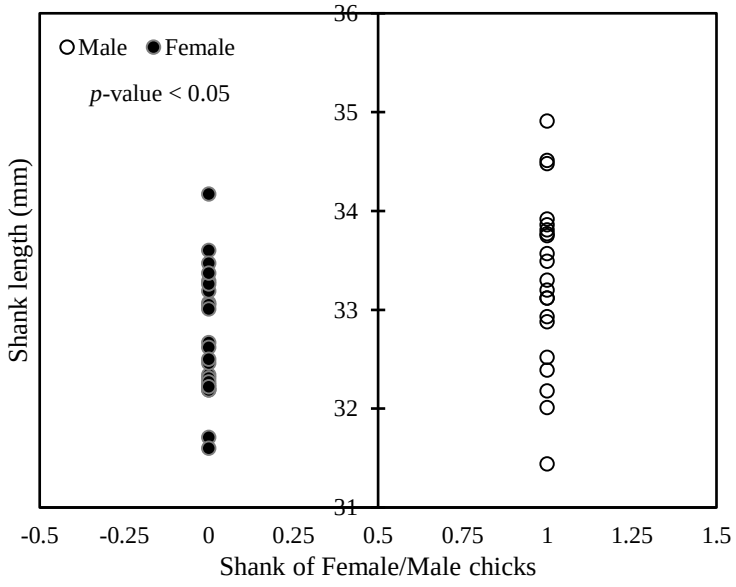


Figure 6-5 Gender differences in shank length of neonatal broiler chicks (♀=25, ♂=21).

Significant sex related differences were also found in post-hatch shank length of neonatal chicks and conversion ratio (Figure 6-4 and Figure 6-5). The higher shank length of male chicks indicates that male embryos absorb and utilize more calcium from the shell, and have a higher metabolic activity and growth rate compared to female embryos. Genotype and gender differences in body composition, body weight and hatching weight of broiler chicken have also been reported by several researchers [41], [94]–[97]. These studies concluded that sex differences exist in food conversion efficiency, growth, embryo weight, chick hatching weight, carcass composition and skeletal muscle characteristics, including muscle fiber size, number and types and protein metabolism. The unit protein content in carcass composition of broiler is higher in male, while fat is higher in the female [96]. The gender differences in chick hatching weight and chick weight : egg ration is due to genetic background associated variation [98]. They found that percent hatch weight of boiler male chicks is significantly (p-value <0.5) higher than female chicks and attributed this to the effect of the Dwarfing gene.

6.3.3 Gender classification models

Discrimination between male and female embryos using opacity was obtained at days 16-18 of incubation, as well as other selective days of incubation. The best observed model was obtained with opacity values on incubation days 16,17 and 18 to classify the gender of embryos. Various classification methods (DA, LR, SVM and KNN) were used to discriminate between male and female embryos (Table 6-1). The results indicate that discrimination between male and female embryo groups had an 84.0 % accuracy using the LDA and LR models (Table 6-2). The male group was considered as a positive class in the data set whereas the female was negative class. Recall and precision were obtained of 0.67 and 1.0 respectively for both the models, LDA and LR. In this case, a higher recall value indicates better performance of the model to identify males. On the other hand, a higher precision value (close to 1.0) indicates the model is detecting female chick embryos accurately. Precision value was mostly influenced by F_p which indicated the miss classification of female embryos as male embryos. This means lower number of miss classifying female embryos will increase the precision value. On the other hand, as only male embryos are input variable in recall calculation, lower number of miss classifying male embryos will increase the recall value. Therefore, recall value was independent of female embryos whether accurately classified or not. A higher precision value here was due to less variation in growth within female embryos, whereas a lower recall value indicates a higher variation in growth among male embryos. This might incorporate be associated with some distinctive biologically associated activity patterns between male and female embryos during incubation.

Table 6-1 Evaluation of models in terms of contingency matrix (male is positive class)

Model		LDA		Logistic Regression		SVM Linear		KNN	
Sex		True Class		True Class		True Class		True Class	
		Male	Female	Male	Female	Male	Female	Male	Female
Predicted Class	Male	14	0	14	0	17	4	18	7
	Female	7	21	7	21	4	21	3	18

Table 6-2 Comparison of models in terms of precision, recall, accuracy and F-score

Model	Classification			
	Precision	Recall	Accuracy (%)	F-Score
DA-Linear	1.0	0.67	84.0	0.80
Logistic Regression	1.0	0.67	84.0	0.80
SVM-Linear	0.8	0.8	81.8	0.80
KNN	0.72	0.85	78.2	0.77

6.4 Conclusions

From a commercial viewpoint, detection of male and female chick embryos before hatching is a crucial challenge for poultry farmers worldwide. This research demonstrates opacity of incubated hatching egg is a distinctive physio-optical parameter of the egg, which is closely associated with embryonic development, and influenced by the gender of the embryo in broiler strains. Sex related opacity was most distinctive in the last half of incubation, which is tentatively associated with the presence of sex hormones following gonadal differentiation, embryonic growth and embryonic structures. This feature offers the potential to detect embryo gender; thus contributing to the optimization of productivity in poultry hatcheries. This detection of chick embryo gender is also important from a humane treatment viewpoint. Although this method needs to be more fully developed with regard to accuracy and data of multiple days, these results represent an important step towards finding an industrial sexing method in the near future, thereby opening the door on a new era of precision poultry production, maximizing meat production; minimizing energy and space consumption, and reducing biological waste production. Thus, this technique can help to achieve Sustainable Developmental Goals (SDGs) by supporting technology to minimize greenhouse gas emission (i.e. climatic action) and zero hunger (i.e. higher animal meat production).

Chapter 7

A non-invasive diagnosis technique of chick embryonic cardiac arrhythmia

Abstract: Cardiac arrhythmia is considered important cause of cardiovascular disorder in birds. Little is known, however, about arrhythmia during embryonic stages, chiefly because of their relative rarity and difficult diagnosis. Electrocardiogram (ECG) can't be applied in avian samples non-destructively due to the eggshell. Therefore, an alternative method is an important tool if avian or reptilian embryo research in this area is to advance. Here, we addressed, a non-invasive method to diagnosis cardiac arrhythmia based on optical sensor together with signal processing. In such a system, the intensity of transmitted light is mostly affected by pulsatile blood volumes and mechanical activity of the heart during blood pumping. In our measurement procedure, the time domain transmissive signal was transformed into the frequency domain using Fast Fourier Transform (FFT). We interpreted the cardiac signal of embryo with a naturally occurred bradycardia throughout the incubation. We found a normal heart rate (HR) during first half of incubation, but HR had two frequency components in the mid incubation and finally a much lower frequency heart rate up until hatching. Early detection of potentially abnormal chicks with cardiovascular defects could significantly contribute to the humane treatment of these embryos and increase production efficiencies by identifying high quality embryos. Moreover, this method could be used in developmental physiology, and cardiovascular medicine in avian and reptile protocols in the future.

Keywords: Cardiac cycle; embryo arrhythmia; near-infrared; animal welfare

7.1 Introduction

7.1.1 Motivation

Cardiac arrhythmia (any abnormal rhythm of heart) is considered an important cause of cardiovascular disorder in vertebrates, which may cause various further consequences in adult hood. [35]. But the availability of non-invasive protocols for the study of cardiovascular system in avian models are still a great challenge to scientist and other stakeholders. Besides, chicks or chick embryos are considered as ideal laboratory animals for vertebrate studies. The hearts of birds are morphologically similar to mammals, containing a full atrial and ventricular septum [31]. Although ECG, a non-invasive technique, is widely used in humans, such a method cannot be applied to avian or reptile embryos due to the presence of the eggshell. Therefore, a non-invasive method together with signal processing is keenly sought for advancing cardiovascular studies of avian or reptilian embryos. Some researchers have recently used the Buddy System and Embryonic Vital Scope for heart rate (HR) measurement of chicken or reptile embryos [18], [30], [32], [33]. A limitation of the Buddy egg monitor is that its readings can be obstructed by embryonic activities; a very common occurrence in avian embryos [18], [30]. For example, heartbeat signals can be obstructed by body motility during signal acquisition. Thus, the Buddy Digital Egg Monitor can't isolate heart beats from the mixed signal. This device can be considered a first generation, non-invasive HR measurement system.

Arrhythmia, which has its origin in the heart, can be classified into sinoatrial (SA) arrhythmia, atrioventricular (AV) arrhythmia and ventricular arrhythmia [34]. Based on the heart rate, it is also classified as bradycardia or bradyarrhythmia when the heart rate is below the normal range and tachycardia or tachyarrhythmia when the heart rate is above the normal range. If the heart rate is much higher it is called flutter or cardiac fibrillation. When the heart rate is much lower, it is called severe bradycardia. Relatively little is known about these disorders in avian, chiefly because of their relative rarity and difficulty of diagnosis [35], [36]. Very few cardiac arrhythmias studies (of those, mostly for tachycardia) have been carried out to date for avian embryos and all of them were invasive (e.g. ECG). Moreover, in all cases the arrhythmias were drug induced or under stress conditions, which can't explain many naturally occurring incidences [37]–[40]. Therefore, it is imperative to develop a non-invasive diagnosis of chick embryo cardiac arrythmias at an earlier stage (during incubation) of their life (e.g. onset time, types, mechanism etc.) for a wide range of studies: precision poultry production system (e.g. embryo grading system); cardiovascular development; cardiovascular medicine, and for future references.

The potential importance of such a non-invasive methodology is heightened by the presumption that complications in post-hatch life have their origin during development in the incubation period. The reasons for the absence of such a non-invasive technique for studying of embryonic arrhythmias are numerous. Firstly, there are methodological difficulties inherent with avian embryos that are

shielded by the eggshell and the eggshell pigment. Perhaps, it makes it more challenge and difficulties to avian scientist and other stakeholders. Second is the relative rarity of such events during incubation. Thirdly, the complexity of the various embryonic movements (e.g. heart beats; body, wing and leg motility) incorporated into the frequency signal is another reason making it difficult to distinguish individual events.

7.1.2 Objectives

In these circumstances, the present research was designed to develop a novel non-invasive method to diagnosis cardiac arrhythmia, as well as investigate the incubation history (cardiac activity behavior) of chick embryo with naturally induced cardiac bradycardia using near infrared (NIR) light. In our previous study, we used EVS (870 nm) for heart beat measurements of chick embryos during incubation [33]. In this study, we updated and built a new device with finer signal resolution, a continuous input voltage control system, and, finally, reduced cost by using an oscilloscope. A different wavelength (810 nm) in the NIR region was used for this device, as well. This is because there are no differences in absorbance for oxyhemoglobin (HbO_2) and deoxyhemoglobin (Hb) blood at this wavelength.

7.2 Materials and Methods

7.2.1 Materials

This research was carried out in strict accordance with the animal experiment regulations of Kyoto University (Animal experiment approval number: 29-59). A total of 155 light brown eggs laid by a 45-week old parent flock (ROSS 308) from a commercial poultry hatchery (Yamamoto Co. Ltd., Kameoka, Kyoto) for incubation studies were selected. Prior to incubation, all eggs were stored for 5 days at 18.0 (± 0.5) °C and 80 (± 5) % relative humidity (RH). One incubator (SSH-02, Showa Furanki, Saitama) consisting of six egg trays was used for incubation of the fertile eggs (30x5+5=155 eggs) at 37.8 °C and 55% RH. Prior to setting the eggs into the incubator, eggs were preheated for 16 h (first 6 h at 28 °C and for the remaining 10 h at 30°C) to reduce thermal shock on blastoderm and to reduce the early embryo mortality. After day 18, the eggs were transferred to hatcher trays, where they were maintained at 37.8 °C and 60 % RH. The embryo cardiac signal of all incubated eggs was measured using the prototype near-infrared sensor from day 8 to 18 of incubation. Although [99] reported that the SA node (pacemaker cells) is established at day 6 of incubation in chick embryos, our measurement began from day 8, as the amplitude of the cardiac rhythm is small before this date due to incomplete septation of cardiac chambers (ventricle) and valve formation [100]. The sinoatrial node, a multicellular and bioelectric signaling center, generates impulses for the rhythmic contraction of the heart. It consists of a network of electrochemically oscillating pacemaker cells encased in a heterogeneous connective tissue microenvironment [99]. The experiment was conducted from 27 June to 27 July 2018, with incubation 4-24 July 2018.

7.2.2 Fabrication of Near Infrared Sensor

An NIR sensor was designed and fabricated to acquire vital signals from chick embryos in a vertical optical configuration. The device consists of light emitting diodes (LEDs) as light sources and a pre-amplifier photodiode (Model: S9269, Hamamatsu, Japan). The dominant emitting wavelength of the LEDs (Model: L810-33AU, epitex, Japan) is 810 nm (spectral range: 800-820 nm). The light received is converted into current by the photodiode that is further converted into a voltage signal by amplification using an integrated amplifier (op-amp) (Table 7-1). During measurement, the average output voltage was maintained at a relatively constant volts (V) for eggs for same day at vertical optical direction (Bottom to top) using a variable LEDs intensity controller (ranges was 1.7 -12.0 V). When the embryo inside the egg starts becoming larger in the latter half of incubation, the input current intensity needs to be increased to keep the output voltage similar. The amplified voltage signal was connected to an Oscilloscope (PC-interface type) through BNC cables. The Oscilloscope was then connected through a user interface to a PC with a USB connector. The user interface was developed based on the software development kit (SDK) in Microsoft excel using a Visual Basic for Application (VBA). The sampling rate per second of the signal was 1000 (sampling points per second). The high frequency signal is essential to observe precise shape of the mechanical work been taking by the heart during pumping. The mechanical work done by heart is the results of an electrical impulse generated by the heart in the SA node. The electric impulse is normally generate from SA Node and propagate from atrium to ventricle through the AV node, Bundle of His and finally Perkinje Fibers [34], [51], [52]. But the optical sensor works on the mechanical movement of the heart and the pulsatile blood flow that result from the electrical impulse. The oscillation due to embryonic movements and heart beats was 1-2 % of the average output voltage and centered around the mean value.

7.2.3 Signal Acquisition

From incubation day 8 to 18, each tray containing 30 eggs was taken out of the incubator every once for signal (8.2 s per egg) acquisition from bottom to top optical configuration. The ambient temperature was around 27 ^{o}C and the temperature of the sample eggs outside the incubation were carefully maintained properly using a warm electric blanket to avoid the influence of ambient temperature on activity signal of embryo (about 36-37 ^{o}C). To minimize the exposure time of the egg outside the incubator, eggs were immediately placed back into the incubator after the measurements. We put the sample egg in egg holder of the fixed Measurement Unit, thus ensuring no tilting of the egg. We also maintained dark conditions, because outside light can influence the signal.

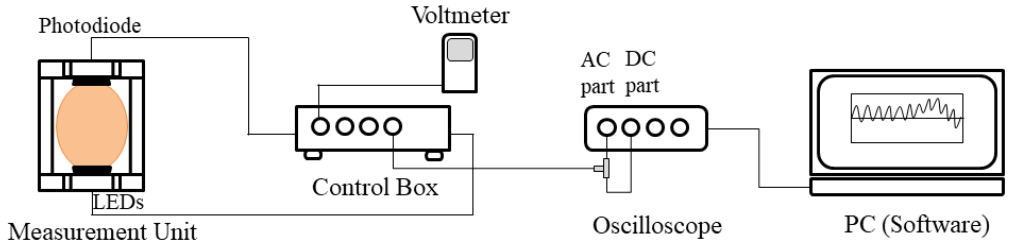


Figure 7-1 Schematic diagram of NIR sensor during signal acquisition in vertical optical configuration. The oscilloscope was connected to control box and PC with BNC cable and USB cable respectively.

7.2.4 Processing of Cardiac Activity Signal

The time domain embryonic activity signals obtained by the sensor were transformed into the frequency domain using a Fast Fourier Transform (FFT) of 8192 (i.e. $2^n = 2^{13}$) sampling points equivalent to 8.192 s for peak frequencies and power spectrum. Before doing FFT, all the signal was normalized in the time domain dividing by the output voltage (static part). In the FFT signal, the peak around 4-6 Hz represents the cardiac movement of the chick embryo whereas the low-frequency peak (<1.0 Hz) represents embryo body movement [88]. Body movement is outside the scope of this paper.

The area under the respective peaks represented by cardiac activity was also calculated as a measure of signal energy, which represents heart beat strength (HBS) using trapezoidal numerical integration after mean (high frequency part, > 7 Hz) detrending of the FFT signal (eq.(25)-(26)). De-trending is normally used for baseline removal of low magnitude fluctuations of the FFT signal. The physical meaning of the signal power is normally equated with the energy or strength of the signal, where the amplitude for a particular frequency contributes a vital part. The area under the Fourier transformed signal curve is proportional to the power of the signal, which can be calculated by summation or integration [101]. Prior calculation of amplitude calculation necessary to calculate cardiac output. This is because cardiac output depends on both heart rate and amplitude of cardiac contraction (i.e. systole and diastole).

$$\text{Area under peak, } A \approx \int_a^b (f(x) - m)dx$$
$$\approx \frac{b-a}{2N}\sum_{n=1}^{N}(f(x_n) + f(x_{n+1}) - 2m) \qquad \text{[eq.23]}$$

Where, $y=f(x)$, a is frequency value (x) at (n-1) sampling position (index) and n is sampling index at peak position, b is frequency value at n+1 sampling position, N=2 as three consecutive points is considered and spacing is (b-a)/N.

$$m = \frac{1}{4036} \sum_{n=59}^{4096} y(n) \qquad \text{[eq.24]}$$

Where, $y(n)$ is magnitude (value of y-axis) of FFT signal of real part. The average magnitude represents (m) the mean of the high frequency part (> 7.0 Hz).

All the signals were processed using MATLAB R2016a software. Since the sampling frequency was finer, the time domain signal was filtered digitally by a classical low pass Butterworth filter with a cut off frequency of 25.0 Hz to visualize the signal pattern.

7.2.5 Hatching time calculation

The hatching process for all embryos were recorded by an automatic image capturing algorithm. From incubation day 19, eggs in the hatcher trays were monitored by two web cameras to record the time of hatching for each egg until the hatching process was ended. During the monitoring period, images were captured at 5 min intervals and saved automatically on a hard disk of the personal computer. The time of capture was also recorded simultaneously. Later, the period of incubation required for each egg to hatch was calculated as the duration from the beginning of incubation to the time of chick emergence [47]. Post-hatch measurement of chicks, such as shank length and chick mass for calculation of conversion ratio (chick mass/egg mass) also measured using a digital slide caliper and electronic balance.

7.3 Results and discussion

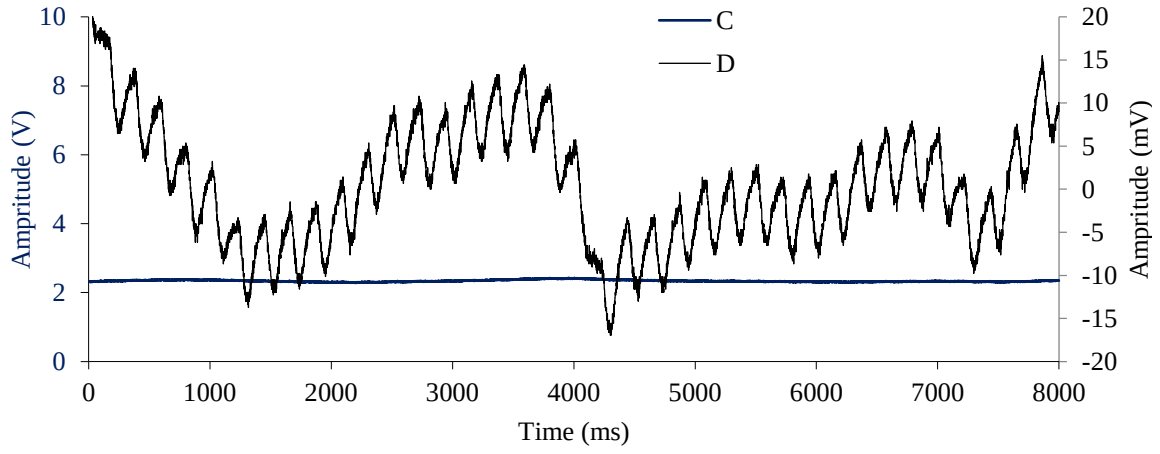
The purpose of this research was to develop a novel non-invasive technique to measure and compare abnormal cardiac rhythms with normal cardiac rhythms of chick embryos based on the mechanical work done by the embryonic heart. And to provide further explanation of the cardiac cycle using the NIR sensor together with signal processing. The sensor was successfully developed and used to capture the cardiac rhythm of embryos during the incubation period. This diagnosis of cardiovascular defects and the explanation of cardiac signals have at least several potentially important functions: the study of cardiovascular development in avian embryos with and without arrhythmias; application in cardiovascular medicine (e.g. recovery of normal rhythm, drug formulations), better understanding of naturally induced cardiac bradycardia at embryonic stages; and in precision poultry production systems by diagnosis of the embryos with cardiovascular disorder. Embryonic bradycardia is a rare event and we are lucky that we were fortunate to observe one ideal embryo out of the 155 incubated eggs with naturally occurred bradycardia during incubation. This device could be equally applied in all avian and reptile protocol.

Some of the limitations of the Buddy egg monitor overcome by this device are:

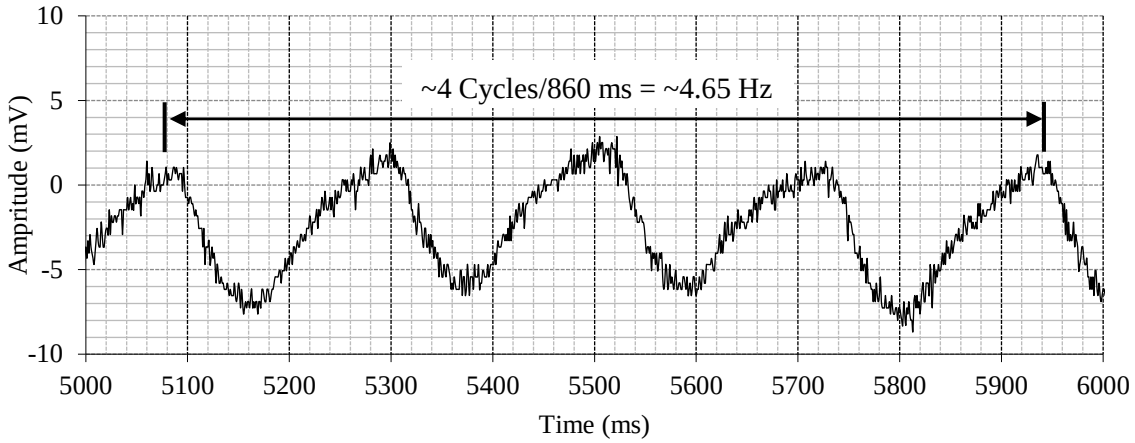
1. Buddy system provides only frequency information (heart rate only). We can't find the actual shape of the signal.
2. Unable to use or separate heartbeat signals from obstructing body movements.
3. Can be applied only during the second half of incubation for heart rate measurement.
4. Includes any motility activity in readings with more than 50 beats per min. Therefore, body motility can be mistakenly included in heart rate measurements. The heart rate of embryos with severe cardiac bradycardia (when HR is less than 50 beats per min) cannot be measured. Thus, the Buddy system can be considered a first generation, non-invasive HR measurement system.

7.3.1 Physical Meaning of Cardiac Optical Signal

The intensity of transmitted light is largely affected by both blood volumes released into the system and mechanical activity of the heart pumping blood (i.e. systole and diastole) (Figure 7-2). This changing pattern in transmitted light intensity due to pulsatile motility of the heart gives a small oscillation in the signal representing heart beats [88]. In contrast, the ECG signal is based on electrical impulse of the heart whereas the optical sensor is based on mechanical work that results from electrical stimulation. The electric impulse is normally generated from the SA node and propagated from atrium to the ventricle through the AV node, bundle of His and finally Purkinje fibers. But the developed optical sensor works on the mechanical movement of heart and pulsatile blood flow the results from an electrical impulse. As a consequence, the peak (representing a cardiac cycle event) due to an electrical impulse should appear earlier than the peak in the optical signal of the same event.



(a) Time domain signal of embryonic activity of normal embryo consists of oscillating part (B) and static part (C). The oscillating part contains two types of information: body motility (low frequency part: bigger movement) and heart beats (high frequency part: saw tooth like small oscillation). The static part (C) is the mean average output voltage, representing a transmission parameter of the sample egg.

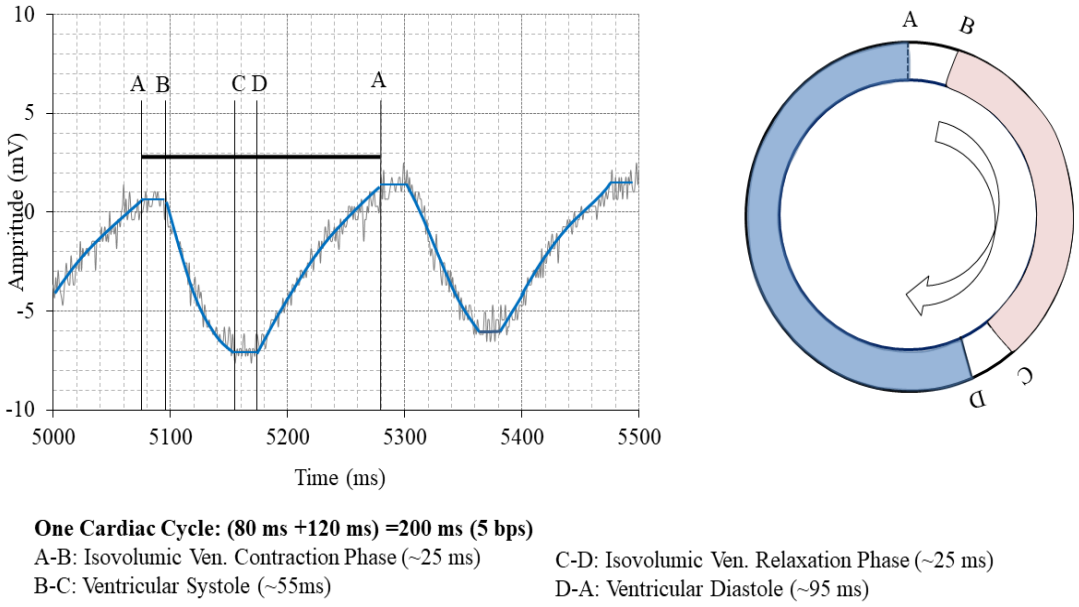


(b) A part of the signal explaining pattern of heart beats and cardiac cycle time.

Figure 7-2 The time domain signal of the cardiac cycle of a normal chick embryo. The mean average output voltage was deducted from the signal; hence the signal baseline is zero.

The ventricular depolarization generally takes a shorter time than ventricular repolarization [51], [102]. The atrium motility cycle didn't appear in the signal due to ventricular dominancy. The activation of the sinus venosus in birds coincides with activation of the atria [103]. Ventricular activation is almost simultaneous and local repolarization is predominantly determined by local action potential duration in chicken [102]. The high transmission point represents the end of ventricular filling isovolumetric point, whereas the lower transmission point represents the end of the ventricular contraction (Figure 7-3). The absorbance is higher at ventricular contraction end isovolumetric point due to light absorbed by the ejected blood at end of the systole.

Electrocardiogram studies of chick embryos conducted by Bogue in the early twentieth century, where the ECG signal was taken after opening the eggshell from day 2 to 5, examined the very early stages of incubation [104]. He reported slow and rapid components in the ECG signal after day 3. He found the P-R and R-S-T intervals for day 5 embryo were 90-100 and 210-240 ms, respectively. Thus, the total time for a cardiac cycle was 300 to 330 ms (200 to 182 bpm). In our study, we made measurements from incubation day 8 to 18. We found the total time for a cardiac cycle for a normal chick embryo was about 200 (80+120) ms (300 bpm) after day 8 (Figure 7-3). This is because the heart rate increases gradually during first half of incaution.



One Cardiac Cycle: (80 ms +120 ms) =200 ms (5 bps)

A-B: Isovolumic Ven. Contraction Phase (~25 ms)
B-C: Ventricular Systole (~55ms)
C-D: Isovolumic Ven. Relaxation Phase (~25 ms)
D-A: Ventricular Diastole (~95 ms)

Figure 7-3 Cardiac cycle signal pattern of normal chick embryo. The larger part (C-A) is for cardiac diastole (~120 ms) and smaller (A-C) is for systole (~80 ms). The right diagram represented the ventricular (outer cycle) contraction and relaxation.

The signal resolution was 500 Hz (sampling rate 1 millisecond per sampling point); a much finer resolution than that of the heart rate (4-7 Hz) of the chick embryos. A coarser frequency resolution would not be able to examine the various phases (events) of cardiac cycle. For example, in a previous study [88], we only used 16.5 Hz (Nyquist Frequency) and were not able to observe the shape of heart beats.

The working principle of ECG is totally different from the current prototype device (light sensor). The ECG technique is based on an electrical impulse generated from differences in cation and anion concentrations (positive charged and negative charged ion) inside and outside of the node. The light sensor is not influenced by such an impulse. Light is only influenced by absorbance and motility. The sequence of phases in the cardiac cycle are well established in the literature [34], [51], [52]. Hence, the detection of systole and diastole phases in the optical signal can provide clear information about other phases of the cardiac cycle, if the signal has a high resolution (e.g. 1-2 milli second sampling). Therefore, the heartbeat events captured by the light sensor can be observed simply based on the motility and absorbances due to pulsatile blood flow. Absorbance is higher (point C or D) just after ventricular contraction because of ejected blood absorbance. In contrast, absorbance is lower because of less exposed of blood during ventricular filing, which is not pulsatile blood.

7.3.2 Cardiac signal pattern of embryo with bradycardia

Cardiac arrhythmia can be defined as any abnormal rhythm in frequency and/or pattern of heart beats. There are many types of arrhythmia based on the origin in the heart such as sinus arrhythmia, AV bock, ventricular arrythmia [34]. The incident of arrhythmias in chicks is probably much higher during post-hatch growth stages than in the incubation stage. We assumed that this is because (among other factors): (1) the post-hatch growth period is much longer than the incubation period; (2) a more diversified environment (more variables are involved), hence less control of environmental variables during post-hatch stages; (3) better control of environmental factors during incubation; and (4) feeding influences post-hatch physiology of hatchling.

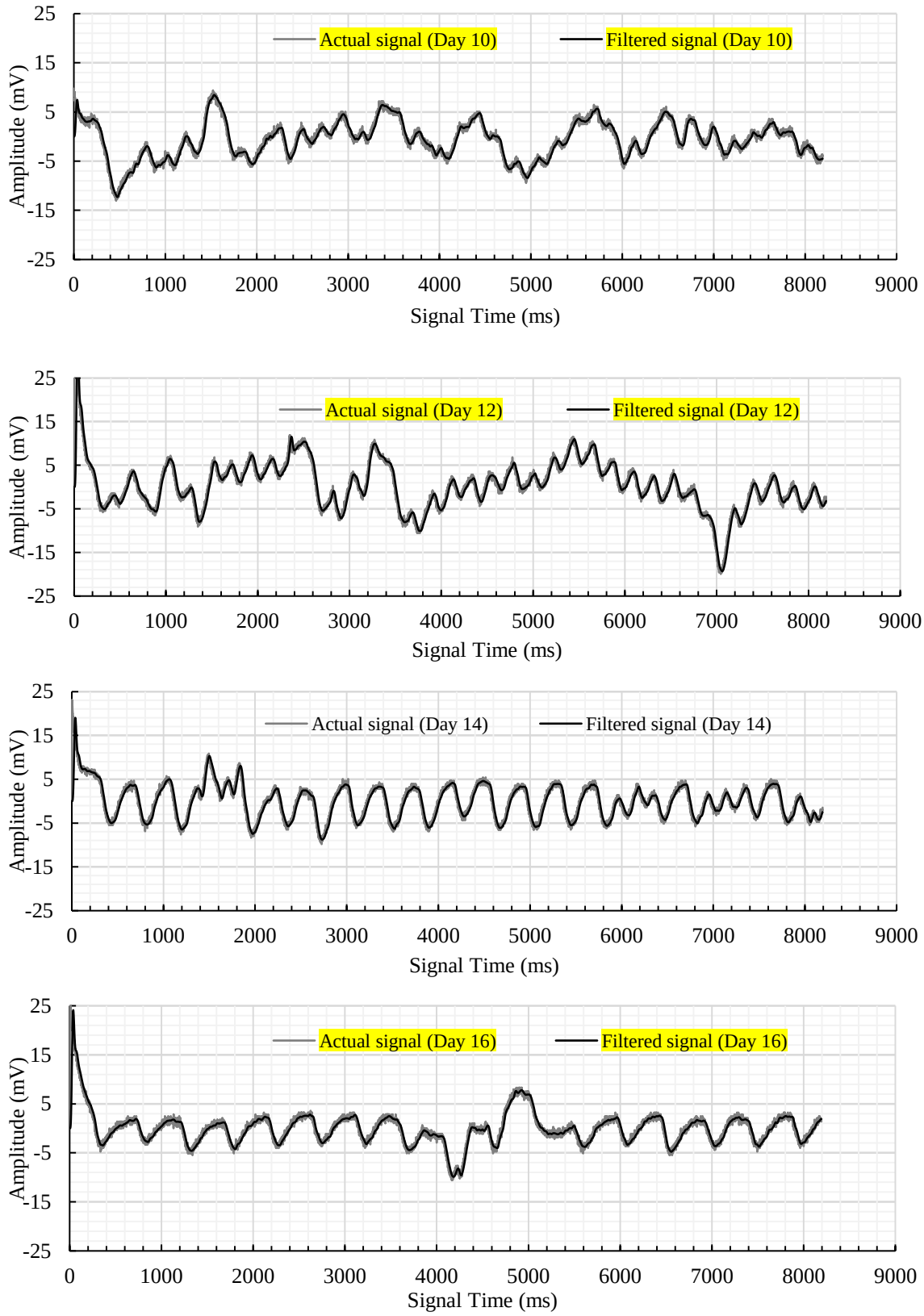


Figure 7-4 Time domain signal of a chick embryo with bradycardia at incubation days 10, 12, 14 and 16. The dark black line represent the activity signal after digital filtering with cut off frequency 25.0 Hz.

Although the heart rate of avian embryos during incubation changes over time and is influenced by incubation conditions, the normal heart rate of chick embryos is 200-320 bpm from day 8 to day 18 [17], [18], [33], [85]. Based on the heart rate, it can be classified as bradycardia or bradyarrhythmia when the heart rate is under the normal range (<<200 bpm) whereas it is called tachyarrhythmia or tachycardia when the heart rate is more than the normal range (>> 320). Heart rate depends on activity of the autonomic nervous system. Vagus and sympathetic nerves control the heart rates by slowing and accelerating the rates, respectively [75].

In this case, chick embryo with bradycardia had lower heart rates from day 14-18 (Figure 7-5 *and* Figure 7-6). The arrhythmia initiated, induced and propagated after first half incubation. The heart rate and cardiac strength provide evidence that cardiac arrhythmia was not a sudden event rather gradually induced over time. In such a case, it is perhaps good news that the time delay might be useful to apply any cardiovascular therapy to recover the normal rhythm in the future. Although the heart rate is much lower than normal range, the higher strength kept the total cardiac output similar with normal embryo (Figure 7-6). For this reason, the embryo with bradycardia was hatched in middle of the hatch window (hatching time was 489.5 h). The frequency and amplitude (vital part of signal strength) differences were also visible in time domain signal (Figure 7-4 *and* Figure 7-5).

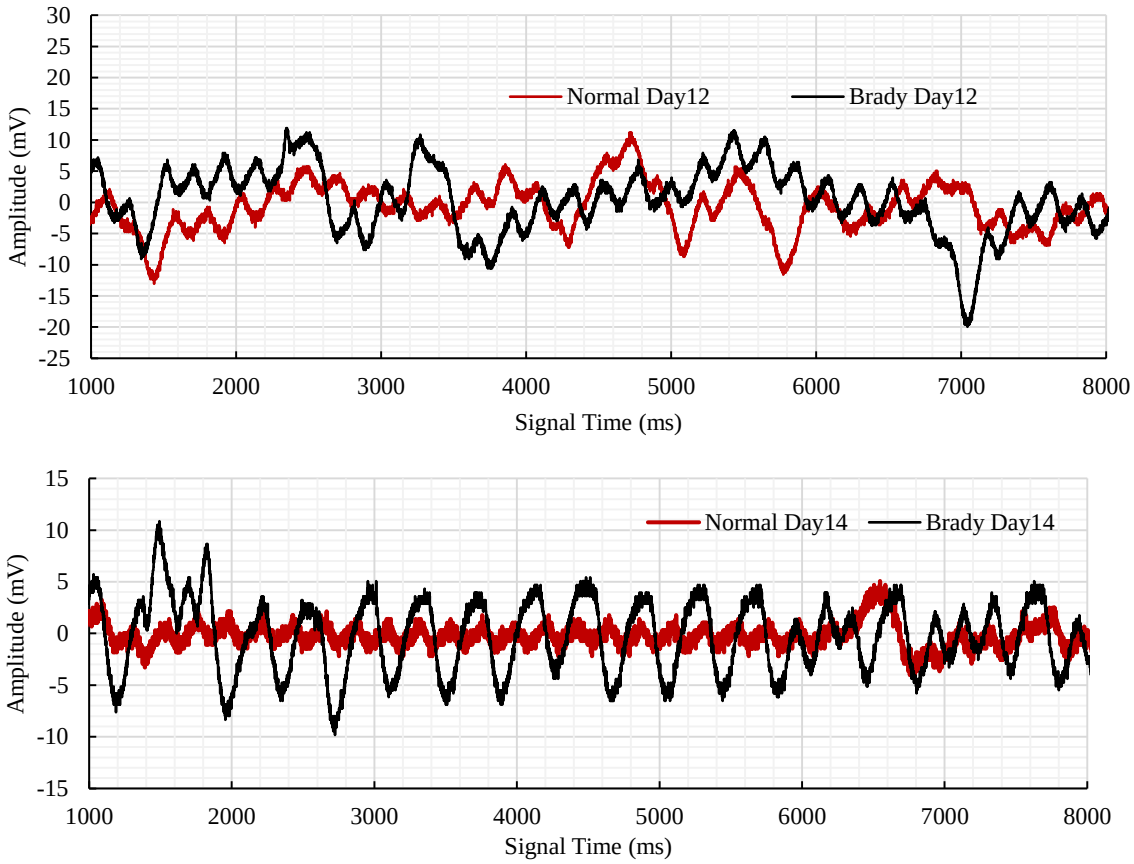
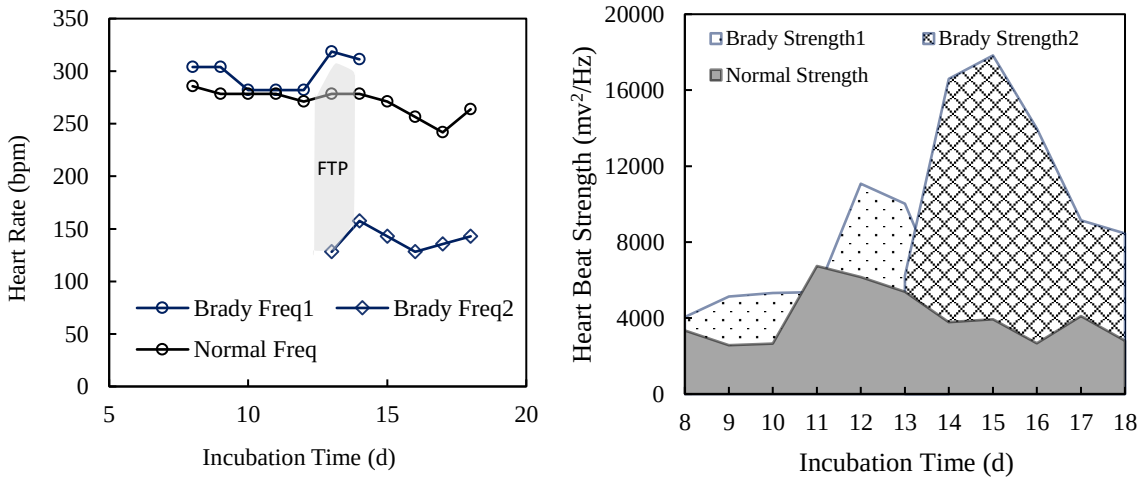


Figure 7-5 Time domain signal of a normal chick embryo and embryo with bradycardia at incubation days 12 and 14. The difference is clearly visible in the time domain signal at day 14. In this case, the bradycardia appears after day 12 of incubation.



(a) Heart rate (HR) pattern during incubation (b) Heartbeat strength (HBS) pattern

Figure 7-6 Comparative HR and HBS of normal embryo and embryo with bradycardia from day 8-18. The chick with bradycardia showed two frequencies. FTP means frequency transition period.

In humans, the normal range of heart rate for an adult is 60-100 bpm and 80-130 bpm for a child. Whereas, the normal fetal heart rate usually varies between 120 and 160 bpm throughout pregnancy [35]. In case of the observed chick embryo, the normal heart rate was 200-320 bpm after day 8 of incubation. The birds require much higher heart rates to maintain high metabolic rates associate with homeothermic endothermy , the ability to maintain body temperature above that of the environment [51]. Heart beats of the endothermic mammals and birds are faster than heart beats of similarly sized ectothermic vertebrates even at similar body temperatures. Higher heart rate permits larger cardiac output in mammals and birds to sustain the metabolic demands related to endothermy [105]. The time of ventricular contraction and relaxation in human is 310 to 490 ms respectively, when the heart beat is around 75 bpm [106]. We found the ventricular contraction and relaxation time in normal chick embryos was around 80 to 120 ms (at 300 bpm), respectively. The AV node in endotherms connects to the fast conducting His bundle and Purkinje system allowing rapid propagation of the activation front toward the ventricular myocardium [51]. But for the chick embryo with bradycardia, the ventricular contraction and relaxation time was much longer, ~140 to ~320 ms (at ~130 bpm), respectively. Time differences were greater in ventricular relaxation, which might indicate a problem in the SA node or AV node. Perhaps, the SA node wasn't generating a regular impulse; resulting in two frequency components in the signal. On the other hand, if not all electrical signals had reached the ventricles, even though the SA node had generated a regular impulse (i.e. some beats were dropped), this could result in a slower and sometimes irregular rhythm. If one beat drops, it is called a second-degree heart block. To ascertain the root cause of the defect in SA node activity we observed the chick after hatching and found symptoms of hypothyroidism. An excess or deficit of thyroid hormones has been established to affect the cardiovascular system in hypothyroidism leading to bradycardia. Profound hypothyroidism and decreased expression of active hormone triiodothyronine (T_3) in the heart cell can result in worsening of cardiac contraction ability and a decrease in heart rate, as well as slowing of the conduction of electrical stimuli in the heart muscle [107]. Thyroxine (T4) is the primary secretory product of the thyroid which is inactive and is converted to the active compound T_3.

In comparison to the ECG signal, which is based on the electrical impulse of the heart, the optical sensor works on mechanical movement of the heart and the pulsatile blood flow that results from the electrical impulse. The source of electrical impulse is derived from differences in cation and anion concentrations (positive changed and negative charged ion) inside and outside of the node. Although ECG contains clearer information regarding various events in the cardiac cycle, such principles are not at work in avian and reptile samples due to the eggshell. Therefore, an alternative method is essential for the study of avian or reptilian embryos. Moreover, the study of chick embryo is also important as it considered as ideal laboratory animal for the study of vertebrates.

7.3.3 Actual values and practical challenges of the system

Although this research has set the stage for development of a non-destructive protocol in precision livestock and biomedical engineering research, addressing the following actual values and practical challenges in future research would help to advance this research area.

- Non-invasive methods;
- Alternative to EGC, can be used for all avian and reptile protocol;
- Reduced costs: cost of LEDs and Oscilloscope is low compare to current instrumentation;
- Availability of parts: Oscilloscope and LEDs and Photodiode are readly available;
- This device can be used for multiple objectives: Embryo mortality, normal and abnormal chick embryos can be detected quickly;
- Quick detection (several seconds only) method. Large number of eggs can be used in a short time if a multiple slot setup is used;
- Although skilled personnel are required for building the device, non-technical personnel can operate the device;
- Chick embryonic cardiac abnormalities, other than bradycardia, need to be investigated from the signal in the future. Although the bradycardia can clearly be identified, how the signal changes with other cardiac abnormalities is yet to be investigated.
- Single slot measurement used in current experiment, takes a relatively long time per sample;
- High frequency electrical noise was added to the signal, hence the use of a filter circuit may improve signal to noise ratio;

7.3.4 Properties of incubated eggs and chicks

Basic data prior to incubation for all eggs used (e.g. egg mass, shape: major and minor diameter, shell color image) and after hatching (e.g. conversion ratio, shank length, time of hatch, gender) were compared to detect any biased or differences in chick embryos without (normal rhythm) and with arrhythmias (Figure 7-7).

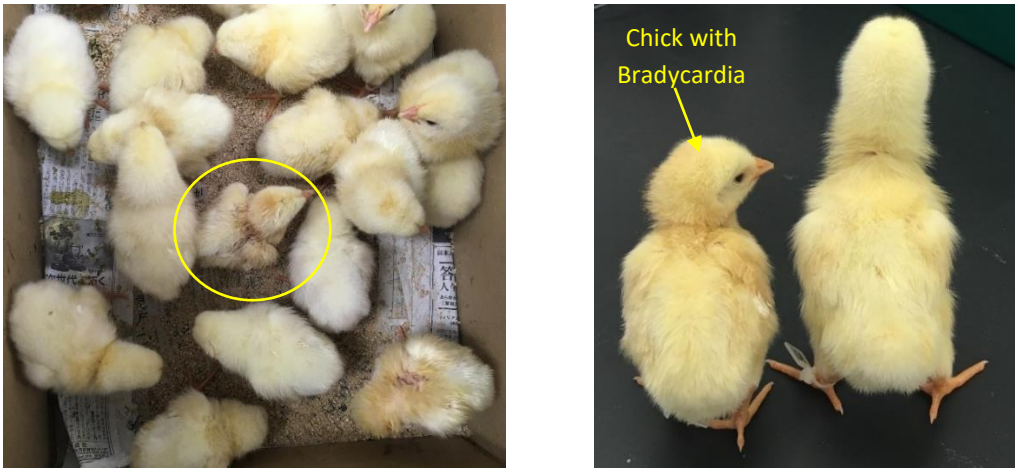
Table 7-1 Pre-incubation and post-hatch information of normal embryos and embryo with severe bradycardia

Parameter	Egg Mass, g	Shape Index	Shell Color	Conversion Ratio	Shank Length, mm	Hatching Time, h
Bradycardia	65.28	0.75	0.380	0.713	27.33	489.5
Normal Embryo	65.86	0.75	0.363	0.720	29.08	488.5
Normal Embryos (Average ± SD)	63.88±3.45	0.77±0.025	0.37±0.11	0.715±0.09	28.68±1.7	485.68±7.3

SD: Standard deviation.

The preincubation parameters and post-hatch evaluation parameters for the chick with bradycardia was not distinguishable different from the normal chicks; they were within the range observed,

except for a lower conversion ratio and shank length, an indicator of retarded growth (Table 7-1). The chick was hatched around the middle of the total hatch window. Although the heart rate of the chick with bradycardia was much lower during the incubation period, the total cardiac output was similar since the stroke volume (e.g. HBS) was higher than for normal embryos. Because cardiac output has relation with hatch window and higher cardiac output makes the incubation period shorter.



(a) Chick with bradycardia (yellow circled) (b) Chick with bradycardia and normal chick

Figure 7-7 Actual pictures of chicks (day-old): chick with bradycardia (circled) and normal chicks. Chick with bradycardia was relatively weak, small and other differences in appearance.

7.4 Conclusions

Naturally induced cardiac arrhythmias are a rare event in the embryonic stage of high-quality eggs from a healthy parent flock. However, we were fortunate enough to be able to observe such an event; an embryo with severe bradycardia. The heart rate and cardiac strength patterns observed indicate that cardiac arrhythmia is not always a sudden event, but rather is an event that may gradually propagated over time. In this case of bradycardia, the ventricular filling took a much longer time than usual ventricular contraction time. Since very little is known about arrhythmias in avian embryo, this research provides a rare insight into such an event (e.g. incubation behavior and mechanism) and could be a steppingstone towards non-invasive diagnosis of cardiovascular disorders in all avian and reptilian protocol. From a commercial production perspective, early detection of chick embryo cardiovascular problems a potentially valuable tool use in precision poultry production systems and humane treatment of embryos or chicks (animal welfare). Moreover, this could help to reduce costs by reducing the rearing time of unwanted embryos (space, energy and labor costs), as well as minimizing post-hatch handling for sorting of unwanted embryos. This research could also open many new dimensions of non-destructive research in precision livestock and biomedical engineering fields such as embryo grading system, indirect blood pressure measurement in avian and reptile models; cardiovascular drug design and in many other areas.

Chapter 8 Conclusions and Future Perspectives

8.1 Introduction

The purpose of this research work was to explore the potential of a near infrared sensor prototype for non-invasive characterization and monitoring of chick embryonic active body movements, passive cardiac movements, as well as chick embryonic growth in relation to hatch-window, gender and arrhythmias detection for sustainable and precision poultry production systems; specifically, for delivery of uniform and high quality day old chicks and to minimize production cost due to energy losses, labour costs and management complexity. The advantages of this approach of using a single wavelength NIR sensor over non-invasive methods that have been developed in the last decade for monitoring of chick embryo heartbeat are less complexity in data processing, higher transmission compared to visible wavelengths, independent of eggshell colour, high sensitivity and lower noise. This approach also overcomes other limitations of previous research of heartbeat measurement, it is not affected by embryo movement, and can be used in the second half of incubation, making this method more robust. Besides, the methods developed for late-hatch chick embryos, gender prediction and cardiac arrhythmia detection of chick embryos before hatching were non-invasive and reported for the first time in avian and reptile protocol.

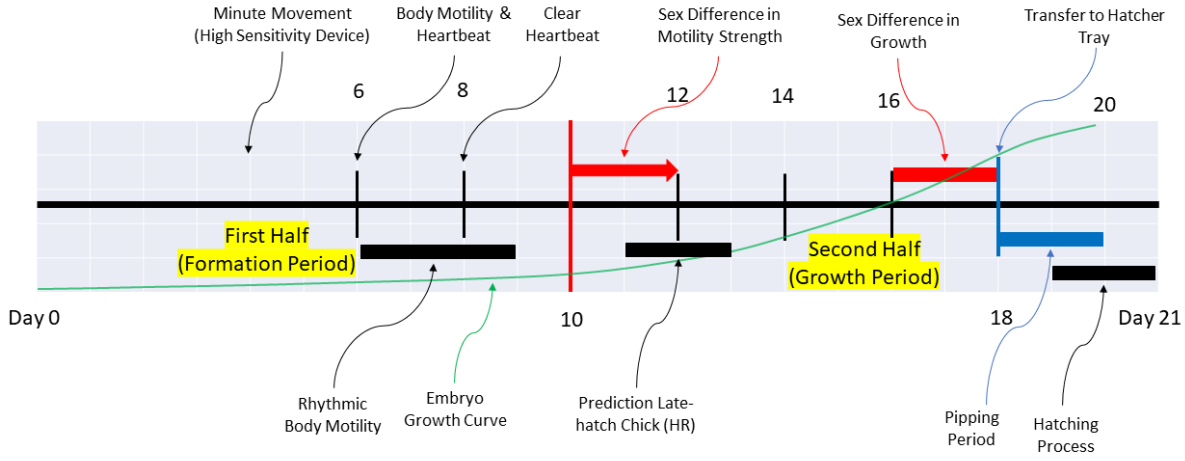


Figure 8-1 Schematic diagram explaining overview of NIR device potentiality to study incubation insight of broiler eggs.

8.2 Main Contributions

1. Chick embryo body and cardiac movements (e.g. frequency and strength) can be non-invasively characterized during incubation
   - This information is useful for detection and disposal of dead embryos, thus minimizing the chances of pathogen contamination during incubation; reducing energy and labour

costs; maximizing incubator space utilization; and for developing better management systems.

- This can also be used as important criteria for achieving uniform embryos cohorts during incubation with respect to body activity strength and cardiovascular performance (e.g. heart rate and heartbeat strength patterns).
- This signal characterization can be used for identification of weak and abnormal embryos with respect to cardiovascular performance and embryo activeness.

2. Cardiac signal behaviour of early and late hatch chick embryos during incubation
   - This can be used as important criteria of hatch-window based embryos sorted with respect to heart rate and heartbeat strength patterns.
   - This signal characterization can be applied for identification of weak embryos with an overall accuracy of 93% (e.g. late-hatched embryos) regarding cardiovascular performance. This is also important with respect to animal welfare issues.
3. Non-invasive detection of chick embryo gender based on body motility
   - Physiological differences were found in chick embryo body motility strength for male and female embryos.
   - This can help to solve the big ethical problem of culling the less preferable gender chick after hatching.
   - This technique is useful to separate male and female embryos (accuracy 84.0%) for various purposes (e.g. separate rearing).
4. Non-invasive broiler chick embryo sexing based on opacity value of incubated eggs
   - Gender specific growth differences were found in chick embryos during the second half of incubation.
   - This can help to solve the big ethical problem of culling the less preferable gender chick after hatching.
   - This technique is useful to separate male and female embryos with an accuracy of 84.0%.
5. A non-invasive diagnosis technique of chick embryonic arrhythmia
   - A technique was developed to diagnosis chick embryonic arrhythmia based on heartbeat signal patterns in the frequency domain. This is an alternative way to using ECG. ECG is destructive for avian and reptile protocols.
   - This can also be used as an important criterion for precise sorting of embryos during the incubation period based on cardiovascular performances (e.g. eliminating chick embryos with cardiac disease).
   - This novel technique could also be applied to cardiovascular drug design research on avian and reptile embryos. This is also important with respect to animal welfare issues.

8.3 Limitations of the Work

1. Some physiological phenomena, such as sleeping (e.g. inactive session) by the embryo, is not considered during signal acquisition. Sleeping may affect the degree of chick embryo body motility strength and hence data variability.
2. Lighting effects on chick embryo movements were not considered in this experiment, although light may not stimulate the embryo before day 18, as reported by a few researchers (Table 1-1 in section 1.3.1).
3. Data was taken at 24 h intervals for all incubated eggs. Therefore, twelve-hour intervals for data acquisition could give clearer indication of embryonic behaviour during incubation.
4. Although an adequate number of samples was used, increased repeatability may increase the accuracy of the results.
5. The eggs used in the experiments were graded based on only size, mass and shell colour; other grading criteria, including internal contents, may help to improve the research sensitivity.
6. The NIR system has less potentiality to study hatching eggs during last few days of incubation. Hence, another technique (e.g. different wavelength) could be used in the future for wider application.
7. For all measurements using the NIR sensor the eggs were taken out of incubator. The sensor could be designed to be put inside the incubator for better results in the future. Thus, big data of time series could be accumulated in cloud for real time analysis and decision display at incubator monitor.

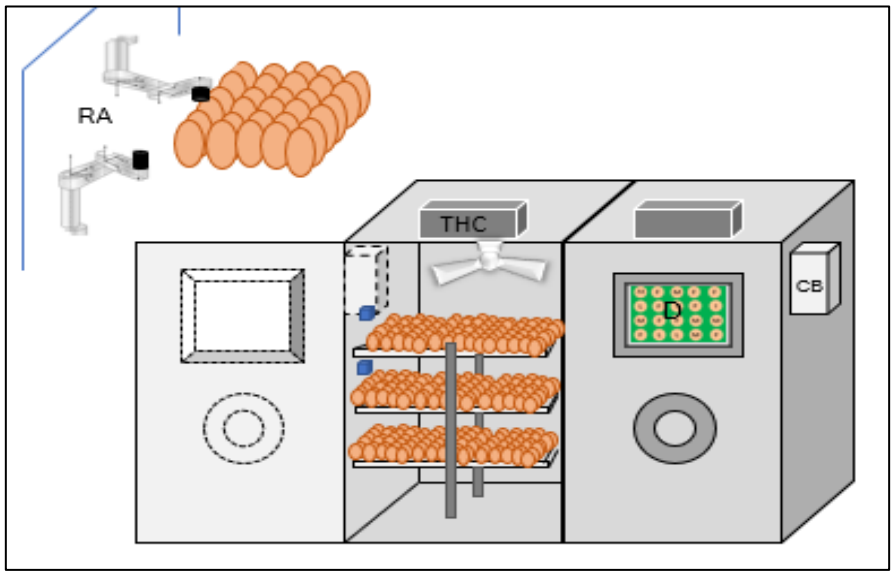


Figure 8-2 Schematic diagram of future commercial incubator. CB: Control Box, THC: Temp. & Humidity Control, D: Display. RA: Robotic arm with 2 degrees (Up-down and Yawing) freedom containing LED at bottom and Photodiode at upper side.

8.4 Future Studies

This dissertation introduces an improved approach and new techniques for monitoring of chick embryo movements, both whole body and cardiac movements, and novel identification of late-hatch chick, gender differences based on body activity strength and growth (opacity) as well as detecting cardiac arrhythmias using a single wavelength near infrared sensor combined with signal processing. The research findings have also proved clues for further research areas that could be explored. The major directions for future research work in this context are listed below, which some researchers may be interested in pursuing.

1. Embryonic behavior, such as body motility, heartbeat, opacity (growth) patterns of broiler breeder (ROSS 308) were investigated. But how do these features change for other bird and reptile protocols? They have yet to be investigated.
2. The features (e.g. growth and activity signal) introduced in this dissertation could also be used for non-destructive gender prediction of embryo of other species protected by an eggshell.
3. A hatch-window based classification model of chick embryos was developed in this dissertation. A regression model could also be developed for precise estimation of hatching time.
4. As most of the inborn characters of animals are determined and fixed during the embryonic stage, these parameters could be used to open new domains of research for more precise prediction of post-hatch chick performance and grading of chicks. This is because small changes at embryonic growth stages could cause big changes in the post-natal period.

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Chapter 10 Appendices

Appendix A: LED of NIR sensor (Prototype I)

General Specifications

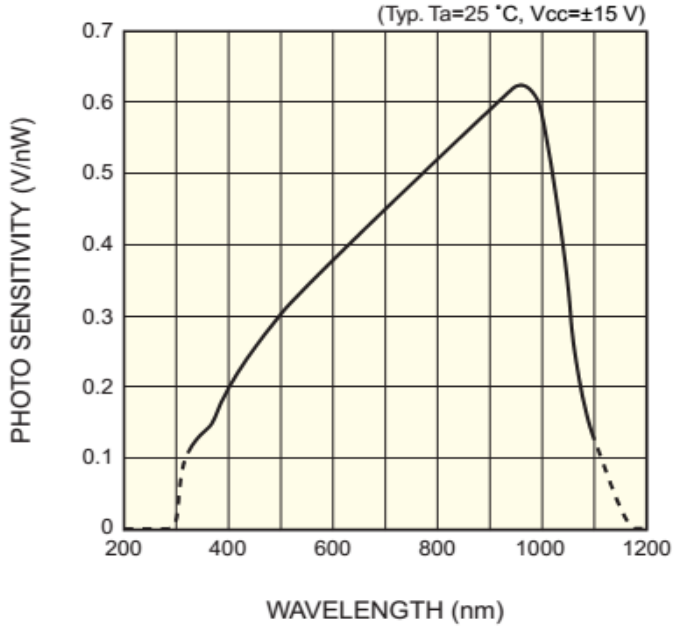
Product name	: Infrared LED Lamp
Type no.	: L870-06UP
Producer	: Epitex Inc.
Chip material	: AlGaAs
Peak wavelength	: 870 nm
Package type	: 5mm clear moulding
Resin material	: Epoxy Resin
Lead frame	: Soldered
Forward current	: 100 mA
Operating temperature	: -30 to + 85 ^{o}C

Appendix B: Photodiode of NIR sensor

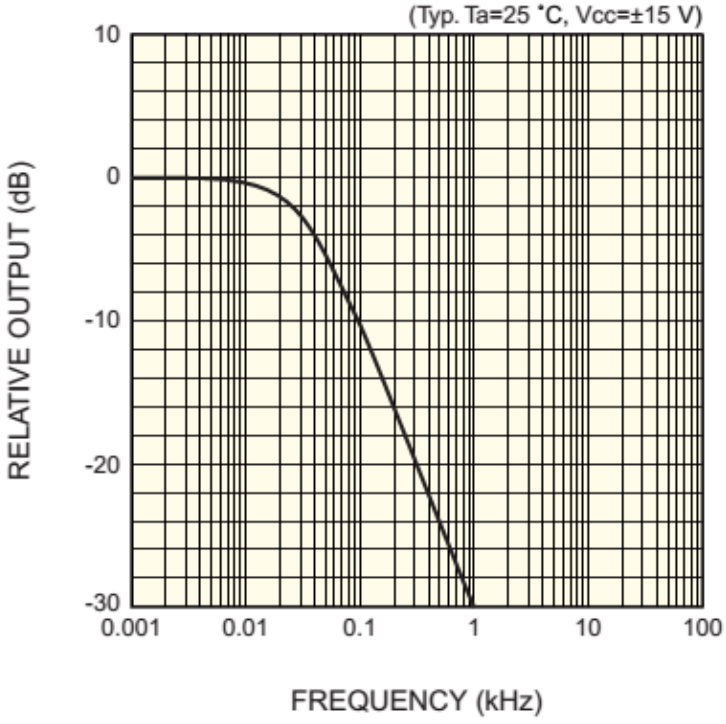
General Specifications

Product name	: Si Photodiode
Producer	: Hamamatsu Photonics K.K.
Type no.	: S9270
Active area	: 10 x 10 mm
Spectral response range	: 320 to 1100 nm
Peak sensitivity wavelength	: 960 nm
Resin material	: Epoxy Resin
Lead frame	: Soldered
Forward current	: 100 mA
Operating temperature	: -20 to + 60 ^{o}C
Rise time	: 15 ns
Fall time	: 10 ns

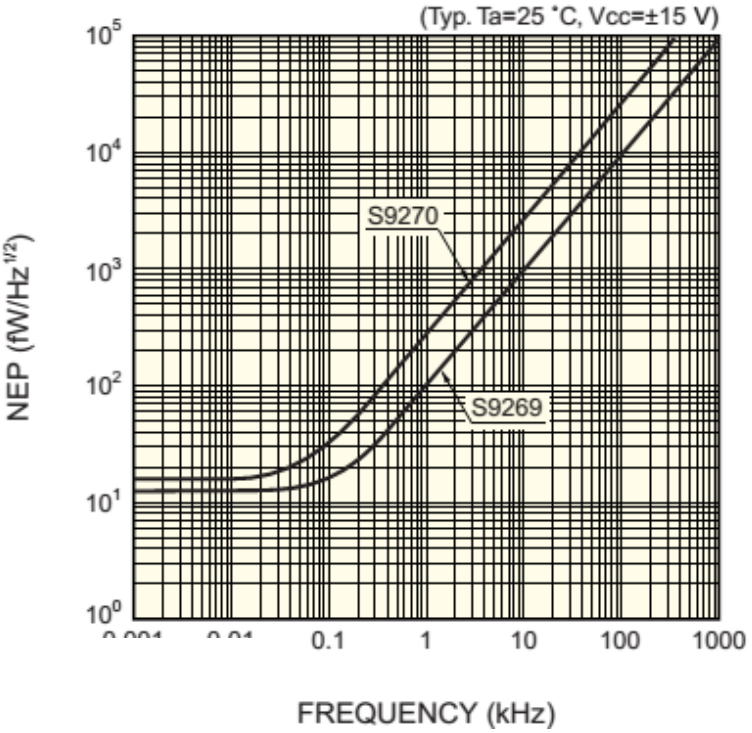
■ Spectral response



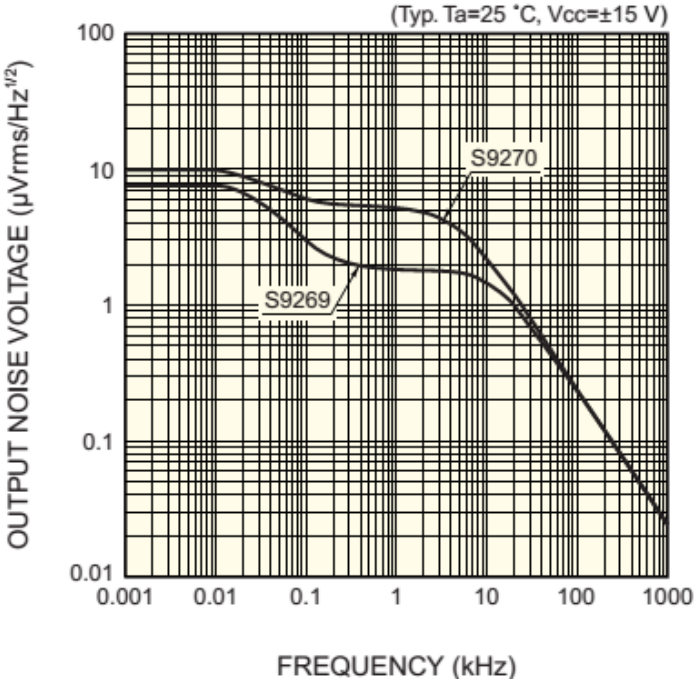
■ Frequency response



■ NEP vs. frequency



■ Output noise voltage vs. frequency



Appendix C: List of Papers and Conferences Presentations

Original Paper:

1. **Khaliduzzaman**, Fujitani, S., Kondo, N., Ogawa, Y., Fujiura, T., Suzuki, T., Kashimori, A., Syduzzaman, M., Rahman, A. Non-invasive characterization of chick embryo body and cardiac movements using near infrared light. Eng. Agric. Environ. Food. 12 (1), 32–39. July 2018. https://doi.org/10.1016/j.eaef.2018.09.002

2. **Khaliduzzaman**, Fujitani, S., Kondo, N., Syduzzaman, M., Rahman, A., Suzuki, T., Ogawa, Y., Kashimori, A., Fujiura, T. Cardiac signal behavior of early and late hatch chick embryos during incubation. Comput. Electron. Agric. 148, 188–196. May 2018. https://doi.org/10.1016/j.compag.2018.03.020

3. **Khaliduzzaman**, Fujitani, S., Kondo, N., Suzuki, T., Ogawa, Y., Kashimori, A. Non-invasive broiler chick embryo sexing based on opacity value of incubated eggs. Comput. Electron. Agric. 158, 30–35. March 2019. https://doi.org/10.1016/j.compag.2019.01.029

4. **Khaliduzzaman**, Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. A non-invasive diagnosis technique of chick embryonic arrhythmia using near infrared light. Comput. Electron. Agric. 158, 326–334. March 2019. https://doi.org/10.1016/j.compag.2019.02.014

5. Syduzzaman, M., Rahman, A., **Khaliduzzaman**, Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. Noninvasive quantification of yolk content using Vis-NIR spectroscopy and its effect on hatching time and gender of broiler chicken. Eng. Agric. Environ. Food. February 2019. https://doi.org/10.1016/j.eaef.2019.02.006

6. Syduzzaman, M., Rahman, A., **Khaliduzzaman**, Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. Noninvasive quantification of yolk content using Vis-NIR spectroscopy and multivariate regression. Journal of the Japanese Society of Agricultural Machinery and Food Engineers. 81(3), 168-176. May 2019.

7. **Khaliduzzaman**, Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. Non-invasive detection of chick embryo gender based on body motility and a near infrared sensor. Eng. Agric. Environ. Food. 2019. [Decision Pending]

8. **Khaliduzzaman**, Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. A novel chick embryo growth model using non-linear least square fitting of egg opacity values during incubation. Eng. Agric. Environ. Food. 2019. [Under review]

9. Syduzzaman, M., **Khaliduzzaman**, Rahman, A., Fujitani, S., Kashimori, A., Suzuki, T., Ogawa, Y., Kondo, N. Noninvasive classification of single and double yolk eggs using Vis-NIR spectroscopy combined with Genetic Algorithm. Eng. Agric. Environ. Food. May 2019. [Under review]

10. Rahman, A., Syduzzaman, M., **Khaliduzzaman**, Fujitani, S., Kashimori, A., Fujiura, T., Suzuki, T., Ogawa, Y., Kondo, N. Nondestructive preincubation sex determination of broiler eggs using visible transmission spectroscopy and multivariate analysis. Eng. Agric. Environ. Food. May 2019. [Under review]

11. Rahman, A., Syduzzaman, M., **Khaliduzzaman**, Fujitani, S., Kashimori, A., Fujiura, T., Suzuki, T., Ogawa, Y., Kondo, N. Nondestructive sex-specific monitoring of early embryonic development rate in white layer chicken eggs using visible transmission. British Poultry Science Journal. May 2019. [Under review]

Conference Proceedings

1. **Khaliduzzaman**, Shinichi Fujitani, Naoshi Kondo, Yuichi Ogawa, Tateshi Fujiura, Tetsuhito Suzuki, Ayuko Kashimori, Shusaku. Nakajima. **2016**. Non-Invasive Characterization of Body and Cardiac Movements of Chicken Embryos Using NIR Light. Proceedings of the International Conference on Agricultural and Biosystems Engineering (CIGR AgEng): Automation, Environment and Food Safety, 26-29 June 2016, Aarhus, Denmark (abstract no 34).
2. **Khaliduzzaman**, Shinichi Fujitani, Tateshi Fujiura, Ayuko Kashimori, Md Syduzzaman, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2016**. A Non-Invasive Technique of Chicken Embryonic Growth Observation: Opacity Using NIR Sensor. Extended abstract for the 75th JSAM Annual Meeting, 27-30 May 2016, Kyoto University, Kyoto, Japan. 6-23, p:222.
3. **Khaliduzzaman**, Md Syduzzaman, Afzal Rahman, Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, Tateshi Fujiura and Naoshi Kondo. **2017**. Cardiac Signal Behavior of early and late hatch chick embryo. JPSA Spring Meeting 2017 (30 March), I-7. Kobe University, Kobe, Japan. ISSN 0029-0254, p:4.
4. **Khaliduzzaman**, Shinichi Fujitani, Afzal Rahman, Md Syduzzaman, Ayuko Kashimori, Tateshi Fujiura, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2017.** Sex differences in Chick Embryo Dynamic Activity. Proceeding of the 76th JSAM Annual Meeting 2017. Tokyo, Japan. p: 94.
5. **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tateshi Fujiura, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018**. Gender differences in opacity of chicken hatching eggs during incubation. 11 th Asia Pacific poultry conference March 2018, Thailand.

6. **Khaliduzzaman**, Shinichi Fujitani, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018**. Non-invasive sexing of chick embryo: A tool for precision poultry production towards global food security. Int. Conference on Challenges for Future Agriculture 2018. Bangladesh Agricultural University 2018, Bangladesh
7. **Khaliduzzaman,** Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018.** Non-invasive Life History Variables of Chick Embryos and Future Grading Systems. The JSAM (Japanese Society of Agricultural Machinery and Food Engineers) Joint Conference 2018 (10-14 September), OS3-6. Ehime University, Ehime. Japan
8. **Khaliduzzaman,** Shinichi Fujitani, Ayuko Kashimori, Afzal Rahman1, Md Syduzzaman, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018**. Chick embryo motility triggers eggshell to release calcium for its development. International Egg Symposium 2018 (October 16-17). Kyoto, Japan. Presentation No.: 16.
9. **Khaliduzzaman,** Tetsuhito Suzuki, Ayuko Kashimori, Shinichi Fujitani, Naoshi Kondo, Yuichi Ogawa. **2019**. A Non-invasive Measurement of Eggshell Thickness Using Terahertz Time-domain Spectroscopy. Spring meeting of Japan Poultry Science Association at Azabu University, Kanagawa, 252-5201, Japan. ISSN 0029-0254, p:3.
10. **Khaliduzzaman,** Shinichi Fujitani, Ayuko Kashimori, Afzal Rahman, Tetsuhito Suzuki, Naoshi Kondo, Yuichi Ogawa. **2019**. Detection of Chick Embryo Respiratory Movement Using Near Infrared Sensor. International Joint Conference on JSAM, SASJ and 13th CIGR VI Technical Symposium, Hokkaido University, Sapporo, Japan
11. Afzal Rahman, Md Syduzzaman, **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tateshi Fujiura, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2017**. Noninvasive classification of broiler chicken eggs gender before incubation using UV-Vis-NIR spectroscopy. Proceeding of the JPSA Spring Meeting 2017 (30 March), I-5. Kobe University, Kobe, Japan. ISSN 0029-0254, p:3.
12. Md Syduzzaman, **Khaliduzzaman**, Afzal Rahman, Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, Tateshi Fujiura and Naoshi Kondo. **2017**. Noninvasive Identification of Double Yolk Egg Using UV-Vis-NIR Spectroscopy Combined with Genetic Algorithm. JPSA Spring Meeting 2017 (30 March), I-6. Kobe University, Kobe, Japan. ISSN 0029-0254, p:3.
13. Md Syduzzaman, Afzal Rahman, **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tateshi Fujiura, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2017.** Noninvasive Quantification of Egg Yolk Using UV-Vis-NIR spectroscopy Combined with Multivariate Analysis. Proceeding of the 76th JSAM Annual Meeting 2017. Tokyo, Japan.p:95
14. Afzal Rahman, Md Syduzzaman, **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tateshi Fujiura, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2017.** Gender Identification

of Unincubated Broiler Chicken Eggs: A Feasibility Study Using UV-Vis-NIR spectroscopy. Proceeding of the 76th JSAM Annual Meeting 2017. Tokyo, Japan.p:96

15. Md Syduzzaman, Ayuko Kashimori, Shinichi Fujitani, Afzal Rahaman**, Khaliduzzaman**, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018.** Nondestructive identification of pale and dark yolk eggs using visible transmission spectroscopy. Proceedings of the JSAM (Japanese Society of Agricultural Machinery and Food Engineers) Joint Conference 2018 (10-14 September) (OS3-7). Ehime University, Ehime. Japan
16. Afzal Rahman, Md Syduzzaman, **Khaliduzzaman,** Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018**. Pre-incubation Non-invasive Sex Determination of Broiler Eggs Using Visible Transmission Spectroscopy and Multivariate Analysis. International Egg Symposium 2018 (October 16-17). Kyoto, Japan. Presentation No. 15.
17. Md Syduzzaman, Afzal Rahman, **Khaliduzzaman,** Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, Naoshi Kondo. **2018**. Chick embryo motility triggers eggshell to release calcium for its development. International Egg Symposium 2018 (October 16-17). Kyoto, Japan. Presentation No.: 17.
18. Rahman Afzal, Syduzzaman Md, **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, and Naoshi Kondo. 2019. Sex-specific Monitoring of Early Embryonic Development in Broiler Eggs Using Visible Transmission Spectroscopy. Spring meeting of Japan Poultry Science Association at Azabu University, Kanagawa, 252-5201, Japan. ISSN 0029-0254, p:2.
19. Syduzzaman Md, Rahman Afzal, **Khaliduzzaman**, Shinichi Fujitani, Ayuko Kashimori, Tetsuhito Suzuki, Yuichi Ogawa, and Naoshi Kondo. 2019. Noninvasive Quantification of Yolk Content Using Vis-NIR Spectroscopy and its Effect on Hatching Time of Broiler Chicken. Spring meeting of Japan Poultry Science Association at Azabu University, Kanagawa, 252-5201, Japan. ISSN 0029-0254, p:2.
20. Yuichi Ogawa, **Khaliduzzaman,** Keiji Konagaya, Tetsuhito Suzuki, Ayuko Kashimori, Naoshi Kondo, Yuichi Ogawa. **2019**. Non-invasive Eggshell Measurement Using Terahertz Time-domain Spectroscopy. International Joint Conference on JSAM, SASJ and 13th CIGR VI Technical Symposium, Hokkaido University, Sapporo, Japan

Awards Received

1. Best Presentation Award at the Spring meeting of Japanese Poultry Science Association, Azabu University, Kanagawa, 2019
2. Golden Egg Award for Poster Presentation at the International Egg Symposium in Kyoto 2018