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その他（別言語等）のタイトル	ニワトリ卵心拍数の長期計測
著者	AKIYAMA Ryuichi, MATSUHISA Akira, PEARSON James, TAZAWA Hiroshi
journal or publication title	Memoirs of the Muroran Institute of Technology
volume	49
page range	31-38
year	1999-11-30
URL	http://hdl.handle.net/10258/151

Mem. Muroran Inst. Tech.,49(1999)31~38

Long-Term Measurement of Heart Rate in Chicken Eggs

Ryuichi AKIYAMA*, Akira MATSUHISA**, James T. PEARSON** and Hiroshi TAZAWA**

(Accepted 31 August 1999)

Taking advantage of acoustocardiogram (ACG), we measured the heart rate (HR) of chick embryos continuously from day 12 until hatching and then investigated the development of HR irregularities (HRI), HR variability (HRV), and the existence of a circadian rhythm in mean HR (MHR). HRI comprised transient bradycardia and tachycardia, which first developed on day 14 and 16 in most embryos, respectively. Transient bradycardia increased in frequency and magnitude with embryonic development and occurred over periods of up to 30 min in some embryos. MHR was maximal on around days 14-15 and thereafter decreased to about 250-260 bpm on days 16-18. Baseline HRV, which is an oscillation of the MHR baseline, occurred as HR decreased from days 15-16 and became predominant on days 17-18. The magnitude of the baseline oscillations reached up to 50 bpm in some embryos and the period ranged between about 40-90 min (ultradian rhythm). A circadian rhythm of MHR was not found in late chick embryos. On days 18-19, embryonic activities were augmented and then breathing movements began to occur, disturbing ACG signals and thus making it difficult to measure the HR. Instead, the development of breathing activities was recorded. Breathing frequency was irregular at first and then increased to a maximum of about 1.5 Hz prior to hatching.

Keywords: acoustocardiogram, breathing activity, chick embryo, continuous measurement, heart rate irregularities, heart rate oscillations, internal pipping, mean heart rate, respiratory movements

1 INTRODUCTION

Cardiogenic signals of avian embryos are detectable by various sensors noninvasively, semi-invasively or invasively while maintaining adequate gas exchange through the eggshell [18]. These signals can be categorized by the kind of sensor used; the ballistocardiogram (BCG) and acousto-cardiogram (ACG) are measured noninvasively from outside of the eggshell [1,3,5,11, 12,19,21]. Thus the measurement of BCG and ACG are referred to as noninvasive methods. In contrast, the electrocardiogram (ECG) and impedance-cardiogram (ICG) are measured with electrodes inserted into the egg [2,3,5,7,8,19] or pasted on the shell membrane after a partial removal of the eggshell[10]; i.e., the measurement of ECG and ICG are semi-invasive methods. In addition, pulse oximetry is used to measure the pulsatile oxygen saturation of chorioallantoic capillary blood from outside of the outer shell membrane and may also be referred to as a semi-invasive method [9]. Besides these cardiogenic signals, the blood pressure of the allantoic artery is measured with a catheter implanted into the artery; i.e., catheterization is a invasive method [6,13,16,19]. Although embryonic heart rate (HR) is determined from the measurement of all of these cardiogenic signals, embryonic activities often disturb the signals, making accurate and clean recording of HR difficult. The noninvasive measurement of BCG and the semi-invasive measurement of ECG, ICG and capillary blood oxygen saturation are susceptible to this contamination. The ACG and blood pressure measurements are relatively less disturbed by embryonic activities [1,6,13,18,19]. In order to make long-term, continuous measurement of instantaneous heart rate (IHR) which is calculated from the beat-to-beat time interval of the heart, the measuring method needs to be noninvasive and contaminated as little as possible by embryonic activities. The ACG signal is detected as acoustic pressure changes across the eggshell, late in incubation, by a condenser microphone attached hermetically to the eggshell and was found to be synchronous with embryonic heart

*Division of Pediatric Cardiology, University of Kentucky;
**Department of Electrical and Electronic Engineering

beats [1,5,11,21]. Therefore, the ACG method is considered to be the best suited for long-term measurements of IHR in avian embryos. Employing the ACG method, we attempted to determine the embryonic HR of the domestic fowl continuously throughout the last half of incubation, for more than 200 hours, without any break in measurement.

2 MATERIALS AND METHODS

Fertile broiler chicken eggs were incubated at a temperature of 38 °C in a forced-draught incubator until day 12, and for the remaining period of incubation measurement was made in a still-air incubator. Eggs were turned automatically every hour for the first 12 days of incubation. On day 12, randomly selected eggs were candled to examine embryonic development and fitted with a condenser microphone glued to the eggshell as described elsewhere [1,5]. In order to attenuate external electrical noises (i.e., line frequency of 50 Hz) entering the leads and connectors of the measuring system, eggs were placed in a metal mesh box put in the still-air incubator at 38°C. The output signal from each microphone was checked on an oscilloscope and eggs whose ACG waves were recognized well were used for measurement. Once the microphone was fitted on the eggshell, it was kept in the same fixed position throughout the measurement.

The ACG signal was amplified by about 40 dB, and then passed through a band-pass filter which had a 3 dB bandwidth of 2-12 Hz with attenuation slope of 48 dB/oct. The signal was stored on a computer after digitization by a 12 bit analog-digital converter. To calculate IHR with an error in accuracy of less than 1 beat/min (bpm) for a maximum HR value of 350 bpm, the signal must be sampled at a frequency of over 4,000 Hz. On the other hand, the signal needs to be sampled at a low frequency to achieve long-term continuous measurement. For these reasons the signal was sampled at 100 Hz and later the ACG waves were restored by interpolation using a sinc function on the computer. The interpolated, restored ACG waves were equivalent to the signal sampled at a frequency of 4,000 Hz [1]. After wave restoration, the peak-to-peak time interval was converted to IHR to investigate development of heart rate irregularities (HRI) and heart rate variability (HRV). Changes in baseline HR were then examined by averaging IHR to condense the vast amount of IHR data. IHR was averaged over 1-min and 5-min intervals, and the mean HR's for 1-min and 5-min periods were referred to as MHR_{1m} and MHR_{5m}, respectively. Although these condensing procedures remove the short-term fluctuations in HR, the long-term HR trends can be presented. The baseline HR oscillations found late in incubation were analyzed by a fast Fourier transform (FFT) for their power spectrum analysis to determine their periodicity. Spectrum analysis was made for HR data averaged over 20-sec intervals (MHR_{20s}) rather than MHR_{1m} or MHR_{5m}, to increase the number of data points for better resolution.

During ACG measurements signals were inspected daily on the oscilloscope. The ACG signals were generally sinusoidal like waves and tended to increase in their magnitude with embryonic development. When it was found that the signals were reduced in magnitude and became irregular waves for a prolonged period, the time was noted and the measurement was continued.

Toward the end of incubation, the embryonic activities became augmented and respiratory movements began, disturbing the ACG signals from the microphone. Instead, the microphone detected embryonic and ventilatory activities, which were much larger in magnitude than the cardiogenic signal (i.e., ACG). Accordingly, the amplification of the amplifier was reduced to match the input level of the analog/digital converter and these signals were stored consecutively on the computer for analysis of ventilatory rhythms.

3 RESULTS

Measurement was made continuously for 216 hours (9 days) from day 12 of incubation in 15 eggs. Figure 1 presents examples of IHR patterns from an embryo that hatched on day 20, showing the development of HRI. Panels from the top to bottom show 30-min periods of IHR on days 12, 14, 16 and 18 of incubation. In general, on day 12 the HR baseline was stable except two embryos (including one shown in Fig. 1), which had HR with intermittent episodes of transient bradycardia. Then, HR began to fluctuate on day 14, and on day 16 transient tachycardia first appeared and baseline fluctuations were augmented. Both bradycardia and tachycardia appeared frequently and the frequency and magnitude of HRI increased with embryonic development. Spontaneous deceleration of HR sometimes

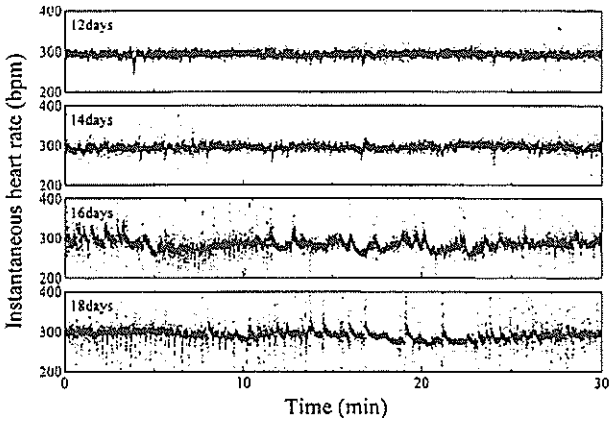

Fig. 1. Instantaneous heart rate (bpm) of a chick embryo during 30-min periods on days 12, 14, 16 and 18 of incubation.

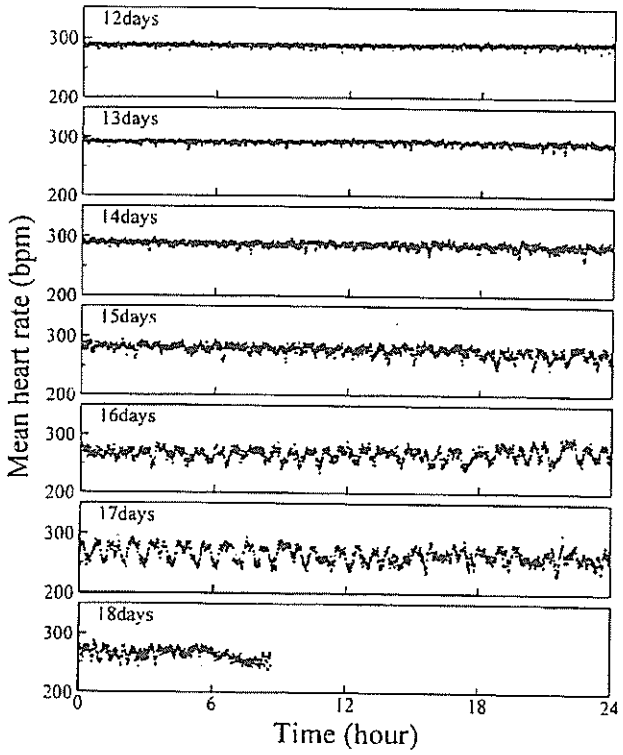

Fig. 2. Mean heart rate (MHR_{1m}), averaged over 1-min intervals, calculated from a continuous recording of instantaneous heart rate in a chick embryo illustrating the development of oscillations in heart rate baseline. On day 18, heart rate was no longer determinable after the eighth hour when the signal was disturbed by somatic activities immediately prior to internal pipping of the aircell.

became greater than 100 bpm. In the embryo shown in Figure 1, the duration of repeated decelerations was about 2 min on day 16, then became more prolonged with development and extended over 15 min period on day 18. Similar patterns were found to last as much as 30 min in other embryos. Combined patterns of repeated HR decelerations and repeated accelerations were also observed.

In order to compress the prolonged recording of IHR data and present it in a single figure for each embryo, IHR was averaged every minute and MHR_{1m} is shown by a single point (Fig. 2). Figure 2 shows an example of the whole continuous recording of MHR_{1m} from day 12 to day 18. From the top to bottom, HR over 24-hour period on each day of incubation is presented in an individual single panel. HR baselines were stable days 12-13, and began to oscillate late on day 15, and the magnitude of baseline changes increased with development. On day 16, cyclic oscillation became obvious and their magnitude extended to about 50 bpm on day 17. Such cyclic oscillations were also observed in other embryos and the magnitude of the oscillations was dependent upon individuals. Previously, IHR was categorized as HRI and HRV; HRI was defined as transient, non-cyclic accelerations and decelerations of more than 10 bpm above or below the HR baseline (Fig. 1) and HRV as cyclic changes in baseline HR of less than 10 bpm [6]. However, as shown in Figure 2, cyclic baseline changes of more than 10 bpm were newly found in the MHR patterns, which may also be categorized as HRV. On day 18, we terminated MHR_{1m} at about 8-hour, because artefacts caused by embryonic activities prior to penetration of the beak into the air cell through the chorioallantoic membrane and inner shell membrane (internal pipping, IP) disturbed ACG signals.

The period of HRV was calculated by analyzing the power spectrum of the HRV intervals. As the number of points for power spectrum analysis needs to be an exponential of 2, we increased the number of points by averaging IHR every 20 sec rather than every 1 min to achieve better resolution. Figure 3 shows two examples of MHR_{20s} (left panels) over 24-h on day 17 and their power spectra calculated by FFT (right panels). The horizontal axes in the right panels indicate the periods of baseline oscillations in minutes converted from frequency. Spectral peaks from these analyses were found at about 85 min and 65 min, respectively. Power spectra of other embryos were also analyzed, and it was found that MHR in other embryos oscillated with periods of 40-90 min during the late incubation period.

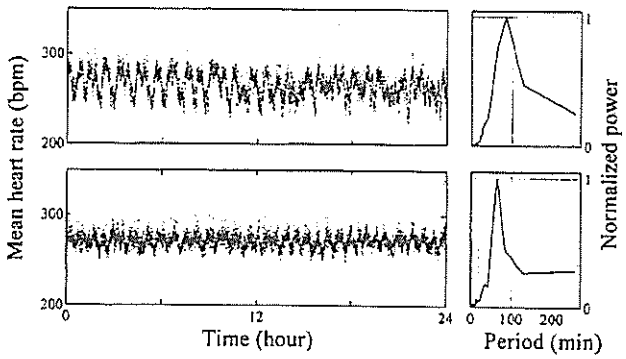

Fig. 3. Spectral analysis of the periodicity of oscillations in mean heart rate (MHR_{20s}) for 24-h periods in two chick embryos. Right panels illustrate the power spectra of each MHR_{20s} interval. Peaks indicate significant oscillation periods of 85 min and 65 min, respectively.

Figure 4 presents MHR_{5m} in two embryos, showing the entire HR recording in a single figure. No circadian rhythm in MHR was found. HR increased from day 12 to a maximum HR on around day 14-15 and then decreased. The HR baseline became wide because of the cyclic oscillation as shown in Figures 2 and 3. These two embryos hatched at times indicated by

arrows, respectively. Because the embryonic activities augmented toward IP and respiratory movements occurred, both disturbed the ACG

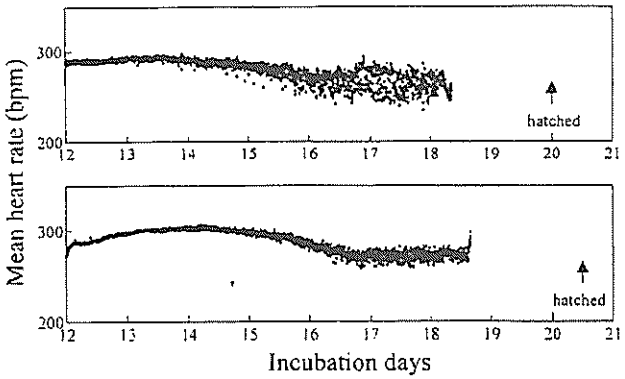

Fig. 4. Mean heart rate (MHR_{5m}) averaged over 5-min intervals in two embryos, recorded continuously from day 12 of incubation. The egg in the top panel was the same as in Figure 2. The gap between the last data point on day 18 and the arrow was due to ACG signal disruption with somatic and breathing activities prior to internal pipping and during hatching.

signals and made it impossible to recored the HR further. Instead, the microphone recorded the embryonic activities and respiratory movements during these periods.

Four embryos died due to unknown reasons prior to IP or during pipping the shell (external pipping, EP). Figure 5 presents an example of MHR_{1m} from an embryo that failed. The upper panel shows the 24-h period MHR_{1m} prior to death on day 17. The lower panel presents the same HR pattern expanded from the upper panels. The HR pattern was normal over the first 6 hours on day 17. Then the HR baseline frequently decreased to about 130 bpm with subsequent recovery to the normal level, and at last decreased consistently prior to death. During the period of failing HR (bottom panel), HR changed swiftly between about 130 bpm and 240 bpm. In another embryo which died, HR decreased from control value of about 270 bpm to a new level of about 180 bpm and then decreased again to an even lower level, without returning to the normal level. The HR fluctuated between the new levels both of which decreased gradually for about 2-hour to death. Fluctuations in HR level were dependent upon individual embryos. During HR fluctuations, the ACG signal changed in amplitude; when HR dropped, the amplitude of the ACG became large. In other embryos, the HR decreased from the normal values to zero during short periods and heart beats never recovered.

Figure 6 shows examples of recordings of embryonic activities and respiratory movements during the last period of incubation. At day 18 10 hours (top panel), the ACG signals, which were recorded during the first few seconds, were disturbed by irregular deflections 5-6 times the amplitude of the ACG. The amplification of the amplifier was reduced to record such large deflections as they increased in magnitude.

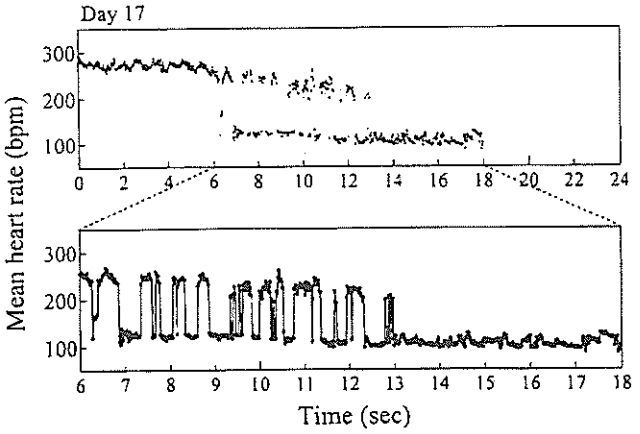

Fig. 5. Mean heart rate (MHR_{1m}) of a chick embryo on day 17, decreasing intermittently over a period of 12 hours prior to death. The lower panel indicates that embryonic heart rate was periodically maintained at a lower level and returned to a higher level many times before eventual death.

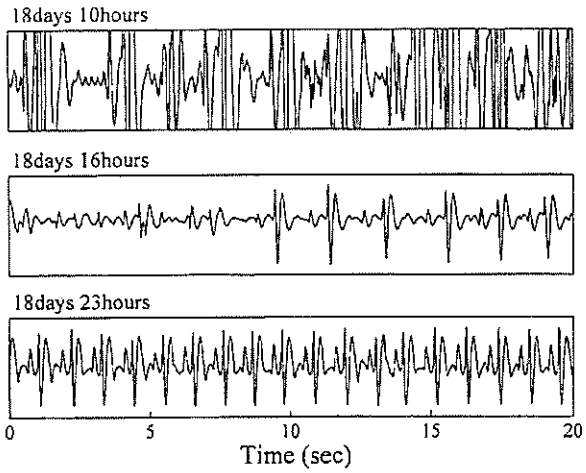

Fig. 6. The microphone signal of a chick embryo on day 18 when somatic activities disturbed the cardiogenic signal. The ACG was recorded for a short period between somatic activities prior to internal pipping (top panel). With the initiation of pulmonary ventilation, intermittent pressure pulses disturbed the ACG (middle panel), and finally became periodic (bottom panel). Minute signals due to heart beats were often recorded in the baseline between intervals of breathing deflections, but they were not seen in this recording, because the amplification of the amplifier was matched for large breathing signals.

About 6 hours later in this embryo (middle panel), the microphone began to intermittently record new signals which were larger than the irregular deflections (shown in the top panel) and occurred over various

intervals of about 1-2 sec. The signals were often distorted and reliable calculations of the time between intervals of the peaks were not possible during early period of the occurrence. Then, they increased in magnitude and became cyclic (last half of the recording in the middle panel). With the lapse of time, the signals became regular patterns and their frequency increased from about 0.5-1 Hz to a maximum of 1-1.5 Hz, shortly before hatching (bottom panel).

4 DISCUSSION

4.1 Long-term HR measurements

Various methods have been developed to determine the HR of the avian embryo through the eggshell, taking advantage of the fact that the avian embryo develops within an egg and is isolated from the external environment only by the eggshell and shell membranes. Previously, the long-term recording of the avian embryo HR was attempted using ECG in the Muscovy duck embryo during the last 10 days of incubation [10]. Because the ECG was frequently disturbed by embryonic movements, IHR was not determined throughout the measuring period, but mean HR was shown based on signal processing by a computer. Meanwhile, blood pressure can be measured relatively free from embryonic activities by catheterization of the allantoic artery [6,13,19], which makes it possible to investigate the development of HRV and HRI. Höchel *et al.* [6] were recently able to compare the development of HRI patterns in the chick embryo with the better known mammalian fetuses. However, catheterization is an acute, invasive method and is not suitable for long-term IHR measurements. With regard to the accuracy of IHR measurement, artefacts can not always be eliminated completely from the IHR determined by the ACG method in contrast to the catheterization, but the ACG method has undoubtedly advantages for noninvasive measurement and long-term measurement of HR.

The ACG is a record of pressure changes occurring outside the eggshell, which is synchronized with heart beats of the embryo developing within the egg. In chicken eggs, the ACG is recorded with the condenser microphone attached hermetically to the eggshell after about the first half of incubation [1,11]. The ACG is also detectable with the microphone or pressure transducer installed in a hermetically sealed box containing the egg [9,21]. The ACG signals became large in magnitude when HR decreased as seen in the failing embryos of this study. Similarly, the ACG magnitude decreased when HR increased as previously reported for the ostrich eggs [15]. Rahn and associates [11], using chicken eggs, suggested that the ACG signal probably originates from a tissue (egg) pressure pulse impinging the eggshell through changes in heart volume. The increased magnitude of ACG signals at lower HR or vice versa seems to be causally related to changes in cardiac output via a Starling mechanism. However, studies on embryonic HR in domesticated birds and recently in emu eggs showed that ACG signals were detectable from eggs, which were pipped externally and eventually had pip-hole through the eggshells [14,17]. Thus, the tissue pressure pulse mechanism is unlikely to explain the origin of ACG signals. Wang *et al.* [21] put forward an alternative mechanism due to a gas kinetic effect on oscillations of gas pressure around the eggshell with each heartbeat (i.e., ACG signal). The diffusive resistance from capillary blood to atmosphere and the capacity of capillary blood for gases form a low-pass filter that attenuates oscillations in O_2 and CO_2 exchanges differently due to their different time constants (resistance x capacitance) [21]. If the time constant is different between O_2 and CO_2 oscillations, the difference in attenuations between them becomes large at lower HR levels, thus increasing the ACG magnitude or vice versa. Even during the pipping period, the gas exchange through the eggshell and chorioallantoic membrane is maintained locally and thus detection of ACG signals is possible when the embryo is quiescent and the microphone is relocated on the eggshell to an area through which the gas exchange continues to take place.

In the present experiment, the microphone was glued to the eggshell so as not to be disengaged during incubation. Therefore, the microphone was not relocated on the eggshell when the ACG signals were disturbed by augmented embryonic activities prior to IP, and the HR calculation was discontinued during the last stages of incubation. However, it is still possible to measure the HR during the pipping period, if the position of the microphone is replaced on an area of the eggshell through which gas exchange takes place between the environmental atmosphere and the chorioallantoic capillary blood.

4.2 Development of HRI

The present continuous measurements of IHR substantiated the findings concerning the development of HRI in chick embryos which were made by acute, short-term measurement of IHR by means of catheterization [6] (Fig. 1). Both studies showed that spontaneous bradycardia occurred first on around days 13-14, whereas spontaneous tachycardia occurred for the first time on around day 16. After the initial infrequent, occasional appearance of HRI, their frequency of occurrence and the magnitude increased with development. After day 16 transient bradycardia often reached 50 bpm or more in magnitude with subsequent swift recovery to baseline levels within a few seconds, and began to occur repeatedly over periods of 30 min. Meanwhile, the repetition

of transient tachycardia occurred on day 16, soon after their first development, and HRI comprised both bradycardia and tachycardia. On around day 18, the magnitude of these HRI increased up to 100 bpm in some cases. Thereafter, the ACG signals were disturbed by embryonic activities and respiratory movements, and the HR measurement could not be made unless the microphone was relocated on the eggshell.

The experiment by Höchel *et al.*[6] demonstrated convincingly that transient bradycardia was vagally mediated, since these decelerations were blocked by administration of atropine, accompanied by a raising of the HR baseline. Parasympathetic innervation of the chick embryo's heart appeared to be functional on around day 14, and its activity increased with embryonic development as noted by the repeated bradycardia, for periods as long as 30-min in some embryos. Although HR increases in many physiological situations are known to be mediated by sympathetic activity, its role in the development of transient tachycardia in chick embryos is not so clear and remains to be investigated [6].

4.3 Baseline HRV

MHR baseline was stable up until days 13-14. Small fluctuations in the baseline of a little over 10 bpm became evident on day 14, but were very brief in duration (Fig. 2). Larger changes in the baseline were found in all embryos by day 16. Averaging of the IHR has the effect of removing short-term HR fluctuations, which comprise rapid accelerations and decelerations of IHR, but it emphasizes the HRV trends in the baseline. It makes it possible to examine the development of cyclic rhythms in HR during incubation. The cyclic oscillations were found to occur continuously from about day 16 in all embryos. The magnitude of oscillations in MHR_{1m} increased to a maximum of about 50 bpm up until day 17-18 in some embryos. Power spectrum analysis of MHR_{20s} over a 24-h period confirmed that the baseline HR oscillations were in fact periodic (Fig. 3), with periods of 40-90 min. The physiological origins of these ultradian rhythms in HR remain to be studied. Meanwhile, circadian rhythms in HR were not found in any of the embryos examined (Fig. 4). It should be noted that even dim light entering the incubator was unlikely to reach the eggs contained in the metal mesh cage and the measurements were made during near darkness throughout the last half of incubation.

4.4 HR during failure

Four embryos failed during incubation in the present experiment. The ACG signals of the failed embryos were reduced in magnitude and became irregular waves during the measurement. The time of death was noted. In two embryos, HR developed normally until failure and there were no apparent warnings in HR patterns prior to death. In other embryos, HR regulation rapidly developed abnormalities several hours to half a day prior to death. For instance, HR decreased to half the normal level and was maintained at the lower HR level for periods between tens of seconds and minutes until HR returned to near normal levels (Fig. 5). Readjustments of embryonic HR were most likely due to changes in pacemaker activity. The changes were achieved within the interval of a few beats and sustained for variable periods, but the cause of failure was unclear.

4.5 Initiation of breathing rhythms

Continuous measurement of a microphone's output during the last stages of incubation allows us to infer about the initiation of breathing. Respiratory related somatic activities of embryos, causing large air pressure changes within the egg, have been observed about 1 day to half a day prior to IP [4, 20]. Figure 6 shows typical changes in signal waves of microphones that occurred in embryos on day 18 shortly before and after IP. The first 20-sec recording at 18 day 10 hours shows large and very rapid pressure changes (top panel), which are considered to reflect embryonic activities prior to the piercing of the beak into the air cell through the chorioallantoic membrane and inner shell membrane (IP). Several hours later, after IP, ACG was disturbed by intermittent breathing movements, which became more regular with development (middle panel). In an additional experiment, it was observed that the large breathing movements first occurred before IP and that the smaller breathing movements, which were initially bouts of intermittent breathing, were correlated with IP. Other studies using different methods, which differed in the transducer used to measure the air pressure changes, also demonstrated similar patterns of change in respiration shortly before IP [4, 20]. Once regular breathing has been established, the breathing frequency increased from about 1 Hz to 1.5 Hz shortly before hatching, similar to the reported values for chickens [4, 20].

In the present experiment which was designed to measure continuously embryonic HR throughout the last half of incubation, it was found that the noises disturbing the cardiogenic signals (ACG) originated in embryonic movements augmenting prior to IP and breathing activities developing during the pipping period. Further experiments using the microphone will elucidate the ontogeny of breathing movements in hatching embryos.

ACKNOWLEDGMENT

This research was supported in part by a Grant-in-Aid for International Collaborative Research (awarded to H.T., No.0704410) and a Grant-in-Aid for Scientific Research (H.T., No.08650471) of the Ministry of Education, Science and Culture; the Monbusho.

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ニワトリ卵心拍数の長期計測

秋山 龍一[1]、松久 晶[2]、ピァーソン ジェームズ[2]、田澤 皓[2]

概要

アコーストカーディオグラムを利用して、ニワトリ胚の心拍数を孵卵 12 日目から孵化するまで連続測定し、心拍不規則性と心拍変動性の発達を明らかにすると共に、平均心拍数にサーカディアンリズムがあるか検証した。心拍不規則性はそれぞれ孵卵 14 日及び 16 日頃に現れる一過性の徐脈と頻脈から成っていた。一過性の徐脈は胚の成長と共にその頻度と振幅が増し、幾つかの胚では 30 分以上継続して現れた。平均心拍数は孵卵 14-15 日に最大になり、その後減少して孵卵 16-18 日には 250-260 回/分になった。平均心拍数ベースラインの振動である心拍数変動性は、心拍数が孵卵 15-16 日頃から減少するにつれ現れ始め、17-18 日には顕著になった。ベースライン変動の大きさは幾つかの胚では 50 回/分にも達し、その周期は 40-90 分（ウルトラディアンリズム）であった。サーカディアンリズムは孵卵後期の胚にも現れなかった。孵卵 18-19 日になると胚の活動が増して呼吸運動が発現し、そのためアコーストカーディオグラム信号は乱されて心拍数計測は困難になった。そのかわりに呼吸活動の発達が記録できた。最初、呼吸周波数は不規則であり、次第に増して孵化前には 1.5 Hz の最大値に達した。

キーワード：アコーストカーディオグラム、呼吸活動、ニワトリ胚、連続測定、心拍不規則性、心拍振動、内嘴打ち、平均心拍数、呼吸運動

[1]ケンタッキー大学、[2]電気電子工学科