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Cardiac rhythms of late pre-pipped and pipped chick embryos exposed to altered oxygen environments

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Abstract

During the final stages of embryonic development in chickens, diffusive gas exchange through the chorioallantoic membrane (CAM) is progressively replaced by pulmonary respiration that begins with internal pipping (IP) of the CAM. Late chick embryos going through the transition from CAM respiration to pulmonary respiration were exposed to hyperoxic (100% O_2) and hypoxic (10% O_2/N_2) environments for 2-h and the responses of baseline heart rate (HR), and HR fluctuation patterns were investigated. 16- and 18-day-old (referred to as 18-d) embryos and 20-d externally pipped (EP) embryos were examined as pre-pipped embryos and pipped embryos, respectively. 19-d embryos were divided into two groups: embryos that had not yet internally pipped (Pre-IP embryos) and embryos that had internally pipped (IP embryos). IP was identified by detecting the breathing signal with a condenser microphone attached hermetically on the eggshell (i.e. acoustorespirogram) on day 19 of incubation. In the hyperoxic environment, HR baseline of pre-pipped embryos remained unchanged and that of pipped embryos was depressed. In the hypoxic environment, HR baseline of 16-d pre-pipped embryos was depressed and that of pipped (IP and EP) embryos elevated. These different responses in pipped embryos might be partially attributed to increased cholinergic input from the vagus nerve in hyperoxia and increased adrenergic response in hypoxia. While hyperoxia did not induce marked modification of instantaneous heart rate (IHR) fluctuation patterns, hypoxia tended to augment transient decelerations of IHR in late pre-pipped embryos and markedly depressed HR fluctuations in pipped embryos.

Keywords: Gas exchange; Heart rate baseline; Heart rate fluctuation; Hyperoxia; Hypoxia; Instantaneous heart rate; Pre-pipped embryos; Pipped embryos; Response of heart rate

1. Introduction

Because chick embryos develop within the confines of hard porous eggshell, gas exchange takes place by diffusive transfer between environmental air and capillary blood of the area vasculosa early in incubation and of the chorioallantoic membrane (CAM) later through the eggshell and shell membranes (Wangensteen et al., 1970/71; Wangensteen, 1972; Ackerman and Rahn, 1981; Tazawa and Whittow, 2000). During the last stages of incubation, embryos begin to clap their beaks and pip internally the CAM (internal pipping, IP) and externally the eggshell (external pipping, EP) (Visschedijk, 1968; Dawes, 1973; Vince and Tolhurst, 1975; Chiba et al., 2002). Upon beak penetration into the air cell at IP, convective gas exchange by the lungs is ready to initiate with subsequent progress towards EP and hatching (Visschedijk, 1968; Rahn et al., 1974; Ar et al., 1980; Tazawa and Whittow, 2000; Menna and Mortola,

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2002). Thus, late embryos prior to IP (referred to as pre-pipped embryos) exchange O_2 and CO_2 with atmospheric air by diffusion through the CAM, while the gas exchange of internally pipped and externally pipped embryos (referred to as pipped embryos) takes place by some combination of CAM respiration and convection through the lungs.

Meanwhile, cardiac rhythm of chick embryos begins with the formation of a primordial tubular heart and daily mean heart rate (MHR) increases rapidly early in incubation, stabilizes during middle incubation with subsequent elevations during the pipping periods (Cain et al., 1967; Van Mierop and Bertuch, 1967; Girard, 1973; Laughlin, 1978; Hu and Clark, 1989; Van Golde et al., 1996; Akiyama et al., 1999b; Crossley and Altimiras, 2000; Crossley et al., 2002; Tazawa et al., 2002). Instantaneous heart rate (IHR) begins to fluctuate with the appearance of rapid transient decelerations on day 13–14 of incubation and is augmented further with the additional appearance of HR accelerations towards pipping (Akiyama et al., 1997; Tazawa and Hou, 1997; Höchel et al., 1998; Tazawa et al., 1999). During pipping periods, distinctive patterns of IHR fluctuations appear: irregular intermittent accelerations, relatively long-lasting cyclic small accelerations and short-term repeated large accelerations indicating imminent hatching (Tazawa et al., 1999). Besides these acceleration patterns, respiratory sinus arrhythmia (RSA) appears during the EP period (Tazawa et al., 1999).

These patterns of IHR fluctuations have been investigated for the embryo developing normally in an intact eggshell in air. If environmental O_2 is altered, alteration of O_2 may modify IHR fluctuations as well as MHR. In addition, different modes of gas exchange before and after initiation of pipping may influence the effect of altered O_2 on the modification of HR. Effects of ambient hypoxia and hyperoxia on HR of chick embryos have been partially investigated in relation to responses of MHR to altered O_2 in non-fenestrated eggs and fenestrated eggs (Tazawa, 1981; Van Golde et al., 1997; Akiyama et al., 1999b; Crossley et al., 2002). Short-term (e.g. 5-min) hypoxia depressed the HR baseline of pre-pipped embryos (Van Golde et al., 1997; Crossley et al., 2002) and pipped embryos (Crossley et al., 2002) in fenestrated eggs. Effects of hyperoxia as well as hypoxia were determined in early (days 3–9) and young (days 14–16) pre-pipped embryos in non-fenestrated eggs (Tazawa, 1981; Akiyama et al., 1999b). Hypoxia depressed MHR in young embryos according to its magnitude and hyperoxia up to 100% O_2 did not induce significant changes in MHR (Tazawa, 1981). In very early embryos (days 3–5), MHR showed a variety of moderate responses to prolonged hypoxia (10% O_2), but as development progressed, hypoxic exposure induced profound decrease in MHR that often resulted in death during 4-h measurement (Akiyama et al., 1999b). In contrast, MHR did not respond to hyperoxia (100% O_2) in early and young embryos, suggesting that pre-pipped embryos developing inside the intact eggshell beat maximally the heart in air; that is, absence of a 'hypoxic drive' to MHR (Akiyama et al., 1999b).

Here, we examined systematically how altered environmental O_2 (i.e. hyperoxia and hypoxia) would modify MHR and IHR fluctuations before and after onset of pulmonary respiration. Taking into consideration the previous studies, the present experiment was designed with three goals based on a longer period of exposure (2-h) of non-fenestrated eggs to altered ambient O_2. The first goal was to confirm previously obtained evidences of the hypoxic depressant effects on MHR in pre-pipped and pipped embryos and the absence of any hyperoxic effects on MHR in pre-pipped embryos. The second was to examine newly hyperoxic effects on MHR in pipped embryos. The third was to investigate the effects of hyperoxia and hypoxia on the IHR fluctuations in pre-pipped and pipped embryos.

2. Materials and methods

2.1. Embryos

Fertile broiler chicken (*Gallus gallus domesticus*) eggs were obtained from a local hatchery. Eggs were placed in horizontal position in a forced-draught incubator and rotated automatically through 90° every 3-h. The first day of incubation was designated as day 0. The temperature of the incubator was maintained at 38°C with relative humidity of approximately 60%. On day 15 of incubation, all eggs were candled to check viability of embryos and the living eggs were transferred to a still-air incubator where measurements of IHR were made at the temperature of 38°C. The measurements were made on days 16, 18, 19 and 20 of

incubation. Embryos on day 19 were divided into two groups depending upon whether or not they had pipped internally the CAM; i.e. embryos prior to IP were designated as Pre-IP embryos, and those with IP were IP embryos. Eventually, 16- and 18-d embryos and 19-d Pre-IP embryos were designated as pre-pipped embryos and 19-d IP and 20-d EP embryos were pipped embryos. Each embryo was subject to a single measurement.

2.2. Distinction between Pre-IP and IP embryos

On day 19 of incubation, a condenser microphone was attached hermetically on the eggshell with clay. An output signal from the microphone was amplified and displayed on a computer monitor as described elsewhere (Akiyama et al., 1997, 1999a; Chiba et al., 2002). Waveforms and magnitude of the microphone signal were altered according to developmental stages of the embryos whether or not they had pipped internally the CAM (Akiyama et al., 1999a; Chiba et al., 2002). The microphone detected cardiogenic signals with minute magnitude (i.e. acoustocardiogram, ACG) in the egg where the embryo had not pipped the CAM. If the microphone detected the beak clapping signals, the embryos soon began to penetrate the CAM and breathing signals with large magnitude that occurred at first intermittently and later repeatedly were detected. The microphone signal attributable to embryonic breathing was previously designated as acoustorespirogram (ARG) (Chiba et al., 2002). Thus, if the signal that was displayed on the monitor was ACG, eggs were used provisionally as Pre-IP embryos for measurement of IHR. After HR measurement, the eggs were again measured for the microphone signal. If the signal was still ACG, the result of experiment was adopted as that of Pre-IP embryos. IP embryos were eggs of which microphone signal was ARG.

2.3. Measurement of instantaneous heart rate

IHR was determined in real time with the aid of a computer from the electrocardiogram (ECG) of embryos as described previously (Moriya et al., 1999; Tazawa et al., 1999). Three wire electrodes (3 cm long and 0.1 cm diameter) were inserted into the egg at a depth of 0.5 cm through three pinholes opened in the eggshell by a tip of hypodermic needle. The electrodes were glued onto the eggshell by epoxy cement that also closed the pinholes. The egg attached with the electrodes was put in an air-tight cylindrical chamber (vol. 1.2-l) that was installed with an electrode socket on the lid and two gas conduits on the wall. The electrodes were connected to the socket and further to an amplifier from the socket with electrically shielded wires. The amplified and band-passed ECG signals were sampled at a frequency of 12,000 Hz by a 16-bit analog to digital converter (sound card). The sampled ECG signals were displayed on the computer monitor and a threshold of the voltage was set on the monitor so that it could cross individual R waves above other signals and background noises on the ECG. IHR (beats per min, bpm) was calculated from time intervals between two adjacent R waves which crossed the threshold line. Each value of IHR was plotted in a graph on the monitor for provisional display and simultaneously saved in time sequence on the computer.

2.4. Altered oxygen environments

The measuring chamber was placed in the still-air incubator warmed at 38°C and a conduit built on a bottom part of the chamber was connected to a 4-way stopcock by a vinyl tube 30-cm long. Three other vinyl tubes approximately 5-m long were connected to the stopcock and the ends of tubes approximately 1-m long were extracted from the incubator to connect air and test gas cylinders, respectively. Another conduit built on an upper part of the chamber was connected with a vinyl tube approximately 1-m long and the end of the tube was extracted to the outside of the incubator. The measuring chamber was at first ventilated with air (20.9% O_2) for 2-h before start of measurements in order to ensure temperature equilibrium of the egg with the 38°C environment. The temperature in the measuring chamber was determined with a thermister probe and assured to be identical with that of the incubator. The inflow O_2 fraction of the test gas mixtures was assured to be 10 and 100%, respectively. Humidification of the air and test gas mixtures was not made.

After temperature equilibration, the measuring chamber was ventilated in sequence by air, a test gas mixture and air each for 2-h periods at a flow rate of approximately 250 ml/min. Thus, embryos were exposed to an altered O_2 environment (hypoxia or hyperoxia) during the second 2-h period (referred to as exposure period). The first

and the last 2-h periods were referred to as pre-exposure control and post-exposure control periods, respectively.

2.5. Power spectrum analysis

When IHR fluctuations appeared to be oscillating for more than a 20 min-period, power spectrum analysis (PSA) of IHR was made by fast Fourier transform to ascertain whether or not they were HR oscillations and to determine the oscillatory frequency, when they oscillated. Because HR oscillation with a period of 1–1.5 s was found in EP embryos and suggested to be RSA (Tazawa et al., 1999), PSA was made on IHR fluctuations that appeared to oscillate in EP embryos.

2.6. Statistical analysis

All the values of IHR for three 2-h segments (pre-exposure control, exposure and post-exposure control periods) were averaged in individual embryos to yield mean heart rate (i.e. $MHR_{2\text{-}h}$). The individual values of $MHR_{2\text{-}h}$ in all the embryos at the same developmental stages were averaged to yield mean value of $MHR_{2\text{-}h}$ (i.e. MHR_{mean}) for pre-exposure control, exposure and post-exposure control, respectively. The difference of MHR_{mean} between three groups was examined for the significance by repeated ANOVA with pairwise comparisons of group means adjusted by the Bonferroni procedure to indicate which groups differed from others at the critical level of 0.016 (0.05 divided by number of groups (3)).

3. Results

3.1. Pre-IP and IP embryos

Fig. 1 presents an example of the output signal from the microphone attached hermetically on the eggshell of a 19-d embryo. In (A), the baseline oscillated with minute magnitude as indicated in an enlarged inset. The oscillating signal was ACG originating from heartbeat of the embryo. Simultaneously, the embryo clapped its beak intermittently, which produced clapping signals with large magnitude compared with ACG. Approximately 1-h later, the embryo began to produce another signal that was different in configuration from ACG and clapping signals (B). The signals indicated by arrows in (B) were produced by pulmonary ventilation of the embryo (i.e. ARG). Subsequently, activities of piercing the CAM and inner shell membrane by the beak were recorded as irregularly vibrating deflections together with ARG that was enhanced in frequency and magnitude (C). Afterwards, the microphone did not detect the vibrating deflections but recorded ARG. The embryos that produced ARG were IP embryos and those producing only ACG were Pre-IP embryos.

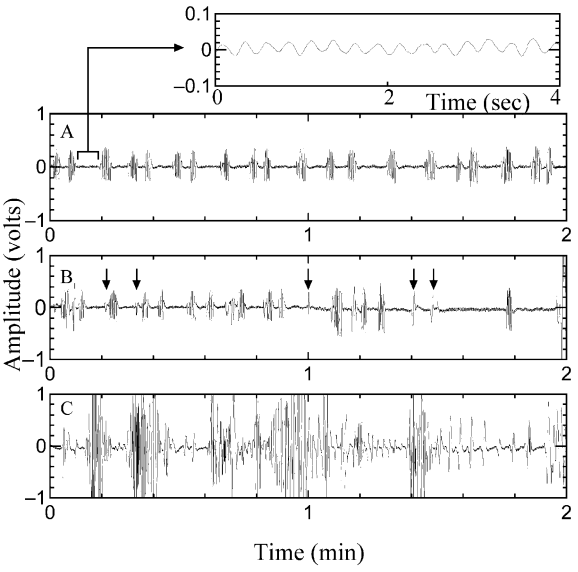

Fig. 1. Changes in output signal from a condenser microphone during beak clapping (A) and IP (B and C). The 4-s recording on the top (inset) enlarges the baseline of the microphone signal indicated by a bar with hook in (A). Arrows in (B) indicate signals produced by pulmonary ventilation. Time (min), and that to the ordinate is, Amplitude (volts).

3.2. Heart rate responses to hyperoxia

Responses of IHR to 100% O_2 were investigated in nine 16-d, eight 18-d, six 19-d Pre-IP, eight 19-d IP and ten 20-d EP embryos. Representative responses of IHR in individual groups of each age are presented in Fig. 2. From top downwards, individual panels show 6-h recording of IHR in a 16-, 18-, 19-d Pre-IP, IP and EP embryos. The first and last 2-h recordings are IHR during control exposure to air (pre- and post-exposure control) and the middle 2-h recording presents IHR responses to hyperoxic exposure. The level of HR baseline did not change in a pre-pipped 16-, 18- and 19-d Pre-IP embryos, while an IP and EP embryos decreased HR baseline during exposure

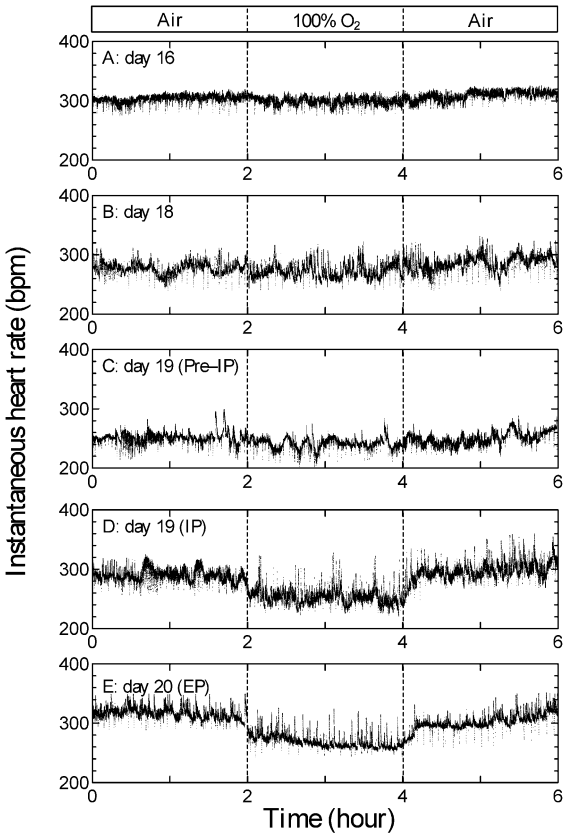

Fig. 2. Examples of IHR response to hyperoxic exposure, examined in a 16-d (A); 18-d (B); 19-d Pre-IP (C); 19-d IP; (D); and 20-d EP (E) embryos. The vertical dotted lines indicate time of change in gaseous environments. The embryos were exposed to 100% O_2 during the middle 2-h period.

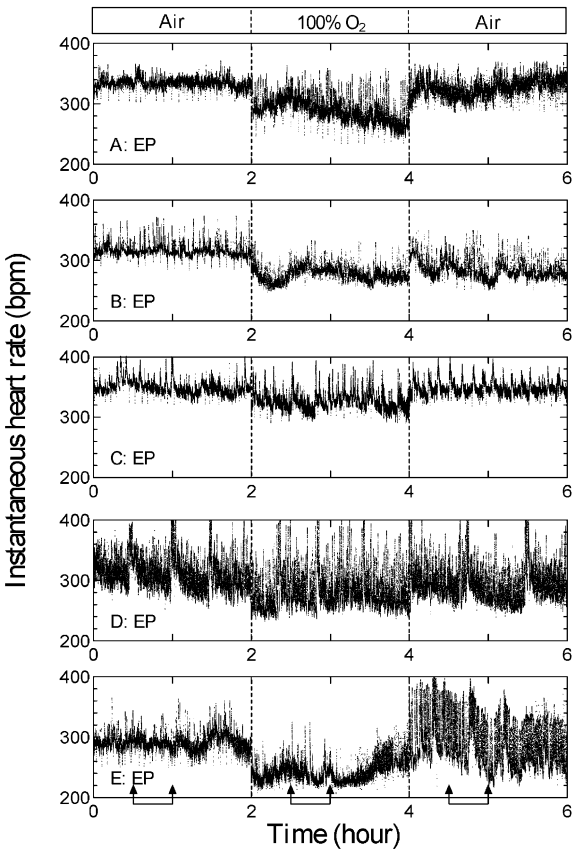

Fig. 3. IHR responses to hyperoxic exposure, examined in five other EP embryos (A–E). Arrows indicate 30-min recordings of IHR that are shown in Fig. 4.

to hyperoxia. The trends showing an absence of changes in HR baseline in pre-pipped embryos and a decline of HR baseline in pipped embryos during hyperoxic exposure were common to all other embryos investigated (e.g. Fig. 3).

Fig. 3 presents IHR responses of five other EP embryos to hyperoxic exposure, showing that embryos at the same developmental stage responded similarly; i.e. HR baseline dropped during hyperoxic exposure. Table 1 summarizes MHR_{mean} of pre-exposure control, hyperoxic exposure and post-exposure control in 10 EP embryos and those in other groups of age. The drop of HR baseline during hyperoxic exposure in IP and EP embryos was significant with subsequent recovery to pre-exposure control levels. Meanwhile, in pre-pipped embryos on days 16, 18 and 19 (Pre-IP), the differences of MHR_{mean} between three groups (i.e. air, 100% O_2 and air) were not significant.

IHR fluctuated throughout the 6-h measurement period in all the embryos, showing heart rate irregularities (HRI) comprising of irregular decelerations and accelerations of IHR (Figs. 2 and 3). Patterns of HRI were various and distinctive patterns of HR fluctuations were neither produced nor

Table 1
Mean value ± S.D. of MHR during exposure to air, 100% O_2 and air, determined for 6–10 embryos on individual days of incubation

Day	*N*	Air	100% O_2	Air
16	8	277 ± 20	275 ± 21	281 ± 17
18	8	265 ± 17	261 ± 18	270 ± 15
19 (Pre-IP)	6	264 ± 12	259 ± 13	269 ± 16
19 (IP)	8	266 ± 16	245 ± 15[a]	272 ± 20[a]
20 (EP)	10	320 ± 28	288 ± 32[a]	307 ± 32[a]

[a] Significant difference from the preceding value.

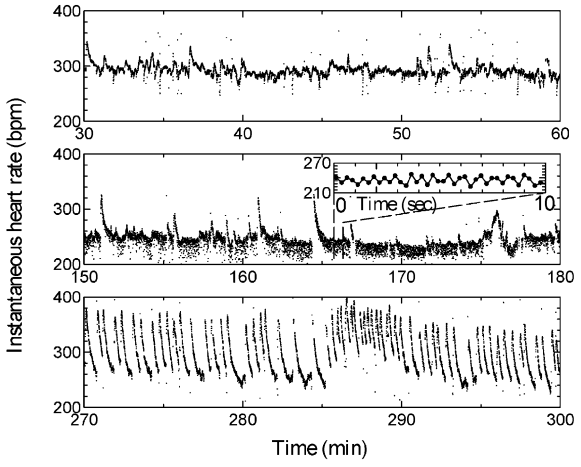

Fig. 4. Time-expanded recordings of IHR, extracted from 30-min period indicated by arrows in (E) of Fig. 3. From top to bottom are shown representative patterns of IHR fluctuations during pre-exposure control, exposure and post-exposure control periods, respectively. The inset in the middle panel shows 10-s time-expanded recording of IHR baseline indicated by broken lines in the lower 30-min recording.

eliminated by hyperoxic exposure irrespective of age (Fig. 2) and of individuals in the same group of age (Fig. 3) except recordings shown in (D) and (E) of Fig. 3. Fig. 4 presents time-expanded 30-min recordings of pre-exposure control, hyperoxic exposure and post-exposure control indicated by arrows in (E) of Fig. 3, showing representative patterns of IHR fluctuations. HR baseline in the middle panel oscillated as shown in inset. The oscillatory frequency was determined 1.4 Hz by PSA.

3.3. Heart rate responses to hypoxia

Responses of IHR to 10% O_2/90% N_2 were investigated in six 16-d, five 18-d, seven Pre-IP, six IP and nine EP embryos. Representative responses of IHR in individual groups of age are shown in Fig. 5. A 16-d embryo dropped HR baseline during hypoxic exposure. $MHR_{2\text{-}h}$ of this embryo was 275 ± 4 (S.D.) ($N = 31{,}231$), 250 ± 5 (29,675) and 256 ± 3 (30,591) bpm for pre-exposure control, hypoxic exposure and post-exposure control, respectively. $MHR_{2\text{-}h}$ decreased with hypoxic exposure in five other embryos and did not return to the initial pre-exposure baseline after returning back to air. The drop by hypoxic exposure without subsequent recovery in 16-d embryos was statistically significant (Table 2). Table 2

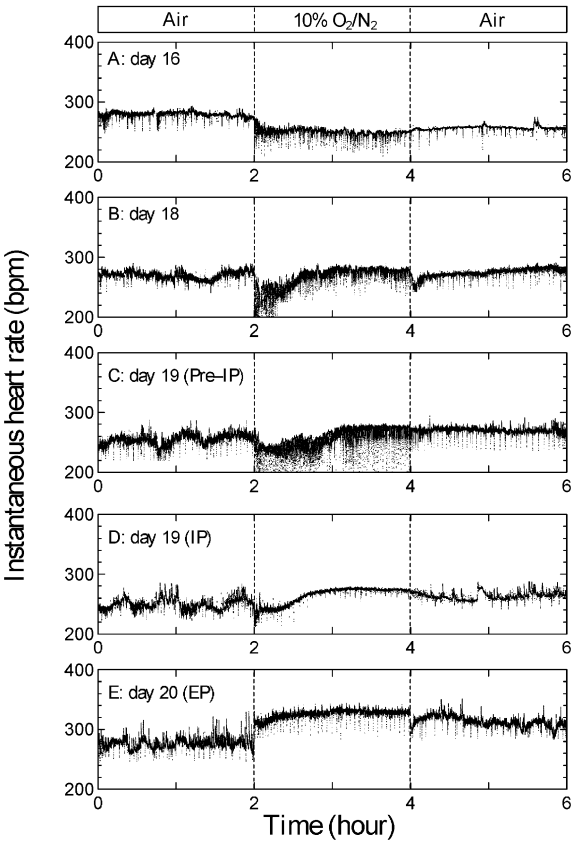

Fig. 5. Examples of IHR response to hypoxic exposure, examined in 16-d (A); 18-d (B); 19-d Pre-IP (C); 19-d IP (D); 20-d EP (E) embryos. Gaseous environments were changed at time indicated by dotted lines. During the middle 2-h period, the embryos were exposed to 10% O_2/90% N_2.

summarizes MHR_{mean} of pre-exposure control, hypoxic exposure and post-exposure control in all ages examined.

HR baseline was also decreased transiently by hypoxic exposure in a 18- and 19-d Pre-IP embryos

Table 2
Mean value $\pm$ S.D. of MHR during exposure to air, 10% O_2 and air, determined for 5–9 embryos on individual days of incubation

Day	*N*	Air	10% O_2	Air
16	6	287 ± 18	261 ± 17^a	268 ± 18^b
18	5	282 ± 24	277 ± 27	282 ± 26
19 (Pre-IP)	7	263 ± 11	269 ± 11	268 ± 11
19 (IP)	6	253 ± 30	271 ± 24^a	262 ± 22
20 (EP)	9	285 ± 45	353 ± 40^a	299 ± 49^a

[a] Significant difference from the preceding value.
[b] Significant difference from pre-exposure control.

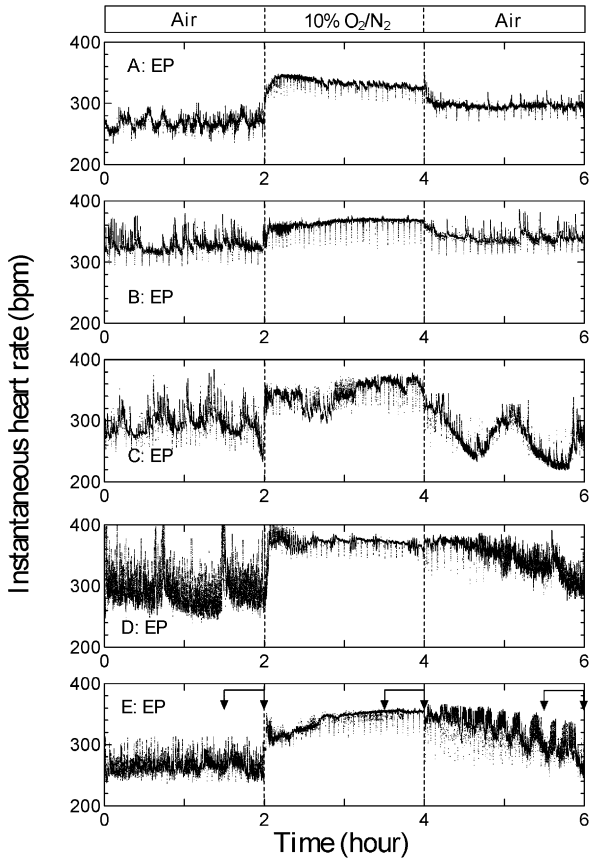

Fig. 6. IHR responses to hypoxic exposure, examined in five other 20-d EP embryos (A–E). Arrows indicate 30-min recordings of IHR that are shown in Fig. 7.

but it returned to the pre-exposure level during hypoxic exposure (Fig. 5B and C). This trend was recorded in some other 18- and 19-d pre-pipped embryos. However, $MHR_{2\text{-}h}$ was not statistically different between three groups (Table 2).

In 18- and 19-d pre-pipped embryos, hypoxic exposure tended to augment the magnitude and frequency of IHR decelerations (Fig. 5B and C). This trend was recorded in other Pre-IP embryos.

When embryos pipped internally the CAM and externally the eggshell, hypoxic exposure caused an elevation in HR baseline (Fig. 5D and E). Fig. 6 presents elevated HR baseline in response to hypoxic exposure in five other EP embryos. This elevation of HR baseline during hypoxic exposure was statistically significant (Table 2).

In IP and EP embryos, hypoxic exposure tended to suppress HR fluctuations (Figs. 6 and 7). Fig. 7 shows time-expanded 30-min recordings of IHR of the EP embryo during the periods indicated by arrows in (E) of Fig. 6. HRI that occurred during pre-exposure control (top panel of Fig. 7) were typical patterns of IHR in late embryos as also shown in Fig. 4 and suppressed during hypoxic exposure that elevated HR baseline and induced HR oscillation as shown in inset (middle panel). The frequency was determined 1.7 Hz by PSA. In this EP embryo, short-term repeated large accelerations of HR, indicating imminent hatching, occurred during post-exposure control period (bottom panel).

4. Discussion

4.1. Identification of IP

Transition from diffusive gas exchange by the CAM to convective respiration by the lungs takes place during the pipping period that begins with IP. In the present study, identification of IP of embryos living in the intact eggshell was made by detecting the breathing signal (ARG) with the condenser microphone (Fig. 1). Although IP could be conventionally identified by candling the egg or by listening for cheeping sound of the embryo, detection of ARG with the microphone was more reliable than sensuous means for identification of IP. Initiation of IP was not always made on day

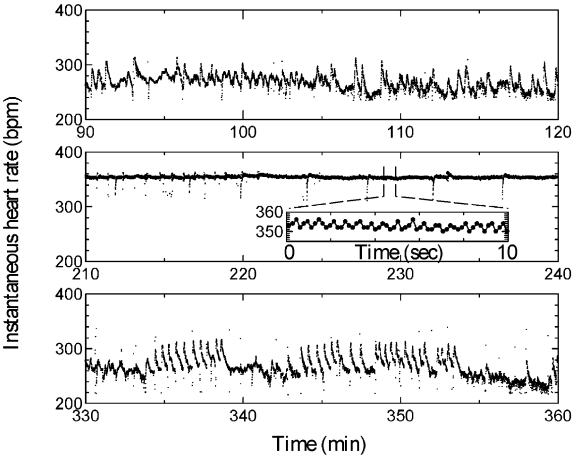

Fig. 7. Time-expanded recordings of IHR, extracted from 30-min period indicated by arrows in (E) of Fig. 6. From top downwards are shown representative patterns of IHR fluctuations during pre-exposure control, exposure and post-exposure control periods, respectively. The inset in the middle panel shows 10-s time-expanded recording indicated by broken lines in the upper 30-min recording.

19 of incubation, but delayed to day 20 in some embryos. Using the microphone detecting system of IP, we successfully collected both Pre-IP and IP embryos on day 19 of incubation.

4.2. Responses of baseline HR to altered O_2 environments

In chick embryos, IHR fluctuates pronouncedly towards hatching, thus it is difficult to determine baseline HR. However, time-compressed recording of IHR implies grossly the baseline HR of which change in altered O_2 environments can be noticed visually. For numerical comparison, $MHR_{2\text{-}h}$ was calculated and used as baseline HR.

4.2.1. Responses to hyperoxia

4.2.1.1. Pre-pipped embryos. Before initiation of convective respiration by the lungs, the baseline HR of 16-, 18- and 19-d Pre-IP embryos remained unchanged in response to hyperoxic exposure as already observed in early (days 3–9) embryos (Akiyama et al., 1999b) and young (days 14–16) embryos (Tazawa, 1981) (Fig. 2A–C, Table 1). During the last periods of incubation prior to IP, the shell gas conductance and the diffusing capacity of the CAM limit oxygen uptake ($\dot{M}_{O_2}$) so that the mass-specific $\dot{M}_{O_2}$ of late pre-pipped embryos tends to decrease (Ar et al., 1987). Hyperoxic exposure increases the O_2 tension gradient across the eggshell, relieving the diffusion-limited gas exchange and increasing $\dot{M}_{O_2}$ in the late pre-pipped embryos (Ar et al., 1991; Stock et al., 1985; Hoiby et al., 1983; Tazawa et al., 1989). Although the O_2 tension gradient across the eggshell and $\dot{M}_{O_2}$ in the present late pre-pipped embryos were also expected to increase in the hyperoxic environment, the baseline HR did not change in response to elevated O_2 level. The absence of depressant responses to hyperoxia in pre-pipped embryos suggests that the heart is beating at a maximum in air and any increase in O_2 levels in the environment will not stimulate an increase in HR. Thus, there is no hypoxic drive to HR in the late pre-pipped embryos as well as in early and young pre-pipped embryos (Tazawa, 1981; Akiyama et al., 1999b).

4.2.1.2. Pipped embryos. During the pipping period when lung breathing began, hyperoxic exposure resulted in a decrease in the baseline HR. This suggests that the hypoxic drive may start with convective gas exchange by the lungs.

In atmospheric air, developmental patterns of baseline HR increased during IP with subsequent temporal decrease during early period of EP and marked increase during the last stage of EP towards hatching (Moriya et al., 2000; Tazawa et al., 2002). The scope of changes in baseline HR during EP extended over 100 bpm. This large change during EP caused large difference between MHR_{mean}s in air of EP embryos shown in Tables 1 and 2.

Some breeds of the domestic fowl including broiler used in the present study exhibited vagal tone as early as 70% of incubation (Höchel et al., 1998; Crossley et al., 2003; Dzialowski, unpublished data), although white leghorn chickens did not possess vagal tone until hatching (Crossley and Altimiras, 2000). Sympathetic nervous function may have started when irregular accelerations of IHR appeared later and the elevated HR baseline during the last stage of EP (Moriya et al., 2000) may be the result of the sympathetic nervous function that preponderates over the vagal tone and contributes to breathing and hatching activities. It may be inferred that hyperoxic exposure of pipped embryos increases cholinergic input from the vagus nerve that then preponderates over the sympathetic nervous function and decreases HR baseline (Fig. 2D and E, Fig. 3, Table 1).

4.2.2. Responses to hypoxia

4.2.2.1. Pre-pipped embryos. In early and young chick embryos in non-fenestrated eggs, HR was decreased by hypoxic exposure (Akiyama et al., 1999b; Tazawa, 1981). In the present study, the baseline HR of 16-d pre-pipped embryos also decreased with hypoxic exposure (A of Fig. 5). Hypoxic bradycardia was also reported for young pre-pipped embryos in fenestrated eggs that were exposed to short-term (i.e. 5-min) hypoxia (Van Golde et al., 1997; Crossley et al., 2002). It was suggested that hypoxia may cause bradycardia due to the direct action of low O_2 on the cardiac muscle.

Meanwhile, in the present experiment, the response of HR baseline in the late pre-pipped embryos (i.e. 18- and 19-d Pre-IP embryos) were different from that in young embryos (Fig. 5B and C). The early depression of HR baseline may be

due to the direct action of low O_2 as in the young embryos and then prolonged hypoxic exposure may increase catecholamine levels in late embryos, elevating HR baseline to pre-exposure control level. Elevated levels of catecholamines have been reported in late chick embryos after 5 min exposure to 10% O_2 (Crossley et al., 2002).

4.2.2.2. Pipped embryos. As embryos developed towards hatching and pipped the CAM and the eggshell accompanied with augmentation of lung breathing, the hypoxia induced more pronounced increase in baseline HR (Fig. 5D and F, Fig. 6), probably due to increases in the adrenergic response or increases in adrenaline (Crossley et al., 2002). As a result, the hypoxic depressant effects on MHR observed in pre-pipped embryos were not induced in pipped embryos in non-fenestrated eggs, while pipped embryos in fenestrated eggs decreased MHR in response to 5-min exposure to 10% O_2 (Crossley et al., 2002). We do not know whether fenestration of the eggshell caused the inconsistent results of hypoxia in pipped embryos. Long period of exposure to hypoxia was another difference from the previous study (Crossley et al., 2002). Eventually, the present experiment did not validate previously obtained evidence of the hypoxic depressant effect on MHR in pipped embryos. The reason for the difference remains to be studied.

4.3. Responses of heart rate fluctuations to altered O_2 environment

4.3.1. Responses to hyperoxia

Hyperoxic exposure did not induce dominant changes in HR fluctuation patterns in either pre-pipped or pipped embryos (Fig. 2) except a few EP embryos (Fig. 3D and E). In these EP embryos, IHR fluctuations changed during hyperoxic exposure (D) or post-exposure control (E). In (D), HR baseline was elevated sporadically during pre- and post-exposure control periods. The large fluctuations with sporadic elevation of HR baseline were caused by short-term repeated large accelerations that were distinctive patterns indicating imminent hatching (Tazawa et al., 1999). Hyperoxia tended to diminish these short-term repeated large accelerations (Fig. 3D). The large fluctuations with transient elevation of HR baseline also appeared during the post-exposure control period (Fig. 3E, and bottom panel of Fig. 4). HR fluctuations with irregular accelerations and decelerations shown in top panel of Fig. 4 were typical patterns of IHR in late embryos. During hyperoxic exposure (middle panel), HR baseline dropped and began to oscillate. The oscillatory frequency of 1.4 Hz (period of 0.7 s) was within a range of breathing frequency determined previously for EP embryos (Chiba et al., 2002). Thus, this HR oscillation might be due to RSA mediated by increased cholinergic input from the vagus nerve in hyperoxia. Upon exposure to air (bottom panel), HR baseline was elevated and large accelerations occurred frequently. The two distinctive patterns of HR fluctuations, i.e. irregular intermittent accelerations during the first 15-min period and last 10-min period and short-term repeated large accelerations during approximately 5 min-period between them, normally occurred in EP embryos at term of incubation (Tazawa et al., 1999).

4.3.2. Responses to hypoxia

Hypoxia tended to diminish irregular accelerations of HR in pre-pipped and pipped embryos (Figs. 5 and 6). In late pre-pipped embryos, in addition to diminution of irregular HR accelerations, hypoxia augmented transient decelerations of IHR (Fig. 5B and C). Particularly, transient HR decelerations were augmented in magnitude and frequency during hypoxic exposure in 19-d Pre-IP embryos.

In pipped embryos, hypoxic diminution of irregular accelerations of IHR appeared to be more pronounced in contrast with large magnitude of accelerations in air (Fig. 5D and E, Fig. 6). The large HR accelerations observed during the pre-exposure control period (Fig. 6D) were the patterns categorized as the same as those shown in (D) and (E) of Fig. 3. These acceleration patterns were thoroughly suppressed by hypoxic exposure.

Although the HR fluctuations were suppressed and HR baseline was elevated by hypoxic exposure, HR oscillated (middle panel of Fig. 7). This HR oscillation might be due to RSA, indicating the function of vagus nerve even during hypoxic exposure.

Because the transient, rapid HR decelerations were blocked by atropine administration (Höchel et al., 1998), the augmented HR decelerations in Pre-IP embryos during hypoxic exposure might be mediated by vagus nerve function. Simultaneously, adrenaline or adrenergic response in pipped embryos increased in hypoxia (Crossley et al., 2002),

causing pronounced increase in HR baseline of hypoxic pipped embryos. The elevated HR baseline seen in hypoxic EP embryos might reach nearly maximum, thus IHR fluctuations originated from HR accelerations appeared to be depressed by hypoxia.

5. Conclusions

A primary goal of the present study was to determine the HR responses to prolonged hyperoxic and hypoxic exposures of pre-pipped and pipped chick embryos living in the intact eggshell (non-fenestrated egg). In order to divide 19-d embryos into Pre-IP and IP embryos, the IP detecting system employing the condenser microphone was for the first time successfully used. HR responses to altered O_2 environments were different between pre-pipped and pipped embryos. In hyperoxic environment, HR baseline of pre-pipped embryos remained unchanged, as found previously in embryos between 3 and 16 d of age (Akiyama et al., 1999b; Tazawa, 1981). For pipped embryos, hyperoxia depressed HR baseline with inducement of RSA, probably due to increased cholinergic input from the vagus nerve. In hypoxic environment, HR baseline of 16-d pre-pipped embryos was depressed, that of 18-d and Pre-IP embryos remained unchanged and pipped (IP and EP) embryos elevated HR baseline. Depression of HR baseline in 16-d embryos was the same as reported previously (Van Golde et al., 1997; Crossley et al., 2002), which might be due to the direct action of low O_2 on the cardiac muscle. Low O_2 depressed HR baseline in 18- and 19-d pre-pipped embryos during early period of exposure and afterwards tended to elevate it, probably due to adrenergic responses induced by prolonged exposure. In EP embryos, HR baseline was markedly elevated during 2-h hypoxic exposure, which was different from the previous result obtained from 5-min exposure of 20-d pipped embryos in fenestrated eggs (Crossley et al., 2002). Exposure to longer periods of hypoxia might result in increased levels of catecholamines in advanced pipped embryos. An emphasized feature of the present study was to present cardiac rhythms as fluctuation patterns of IHR. In comparison with hyperoxia, hypoxia caused pronounced changes in IHR fluctuations that may be related to status of embryonic development and amenity.

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