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Heart rate responses to altered ambient oxygen in early (days 3–9) chick embryos in the intact egg

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Abstract Normal heart rate (HR), and the HR responses to hypoxia and hyperoxia during early heart development in chick embyros have not been studied in detail, particularly in undisturbed embryos within the intact egg. HR was measured in day 3–9 chick embryos at 38 °C using relatively noninvasive impedance cardiography. Embryos were exposed to air (control) and to hypoxic (10% O_2) or hyperoxic (100% O_2) gas for a 2-h or 4-h period, during which HR was continually monitored. Control (normoxic) HR increased from about 150 beats per min (bpm) on day 3 to about 240 bpm on days 7–9. HR in very early embryos showed a variety of moderate responses to hypoxia (all survived), but as development progressed beyond day 6, hypoxic exposure induced a profound bradycardia that frequently terminated in death before the end of the measurement period. In contrast to the marked developmental changes in hypoxic sensitivity, HR showed little response to hyperoxia throughout development, suggesting no "hypoxic drive" to HR. We speculate that hypoxia has little effect early in development because of the embryo's small absolute O_2 demand, but as the embryo grows, hypoxia represents a progressively more severe perturbation. Although general trends were identified, there was considerable variation in both HR and HR responses to ambient O_2 changes between individuals of the same developmental stage.

Key words Early chick embryos · Heart rate/heart rate responses · Hypoxia/hyperoxia · Development · Bradycardia

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Abbreviations *ACG* acoustocardiogram · *BCG* ballistocardiogram · *bpm* beat per min · *ECG* electrocardiogram · *FFT* fast Fourier transform · *HR* heart rate · HR_1 mean heart rate for 1-min period · HR_{air} averaged value of HR_1 for 30-min period in air · HR_{hyper} averaged value of HR_1 for 2-h measurement period in 100%O_2 · *ICG* impedance-cardiogram · *IHR* instantaneous heart rate · *NF* Nieuwkoop-Faber stage · *SD* standard deviation

Introduction

The heart of chick embryos changes from a primordial tubular structure into its four-chamber configuration during the first 8 days of incubation. The heart begins to beat after about 30 h of incubation and the HR increases asymptotically with embryonic development early in incubation to a plateau middle in incubation (Tazawa and Hou 1997). Previous measurements of HR in early (< 8 days) embryos were made after opening the eggshell, because other cardiovascular parameters were being measured through invasive techniques with HR only being measured secondarily (Van Mierop and Burtuch 1967; Girard 1973; Clark and Hu 1982; Hu and Clark 1989, Zahka et al. 1989; Keller et al. 1990; Van Golde et al. 1996). These invasive measurements have helped elucidate development of cardiovascular function and HR in very early embryos soon after the heartbeat is initiated, under both normal and altered ambient conditions. However, gas exchange of embryos takes place by molecular gas diffusion through porous eggshell between surrounding air and blood in the area vasculosa early in incubation and in the chorioallantoic membrane later. Thus, partial removal of the eggshell and inner shell membrane and subsequent rupture of the chorioallantoic membrane, employed in cardiovascular measurements in embryos, may directly disturb gas exchange (or indirectly disturb gas exchange through the effects of disrupted vasculature and hemodynamic

effects) even during early incubation when area vasculosa serves as the primary gas exchanger.

The important contribution of the undisturbed eggshell to embryonic gas exchange suggests an investigation of normal daily changes in embryonic heart rate (HR) during early development, with gas exchange occurring through the intact eggshell and area vasculosa/chorioallantoic membrane. In addition, investigation of embryonic HR responses to altered gaseous environment also needs to be made under natural, undisturbed conditions of gas exchange through the eggshell and area vasculosa/choriallantoic membrane. Noninvasive measurements of HR in avian embryos have been previously made by detecting cardiogenic ballistic movements of the egg (BCG) and cardiogenic acoustic pressure changes outside the eggshell (ACG) (see references: Akiyama et al. 1997; Ono et al. 1997). Because of technical difficulty in detecting the BCG and ACG from early embryos, such measurements have been made primarily during the last half of incubation. Although the electrocardiogram (ECG) and impedance-cardiogram (ICG) are measured somewhat invasively by wire or needle electrodes implanted inside an egg (Laughlin et al. 1976; Laughlin 1978; Vince et al. 1979; Tazawa and Rahn 1986; Burggren et al. 1994; Haque et al. 1994; Tazawa et al. 1994; Howe et al. 1995; Pirow et al. 1995), the mean HRs determined from ECG and ICG are not different from those determined from BCG and ACG in chicken eggs (Haque et al. 1994). Although the use of ECG and ICG thus appears suitable for the investigation of early HR, with the exception of Cain et al. (1967) all studies employing this technique have focused exclusively on later embryos. Morevoer, Cain et al.'s (1967) measurements began on day 4 which is 2–3 days after the heart beat has begun, and during a developmental phase in which the HR appears to be increasing relatively rapidly. Thus, measurement of the complete developmental pattern of HR during the very early period of incubation for day 3 onwards in the intact egg remains to be made.

The present study employs ICG to extend our knowledge of embryonic HR and its changes in unfenestrated eggs into the period encompassing day 3, and covering the period of days 3–9 of incubation. In addition, we investigate the HR responses of these very early embryos to both hypoxic and hyperoxic exposure at the time that the premature heart is forming into a more mature four-chambered heart. We hypothesize that very early embryos will show little or no reflex-HR changes because of the immaturity of their neural and endocrine cardiac regulatory mechanisms. Additional important components of this study involve both the dynamics (time course) of the HR responses to ambient O_2 changes and the HR variations between individuals at a given developmental stage. Previous studies of HR have not generally described differences in time course or values between individuals within a given stage. Such physiological variation within populations is becoming of increasing interest to physiologists, particularly as it pertains to physiological development (see Burggren et al. 1994), and the present study indicates interesting patterns of such variability.

Materials and methods

Fertile eggs of chickens (*Gallus domesticus*) were incubated at 38 °C and about 60% of relative humidity in a forced-draft incubator. The eggs were turned automatically every 3 h. The stage of embryonic development was based upon the Hamburger-Hamilton method (Hamburger and Hamilton 1951) after postmortem examination.

On the day of HR measurement, eggs were candled to locate the embryo and two sites on the eggshell over the embryos were marked. Because it was difficult to locate the embryo by candling the egg before 2 days of incubation when the ICG could not be detected, the measurement of ICG was made from 3 days of incubation (referred to as day 3). A pair of needle electrodes were inserted into the egg marked previously and fixed onto the shell with an epoxy glue, as described by Tazawa et al. (1994). The egg with electrodes was put in a small, gas-tight measuring chamber (150 ml) having an electrode socket for making external electrical connections. The chamber also had gas conduits for introducing air or a test gas mixture through a Y-conduit at a rate of about 30 ml/min via a vinyl tube 2-m long connected to a gas cylinder. The measuring chamber was placed in a still-air incubator warmed at 38 °C and vented with air by a gas pump that was put in the incubator before and during the control measurement. The vinyl tube from the gas cylinder was also put in the incubator to warm the test gas mixture. A vinyl tube from the chamber was extended outside the incubator. No particular humidification of the test gas mixture was made. The electrode leads from the electrode socket were connected to an impedance converter (model 2991, UFI, USA). The output from the converter was amplified, bandpass-filtered, and digitized by an A/D converter at 50 Hz and entered into a micro-computer for calculation of HR.

The mean HR measured over a 1 min period (HR_1) was determined by a power spectral analysis employing fast Fourier transform (FFT). In order to determine the HR_1 with an error less than 1 bpm, the power spectrum had to be analyzed by FFT for $2^{12} = 4096$ sampled points. Because the ICG waves were sampled at 50 Hz and thus 3000 points taken for 1 min, the remaining 1096 points were substituted by zero and then the power spectrum was analyzed. A frequency corresponding to a maximum peak of the power sepctrum was converted to HR_1. Similarly, HR_1 was calculated for each successive 1-min period for as long as 120 or 240 min, and then plotted as a series of points on a graph against time.

The egg in the chamber was vented by air during the 1st 1 h to ensure temperature equilibration of the egg with the 38 °C environment. HR was measured for the next 30-min period and 30 HR_1's were averaged to yield HR_{air}. Then, the air in the chamber was replaced with 10% O_2/N_2 (hypoxia) or 100% O_2 (hyperoxia), a replacement taking 2–4 min, and HR was continuously determined either for 4 h (hypoxia) or 2 h (hyperoxia). Though chronic exposure to hyperoxia at this level can be toxic through free-radical elevation and other mechanisms, the highly acute exposure employed here is unlikely to have produced any toxic effects.

As the embryos grew, somatic movements and posture changes became larger and more frequent, disturbing ICG detection and limiting HR measurement as day 9 of incubation approached. Even during the above measurement period (days 3–9), ICG signals were frequently contaminated by embryonic movements. Embryos whose ICG signals were contaminated by embryonic movements were measured for only control HR_{air}. Exposures to 10% O_2/N_2 or 100% O_2 were made for the embryos whose ICG signals were relatively uncontaminated by embryonic movements during measurement in air. Eventually, eight and three tests of hypoxic and hyperoxic exposures, respectively, were made for each day except

for day 9 when five tests were performed for hypoxic exposure. A total of 119 embryos were employed in measurement of HR_{air} and the time course of the HR response.

Significance of the difference between control HR (HR_{air}) and HR responded to a test gas mixture was examined by unpaired Student *t*-test with significant level of $P < 0.05$.

Results

Developmental pattern of control heart rate (HR_{air})

Figure 1 shows the developmental pattern of HR_{air} during early stages of embryonic development. The mean HR increased steadily from about 150 bpm on day 3 to 240 bpm on day 7 of incubation, showing small daily changes subsequently. HR_{air} varied among individual embryos on any given day of incubation. The coefficient of variation in HR was 9.5, 8.3, 9.8, 8.0, 5.9, 7.0 and 4.4% for incubation days from day 3 to day 9, respectively. Figure 2 presents HR_{air} plotted against developmental stages of embryos.

HR responses to hypoxia

The time sequence patterns of HR_1 during 4 h of hypoxic exposure varied considerably even among embryos of identical age. Figure 3 presents one example of HR responses for each age, with the specific nature of the variation described below for each age.

During the first 40-min period of hypoxic exposure, the 3-day embryo depicted in the top panel of Fig. 3 decreased its HR gradually from about 140 bpm to about 80 bpm and maintained it at almost the same level

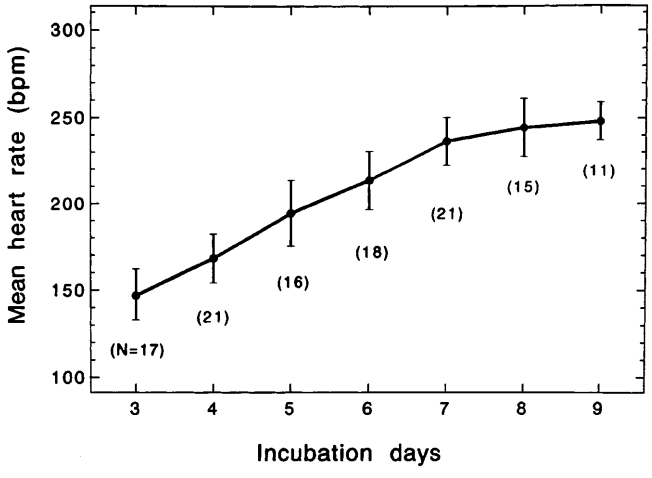

Fig. 1 The developmental pattern of embryonic heart rate (HR) in air (HR_{air}) determined from 119 embryos in total. The number of embryos determined for HR_{air} on each day of incubation is shown in the *parentheses* and a *closed circle* with *vertical line* indicates the averaged value of HR_{air} of developing embryos ± SD. The averaged HR with SD is 147 ± 14 (n = 17), 168 ± 14 (n = 21), 194 ± 19 (n = 16), 213 ± 17 (n = 18), 236 ± 14 (n = 21), 244 ± 17 (n = 15) and 248 ± 11 (n = 11) bpm for days 3–9, respectively

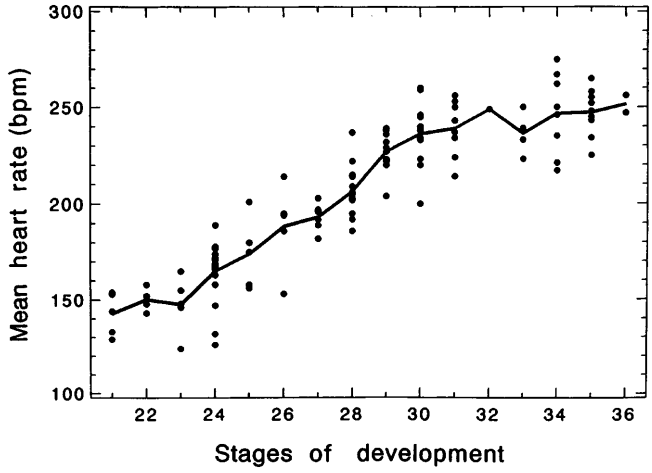

Fig. 2 The developmental pattern of embryonic HR in air plotted against stages of embryonic development. The *solid line* connects averaged value of HR_{air} at each stage of development

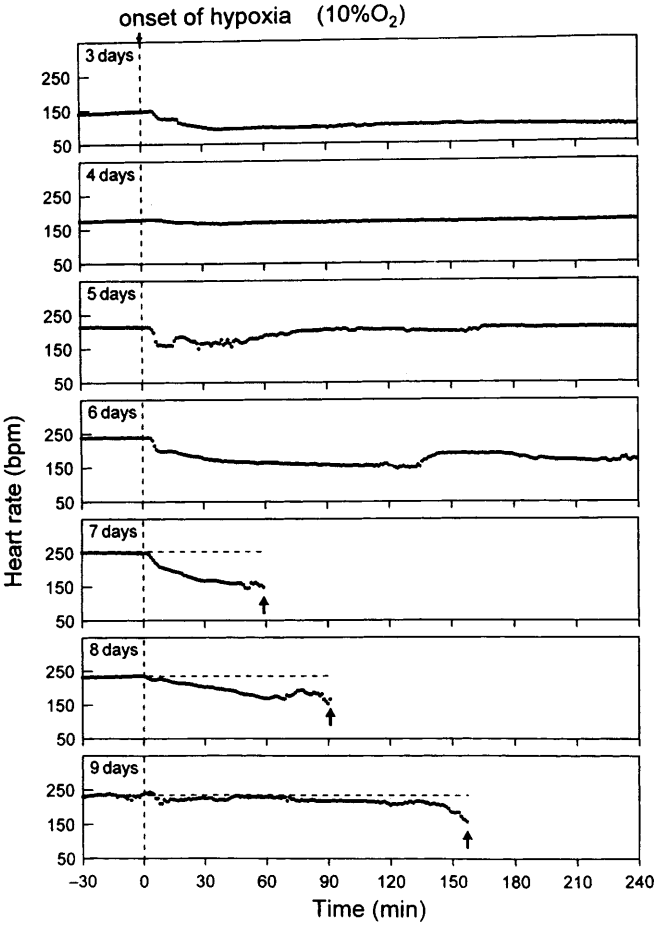

Fig. 3 Time-sequence patterns of embryonic HR before and during exposure to 10% O_2/N_2. A result from one embryo on each incubation day is shown. HR was measured in air for 30 min (HR_{air}) and then the chamber was vented with 10% O_2/N_2 at time 0, shown by "onset of hypoxia". Mean HR_{air} is 143, 170, 214, 238, 250, 235 and 235 bpm for each embryo shown from the top to the bottom panels, respectively. The *horizontal dotted line* indicates the level of HR_{air}, and the *upward pointed arrow* shows the time of embryonic death

until the end of 4-h exposure. During the first 6–7 min exposure, the HR was the same level as in air, which might be due to the time required for replacement of gas mixtures in the measuring chamber. Seven other embryos (HR_{air} = 148 ± 9 SD bpm) showed similar gradual decreases in HR with various changes ranging from about 30 bpm to 60 bpm. All 3-day embryos survived the 4-h hypoxic exposure.

On day 4 there was considerable variation in response to hypoxic exposure. The embryo (second panel of Fig. 3) decreased its HR only slightly from about 170 bpm in air to about 160 bpm during the first 1-h period and maintained it until the end of exposure. Two other embryos (HR_{air} = 132 bpm and 201 bpm, respectively) showed similar insensitivity to 10% O_2. However, four other embryos (HR_{air} = 176 ± 11 bpm) decreased their HRs by about 35–60 bpm during the first 10–20-min period; two of them maintained their HRs at the same level of about 130 bpm and 150 bpm, respectively, during the remaining period; two other embryos gradually increased their HRs from a minimum level of about 110 bpm and 120 bpm 10–20 min after hypoxic onset back towards the air level during further exposure. The remaining one embryo gradually decreased its HR from about 175 bpm in air to about 100 bpm during the first 100 min, then increased its HR by about 20 bpm during the next 100-min period, and finally decreased its HR during the remaining 40-min period. All eight embryos survived the 4-h exposure.

On day 5, HR responses to hypoxia were more consistent, although interindividual variation was still apparent. The embryo in the third panel of Fig. 3 responded to hypoxic exposure with a steep drop in HR from 214 bpm in air to about 160 bpm during the first 10-min period. Then, the HR changed in an irregular fashion and returned to almost the same level as the air control after about 3 h of exposure. However, six other embryos decreased their HR. In four embryos this HR decrease was marked during the first 10 min of exposure, dropping by about 20–40 bpm (HR_{air} = 200 ± 4 bpm). In two embryos it decreased gradually by about 30 bpm and 40 bpm during about 1 h from air control values of 191 bpm and 214 bpm and showed a similar trend of partial recovery towards control HR during the remaining exposure period. The remaining embryo (HR_{air} = 203 bpm) was insensitive to 10% O_2 and did not show a marked change in HR during the 4-h exposure period. All eight embryos tested survived the 4-h exposure.

On day 6, embryos generally responded to hypoxic exposure with an initial steep drop in HR. The embryo in the fourth panel of Fig. 3 responded to hypoxic exposure with a steep drop in HR from about 240 bpm in air to 195 bpm, followed by a gradual decrease to about 155 bpm. A transient increase to about 185 bpm then occurred around 140 min with a subsequent slight decrease. Three other embryos (HR_{air} = 212 ± 8 bpm) showed similar sharp drops in HR by about 40, 50 and 60 bpm soon after exposure, followed by a small transient increase with subsequent plateau (one embryo) or by a plateau without marked change during the remaining period (two embryos). Two other embryos (HR_{air} = 192 bpm and 237 bpm) decreased their HRs more gradually by about 30 bpm and 40 bpm during the first 2 h followed by a small increase (about 15 bpm) or a plateau during the next 2 h. The remaining two embryos (HR_{air} = 200 bpm and 195 bpm) also decreased their HRs by about 30 bpm and 45 bpm soon after exposure. These two embryos died after about 55 min and 65 min of hypoxic exposure.

On day 7, a 4-h hypoxic exposure period proved lethal to all embryos. The embryo in the fifth panel of Fig. 3 decreased its HR by about 100 bpm from 250 bpm in air during the 1st h of exposure. Then, the amplitude of the ICG signal became too small to be detected at the time indicated by the arrow. After 4 h of exposure, the egg was opened and the embryo was dead, showing cyanosis. The remaining seven embryos (HR_{air} = 240 ± 11 bpm) also decreased their the HRs; in five of them a decrease in HR occurred soon afer exposure and was large (50–150 bpm) compared with younger embryos. The ICG disappeared (indicating death) at about 25, 45, 60, 130, 150 (two embryos), 160 and 165 min after the onset of exposure for eight embryos, respectively.

On day 8 all embryos responded to hypoxic exposure with deceased HR and then death. The embryo in the sixth panel of Fig. 3 decreased its HR from 235 bpm in air to about 170 bpm followed by irregular changes during about a 30-min period and died at the time indicated by the arrow. The decrease in HR in six embryos (HR_{air} = 245 ± 16 bpm) was large, ranging from about 60 bpm to 110 bpm, during the survival period of about 60, 90, 95 (two embryos), 100 and 190 min. The remaining embryo decreased its HR by a small amount from 235 bpm in air to about 215 bpm soon after exposure, but suffered from cardiac arrest about 10 min later.

The 9-day-old embryo (bottom panel of Fig. 3) decreased its HR slightly from 235 bpm in air to about 215 bpm soon after hypoxic exposure. Small irregular changes followed during the next 140 min with subsequent decrease to about 150 bpm at around 160 min, after which the heart beating stopped at the time shown by the arrow. Four of five embryos (HR_{air} = 245 ± 14 bpm) responded to hypoxic exposure with relatively small decreases in HR, ranging from 15 bpm to 40 bpm, followed by subsequent additional decreases of 20–50 bpm until the heartbeat stopped at around 10, 45, 85 and 105 min of hypoxic exposure.

HR responses to hyperoxia

In contrast to the marked effects of hypoxic exposure, HR was slightly increased or showed no change in response to hyperoxic exposure on any of the incubation days tested. Figure 4 shows an example of HR responses

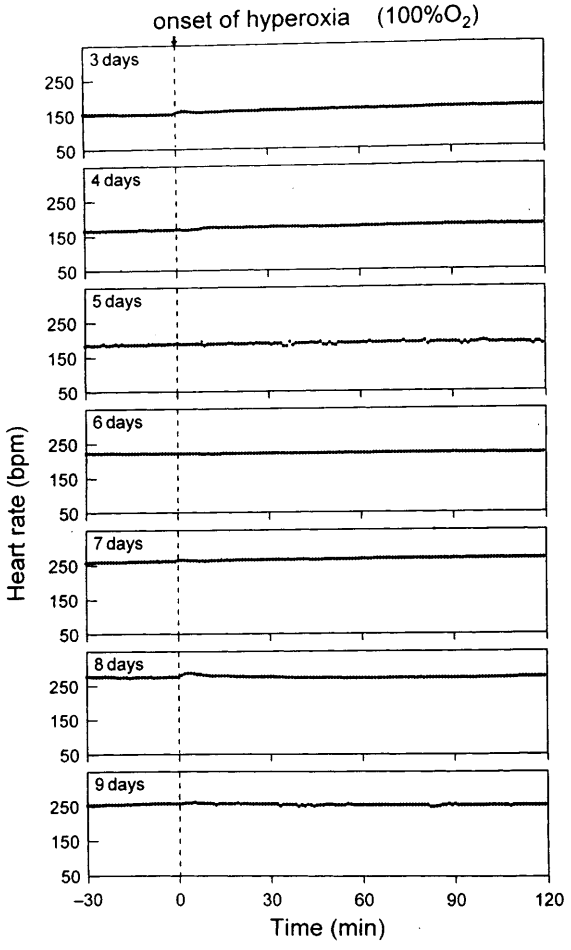

Fig. 4 Time-sequence patterns of embryonic HR before and during exposure to 100% O_2. A result from one embryo on each incubation day is shown. After measurement of HR in air during a 30-min period, embryos were exposed to 100% O_2 at time 0, shown by "onset of hyperoxia"

of embryos to 100% O_2 exposure for 2 h; the HR was averaged for a 2-h period of hyperoxic exposure and referred to as HR_{hyper}. Table 1 summarizes the averaged HRs before and during exposure; i.e., HR_{air} and HR_{hyper}, detemined for three embryos on each day of incubation from day 3 to day 9. The HR_{air} and HR_{hyper} calculated for Fig. 4 are shown on the first line in Table 1. Because the variation of HR_1 in air and in 100% O_2 was small, the slight difference between HR_{air} and HR_{hyper} tended to be significant.

Embryonic mortality during hypoxic and hyperoxic exposures

Figure 5 indicates the mortality associated with the experimental conditions imposed during the experiments. Essentially, measurement of HR by impedance techniques caused no mortality during either normoxic or acute hyperoxic exposure at any developmental stage. From day 3 to day 5, 10% hypoxic exposure similarly

Table 1 The averaged value of heart rate (HR) ± SD determined for 30 min in air (HR_{air}) and for 120 min in 100% O_2 (HR_{hyper}). HR_{air} ($n = 30$), HR_{hyper} ($n = 120$)

Days	3		4		5		6		7		8		9	
Egg	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}	HR_{air}	HR_{hyper}
1	152 ± 0	162 ± 3	166 ± 0	175 ± 2	186 ± 1	189 ± 2	221 ± 0	221* ± 0	259 ± 1	266 ± 2	265 ± 0	265* ± 2	254 ± 1	254* ± 1
2	147 ± 1	157 ± 3	165 ± 0	165* ± 2	193 ± 0	193* ± 0	204 ± 1	216 ± 2	230 ± 1	232* ± 2	246 ± 2	253 ± 2	248 ± 2	252 ± 1
3	147 ± 2	153 ± 1	168 ± 2	177 ± 2	202 ± 0	206 ± 1	214 ± 1	218 ± 0	232 ± 4	241 ± 3	250 ± 1	251* ± 0	259 ± 1	266 ± 2

* Insignificant difference ($P > 0.05$) determined by unpaired *t*-test

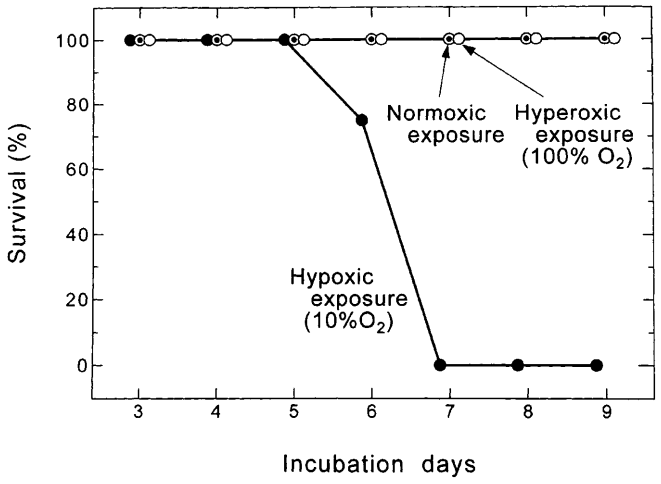

Fig. 5 Mortality during HR measurement in normoxia (control, 21% O_2), as well as acute exposure to hypoxia (10% O_2, 4 h) and hyperoxia (100% O_2, 2 h) as a function of development in day 3–9 chick embryos

caused no mortality, but by day 7 and above the hypoxic exposure for 4 h proved lethal to all embryos.

Discussion

Critique of the method

Because ICG is measured by supplying an electrical current and detecting impedance changes in nearby tissues, the pair of electrodes should be placed close to the heart. Thus, it is important to locate the embryo within the eggshell before electrode implantation, particularly early in incubation when the embryo is small. Because it was difficult to locate the embryo and thus to find adequate sites for electrode insertion earlier than 3 days of incubation, we could not detect the ICG from these early embryos through the eggshell. This may be an ultimate limitation of HR measurement with intact eggs using ICG.

ICG was recorded every heartbeat, so that instantaneous HR (IHR) (i.e., beat-to-beat) could be determined. However, ICG, which is slow wave, was distorted by high source line frequency, somatic movements of embryos and other unknown noises and consequently IHR sometimes scattered. Thus, we carried out power spectral analysis of recorded signals and determined the mean HR for each 1-min period. As the embryos grew, somatic movements became large and frequent, which further contanimated the ICG signals. Consequently, it became difficult to determine the mean HR for each min in embryos older than 9 days. In addition, interruption by somatic movements became more serious during hypoxic exposure than in air, thus limiting the measurement of HR responses to hypoxic exposure.

Implantation of impedance electrodes in the egg through the shell and the associated electrical current flowing through older embryos does not affect HR determination (Haque et al. 1994). Possible adverse effects of these factors on the HR for early embryos are not known. However, the extremely small size of the embryos relative to the area in which electrodes were inserted, combined with the very high survival rate of the embryos, suggests that embryos were not directly affected by electrode insertion through the eggshell.

Changes in HR with development

Considerable variation existed between individuals on each incubation day (Fig. 1). Because the developmental stage of embryos differed between individual eggs even on the same incubation days, the HR_{air} of individual embryos was plotted against their developmental stage (Fig. 2). The variation was still dominant, indicating that the considerable variation of HR_{air} on the same incubation day was not due to the developmental stages which varied between the individual embryos on the same incubation days. Mean HR_{air} was almost identical with the averaged values of HR measured previously in fenestrated eggs on days 3 and 4, but became larger than the latter as incubation advanced. The averaged HR values of fenestrated eggs on 3–9 days of incubation in seven previous reports are summarized in Table 2 (Van Mierop and Bertuch 1967; Girard 1973; Clark and Hu 1982; Clark et al. 1986; Hu and Clark 1989; Zahka et al. 1989; Keller et al. 1990; Van Golde et al. 1996). A common trend in all reports is for HR to increase as the primordial heart forms a more mature four-chambered structure. However, with further development after the first half of incubation, the difference in HR between non-fenestrated eggs determined with ECG, ICG and BCG (Cain et al. 1967; Laughlin et al. 1976; Tazawa et al. 1991; Howe et al. 1995) and fenestrated eggs be-

Table 2 The averaged HR values (in bpm) of fenestrated eggs reported previously (*A* Girard 1973; *B* Van Mierop and Bertuch 1967; *C* Clark and Hu 1982; *D* Clark et al. 1986; *E* Zahka et al. 1989; *F* Keller et al. 1990; *G* Van Golde et al. 1996)

Days	3	4	5	6	7	8	9
A	138	–	191	200	222	213	212
B	141	160	179	188	203	217	213
C	165	183	188	–	–	–	–
D	146	172	183	208	–	–	–
E	148	169	174	193	–	–	–
F	131	162	–	–	–	–	–
G	–	–	–	–	–	195	190
Average ± SD	145 ± 11	169 ± 8	183 ± 6	197 ± 8	213 ± 10	208 ± 10	205 ± 11

came large and distinct (Tazawa and Hou 1997). The partial removal of the eggshell in the fenestrated eggs improves O_2 availability through the outer diffusion barrier (i.e., the eggshell and outer shell membrane), but subsequent injury or rupture of the chorioallantoic membrane made for experimental procedures to investigate cardiovascular functions reduces the diffusing capacity of the inner diffusion barrier (mainly the chorioallantoic membrane and O_2 reaction with capillary blood, Tazawa et al. 1976) and thus the arterialized blood oxygen tension. In late chick embryos, the restricted O_2 supply decreased the HR (Laughlin et al. 1976; Tazawa 1981). Possible evaporative heat loss through a hole opened in the eggshell and the chorioallantoic membrane may be another factor responsible to lower HR in fenestrated eggs. The drop of egg temperature by 1 °C decreased the HR of late chick embryos by about 15–20 bpm (Tazawa and Nakagawa 1985). Additionally, there are large differences in embryonic HR even among studies with non-fenestrated eggs. The HR of early embryos determined by BCG was considerably higher than the present result; 197, 223, 243, 266, 273 and 274 bpm for 4–9 days of incubation (Cain et al. 1967). The HR of embryos 7 days old and older determined by ICG (Howe et al. 1995) was even larger than that determined with ballistocardiography (Cain et al. 1967). Perhaps the lower rates reported in the present study result from the more sophisticated power spectral analysis to generate mean HR. Certainly, the measurement of embryonic HR in non-fenestrated eggs during the early period of incubation still remains to be made with different techniques of both heart beat measurement and mean HR calculation.

HR responses to hypoxia and hyperoxia

The HR response to 10% O_2 exposure varies among individual embryos. Early embryos having a primordial heart (3–5 days old) were less sensitive to hypoxia in terms of HR response and could stand the hypoxic exposure for a prolonged period (4 h). On day 6, six out of the eight embryos could tolerate a 4-h hypoxic exposure. In contrast, embryos whose heart had developed to a more complete form (7–9 days old) ceased heart beat during 4 h exposure. During the first 8–9 days of incubation, the yolk sac is well vascularized in its medial region forming the area vasculosa, which is a gas exchanger in early embryos. During this period, the blood O_2 affinity decreases with embryonic development (Lapennas and Reeves 1983). The high O_2 affinity of 4- to 6-day-old embryos may partially contribute to hypoxic tolerance. In addition, early embryos having an immature heart may be more able to utilize anaerobic metabolism in order to form the heart with less risk, given the immaturity of the developing gas exchanger. Premature embryos may be provided with a capacity of tolerance to hypoxic environment to protect the vulnerable developing tissues from the damages caused by the action of reactive O_2 species (Ar and Mover 1994).

Hyperoxic exposure tended to increase HR, but the increases were small (10 bpm at the most, Table 1). Tazawa (1981) reported similar findings in late embryos in the range of 14–16 days, where HR determined from arterial blood pressure recordings changed little during 15 min exposure to various degrees of hyperoxia including 100% O_2.

In conclusion, our data collectively indicate that early in development there is only a very slight HR-sensitivity of embryos to changes in ambient O_2 (particularly to hypoxia). However, as the mass of the embryo increases with development, embryos become more and more sensitive to hypoxia, until it becomes a lethal experience after about 6–7 days of incubation, as indicated in Fig. 5. Certainly, the increasing mass and O_2 demand of the embryos with continuing growth, combined with a possible less rapidly growing surface area for gas exchange, prevents adequate gas exchange from occurring in the face of hypoxia. To what extent the bradycardia induced by hypoxia represents an active depression of the heart via neural or hormonal means, as distinct from a direct hypoxic inhibition of cardiac tissue, remains unknown. In this regard, it is interesting to consider the ontogeny of passive vs. active cardiovascular responses to hypoxia in embryos of the clawed frog, *Xenopus laevis*. In early development (NF 45–51) in the frog *Xenopus laevis*, the heart responds to hypoxia with direct depressions of HR, stroke volume and blood pressure (Fritsche and Burggren 1996). However, as development proceeds and cardiac innervation develops, hypoxia actually stimulates stroke volume and blood pressure, although bradycardia remains, for no net change in cardiac output. These findings appear to differ from those found in the present study in the chick embryo, in which there is no evidence of direct metabolic depression of the heart by hypoxia early in development, but significant and even lethal depression of cardiac development later in embryonic development. More detailed studies evaluating lactate concentration in cardiac tissue, along with physiological measures and pharmacological intervention, will be required to determine the root cause of the hypoxic bradycardia in chick embryos during development. Finally, the absence of any significant response to hyperoxia suggests that there is no "hypoxic drive" to embryonic HR, since there is no depression of HR at elevated O_2 levels.

The present study has revealed general trends of change in HR and its response to hypoxia and hyperoxia during development. Yet, there are equally interesting significant variations in both the time course and extent of the response between individuals at the same stages of development. Burggren et al. (1994) have described a "sibling effect" for HR in bird embryos, in which the HR changes during development of siblings are much more similar than in non-siblings. This effect could have either a genetic basis and/or result from so-called "maternal effects", and these phenomena could also be

responsible for the interindividual variation noted in the present study. Ascribing a cause for variations in the cardiac response to hypoxia is beyond the scope of this study, but nonetheless we pose speculative questions such as: "is greater hypoxic survivability conferred upon those individual animals with greater choriallantoic vascularization, greater blood hemoglobin concentration, or lower body mass and overall metabolic rate?" We hope that by highlighting physiological differences in the present study we will stimulate future studies designed specifically to investigate the issue of interindividual physiological variation in developing embryos.

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