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Review

Embryonic control of heart rate: Examining developmental patterns and temperature and oxygenation influences using embryonic avian models☆

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ABSTRACT

Long-term measurements (days and weeks) of heart rate (HR) have elucidated infradian rhythms in chicken embryos and circadian rhythms in chicken hatchlings. However, such rhythms are lacking in emu embryos and only rarely observed in emu hatchlings. Parasympathetic control of HR (instantaneous heart rate (IHR) decelerations) occurs at ~60% of incubation in both precocial and altricial avian embryos, with sympathetic control (IHR accelerations) becoming more prevalent close to hatching. A large increase in avian embryonic HR occurs during hatching (presumably an energetically expensive process, i.e. increased oxygen consumption ($\dot{M}_{O_2}$)), beginning during pipping when a physical barrier to O_2 conductance is removed. Alterations in ambient O_2 have little effect on early embryonic HR, likely due to the low rate of $\dot{M}_{O_2}$ of early embryos and the fact that adequate O_2 delivery can occur via diffusion. As $\dot{M}_{O_2}$ increases in advanced embryos and circulatory convection becomes important for O_2 delivery, alterations in ambient O_2 have more profound effects on embryonic HR. Early embryos demonstrate a wide ambient temperature (T_a) tolerance range compared with older embryos. In response to a rapid decrease in T_a, embryonic HR decreases (stroke volume and blood flow are preserved) in an exponential fashion to a steady state (from which it can potentially recover if re-warmed). A more severe decrease in T_a results in complete cessation of HR; however, depending on developmental age, embryos are able to survive severe cold exposure and cessation of HR for up to 24 h in some instances. The development of endothermy can be tracked by measuring baseline HR during T_a changes. HR patterns change from thermo-conformity to thermoregulation (reverse to T_a changes). Further, IHR low frequency oscillations mediated by the autonomic nervous system are augmented at low T_as in hatchlings. Transitions of baseline HR during endothermic development are unique to individual avian species (e.g. chickens, ducks and emu), reflecting differences in life history.

1. Introduction

Avian embryos have been used extensively in physiological developmental research (see e.g. Burggren and Warburton, 1994; Phoon, 2001; Tazawa, 2004, 2005 for reviews). The model has proved invaluable as eggs are relatively inexpensive and the development of the avian cardiovascular system follows the same ontogenic patterns when compared with humans and mice (see e.g. Phoon, 2001 for review). Further, avian embryos develop relatively free from direct maternal influences (although maternal influences through egg environment persist – e.g. Ho et al., 2011) compared with mammalian embryos and similar questions can be answered using less complex measurement techniques and less invasive surgical techniques (thus minimizing potential effects on physiology) (see Tazawa, 2005 for review). It is also easy to manipulate the surrounding environment of avian embryos compared with mammalian models *in utero*. Prolonged *in situ* instantaneous heart rate (IHR) measurements of avian embryos with unaltered eggshell gas exchange during altered environmental O_2 and ambient temperature (T_a) exposure have allowed insight into the development of IHR fluctuations and the development of HR control (e.g. Tazawa and Hou, 1997; Tazawa et al., 2002b; Tazawa, 2005). Thus, this review will primarily focus on insight into heart rate (HR) regulation gained using the avian model with comparative discussion where appropriate.

2. Determination of avian embryonic heart rate

The ontogeny and development of HR, its fluctuations and patterns have been determined via impedance-cardiogram (ICG) (Akiyama et al., 1999b), acoustocardiogram (ACG) (Tazawa and Hou, 1997; Akiyama et al., 1999a; Pearson and Tazawa, 1999;

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Pearson et al., 2000; Tazawa et al., 2001a; Chiba et al., 2004b; Tazawa, 2005), allantoic arterial blood pressure (Höchel et al., 1998) and electrocardiogram (ECG) (Pearson et al., 1998; Höchel et al., 1999; Moriya et al., 1999, 2000, 2002; Tazawa et al., 1999, 2001b, 2002a; Kato et al., 2002; Chiba et al., 2004a; Fukuoka et al., 2006; Yoneta et al., 2006c; Shah et al., 2010) analysis. HR of embryos grown in shell-less cultures can be assessed visually or with microscopic imaging and software analysis (e.g. Ho et al., 2011). Further, HR can be determined *in ovo* using a micro-camera inserted through a small hole in the eggshell overlying the air cell and sealed in place to minimize effects on eggshell conductance (e.g. Akiyama et al., 2006). For the purposes of this review, HR will be referred to as MHR or IHR depending on the respective (mean heart rate or instantaneous heart rate) data collection method. Further, changes in baseline HR indicate HR change trends (either MHR or IHR) for a specific developmental stage, time period or environmental challenge. A wide HR baseline is indicative of high HR fluctuation/variability; conversely, a narrow baseline indicates a stable HR with low fluctuation. See Tazawa (2005) for a thorough review of the methodologies utilized to determine HR in avian embryos and hatchlings.

It is relatively easy to determine developmental patterns from daily measurements of embryonic MHR obtained at a given time of day (see e.g. Tazawa and Hou, 1997). However, some subtle, yet persistent cardiac rhythms that may be expressed over a relatively long period of time such as infradian, circadian and ultradian rhythms, may fail to be detected. Instead, long-term, continuous measurements of MHR are extremely advantageous as they allow long-term monitoring of HR across development and the elucidation of broad scale developmental patterns. For example, MHR data obtained via continuous acoustocardiography over a 1-min period ($MHR_{1\,min}$) throughout the last half of chicken embryo incubation reveal an infradian rhythm not previously detected in avian embryos (e.g. Fig. 1) (Akiyama et al., 1999a). Measurements of beat-to-beat HR (i.e. instantaneous heart rate; IHR) are required to gain an understanding of how HR fluctuates over shorter time periods.

Having explored methodology behind HR measurement, let us now turn to the physiology of embryonic heart rate and its control.

3. Onset of heartbeat

Beating of the heart is an inevitable requirement to produce convective blood flow for the transport of respiratory gases, nutrients, hormones and metabolic wastes. However, prior to the full formation of the vertebrate heart, and even initially after the forming heart is beating, oxygen (O_2) can be adequately supplied from the environment to the proliferating embryonic cells via diffusion. Thus, convective blood flow and HR are not required for the transport of blood gases between the environment and early embryonic tissues. In fact, early chicken embryos maintain normal O_2 consumption ($\dot{M}_{O_2}$) without blood convection and body development continues normally after cardiac output is interrupted (ligation of the main vessel from the heart) or hemoglobin is rendered functionless (carbon monoxide exposure) (Burggren et al., 2000, 2004). Accordingly, despite the early generation of HR and blood flow, heart rate is initially not required for convective blood flow to the tissues, but likely plays a role in angiogenesis (Burggren, 2004; Ruck, 2010). The appearance of heartbeat prior to the absolute requirement for convective blood flow required for bulk transport early in vertebrate development, termed "prosynchronotropy" (Territo and Burggren, 1998), is accompanied in early avian embryos (<~day 6 (d6)) by an HR that is relatively insensitive to changes in environmental O_2 (Akiyama et al., 1999b; Mortola et al., 2010).

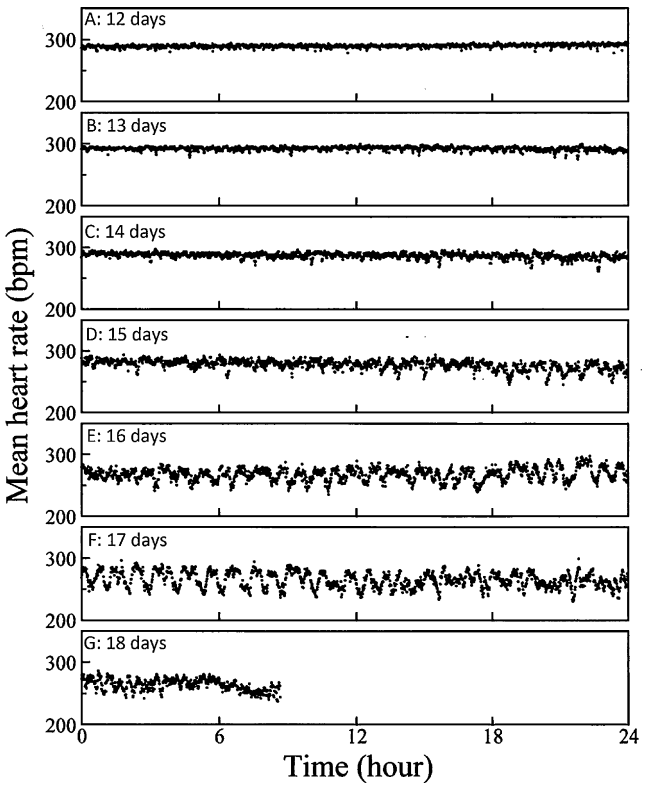

Fig. 1. Continuous measurement of mean heart rate (MHR) in the same chicken embryo from d12 to 18 of incubation. An infradian $MHR_{1\,min}$ rhythm is apparent during embryonic d16–17. By the 8th hour on d18, HR is no longer clearly discernable by acoustic methods as somatic activity and breathing signals are much larger than the ACG (Akiyama et al., 1999a).

As embryos continue to develop, the increase in total $\dot{M}_{O_2}$ of the maturating tissues and organs must be met with an increase in O_2 delivery, and ultimately, convection via the circulatory system is required. In the mature cardiovascular system, $\dot{M}_{O_2}$ is the product of total cardiac output (the product of HR and stroke volume) and O_2 extraction (O_2 content difference between arterial blood and mixed venous blood). Thus, $\dot{M}_{O_2}$ and HR are inextricably linked in advanced embryos as the heart provides the mechanical thrust for O_2 convection around the body. Although HR values alone provide little insight into underlying developmental mechanisms, HR is relatively easily measured, particularly in avian embryo models, and can be used to provide a wealth of information concerning nervous system and thermoregulatory development.

4. Overall patterns of heart rate change during avian ontogeny

The change in HR across development is highly variable between vertebrate classes and sometimes even within genera (see e.g. Fig. 1 in Burggren and Warburton, 1994). For example, in embryonic mice HR increases during development while in the human fetus HR decreases across gestation (e.g. Schifferli and Caldeyro-Barcia, 1973; Gui et al., 1996). Chickens show a complex pattern of an initially increasing HR that then slows towards pipping. Once internal pipping (IP) occurs and lung ventilation is initiated, the physical barrier to O_2 delivery is removed and HR increases (Tazawa and Hou, 1997; Tazawa, 2005). A subsequent temporal decrease in HR occurs during early external pipping (EP) followed by a marked elevation in HR baseline during the later stage of EP towards hatching (Moriya et al., 2000; Kato et al., 2002). These changes in HR are likely the result of the sympathetic nervous function overriding the vagal

tone and contributing to breathing and hatching activities. There are also mutations that affect HR development in avian embryos. In embryonic chickens, for example, mutation of the *C*-locus, which contains the structural gene for tyrosinase, translates into a chronic bradycardia late in embryonic development (Howe et al., 1994).

5. Development of heart rate fluctuations

Beyond the study of HR and its daily changes during avian development (which reflect amongst other things development of the cardiac pacemaker), analyses have also been made of HR fluctuations. Fluctuations in HR are the result of complex interactions between the suite of control mechanisms that influence HR and are indicative of the initiation of neural and hormonal control over pacemaker function. The control mechanisms may not develop synchronously during ontogeny; for example, parasympathetic nervous innervation of the heart occurs by d5 in chicken embryos, with sympathetic innervation occurring later by d16 (see Burggren and Crossley, 2002; Crossley et al., 2002; Altimiras et al., 2009 for reviews). Thus, the fluctuation patterns that are under sympathetic or cholinergic mediation may also develop asynchronously. Moreover, once nerve fibers have reached their respective targets, further maturation is required before fully functional neurotransmitter release occurs (see Altimiras et al., 2009 for review). Embryonic HR may demonstrate a difference in sensitivity to, e.g. cholinergic or adrenergic neuro-blockers across development, allowing insight into the relative timing of the development of the various control mechanisms. Further, specific fluctuation patterns become more prevalent as embryos develop thermoregulatory competence and are likely correlated with body movement, i.e. behavioral thermoregulation (Yoneta et al., 2006a).

5.1. Heart rate fluctuations in prenatal (pre-pipped) embryos

5.1.1. Rhythmic fluctuations – heart rate variability

In chicken embryos, baseline HR is stable until d12–13 of incubation (Figs. 1A, B and 4) (Höchel et al., 1998; Akiyama et al., 1999a; Tazawa et al., 1999; Chiba et al., 2004a), but begins to fluctuate around d14 (Figs. 1C, 4B and C), with subsequent increase in the magnitude of fluctuations from d15 onward (Fig. 1D). The MHR baseline begins to rhythmically oscillate on d16 and the magnitude of the oscillation increases to ~50 bpm with a periodicity of $\sim 2 \times 10^{-4}$ Hz (~85 min period^{-1}; determined from power spectrum analysis) (Fig. 1E and D). Similarly, an oscillation ranging from ~40 to 90 min period^{-1} was observed for the cyclical infradian oscillations of MHR in a separate cohort of late chicken embryos (Akiyama et al., 1999a). The magnitude of the infradian HR oscillations is highly variable amongst individual embryos; the magnitude exceeds 50 bpm in some embryos and is barely distinguishable in others from the same cohort.

Contrary to that in chicken embryos, the MHR in ratites (e.g. emu, ostriches) decreases from mid incubation until hatching (Tazawa et al., 1998; Tazawa, 2005). $MHR_{1\,h}$ of developing emu embryos decreases from ~200 bpm during the middle of incubation to 100–120 bpm close to hatching (Fig. 2) (Tazawa, 2005). The HR of emu embryos lacks circadian rhythm and, in contrast to chickens, lacks infradian rhythms (Fig. 3) (Kato et al., 2002). Non-rhythmic fluctuations in the HR baseline, however, become apparent at ~d34 and increase towards hatching (Fig. 2).

Both cholinergic and sympathetic control mechanisms are active in controlling HR by these stages in avian development, and the observed fluctuations are likely a result of the onset of, and interplay between, vagal and sympathetic tone and cardiovascular reflexes (see below), as they are in mammals (e.g. Dunster, 1999; Suzuki et al., 2003). However, the full physiological explanation for the presence of an infradian HR oscillation is yet to be elucidated.

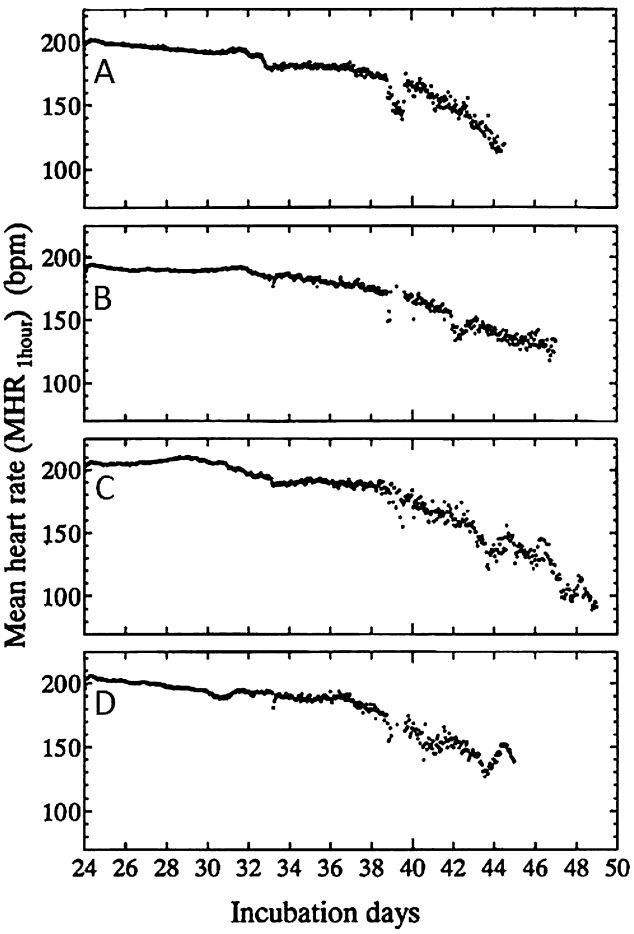

Fig. 2. Continuous measurement of mean heart rate ($MHR_{1\,h}$) in four emu embryos that successfully hatched during the last half of incubation until the ACG is disrupted by breathing activity at IP. No circadian rhythm of HR exists in emu embryos (Tazawa, 2005).

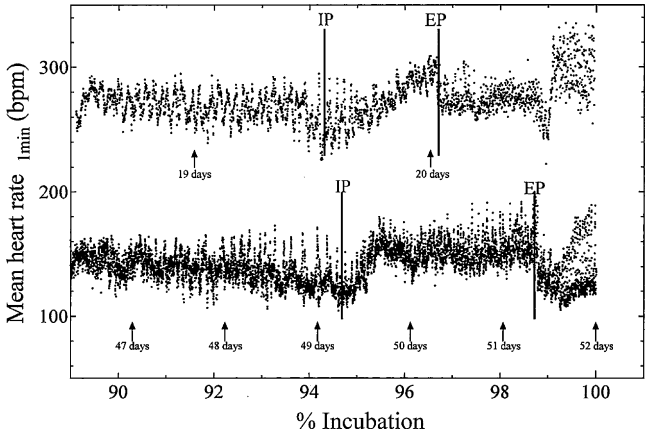

Fig. 3. Representative developmental patterns of mean heart rate (MHR) during the last 10% of incubation in a chicken embryo (top line) and an emu embryo (bottom line). The initiation of internal pipping (IP) and external pipping (EP) are indicated with verticals. The chick embryo hatches at d20 and 16-h and the emu embryo hatches at d52. In both species MHR increases and the amplitude of the baseline becomes wider (i.e. increased HR variation) during the last 1% of incubation (Kato et al., 2002).

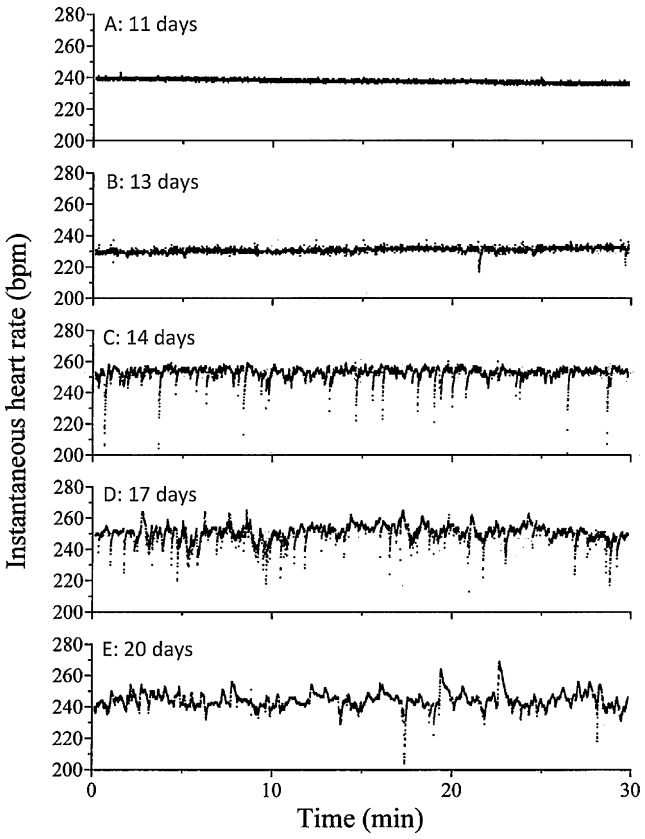

Fig. 4. Representative traces of instantaneous heart rate (IHR) recorded over a 30-min period from d11, 13, 14, 17 and 20 chicken embryos. No variation in HR is seen at d11. Spontaneous rapid decelerations of IHR begin on d13 and increase in frequency on d14. IHR decelerations are recorded together with accelerations on d17 and 20, and IHR accelerations become dominant (Höchel et al., 1998).

5.1.2. Non-rhythmic fluctuations – heart rate irregularities

Transient, rapid IHR decelerations begin on d12–13 in chicken embryos with subsequent increase in frequency and magnitude (Fig. 4) (Höchel et al., 1998; Akiyama et al., 1999a; Tazawa et al., 1999; Chiba et al., 2004a). Rapid IHR decelerations have also been reported to first appear on d11 (Yoneta et al., 2006c) and d14 (Tazawa and Hou, 1997). Prominent *irregular intermittent large accelerations* of IHR follow on d16–17, and IHR becomes increasingly irregular, with additional, spontaneous deceleration and acceleration patterns developing towards pipping and hatching (e.g. Höchel et al., 1998).

IHR decelerations can be eliminated, and HR baseline elevated, by intravenous administration of atropine in d13–14 embryos (Chiba et al., 2004a). These changes, produced by parasympathetic blockage, indicate that the transient IHR decelerations are mediated by parasympathetic nervous function, and that vagal tone in chicken embryos begins to appear at around the end of the second week (~60%) of incubation. Further, IHR decelerations in advanced embryos (e.g. d18) increase in magnitude and frequency with the additional appearance of transient, irregular accelerations. Administration of atropine eliminates the IHR decelerations completely and elevates the HR baseline even more markedly than in young embryos, indicating further maturation of the vagal tone late in incubation (Chiba et al., 2004a; Yoneta et al., 2006c). Although broiler embryos have a higher HR than layer (White Leghorn) embryos, early cholinergic chronotropic control of HR develops similarly in the two strains, with HR decelerations appearing at similar developmental stages and no strain differences in spectral power analysis of IHR (Yoneta et al., 2006c). Sympathetic nervous control is likely involved in mediating HR acceleration. However, intravenous administration of sympathomimetic and sympathetic blocking agents did not remove acceleration patterns in all tested embryos (Höchel et al., 1998), so further validation is required.

A similar developmental pattern of IHR fluctuation is found in other species of precocial birds. HR decelerations, indicating parasympathetic nervous input, first appear at ~60% of incubation in muscovy duck embryos. Later, IHR accelerations develop gradually on d25–30 until at d33 (~1 d pre-hatching) HR is dominated by accelerations; i.e. sympathetic influences increase progressively (Höchel et al., 1999). Emu eggs are ~10-fold heavier (~600 cf. ~60 g) than chicken eggs and their incubation duration is ~2.5-fold longer (~50 cf. ~20 d). Prior to pipping, the embryonic IHR fluctuations mainly comprised irregular accelerations with two distinctive patterns: *irregular intermittent large accelerations* (also common in chicken embryos throughout the final stages of development) and *wide accelerations* lasting for 10–20 min (unique to emu embryos). The distinctive IHR decelerations have not been reported in late (last 2–7 d of incubation) emu embryos; HR is dominated by accelerations only (Kato et al., 2002), although they appear only rarely in hatchlings (Tamura et al., 2003; Shah et al., 2010). Spontaneous decelerations and accelerations of IHR have also been recorded in late (80–90% of incubation) embryos of the smallest precocial bird, king quail (*Coturnix chinensis*) (Pearson et al., 1998), demonstrating that sympathetic (accelerations) and parasympathetic (decelerations) control of HR is established even in the smallest precocial birds by late incubation. Both the frequency and the amplitude of HR fluctuations increase with incubation time in all species (e.g. Höchel et al., 1999; Pearson et al., 2000; Kato et al., 2002).

In altricial crows (*Corvus corone* and *Corvus macrorhynchos*), spontaneous HR decelerations are also first to appear, with HR accelerations developing later and occurring with less frequency compared with chicken embryos. The development of parasympathetic control of HR in crows occurs at ~60% of incubation, similar to precocial embryos. However, sympathetic control is relatively delayed and suppressed in contrast to chicken embryos (Pearson and Tazawa, 1999).

5.2. Heart rate fluctuations in perinatal (pipped) embryos

Embryonic chicken and emu MHR fluctuations are further augmented during the last 10% of the incubation period as the embryos develop towards pipping and hatching (Fig. 3) (Kato et al., 2002). Across development, chicken embryo HR is higher than the emu embryo developmental pattern because the allometric relationship between HR and egg mass results in faster HR (i.e. emu) (Tazawa et al., 2001a). In addition, there are some differences between the developmental pattern of MHR in emu and chickens, i.e. the decrease in MHR towards the end of pre-pipping is greater in emu than in chickens. Regardless, in both species the overall MHR decreases slightly towards internal pipping (IP), which begins around 94–95% of incubation before increasing during IP. Initiation of external pipping (EP) again decreases the MHR baseline which increases once more during the last ~1% of incubation (Fig. 3). The wide MHR baseline during the last period of incubation in both species is attributed to distinctive, large, repeated, short-term IHR accelerations, which are indicative of imminent hatching. Similar large, distinctive HR accelerations are also reported in altricial black-tailed gull (*Larus crassirostris*) embryos just prior to hatching (Pearson et al., 2000). These accelerations occurring in both precocial and altricial species likely result from short-term activity-associated increases in $\dot{M}_{O_2}$ as embryos become active towards hatching; i.e. increase of HR due to O_2 supply matching with O_2 demand. Baroreflex regulation also appears during the last 3 d of incubation (Altimiras

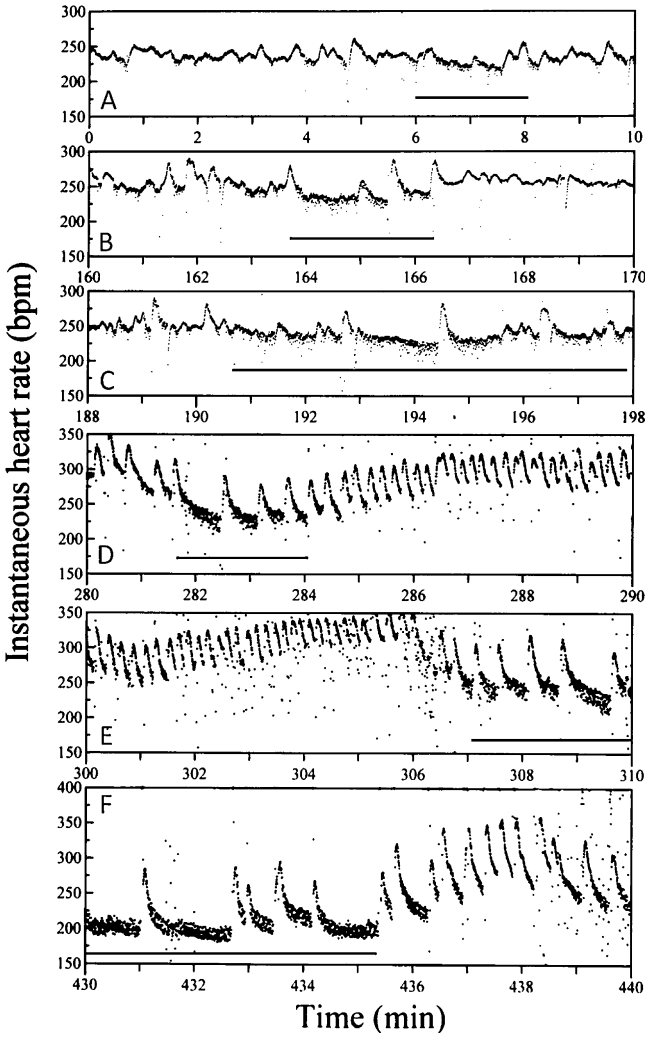

Fig. 5. Six 10-min recordings of instantaneous heart rate (IHR) taken from an 8-h continuous measurement in an externally pipped chicken embryo. Three patterns of IHR accelerations are recorded: (1) *irregular intermittent large accelerations* (A, B, C); (2) *relatively long-lasting cyclic small accelerations* (D, E); and (3) *short-term repeated large accelerations* (F). The underlined regions indicate respiratory sinus arrhythmia, which is characterized by wide HR baselines (Tazawa et al., 1999).

and Crossley, 2000) with potential consequences for HR regulation.

Although somewhat obscured, the distinctive IHR fluctuation patterns can be seen overlying the broad MHR baseline (e.g. Fig. 3). Cyclic infradian oscillations (period of 40–90 min) occur until early in the perinatal period when the HR baseline begins to increase. The origin of the infradian oscillations is currently unconfirmed and may result from sympathetic control of the heart as well as movement of the embryos, which likely increases towards hatching. During early stages of EP of the eggshell HR drops. Then, during the final stage of EP, when the HR baseline increases again prior to hatching, three patterns of IHR accelerations are evident; i.e. *irregular intermittent large accelerations* (continuing from prenatal period), *relatively long-lasting cyclic small accelerations* and *short-term repeated large accelerations* (both of which are unique to the EP period) (Fig. 5). Respiratory sinus arrhythmia (RSA), a cyclic oscillating pattern (frequency of ~1–3 Hz in chickens) associated with ventilation, also appears during the EP period (Fig. 5). Simultaneous measurement of IHR and breathing signals determined by a condenser microphone (Acoustorespirogram, ARG) has confirmed the coupling of RSA (distinctive wide HR baseline) and the onset of embryonic ventilation during pipping (Chiba et al., 2004b). Further, repeated alternate occurrences of *short-term repeated large accelerations* and *irregular intermittent large accelerations* signal imminent hatching in chickens and emu (Tazawa et al., 1999; Moriya et al., 2000; Kato et al., 2002).

5.3. Hatchlings

The IHR of avian embryos fluctuates distinctively as they approach hatching (e.g. Moriya et al., 2000; Kato et al., 2002), and after hatching, the HR fluctuations increase in magnitude. Hatchling HR is substantially more arrhythmic than embryonic HR, with spontaneous accelerations becoming the predominant fluctuation pattern. Continuous measurements of chicks throughout their first week at thermoneutrality (natural photoperiod and with feed available) clearly reveal circadian MHR rhythms (Moriya et al., 1999, 2004). MHR is highly variable during the day (250–500 bpm), partly due to feeding and activity, and decreases to a diurnal low (200–350 bpm) at night when MHR is relatively stable, although MHR fluctuations persist throughout the diurnal cycle. While chicks develop an endogenous circadian MHR rhythm soon after hatching (Moriya et al., 1999, 2000, 2004; Tazawa et al., 2002b), emu hatchlings lack a circadian MHR rhythm (Moriya et al., 2002; Tazawa, 2005).

IHR fluctuations of chicken hatchlings are categorized into three types (partially similar to embryos; Fig. 5); Type I is high-frequency (HF) oscillation due to RSA and is characterized by a wide HR baseline (20–50 bpm) with a mean oscillatory frequency of 0.74 Hz (range 0.4–1.2 Hz), Type II is low-frequency (LF) oscillation of the HR baseline at a mean frequency of 0.07 Hz (range 0.04–0.10 Hz) (unique to hatchlings), and Type III are non-cyclic irregularities, dominated by frequent transient accelerations (Moriya et al., 1999). Not surprisingly, the vagus nerve (an important mediator of ventilation in adults) mediates the Type I HF oscillation, which is associated with RSA and tends to appear when the HR baseline is low due to vagal tone (~220–300 bpm). On the other hand, Type II LF oscillations are produced over a wide range of HR baselines (~250–450 bpm) during both day and night, and are produced (or augmented) when hatchlings are exposed to lowered T_a and eliminated during elevated T_a exposure (Tazawa et al., 2001b, 2002a; Khandoker et al., 2004).

The increase in LF oscillation and HR baseline in response to cooling is likely a result of increased thermoregulatory ability (Fig. 10; see Section 7.3). Further, there is some evidence of potential behavioral thermoregulation as wing and body movements occur synchronously with Type II and Type III fluctuations (Yoneta et al., 2006a). Frequently, HF and LF HR oscillations (Type I and Type II) and irregularities (Type III) occur simultaneously. This is not surprising considering that Type I fluctuations are respiratory in origin and Type II and Type III fluctuations are likely due to movement.

HR oscillations (Type I and Type II) and irregularities (Type III) are also observed in emu hatchlings with additional distinctive accelerations (Moriya et al., 2002; Tamura et al., 2003; Shah et al., 2010). Emu hatchling baseline HR (~100–200 bpm) is lower than chicken hatchlings (Tazawa et al., 2001a), but the IHR fluctuates more markedly. Type I oscillations associated with RSA have a mean frequency of 0.37 Hz (range 0.2–0.7 Hz) (Moriya et al., 2002) or 0.22 Hz (0.1–0.7 Hz) (Shah et al., 2010), and accordingly breathing frequency in emu hatchlings is estimated to be 1/2 that in chickens. Type II oscillations in normoxic emu hatchlings are rare in occurrence and have a mean frequency of 0.06 Hz (range 0.04–0.2 Hz). In addition to Type III HR irregularities (as seen in chickens), irregular HR accelerations with variable duration and amplitude are characteristic of emu hatchlings (Moriya et al., 2002). LF oscillations (range 0.01–0.1 Hz) appear in most emu hatchlings exposed to hypoxia and can be abolished by cholinergic blockade, suggesting that the sympathetic system contributes to LF oscillations by suppressing

the influence of the parasympathetic nervous system (Shah et al., 2010).

6. Influence of oxygen on heart rate in embryonic birds

6.1. Heart rate control in hypoxia

Embryos of some bird species may encounter a reduction in ambient O_2 levels (hypoxia) in the natural environment if, for example, submersed in water during periods of heavy rain, or covered by O_2 impermeable waste materials when the parental birds are absent. Additionally, many species (e.g. cliff swallows) are fossorial, laying their eggs at the end of long, narrow burrows or within mounds. Such situations may result in a decrease in O_2 supply to the embryo, and consequently a decrease in $\dot{M}_{O_2}$ and HR, potentially resulting in embryonic death. Chicken embryos can survive chronic exposure to 14–15% O_2 during incubation (e.g. Dzialowski et al., 2002; Miller et al., 2002; Villamor et al., 2004; Chan and Burggren, 2005; Azzam and Mortola, 2007; Ferner and Mortola, 2009). Additionally, exposing embryos to CO_2 (hypercapnic hypoxia) shortens survival duration with only half of d15 embryos surviving 1 d exposure to 14% O_2, 6% CO_2 and no embryos surviving 1 d exposure to 13% O_2, 7% CO_2 (H. Tazawa, unpublished data). However, embryos can survive mild hypercapnia alone (4% CO_2) across the last half of incubation (Everaert et al., 2008, 2011; Szdzuy and Mortola, 2008) and severe hypercapnia (10% CO_2) for 1 d (H. Tazawa, unpublished data).

Unlike cardiac output, which increases in parallel to the growth of the prenatal embryo, MHR increases asymptotically during early development with only small changes occurring during the last half of incubation (Tazawa and Hou, 1997; Tazawa, 2004). Further, HR at normoxic, normothermic rest is well below maximal, ensuring that there is the potential to increase HR during environmental challenges such as changes in ambient O_2 (or T_a, see Section 7). The HR response to lethal hypoxic exposure (10% O_2) is dependent on developmental age (Fig. 6) (Akiyama et al., 1999b). d3–5 chicken embryos can survive 4 h exposure to 10% O_2 with HR generally decreasing only moderately with initial hypoxia onset (Fig. 6). An attenuated response is not surprising considering that HR is relatively unimportant for O_2 transport, which can occur via diffusion in small embryos (Burggren et al., 2000, 2004). Accordingly, hypoxia has little effect early in development, likely due to the relatively small absolute $\dot{M}_{O_2}$ of the embryo (Bartels and Baumann, 1972; Raddatz and Kučera, 1983). As the embryos grow, $\dot{M}_{O_2}$ increases and diffusion no longer suffices to meet the aerobic requirements of the embryo; consequently, hypoxia represents a progressively more severe perturbation as development proceeds (Fig. 6) (Akiyama et al., 1999b). Hypoxia reduces $\dot{M}_{O_2}$ across a broad range of adult, neonatal and embryonic animals (see Mortola, 2005 for review). Exposure to 10% O_2 reduces the $\dot{M}_{O_2}$ of prenatal (d12, 16 and 18) and perinatal (d20, EP) embryos to less than 10% of control (normoxic) values and is lethal with $\dot{M}_{O_2}$ ceasing within a few hours (Tazawa et al., 1992a). The reduction of $\dot{M}_{O_2}$ in hypoxic neonatal mammals is due to a down-regulation in the thermoregulatory set point resulting in less energy expenditure for body temperature (T_b) maintenance (Mortola et al., 1989). Avian embryos have very limited thermogenic ability (see Section 7.3). Thus it is more likely that hypoxia further compounds the O_2 conductance limitations of advanced embryos and, therefore, advanced embryos demonstrate greater O_2 sensitivity than early embryos (see above). Even 15 min of hypoxic exposure of d14–16 chicken embryos induces bradycardia, the magnitude depending on O_2 concentration (Tazawa, 1981).

Hypoxic exposure (10% O_2) for 2 h induces bradycardia in late pre-pipped embryos (d16) and HR does not subsequently recover once the embryos are returned to normoxia (Khandoker et al., 2003). This effect may be the result of the direct action of low O_2 on the cardiac muscle and pacemaker cells (Van Golde et al., 1997; Crossley et al., 2002). d18 and 19 (pre-pipped) embryos demonstrate a transient depression of HR during early hypoxia exposure with prompt recovery during further exposure, likely due to adrenergic response. In contrast, the HR of pipped (IP and EP) embryos elevates in response to hypoxia (Khandoker et al., 2003). Conversely, pipped embryos in fenestrated eggs decrease HR in response to short (5 min) bouts of hypoxia (Crossley et al., 2002). Potentially this highlights the advantages of methodologies that maintain 'normal' gas conductance across the eggshell. However, catecholamine levels may be increased in pipped embryos exposed to longer (>5 min) periods of hypoxia resulting in recovery of HR (e.g. Khandoker et al., 2003).

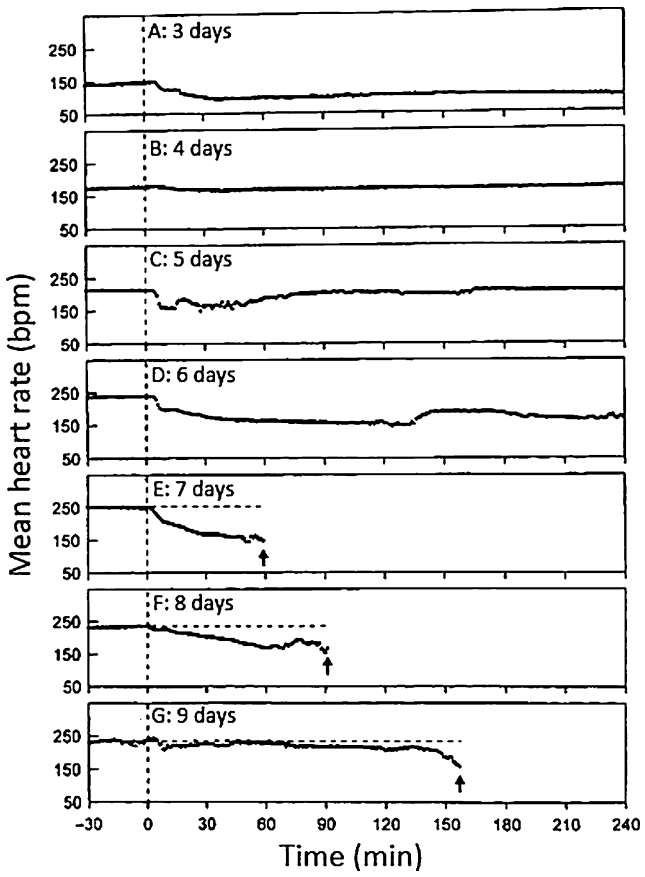

Fig. 6. Mean heart rate ($MHR_{1\,min}$) measurements of representative chicken embryos (d3–9) before and during hypoxia (10% O_2 – onset indicated by vertical dashed line). Mean HR_{air} is 143, 170, 214, 238, 250, 235 and 235 bpm for each embryo from d3 to 9 of incubation, respectively. The horizontal dotted line indicates HR_{air}, and the arrow indicates embryonic death (Akiyama et al., 1999b).

6.2. Heart rate control in hyperoxia

Hyperoxic (100% O_2) exposure of early embryos (d3–9) increases HR. However the increases are small, not exceeding 10 bpm, and are lacking entirely in some embryos (Akiyama et al., 1999b). The absence of hyperoxic influence on HR is not surprising at this early stage, since O_2 demand is small in early development. More surprisingly, similar minor effects of hyperoxia on HR are found in advanced embryos (d14–16) during 20 min exposure to various degrees of hyperoxia including 100% O_2 (Tazawa, 1981). The absence of a hyperoxic response in pre-

pipped embryos suggests that 1) the $\dot{M}_{O_2}$ of the embryo is not O_2-conductance limited (Khorrami et al., 2008), 2) the HR is maximal in air (which is unlikely as HR can be increased in response to, e.g. increased T_a; see Section 7) and any increase in O_2 concentration fails to stimulate an increase in HR or 3) there is no O_2 driven control of HR (termed "hypoxic drive") in either early or advanced pre-pipped embryos (Tazawa, 1981; Akiyama et al., 1999b).

The HR baseline of pipped embryos is depressed during hyperoxic exposure (2 h at 100% O_2) (Khandoker et al., 2003). O_2 sensitivity (hypoxic drive) may be initialized with the onset of mechanical ventilation. During the pipping period, mechanical ventilation begins and hyperoxic exposure decreases baseline IHR (Khandoker et al., 2003; Chiba et al., 2004b). Exposing pipped embryos to hyperoxia may increase cholinergic input from the vagus nerve, which overrides sympathetic nervous function, decreasing the HR baseline (Khandoker et al., 2003). Further, at EP the peripheral chemoreceptors play a role in mediating ventilation during hyperoxic (and hypercapnic) exposure (Menna and Mortola, 2003). In birds, similar to mammals, the carotid body is the major peripheral chemoreceptor involved in O_2 sensing (see Milsom and Burleson, 2007 for review). Avian intrapulmonary chemoreceptors (IPC), innervated by the vagus nerve, can detect CO_2 at IP in ducklings, although full CO_2 sensitivity of the IPCs does not develop until EP (Pilarski and Hempleman, 2007). Further study is required to elucidate the complex interactions between central and peripheral chemoreception, ventilation and HR.

6.3. Heart rate fluctuations in hypoxia and hyperoxia

Hypoxic exposure tends to augment the magnitude and frequency of IHR decelerations in pre-pipped embryos and suppress HR fluctuations in pipped embryos, while hyperoxia has little effect on HR fluctuations (Khandoker et al., 2003). Augmented HR decelerations in pre-IP embryos during hypoxic exposure may be mediated by vagus nerve function, as transient, rapid HR decelerations are blocked by atropine administration (Höchel et al., 1998; Yoneta et al., 2006c). Further, the presence of RSA in some embryos exposed to hypoxia or hyperoxia (e.g. Shah et al., 2010) supports vagus nerve mediation. Blocking muscarinic receptors with atropine on d15–19 and α-adrenergic receptors with phentolamine on d15–21 removes the hypoxic bradycardia, indicating that the muscarinic receptors in the heart and the sympathetic nervous system also play a role in mediating hypoxic bradycardia (Crossley et al., 2002). During hypoxic exposure, IHR accelerations appear to be limited as hypoxia elevates the HR baseline in EP embryos to a level that may be approaching maximum (maximal observed HR of ~500 bpm; Ono et al., 1994), thus preventing further increase through accelerations.

7. Heart rate during environmental thermal challenges

Naturally incubated embryos may encounter thermal hazards during the absence of parental birds from the nest, i.e., exposure to low or high T_a. The duration of these T_a challenges and the degree of tolerance of the embryos is species specific (e.g. Rahn et al., 1977; Carey, 1980; Webb, 1987; Reyna, 2010; see Martin et al., 2007 for review). Further, nest structure and the degree of insulation likely buffer embryos against environmental T_a changes.

7.1. Embryonic thermal tolerance

The HR of cold-exposed chicken embryos decreases exponentially to a T_a-dependent, steady-state value (from which it can potentially recover if re-warmed) at 3–4 h of exposure. Embryonic cardiac stroke volume and blood flow are preserved, however, despite decreased HR (e.g. Wispé et al., 1983; Tazawa et al., 1985). At a T_a of 28–26 °C, the HR of prenatal (d10, 17–18) chicken embryos plateaus at ~100 bpm and can be sustained for up to 100-h (~4 d). Ultimately arrhythmia and cardiac arrest occur, and re-warming the embryos to 38 °C fails to restart heartbeat and death results (Tazawa and Nakagawa, 1985; Tazawa and Rahn, 1986). The HR of perinatal (d19–20) embryos exposed to 28 °C is higher (~30–40 bpm) than prenatal embryos, reflecting greater intrinsic heat production (i.e. an increase in basal metabolic rate). Exposing prenatal and perinatal embryos to a more severe T_a stress of 18 °C results in a steady-state HR of ~30 bpm which can only be maintained for ~60 h (2.5 d). In this instance, after cardiac arrest some embryos autoresuscitate (i.e. heartbeat re-initiates) upon re-warming to 38 °C. This phenomenon is more likely upon re-warming from exposure to T_a of 8 °C. HR decreases to below 10 bpm after 2–3 h and ceases without going through arrhythmia. Cardiac arrest at 8 °C does not result in embryonic death. Instead, embryos enter a state akin to torpor and all embryos recover heartbeat during re-warming to 38 °C. The length of time that the embryos can tolerate cardiac arrest is age dependent. d6 embryos are relatively cold tolerant and are capable of recovering from more than 24 h of cardiac standstill at 8 °C, while the tolerance time of d20 (EP) embryos without a beating heart is ~8 h (Tazawa and Rahn, 1986). Neonatal placental mammals also often resume heartbeat after a short period of cardiac arrest induced by cold (Adolph, 1951) and Tammar wallaby joeys re-initiate ventilation after ventilation cessation upon re-warming from hypothermia (S. Andrewartha, personal observation). Thus the intrinsic cold tolerance of young animals is conserved across taxa, although the underlying mechanisms are still largely unknown.

Temperature tolerance is commonly examined via lethal T_a exposure or lethal exposure time. In lethal T_a exposure protocols, an exposure time (e.g. 5 h) is set and the T_a that results in embryonic mortality after the exposure is determined. Measurement of HR, for example, can conveniently indicate when embryonic mortality occurs. d12, 16, 18 and 20 chicken embryos survive exposure to T_a of 42 °C for 5 h; however, 100% mortality results when embryos are exposed to 46 °C for 5 h (Tazawa et al., 1992b). The lethal T_a that stops the heart of d12, 16–18 and 20 (EP) embryos is 46, 45 and 44 °C, respectively. The mean HR prior to cardiac arrest at lethal T_a reaches ~400, 370 and 315 bpm for d12, 16–18 and 20 embryos, respectively (Tazawa et al., 1992b). Similar to cold exposure, younger embryos demonstrate a greater heat tolerance than older embryos. There are, not surprisingly, very large differences in thermal tolerance between species. Bobwhite quail often lay their eggs on bare ground where surface temperatures can rise well above 40 °C for much of the day. Consequently, the embryos can tolerate temperatures of 40 °C for 6 h and 50 °C for 1 h (Reyna, 2010).

During lethal exposure time protocols, an exposure T_a (e.g. 48 °C) is set, and the exposure time that results in mortality is determined (Ono et al., 1994). In prenatal (pre-pipped) embryos, HR increases in response to rapidly elevated T_a in parallel to the increase of the egg temperature (T_e), then plateaus. Ultimately, the increased HR cannot be sustained, the embryo likely succumbs to heat stress and cardiac arrest occurs. In perinatal (pipped) embryos, HR increases in a relatively irregular fashion with the increase in T_e until ultimate cardiac arrest (Ono et al., 1994). The mean tolerance time shortens significantly with embryonic development from 100 min in d12 embryos to 56 min in EP (d20) embryos. However, the critical internal temperature of the egg (CT_e – lethal temperature) is independent of embryonic development (46–47 °C) and similar to the temperature at which most biological enzymes lose their catalytic abilities due to conformational changes (Hochachka

and Somero, 2002). This indicates that the tolerance time does not correspond to the ability of the embryos to withstand the thermal stress, but merely depends on the CT_e, which in turn depends on the temperature at which enzyme (and other important biological protein) denaturation occurs. Advanced embryos have a more developed blood circulation, higher metabolic heat production and higher T_e compared with early embryos, and therefore reach CT_e faster than early embryos. Thus, although CT_e is identical in all embryos, the tolerance time is shorter in advanced embryos as they reach CT_e faster. The mean HR at CT_e is statistically similar during the pre-pipping stages (440–460 bpm), but decreases in EP embryos (420 bpm). Pre-pipped embryo MHR at control T_e of 38 °C is ~250–260 bpm resulting in a Q_{10} for HR, of ~2. The control HR of 300 bpm at T_e of ~40 °C in EP embryos results in a Q_{10} for HR of <2, indicating the initiation of endothermic capacity (Whittow and Tazawa, 1991; Ono et al., 1994).

7.2. Heart rate thermoconformity in early embryos

Young avian embryos are ectothermic and will readily lose heat to the environment. Consequently they require a heat supply from the external environment to develop. As ectotherms, the HR of prenatal embryos generally varies somewhat proportionally with T_a. In response to mild cold exposure (~26–28 °C), T_e and HR decrease in an exponential fashion (Tazawa and Nakagawa, 1985). However, the relationship between T_e and HR changes across embryonic development. In younger embryos (d12–16) HR decreases more quickly than the change in T_e, whereas in older embryos (d17–18) HR changes in a manner similar to T_e, indicating that the cardiac pacing is becoming resistive to mild cold exposure during development. This phenomenon is likely attributable to increasing endogenous heat production of more advanced embryos (see Mortola, 2009 for review). Despite wide changes in HR in response to altered T_a in late prenatal embryos, the functional capacity of the heart (e.g. stroke volume, blood flow and blood pressure) ensures continued blood convection even after prolonged exposure to low thermal environments (Tazawa et al., 1985; Tazawa and Nakagawa, 1985). The almost constant isometric ventricular contraction period (determined from ballistocardiography) in d15 chicken embryos over a wide T_a range (38–26 °C) demonstrates that cardiac function is well preserved despite wide changes in HR (278–124 bpm, respectively; Q_{10} ~2; Tazawa et al., 1989).

7.3. Heart rate control during development of endothermy in late embryos and hatchlings

In the domestic fowl, a brief period of homeothermic competence occurs at the end of incubation when the embryos are still within the confines of their egg; homeothermic ability then increases greatly soon after hatching (Tazawa and Rahn, 1987; Tazawa et al., 2001b). The initial, eggbound, homeothermic competence is influenced by eggshell gas conductance and the chorioallantoic membrane (CAM). The eggshell gas conductance limits the potential increase in $\dot{M}_{O_2}$ by limiting the rate of O_2 diffusion through the eggshell (the O_2 supply), and the CAM limits the amount of O_2 transported via the blood through limited diffusing capacity. At this stage, the mechanisms required for endothermy are substantially developed and the embryos are at an 'O_2 conductance-limited stage' of homeothermy, evident from the fact that $\dot{M}_{O_2}$ increases when late-stage (pre-pipped) chicken or emu embryos are exposed to hyperoxia. This demonstrates that the eggshell and CAM create a physical barrier to O_2 transport (e.g. Hoiby et al., 1983; Tazawa et al., 1988; Dzialowski et al., 2007). The newly hatched chick cannot sustain a $\dot{M}_{O_2}$ that produces enough heat to offset heat loss, and thus is 'power-limited' prior to reaching 'full-blown' homeothermy (Tazawa et al., 1988; Whittow and Tazawa, 1991). The development of the thyroid gland is important for the control of basal metabolic rate (Hulbert, 2000), resulting in T_b thermal stability. Thyroid hormones increase $\dot{M}_{O_2}$ by a variety of mechanisms including increasing protein, lipid and glucose synthesis, proteolysis, lipolysis and glucose oxidation (see Silva, 2006 for review). More importantly thyroid hormones increase ATP usage by stimulating sarcoendoplasmic reticulum Ca^{2+} activity in the skeletal muscle and produce a decrease in ATP synthesis efficiency through increased membrane permeabilities resulting in more energy expended to maintain ion concentration gradients, and stimulation of the mitochondrial proton leak (Silva, 2003, 2006). Precocial avian thyroid hormone circulation peaks at hatching (see McNabb, 2006 for review) aligning with HR and $\dot{M}_{O_2}$ evidence of development of thermoregulation (e.g. Figs. 7 and 8) (Mortola, 2009). In order to meet the increasing O_2 demand, HR control matures rapidly across the O_2 conductance- and power-limited stages of thermoregulatory development in the domestic fowl. The peripheral and central (deep-body) thermoreceptors are believed to be functional by the first week of hatching (see Baarendse et al., 2007 for review); thus, integrated thermal input will allow for an integrated thermal response.

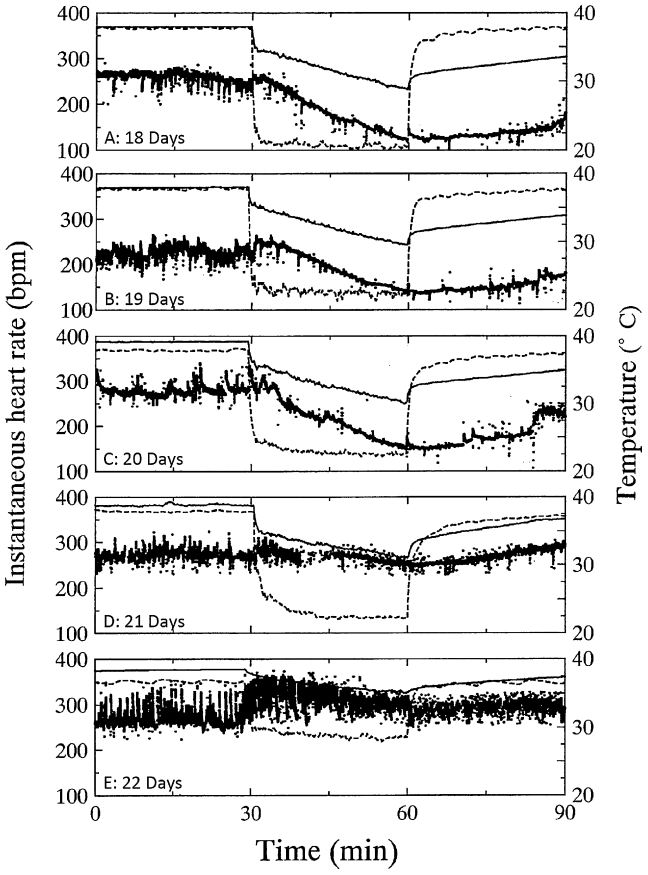

Fig. 7. Representative instantaneous heart rate (IHR) responses to low ambient temperature (T_a) exposure in a developing chicken embryo. The embryo was exposed to lowered T_a (=21 °C on d18, 22 °C on d19–21 and 28 °C on d22) for 30 min once per day from d18 to 22 of incubation and IHR measured before, during and after exposure. The dotted line represents T_a, and the solid line indicates egg temperature (T_e) (Tazawa et al., 2001b).

Thermoregulatory HR strategies may well be species-specific and related to life history strategies. Waterfowl hatchlings must enter the water, a media with high heat conductance, soon after hatching and so demonstrate earlier development of thermoregulatory competence than chickens (Kuroda et al., 1990; Whittow and Tazawa, 1991). Further, egg size, an important determinant of

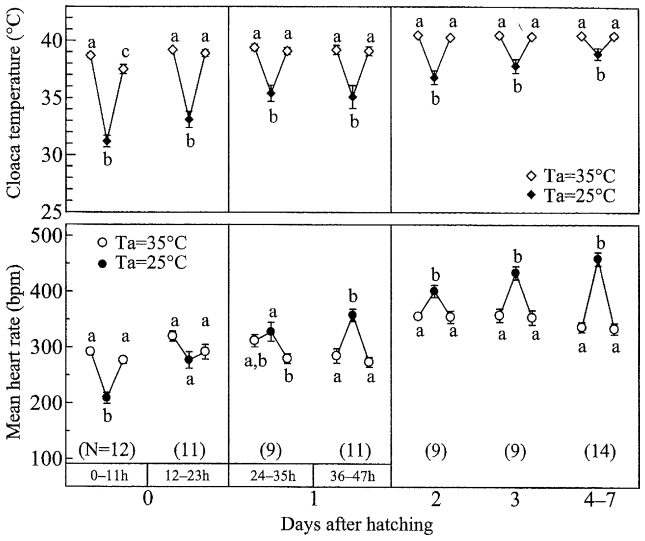

Fig. 8. Mean cloacal temperature ± SE (T_b, upper panel) and mean heart rate (MHR, lower panel) during the last 10 min of 1-h exposure to T_a = 35 °C, then 25 °C and returned to 35 °C. Broiler chicks are separated into 2 groups on day 0 (*group 0–11 h* and *group 12–23 h*), d1 chicks into 2 groups (*group 24–35 h* and *group 36–47 h*), and chicks examined on post-hatch d4, 5, 6 and 7 are pooled into a single group. For each stage of development, data points with different letters are significantly different from each other. Numerical figures in the parentheses represent the number of hatchlings measured in each age group (Yoneta et al., 2006b).

thermal dynamic characteristics, may also play an important role in the development of embryonic thermoregulatory competence (Tazawa et al., 2001b; Tamura et al., 2003). Accordingly it is anticipated that embryonic waterfowl and ratites (large eggs) may attain endothermy earlier than chickens and thus respond to altered T_a with a more advanced thermoregulatory pattern.

7.3.1. Chickens

The HR of pre-pipped and pipped chicken embryos decreases with T_a in typical ectotherm fashion (Fig. 7A–C). HR slowly increases in d18 and 19 embryos upon re-warming from T_a = 22 °C. By EP (d20), HR increases faster upon re-warming (from T_a = 22 °C) (Fig. 7C), due likely to an enhanced ability to increase $\dot{M}_{O_2}$ in response to lowered T_a (see Mortola, 2009 for review). T_e also recovers faster towards T_a in EP embryos as the higher $\dot{M}_{O_2}$ of EP embryos produces more endogenous heat. By d21 (when all embryos are supposed to hatch, but this embryo failed to escape from the eggshell) (Fig. 7D), T_e and HR decline little during cooling (T_a = 22 °C), and on d22 IHR increases in response to cooling to the milder T_a of 28 °C (i.e. a thermoregulatory response) (Fig. 7E) and Type II LF oscillation is present (see Section 5.3; Tazawa et al., 2001b). Thus, homeothermic competence occurs while this embryo is still within the eggshell. However, the majority of thermoregulatory development occurs after hatching.

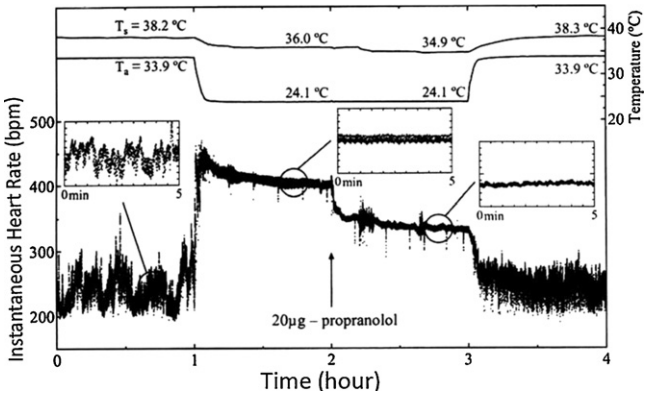

Fig. 9. Representative changes in instantaneous heart rate (IHR) baseline and fluctuations and skin temperature (T_s) during cold exposure (T_a = 24 °C) and administration of the sympathetic blocker propranolol ([20 μg] – subcutaneous injection) in a d2 chick. The left inset (T_a = 38 °C) shows the oscillation of the IHR baseline at a frequency of 0.54 Hz (the frequency determined by power spectrum analysis). The wide IHR baseline apparent in the middle inset (24 °C prior to propranolol) lacks irregularities, and after propranolol administration, no oscillation was recorded (right inset) until re-warming to 38 °C (Tazawa, 2005).

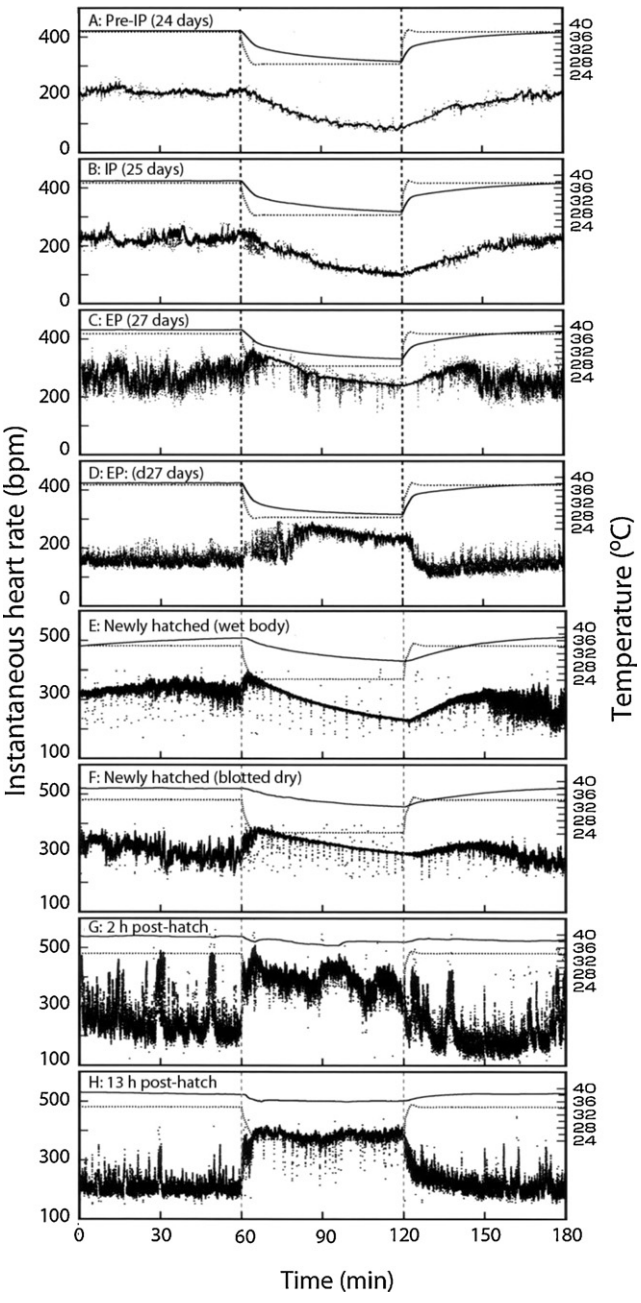

Fig. 10. Representative instantaneous heart rate (IHR; individual points) responses of d24 pre-IP (panel A), d25 IP (B) and two d27 EP (C and D) duck embryos (demonstrating the variation in IHR pattern observed at EP), a wet newly hatched duckling (E), a newly hatched duckling that has been blotted dry (F), a duckling 2-h after hatching whose body has dried naturally (G) and a 13 h duckling (H) during exposure to lowered ambient temperature (T_a = 28 °C for embryos and 25 °C for ducklings; dotted line). The solid line represents egg temperature (T_e; solid line on A–D) or body temperature (T_b; solid line on E–H). The vertical dashed lines indicate the cold exposure period (unpublished data).

Pronounced development of thermoregulatory competence occurs during the first day post-hatching (e.g. Khandoker et al., 2004; Yoneta et al., 2006b). Initially (0–11 h), the MHR of newly hatched broiler chicks decreases during cold T_a exposure (from brooding T_a of 35 °C to 25 °C) (Fig. 8). Later on day 0 (12–23 h), chick hatchlings are able to maintain constant MHR in the face of the same T_a challenge. By the second half of day 1, broiler chick hatchlings demonstrate a fully functional endothermic HR response with HR increasing during cold exposure. White leghorn chickens hatch at an identical mass to broilers, but they develop more slowly and by day 2, broiler hatchlings are larger than white leghorn hatchlings. The endothermic HR response in white leghorn hatchlings is delayed by ~1 d compared with broiler hatchlings (Yoneta et al., 2007). Genetic selection for fast growth in broiler chickens is the most likely cause for physiological heterochrony between the two strains. Across the first few days post-hatching, the endothermic capacity of the hatchlings (broiler and layer) increases and they are able to maintain a more constant cloacal temperature during cold exposure likely through increased $\dot{M}_{O_2}$, which is reflected through increased HR baseline (Fig. 8) (see Mortola, 2009 for review).

In contrast to the development of the endothermic HR response (which occurs across the first few days post-hatching), an endothermic $\dot{M}_{O_2}$ response (increase with cold exposure) has been observed in layer strain chicks immediately after hatching when exposed to the same T_a challenge (Tazawa et al., 2004). Potentially, alterations in O_2 extraction are responsible for the uncoupling of $\dot{M}_{O_2}$ and HR. Conversely, the ΔT_b observed in layer chicks is smaller than that in broiler chicks exposed to a very similar cooling regime (Khandoker et al., 2004 cf. Tazawa et al., 2004) suggesting that, in contrast to the development of the endothermic HR response (Yoneta et al., 2007), layer chicks have a more advanced endothermic response than broiler chicks. These studies took place in two very different localities and perhaps highlight the sensitivity of the development of endothermy and the need for further concurrent, between-strain investigations.

During the development of endothermy, changes in IHR occur in addition to changes in MHR baseline. HF (Type I, RSA) and LF (Type II) oscillations occur in broiler chicks in response to lowered T_a, and both HF and LF oscillations become present in more hatchlings throughout development and are indicative of thermoregulatory competence and maturation of the autonomic nervous system (Khandoker et al., 2004). Simultaneous IHR and video analysis of body movement have demonstrated a link between wing movement and Type II fluctuations during cold exposure, likely resulting from behavioral thermoregulation (Yoneta et al., 2006a). Similarly, LF and very LF oscillations appear during cooling in neonatal mammals and are under sympathetic and parasympathetic mediation with possible thermoregulatory functions (Kitney, 1980; Askelrod et al., 1981).

Brooding broiler chicks at 25 °C (10 °C lower than preferred brooding temperature) and then exposing them to a relative increase in T_a (35 °C) results in a similar change in HR from thermoconformity during early hatching-day 0, to thermoregulation during late hatching-day 0. Thus, neither time sequence of T_a exposure nor brooding T_a affects the endothermic HR response development (Khandoker et al., 2004; Yoneta et al., 2006b). Thermoregulation and the HR response to cold exposure in hatchlings are likely mediated via sympathetic nervous function. Blocking β-receptors (propranolol) in 2-day-old hatchling during cooling partially depressed the increased HR and skin temperature (T_s) by −1.1 °C (Fig. 9). Further, administration of the β-blocker transiently eliminates RSA (i.e. Type I HF HR oscillation), which begins again upon re-warming. As β-blocker administration only diminishes the HR response to cold exposure, the thermoregulatory responses of HR to low T_a are only partially attributed to β-receptor mediation, and other mechanisms, such as autonomic nervous mediation, are likely involved.

7.3.2. Ducks

Ducklings must attain thermoregulatory competence early (relative to the terrestrial chick) during perinatal development to live in an aquatic environment soon after hatching (Kuroda et al., 1990; Whittow and Tazawa, 1991). Even at the pre-IP stage, ducks demonstrate a greater ability than chicks to recover T_e and HR when returned to incubation T_a after cold exposure (Fig. 10). By EP, duck embryos demonstrate an endothermic HR response (increase during cold exposure from incubation T_a of 38 to an exposure T_a of 28 °C) that is similar to, although larger than, the HR response seen in EP chicken embryos (Fig. 10 cf. Fig. 7). Immediately post-hatching, the wet duckling cannot maintain constant T_b during cold exposure (from brooding T_a of 35 °C to 25 °C; i.e. a 10 °C change similar to embryos) (Fig. 10E). Interestingly, although the late EP duck

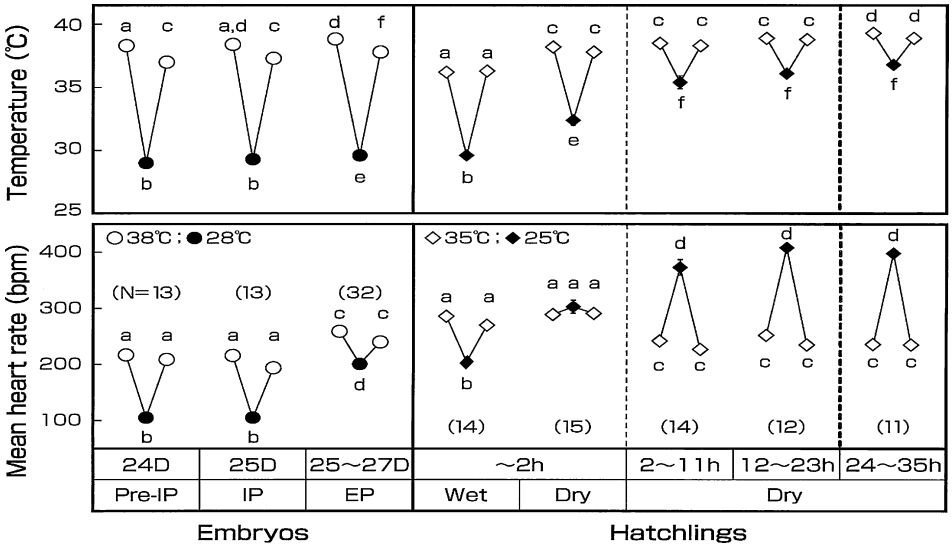

Fig. 11. Mean egg or cloacal temperatures (T_e or T_b, upper panel) and heart rate (MHR, lower panel) during the last 10 min of 1 h cold exposure (28 °C) in d24 pre-IP, d25 IP, d25–27 EP duck embryos, newly hatched wet (wet), newly hatched blotted dry (dry) and naturally dried d0 (2–11 h and 12–23 h) and d1 (24–35 h) ducklings. Values are means ± S.E.M. with some errors too small to visualize. *N* values are in parentheses. Different lowercase letters represent significant effects of T_a on T_e or T_b and MHR for each developmental stage (unpublished data).

embryos demonstrate an ability to increase HR during cold exposure (Fig. 10D), the HR of the newly hatched wet ducklings increases only transiently before the ducklings succumb to the cold and HR decreases. In newly hatched ducklings that have been blotted dry, both HR and Tb decrease to a lesser extent (e.g. $\Delta T_b = -6.8\,^{\circ}C$ cf. $-5.4\,^{\circ}C$) during cold exposure, highlighting the importance of thermal conductance, which is higher in wet ducklings. Presumably, the higher overall baseline HR of ducklings (compared with embryos) is indicative of higher baseline $\dot{M}_{O_2}$ resulting from increased obligatory thermogenesis (see Section 7.3). Although EP duck embryos are able to maintain HR during cold exposure, the lower overall baseline HR is indicative of lower basal metabolic rate. This lower level is potentially sustainable during cold exposure, whereas the higher basal HR and $\dot{M}_{O_2}$ observed in newly hatched ducklings is initially unsustainable. By 2 and 13 h post-hatching the ducklings have naturally dried and cold exposure ($T_a = 28\,^{\circ}C$) results in only a small deviation in T_b ($\Delta T_b = -1.9$ and $-2.0\,^{\circ}C$ respectively). Further, HR demonstrates an endothermic response, increasing during cold exposure (Fig. 11).

7.3.3. Emu

The newly hatched emu increases HR in response to cooling on the first day of hatching (Tamura et al., 2003). Thus, compared with the chick, the developmental pattern is shifted with the emu hatching at a more precocial stage having attained a higher level of endothermic competence. The T_e of pre-IP and IP emu embryos decreases during cold exposure and HR initially increases, although the embryos are unable to maintain the sustained HR (Fig. 12). Recovery of T_e and HR towards control levels occurs upon re-warming (similar to day 0 chick hatchlings e.g. Figs. 7 and 8). T_e decreases less in EP compared with pre-IP and IP embryos and embryos are able to re-warm once the cold stimulus is removed (Fig. 12). The HR response of EP embryos to cold exposure is variable, with some embryos exhibiting an endothermic thermoregulatory response, and others initially increasing HR (and presumably $\dot{M}_{O_2}$) and being unable to sustain these elevated levels. Overall, however, HR increases during cold exposure (Fig. 12). In summary, the endothermic HR response develops prior to hatching in the emu, providing EP embryos with a marked endothermic response of HR during cold exposure (Fukuoka et al., 2006). Type II HR oscillation, a LF oscillation, linked to thermoregulation in chicken embryos, is at times present (although uncommon) in emu hatchlings, although the association with thermoregulation in emu hatchlings is not as clear (Moriya et al., 1999, 2002; Tamura et al., 2003; Tazawa et al., 2001b).

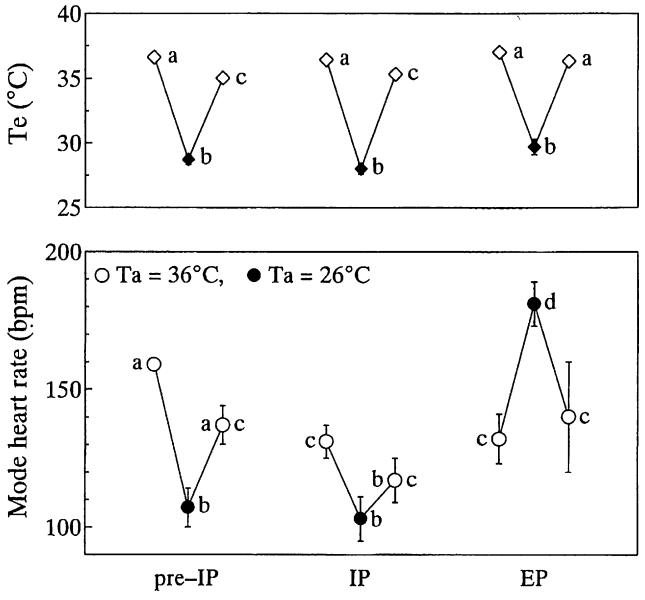

Fig. 12. Egg temperature (T_e; top panel) and mode heart rate during the last 10 min of 90-min cold exposure ($T_a = 26\,^{\circ}C$) of pre-IP, IP and EP emu embryos. Different lowercase letters represent significant effects of T_a on T_e and MHR for each developmental stage (Fukuoka et al., 2006).

8. Concluding remarks and perspectives

Although developments in mammalian *in utero* HR measurement will allow for a more thorough understanding of the development of HR and HR control in mammals (see e.g. Phoon, 2001 for review), the importance of the avian model should not be overlooked. Avian embryonic models allow for environmental manipulation and the separation of maternal factors that are just not possible with mammalian models *in utero*.

The use of relatively long-term measurements (days and weeks) of MHR has been integral in revealing infradian rhythms in chicken embryos and circadian rhythms in chicken hatchlings. These rhythms are not found in all bird species, with infradian rhythms lacking in the more precocial emu embryo and circadian rhythms only rarely observed in emu hatchlings.

Alterations in ambient O_2 levels have little effect on early embryonic HR, due likely to the low $\dot{M}_{O_2}$ of early embryos and the fact that O_2 delivery can occur via diffusion alone. As $\dot{M}_{O_2}$ increases in advanced embryos and circulatory convection becomes important for O_2 delivery, alterations in ambient O_2 have more profound effects on embryonic HR. Further, by ~60% of incubation parasympathetic control is present in precocial and altricial avian embryos with sympathetic control (IHR accelerations) becoming more prevalent close to hatching.

Temperature tolerance decreases with embryonic age. Embryonic HR decreases (blood flow is preserved) in an exponential fashion to a steady-state rate in response to a rapid decrease in T_a. A more severe decrease in T_a results in complete cessation of HR. However, depending on developmental age, embryos are able to survive severe cold exposure and cardiac arrest. Later as embryos begin to gain endothermic competence, the baseline HR and IHR fluctuations start to contribute substantially to endothermic development.

The allantoic arteries and veins can be simultaneously catheterized while adequate eggshell gas conductance is preserved. This allows measurement of arterial blood pressure and IHR during the administration of autonomic reagents (e.g. Fig. 9) at altered gaseous environments. Further, $\dot{M}_{O_2}$ of embryos can be simultaneously measured with IHR to further disentangle HR responses utilized for O_2 transport from other potential functions (e.g. angiogenesis) and to help understand the biological reasons for heart rate variability and heart rate irregularities. Holistic approaches such as these (and others that look at multiple organ systems) will be integral in revealing the complex interactions that contribute to HR control.

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