The Physiology of the Avian Embryo

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ABBREVIATIONS

A_p effective pore area
CAM chorioallantoic membrane
CO cardiac output
CO_2 carbon dioxide
dCO_2 carbon dioxide diffusion coefficient
dH_2O water vapor diffusion coefficient
dO_2 oxygen diffusion coefficient
DO_2 oxygen diffusing capacity
EP external pipping
$\overline{Fc}_{OX}$ mean corpuscular oxygenation velocity
GCO_2 carbon dioxide conductance
GH_2O water vapor conductance
GO_2 oxygen conductance
Hb hemoglobin
Hct hematocrit
HR heart rate
I incubation period
IHR instantaneous heart rate
IP internal pipping
IRR instantaneous respiratory rate
L shell thickness
$\dot{M}CO_2$ carbon dioxide elimination rate
MHR mean heart rate
$\dot{M}H_2O$ rate of water loss
$\dot{M}O_2$ oxygen consumption rate
O_2 oxygen
P_aCO_2 arterialized blood carbon dioxide partial pressure
P_aO_2 arterialized blood oxygen partial pressure
$PACO_2$ airspace carbon dioxide partial pressure
PAO_2 airspace oxygen partial pressure
P_B atmospheric pressure
$P\overline{c}_{O_2}$ mean capillary oxygen partial pressure
PH_2O water vapor pressure
$PICO_2$ effective environmental carbon dioxide partial pressure
PIO_2 effective environmental oxygen partial pressure
P_a arterial blood pressure
P_{sys} systolic blood pressure
$\dot{Q}_a$ allantoic blood flow
Q_{10} temperature coefficient
R gas constant
T_a ambient temperature

T_{egg} egg temperature
t_c contact time of erythrocytes in chorioallantoic capillary with O_2
V_c capillary volume
$[HCO_3^-]$ bicarbonate concentration
$[La^-]$ lactate concentration

32.1 INTRODUCTION

The freshly laid avian egg contains most of the materials needed for embryonic growth and development, but lacks the oxygen and heat needed for successful development. Microscopic pores in the eggshell allow O_2 to diffuse into the egg from the environment and water vapor and CO_2 produced by the embryo to diffuse out. The adult bird has a key role in incubation, providing not only the heat necessary for embryonic development but also controlling the microclimate of the egg. In the poultry industry and for research purposes, the adult bird can be conveniently replaced by an incubator. The majority of research on avian incubation is undertaken using artificially incubated chicken (*Gallus gallus domesticus*) eggs. Thus, in this chapter, the chicken embryo is used to elucidate the development of physiological function during avian incubation, supplemented by additional species when data are available. Developmental physiology of the gas exchange, acid–base, cardiovascular, osmoregulatory and thermoregulatory systems are examined. The optimal conditions for artificial incubation are outlined and embryonic responses to incubation extremes are described.

32.2 THE FRESHLY LAID EGG

The mass of the freshly laid bird egg ranges from ~0.8 g in the bee hummingbird (*Mellisuga helenae*) to ~2 kg in the ostrich (*Struthio camelus*). The egg is composed of the eggshell and outer and inner shell membranes that encompass the albumen, which serves as a source of water and protein; and yolk, a source of necessary nutrients. The composition of the freshly laid egg is related to the maturity of the hatchling, which differs considerably between species.

Hatchlings are divided into four major categories based on criteria such as mobility, amount of down, ability to feed, and locomotion. Precocial species are the most mature, and can walk, swim, or dive soon after hatching, while altricial species are the least mature and hatch naked, with eyes closed and are incapable of coordinated locomotion. Two intermediate categories—semi-precocial and semi-altricial—describe species that are within these extremes. The amount of yolk in the freshly laid egg is much greater in precocial species than in altricial species (Sotherland and Rahn, 1987). The yolk is only 16% of egg mass in the altricial red-footed booby (*Sula sula*), while in the highly precocial kiwi (*Apteryx australis*) it is 69% of egg mass. As most of the energy contained in the egg is in the lipid fraction, most of which is in the yolk, the energy content of precocial eggs is higher than that of altricial eggs. Furthermore, most of the water in the egg is in the albumen, and as the yolk/albumen ratio is lower in altricial than in precocial eggs, altricial eggs have relatively higher water compared with precocial eggs (Sotherland and Rahn, 1987).

32.3　INCUBATION

32.3.1　Incubation Period

Incubation encompasses the prenatal period, prior to "internal pipping" (IP), when the embryo penetrates its beak through the chorioallantoic and inner shell membranes into the air cell; and the perinatal or paranatal period from IP to when the embryo fractures the shell ("external pipping," EP) and hatches. The incubation period (*I*, days) increases with egg size and can be represented as follows:

$$I = 12 \cdot (\text{Egg mass})^{0.22} \tag{32.1}$$

where egg mass is in g (Rahn and Ar, 1974). In addition, as a first approximation, the product of incubation period and O_2 consumption $(\dot{M}O_2)$ at the stage where $\dot{M}O_2$ remains unchanged (i.e., plateau) is proportional to egg mass (Rahn et al., 1974):

$$I \cdot \dot{M}O_2 = c \cdot (\text{Egg mass}) \tag{32.2}$$

where *c* is a constant. Thus, for a given egg mass an egg that consumes less O_2 at the plateau stage needs a longer incubation period. Furthermore, because the $\dot{M}O_2$ at the plateau stage is matched to the shell gas conductance, for a given egg mass an egg with lower shell conductance requires a longer incubation period.

　　Apart from these general trends, eggs that are left unattended by the incubating parent for a period of time will exhibit cooling, and this results in slower embryonic growth and a longer incubation period. Some birds, notably tropical seabirds and petrels (Procellariiformes), have much longer incubation periods than that suggested by the general relationship (Whittow, 1980), even though they are incubated continuously.

32.3.2　Egg Water Content and Shell Conductance

As the embryo grows within the egg, water is lost and both the yolk and albumen diminish in mass. However, metabolic water is produced when fat in the yolk is oxidized. Part of the water is inevitably lost by diffusion through the pores in the shell to the microclimate of the egg. The rate of water loss $(\dot{M}H_2O,\ \text{mg /day})$ is determined by two factors: the water vapor conductance of the shell and shell membranes (GH_2O, mg/day/kPa) and the difference in water vapor pressure between the contents of the egg and the environment (ΔPH_2O, kPa) (Rahn and Ar, 1974):

$$\dot{M}H_2O = GH_2O \cdot \Delta PH_2O \tag{32.3}$$

GH_2O is a function of multiple factors, including shell geometry (effective pore area, A_p, thickness of the shell combined with shell membranes, *L*); water vapor diffusion coefficient (dH_2O); and the inverse product of the gas constant (*R*) and absolute temperature (*T*):

$$GH_2O = [(A_p/L) \cdot dH_2O]/RT \tag{32.4}$$

Both pore area and shell thickness increase in larger eggs (Ar et al., 1974). Although an increase in pore area increases GH_2O, an increase in shell thickness has the opposite effect, because it lengthens the diffusion pathway for water vapor. The water lost from the egg is replaced by air, enlarging the air cell at the blunt pole of the egg during development. Upon IP and EP, the rate of water loss from the egg increases, as water vapor can diffuse through the cracks (pip-hole) in the shell. The water loss from the egg over the entire incubation period amounts to ~18% of the mass of the freshly laid egg.

32.3.3　Heat Transfer

Many adult birds (typically but not always the female) play an important role in incubation of their eggs, by using their body and nesting material to alter the environment of the eggs. Most birds develop a seasonal bare patch of skin, the brood patch, on part of the thorax and abdomen. This directly contacts with the eggs, permitting a greater rate of heat transfer than if the patch were covered with plumage. Accompanying the loss of feathers is an increase in the size and number of blood vessels in the bare skin. The adult can adjust the rate of heat transfer to the egg by standing over or leaving the vicinity of the egg, but also by the closeness with which the bird applies its patch to the egg. The bird responds physiologically to variations in egg temperature (T_{egg}), increasing its metabolic heat production in response to cooling of the egg (Tøien et al., 1986; Rahn, 1991).

　　The amount of heat transferred to the eggs is directly proportional to the increase in heat production (Tøien et al., 1986). The efficiency of heat transfer to the eggs diminishes

with decreasing ambient temperature and clutch size. Heat stored in the incubating bird while flying and foraging (i.e., while not incubating the eggs) can be transferred to the eggs on return to the nest (Biebach, 1986). If the egg is very cold, "cold vasodilation" occurs in the brood patch, increasing the patch blood flow and temperature and therefore heat transfer to the egg (Mitgard et al., 1985). The brood patch temperature varies from 34.9 °C in the bonin petrel (*Pterodroma hypoleuca*) to 42.4 °C in the dusky flycatcher (*Empidonax oberholseri*). The brood patch temperature is 1.1–5.5 °C higher than the T_{egg} in numerous species (Rahn, 1991).

At the beginning of incubation, heat is transferred through the egg by conduction, and the surface of the egg on the opposite side to the brood patch may be 4 °C or more below the brood patch temperature (Rahn, 1991). As incubation proceeds, this temperature difference diminishes as the embryo's developing circulation assists in the distribution of heat and its increasing metabolism provides additional heat (Turner, 1987; Rahn, 1991). This effect of blood flow on heat flow is more important in large eggs than in small ones (Tazawa et al., 1988a). The main barrier to heat loss from the egg is a thin layer of air immediately adjacent to the shell, the boundary layer (Sotherland et al., 1987). If the egg is in a nest, the nest itself imposes an additional resistance to heat loss.

As the embryo grows, incubation likely becomes more a matter of regulating the balance between heat gain from the mother and heat loss through radiation from the egg surfaces, because the near-term embryo is producing considerable heat itself. The rate of heat loss from the egg is going to depend on many factors, though one of the more important ones may be the surface area-to-volume ratio of the egg. Thus, larger eggs (e.g., emu, ostrich) may have lower capacity for heat loss because of their smaller surface area-to-volume ratio. However, larger embryos may also show conventional metabolic scaling, with lower per-gram body mass rates of temperature production than smaller embryos. A study that integrates egg size, embryo metabolic rate, and incubation behavior changes during embryonic development is highly warranted.

32.3.4 Energy Use

As chicken embryos grow, their mass increases geometrically until growth rate slows during the last stages of development (Romanoff, 1967; Van Mierop and Bertuch, 1967; Tazawa et al., 1971a; Lemez, 1972; Clark et al., 1986; Haque et al., 1996). From the mean values of the above references, the geometrical increase in embryo wet body mass to day 16 of incubation is expressed by:

$$\text{Body mass (mg)} = 0.24 \cdot I^4 \qquad (32.5)$$

Of the energy measured as oxygen uptake $(\dot{M}O_2)$, the majority is utilized by the embryo to synthesize new tissues for growth and to meet the physiological demands for maintenance during development. In addition, toward the end of incubation, the embryo uses energy for active control of body temperature and hatching. Prior to this, it is assumed that prenatal $\dot{M}O_2$ is used for maintenance, which is proportional to body mass, and for growth, which is proportional to growth rate ($GR = \Delta Body\ mass/time$) as follows (Vleck et al., 1980; Mortola and Cooney, 2008):

$$\dot{M}O_2 = a \cdot (\text{Body mass}) + b \cdot GR \qquad (32.6)$$

where coefficients a and b express the daily average cost of maintaining 1 g of tissue (mL O_2/g/day) and the cost of growing it (mL O_2/g), respectively. During days 9 to 18 of the ~21 day incubation period of chickens, and excluding early development when $\dot{M}O_2$ of small embryos does not reflect total egg $\dot{M}O_2$ because of uptake by extra-embryonic tissues, a and b are estimated as ~15 mL O_2/g/day and ~41 mL O_2/g, respectively (Mortola and Cooney, 2008). Therefore, the cost of growing 1 g of tissue averages ~3 times the cost of maintaining it. In addition, cooling due to low temperature incubation (35 °C) only decreases GR with a decrease in the cost of growth, but hypoxic incubation decreases not only GR but also the cost of maintenance. That is, cold incubation decreases GR without altering the partitioning of energy expenditure while hypoxia decreases GR and alters the partitioning of the energy.

Daily energy use increases as the embryo grows and is reflected in an increase in $\dot{M}O_2$. However, precocial and altricial birds show a different developmental pattern in embryonic $\dot{M}O_2$ (e.g., Vleck and Vleck, 1987). In altricials, $\dot{M}O_2$ increases at an increasing rate throughout incubation, lacking a plateau. In precocials, the rate of increase in $\dot{M}O_2$ plateaus before the egg is pipped and then increases again after pipping. For a given egg size and incubation period, altricials use less energy, and so have a lower incubation energy cost to hatching than precocials (Vleck et al., 1980). The high cost of incubation in precocials is due to their rapid growth early in development, which results in higher maintenance costs—that is, there is more tissue to maintain for a longer time (Vleck et al., 1980).

Based on the assumption that both precocials and altricials have an identical basic pattern of increase in $\dot{M}O_2$, including a plateau phase attributable to the shift of the gas exchange from the chorioallantoic membrane (CAM) to the lungs (Rahn and Ar, 1974), Prinzinger et al. (1995) defines the plateau phase as a clear interruption of the continuous exponential increase in $\dot{M}O_2$. This demonstrates no fundamental difference between both modes with respect to the presence of a plateau (Figure 32.1) (Prinzinger et al., 1995; Prinzinger and Dietz, 1995). They suggest that in small altricials the plateau lasts only a few hours and can be overlooked when $\dot{M}O_2$ measurement is not continuous or when many individual measurements are averaged. The extremely precocial mound-building birds (Megapodiidae) are one exception having no plateau phase as they lack the CAM-pulmonary transition within the egg (Vleck et al., 1984).
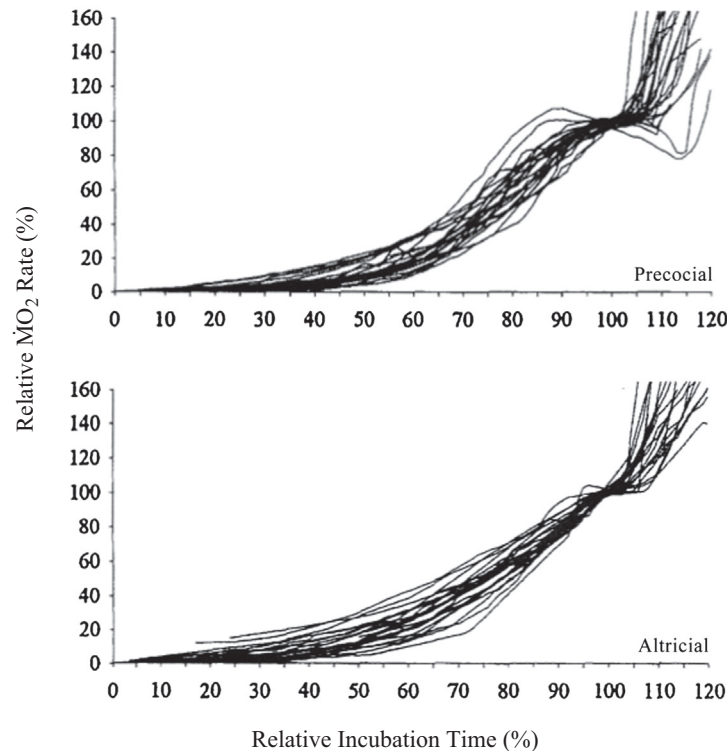
FIGURE 32.1   Developmental patterns of relative oxygen uptake ($\dot{M}O_2$, %) in precocial and semiprecocial species ($N=27$) (top) and altricial and semi-altricial species ($N=24$) (bottom) plotted against relative incubation time (%). The O_2 uptake and incubation time at the middle of plateau stage are both set at 100%. *Modified from Prinzinger and Dietz (1995), with permission from Elsevier.*

32.4 DEVELOPMENT OF PHYSIOLOGICAL SYSTEMS

32.4.1 Gas Exchange

The egg with its hard shell does not enable embryonic ventilatory movements, and thus there is no convective gas exchange until the embryo's lungs begin to function. Early in incubation, prior to a formation of the heart and even initially after the forming heart beats, O_2 can be adequately supplied from the environment to the embryo through diffusion. $\dot{M}O_2$ is normally maintained without blood convection, even in embryos whose main vessel from the heart is ligated or hemoglobin (Hb) is rendered functionless due to carbon monoxide exposure (Burggren et al., 2000, 2004; Mortola et al., 2010; Burggren, 2013). When gas transport by diffusion alone becomes inadequate, blood convection begins to meet O_2 demand during development, and three different gas exchangers sequentially function in the egg; the area vasculosa, the chorioallantois, and the lungs. Figure 32.2 shows the growth rate of the functional surface area of the area vasculosa and the chorioallantois (Ackerman and Rahn, 1981) and the associated $\dot{M}O_2$ of the chicken embryo. The area vasculosa is a well-vascularized region of the yolk sac that fans out from the embryo and rapidly grows around the yolk during days 3 to 5 of incubation. The blood vessels of the yolk sac connect with the dorsal aorta of the embryo by day 2 and blood begins to circulate through the embryo and the area vasculosa. The fine reticulation of the vitelline circulatory system plays the role of the main gas exchanger until the chorioallantois makes contact with the inner shell membrane around day 6 (Ackerman and Rahn, 1981). Subsequently, respiratory function transitions from the area vasculosa to the chorioallantois.

Beginning on day 5 of incubation, the mesenchyme covering the fundus of the allantoic sac comes into contact with the mesenchyme lining the chorion. The two membranes begin to fuse, and the growing allantoic sac flattens out beneath the chorion, which lies close to the eggshell. The outer limb of the flattened allantois, composed of the cohesive chorion and allantois, is the chorioallantois. The chorioallantois grows rapidly (Figure 32.2). It reaches almost the same size as the embryo by the time it makes contact with the shell membranes on day 6. By day 12, the chorioallantois extends to envelop the contents of the whole egg, lining the entire surface of the inner shell membrane.

The outer surface of the chorioallantois, the chorioallantoic membrane (CAM), is well vascularized (Wangensteen et al., 1970/71; Tazawa and Ono, 1974; Wangensteen and Weibel, 1982). Early in incubation, the capillaries lie on the mesenchymal surface of the CAM. They begin to migrate through the ectoderm on day 10 and lie on its thin layer late in incubation. In addition, relocation of the nuclei of the
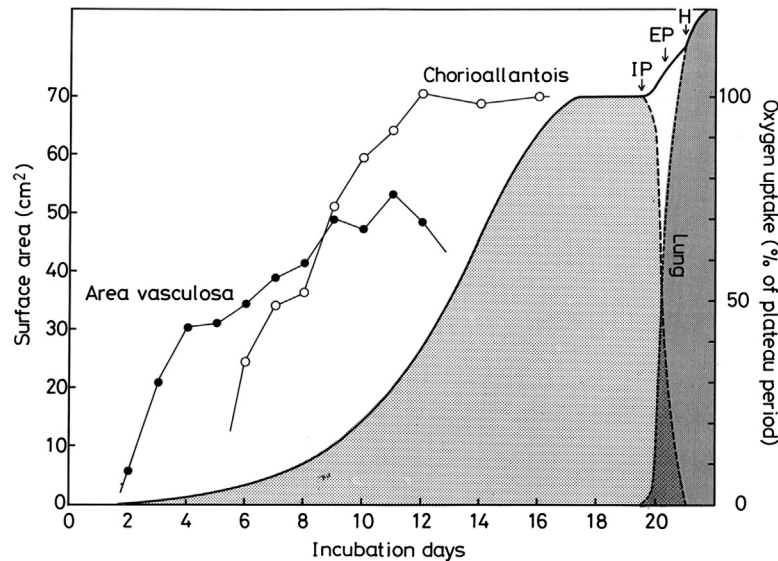
FIGURE 32.2 Daily changes in functional surface area of the area vasculosa and the chorioallantois (left ordinate), and developmental pattern of oxygen uptake ($\dot{M}O_2$) during the prenatal period (until internal pipping, IP) and perinatal period (from IP to hatching, H) (right ordinate). External pipping (EP) occurs during the perinatal period. $\dot{M}O_2$ is drawn diagrammatically with the plateau set at 100%. The lightly shaded area indicates $\dot{M}O_2$ by diffusion through the area vasculosa/chorioallantois and the heavily shaded area $\dot{M}O_2$ by the lungs. *From Ackerman and Rahn (1981), with permission from Elsevier.*

CAM capillaries occurs (Mayer et al., 1995). The endothelial nuclei randomly distribute around the capillary lumen early in incubation and are located progressively on the portion of the capillaries away from the shell membrane after the chorioallantois envelops the whole contents of the egg. Together with capillary migration, the relocation of endothelial nuclei results in progressive thinning of the gas diffusion pathway between the interstices of the inner shell membrane (airspace) and the capillary blood.

The CAM functions as a gas exchanger during prenatal development (prior to IP) until it degenerates when the embryo pips it and the inner shell membrane with the beak. The increase in $\dot{M}O_2$ of the chicken embryo during the first ~16 days of incubation is enabled by the extension of the CAM (Figure 32.2). The gas exchange of embryos before IP takes place by diffusive transport across the porous shell, two shell membranes, and capillary blood of the CAM (Wangensteen and Rahn, 1970/71; Wangensteen et al., 1970/71, Wangensteen and Weibel, 1982). Then, the chick breaks the shell and breathes environmental air by lung ventilation (EP). As the near-term embryo begins ventilating the lungs, they replace the gas exchange function of the CAM.

When the CAM envelopes all contents of the egg, the gas exchange by diffusive transport is expressed by:

$$\dot{M}O_2 = GO_2 \cdot (PIO_2 - PAO_2) \tag{32.7}$$

$$= DO_2 \cdot (PAO_2 - P\bar{c}O_2) \tag{32.8}$$

where $\dot{M}O_2$ is oxygen uptake (mL/min), GO_2 is oxygen conductance of the shell and shell membrane (mL O$_2$/min/

kPa), DO_2 is the diffusing capacity of the CAM and capillary blood (mL O$_2$/min/kPa), PIO_2 is the effective environmental oxygen partial pressure (kPa); PAO_2 is the airspace oxygen partial pressure (kPa), and $P\bar{c}O_2$ is mean capillary oxygen partial pressure (kPa).

GO_2 is related to GH_2O by the diffusion coefficient ratio (dO_2/dH_2O =0.23/0.27). GO_2 (in mL/min/kPa) is derived from GH_2O (in mg/min/kPa) by multiplying by a factor of 1.06. Because GO_2 in air is nearly constant during the prenatal incubation, PAO_2 decreases as the embryo grows and consumes more O$_2$ (Table 32.1). The increased difference of O$_2$ partial pressure between the environmental air and airspace is the driving force that meets the increased O$_2$ demand of the developing embryo. Concurrently with the decrease in PAO_2, $P\bar{c}O_2$ also decreases. The O$_2$ flux from the airspace to the Hb of capillary blood (inner diffusion barrier) is facilitated by an increase in DO_2, which is expressed by:

$$DO_2 = 60 \cdot V_c \cdot F\bar{c}_{OX} \cdot Hct \tag{32.9}$$

$$= \dot{Q}_a \cdot t_c \cdot F\bar{c}_{OX} \cdot Hct \tag{32.10}$$

where V_c is capillary volume of the CAM (µL), $F\bar{c}_{OX}$ is mean corpuscular oxygenation velocity during the contact time per s per kPa, Hct is hematocrit, $\dot{Q}_a$ is blood flow through the CAM (mL/min), and t_c is the contact time of erythrocytes with O$_2$ when they pass through the CAM capillaries (s) (Tazawa et al., 1976b; Tazawa and Mochizuki, 1976) (Table 32.1).

DO_2 is increased by an increase in V_c, which depends on $\dot{Q}_a$ and t_c; $V_c = (\dot{Q}_a/60) \times t_c$ (Table 32.1). While $\dot{Q}_a$ increases about five-fold from days 10 to 18, the t_c halves,

TABLE 32.1 Gas Exchange Variables Used to Determine the Diffusing Capacity of the Inner Diffusion Barrier (Chorioallantoic Membrane and Capillary Blood) in Developing Chicken Embryos

Age (days)	10	12	14	16	18
Body mass	2.4	5.2	9.3	16.0	22.9
PAO_2	18.5	17.2	16.3	14.7	14.4
$PACO_2$	1.5	2.1	3.3	3.8	4.2
P_aO_2	10.9	10.7	9.9	8.6	7.8
pH_a	7.64	7.62	7.54	7.50	7.48
S_aO_2	87.2	88.2	87.0	88.0	84.5
PvO_2	4.5	3.7	3.4	2.5	2.4
$PvCO_2$	2.5	3.3	4.9	5.4	5.5
S_vO_2	10.4	11.2	17.7	17.3	25.2
O_2 capacity	9.4	10.1	10.5	11.4	12.3
$\dot{M}O_2$	0.08	0.15	0.25	0.36	0.42
$\dot{Q}_a$	1.11	1.93	3.42	4.48	5.78
t_c	0.87	0.74	0.57	0.49	0.36
V_c	16.1	23.8	32.5	36.6	34.7
$F\bar{c}_{OX}$	41.9	51.0	60.8	62.3	65.3
Hct	20.5	22.6	27.6	32.8	36.5
DO_2	0.83	1.65	3.27	4.49	4.96

Body mass (g); from Tazawa and Mochizuki (1976); PAO_2 (airspace PO_2, kPa), $PACO_2$ (airspace PCO_2, kPa), PvO_2 (mixed venous blood PO_2, kPa), $PvCO_2$ (mixed venous blood PCO_2, kPa); calculated from data in Tazawa (1973) and Wangensteen and Rahn (1970/71). Note: Unit of partial pressure is converted from 'mmHg' in original sources to 'kPa' in current text; P_aO_2 (arterialized PO_2 in the allantoic vein, kPa); from Tazawa (1973); pH_a (arterialized blood pH corresponding to the allantoic vein); from Tazawa et al. (1971a); S_aO_2 (oxygen saturation of arterialized blood, %); calculated by substituting P_aO_2 and pH_a into the modified Hill's equation of the O_2 dissociation curve (Tazawa et al., 1976a); S_vO_2 (oxygen saturation of mixed venous blood, %); determined by micro-photometer (Tazawa and Mochizuki, 1976); O_2 capacity (vol%); determined from Tazawa (1971) and Tazawa and Mochizuki (1977); $\dot{M}O_2$ (oxygen uptake, mL/min); from Tazawa (1973); $\dot{Q}_a$ (allantoic blood flow, mL/min); calculated by $\dot{M}O_2/[O_2$ capacity $(S_aO_2 - S_vO_2)]$; t_c (contact time, sec); determined by micro-photometer (Tazawa and Mochizuki, 1976); V_c (capillary volume, 10^{-3} mL), calculated by $\dot{Q}_a$ /60 × t_c; $F\bar{c}_{OX}$ (oxygenation velocity factor per s per kPa); from Tazawa et al. (1976b); Hct (hematocrit, %); from Tazawa et al. (1971a); DO_2 (diffusing capacity of inner diffusion barrier, mL/min/kPa); calculated by $60 \cdot V_c \cdot F\bar{c}_{OX} \cdot Hct$ or $\dot{Q}_a \cdot t_c \cdot F\bar{c}_{OX} \cdot Hct$.

days 14 and 15. Nevertheless, DO_2 increases further after day 14. This is largely due to the increase in $\dot{Q}_a$ and Hct, as both variables increase after the CAM spreads over the inner shell membrane. Towards the end of prenatal development, the increase in DO_2 slows down and $\dot{M}O_2$ reaches a plateau. This suggests an important contribution of the inner diffusion barrier to gas exchange, which contributes, along with the outer diffusion barrier (GO_2), to the plateau status of $\dot{M}O_2$. This results in the developmental pattern of $\dot{M}O_2$ paralleling the daily changes in DO_2 (Table 32.1).

In chicken eggs, the variability of shell GO_2 is large, higher than that of egg mass. Mass-specific $\dot{M}O_2$, measured on days 16 to 19 of incubation, is maximal at medium GO_2, decreasing at both lower and higher GO_2 (Visschedijk et al., 1985). The maximum $\dot{M}O_2$ at medium GO_2 values is considered to be optimal for embryo development, and the decrease in $\dot{M}O_2$ at both higher and lower GO_2 is a sign of compromised development. When GO_2 is decreased by partially covering the shell with impermeable material or increased by partially removing the shell over the air cell at the beginning of incubation, $\dot{M}O_2$ of day 16 embryos increases hyperbolically with increasing GO_2, reaching a maximum at the control GO_2 of intact eggs and decreasing with further increase in GO_2 (Okuda and Tazawa, 1988). When part of the shell is covered and the other is exposed to hyperoxia, $\dot{M}O_2$ and growth rate do not change, indicating uniform GO_2, and therefore uniform chorioallantoic perfusion is not required to maintain $\dot{M}O_2$ (Wagner-Amos and Seymour, 2002).

Because the oxygen diffusion coefficient (dO_2) affects shell conductance, GO_2 can be changed by replacing N_2 in air with an inert background gas whose density is different from that of N_2 (e.g., He or SF_6), thus changing dO_2 (Erasmus and Rahn, 1976; Ar et al., 1980; Tazawa, 1981a; Tazawa et al., 1981). Accordingly, the gas exchange of the egg can be manipulated by changing GO_2 with He or SF_6. The dO_2 is also inversely related to atmospheric pressure (P_B), and thus gas exchange of the egg is increased at high altitude because the shell GO_2 increases inversely with P_B. The reduction of GO_2 in eggs laid by birds incubating at high altitude occurs as a natural adaptation to altitude (Rahn et al., 1977).

As incubation proceeds, diffusive gas exchange governed by GO_2 and DO_2 is gradually replaced by convective gas exchange through the lungs during the perinatal period, beginning at IP (Figure 32.2). Breathing movements of perinatal embryos can be recorded as pressure changes using an optical system or pressure transducer (Romijn, 1948; Vince and Salter, 1967; Dawes, 1976). Conveniently, prenatal cardiac rhythms at the onset of IP and EP and the subsequent development of respiratory rhythms until hatching can be demonstrated by continuous measurements of cardiogenic, ventilatory and hatching activities in chickens by means of a condenser-microphone measuring
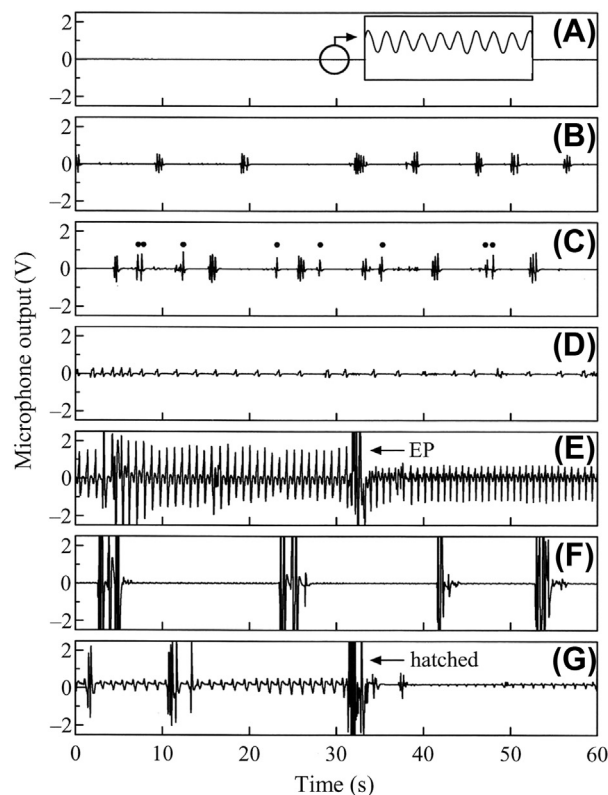
probably because of shortening of the blood circulation time, as cardiac output increases more rapidly than total blood volume. Consequently, V_c increases even after the CAM spreads over the whole surface of the inner shell membrane on day 12 and reaches a maximum volume on

FIGURE 32.3 **The cardiogenic and breathing activities of a chicken embryo from the prenatal through perinatal period to hatching, recorded by a condenser microphone attached hermetically on the shell.** A 60 s recording is shown in each panel. (A) Cardiogenic signal (acoustocardiogram, ACG) during the final stages of the prenatal period; ACG is evident in the enlarged inset. (B) Beak-clapping signals just prior to IP (C) Onset of breathing signals occurring as single, irregular deflections (approximately once per 5 s) among beak clapping after IP (acoustorespirogram, ARG). (D) Rhythms become fast and somewhat irregular. (E) Breathing signals are more regular before and after EP. The onset of EP is evident by a sudden decrease in magnitude of ARG. (F) Approximately 1 h prior to hatching, large deflections due to hatching activities (e.g. climax) begin to occur and the breathing becomes intermittent. (G) The hatching activities continue before breathing regulates and finally the embryo hatches. Breathing signals are recorded after hatching, because the microphone is located close to the beak of hatchling. *From Chiba et al. (2002), with permission from Elsevier.*

system (Figure 32.3) (Chiba et al., 2002). Using the breathing patterns of chickens from the onset of IP through EP to hatching, the length of the IP, EP, and whole perinatal periods are determined as 14.1 ± 2.0 (SEM) h, 13.6 ± 1.3 h, and 27.6 ± 1.7 h long, respectively. The duration of climax (hatching) takes 48 ± 6 min. The instantaneous respiratory rate (IRR in breaths per min) is approximately 10 to 15 breaths/min at the beginning of IP; IRR baseline increases up to 80–100 breaths/min with large fluctuations of IRR of 60 to 180 breaths/min after the onset of EP.

Pulmonary ventilation and gas exchange during the perinatal period has been quantified using a barometric plethysmograph or pneumotachograph (Pettit and Whittow, 1982a;

Menna and Mortola, 2003; Sbong and Dzialowski, 2007; Szdzuy and Mortola, 2007) and by measurement of gases in air cell and allantoic blood (Pettit and Whittow, 1982b; Tazawa et al., 1983b). The embryonic development of pulmonary ventilation and its regulation has been reviewed by Mortola (2009).

32.4.2 Acid–Base Regulation

While the embryo consumes O_2, it produces CO_2, which is partially dissolved and stored in the blood and body fluids, but mostly eliminated through the eggshell. As with O_2, CO_2 elimination $(\dot{M}CO_2)$ depends upon the eggshell CO_2 conductance (GCO_2) and CO_2 partial pressure difference between air cell ($PACO_2$) and atmosphere ($PICO_2$):

$$\dot{M}CO_2 = GCO_2 \cdot (PACO_2 - PICO_2) \qquad (32.11)$$

GCO_2 is a function of eggshell geometry (A_p and L), CO_2 diffusion coefficient (dCO_2), and the inverse product of R and T. $PICO_2$ is very close to zero when the egg is in air.

As embryos develop and their body mass increases, they produce more CO_2, which accumulates in the egg. $PACO_2$ increases, thus increasing arterialized blood PCO_2, P_aCO_2. Consequently, arterialized blood pH is lowered (Figure 32.4) or stays relatively constant in the allantoic vein during the last half of prenatal development (Dawes and Simkiss, 1969; Girard, 1971; Erasmus et al., 1970/71; Boutilier et al., 1977; Everaert et al., 2011). The rate of decrease in pH during embryonic development slows, however, late in incubation. Although the plasma bicarbonate concentration ([HCO_3^-]) increases with development, the increase is more than would be expected from changes in pH and the buffer value (-16 mmol/L, Burggren et al., 2012). The pH change due to CO_2 accumulation is mitigated by an increase in nonrespiratory HCO_3^- (Figure 32.4) (Tazawa, 1986, 1987). Hemoglobin (Hb), which serves as the noncarbonate buffer in blood, increases during the last half of incubation and thus is partly responsible for the mitigated change in pH. Reflecting the developmental increase in Hct and [Hb], the buffer value increases from ~ -8 to -10 mmol/L on days 9 to 10 to ~ -17 mmol/L on days 15 to 18 (Erasmus et al., 1970/1971; Tazawa and Piiper, 1984). In day 16 embryos, values ranging from -12.7 to -15 mmol/L are reported (Tazawa et al., 1981; Tazawa, 1980a, 1981b, 1982, 1986). Other studies have not found an increase in buffer value during development (Tazawa et al., 1983a; Andrewartha et al., 2011b; Burggren et al., 2012). The determination of the buffer value is a controversial issue, and clearly not all sources of variation have been identified. However, the value of -16 mmol/L reported recently (Burggren et al., 2012) is depicted in a pH-[HCO_3^-] diagram (Davenport diagram, Figure 32.4) to show and estimate respiratory changes in acid–base balance.
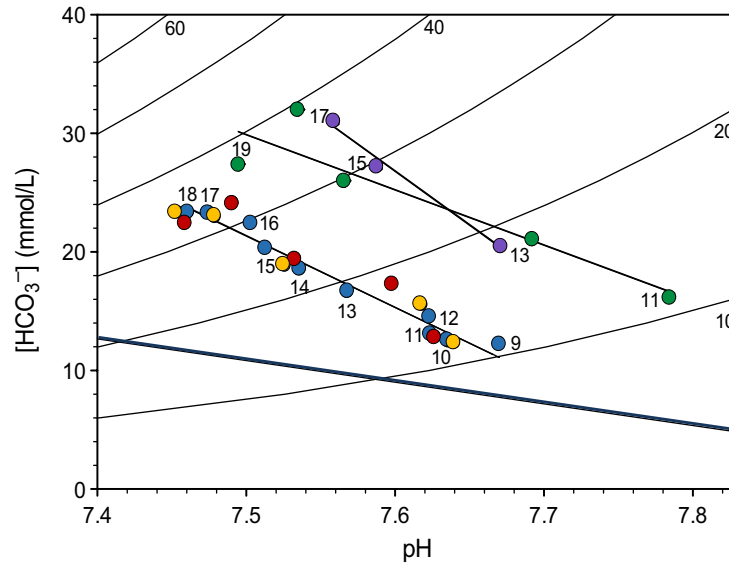
FIGURE 32.4 **Davenport diagram illustrating the daily changes in control acid–base balance of arterialized blood in chicken embryos developing in air.** The dark blue line is an arbitrary buffer line with the determined slope of −16 mmol/L pH/unit. Numbered curves are PCO_2 isopleths in mm Hg. The numbers near the data points represent the ages of the embryos. *Data are collected from the following studies; blue circles: Tazawa et al. (1971a), red circles: Tazawa et al. (1971b), yellow circles: Tazawa (1973), purple circles: Burggren et al. (2012), green circles: Tazawa et al. (2012). The solid line drawn through the data points corresponds to the following regression equation; (1) $[HCO_3^-] = -60.2\ pH + 472.6$ for combined data from Tazawa et al. (1971a, 1971b) and Tazawa (1973), (2) $[HCO_3^-] = -90.9\ pH + 717.4$ from Burggren et al. (2012), and (3) $[HCO_3^-] = -46.6\ pH + 379.1$ from Tazawa et al. (2012).*

Responses in acid–base balance to 1 day exposure to altered environmental gas mixtures differ depending on the gas mixture and age of chicken embryos (Figure 32.5) (Burggren et al., 2012). One day of hypercapnic exposure (5% CO_2, 20% O_2) increases P_aCO_2 and decreases pH_a, producing respiratory acidosis that is partially (~50%) compensated by metabolic alkalosis at all embryonic stages examined (days 13, 15, and 17) (Figure 32.5). Similar patterns of partially compensated respiratory acidosis have been reported in embryos exposed to 9% CO_2 in air for >3 days (Dawes and Simkiss, 1969). One day of exposure to hypercapnic hypoxia (5% CO_2, 15% O_2) abolishes compensatory metabolic alkalosis in day 15 and day 17 embryos, but a metabolic compensation of ~37% still occurs in day 13 embryos (Figure 32.5). This suggests that a relatively high O_2 level is required for metabolic compensation to occur. The lower $\dot{M}O_2$ and overall higher allantoic P_aO_2 in air (Tazawa, 1971, 1980a; Tazawa et al., 1971a,b) in day 13 embryos compared with more advanced embryos preserves the metabolic compensation during hypoxia. One day of hypoxic exposure (15% O_2) creates a metabolic acidosis in day 15 and day 17 embryos, but not in day 13 embryos (Figure 32.5). Hypoxia produces hypometabolism without anaerobic energy compensation (Bjønnes et al., 1987; Mortola and Besterman, 2007). However, once a lower threshold $\dot{M}O_2$ is reached, embryos turn to anaerobic glycolysis and blood lactate concentration ($[La^-]$) increases (Grabowski, 1961, 1966; Bjønnes et al., 1987). Therefore, embryos exposed to severe hypoxia (e.g., 10% O_2)

encounter metabolic acidosis caused by glycolysis (Tazawa et al., 2012). However, metabolic acidosis occurring in moderate hypoxia is attributed only slightly to glycolysis and other unverified mechanisms, such as O_2 level influencing HCO_3^- transfer across the CAM. One day exposure to hyperoxia (40% O_2) causes respiratory acidosis that varies with embryonic age (Figure 32.5). Hyperoxia causes hypermetabolism (and a consequent increase in $\dot{M}CO_2$) (Visschedijk et al., 1980; Høiby et al., 1983; Stock et al., 1985; Tazawa et al., 1992b). Increased CO_2 is accumulated in the blood and hydrated to create H^+ and HCO_3^-. Coupled with a nearly fixed eggshell gas GCO_2, this results in respiratory acidosis. Age-specific differences in the magnitude of respiratory acidosis are due to hyperoxia causing a greater hypermetabolism in advanced embryos (Stock et al., 1985; Tazawa et al., 1992b).

Because eggshell GCO_2 is governed by dCO_2, respiratory acidosis also occurs in embryos exposed to a SF_6/O_2 gas mixture, which reduces GCO_2. Inversely, respiratory alkalosis occurs in embryos exposed to a He/O_2 atmosphere, which increases GCO_2 (Tazawa et al., 1981). Partially covering the eggshell with a gas-impermeable material or opening the eggshell over the air cell also produce respiratory acidosis and respiratory alkalosis, respectively. These respiratory disturbances to the acid–base balance occur rapidly and blood P_aCO_2 reaches a plateau ~10 min after changing GCO_2 (Tazawa, 1981a). Concurrently, blood pH changes to the value predicted by buffer capacity during the following 30–60 min (i.e., noncompensated respiratory
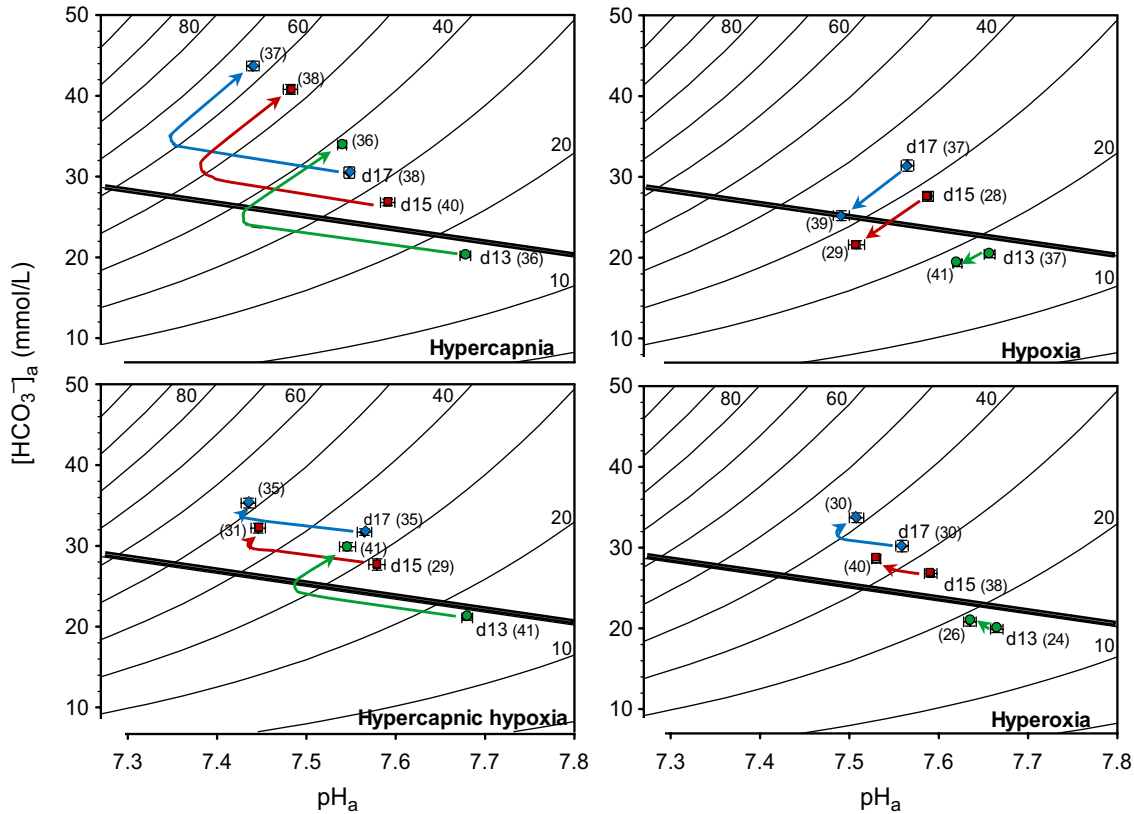
FIGURE 32.5 Regulation of arterialized blood acid–base balance of chicken embryos exposed to air (control) or altered $[CO_2]$ and/or $[O_2]$ for the last day of 13 (green), 15 (red), and 17 (blue) days of incubation. The colored arrows indicate changes in acid–base status from control. The dark blue line is the same as in Figure 32.4. *N* values are presented in parentheses. Mean values ±1 S.E.M. are shown. *Modified from Burggren et al. (2012), with permission from Elsevier.*

acidosis/alkalosis) with a subsequent (2–6 h), but incomplete, change toward the control level, while P_aCO_2 is maintained at a constant value. Once partial metabolic compensation is achieved at ~4–6 h, the state of acid–base disturbance remains constant over the next 24 h in normoxia or hyperoxia (e.g., 5% CO_2/20% O_2 or 5% CO_2/40% O_2), but metabolic compensation is not preserved after 24 h in hypoxia (5% CO_2/15% O_2) (Mueller et al., 2013b). Therefore, preservation of metabolic compensation requires an O_2 concentration above 20%.

Although partial metabolic compensation in response to hypercapnia or moderate hypoxia with hypercapnia (e.g., 15% O_2/5% CO_2) proceeds over the course of several hours, responses to severe hypoxia (10% O_2), with or without CO_2 (e.g., 5%), progress more quickly (Figure 32.6) (Tazawa et al., 2012). This is due to anaerobic glycolysis and the attendant rapid increase in $[La^-]$ that creates severe metabolic acidosis. If hypoxic embryos can preserve $[HCO_3^-]$ above ~10 mmol/L (in the case of day 15 embryos), which is generally reached beyond 2 h, embryos can survive and recover to the control state of acid–base balance in ~2 h after being returned in air (Figure 32.6) (Tazawa et al., 2012). Accordingly, hypoxia-induced acid–base disturbances are only transient and may not affect long-term survival.

In nature, the natural variations in eggshell conductance cause large differences in P_aCO_2 among eggs, but blood pH variations are kept to a minimum (Tazawa et al., 1983a). In eggs with low GCO_2, the Hct (and thus Hb) increases. In part, increased [Hb] may be responsible for the minimum change in pH.

The acid–base balance of chicken embryos also reacts promptly to metabolic disturbances, as shown by the time course changes in metabolic acid–base alterations created by infusion of electrolyte solution ($NaHCO_3$ and NH_4Cl) (see Figure 32.5 in Tazawa, 1982). For instance, the infusion of 15 μL 1 M $NaHCO_3$ increases plasma $[HCO_3^-]$ and blood PCO_2. Because the embryo has no convective ventilation, the metabolic disturbance is not subjected to respiratory compensation. The increase in PCO_2 1 h after infusion indicates that the infused HCO_3^- is partly eliminated as dissolved CO_2. The acid–base status returns to control values 6 h after infusion. Besides elimination of CO_2 from the CAM, the increased fluid volume and hypertonicity of the infused $NaHCO_3$ solution may be partially responsible for the decrease in $[HCO_3^-]$. Additionally, penetration of HCO_3^- into the intracellular space and excretion of HCO_3^- into allantoic fluid through the CAM may contribute to the regulation (Tazawa, 1982, 1986).
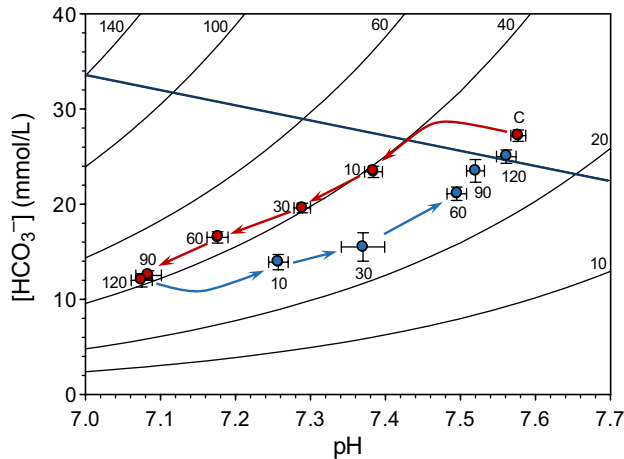
FIGURE 32.6 **Time-course of acid–base disturbance in day 15 chicken embryos exposed to hypercapnic hypoxia (5% CO_2, 10% O_2) for 120 min (red circles) with recovery in air for 120 min (blue circles).** Other details as in Figures 32.4 and 32.5. *Modified from Tazawa et al. (2012), with permission from Elsevier.*

In summary, acid–base regulation develops in concert with the increased CO_2 production as avian embryos grow. The ability of chicken embryos to tolerate and respond to acid–base disturbance, induced by environmental gas challenges, increases or decreases in GCO_2, or infusion of electrolytes, increases with development. Comparative approaches are required to examine species differences in acid–base regulation, and how tolerance to perturbations may be related to the incubation environment.

32.4.3 Cardiovascular System

32.4.3.1 Basic Cardiovascular Parameters

The primordial bird embryo's heart is a paired tubular structure that soon becomes a single tube. The heart begins to elongate more rapidly than the pericardial cavity containing it, and this space limitation forces the tubular heart to bend. Only the ventricle and bulbus are present on days 1.5 to 2 of chicken incubation. The impact of bloodstreams upon the inner surface of the contorted tube forms the external configuration and internal structure of the heart (Taber, 2001; Alford and Taber, 2003; Tobita et al., 2005) as well as contributing to blood vessel formation (le Noble et al., 2008; Burggren, 2013), although the specific role of pressure and flow fluctuations to the contribution of developing blood vessels remains somewhat enigmatic (Branum et al., 2013).

The structural alterations that separate atrium from ventricle, ventricle from aorta, and the left from the right chambers take place during days 3 to 8 of incubation, resulting in a four-chambered heart by days 8 to 9 (Pattern, 1951; see also multiple papers in Burggren and Keller, 1997).

The mass of the heart increases as a power function of incubation day (I) (Romanoff, 1967; Clark et al., 1986):

$$\text{Heart mass } (\mu g) = 12.62 \cdot I^{3.26} \qquad (32.12)$$

The growth of the heart relative to the whole body is greatest early in development and declines during incubation. The ratio of the heart mass to the whole body mass falls from 1.8% on day 4 to 0.7% on day 18 of incubation.

The heart begins to beat at about 30 h of incubation and blood begins to circulate after about 40 h, when the connections between the dorsal aorta and the vessels of the yolk sac complete the circulation (Pattern, 1951). Despite the early generation of heartbeat and blood flow, heart rate is not initially required for convective blood flow to the tissues, but likely plays a role in angiogenesis (Burggren et al., 2000, 2004; Burggren, 2004, 2013; Branum et al., 2013).

Blood volume increases with incubation day (Figure 32.7(B)) (Yosphe-Purer et al., 1953; Barnes and Jensen, 1959; Lemez, 1972; Kind, 1975), and can be approximated by the following function (Tazawa and Hou, 1997):

$$\text{Blood volume } (\mu L) = 1.85 \cdot I^{2.64} \qquad (32.13)$$

The increase in blood volume during development is not as rapid as growth rate (Figure 32.7(A)), and therefore embryo mass-specific blood volume decreases during development from about 1 mL/g on day 4 to 0.15 mL/g on day 18.

As both the heart mass and blood volume increase, the stroke volume of the heart increases (Hughes, 1949; Faber et al., 1974; Hu and Clark, 1989). During early development, prior to the left and right separation of the heart, stroke volume depends on blood volume and increases in parallel with embryonic growth (Faber et al., 1974). The relationship between stroke volume and incubation day (I, days 2–6) (Hu and Clark, 1989) can be described as:

$$\text{Stroke volume } (\mu L) = 0.002 \cdot I^{3.46} \qquad (32.14)$$

Even after the second week of incubation, stroke volume seems to increase as a power function of incubation day (Figure 32.7(C)) (Hughes, 1949).

For early embryos, the dorsal aortic blood flow increases as a power function of incubation day during days 2 to 6 (Hu and Clark, 1989). The mass (embryo)-specific value is 0.5–1 mL/min/g. The mass-specific cardiac output of day 3 to 5 embryos, determined from the stroke volume, is similar at 1 mL/min/g (Faber et al., 1974). Cardiac output calculated from the stroke volume of young embryos and that of day 16 chicken embryos estimated by model analysis and blood O_2 measurement (White, 1974; Rahn et al., 1985; Tazawa and Johansen, 1987) indicate that the mass-specific cardiac output of young/late embryos is in the narrow range of 0.5–1.5 mL/min/g, and thus the cardiac output seems to increase as a power function of incubation day (Figure 32.7(D)). Eventually, cardiac output may increase almost in parallel with embryonic growth. If it is assumed that the
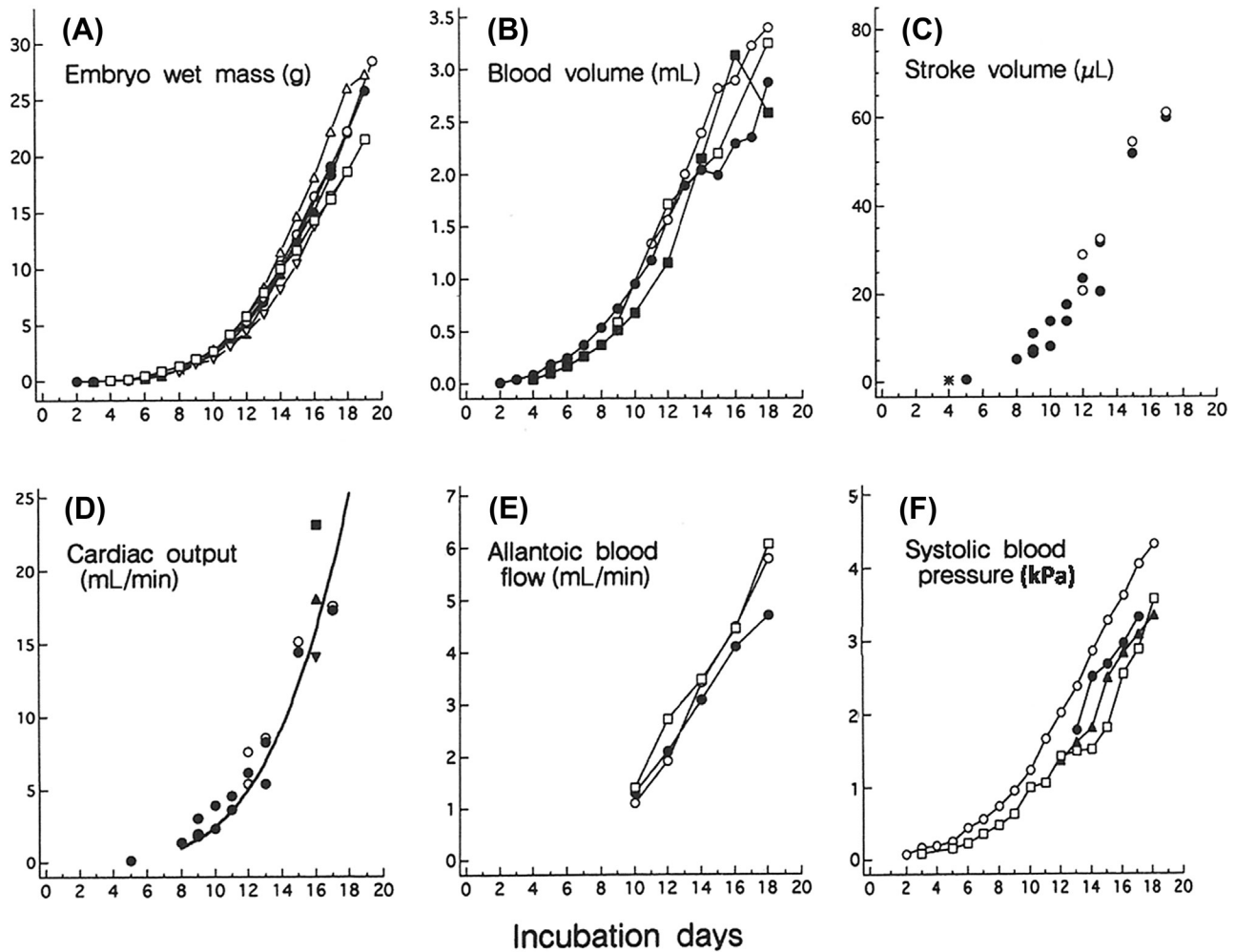
FIGURE 32.7 Developmental patterns of embryo wet mass (A), total blood volume (B), cardiac stroke volume (C), cardiac output (D), allantoic blood flow (E), and arterial systolic blood pressure (F) during the prenatal stage. Symbols connected by solid lines in (A), (B), (E), and (F) indicate the developmental patterns from the papers cited in the text.

mass-specific cardiac output during the last 2 weeks of prenatal development is 1 mL/min/g, the cardiac output of young embryos is related to incubation day (I) as follows,

$$\text{Cardiac output } (\mu L/min) = 0.24 \cdot I^4 \qquad (32.15)$$

which is the same expression as that relating body mass of developing embryos to incubation day (Eqn (32.5), Figure 32.7(A), (D)).

Blood flow through the CAM, determined from $\dot{M}O_2$, blood gas analysis, or via flow probe (Tazawa and Mochizuki, 1976, 1977; Bissonnette and Metcalfe, 1978; Van Golde et al., 1997), increases with embryonic growth (Figure 32.7(E)), but the mass-specific value decreases from ~0.5 mL/min/g on day 10 to ~0.25 mL/min/g on day 18. Although cardiac output increases in parallel with embryonic growth, its partition to the CAM decreases as embryos grow. On days 10 to 13, about half of the cardiac output goes to the CAM and this decreases to ~40% on days 17 to 19 (Mulder et al., 1997; Dzialowski et al., 2011 for review)

or to ~17–20% on days 16 to 18 (Tazawa and Johansen, 1987). Consequently, the percentage of cardiac output to the organs and tissues increases as development proceeds.

Arterial pressure (P_a) is measured via a vitelline vessel in early embryonic chick development and an allantoic artery thereafter. Arterial systolic pressure (P_{sys}), measured by micropipette (Van Mierop and Bertuch, 1967; Girard, 1973) or needle-catheter techniques that maintain adequate gas exchange through the eggshell (Tazawa, 1981c; Tazawa and Nakagawa, 1985), increases with incubation day (I) in the following manner (Figure 32.7(F)):

$$P_{sys} \text{ (kPa)} = 0.015 \cdot I^{1.86} \qquad (32.16)$$

P_a of the vitelline artery is pulsatile by day 2 of chicken incubation (Van Mierop and Bertuch, 1967; Hu and Clark, 1989; Taber, 2001). While the presence of a dicrotic notch is reported in early embryos (Hu and Clark, 1989; Yoshigi et al., 1996), a clear dicrotic notch is absent from allantoic P_a waves determined by others (Van Mierop and Bertuch,

1967; Tazawa and Nakagawa, 1985). However, the configuration of the P_a tracings and the ventricular pressure waves suggest that an arterial valve mechanism is present in early embryos, which lack true cardiac valves (Van Mierop and Vertuch, 1967; Faber, 1968).

32.4.3.2 Mean Heart Rate

According to the equations expressing the relationship between incubation day and body mass (Eqn (32.5)) or cardiac output (Eqn (32.15)), both body mass and cardiac output in day 16 chicken embryos are ~16 g and ~16 mL/min. Assuming a mean heart rate (MHR) of 280 beats per min (bpm), the stroke volume and mass-specific stroke volume are estimated as ~60 μL and ~4 μL/g. The cardiac contraction generating this amount of stroke volume physically moves the body of the embryo by an almost imperceptible amount, and these movements are transferred to the whole egg, producing cardiogenic ballistic movement of the egg, referred to as a ballistocardiogram (BCG) of the egg (Tazawa et al., 1989b). The egg movement determined by a laser displacement meter is ~1 μm (Sakamoto et al., 1995). The BCG measured by various means offers a convenient way to noninvasively determine MHR of avian embryos (Tazawa et al., 1999; Tazawa, 2005). Additionally, the heartbeat of the embryo inside the eggshell produces not only the ballistic movements of the egg, but also acoustic pressure changes outside the eggshell. A conventional condenser microphone hermetically fixed on the eggshell detects the cardiogenic acoustic pressure changes, designated as an acoustocardiogram (ACG) (Rahn et al., 1990), which can conveniently determine MHR of avian embryos.

In contrast to developmental patterns of the circulatory variables that increase steadily with embryonic growth, the embryonic MHR of chickens increases rapidly after the heart commences beating and then becomes asymptotic until early in the second week of incubation (Cain et al., 1967; Van Mierop and Bertuch, 1967; Girard, 1973; Hu and Clark, 1989; Tazawa et al., 1991a; Burggren and Warburton, 1994; Howe et al., 1995). During the last half of incubation, the daily change in MHR is small, with a temporal increase at 60–70% of incubation followed by a decrease toward IP and increase during hatching (Tazawa et al., 1991a). The change in MHR near the end of development in other precocial birds is shown in Table 32.2. Precocial species, from the smallest (king quail, *Coturnix chinensis*) to the largest (ostrich), show either a plateau or decrease in MHR prior to IP (Tazawa et al., 1991a, 1998a; 1998b, 2000; Pearson et al., 1998; Kato et al., 2002).

In comparison, HR increases rapidly toward hatching in small altricial embryos (Table 32.3) (Tazawa et al., 1994; Pearson et al., 1999). However, the increase in MHR during pre-pipping development slows in larger altricial eggs, such as those of crow (*Corvus corone*) and cattle

egret (*Bubulcus ibis*), tending to remain unchanged during the last half of the prenatal period (Pearson and Tazawa, 1999; Tazawa et al., 2001b). The trend for MHR to remain unchanged during the end of prenatal development also occurs in semi-precocial seabirds such as the brown noddy (*Anous stolidus*) and laysan albatross (*Phoebastria immutabilis*) (Table 32.2) (Tazawa et al., 1991b; Tazawa and Whittow, 1994; Pearson et al., 2000). Hence, the pattern of change in MHR during development, particularly at the end of incubation, can be somewhat predicted by the degree of development at hatching. Precocial species tend to show a plateau or decrease in MHR near hatching, while MHR tends to increase in altricial species.

These determinations of embryonic MHR along with egg mass of various species (Tables 32.2 and 32.3) reveal a significant relationship between MHR at 80% of I and egg mass (Tazawa et al., 2001b). The allometric relationship derived for 20 species of altricial and semi-altricial (ASA) birds with egg mass ranging from 0.96 g of the zebra finch to ~41 g of the lanner falcon (*Falco biarmicus*) is:

$$\text{MHR at 80\% of } I = 371 \cdot (\text{Egg mass})^{-0.121} \atop (r = -0.846 ,\ P < 0.001) \tag{32.17}$$

The relationship for 13 species of precocial and semi-precocial (PSP) birds with egg mass ranging from 6 g of the king quail to ~1400 g of the ostrich is:

$$\text{MHR at 80\% of } I = 433 \cdot (\text{Egg mass})^{-0.121} \atop (r = -0.963 ,\ P < 0.001) \tag{32.18}$$

The slopes for MHR are parallel, but the MHR of ASA embryos is low compared with PSP embryos from the same egg mass. In ASA embryos, HR becomes maximal during the pipping period, and the maximum HR is significantly related to egg mass. Consequently, the allometric relationship of maximum HR and egg mass in ASA and that of 80% of I in PSP is statistically identical, expressed by a single allometric equation:

$$\text{MHR} = 437 \cdot (\text{Egg mass})^{-0.123} \atop (r = -0.948 ,\ P < 0.001 ,\ N = 33) \tag{32.19}$$

In addition, determination of MHR in embryos from the same clutch (siblings) in pigeons and bank swallows demonstrates that developmental patterns of MHR in siblings are more alike than those of embryos from other clutches of the same species (Burggren et al., 1994). These findings may be termed the "sibling effect," in which siblings are predisposed to show particular physiological patterns. Whether these effects are genetically based (i.e., resulting from the common genetic heritage of the F_1 offspring) or involve epigenetic influences is uncertain. Certainly, physiological process can be transferred across generations (see Ho and Burggren, 2010, 2012; Burggren, in press) through

TABLE 32.2 Egg Mass (g), Incubation Period (*I*, days), Heart Rate (HR, beats/min) at 80% of Incubation and Maximum Heart Rate (Max HR) during Prenatal Development in Precocial Birds and Heart Rate Prior to Internal Pipping (Pre-IP) and at the First Star Fracture of the Eggshell in Semi-Precocial Birds

	Species	Egg Mass (g)	Incubation Period (*I*, days)	HR at 80% of *I* (beats/min)	Max HR (beats/min)
Precocial	King quail (Pearson et al., 1998) *Coturnix chinensis*	6.0±0.4	16	341±8 (81%)	341±8 (81%)
	Japanese quail (Tazawa et al., 1991a) *Coturnix coturnix japonica*	10.7±0.7	17	319±8 (76%)	326±7 (76%)
	Chicken (Tazawa et al., 1991a) *Gallus gallus domesticus*	64.9±2.5	21	287±9 (81%)	287±9 (81%)
	Duck (Tazawa et al., 1991a) *Anas platyrhynchos*	79.0±2.5	28	247±15 (82%)	258±11 (61%)
	Turkey (Tazawa et al., 1991a) *Meleagris gallopavo*	82.9±2.6	28	246±10 (79%)	248±10 (75%)
	Peafowl (Tazawa et al., 1991a) *Pavo cristatus*	111.3±9.3	28	262±12 (79%)	267±9 (86%)
	Goose (Tazawa et al., 1991a) *Anser cygnoides*	158.3±11.3	30	224±8 (80%)	248±10 (60%)
	Emu (Tazawa et al., 2000) *Dromaius novaehollandiae*	634±9	50	192±7 (80%)	199±11 (72%)
	Ostrich (Tazawa et al., 1998a) *Struthio camelus*	1395±199	42	185±12 (81%)	208±9 (55%)
				Pre-IP HR (beats min^{-1})	**HR at First Star Fracture of Shell (beats min^{-1})**
Semi-precocial	Brown noddy (Tazawa et al., 1991a) *Anous stolidus*	37.9±2.2	35	298±7	303±13
	Wedge-tailed shearwater (Tazawa and Whittow, 1994) *Puffinus pacificus*	57.2±2.3	52	244±10	252±11
	Laysan albatross (Tazawa and Whittow, 1994) *Diomedea immutabilis*	288±18	65	232±15	233±15

Values were measured at 38 °C or converted to that at 38 °C using HR(38 °C)=HR(*T*°C)e$^{[0.0639(38 − T)]}$; For precocial species: if HR was not measured at 80% of *I*, the value closest to 80% of *I* was used with the % of *I* at which HR was measured shown in parentheses. The % of *I* of max HR is shown in parentheses; Data are mean ±S.D.
Modified From Tables 32.1 and 32.2 in Tazawa et al. (2001b).

TABLE 32.3 Egg Mass (g), Incubation Period (*I*, days), and Heart Rate (HR, beats/min) at 80% of Incubation and during Internal Pipping (IP) and External Pipping (EP) in Altricial and Semi-altricial Birds

Species	Egg Mass (g)	Incubation Period (*I*, days)	HR at 80% of *I* (beats/min)	HR during IP (beats/min)	HR during EP (beats/min)
Zebra finch (Pearson et al., 1999) *Taeniopygia guttata*	0.96±0.13	14	335±10	376±20	405±12
Bengalese finch (Pearson et al., 1999) *Lonchura striata* Var. *domestica*	1.10±0.12	15	404±36	409±25	448±35
Marsh tit (Pearson et al., 1999) *Parus palustris*	1.39±0.04	14	363±17	409±19	–
Bank swallow (Tazawa et al., 1994) *Riparia riparia*	1.42±0.10	14	298±12	–	352±16
Great tit (Pearson et al., 1999) *Parus varius*	1.59±0.14	14	348±11	432±13	495±14
Varied tit (Pearson et al., 1999) *Parus varius*	1.69±0.01	14	356±7	434±11	–
Tree sparrow (Pearson et al., 1999) *Passer montanus*	2.09±0.07	12	335±13	411±32	–
Budgerigar (Pearson et al., 1999) *Melopsittacus undulates*	2.19±0.19	18	314±14	339±15	364±12
House martin (Pearson et al., 1999) *Delichon urbica*	2.25±0.04	15	357±7	369±8	367±11
Japanese bunting (Pearson et al., 1999) *Emberiza spodocephala*	2.56±0.09	13	370±5	426±1	–
Red-cheeked starling (Pearson et al., 1999) *Sturnus philippensis*	4.14±0.01	14	358±1	409±5	–
Cockatiel (Pearson et al., 1999) *Nymphicus hollandicus*	5.08±0.18	20	300±8	318±25	344±19
Brown-eared bulbul (Pearson et al., 1999) *Hypsipetes amaurotis*	6.4±0.5	16	333±7	402±8	–
Domestic pigeon (Tazawa et al., 1994) *Columba domestica*	17.1±1.0	18	247±17	–	276±13
Fantail pigeon (Tazawa et al., 1994) *Columba domestica*	19.7±2.4	18	267±10	–	293±6
Homing pigeon (Tazawa et al., 1994) *Columba domestica*	19.8±1.2	18	230±16	–	273±4
Crow (Pearson and Tazawa, 1999) *Corvus corone*	20.5±2.2	20	297±11	348±35	366±22
Barn owl (Tazawa et al., 2001b) *Tyto alba*	20.1±0.6	30	219±11	–	276±13
Cattle egret (Tazawa et al., 2001b) *Bubulcus ibis*	27.5±3.3	23	251±8	–	283±12
Lanner falcon (Tazawa et al., 2001b) *Falco biarmicus*	41.2±0.4	33	242±9	–	276±6

Values were measured at 38°C or converted to that at 38°C using HR(38°C)=HR(*T*°C)e$^{[0.0639(38-T)]}$; Data are mean ±S.D.
Modified From Tazawa et al. (2001b).

epigenetic mechanisms. However, environmental effects on the mother may also cause siblings to undergo similar patterns of physiological development through a "maternal effect" or even direct effects on the gamete cells of the mother (Burggren et al., 1994; Dzialowski and Sotherland, 2004; Ho et al., 2011; Burggren, in press).

32.4.3.3 Instantaneous Heart Rate

In addition to daily changes in MHR during development, HR varies from beat to beat (i.e., instantaneous HR; IHR). At rest, the baseline IHR in chicken embryos is more or less constant before ~day 11, indicating that neither IHR accelerations of adrenergic origin nor IHR decelerations of cholinergic origin occurs during early development. The flat baseline of IHR begins to fluctuate with the appearance of rapid, transient decelerations on ~days 12 to 13, with a subsequent augmented frequency of appearance (Figure 32.8) (Höchel et al., 1998; Tazawa et al., 1999; Chiba et al., 2004). Thereafter, IHR becomes arrhythmic with the appearance of transient accelerations on ~days 15 to 16 and more arrhythmic with augmented decelerations and accelerations toward hatching (Höchel et al., 1998; Tazawa et al., 1999, 2002a; Moriya et al., 2000; Khandoker et al., 2003). These IHR decelerations are eliminated, and baseline HR is elevated, by intravenous administration of atropine (Figure 32.8). In
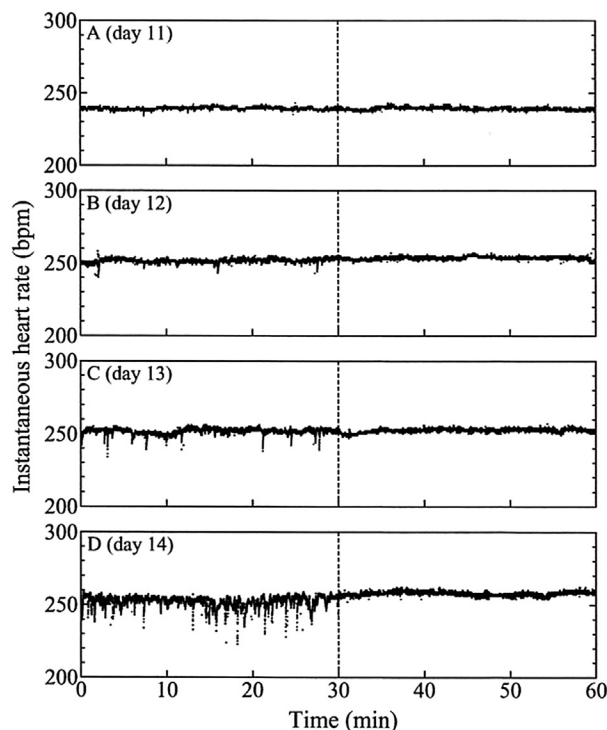

FIGURE 32.8 Instantaneous heart rate fluctuations before and after intravenous infusion of 20 μg atropine at 30 min in day 11 to day 14 chicken embryos. *From Chiba et al. (2004), with permission from Elsevier.*

late embryos whose IHR fluctuations consist of complicated decelerations and accelerations with augmented magnitude and frequency, atropine diminishes decelerations only but preserves accelerations, while the HR baseline is markedly elevated (e.g., Figure 32.3 of Chiba et al., 2004), indicating that IHR decelerations are mediated by the vagus nerve. Accordingly, vagal tone begins to appear on around days 12–13 of incubation in chickens, maturing with development, correcting previous reports on no vagal control of HR determined from blood pressure signals (Tazawa et al., 1992a). Furthermore, cholinergic chronotropic control of HR occurs on days 12 to 13 of incubation in both broiler and White Leghorn embryos (Yoneta et al., 2006c). These events coincide with the presence of a complete cholinergic effector pathway from 60% of chicken incubation (Pappano and Löffelholz, 1974).

Some controversy exists as to whether cholinergic tone is present at this developmental timepoint; blood pressure studies indicate no vagal tone on the heart during embryonic development (Crossley and Altimiras, 2000). Further, blood pressure studies using adrenoreceptor stimulation via pharmacological manipulation indicate that a positive β-adrenergic chronotropic tone can be induced from 60% of incubation (Crossley and Altimiras, 2000). The effect is enacted by circulating catecholamines, as ganglionic blockade with hexamethonium has no impact on HR. In comparison, pharmacological manipulation of blood pressure indicates that the emu heart is under both β-adrenergic and cholinergic control by 70% of incubation (Crossley et al., 2003).

Long-term measurement of IHR reveals the occurrence and development of various cardiac rhythms and irregularities. The developmental pattern of MHR (constructed from IHR measurement) of an embryo before and after hatching demonstrates the occurrence of infradian rhythm before and during IP, a gradual increase in HR baseline during IP, a sudden drop with a subsequent sudden increase in HR baseline during EP, and the first occurrence of circadian rhythm of HR after hatching (Figure 32.9) (Moriya et al., 2000). Recordings of IHR reveal respiratory arrhythmia associated with EP, together with three unique patterns of IHR during the final stage of EP (i.e., relatively long-lasting cyclic small accelerations, irregular intermittent large accelerations, and short-term repeated large accelerations) (Tazawa et al., 1999; Moriya et al., 2000). Short-term repeated large accelerations also occur in EP emu embryos, indicating imminent hatching (Figure 32.10) (Kato et al., 2002).

After hatching, three types of IHR fluctuations (Types I, II and III) are demonstrated in chickens (Moriya et al., 1999, 2000; Tazawa et al., 2002a). Type I is characterized as a widespread baseline HR (20–50 bpm) due to respiratory arrhythmia with a mean oscillatory frequency of ~0.7 Hz. Type II is evident by low-frequency oscillations of baseline HR at ~0.07 Hz, occurring at low T_a or when hatchlings are
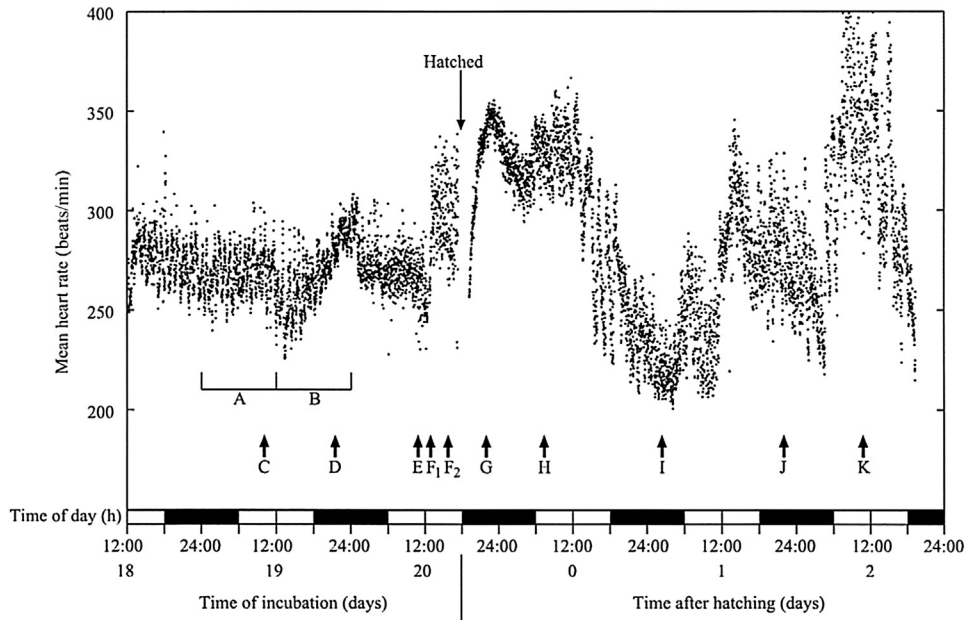
FIGURE 32.9   Typical developmental pattern of mean heart rate (MHR) of a chick, recorded from day 18 of incubation through hatching on day 20 to day 2 of post-hatching. Each point indicates the MHR over a 1 min period determined from the continuous recording of instantaneous heart rate (IHR). IP begins at around 12:00 on day 19 when MHR temporarily decreases with subsequent gradual increase during IP. The MHR oscillates with a period of about 42 min from the beginning of recording to middle of IP, i.e. infradian rhythm. EP begins at the start of day 20 when MHR suddenly drops. Following the drop, which lasts for the first half of day 20, MHR increases sharply at 12:00 and maintains high baseline until hatching. During the last period of EP, characteristic patterns of IHR occur, e.g., a sharp increase in MHR (pointed by F_1) consisting of relatively long-lasting cyclic small accelerations. The wide baseline of MHR during the last 1% of incubation is composed of repeated alternate occurrence of irregular intermittent large accelerations and short-term repeated large accelerations indicating imminent hatching (see Figure 32.10). After hatching, circadian rhythms occur on days one and 2. *Reproduced from Moriya et al. (2000), with permission from The Company of Biologists.*
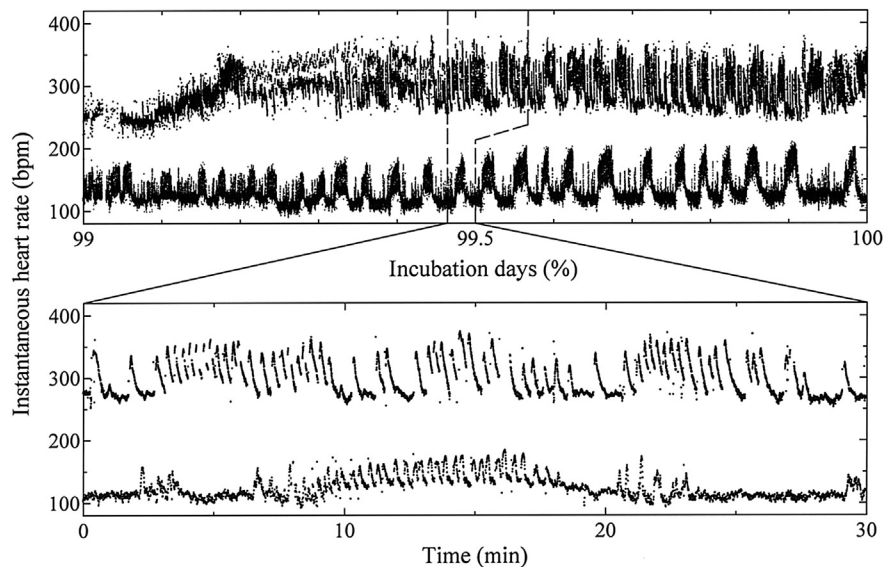

FIGURE 32.10   Instantaneous heart rate (IHR) during the last 1% of incubation of chicken (upper recording) and emu (lower recording) embryos (top panel) and the 60-min recordings taken from the middle of the last 1% of incubation indicated by broken lines (bottom panel). Short-term repeated large IHR accelerations with intermittent large accelerations continuously appear towards hatching in both embryos. HR of small chicken embryos is higher than that of large emu embryos (allometry). *From Kato et al. (2002), with permission from Elsevier.*

exposed to lowered T_a, and is thus related to thermoregulation. Type III is characterized as noncyclic irregularities, dominated by frequent transient accelerations. The image-processing system developed to capture the movements of wing or whole body of the hatchling reveals that Type I and Type II HR oscillations are related to the periodic movements of the wing and Type III HR irregularities occur simultaneously with spontaneous whole body movements (Yoneta et al., 2006a). Consequently, it is likely that the chick moves the body at the same frequencies as Type I and Type II oscillations and simultaneously with Type III HR irregularities, and these HR fluctuations and body movements are likely to be attributed to the same origins.

32.4.3.4 Blood Pressure Regulation

The sympathetic nervous system is heavily involved in regulation of avian embryonic blood pressure (Altimiras et al., 2009). Injection with the α-adrenereceptor antagonist phentolamine causes hypotension from 60% of chicken incubation, indicating α-adrenergic tone on the vasculature (Girard, 1973; Saint Petery and Van Mierop, 1974; Tazawa et al., 1992a; Crossley and Altimiras, 2000). Phentolamine also causes bradycardia, a likely indirect effect of vasodilation, and reduced venous return. β-adrenergic tone on the vasculature is also present from 60% of incubation, as evident by a P_a increase in response to the β-adrenereceptor antagonist propranolol (Girard, 1973; Saint Petery and Van Mierop, 1974; Tazawa et al., 1992a; Crossley and Altimiras, 2000). The magnitude of both α- and β-adrenergic tone is maximal at 90% of incubation, just prior to IP (Crossley and Altimiras, 2000). The increase in adrenergic tone is matched to the maximal plasma catecholamine release on day 19. Likewise, emu embryos show signs of increasing α- and β-adrenergic tone on the vasculature matched to increasing levels of catecholamines as incubation proceeds (Crossley et al., 2003).

In addition to regulation via catecholamines, other hormones may also influence baseline cardiovascular function of avian embryos. The most extensively studied in chicken embryos is angiotensin II (ANG II), a strong vasoconstrictor and the active peptide of the renin-angiotensin system (RAS). Components of the RAS, including renin, angiotensin-converting enzyme, ANG II and its receptors, are present early in chicken ontogeny (Nishimura et al., 2003; Savary et al., 2005; Crossley et al., 2010). ANG II levels are elevated in embryos compared to adults; the peptide produces hypertension from at least 60% of incubation (Crossley et al., 2010), and contributes to baseline P_a at 90% of incubation (Mueller et al., unpublished). Despite an increase in P_a, ANG II does not alter MHR as it instead attenuates the embryonic cardiac baroreflex. The baroreflex is an important compensatory mechanism that buffers short-term changes in P_a and is composed of a peripheral limb adjusting vascular resistance and a cardiac limb that changes HR. A functioning baroreflex is present in chicken embryos from 80% of incubation (Altimiras and Crossley, 2000; Elfwing et al., 2011). ANG II decreases the sensitivity of the cardiac baroreflex at 90% of incubation so that reflex changes in HR in response to P_a changes are blunted (Mueller et al., 2013a). ANG II also raises the operating P_a of embryos, and it is via these short-term changes that the hormone partly enacts some of its influence on long-term P_a.

Other potentially important moderators of cardiovascular function include endothelin-1 (ET-1), a potent vasoconstrictor produced primarily in the endothelium, and natriuretic peptides (NP), strong vasodilators excreted by heart muscle cells. Components necessary for functional ET-1, including mRNA and converting enzymes, are found in chicken embryos from 15% of incubation (Hall et al., 2004; Groenendijk et al., 2008) and ET-1 alters chicken embryo hemodynamics (Groenendijk et al., 2008; Moonen and Villamor, 2011). Likewise, NP is present in the chicken heart and most likely contributes to hemodynamics from at least day 14 (Maksimov and Korostyshevskaya, 2013). Further research from the molecular to whole-organism level is required to understand the contribution of these hormones to P_a and HR control, and therefore embryonic cardiovascular regulation.

32.4.4 Osmoregulation

During development, various avian embryos face one of two osmoregulatory challenges: water loss through the pores of the eggshell, in desiccating arid environments; or excess water gain from the metabolic production of water as part of metabolizing the yolk stores (Ar and Rahn, 1980). Irrespective of the species-specific challenge, the developing kidney and extra-embryonic structures, including the yolk, CAM, and allantoic and amniotic fluids work in concert to regulate ion and water balance. The embryonic kidney of chickens has three stages, actually comprising separate structures: the pronephros, mesonephros, and metanephros. The pronephros appears first and functions until day 5 to 6 of incubation (Abdel-Malek, 1950; Himura and Nakamura, 2003). Mesonephros function takes over from day 5, is maximal on days 10 to 15 (Romanoff, 1960), and then degrades between days 18 and 19 (Atwell and Hanan, 1926). The mesonephros functions simultaneously with the metanephros, which begins developing from day 4 (Abdel-Malek, 1950) and continues to develop post-hatching. The metanephros is the most complex of the three embryonic kidney structures, and comprises the functioning structure in adults. The allantoic sac first appears at about days 3 to 4 of incubation, and acts as a repository for kidney secretions, as evident by the increase in uric acid content throughout development (Romanoff, 1967). The allantoic

epithelium actively transports sodium across its membrane from days 12 to 19 (Stewart and Terepka, 1969; Graves et al., 1986; Gabrielli and Accili, 2010), thus maintaining the fluid hypo-osmotic to the blood and permitting reabsorption of water by the embryo (Hoyt, 1979).

Osmoregulation is strongly tied to cardiovascular function through the synergistic maintenance of blood pressure and osmotic homeostasis. Regulators of blood pressure, such as ANG II and NP (see above), may also contribute to ion and water balance in embryos. Chronic removal of ANG II from chicken eggs not only decreases P_a but also lowers osmolality, decreases Na^+, and increases K^+ concentration of the blood at 90% of incubation. These actions remove the osmolality gradient between the blood and allantoic fluid (Mueller et al., unpublished). Therefore, ANG II appears to influence osmotic balance and may do so by direct vasoconstrictive action or by stimulating the release of aldosterone and arginine vasotocin, which promote proximal tubular sodium reabsorption. Aldosterone is present in chicken embryo adrenal glands from day 15 (Pedernera and Lantos, 1973) and arginine vasotocin is present in the brain from day 6 and the plasma from days 14 to 16 (Klempt et al., 1992; Mühlbauer et al., 1993). Both aldosterone and arginine vasotocin alter allantoic fluid volume, allantoic salt content, and renal enzyme activity in chicken embryos (Doneen and Smith, 1982). Prolactin and growth hormone also have osmoregulatory effects in chicken embryos (Doneen and Smith, 1982; Murphy et al., 1986). The function of these hormones in osmoregulation requires further study, including how they may contribute to interactions between the developing cardiovascular and osmoregulatory systems and the maintenance of homeostasis.

32.4.5 Thermoregulation

Embryos produce metabolic heat, which increases as development progresses. Metabolic heat production of the chicken embryo increases from about 35 mW on day 12 to 130–140 mW on days 17 to 18 of incubation, and reaches 160–170 mW at EP (Tazawa et al., 1988b). Over the same developmental range, the egg's thermal conductance is ~70 mW/°C (Tazawa et al., 2001a). This means that metabolic heat production at 38 °C elevates egg temperature (T_{egg}) above ambient temperature (T_a) by ~0.5 °C on day 12 and ~2.5 °C in EP eggs. When eggs are cooled to 28 °C, a new quasi-equilibrium state is reached in 5 h (defined as a change in T_{egg} of less than 0.2 °C/h) (Tazawa and Rahn, 1987). The new difference between T_{egg} and T_a is lower, so that even in EP embryos T_{egg} is only 1.2 °C above T_a. In addition, during the quasi-equilibrium state at lowered T_a, embryos consume the amount of O_2 predicted by a temperature coefficient (Q_{10}) of 2. On the other hand, hatchlings, which are subjected to the same test, have body temperatures ~6 °C above T_a, even right after hatching, and consume

much more O_2 than that predicted by Q_{10} of 2 (Tazawa and Rahn, 1987). These observations suggest that chickens are essentially poikilothermic during embryonic stages, even at EP, and rapidly develop the capacity to maintain body temperature upon cold exposure soon after hatching.

When eggs incubated at 38 °C are exposed to a T_a only 2 °C lower, the heat loss from the egg is greater than the heat produced by young embryos and comparable with the heat production in late embryos. If the eggs are exposed to air 10 °C cooler than the egg, the embryos would have to generate heat of about 800 mW to keep T_{egg} steady (Turner, 1986). Yet, even EP embryos can generate at most 170 mW. Consequently, heat loss during cooling exceeds the embryo's maximum rate of heat production. A feeble compensatory capacity, if any, may be overwhelmed by the much larger losses of heat. The egg cools and its metabolic rate decreases as a result of the van't Hoff-Arrhenius effect. The detection of homeothermic capacity thus requires a procedure in which the heat loss from the egg does not overwhelm the heat production of the embryo. The gradual cooling test fulfills this requirement, and it shows that prenatal chicken embryos near term respond to lowered T_a by maintaining $\dot{M}O_2$ until T_a falls below 35 °C (Tazawa et al., 1988b). This response in late embryos is evidently different from that in young embryos. In the prolonged cooling test, late chicken embryos are exposed to a slightly lowered T_a for a prolonged period, the $\dot{M}O_2$ is maintained at a level above that predicted by a Q_{10} of 2. This response also differs from that shown by young embryos (Tazawa et al., 1989a). Consequently, precocial chickens exhibit a feeble, incipient metabolic response to cooling, indicating endothermic homeothermy before hatching. This coincides with enhanced activity of the thyroid gland and increasing concentrations of peripheral thyroid hormones during the last stages of incubation (Decuypere et al., 1979; McNabb, 1987). In fact, while late prenatal embryos in eggs injected with saline show a feeble homeothermic metabolic response to gradual cooling, this response is absent in eggs treated with thiourea, which antagonizes the metabolic effect of thyroid hormones (Tazawa et al., 1989c). Additionally, while the compensatory metabolic response disappears in embryos exposed to hypoxia, it is augmented in perinatal embryos in hyperoxia or following improved oxygenation by opening the shell over the air cell (Tazawa et al., 1989c; Dzialowski et al., 2007; Szdzuy et al., 2008). These results indicate that the homeothermic metabolic response in late embryos is "O_2 conductance limited" in precocial chickens (Tazawa et al., 1988b).

The development of homeothermy in precocial and altricial birds has been modeled (Figure 32.11) (Tazawa et al., 1988b; Whittow and Tazawa, 1991; Tzschentke and Rumpf, 2011 for review). The transition for a precocial bird takes place in four stages; (1) an Arrhenius-limited stage in which $\dot{M}O_2$ is directly related to the temperature with a Q_{10} value

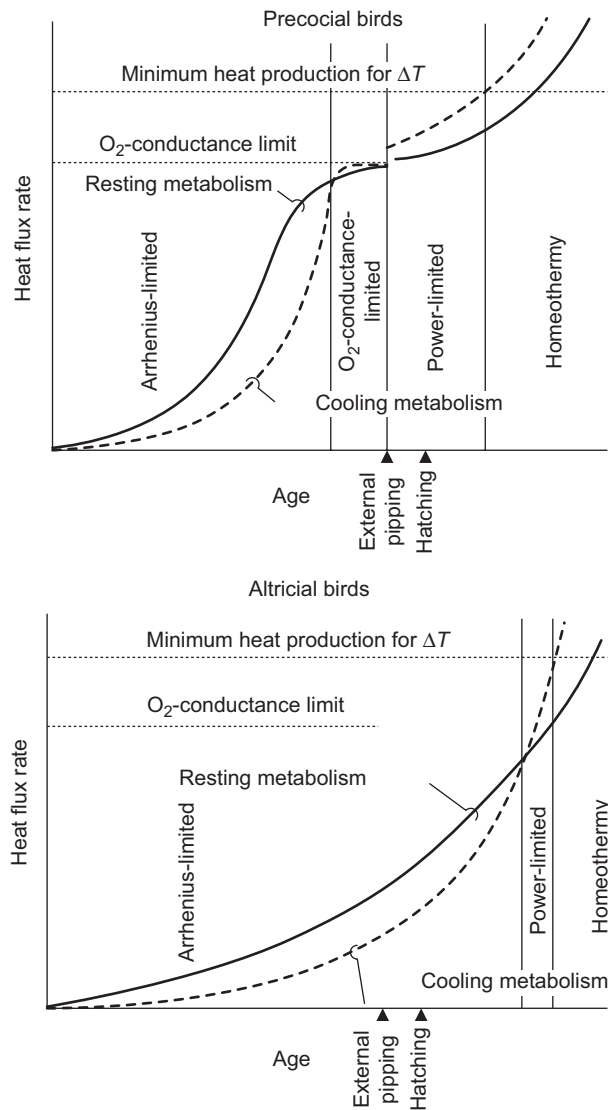FIGURE 32.11   **Models of the development of homeothermy in precocial and altricial birds.** Minimum Heat Production for ΔT equals the amount of heat needed to keep the egg temperature warmer than ambient temperature by ΔT and homeothermy occurs when the embryo heat production reaches this level. *From Tazawa et al. (1988b), with permission from Elsevier.*

of approximately 2; (2) an O_2-conductance-limited stage in which $\dot{M}O_2$ is limited by the rate of diffusion of O_2 through the shell and CAM; (3) a power-limited stage in which the embryo has a limited capacity to generate heat in response to cooling, which is a function of the maturity of the tissues and development of thyroid activity; and (4) "full-blown" homeothermy. An altricial species never passes through the O_2-conductance-limited stage and is subject to the Arrhenius limitation until after it hatches.

The comparative metabolic responses to prolonged cooling have been examined in the precocial duck, semi-precocial brown noddy, and altricial pigeon (*Columba livia domestica*) (Matsunaga et al., 1989; Kuroda et al.,

1990; Tazawa et al., 2001a). Incipient homeothermic ability appears in the duck during prenatal development, but it is not evident in the pigeon even after emergence from the shell. The precocial chicken and semi-precocial noddy are intermediate in their metabolic response between the duck and the pigeon.

The development and maturation of homeothermy is expected to occur and progress even during the prenatal period in the highly precocial emu. In addition to the precocial nature of the emu, its large egg with a smaller surface area-to-volume ratio and greater contribution of blood circulation compared to smaller eggs most likely enhances thermoregulatory capacity. For emus, a more apparent metabolic response test is used in which embryos and hatchlings are exposed for a prolonged period (e.g., 1.5 h) to T_a altered sequentially by 10 °C, for example, 35-25-35 °C for embryos or 25-35-25 °C for hatchlings (sequential cooling-warming test, with ΔT_a of ±10 °C) (Dzialowski et al., 2007). Hatchlings and EP embryos respond to ΔT_a with an endothermic change in $\dot{M}O_2$, showing an inverse metabolic response with marked increase and decrease in $\dot{M}O_2$ in response to sequential cooling and warming bouts. Late prenatal (day 45) and IP (day 49) embryos do not change $\dot{M}O_2$ in response to ΔT_a in air, but demonstrate partial (day 45) or apparent (IP) endothermic change in $\dot{M}O_2$ when the test is run in 40% O_2. This suggests that the late prenatal emu embryo already possesses homeothermic ability, but it is limited by the eggshell gas GO_2.

Changes in IHR, with apparent increases or decreases in HR baseline with oscillating pattern, in response to ΔT_a, are also effective in investigating endothermic capacity in precocial embryos and hatchlings (Tazawa et al., 2001a, 2004; Tamura et al., 2003; Khandoker et al., 2004). In the sequential cooling-warming test with ΔT_a of ±10 °C, the sequence of exposure (cooling-warming and vice versa) does not affect the endothermic HR response (Yoneta et al., 2006b). In chick hatchlings the endothermic HR response is demonstrated to be advanced by ~1 day in broiler compared with White Leghorn during 2 days of post-hatch life (Yoneta et al., 2007).

In the chicken embryo, the baseline of IHR shows a thermo-conformity pattern in response to a decrease in T_a during EP (day 20), but it changes little on day 21. IHR rises accompanying HR oscillation on day 22, when the embryo stays inside the egg (Tazawa et al., 2001a; Andrewartha et al., 2011b). Although the embryo fails to hatch after EP, it matures equivalently to a hatchling on day 21 and day 22. In newly hatched chicks, HR oscillations with a period of 10–25 s occur frequently in air, reported as Type II HR fluctuation (Moriya et al., 1999, 2000). In addition, IHR oscillates with lowering T_a, resulting in Type II low-frequency HR oscillation, which disappears when hatchlings are transferred to high T_a (Tazawa et al., 2002b; Khandoker et al., 2004). Consequently, chicken hatchlings

possess low-frequency HR oscillation (Type II HR fluctuation) in relation to their thermoregulation (Tazawa, 2005 for review).

In duck embryos (Figure 32.12 (A); day 24), the HR response during pre-IP stage indicates thermoconformity (Andrewartha et al., 2011b). However, the recovery of HR (and T_{egg}) at T_a of 38 °C is faster than chicken embryos (cf. Figure 32.1 of Tazawa et al., 2001a). By EP (Figure 32.12(C), (D); day 27), duck embryos demonstrate an endothermic HR response (increase during cold exposure) larger than chicken EP embryos. Immediately after hatching, the wet duckling cannot maintain the increased HR, succumbs to the cold, and HR decreases (E). However, the HR of ducklings blotted dry within 2 h is maintained at similar values at T_a of 35 °C (F), demonstrating incomplete endothermic capacity. By 2 and 13 h post-hatching (Figure 32.12(G), (H)), the plumage of the ducklings has naturally dried and their HR shows an apparent endothermic response with a small decrease in body temperature, T_b ($\Delta T_b = -1.9$ and -2.0 °C, respectively), close to full-blown homeothermy. Ducklings must attain thermoregulatory competence early (relative to the domestic chicken) during perinatal development to live in an aquatic environment soon after hatching.

In the more highly precocial emu, pre-IP and IP embryos exhibit thermoconformity and incomplete endothermic response of HR, respectively, but the apparent endothermic HR response occurs in EP embryos just like the endothermic metabolic response (Fukuoka et al., 2006; Dzialowski et al., 2007; Andrewartha et al., 2011b).

Assessment of $\dot{M}O_2$ and HR responses to various tests that alter incubation temperature have been successfully utilized to assess thermoregulatory ability of avian embryos. The timing of endothermic responses differs along the precocial to altricial continuum, with precocial species generally showing thermoregulatory ability earlier than altricial species. Examination of mitochondrial, cellular, and tissue-level mechanisms are now required to further our understanding of how thermoregulatory ability develops in embryos and hatchlings.

32.5 ARTIFICIAL INCUBATION

32.5.1 Preincubation Egg Storage

Many precocial birds laying multiple eggs in a clutch start their incubation with the penultimate or ultimate eggs. Consequently, first-laid eggs are stored in the nest until incubation starts, sometimes for many days. Egg storage is not just a phenomenon of nature, of course. Egg storage commonly occurs in commercial conditions of artificial incubation. If the storage temperature for freshly laid chicken eggs is kept below physiological zero (25–27 °C), dormancy of the embryo is maintained and fertile eggs can be stored for several days without a major loss of hatchability (Butler, 1991; Wilson, 1991). The optimal temperature for 3 to 7 days storage of chicken eggs is 16–17 °C and it drops to 10–12 °C for eggs stored for more than 7 days (Butler, 1991; Wilson, 1991). However, prolonged preincubation egg storage results in malformations and retarded growth of the embryos, decreased hatchability, and increased incubation period, and it even influences hatchling growth (Arora and Kosin, 1966; Mather and Laughlin, 1979). The hatchability of the northern bobwhite quail (*Colinus virginianus*) remains above 70% after 14 days of preincubation storage at 20–22 °C, but decreases to below 30% after 21 days of storage (Reyna, 2010). These deleterious effects are related to not only the length of storage but also the environmental and physical conditions such as temperature, relative humidity, atmospheric gas composition, orientation, and positional changes during storage (Brake et al., 1997).

Prolonged preincubation egg storage also affects the physiological function of developing chicken embryos (Haque et al., 1996; Fasenko, 2007). Albumen quality, an indicator of overall egg quality, decreases with storage (Scott and Silversides, 2000; Reyna, 2010). Furthermore, while the developmental patterns of $\dot{M}O_2$ are consistent among unstored (control) eggs, those stored for 20 and 30 days at 10–11 °C are varied and depressed among eggs; the depression of the $\dot{M}O_2$ increases in severity as the storage duration increases. The developmental trajectories of HR in stored eggs are flattened compared with those of control eggs. As a result, the O_2 pulse (O_2 uptake every heartbeat) is markedly lowered by preincubation storage, decreasing blood O_2 transport and retarding embryo growth in stored eggs, resulting in death during the last days of incubation (Haque et al., 1996).

32.5.2 Egg Turning

In many avian species, incubating adults actively move their eggs in the nest (egg-turning). The critical period for lack of egg-turning in artificial incubation of the chicken egg ranges from days 3 to 7 of artificial incubation (New, 1957; Deeming, 1989a). Chicken eggs should be turned minimally 3 times a day. Turning more than 24 times a day does not further enhance hatchability. Lack of egg-turning is detrimental not only to hatchability, but can slow incubation, the development of the CAM, and embryo growth (New, 1957; Tazawa, 1980b; Deeming et al., 1987; Tullet and Deeming, 1987; Deeming, 1989a,b).

Failure to turn eggs during incubation also produces adverse effects on gas exchange through the CAM (Tazawa, 1980b). The movement of albumen into the amnion is retarded, and the unabsorbed albumen, which becomes more viscid and heavy as it loses water early in incubation, sinks towards the lower end of the egg, where it remains. The chorioallantois fails to fold around the unabsorbed
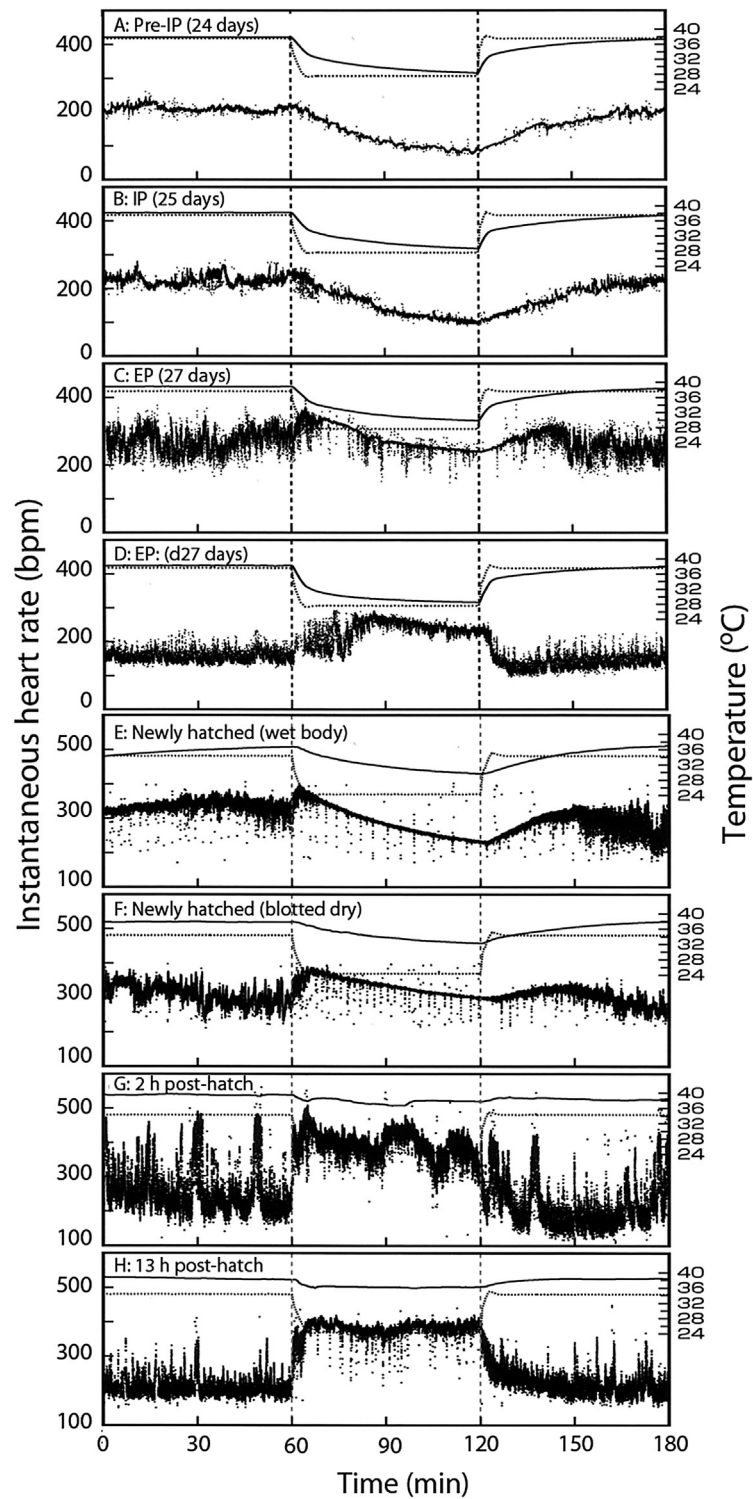
FIGURE 32.12   Responses of instantaneous heart rate (IHR) in duck embryos and hatchlings exposed sequentially to high ambient temperature ($T_a = 38\,°C$ for embryos and $35\,°C$ for hatchlings; dotted line), low ambient temperature ($T_a = 28\,°C$ for embryos and $25\,°C$ for ducklings), and high temperature again for 60 min, respectively. The solid line represents egg temperature or body temperature. *From Andrewartha et al. (2011a), with permission from Elsevier.*

albumen, and the interposition of the albumen between the CAM and inner shell membrane reduces gas exchange. These effects cause a pronounced fall in the arterialized blood PO_2 of late embryos, which is accompanied by an increase in Hct. The inhibition of gas exchange is reflected in the decreased $\dot{M}O_2$ of unturned eggs compared to turned eggs (Pearson et al., 1996).

32.5.3 Ambient Temperature and Incubation

Freshly laid eggs can be stored at a low temperature to maintain dormancy of embryos. However, once incubation starts, T_a must be kept within a certain range so that the embryo temperature is maintained and cell proliferation can proceed. In artificial incubation of chicken eggs at constant T_a, hatchability decreases when T_a is lowered to 35 °C or elevated to 40 °C from the optimal temperature of 37.5 °C. At 35 °C, incubation period and embryonic development of chickens are ~3 days longer than at 38 °C (Tazawa, 1973; Black and Burggren, 2004a). Survival during total incubation period is reduced at 35 °C (Black and Burggren, 2004a) and temperatures below this threshold are lethal. Hypothermal incubation decreases $\dot{M}O_2$ in proportion to retarded development of embryos and preserves the relative timing of IP and EP (Tazawa, 1973; Black and Burggren, 2004a). However, at the final stages of hypothermal incubation, hematological development and blood O_2-carrying capacity are retarded (Black and Burggren, 2004b), and eventually hypothermal incubation causes a significant delay of the relative timing of the onset of thermoregulatory ability (Tzschentke et al., 2001; Nichelmann, 2004; Black and Burggren, 2004b; Mortola, 2006).

The tolerance limits of developing embryos to acutely lowered and elevated T_a have been investigated in chickens in reference to their HR (Tazawa and Rahn, 1986; Ono et al., 1994). When day 10 embryos are exposed to a T_a of 28 °C or 18 °C, HR decreases in an exponential fashion to reach plateau values during 2–3 h of exposure. The plateau values are ~100 bpm at 28 °C and ~30 bpm at 18 °C, which are maintained until irreversible cardiac arrest occurs at ~100 and ~60 h after exposure to 28 °C and 18 °C, respectively. Accordingly, day 10 embryos survive exposure to 28 °C for no less than 4 days and to 18 °C for about 2.5 days. Reduction of T_a to 8 °C forces the heartbeat of day 10 embryos to cease after ~3 h. However, the cardiac arrest at this low T_a (8 °C) does not mean the death of embryos. Ten-day embryos survive the low T_a, without a heartbeat, for 18 h more, and the heart begins to beat again after rewarming at 38 °C. The survival time at 8 °C is reduced as the embryos grow. While the heart of day 6 embryos begins to beat even after 1 day exposure to 8 °C, the heart of day 20 embryos fails to beat after an 8 h exposure (Tazawa and Rahn, 1986). Although chicken embryos can withstand a lowered T_a for a prolonged period without a heartbeat, at an increased T_a the cardiac arrest following arrhythmia is irreversible and the embryos cannot withstand exposure to an increased T_a for a prolonged period (Ono et al., 1994). The HR of embryos increases in an exponential fashion at increased T_a. When T_{egg} reaches 46–47 °C, regardless of the developmental stage of embryos, the HR becomes arrhythmic and irreversible cardiac arrest follows. The lethal T_a and tolerance time of chicken embryos depend upon the time T_{egg} takes to reach a lethal critical value of 46–47 °C. At a T_a of 48 °C, the tolerance time of day 12 embryos is ~100 min and that of day 20 embryos shortens by about half. The tolerance time to 48 °C exposure is shortened as the embryos grow. This may be in part due to the higher metabolic heat produced by late embryos compared to earlier embryos. The HR reaches ~450 bpm at the critical T_{egg} (46–47 °C) (Ono et al., 1994).

The embryos of ground-nesting birds in semiarid and arid areas, in particular, experience environmental temperatures well in excess of typical avian incubation temperatures. For example, the northern bobwhite quail can experience nest temperatures as high as 45 °C in the wild. Not surprisingly, this species shows remarkable tolerance to brief preincubation hyperthermia. Bobwhite quail embryos can survive 6 h exposure to 46 °C, 3 h exposure to 49 °C, and, remarkably, 1 h exposure to 50 °C (Reyna and Burggren, 2012). While these temperatures certainly increase mortality and decrease hatching rates, the high temperature tolerance of the bobwhite quail highlights the importance of a comparative approach to examining temperature tolerance. Extreme temperatures in the natural environment may result in increased tolerance in avian species, which warrants further investigation.

32.5.4 Humidity

Successful artificial incubation of chicken eggs occurs over a relative humidity (RH) range of 40–70% (Robertson, 1961). Within the successful incubation range, 53% RH is optimal for embryo survival and hatchability (Bruzual et al., 2000).

High (>85%) and low (<30%) humidity increases mortality in chicken embryos (Ar and Rahn, 1980; Bolin, 2009). Under low humidity, inadequate water content can result from increased water loss across the porous eggshell due to a high vapor pressure gradient between the inside of the egg and surrounding air. Under high humidity, water loss is reduced and this can exert its osmotic stress within the embryo. Allantoic fluid volume, which is a balance between renal filtrate production and water reabsorption into the embryo, is reduced under high water loss conditions, and conversely increased when water loss is low (Davis et al., 1988). Under high water loss, water is reabsorbed from the allantois, uric acid levels in the allantois increase, and sodium is actively transported to maintain the allantoic fluid hypotonic to the blood and aid water reabsorption (Hoyt, 1979; Davis et al., 1988). Plasma calcium, sodium, and potassium levels are elevated

in late-stage embryos, a sign of osmotic stress (Davis et al., 1988). After exposure to <30% RH, the kidneys of day 18 chicken embryos have additional glomeruli, more functioning glomeruli, and high cloacal fluid osmolality, illustrating an increased filtering capacity (Bolin, 2009).

Extremes in humidity or water loss reduce hatching success (Davis et al., 1988). Water balance appears to have the greatest effects on hatchability early in incubation, with hatchability related to water loss during the first half of incubation, rather than total water loss (Snyder and Birchard, 1982). Humidity extremes also affect late-stage embryo wet mass; that is, embryos from low-humidity or high water loss environments have smaller wet masses while those from high-humidity environments have higher wet masses (Bruzual et al., 2000). In low water loss environments, excess water is not always incorporated into wet mass but is instead left behind in the albumen (Davis et al., 1988). Growth and oxygen consumption can be retarded after exposure to extreme humidity (Bolin, 2009), but embryos that do hatch under extreme humidity reach normal weight within 7 to 10 days post hatch (Davis et al., 1988).

32.6 CONCLUSIONS AND FUTURE DIRECTIONS

Incubation is the first important process experienced by the embryonic life stages of any avian species. The characteristics of the egg itself and the surrounding environment determine the success and timing of embryonic development. As this chapter has demonstrated, the study of avian embryonic physiology has provided extensive information on the complex processes that occur during avian development. We examined the physical processes of incubation, including the transfer of water and heat between the egg and environment. We provided an overview of the development of the form and function of the major physiological systems, including the gas exchange, acid–base, cardiovascular, osmoregulatory, and thermoregulatory systems. The conditions for successful artificial incubation were discussed, as well as the effects of incubation extremes. However, numerous possibilities still exist to expand our knowledge of avian development by utilizing new physiological techniques and undertaking studies using a cross-discipline approach. Molecular and cellular studies will be important in revealing the mechanistic underpinnings of system- and organism-level observations. Furthermore, many fruitful areas of research at all levels, from genes to the whole organism, remain. These include the timing and control of important physiological events during development, how and when systems are perturbed during development and if recovery is possible, and how systems work together during development. Comparative approaches are also needed to place physiological processes in an ecological and evolutionary context. Addressing these research areas will allow us to further understand avian embryos for the complex, multi-system organisms that they are, while also contributing to the broader field of vertebrate development.

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