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INTERRUPTION OF CARDIAC OUTPUT DOES NOT AFFECT SHORT-TERM GROWTH AND METABOLIC RATE IN DAY 3 AND 4 CHICK EMBRYOS

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Summary

The heart beat of vertebrate embryos has been assumed to begin when convective bulk transport by blood takes over from transport by simple diffusion. To test this hypothesis, we measured eye growth, cervical flexure and rates of oxygen consumption ($\dot{V}_{O_2}$) in day 3–4 chick embryos denied cardiac output by ligation of the outflow tract and compared them with those of embryos with an intact cardiovascular system.

Eye diameter, used as the index for embryonic growth, increased at a rate of approximately 4.5–5 % h^{-1} during the observation period. There was no significant difference (P>0.1) in the rate of increase in eye diameter between control (egg opened), sham-ligated (ligature present but not tied) and ligated embryos. Similarly, the normal progression of cervical flexure was not significantly altered by ligation (P>0.1).

$\dot{V}_{O_2}$ (ml O_2 g^{-1} h^{-1}) at 38 °C, measured by closed respirometry, was not significantly different (P>0.1) on day 3 in sham-ligated (14.5±1.9 ml O_2 g^{-1} h^{-1}) and ligated 17.6±1.8 ml O_2 g^{-1} h^{-1}) embryos. Similarly, on day 4, $\dot{V}_{O_2}$ in sham-ligated and ligated embryos was statistically the same (sham-ligated 10.5±2.9 ml O_2 g^{-1} h^{-1}; ligated 9.7±2.9 ml O_2 g^{-1} h^{-1}). Expressed as a linear function of body mass (M), $\dot{V}_{O_2}$ in sham-ligated embryos was described by the equation $\dot{V}_{O_2}$=–0.48M+24.06 (r^2=0.36, N=18, P<0.01), while $\dot{V}_{O_2}$ in ligated embryos was described by the equation $\dot{V}_{O_2}$=–0.53M+23.32 (r^2=0.38, N=16, P<0.01). The regression line describing the relationship between body mass and $\dot{V}_{O_2}$ for pooled sham-ligated and ligated embryos (the two populations being statistically identical) was $\dot{V}_{O_2}$=–0.47M+23.24. The slope of this regression line, which was significantly different from zero (r^2=0.30, N=34, P<0.01), was similar to slopes calculated from previous studies over the same range of body mass.

Collectively, these data indicate that growth and $\dot{V}_{O_2}$ are not dependent upon cardiac output and the convective blood flow it generates. Thus, early chick embryos join those of the zebrafish, clawed frog and axolotl in developing a heart beat and blood flow hours or days before required for convective oxygen and nutrient transport. We speculate that angiogenesis is the most likely role for the early development of a heart beat in vertebrate embryos.

Key words: development, heart, embryo, cardiac output, blood flow, chick.

Introduction

Cardiovascular physiologists have long assumed that the initiation of cardiac output in the developing embryo occurs concomitant with the need for convective delivery of nutrients and oxygen and the removal of wastes and carbon dioxide. Certainly, the eventual replacement of diffusive with convective transport of materials is inevitable as a developing animal grows too large for simple diffusion to suffice, especially if tissue metabolic rates are high. Unresolved, however, is whether the early initiation of a heart beat and subsequent cardiac output occur on a ‘just in time’ basis when diffusive transport fails. Recent data from lower vertebrate embryos indicate that the heart may begin to generate blood flow well before convective transport of materials is required. These data derive from studies in which convective transport of oxygen by the blood is eliminated by surgical removal of the pre-cardiac mesoderm (eliminating heart development) in salamander (*Ambystoma mexicanum*) embryos (Mellish et al., 1994, 2000) or by elimination of hemoglobin oxygen-transport by carbon monoxide exposure and/or destruction of red blood cells in embryos of *Xenopus laevis* (Territo and Burggren, 1998) or zebrafish (*Danio rerio*) (Pelster and Burggren, 1996). These studies refute the hypothesis of ‘synchronotropy’ (that the heart starts to beat just as convective transport is required) and instead support the hypothesis of ‘prosynchronotropy’ (that heart beat and convective flow of blood develop well before the metabolic needs of the embryo outstrip material transport by diffusion (Burggren and Territo, 1995; Burggren and Fritsche, 1997).

The relative importance in embryos of diffusive and convective transport, and the timing of the absolute need for

the transition from the former to the latter, will surely be affected by the metabolic rate of the embryonic tissue being examined. In this regard, the applicability of the 'prosynchronotropy' hypothesis to all vertebrates, as opposed to lower vertebrate ectotherms with intrinsically low metabolic rates, requires that this hypothesis be tested in a vertebrate endotherm with a high metabolic rate. Early experiments exploring the process of angiogenesis in chick embryos suggest that elimination of cardiac output by surgical ligation of the outflow tract is non-lethal in early chick embryos (Thoma, 1893; Chapman, 1918), but does eventually result in developmental abnormalities. Metabolic rate and overall embryonic growth were not, however, assessed in these early experiments. Männer et al. (1995) have shown that complete removal of the heart in Hamilton–Hamburger (HH) stage 12 chick embryos results in cranial and other abnormalities, and that these abnormalities can be partially reversed by exposure to long-term hyperoxia. They suggest that these data indicate the importance of blood circulation in the development of the chick embryo. Is, then, prosynchronotropy applicable only to vertebrate embryos with modest oxygen demands?

To test the prosynchronotropy hypothesis in chick embryos at later, presumably more convection-dependent, stages than examined by Männer et al. (1995), we have examined the influence of outflow tract ligation and the ensuing elimination of cardiac output and convective blood transport on growth and oxygen consumption of chick embryos at day 3 and day 4 (HH stages 18–25). Convective blood flow normally starts at approximately the end of the second day of development and has increased greatly from approximately 0.05–0.1 $\mu l\,s^{-1}$ at the beginning of day 3 to more than 1 $\mu l\,s^{-1}$ by the end of day 4 (see Clark, 1991). Thus, there is a major and increasing perfusion of peripheral tissues during days 3–4, and elimination of cardiac output during day 3 and day 4 of development will rigorously test the dependence of metabolic rate upon blood flow.

Materials and methods

Animals

Fertile Rhode Island Red chicken eggs were obtained from a local breeder and transported to the laboratory in Las Cruces, NM, USA. Eggs were set in commercial incubators (Turbo Hova-bator) with continuous mechanical rotation through 90° and incubated at 38 °C. Experiments were performed on a total of 66 day 3 and day 4 embryos. Observations were confined to 4 h periods on each day of development to limit the number of developmental stages over which the experiments were performed. Following experiments, the wet body mass of the embryo (minus yolk and extraembryonic membranes, which were first dissected away) was carefully measured. Since there was considerable variation in embryonic body mass irrespective of incubation day, all subsequent data are reported on the basis of body mass rather than hour or day of development.

Measurement of P_{O_2} in the air cell

Opening the egg shell to access the heart of the embryo may result in an artificial elevation of the partial pressure of O_2 (P_{O_2}) in the air cell, and thus a non-physiological state of relative hyperoxia for the developing embryos. This concern is predicated on air cell P_{O_2} being significantly below that of air at the stages in which our experiments were conducted. To determine this, air cell P_{O_2} in intact, normally incubated eggs was determined each day from day 1 to day 10. To sample air cell gas, an egg was removed from its incubator and completely immersed in water at 37° C to eliminate possible contamination of the air cell sample with room air. The tip of a 22 gauge needle connected to a 5 ml glass syringe was used to penetrate the egg shell over the air cell and inserted into the center of the air cell. A 1–2 ml sample of air cell gas was then withdrawn from the air cell and immediately analzyed for P_{O_2} using a Cameron Instruments BGM200 blood gas meter.

Measurement of eye diameter and cervical flexure as an index of normal growth

The diameter of the embryonic eye is an excellent indicator of early embryonic growth, showing large increases during days 3–5 (Romanoff, 1960). Eye diameter is also readily measured because of the steady increase in eye pigmentation. Cervical flexure has also been measured in chick embryos as an index of normal growth (Männer et al., 1995). We employed both morphological measurements in two distinct experimental series.

On the day of the experiment, eggs were removed from the incubator and temporarily placed in a sand bath heated to 38 °C. In all eggs, a portion of the shell covering the air cell was removed, and the inner shell membrane was carefully peeled back to expose the embryo. Three distinct groups of embryos were then generated: control, sham-ligated and experimental. The control group, comprising 12 individuals, consisted of embryos with opening of the egg, as described above, being the sole disturbance. In 19 sham-ligated embryos, a 10-0 silk ligature was passed around the outflow tract distal to the ventricle and tied loosely so as to not interfere with blood flow. In the ligated group, consisting of 19 embryos, the cardiac outflow tract was completely ligated by tightening the knot in the silk ligature, thus blocking all flow of blood from the heart. Total occlusion was verified visually.

Embryos in eggs treated as described above were imaged at 16× using a Zeiss Stemi 2000-C dissecting microscope and a Samsung color video camera (SAC-410NA). Images were captured using a Matrox Comet video capture card and stored for later analysis using Matrox Inspector software. Three groups of embryos were examined: control, sham-operated and ligated. Following surgery, the eggs were imaged as described above, sealed with oxygen-permeable plastic film held in place with adhesive tape. These eggs were then returned to the incubator. After 4 h of further incubation, the eggs were removed from the incubator, unsealed and a second image of the embryo was acquired. Following treatment appropriate for its assigned group, each embryo was replaced in the incubator.

After a further 4 h observation period in the container at incubation temperature, the embryo was removed from the container and imaged a third time. Embryonic growth during the observation period was analyzed both for degree of cervical flexure, using a modification of the technique described by Männer et al. (1995), and for eye diameter. Determination of flexure angle requires the establishment of two reproducible reference lines. In our study, the first reference line was established by drawing a line that overlay the linear portion of the dorsal aorta posterior to the confluence of the posterior and common cardinal veins. The second reference line was drawn from the point of confluence of the posterior and common cardinal veins to the center of the eye. The obtuse angle created by the intersection of these two line was used to define the degree of cervical flexure. Thus, an increasing amount of cervical flexure is indicated by a decrease in this angle.

Measurement of metabolic rate

Since preliminary analysis revealed no difference in growth between embryos in eggs that had been opened and those that had additionally had a silk ligature loosely passed around the outflow tract (sham-ligated), metabolic experiments were subsequently performed on sham-ligated eggs (N=16) and ligated eggs (N=18). After appropriate preparation, sham-ligated and experimental eggs were individually placed upright in a plastic or glass jar that served as a closed-chamber respirometer (volume approximately 250 ml). Carbon dioxide absorbent (moist KOH) was placed in the respirometer remote from the egg. An airtight lid containing fittings for a water manometer to measure pressure in the respirometer and an air-filled syringe for adjusting respirometer volume/pressure was then screwed onto the respirometer, which was then immersed in a circulating water bath maintained at 38 °C. The embryo in the respirometer was allowed to acclimate and thermally equilibrate for 20–45 min. A flow of water-saturated air at 38 °C was maintained through the respirometers during the acclimation period. After equilibration, a syringe was placed in one of the lid fittings, and this injection port was closed. A water-filled manometer was attached to the other port in the lid, and the manometer level was measured. After a significant and measurable change in respirometer volume had occurred (typically 1–2 h), a measured volume of air was injected to return the level in the manometer to the volume at the start of the respirometer run. The volume of air injected was, therefore, equal to the amount of oxygen consumed by the embryo. Most determinations were duplicated, and the average value was used in all further calculations. Mass-specific rates of oxygen consumption were calculated from whole-egg oxygen consumption and embryonic mass. We chose not to include the mass of the extraembryonic membranes, given their very low mass-specific rate of oxygen consumption (Romanoff, 1941).

After oxygen consumption measurements had been completed, the egg was carefully removed from the respirometer and weighed. The embryo was then removed from the egg and fixed in 10 % neutral buffered formalin. Embryos were later blotted dry and weighed to the nearest 0.1 mg.

Statistical analyses

The rate of oxygen consumption changes non-linearly over the entire range of development in chick embryos (Romanoff, 1941; Romijn and Lokhorst, 1960; Howe et al., 1995). However, over the range of days 3 and 4, the relationship between metabolic rate and body mass is accurately represented by a linear regression. Consequently, we have employed linear regressions over this narrow developmental range for both our own data and those of previously published studies.

Differences in rates of oxygen consumption and eye diameters in the first set of experiments were assessed using a one-way analysis of variance (ANOVA; Jandel SigmaStat 2.03). Differences between eye diameters and differences between flexure angles in the second set of experiments were analyzed by a repeated-measures general linear model ANOVA (GLM; SPSS). No *post-hoc* tests were required because there were no significant differences between treatments. A fiduciary level of 0.05 was adopted for all tests.

Results

Role of the egg shell in embryonic gas exchange

Changes in P_{O_2} of air cell gas are shown in Fig. 1. Essentially, the egg shell presents no significant boundary to inward oxygen diffusion in days 3–5, since air cell P_{O_2} was no more than 0.2 kPa below incubator air P_{O_2} during the period of development in which metabolic and growth measurements were made. Even by day 10, the gradient between the air and the air cell was only approximately 0.8 kPa.

To ensure that opening the egg shell had no effect on oxygen exchange dynamics, measurements of oxygen consumption were made in day 3 and day 4 embryos before and after opening the egg shell. There were no significant differences in the rate of oxygen consumption after the shell had been opened (P=0.48; paired t-test).

Embryonic body mass

Embryonic body mass (minus yolk) is presented in Table 1. There was considerable variation in body mass for each day of

Table 1. *Mean values of body mass and rate of oxygen consumption in day 3 and day 4 chick embryos either sham-ligated or with their ventricular outflow tract completely ligated*

	Body mass (mg)		Oxygen consumption (ml O_2 g^{-1} h^{-1})	
Condition	Day 3	Day 4	Day 3	Day 4
Sham-ligated	16.5±2.0 (11)	24.9±3.0 (5)	14.5±1.9 (11)	10.5±2.9 (5)
Ligated	14.2±1.9 (13)	27.3±3.0 (5)	17.6±1.8 (13)	9.7±2.9 (5)

Values are means ± S.E.M. (N).

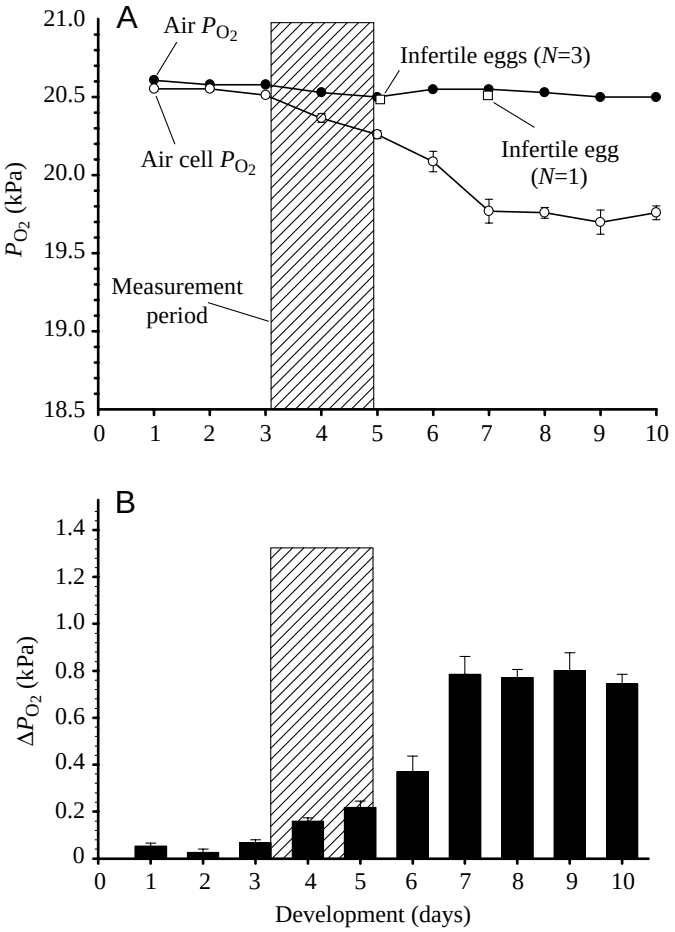

Fig. 1. Development-related changes in air cell P_{O_2} in the chick embryo. (A) Actual air cell P_{O_2} (water-saturated, at 37 °C) (filled symbols show air P_{O_2}), (B) P_{O_2} differential (ΔP_{O_2}) between incubator air and air cell gas. The vertical hatched column shows the period during which metabolic and growth measurements were made. Note that data from four infertile eggs indicate that air cell and incubator air were in equilibrium. Values are means ± S.E.M.

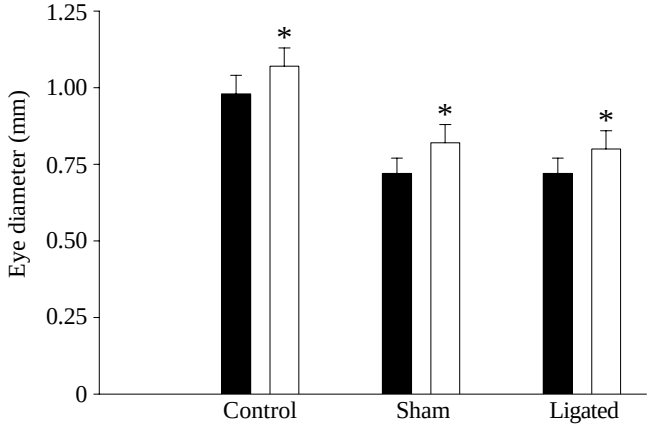

Fig. 2. Growth in eye diameter during incubation at 38 °C in day 3 chick embryos. Data for three conditions (control, sham-ligated and ligated) are shown. For each condition, diameters before (open columns) and after (filled columns) the observation period are presented. An asterisk indicates a significant increase in eye diameter during the measurement period. Values are means + S.E.M.

Table 2. *Mean values of eye diameter and cervical flexure before and after surgery (opening of the egg shell for suture placement) in day 3 chick embryos*

	Eye diameter (mm)		Cervical flexure (degrees)	
	Before surgery	After surgery	Before surgery	After surgery
Sham-ligated	0.68±0.06 (20)	0.82±0.05 (20)*	103.8±6.0 (20)	101.7±1.8 (20)*
Ligated	0.72±0.05 (15)	0.80±0.06 (15)*	104.3±7.9 (19)	101.7±2.5 (19)*

Values are means ± S.E.M. (*N*).

* indicates a value significantly different from that before surgery as indicated by repeated-measures ANOVA. There were no significant differences between sham-ligated and ligated groups.

development and, consequently, there was no significant difference (P>0.1) between body mass on days 3 and 4.

Embryonic eye growth and cervical flexure

Changes in eye diameter during early embryonic growth and development of the three populations are shown in Fig. 2. There were no significant differences (P>0.1) in eye diameter before treatment between control, sham-ligated and ligated embryos. All three populations showed highly significant and almost identical levels of eye diameter growth during the observation period, eye diameter increasing by approximately 0.15–0.20 mm (Fig. 2). These increases were significant (P<0.01) in all three populations. Importantly, eye diameter at the end of the measurement period was not significantly different (P>0.1) among the three groups.

In a second set of experiments that included cervical flexure measurements, there were also no significant differences in eye diameter growth imposed by ligation (P>0.1), and eye diameter increased over 4 h of development in both ligated and sham-ligated embryos (P<0.01; Table 2). Similarly, there were no significant differences between the two groups in cervical flexure (P>0.2) and, in both groups, flexure decreased with time (P<0.01; Table 2). In no case was there a significant interaction between experimental group and time. There was a strong interaction between eye diameter and flexure (P<0.01).

Embryonic oxygen consumption

There were no significant differences (P>0.1) in mass-specific metabolic rate between sham-ligated embryos on day 3 (14.5 ml O_2 g^{-1} h^{-1}) and day 4 (10.5 ml O_2 g^{-1} h^{-1}) (Table 1). As indicated above, there was considerable variation in body mass within a given day of development. Fig. 3 shows data for metabolic rate plotted as a function of embryonic body mass. The slopes and intercepts of the regression lines describing the mass-specific rate of oxygen consumption for

sham-operated and ligated embryos were very similar, indicating that ligation of the outflow tract had no effect on the mass-specific rate of oxygen consumption. However, in sham-ligated embryos, body mass *per se* accounted for less than half of the total variance in mass-specific metabolic rate (r^2≈0.36). The correlation coefficient was similarly low for ligated embryos (r^2≈0.38).

The regression line describing the relationship between body mass (M) and the rate of oxygen consumption for pooled sham-ligated and ligated embryos (there being no statistical reason to maintain their individuality) was $\dot{V}_{O_2}$=–0.47M+23.24. The slope of this regression line was significantly different from zero (r^2=0.30, N=34, P<0.01).

Discussion

Critique of 'open' versus 'closed' egg shell preparations

The avian eggshell provides a barrier to the flux of oxygen, carbon dioxide and water vapor between embryonic tissues and the atmosphere surrounding the egg. Opening the egg shell, and thus removing this potential diffusion barrier might, therefore, result in an artificial elevation of air cell and embryonic tissue P_{O_2} if the embryo acts as a major O_2 sink. However, our demonstration that P_{O_2} in the air cell on days 3 and 4 is in equilibrium with air indicates that the egg shell is not a significant barrier to O_2 movement in this early stage of development when $\dot{V}_{O_2}$ is extraordinarily low in absolute terms. Consequently, opening the egg shell to provide access to the early embryo and its blood vessels does not artificially elevate air cell P_{O_2}. Thus, we could work with eggs through a small opening without altering normal gas exchange dynamics. Only during the later stages of chick development does air cell P_{O_2} begin to deviate significantly from atmospheric, for example, reaching a very mildly hypoxic value of approximately 19.5–20 kPa by day 10 (Fig. 1) and approximately 17–18 kPa by day 12 of development (Tazawa et al., 1980).

Opening the egg shell could also result in dehydration of the early embryo if the infused air were less than fully saturated with water vapor and the shell was opened for long periods. However, our metabolic measurements were made on embryos in opened eggs that were maintained in air saturated with water vapor over a period no longer than 4 h. Moreover, the embryos, weighing less than 50 mg, were essentially floating in a relatively enormous aqueous reservoir. The cervical flexure and eye diameter measurements were performed on embryos maintained in incubators with 70 % relative humidity, so these egg shells were closed following initial surgery, eliminating any concerns regarding embryo dehydration.

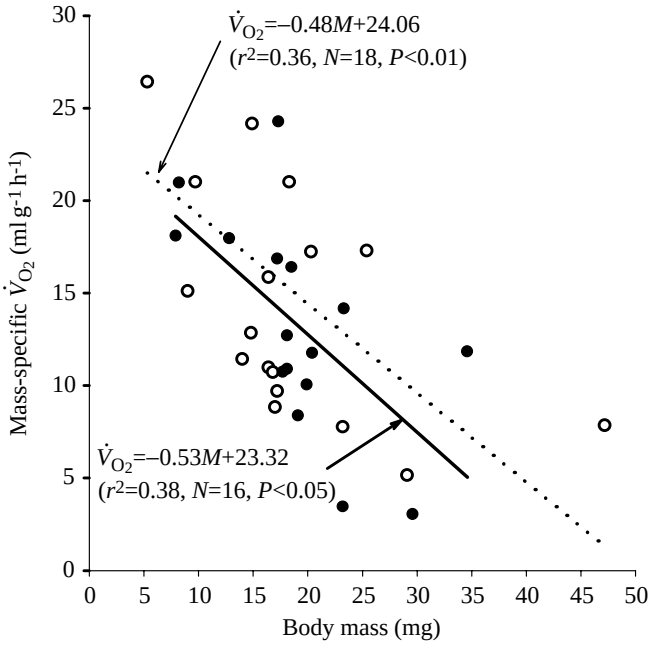

Fig. 3. Mass-specific rates of oxygen consumption ($\dot{V}_{O_2}$) as a function of body mass (M) in day 3–4 chick embryos that were either sham-ligated (open circles, dotted lines) or had their aorta ligated (filled circles, solid lines). Equations and correlation coefficient for each regression line are shown. See text for statistical interpretation of the data.

Metabolic rate in control and sham-ligated embryos

Metabolic rate ($\dot{V}_{O_2}$) measured in the present study in day 3 and 4 embryos over a body range of 10–47 mg was approximately 25–35 % lower than that over the same developmental range calculated by the present authors from Romijn and Lokhorst (1960) and Romanoff (1967). Regression lines are shown in Fig. 4. These studies show similar rates of decline of mass-specific metabolic rate with increasing body mass. However, the y-intercept in the present study reveals a rate of oxygen consumption 25–35 % lower than that reported previously. The reason for these differences in rates of oxygen

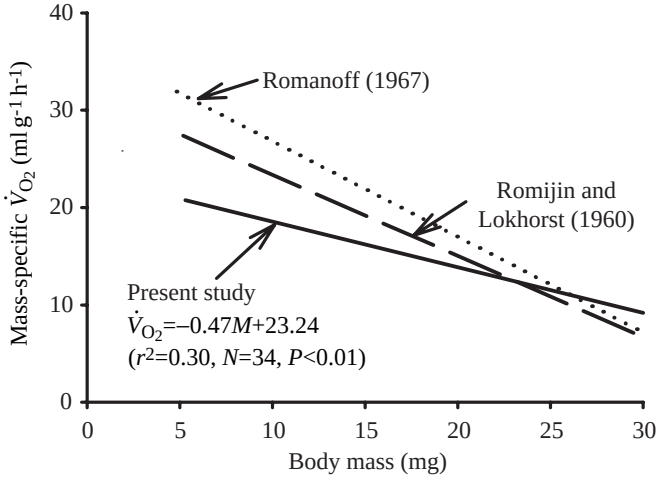

Fig. 4. Mass-specific rates of oxygen consumption ($\dot{V}_{O_2}$) as a function of body mass (M) in chick embryos up to 30 mg body mass (i.e. up to approximately day 4 of development). Regression lines for Romanoff (1967) and Romijn and Lokhorst (1960) were calculated by the present authors from several tables presented by Romanoff (1967) and in the original study of Romijn and Lokhorst (1960) (see these references for original citations).

consumption are not clear. Metabolic differences between studies in adults can often be attributed to differences in activity levels and stress, but this seems an unlikely source of variation in such early embryos. Possible explanations for our lower metabolic rates are systematic methodological differences in either metabolic measurements or in separating the yolk from the embryo during body mass determination.

Mass-specific metabolic rate decreased at a similar rate with increasing body mass in all the studies, as would be predicted from allometric metabolic equations. Allometric relationships are best revealed by measuring the variable of interest over at least a 10-fold range of body mass, especially if there are developmental changes occurring alongside changes in body mass. The linear regressions shown in Fig. 4 for other studies over a very narrow body mass range (25 mg) were derived from regressions based on the entire range of chick development (see above). Even with other significant sources of variation, the similarities in slope suggest that there are no major differences between the allometry of growth during early development in days 3–4 and during the rest of development.

Although an allometric component of growth can be demonstrated during days 3–4, the low correlation coefficient suggests that other factors are exerting a strong effect on metabolic rate. Although determination of these factors is beyond the scope of the present study, we now know that genetic effects on physiological variables can be important throughout the development of bird embryos (Burggren et al., 1994; Burggren, 1999).

Lack of dependence of growth on cardiac output

Normal growth (measured by eye diameter), development (measured by cervical flexure) and $\dot{V}_{O_2}$ in day 3–4 chick embryos are not affected by experimentally eliminating cardiac output and all associated peripheral blood flow. These data would seem to be at odds with those of Männer et al. (1995), but there are several significant differences between the two studies that preclude their direct comparison. In their study, the heart was completely excised at HH stage 12, and the embryos were incubated for 27–29 h before morphometric examination. In our study, the outflow tract was ligated (but the heart was otherwise left intact) in older embryos with greater rates of oxygen consumption (HH stage 18–25), and incubation was for only 2–4 h. Although the experimental studies of Männer et al. (1995) seem to indicate that a convective supply of oxygen is required for normal morphological development, our data clearly show that, even in later stages, convection is not required. Their more radical intervention of heart removal and perhaps concomitant removal of mechanical forces induced by the growing heart may explain the differences in results.

Our data on older embryos with greater rates of oxygen consumption than those used in Männer et al. (1995) clearly show that the delivery of oxygen and nutrients and the removal of carbon dioxide and other byproducts of metabolism are not dependent upon convection produced by blood flow within the developing cardiovascular system on days 3 and 4. Although simple diffusion is a very slow process, the relatively very small distances in day 3–4 chick embryos permit diffusive gas and nutrient transport at rates sufficient to support normal embryonic growth and metabolism.

That heart rate and blood flow begin well before the vertebrate embryo must make the transition from diffusive to convective material transport, a hypothesis termed ‘prosynchronotropy’ (Burggren and Territo, 1995; Territo and Burggren, 1998), is well supported by our current findings on the chick embryo. The chick therefore joins the salamander *Ambystoma mexicanum* (Mellish et al., 1994), the clawed frog *Xenopus laevis* (Burggren and Territo, 1995; Territo and Burggren, 1998) and the zebrafish *Brachydanio rerio* (Pelster and Burggren, 1996) as vertebrates that develop a heart beat, significant blood pressure and measurable blood flow well before the absolute need for convective oxygen and nutrient transport by the blood. The diversity of taxa represented in this group suggests that ‘prosynchronotropy’ is indeed a general vertebrate characteristic, although no examination of the chronology of this diffusion-to-convection transition in developing mammals has been carried out and is warranted. Whether mammals might represent a special case in which the cardiac output develops to meet the immediate needs for convection would depend in part upon whether mammalian embryos have markedly higher metabolic demands than similarly sized bird embryos; we know of no evidence to support this contention. Embryonic body mass and body size continue to increase rapidly after day 4, and the metabolic rate of the embryo increases accordingly (Romanoff, 1941; Romijn and Lokhorst, 1960; Howe et al., 1994). The point at which the transition from diffusive to convective oxygen delivery occurs in the chick is unknown. Unfortunately, by day 5, the heart has subsided into the interior of the egg from its earlier more peripheral location, and we were unable to ligate the outflow tract of the heart at day 5 without damaging surrounding tissues.

Timing of the onset of the embryonic heart beat

Why does the embryonic heart begin to beat ‘prematurely’, i.e. before it is absolutely required for convective respiratory gas nutrient and waste transport? There are a number of possible explanations, including continuing heart formation, angiogenesis and protection against environmental hypoxia.

Current data suggest that cardiac function is directly linked to changes in structure, both in embryos and in adults. For example, increased cardiac work results in cardiac hypertrophy (for a review and additional citations, see Keller, 1997). It was therefore logical to assume that the early beating of the heart contributed to its structural development, with centrifugal and other forces acting to sculpt and shape the forming heart. The heart was thought to beat to aid in its own development, an attractive hypothesis supported by some experimental data (Clark et al., 1989). However, detailed examination of heart formation in a variety of cardiac mutants that fail to develop a heart beat show that grossly normal heart development occurs in the complete absence of heart contraction and blood flow through the interior chambers (Mellish et al., 2000). Thus,

while heart contraction appears to influence heart development, it is not essential for cardiac formation and maturation.

Previously published studies, including some more than a century old, suggest that angiogenesis might be heavily dependent on the embryonic heart beat. Thoma (1893) and Chapman (1918), in a series of experiments involving ligation of the outflow tract of the chick embryo, determined that the growth of peripheral vasculature was somewhat dependent upon the presence of blood flow and pressure associated with uninterrupted cardiac output. At the time of their experiments, the important roles of shear and strain in endothelial cell growth and subsequent angiogenesis were unknown. Recent experiments have shown that the proliferation of endothelial cells in presumptive blood vessels in response to cell stresses is mediated by paracrine effects involving the release of vascular endothelial growth factor (VEGF) (for references, see Tomanek and Ratajska, 1997). *In vivo*, this would mean that the greater the pressure pulse generated by the developing heart, the stronger would be the angiogenic stimulus. This scenario does not rule out the additional possibility that blood-borne factors may contribute to peripheral blood vessel growth. Männer et al. (1995) have shown that surgical removal of the heart of the very young embryonic chick (HH stage 12, 12+) is associated with incomplete formation of yolk sac vasculature. Collectively, the available data strongly suggest that the early embryonic heart beat arises to promote angiogenesis, not bulk transport by the blood.

Finally, although we speculate that angiogenesis may be the primary reason for the first development of the heart beat, a non-mutually exclusive possibility is that the timing of the heart beat in vertebrates is in some way linked to the possible occurrence of embryonic hypoxia. Since diffusive transport of oxygen is dependent upon an adequate driving head pressure, the ability of an embryo to subsist entirely on diffusion for gas exchange will be linked to an adequately high level of ambient oxygen. The lower the ambient oxygen level, the lower the partial pressure gradient for diffusion into the interior tissues of the embryo and the greater the need for a convective oxygen supply. Exposure to environmental hypoxia is a real possibility in many aquatic lower vertebrate embryos (Pelster, 1997) and remains a risk in reptilian, avian and mammalian embryos, especially under physiologically challenging conditions (e.g. nest flooding, an abnormally thick egg shell or a regionally inadequate uterine blood supply). It may be that the 'early' onset of a heart beat (early, that is, from the perspective of needing to transport oxygen by blood convection in a normoxic environment) has been selected for in vertebrate embryos through its highly adaptive advantage in aiding embryonic survival should environmental hypoxia occur. Accelerated angiogenesis is known to accompany hypoxia in chick embryos (Hoper and Jahn, 1995), and this suite of responses could help ensure embryonic survival during the transition from diffusive to convective oxygen supply.

Although the definitive answer or answers to why the embryonic heart beats remains to be determined, the present experiments and other recent studies indicate quite clearly that the early embryo can survive without convective blood transport. Whether other, less readily observed but equally important physiological, processes (e.g. osmoregulation) are similarly facultative rather than obligatory during early development should be the focus of future studies.

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