Fixed patterns of bradycardia during late embryonic development in domestic fowl with *C* locus mutations

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Howe, Robert S., Warren W. Burggren, and Stephen J. Warburton. Fixed patterns of bradycardia during late embryonic development in domestic fowl with *C* locus mutations. *Am. J. Physiol.* 268 (*Heart Circ. Physiol.* 37): H56–H60, 1995.—A predictable late embryonic bradycardia (relative to normal White Leghorn chickens) has been documented in chicken strains with *C* locus mutations. The basis of the bradycardia remains unknown but clearly is related to a mutation at the *C* locus, which contains the structural gene for tyrosinase. When compared with the heart rate of normal White Leghorns (~295–305 beats/min from *day 8* to *day 20* of incubation), c^a/c^a and other *C* locus mutants showed a 10–12% reduction in heart rate during the last 4 days of incubation. Embryonic mortality occurred in both mutant and normal strains at an equivalent rate (~23%); a significant bradycardia (when compared with surviving embryos of the same strain) developed on the day before death in White Leghorn but not mutant strains. The bradycardia did not affect embryonic oxygen consumption (~0.2 ml $O_2 \cdot egg^{-1} \cdot min^{-1}$ at *day 14* and 0.4 ml $O_2 \cdot egg^{-1} \cdot min^{-1}$ at *day 20*), which showed only minor differences between strains that can be attributed to differences in embryonic mass on *days 16–20*.

heart rate; bird; embryo; fetal; cardiovascular; embryonic death

DEVELOPMENT OF THE cardiovascular system is of primary importance in the ontogeny of all animal species. Embryo death may be caused by developmental anomalies of this system, anomalies of other organ systems, failure of adequate nourishment, or other causes. In vertebrates, heart rate developmental patterns are quite variable, and few generalizations can be made (see Ref. 3). However, data from mammals (7, 18), amphibians (3, 4), reptiles (S. Warburton, unpublished data), and birds (21) indicate that for many species there is a relatively slow heart rate initially (that is, when heart rate can first be detected) followed by a steep rise of considerable magnitude to a peak rate. Depending on species, this peak rate may or may not persist for the rest of prenatal development. Even small deviations from this heart rate pattern are predictors of embryonic death in human singleton and multiple pregnancies (7, 10), suggesting that few deviations from the normal pattern of cardiac physiological development can be permitted.

There is currently no animal model for embryonic death associated with heart rate anomalies, nor is there an obvious candidate for an animal model, since studies on "variation" in patterns of heart rate development in nonhuman vertebrate species are few (see Refs. 2, 21).

One of the standard animal models for studying cardiovascular physiological development is the chick embryo. Basic physiological and related anatomic aspects (e.g., onset of heart beat, differentiation of cardiac pacemaker, and myocardium) have been known for some time (11, 22). Hemodynamic measurements on bird embryos have focused on measurements of pressure and flow in cardiac chambers and chorioallantoic arteries and other vessels, usually under normal conditions of development but in some cases during abnormal development (for recent reviews, see Refs. 5, 8, 9). Given an extensive data base on normal cardiac development (including heart rate), we searched for a chicken mutant that might show genetic heart rate anomalies as an embryo. We investigated strains of chickens with a *C* locus pleiotropic mutation, based on published accounts showing increased late embryonic mortality, low birth weight, and lower growth rates as young chicks (14–16). The *C* locus contains the structural gene for tyrosinase, and defects at this site can result in albinism. Preliminary experiments found that this strain has a bradycardia during the last 4 days of embryonic development. Consequently, in this paper we *1*) describe anomalous patterns of heart rate development in this and similar mutant strains of chicken, *2*) consider the predictability of embryonic death based on embryonic heart rate observation, and *3*) investigate whether decreases in heart rate in the late embryonic period are associated with a decreased metabolism before hatching.

MATERIALS AND METHODS

Genetic strains. Experiments were performed on a total of 232 chick eggs during the period from September 1990 to January 1992. All eggs were obtained from the University of Massachusetts Tilson Poultry farm. Six different genetically pure strains of chickens were used to provide eggs. White Leghorn (WL) and Rhode Island Red (RR) represented normal strains. The remaining four strains, designated c^a/c^a, SLA, EB5, and CRE, represented genetically pure mutant strains. The c^a/c^a strain is homozygous for the recessive albino allele. The genetics and developmental biology of these mutant strains have been described in detail by Pardue and co-workers (14–16). Briefly, the various mutant strains all have a mutation at the *C* locus, leading to a host of graded pleiotropisms in adults including albinism and impaired immunocompetence. Numerous pre- and posthatching effects are evident, including increased late embryonic mortality, reduced weight at hatching, reduced feed utilization, and higher incidences of subcutaneous hemorrhage and inflammation.

Holding conditions. Eggs were collected shortly after being laid and were immediately placed in a cold room (10°C) to arrest development. All eggs spent a minimum of 1 day and a maximum of 14 days at 10°C before being set in a commercial incubator at 38°C, after which they were treated identically (unless experimental protocols differed, as otherwise indicated). On *day 6* of development (i.e., 6 days after being set), all eggs were transferred to an experimental incubator maintained at a temperature of 37.5–38.5°C and a relative humidity of 70–95%. Eggs were placed in a tray that tilted through 90° every 4 h, thus ensuring the embryos were uniformly and regularly turned throughout their entire period of develop-

ment. Failure to turn eggs can adversely affect development and oxygen exchange (19).

Embryonic/egg mass measurements. Mass was recorded for each intact egg. In a subset of experiments, intact egg mass as well as embryonic mass (yolk removed) was recorded at external piping.

Heart rate measurement. Heart rate (f_H) in intact undisturbed embryos was measured by an impedance technique (6). On *day 6*, eggs were removed from the incubator for a maximum of 1 min for implantation of heart-monitoring electrodes. A sharp 24-gauge needle was used to make two 0.5-mm holes in the egg shell at diametrically opposed positions across the short axis of the shell, about midway along the egg's long axis. About 1.5–2.0 mm of the insulation was stripped off of a pair of 25-gauge copper wires (length = 0.5 m). The uninsulated tip of each wire was bent at right angles to the rest of the wire and carefully inserted through the hole in the shell. The wire was fixed in place with a small drop of cyanoacrylate glue. The two wires were led together at the tip of the egg, braided together, and led out of the incubator through a small port in its door. Immediately after electrode implantation, eggs were returned to the incubator. The electrode wires were attached to a Biocom impedance convertor, which produced an impedance signal correlated with ventricular contraction in the embryo. The impedance signal was fed into a Narco BT-1200 ratemeter and a Narco MK IV stripchart recorder, from which heart rates were then determined.

This technique allowed heart rate to be measured "remotely" from outside the incubator without any thermal, light, or vibrational disturbance, which can affect chick embryo heart rate (12, 23). Preliminary experiments demonstrated no increased mortality in embryos whose eggs were instrumented as described above when compared with uninstrumented controls or with eggs in which holes were created and sealed but in which no electrode wires were placed. The validity of this technique has been confirmed (6).

In the first experiment, designed to look at the time course of developmental changes in heart rate in WL and c^a/c^a, heart rate was measured daily between 1200 and 1600 until eggs hatched on *days 20–21*. These experiments indicated a significant difference in heart rate between strains in the last 4 days of development. To investigate this phenomenon more thoroughly, in a separate set of experiments eggs of six different strains were instrumented on *day 14*, and heart rate was determined on *day 18* only.

Oxygen consumption measurement. Oxygen consumption was measured in a separate set of experiments on WL and c^a/c^a eggs once daily from *day 7* to *day 20* of development. Standard techniques of closed respirometry for measurement of chicken egg oxygen consumption were used (see, for example, Ref. 20). Briefly, an egg removed from the incubator was very gently placed in a Lucite chamber (volume 231 ml) thermostatted to 38°C. The lid was placed on the chamber, and a gas sample was taken from the respirometer and analyzed for oxygen concentration in a Beckman OM14 gas analyzer. After either a 10-min measurement period (*days 7–14*) or a 5-min measurement period (*days 15–20*), a second gas sample was taken from the chamber and analyzed for oxygen concentration. From the elapsed time, the difference in initial and final oxygen concentration, the volume of the chamber, and the volume of the egg (determined from the formula for a prolate spheroid, $4/3\pi ab^2$, where a is the radius of the shell's long axis and b is the radius of the short axis), the oxygen consumption in milliliters oxygen per egg per minute was calculated. Typical levels of oxygen depletion in the chamber ranged from ~0.05 to 0.8%. Neither this level of oxygen depletion nor the attendant carbon dioxide accumulation was considered large enough to affect oxygen consumption rates.

Water loss during development. In a separate series of experiments, 18 WL, 28 c^a/c^a, and 20 EB5 eggs were incubated under standard conditions, beginning on the day of laying and continuing until piping. Every day, each egg was removed from the incubator and weighed, thus determining the daily weight change, a measure of insensible water losses occurring during incubation. From the elapsed time between weighings, the previous day's weight, and the day-to-day difference, daily water losses were calculated as percent of total egg mass per hour.

Statistical analysis. The significance of difference in heart rate or oxygen consumption on any given day of development between WL and c^a/c^a strains was determined by a Student's t-test. Animals that died during incubation were not included in the statistical treatment or in the figures. Differences in heart rate among two normal and four mutant strains measured at *day 18* were assessed with a one-way analysis of variance. The deviation in heart rate (expressed as percent deviation from control f_H) on any given day in embryos that died before hatching was compared with that in surviving embryos by a Student's t-test. A fudicial level of 0.05 was adopted for all tests of significance. All data are reported as means ± SE.

RESULTS

Heart rate patterns during development. Patterns of heart rate change during development differed between the c^a/c^a and WL strains (Fig. 1). The c^a/c^a strain had a significantly lower mean heart rate on the first day of measurement (*day 7*) but was indistinguishable from

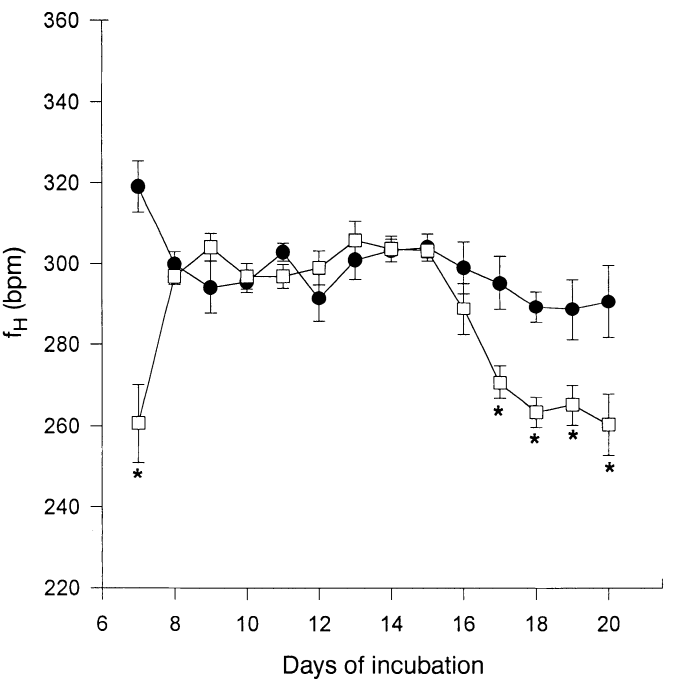

Fig. 1. Changes in heart rate (f_H) during development at 38°C in chicken embryos in intact eggs. Means ± SE are plotted for both normal White Leghorns (WL; ●) and mutant c^a/c^a (□) strains. Number of WL embryos observed is 5, 8, 11, 14, 14, 15, 15, 16, 15, 13, 7, 11, 9, and 9 for *days 7–20*, respectively. Number of c^a/c^a embryos observed is 12, 9, 12, 14, 15, 15, 16, 19, 22, 22, 18, 15, 14, and 16 for *days 7–20*, respectively. *Significant difference (Student's t-test) between mean heart rate for WL and c^a/c^a strains.

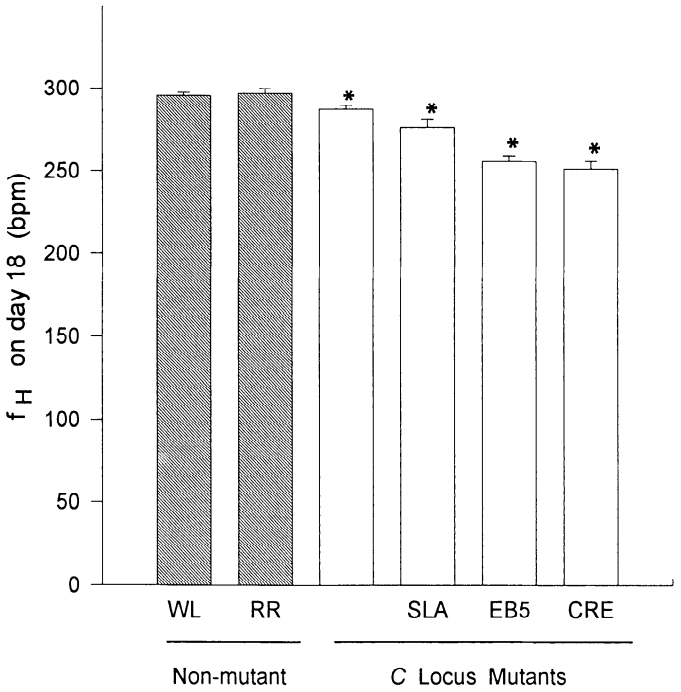

Fig. 2. Mean heart rate (±SE) at 38°C on *day 18* of embryonic development in 2 normal strains (WL, RR) and 4 strains with mutations at *C* locus (c^a/c^a, SLA, EB5, and CRE). Number of observations and mean body mass (±SE) are as follows: WL, n = 20, 59.1 ± 1.0 g; RR, n = 10, 55.4 ± 0.7 g; c^a/c^a, n = 29, 59.3 ± 1.1 g; SLA, n = 14, 46.6 ± 0.8 g; EB5, n = 13, 43.1 ± 0.6 g; and CRE, n = 14, 33.3 ± 0.6 g. *Significant difference (Student's t-test) between mean heart rate for a mutant strain and pooled heart rates for WL and RR.

the WL embryos from *day 8* to *day 16*, ranging from 295 to 305 beats/min. On *day 16*, although statistically indistinguishable from WL, the c^a/c^a embryos were beginning to show a bradycardia, relative to both their earlier f_H and to f_H in WL embryos. This bradycardia was significant from *day 17* to *day 20*, with a f_H depression of ~40 beats/min, 10–12% lower than WL.

Comparison of mutant and nonmutant strains: day 18 heart rate. Heart rates of both nonmutant strains (WL, RR) were virtually identical on *day 18* and were pooled for further analysis (pooled f_H = 296 ± 2 beats/min). Each of the four mutant strains tested displayed a significant bradycardia relative to the nonmutants (Fig. 2). The mutant strains also displayed different degrees of bradycardia relative to each other, with CRE showing the most severe bradycardia on *day 18* (f_H = 256 ± 4 beats/min). The c^a/c^a strain, which was used in the f_H progression experiment (above), showed the smallest degree of bradycardia when measured only on *day 18* (f_H = 287 ± 2 beats/min).

Embryo mortality and heart rate. Death of the embryos could occur at any point during development. Previous studies have indicated that overall mortality rate, ~17% in intact uninstrumented eggs, is not significantly different between the WL and c^a/c^a strains (14–16). We found a rate of mortality of ~23% overall in both strains. The slightly higher rate of embryo mortality in our study compared with previous studies may have resulted from invasive electrode implantation (see Ref. 21), although our preliminary data demonstrated no such deleterious effects.

Figure 3 shows the percent deviation from control f_H exhibited in embryos (both WL and c^a/c^a) that died before hatching. Percent deviations rather than absolute values were presented because of the change in absolute heart rate during development. These data show that there was no "predictive" bradycardia before death in the c^a/c^a embryos that died before hatching; that is, embryos had a f_H that was not significantly different from surviving embryos of the same stage even 1 day before death. In the WL embryos that died before hatching, f_H fell significantly by 7.6% (relative to surviving embryos of the same strain) 1 day before death.

Oxygen consumption in WL and c^a/c^a eggs. Relative to the WL strain, the c^a/c^a strain had significantly lower oxygen consumption (on a per egg basis) on *days 14–16* and *19* of incubation (Fig. 4). However, the differences were not large and, as indicated in the DISCUSSION, could be explained by differences in embryo mass. The mutant strain produces smaller embryos at *day 20* (27.98 ± 2.73 g in WL vs. 25.87 ± 3.23 g in c^a/c^a).

Water loss rates in normal and mutant strains. Rates of water loss (expressed as percent weight loss) in EB5 and WL eggs were not significantly different from each other, with a mean of 0.031%/h. The rate of water loss in c^a/c^a eggs was significantly higher than in WL and EB5 eggs (0.033%/h; Fig. 5).

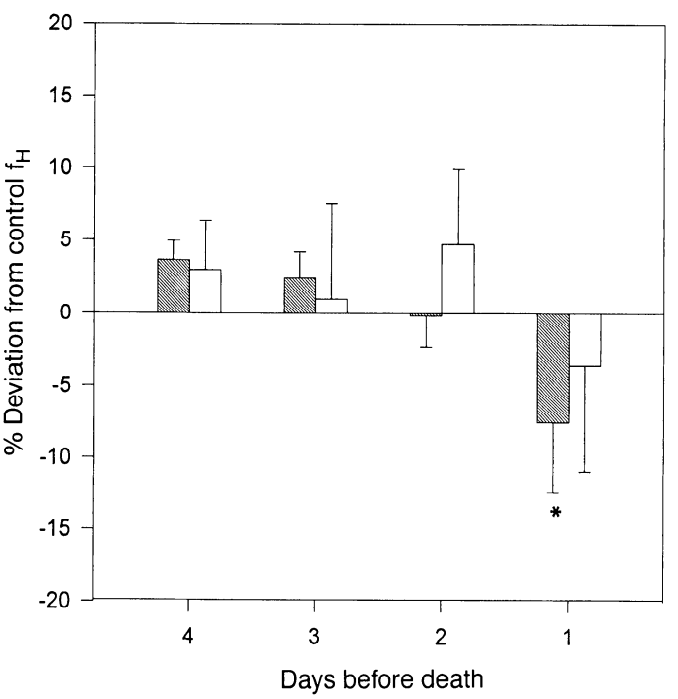

Fig. 3. Heart rate changes in embryos that died during incubation period. Percent deviation in heart rate from that of surviving embryos of same strain shown by embryos dying during incubation period. All data, which are means ± SE, are normalized with respect to day of death; n = 18 for WL (hatched bars) and n = 5 for c^a/c^a (open bars). *Significant difference (Student's t-test) between mean percent deviation in heart rate of dying and surviving embryos for each strain on each of 4 days before death.

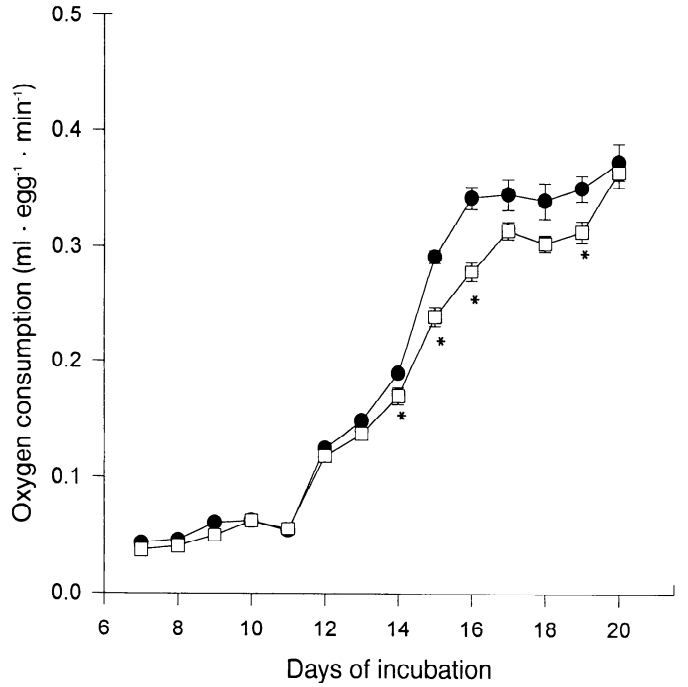

Fig. 4. Oxygen consumption in embryos of WL (●) and c^a/c^a (□) strains. Means ± SE are plotted. Number of embryos observed and mean egg weights (±SE) are as follows: WL, n = 14, 46.4 ± 0.7 g; c^a/c^a, n = 14, 45.9 ± 0.8 g. *Significant difference (Student's t-test) between strains.

DISCUSSION

Embryos from the mutant strain displayed an obviously different pattern of f_H development, which was highly consistent within the breed. This indicates a heritable component to heart rate control. However, to our knowledge, there have been no studies in avian embryos that specifically address mutations that affect heart rate during development. Whether the mechanism causing bradycardia in c^a/c^a and other related mutant strains acts directly on the heart or on a more peripheral component of cardiovascular regulation is unknown at this time. The fact that the deviation is seen at a very predictable time during development may allow us to define which aspect of the cardiovascular system is being affected by the mutation. Functional vagal innervation is first evident on *day 12* (*day 10* with physostigmine inhibition of cholinesterase) (13). This suggests that the onset of parasympathetic control is not responsible for this divergence of heart rates between WL and c^a/c^a embryos beginning at approximately *day 16*. However, abnormally high vagal tone in the maturing parasympathetic nervous system could be responsible for these differences. Abnormal levels of parasympathetic and sympathetic neural activity, cardioactive hormones, or their interactions could explain the differences in heart rate that develop between the two strains. Certainly, tyrosine metabolism is involved in the synthesis of catecholamines, and abnormal catecholamine levels could directly or indirectly account for our observations. However, the underlying mechanism remains unresolved and worthy of future investigation on the timing of receptor and transmitter appearance and the onset of their role in controlling the heart in normal and mutant strains.

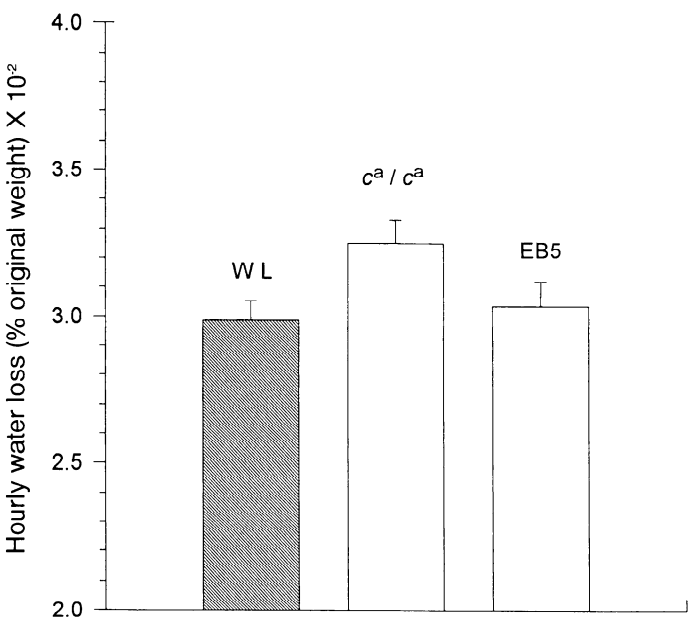

Fig. 5. Mean hourly water loss (as percent of initial egg mass) during entire incubation period in WL, EB5, and c^a/c^a strains. Number of embryos observed and mean embryo weights (±SE) are as follows: WL, n = 18, 63.68 ± 0.95 g; EB5, n = 20, 49.55 ± 1.66 g; c^a/c^a, n = 28, 57.41 ± 0.51 g.

Having determined that there was a difference in f_H between a normal and a mutant strain, we investigated the possibility that the alteration in f_H was indeed a feature of the *C* locus mutation, and not an unrelated feature of one strain. Testing three additional strains (mutants at the same locus) and one additional normal strain indicated that the relative bradycardia of the mutants was ubiquitous, appearing in all mutant strains. Indeed, the c^a/c^a strain evinced the least pronounced bradycardia (on *day 18*) of all mutant strains tested. Thus the developmental bradycardia seems to be associated with a mutation at the *C* locus.

Although the data presented in Fig. 3 suggest it may be possible to predict mortality in chick embryos by increased bradycardia, the variation in day-to-day heart rate is such that the predictive ability of f_H changes was limited in both strains. Perhaps an analysis that compares f_H variability at different times in development may provide a better predictor of embryonic mortality than mean f_H alone. Finally, physiological variables that relate to other aspects of cardiovascular function might have more predictive value, and this needs to be studied experimentally.

If the bradycardia in the mutant strains were due to reduced cardiovascular efficacy, then metabolic limitations might occur. The oxygen consumption data presented in Fig. 4 do not support this contention. Although there was slightly (and significantly) lower oxygen consumption on *days 14–16* and *19* during incubation (Fig. 4), the c^a/c^a strain lays smaller eggs and produces smaller embryos (~8–10% smaller immediately before internal piping). This is similar to the biggest difference in oxygen consumption between the two strains at any

stage, which was only ~14%. A future study assessing whether there are intrinsic differences in metabolic rate associated with these mutations would be of considerable interest.

We evaluated water loss in several strains of eggs as an index of gas exchange. Our values for hourly water loss (~0.03% of total egg weight per hour) are virtually identical to established values of 15% total egg weight over the course of the 21-day development period (1, 17). Because water vapor and oxygen both move by diffusion through eggshell pores and move at a rate that is proportional to differences in their respective partial pressures, water loss can be used to evaluate relative oxygen conductance. Although oxygen consumption in the c^a/c^a strain was equal to the WL strain, the water loss was significantly higher. Therefore, given the higher conductivity in the c^a/c^a eggs, how could the lower oxygen consumption be explained? We speculate that a lower partial pressure gradient across the eggshell exists in the mutant strains. Because the oxygen partial pressure outside the eggs was kept constant, the oxygen partial pressure inside the eggs must have been higher in the c^a/c^a eggs. If this is true, then either *1*) a substantial diffusion barrier exists between the airspace and the blood, or *2*) the Po_2 of arterial blood perfusing the shell should be higher in the c^a/c^a eggs. There are several possible causes for higher blood Po_2 in arterial chorioallantoic blood. There may be *1*) lower tissue oxygen extraction in the mutant eggs, *2*) different shunt fractions of venous and arterialized blood, *3*) different oxygen capacity in the mutant blood, or *4*) higher blood flow in the mutants. These possibilities all remain to be explored.

Finally, tyrosinase mutations occur in other animals. However, we are unaware of studies that have looked at cardiovascular performance and whole animal oxygen consumption in animals with defects in tyrosine metabolism.

Conclusions. These results suggest there are specific alterations in cardiovascular development that are influenced by tyrosine metabolism, the process directly influenced by the mutation at the *C* locus. Although significant, mutation-induced bradycardia was rarely of sufficient magnitude to allow accurate prediction of embryonic death in the c^a/c^a strain. Strains that displayed more pronounced bradycardia may be more predictable. The specific factors that influence heart rate development are not well understood, and the specific parameters that are altered in these mutants remain to be determined. These include blood flow, blood pressure, and minute-to-minute variability in f_H.

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REFERENCES

1. **Ar, A., and H. Rahn.** Water in the avian egg: overall budget of incubation. *Am. Zool.* 20: 373–384, 1980.
2. **Burggren, W., H. Tazawa, and D. Thompson.** Intraspecific variability in avian embryonic heart rate: potential genetic and maternal environment influences. *Isr. J. Zool.* In press.
3. **Burggren, W., and S. Warburton.** Patterns of form and function in developing hearts: contributions from nonmammalian vertebrates. *Cardioscience* 5: 183–191, 1994.
4. **Burggren, W. W.** Central cardiovascular function in amphibians: qualitative influences of phylogeny, ontogeny and seasonality. In: *Mechanisms of Systemic Regulation. Respiration and Circulation*, edited by N. Heisler. Berlin: Springer-Verlag, 1995, vol. 1.
5. **Clark, E. B.** Functional characteristics of the embryonic circulation. In: *The Development of the Vascular System*, edited by F. Feinberg, G. K. Sherer, and R. Auerbach. Basel: Karger, 1991, p. 125–135.
6. **Haque, M. A., W. Watanabe, H. Ono, Y. Sakamoto, and H. Tazawa.** Comparisons between invasive and noninvasive determinations of embryonic heart rate in chickens. *Comp. Biochem. Physiol.* 108A: 221–227, 1994.
7. **Howe, R. S., K. J. Isaacson, J. L. Albert, and C. B. Coutifaris.** Embryonic heart rate in human pregnancy. *J. Ultrasound Med.* 10: 367–371, 1991.
8. **Kirby, M. L.** Cardiac morphogenesis—recent research advances. *Pediatr. Res.* 21: 219–224, 1987.
9. **Kirby, M. L.** Role of extracardiac factors in heart development. *Experientia Basel* 44: 944–951, 1988.
10. **Laboda, L. A., J. A. Estroff, and B. R. Benacerraf.** First trimester bradycardia. A sign of impending fetal loss. *J. Ultrasound Med.* 8: 561–563, 1989.
11. **Manasek, F. J.** The extracellular matrix: a dynamic component of the developing embryo. *Curr. Top. Dev. Biol.* 10: 35–102, 1975.
12. **Ockleford, E. M., and M. A. Vince.** Heart rate response to light in the embryo of the Japanese quail (*Coturnix coturnix*). *J. Exp. Zool.* 201: 439–444, 1977.
13. **Papano, A. J.** Ontogenetic development of autonomic neuroeffector transmission and transmitter reactivity in embryonic and fetal hearts. *Pharmacol. Rev.* 27: 3–33, 1977.
14. **Pardue, S. L., N. M. Ring, and J. R. Smyth, Jr.** Pleiotropism associated with alleles of the *C* locus in the domestic fowl. *Poultry Sci.* 64: 1821–1828, 1985.
15. **Pardue, S. L., N. M. Ring, and J. R. Smyth, Jr.** Autosomal albinism affects immunocompetence in the chicken. *Dev. Comp. Immunol.* 14: 105–112, 1990.
16. **Pardue, S. L., and J. R. Smyth, Jr.** Influence of *C* locus alleles on neonatal development in the domestic fowl. *Poultry Sci.* 65: 426–432, 1986.
17. **Rahn, H.** Why birds lay eggs. In: *Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles*, edited by D. C. Deeming and M. W. J. Ferguson. Cambridge, UK: Cambridge Univ. Press, 1991, Chapt. 21, p. 345–360.
18. **Robinson, H. P., and J. Shaw-Dunn.** Fetal heart rates as determined by sonar in early pregnancy. *J. Obstet. Gynaecol. Br. Commonw.* 80: 805–809, 1973.
19. **Tazawa, H.** Adverse effect of failure to turn avian egg on the embryo oxygen exchange. *Respir. Physiol.* 41: 137–142, 1980.
20. **Tazawa, H., H. Wakayama, J. S. Turner, and C. V. Paganelli.** Metabolic compensation for gradual cooling in developing chick embryos. *Comp. Biochem. Physiol.* 89: 125–129, 1988.
21. **Tazawa, H., W. Watanabe, and W. Burggren.** Embryonic heart rate in altricial birds, the pigeon (*Columba domestica*) and the bank swallow (*Riparia riparia*). *Physiol. Zool.* 67: 1448–1460, 1994.
22. **Van Mierop, L. H. S., and L. M. Kutsche.** Some aspects of comparative anatomy of the heart. In: *Cardiovascular Shunts: Ontogenetic, Phylogenetic, and Clinical Aspects*, edited by K. Johansen and W. Burggren. Copenhagen: Munksgaard, 1985, p. 38–56.
23. **Vince, M. A., J. V. Clarke, and M. R. Reader.** Heart rate response to egg rotation in the domestic fowl embryo. *Br. Poult. Sci.* 20: 247–254, 1979.