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Are chicken embryos endotherms or ectotherms? A laboratory exercise integrating concepts in thermoregulation and metabolism

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Hiebert SM, Noveral J. Are chicken embryos endotherms or ectotherms? A laboratory exercise integrating concepts in thermoregulation and metabolism. *Adv Physiol Educ* 31: 97–109, 2007; doi: 10.1152/advan.00035.2006.—This investigative laboratory exercise uses the different relations between ambient temperature and metabolic rate in endotherms and ectotherms as a core concept to answer the following question: What thermoregulatory mode is employed by chicken embryos? Emphasis is placed on the physiological concepts that can be taught with this exercise, including methods for measuring rates of oxygen consumption, the relation between oxygen consumption and metabolic rate, the influence of temperature on metabolic rate, and the differences between endotherms and ectotherms both in the overall magnitude of metabolic rate and in the shape of the relation between metabolic rate and ambient temperature. Included in this article are respirometer designs suitable for teachers working with a wide variety of budgets and available equipment, specific laboratory protocols for collecting data, sample data, thought questions with sample answers, and suggestions for classroom implementation as a 1-, 2- or 3-wk laboratory exercise that can be taught at a variety of undergraduate levels.

endothermy; ectothermy; respirometry

MOST ADULT BIRDS AND MAMMALS are endotherms, but what thermoregulatory mode is employed by their embryos? The very different responses of metabolic rate to changes in ambient temperature (T_a) by endotherms and ectotherms (29) (Fig. 1) can serve as the basis for a simple but thought-provoking experiment in which students can determine which of these modes is used by chicken embryos. One of the primary purposes of this laboratory exercise is to help students integrate what they know about metabolism and temperature, two physiological variables inextricably linked in the lives of all animals, in a learner-centered, inquiry-based activity. The traditional laboratory exercise on this topic calls for students to measure the rates of respiration in a similarly sized endotherm (e.g., the mouse) and ectotherm (e.g., the frog) at several temperatures. The results of this experiment, however, are known by most students if they have taken an introductory biology course or read their introductory textbook. The exercise described here requires students to understand the shapes of the temperature-metabolism curves for both endotherms and ectotherms at the outset and then to use this information as the basis for an experiment that they will design to answer a question to which most students do not intuitively know the answer. This inquiry-based approach is aligned with the recommendations of a growing literature demonstrating that enhanced learning takes place in the student-centered classroom (12). Although methods are described in the specific context of the chick embryo metabolism experiment, they can be adapted for use with a variety of animals available in the classroom laboratory.

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Endotherms Versus Ectotherms: Underlying Concepts

In ectotherms, metabolic rate varies directly with T_a, as summarized by the Arrhenius relation. This is sometimes referred to as the "Q_{10} effect," where Q_{10} is the multiplier by which the rate of a physiological process increases when the temperature increases by 10°C (2). In a typical biological system, a 10°C increase in temperature results in a two- to threefold increase in the rate of many physiological functions; in other words, Q_{10} is typically 2–3 (2).[1] In endotherms, metabolic rate remains relatively constant within the thermal neutral zone (TNZ) but at T_a below the TNZ rises in response to decreases in T_a so that body temperature (T_b) can be maintained at a relatively constant set point (Fig. 1). At T_a above the TNZ, metabolic rate may increase *1*) as a result of the metabolic cost of efforts to cool the body to the normal T_b set point and *2*) if heating proceeds unchecked, because tissue temperature rises passively. Measurements of metabolic rates at different temperatures can therefore be used to determine the thermoregulatory mode of an animal: as T_a increases, metabolic rate rises in ectotherms but decreases in endotherms as long as either or both T_as lie below the TNZ (Fig. 1). Thus, by comparing the metabolic rates at two appropriately chosen T_as, students can distinguish experimentally between endotherms and ectotherms. A second important difference between endotherms and ectotherms is that ectotherms have much lower metabolic rates (on the order of ~1/5 to 1/10 of the basal metabolic rate of endotherms) even when they are maintaining comparable T_bs (2). Thus, the endotherm lifestyle, while affording a relatively constant T_b at which physiological systems can function at their best, is an energetically costly one. Ectotherms are more limited in the habitats, seasons, and times of day at which they can be active because they rely on heat provided by the environment, but they gain the advantage of requiring much less energy overall.

[1] Pointing out that Q_{10} itself is temperature dependent (i.e., its value depends on the particular temperature range being examined), Gillooly et al. (16) have recently suggested that temperature effects can better be expressed in terms of a "universal temperature dependence factor" (UTD), which allows more direct comparisons between organisms independent of temperature. While potentially useful as a unifying theory, the applicability of the UTD to physiology-scale problems within species remains controversial (8, 9, 15). Q_{10} remains a useful if somewhat imprecise tool that is more intuitive, is easily calculated, and appears widely throughout the literature and in currently available textbooks.

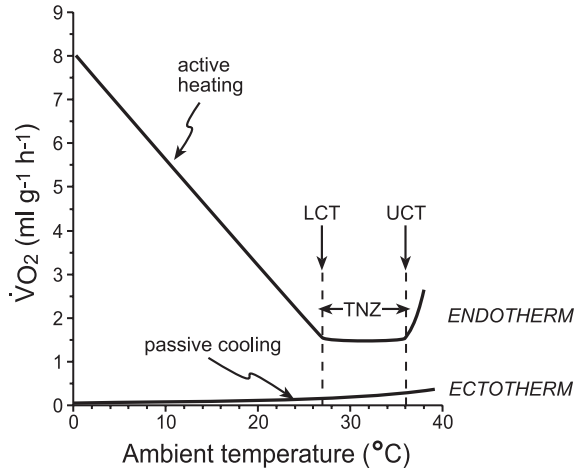

Fig. 1. Comparison of metabolic response to ambient temperature (T_a) by endotherms (*top* curve) and ectotherms (*bottom* curve). The metabolic rate of ectotherms varies directly with T_a and is the result primarily of Arrhenius ("Q_{10}") effects. The metabolic rate of endotherms remains constant within the thermal neutral zone (TNZ), increases with decreasing temperatures below the lower critical temperature (LCT) because of the energy cost of heat production, and increases with increasing temperatures above the upper critical temperature (UCT), first as a result of active cooling and then when tissue temperatures begin to rise as a result of Q_{10} effects. The two curves, based on data from Refs. 3 and 24, show approximate rates of oxygen consumption ($\dot{V}O_2$) for a 15- to 20-g bird or mammal and a 15- to 20-g lizard.

General Principles That Can Be Taught With This Exercise

In the fact-laden realm of biology, it is important for students to understand that specific examples, such as the response of chick embryo metabolism to T_a, are less important in their own right than as illustrations of larger, recurring themes that can help them understand the lives of all organisms. Students may find it challenging but ultimately very useful to generate a list of general principles related to the chicken embryo metabolism experiment. The following are some important concepts that can be taught in this laboratory exercise; the ones you include will depend on the content of your course, the background knowledge of your students, and the time your course devotes to this exercise.

1. The rate of a biochemical reaction is directly related to the temperature of the reactants. Thus, in ectotherms, the decrease in metabolic rate in response to a decrease in T_a is a direct response to passive tissue cooling. In endotherms, on the other hand, the metabolic rate is actively regulated to keep the body at a constant temperature; thus, as T_a decreases, the metabolic rate increases to maintain a constant tissue temperature.

2. Even when an endotherm and ectotherm have the same T_b, the metabolic rate of an endotherm is 2–10 times higher than that of an ectotherm (2). Thus, endotherms incur a much higher energetic cost of living.

3. Metabolic rate, the rate of heat production by an organism and therefore a measure of the overall energy turnover by that organism, is difficult to measure and therefore is rarely measured directly.

4. The rate of oxygen consumption ($\dot{V}O_2$) is an indirect measure of metabolic rate.

5. Converting $\dot{V}O_2$ to metabolic rate requires either knowing which metabolic fuel (carbohydrate, protein, or fat) is being metabolized, making an educated guess, or using an intermediate conversion constant likely to yield the lowest maximum error.

6. Volumes, flow rates, and consumption rates of gasses are expressed at standard temperature (273 K) and standard pressure (760 mmHg) for dry air (STPD) so that they can be compared with one another or converted to their stoichiometric equivalents.

7. As endothermic animals increase in size, the thermal neutral zone (TNZ) broadens, chiefly by a decrease in the lower critical temperature (LCT) (5, 7). (See *Choosing test temperatures* below for the context in which this principle might be discussed.)

8. Plants and animals face some of the same design constraints. For example, eggshell pores and plant stomata perform similar functions (regulating the flow of oxygen, carbon dioxide, and water vapor) and impose similar constraints in desiccating environments: in both systems, increased metabolic rates can result in excess dehydration. (See *Background information on avian incubation and embryo development* below.)

Experiment Design

Experimental designs, suggestions for classroom implementation of a student-centered experiment design process, and specific content pertaining to the chick embryo metabolism experiment are described in detail in the companion article "Teaching simple experimental design to undergraduates: do your students understand the basics?" (19). Briefly, either of the two following designs is recommended. In the first design, the $\dot{V}O_2$ of all embryos is measured at the normal incubation temperature (38°C), and the results are used to create two treatment groups balanced for $\dot{V}O_2$. The experimental group is then placed at 23°C (room temperature), whereas the control group remains at 38°C. After 90 min of temperature equilibration, the $\dot{V}O_2$ of all embryos is measured again, and the final $\dot{V}O_2$ of the two treatment groups is compared with an unpaired *t*-test. In the second design, each embryo is subjected to a measurement of $\dot{V}O_2$ following 90 min of equilibration at each temperature, but half of the embryos are measured first at 38°C, whereas the other half are measured first at 23°C. A paired *t*-test is then used to compare all $\dot{V}O_2$ at 38°C with all $\dot{V}O_2$ at 23°C. For both experimental designs, a total of 12 eggs (~16 days old) is sufficient for clear, easily interpretable results using any of the methods described in this article.

Instructions for Students: Measurements and Calculations

This section contains instructions for the collection and analysis of air samples, calculations, statistical analysis, and interpretation of results. These methods are to be used in conjunction with an experimental design (see *Experimental Design* above) that has been designed by students or chosen by the instructor. At a minimum, this design requires one incubator that is large enough to *1*) maintain the eggs when they are not being used in the experiment and *2*) house respirometers when $\dot{V}O_2$ is being measured at 38°C. The second temperature treatment (23°C) can be achieved by either placing the eggs at room temperature for 90 min or placing them in an incubator at 23°C. After a suitable incubation period in the respirometer, air samples are drawn into a syringe and injected into an oxygen analyzer. Instructions are for respirometers consisting of 500-ml jars, the lids of which have been fitted with a

two-way stopcock (Fig. 2). Several different respirometer designs and low-cost alternatives for measuring $\dot{V}O_2$ without specialized equipment are described in *Equipment: Descriptions and Alternatives*. Students should practice critical aspects of the protocol, such as obtaining air samples and injecting them into the oxygen analyzer, before they begin an experiment with live embryos.

I. Collecting an air sample from an egg in a respirometer.

A. Equilibrate open respirometer at the desired temperature.

B. Carefully place the egg in the respirometer, seal the chamber, and place it in a water bath, incubator, or other location at the test temperature. Record the time at which the chamber was sealed and the following variables for the air *in the room where the respirometer was filled and sealed:* barometric pressure (P_b), temperature, and relative humidity (RH). Note that when weather fronts are moving through an area, P_b may change significantly during the laboratory period, and, thus, an additional reading may be required when $\dot{V}O_2$ is measured the second time.

C. After ~15 min, follow the instructions below to draw a 20-ml air sample from the respirometer into a syringe. Be sure to record the time at which you withdrew the air sample; you will need it for your calculation of $\dot{V}O_2$.

1. Remove the syringe cap and attach the sample syringe to the two-way stopcock.

2. Open the two-way stopcock so that air can flow between the syringe and the respirometer.

3. Withdraw and push in the syringe's plunger three times to mix air in the respirometer.

4. Draw a 20-ml air sample into the syringe and hold on to the plunger so that it is not sucked downward by the reduced air pressure in the respirometer. (Note that because the oxygen analyzer will measure the concentration of oxygen in the air sample, the exact volume of the sample does not matter. It should, however, be large enough to obtain an accurate reading with the oxygen analyzer you are using.)

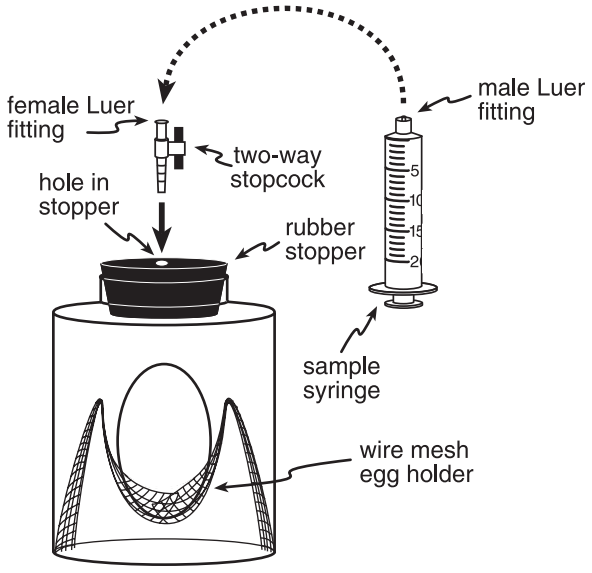

Fig. 2. Jar-type respirometer with a two-way stopcock to permit the removal of air samples. The porous egg holder is made of a bent strip of wire mesh, notched and bent upward on each side to form a cradle for the egg.

5. Close off the two-way stopcock before disconnecting the syringe to prevent your air sample from mixing with room air. Now, push on the plunger until you meet resistance and let go of the plunger to allow it to be pushed outward by the air pressure inside the syringe. At this point, the air pressure inside the syringe is equal to or greater than that of the outside air; it is now safe to disconnect the syringe and quickly cap it.

6. Remove the egg from the respirometer and put it at the temperature needed for your next measurement. If this is your last measurement, return the egg to the 38°C incubator.

II. Measuring the oxygen content of the air sample.

A. Before you inject your air sample into the oxygen analyzer, confirm that the percent oxygen reads 20.96. If not, ask your instructor to adjust the baseline reading.

B. Inject the air sample as quickly as you can without causing the reading on the oxygen analyzer to change (some oxygen analyzers are extremely sensitive to changes in air pressure).

C. Watch the percent oxygen display. As the air sample enters the analyzer, the percent oxygen will begin to decrease. As the trailing edge of the air sample leaves the oxygen analyzer, the percent oxygen will return to 20.96. The lowest value reached during the air sample's transit through the oxygen analyzer is the concentration of oxygen in your sample (F_E); record this value.

III. Calculating the volume of air in the respirometer.

A. *Method I.*

1. With the egg and egg holding rack (Fig. 2) in the respirometer, fill the respirometer with water (a brief submersion of the egg in water should not harm a healthy embryo).

2. Pour the water from the chamber into a graduated cylinder, taking care not to damage the egg. Record the volume.

3. Remove the egg and egg rack from the respirometer. Dry the egg with a paper towel and return it to the incubator.

B. *Method 2.*

1. With the egg holding rack (but not the egg) in the respirometer, fill the chamber with water.

2. Pour the water from the chamber into a graduated cylinder and record the volume.

3. Measure the diameter and length of the egg with calipers (measurements must be in cm). Use the equation below to calculate the volume of the egg (22). Note that 1 cc = 1 cm^3 = 1 ml.

$$\text{Egg volume (in cc)} = \{[\text{length (in cm)} \times [\text{diameter (in cm)}]^2\} \times 0.497 \quad (1)$$

4. To calculate the volume of air in the respirometer, subtract the egg volume obtained in *step 3* from the water volume obtained in *step 2*.

IV. Calculating $\dot{V}O_2$.

Use the following equation to convert percent oxygen in the air sample you measured to $\dot{V}O_2$ at standard temperature (273 K) and pressure (760 mmHg) in dry conditions (STPD) (20). Be sure to enter each of the values in the correct number format as shown below:

$$\dot{V}O_2 = V\left(\frac{273}{T_a}\right)\left[\frac{P_b - (WVP)(RH)}{760}\right]\left(\frac{F_I - F_E}{1 - F_E}\right)\left(\frac{60}{t}\right) \quad (2)$$

where $\dot{V}O_2$ is measured in ml/h; V is the volume of air in the

respirometer (in ml); T_a is the temperature of air in the respirometer at the beginning of the trial (in K; K = °C + 273); P_b is measured in mmHg; WVP is the water vapor pressure in fully saturated air at T_a [in mmHg; e.g., 19.83; values can be found in a table entitled the Vapor Pressure of Water in the *CRC Handbook* (23a)]; RH is the relative humidity of air in the respirometer at the beginning of the trial (e.g., 72% would be entered as 0.72); F_I is the initial fractional concentration of oxygen [the fractional concentration of oxygen in the room air (e.g., 0.2096)]; F_E is the ending fractional concentration of oxygen (e.g., 0.2074); and *t* is the amount of time the egg was closed in the respirometer (in min; e.g., 15.5).

V. Calculating mass-specific $\dot{V}O_2$.

In many cases, it is useful to standardize $\dot{V}O_2$ between individuals of different masses by expressing $\dot{V}O_2$ per gram of tissue. In the case of the chicken embryo, however, using the mass of the whole egg to compute mass-specific $\dot{V}O_2$ is probably not very meaningful because not all of the egg mass consists of embryonic tissue. This is especially true at the earlier stages of development, when much of the egg's mass is made up of the shell, albumen, and unmetabolized yolk.

A. Consult Table 1 to find the approximate mass of the embryo in your egg. To compute the mass-specific $\dot{V}O_2$ of your embryos, divide the $\dot{V}O_2$ you calculated in *step IV* by this embryo mass.

B. Consult Fig. 1. Does the mass-specific $\dot{V}O_2$ you calculated fall within the range expected for an endotherm or for an ectotherm?

VI. Calculating metabolic rate from $\dot{V}O_2$.

$\dot{V}O_2$ (expressed as the volume of oxygen consumed per unit time) is not a direct measure of metabolic rate (expressed as energy turnover per unit time), because the energy equivalent for a given $\dot{V}O_2$ depends on which fuel (carbohydrate, protein, or fat) is being metabolized. In the case of chicken embryos, the primary energy source is the fatty yolk. When fats are broken down for energy, ~19.8 kJ is liberated for every liter of oxygen consumed (21). Thus, for chicken embryos,

Table 1. *Mass of the developing chicken embryo*

Day After Fertilization	Mass, g
1	0.0002
2	0.0003
3	0.02
4	0.05
5	0.13
6	0.29
7	0.57
8	1.15
9	1.53
10	2.26
11	3.68
12	5.07
13	7.37
14	9.74
15	12.00
16	15.98
17	18.59
18	21.83
19	25.62
20	30.21
21	Hatched

Data are taken from the results of Ref. 31.

$$\text{Metabolic rate (in kJ/h)} = \dot{V}O_2(\text{in ml } O_2/\text{h}) \times 19.8(\text{in kJ/l } O_2) \times \frac{1 \text{ liter}}{1{,}000 \text{ ml}} \quad (3)$$

The conversion factor for carbohydrates is ~21.1 kJ/l oxygen and for proteins is ~18.7 kJ/l O_2 (21). When the metabolic fuel is unknown, a conversion factor of intermediate value, 20.2 kJ/l O_2, is used. Note that calculating the metabolic rate from $\dot{V}O_2$ is not needed to answer the question posed in this exercise, because the metabolic rate and $\dot{V}O_2$ are always related by the same constant (19.8 kJ/l O_2). Many published studies have reported $\dot{V}O_2$ instead of metabolic rate unless the authors wanted to quantify energy use per se.

A. Calculate the metabolic rate for the whole embryo using *Eq. 3*.

B. Divide the whole embryo metabolic rate by the embryo's mass to calculate the mass-specific metabolic rate.

C. Convert the whole embryo metabolic rate to watts (W), a unit of energy consumption with which you are probably already familiar from household appliances. 1 W = 1 J/s.

VII. Comparing $\dot{V}O_2$ at the two experimental temperatures.

For the first experimental design, use an unpaired (two-sample) *t*-test to compare the $\dot{V}O_2$ of the embryos at 23°C with the $\dot{V}O_2$ of the embryos at 38°C. For the second experimental design, use a paired *t*-test to compare $\dot{V}O_2$ at 23 and 38°C for all embryos. Is there a significant difference between the $\dot{V}O_2$s measured at the two temperatures? If so, do your embryos show a positive or negative relation between T_a and $\dot{V}O_2$? On the basis of this finding, do you conclude that your embryos are endotherms or ectotherms? If your conclusions in *steps V* and *VII* are different, which do you think provides the most conclusive answer to the question of whether chicken embryos are endotherms or ectotherms? Why?

VIII. Calculating Q_{10}.

Q_{10} is a standardized way of describing how reaction rates change with temperature. It can be calculated not only for simple chemical reactions but also for more complex physiological processes such as metabolic rate or running speed. Q_{10} is the number of times the reaction rate increases when the temperature is increased by 10°C. For example, if the reaction rate doubles when the temperature is increased from 20 to 30°C, then $Q_{10} = 2$ for this temperature range. However, Q_{10} can be calculated even when reaction rates are measured at temperatures that are not exactly 10°C apart using the following formula:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (4)$$

where R_1 is the reaction rate at the first temperature (T_1) and R_2 is the reaction rate at the second temperature (T_2). If you have determined that your embryo is an ectotherm, calculate Q_{10} from the $\dot{V}O_2$s that you measured for the whole embryo. Typically, Q_{10} for biological reactions ranges from 2 to 3. Does the Q_{10} you calculated fall within this range?

IX. Presenting your results.

Primates, including the readers of your report, are especially skilled at interpreting effective visual images. Present a summary of the data you have collected in a single graph that shows all of the important findings you have made in this

experiment. Remember to show all means with some measure of variability (SD, SE, or 95% confidence interval) so that your reader can determine whether any differences between means are meaningful or simply a result of random differences among individuals.

Sample Data

For both experimental designs, measurements of healthy embryos should yield a significantly higher $\dot{V}O_2$ at 38°C than at 23°C, leading to the conclusion that embryos behave as ectotherms (Fig. 3). In over a decade of using this laboratory exercise, our students have always found significant differences in the expected direction for ectotherms with a total sample size of 12 eggs regardless of which experimental design they chose to use.

Thought Questions

These questions address both the laboratory method and conclusions that can be drawn from the data that the students have collected. They are designed to address students' most frequent misunderstandings about this exercise, stimulate class discussion, and prepare students to consider the thermoregulation of avian embryos in a broader physiological, ecological, and evolutionary context.

1. Sometimes, the rate of energy expenditure is expressed as $\dot{V}O_2$, whereas, at other times, the rate of energy expenditure is expressed as metabolic rate.

A. In which units are each of these quantities expressed? *Answer: $\dot{V}O_2$ is expressed in units of volume of oxygen consumed per unit time (e.g., ml O_2/h), whereas metabolic rate is expressed as energy turnover per unit time (e.g., J/h).*

B. What additional piece of information is required to convert $\dot{V}O_2$ to metabolic rate? *Answer: the fuel (carbohydrate, protein, or fat) being metabolized is required, because the relation between $\dot{V}O_2$ and metabolic rate differs for each of these metabolic substrates.*

C. Give an example of an experiment in which expressing energy expenditure as $\dot{V}O_2$ is sufficient to answer the question. Give an example of an experiment in which converting $\dot{V}O_2$ to metabolic rate is necessary to answer the question. *Answer: expressing rates of energy expenditure as $\dot{V}O_2$ is generally suitable for any experiment in which one wants to compare the energy cost of activities (e.g., an animal walking versus running, a hibernating versus normothermic animal, or an animal thermoregulating at different temperatures). This is especially true if the metabolic fuel is 1) the same for each activity or 2) not known for any of the activities, since one would have to use the same conversion constant for each calculation. In these cases, a conversion to metabolic rate would not change the result qualitatively. Expressing rates of energy expenditure as metabolic rate is necessary when one wants to construct an energy budget (e.g., how many kJ does this animal burn per day or year?) or when comparing energy intake and expenditure (e.g., how many acorns are needed to fuel a squirrel's daily activity? or How many additional acorns are needed to fuel a dominant male's territorial and aggressive activities compared with the activities of a subordinate male?). In the latter examples, it would also be necessary to determine the amount of energy available per acorn by bomb calorimetry, a procedure in which one measures the amount of heat released when a known mass of a substance is burned.*

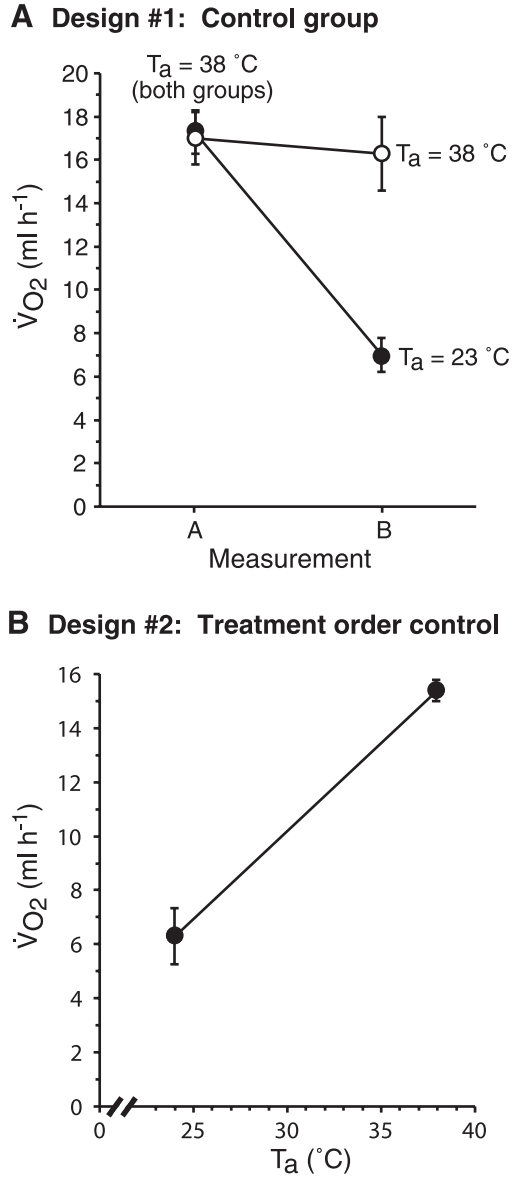

Fig. 3. Sample data from two experimental designs. $\dot{V}O_2$ values in 16-day-old chicken embryos (*Gallus domesticus*) at two T_a values are shown. *A*: *design 1*. After an initial measurement (*measurement A*) at 38°C, experimental eggs (●; $n = 6$) were placed at 23°C for 90 min, whereas control eggs (○; $n = 6$) remained at 38°C for 90 min, before a second measurement (*measurement B*) was taken. $\dot{V}O_2$ was significantly reduced by exposure to $T_a = 23$°C (degrees of freedom = 10, unpaired *t*-test value = 4.974, $P = 0.0006$), suggesting that the embryos are ectothermic. *B*: *design 2*. $\dot{V}O_2$ of all embryos ($n = 12$) was measured after 90-min equilibration at both 38 and 23°C, with 6 embryos experiencing the temperature treatments in each order. $\dot{V}O_2$ was significantly lower at 23°C than at 38°C (degrees of freedom = 11, paired *t*-test value = 9.461, $P < 0.0001$), also suggesting that the embryos are ectothermic. Values are means ± SE.

2. The quantity Q_{10} is a standardized way of describing changes in rates of reaction as the temperature of the reactants changes. In the laboratory exercise, you were asked to calculate Q_{10} if your data supported the conclusion that your embryo was an ectotherm. Why would it not be appropriate to calculate Q_{10} if you had found that your organism was an endotherm? *Answer: endotherms maintain a relatively constant T_b. Thus, as T_a decreases below the TNZ, the temperature of the reactants (T_b) does not change, but the metabolic rate (and thus*

$\dot{V}O_2$) increases because the body must generate more heat to maintain T_b in a cold environment. In this case, a calculation of Q_{10} would tell us not about the direct effect of tissue temperature on metabolic rate but about the active thermoregulatory responses of the animal to cold.

3. Which of the following conclusions can correctly be drawn from the finding that an embryo behaves as an ectotherm? Explain what is different about each statement and what each statement implies about the physiology of the embryo.

A. The embryo is not generating its own heat. *Answer: the embryo might be physiologically able to generate heat, but it is not doing so, perhaps for some adaptive reason (see below).*

B. The embryo is unable to generate its own heat. *Answer: the embryo does not have the ability to shiver or run cellular metabolic processes at a rate that generates significant amounts of heat, or it lacks the neural regulatory machinery to stimulate these processes.*

C. The embryo is unable to regulate its own body temperature. *Answer: the embryo may be able to generate heat, but it is not able to maintain a constant T_b. Note that most ectotherms are able to thermoregulate even though they are unable to generate significant quantities of heat metabolically; however, they do so primarily behaviorally, by moving between warm and cool areas or by changing their orientation with respect to an environmental heat source. Because chicken embryos cannot move, however, the usual means of behavioral thermoregulation are not available. Interestingly, evidence suggests that some avian embryos may be able to thermoregulate by calling to their parents from inside the egg, thus inducing the parent to sit on the eggs and warm them (1).*

4. What might limit the ability of the embryo to generate its own heat? *Answer: insufficient fuel stored in the egg, muscles not developed enough to generate heat by shivering, or the oxygen flow through pores in the egg is too slow to support a high rate of metabolism.*

5. What might limit the ability of the embryo to regulate its T_b? *Answer: insufficient neural development (specifically in the preoptic area of the hypothalamus, the location of the vertebrate thermostat), a heart insufficiently developed to distribute warm blood, or a thyroid gland insufficiently developed to produce the high levels of thyroid hormone that are needed to stimulate increased metabolic activity (42).*

6. Why might an embryo not produce its own heat even if it had the physiological capability to do so? *Answer: rather than using precious stored energy for thermoregulation, embryos would benefit from using stored fuel for developing more quickly and to greater maturity before hatching. A parent can supply heat to an egg-bound embryo, but it cannot provide food that the embryo could use for generating its own heat.*

7. If it is true that the amount of stored fuel limits how far an embryo can develop before hatching or how much heat it can produce, then why doesn't the hen produce eggs with more fuel stored in them? What might be some of the selective pressures for and against adding more fuel to the egg? *Answer: if they contained more yolk, embryos could be more mature at hatching (13) and might be able to produce some of their own heat before hatching, provided the thermoregulatory structures in the brain and heat-producing organs were sufficiently developed. However, eggs with more stored fuel would be larger and would require a larger energy investment by the hen to produce them, with the result that a hen would be able to produce fewer of them or would require longer to produce each egg. Large eggs have a greater thermal mass, thus requiring more heat to return them to incubation temperature when they cool during a temporary absence of the incubating hen from the nest. They might also require a longer incubation period (26) and thus a greater time investment by the hen and would suffer increased exposure to nest predators (9). Longer incubation periods may also be selected against because they increase potential exposure of the egg to microbial pathogens that enter the egg across the shell (10). In addition, birds are limited in the size of egg they can carry while flying. Notably, flightless birds such as the kiwi are able to produce very large eggs relative to their body size (6). However, the success of such flightless birds depends on the absence of ground predators, a special condition that exists in only a few isolated habitats such as islands.*

8. Based on your results, what do you predict happens to an embryo's metabolic rate when the incubating parent leaves the nest unattended? If food is scarce and the parent needs to extend these absences, what would be the effect on embryo development time? *Answer: when eggs are left unattended, they cool and the metabolic rate consequently decreases. The rate of developmental processes also decreases, thus increasing development time. Embryos are less vulnerable to prolonged cooling (13), but excessive cooling in later stages may kill the embryo. It has also been suggested that embryos are more vulnerable to microbial pathogens when they cool (10).*

9. During most of development, a chicken embryo obtains oxygen by diffusion across the chorioallantoic membrane. During the last week of incubation, the embryo gradually switches to using its lungs as the primary gas exchange surface. What effect, if any, would the increased use of the lungs have on metabolic rate? On whether the embryo is endothermic or ectothermic? *Answer*: *students should read "How bird eggs breathe" (31) before answering this question. The increased rate of oxygen exchange made possible by using the lungs permits an increase in metabolic rate that might make it possible for the embryo to become partially endothermic. However, full endothermy is not expressed until after the bird hatches (42).*

10. If metabolic rate and expression of endothermy are limited by the amount of oxygen that can be obtained by diffusion across the eggshell, why aren't eggshell pores larger or more numerous? *Answer: increased pore numbers and/or size would weaken the eggshell, might make it more vulnerable to microbial pathogens (10), and would allow water vapor to escape at a higher rate and thus threaten the embryo with desiccation (31). See also answers to questions 6 and 7.*

Classroom Implementation: Practice and Philosophy

Possible laboratory schedules (based on one 3-h laboratory period/day). 3-DAY SCHEDULE. On *day 1*, students learn the principles behind different methods for measuring metabolic rate and learn how to measure $\dot{V}O_2$, perform calculations, and handle the eggs. On *day 2*, students plan their experiment and discuss the content of a strong-inference protocol. On *day 3*, students turn in their strong-inference protocol for grading, perform their experiment, and collect data. Note that if there is 1 laboratory session/wk, this schedule requires two sets of

experimental embryos: one set for *week 1* and another set for *week 3*.

2-DAY SCHEDULE. On *day 1*, students plan their experiment and discuss the content of a strong-inference protocol. On *day 2*, students turn in the strong-inference protocol for grading, perform their experiment, and collect data.

1-DAY SCHEDULE. Students perform their experiment and collect data.

Rationale. When this laboratory exercise is taught as part of our intermediate-level Animal Physiology course, we use the 3-wk schedule for the chick metabolism experiment because it fulfills a variety of pedagogical and content functions. In relation to content, the exercise bridges the topics of metabolism and temperature relations. The exercise also prepares students for the independent project that they will conduct later in the semester, by guiding them through the process that begins with a question, follows with the design of an experiment to address the question and the formulation of a strong-inference protocol, and concludes with the collection of data, statistical analysis, and preparation of a report in the style of a scientific paper. The student-centered experiment design process and the use of strong-inference protocols are described in detail in the companion articles "Teaching simple experimental design to undergraduates: do your students understand the basics?" (19) and "The strong-inference protocol: not just for grant proposals" (18).

The need for practical instruction in the scientific method has been highlighted in a recent study (33) of secondary science education by the National Academies of Science. According to this report, first-year undergraduates are arriving in the science classroom with little if any practical experience in using the scientific method and sparse experience with any kind of investigative laboratory exercises in which they do not know the outcome before they start the experiment. Even though instruction focusing on the scientific process has been articulated as a major goal of science education, teachers and administrations tend to favor content coverage in the interest of preparing students for standardized examinations (33). Until significant changes are made in high school curricula, your course may be the first (and possibly the last) chance a future well-educated citizen has to acquire this fundamental aspect of science literacy.

Because prior experience in the experimental method is lacking, most of my students are surprised to learn that planning an experiment could fill a 3-h laboratory session. This schedule effectively makes the point that designing a good experiment is at least as important as actually performing it. Instructors may raise the concern that extensive planning will initiate the "expectancy effect," which can color students' interpretation of results, or that it will take away from the enjoyment that students derive from a laboratory exercise when they don't know the outcome in advance (14, 24). Despite the extensive thought, planning, and discussion that precede the experiment, however, most students in our course are surprised by the data they obtain in the chicken embryo metabolism experiment. In addition, the increased clarity regarding data analysis and interpretation offered by the strong-inference approach (28) sets the stage for a more satisfying and rigorous laboratory experience. It is well worth noting that instructors can also benefit from the 3-wk schedule because a different laboratory setup is not required each week, thus reducing preparation time. Sometimes, a better educational experience for students is also less work for teachers.

Educators feeling the constraint of content coverage may be relieved to learn that this laboratory module can be taught in fewer than 3 wk. Each instructor must, of course, respond to the particular needs of his or her own course and/or department, but we urge instructors to consider which skills (specific content knowledge versus the ability to design, execute, and evaluate an experiment) will prove more universally applicable in the longer view. We cannot teach our students everything there is to know; therefore, we might as well teach what we teach in a way that will help students to learn it deeply so that they will retain the information after the exam is over. Some educators may argue that it is the students who demand a new laboratory exercise each week, but the feedback we have received regarding the 3-wk laboratory modules in the Animal Physiology course is extremely favorable; the student who complains about lack of variety is extremely rare.

Experiment Variations

The basic experiment described above may be expanded or modified in a variety of ways. Some possibilities include the following:

1. Examining chicken embryos at a variety of incubation stages. Considerable attention has been paid to the time close to hatching to determine how thermoregulatory abilities might change during this time (for a review, see Ref. 42).

2. Examining eggs of different species. Duck, quail, turkey, or goose eggs may be available in some areas. Note that it is not legal to obtain eggs of native wild birds without federal and state permits. Rock doves (common pigeons), starlings, and house sparrows, however, are introduced species that are not covered by this restriction.

3. Comparing different pairs of temperatures. If partial endothermy developed during later stages of incubation, embryos would demonstrate this ability by maintaining a more constant metabolic rate or even increasing metabolic rate when exposed to small decreases in T_a [e.g., 35 vs. 38°C (42)]. Note that smaller temperature differentials may require larger sample sizes to obtain clear results, since variation among individuals may be larger relative to the differences between temperature treatments.

4. Studying other species, such as insects and other readily available invertebrates. The size of the respirometer and incubation times can be varied according to animal size, T_a, and metabolic rate.

Technical Notes for Instructors

Temperature treatments. MAINTENANCE INCUBATION. For this exercise, we use ~16-day-old chicken eggs. Until used in the experiment, eggs should be incubated at 38°C in a humidified incubator. Any 38°C chamber can be adequately humidified by placing a tray filled with water at the bottom of the incubator; check this tray at least once per day and refill it as necessary. It is easiest to order the eggs so that they will arrive close to the time of use. If you plan to keep the eggs in the laboratory for a day or more, place them in an automatic egg turner or turn them gently by hand 4 times/day. Turning is required to prevent developmental abnormalities (13, 27).

CHOOSING TEST TEMPERATURES. The temperatures stipulated in the procedure for students are known to provide statistically significant differences in $\dot{V}O_2$ and a qualitative result that is easy for students to interpret. You may, however, modify the experiment by choosing different temperatures. Ultimately, this decision will be informed by the precise question students want to answer, the equipment available, the amount of time available for collecting data, the range of temperature tolerance by avian embryos, and a general knowledge of temperature-metabolism relations for endotherms and ectotherms. The goal is to choose physiologically tolerable temperatures that will result in either a positive (ectotherms) or negative (endotherms) relation between T_a and $\dot{V}O_2$. For ectotherms, any two temperatures in the normal range experienced by the animals in nature will show a negative relation between T_a and $\dot{V}O_2$. For endotherms, the higher T_a should be either within the TNZ or below the LCT and the lower T_a should be below the LCT (see Fig. 4 for a graphical explanation).

Although it would be easy for us to provide students with two temperatures that we know would work in this experiment, we believe it is an excellent exercise for students to reason through the choices as much as they can on their own. When students realize they need to know what temperatures the embryo can tolerate, we provide the abstract from Ref. 40, which provides lower and upper limits of 16 and 41°C, respectively. From first principles, we can deduce that if the embryo is endothermic, the normal incubation temperature is likely to lie within the TNZ; if this were not true, normal incubation would stimulate the embryo to expend energy for warming or cooling itself. An upper T_a of 38°C is thus a good choice from both a physiological and logistical perspective, since a 38°C incubator can be used both for maintenance incubation and for respirometry in the experiment. The precise range of the TNZ has been difficult to establish in chickens (26). However,

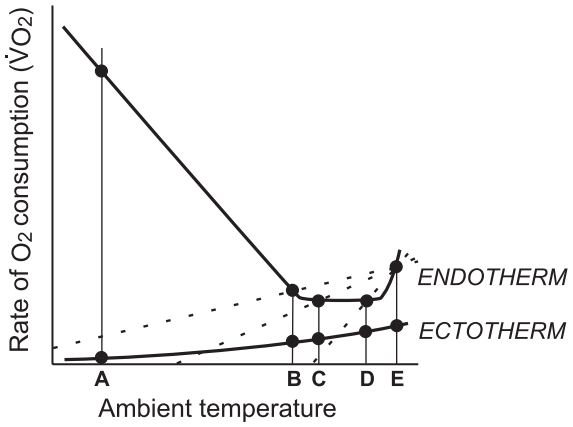

Fig. 4. In an experiment designed to distinguish between endotherms and ectotherms by the positive or negative slope of the temperature-metabolism relation, T_as must be chosen carefully to avoid misleading results (dotted lines). If the embryo is an ectotherm, any pair of temperatures will yield a positive relation between $\dot{V}O_2$ and temperature. If the embryo is an endotherm, any two temperatures below the TNZ (e.g., *temperatures A* and *B*) will yield a negative relation between temperature and $\dot{V}O_2$, as will any temperature below the TNZ paired with any temperature within the TNZ (e.g., *temperatures A* and *C*). If the two temperatures lie within the TNZ (e.g., *temperatures C* and *D*) or if one lies above the TNZ (e.g., *temperatures C* and *E* or *temperatures D* and *E*), it is possible to obtain the ectotherm pattern of a positive relation between temperature and $\dot{V}O_2$. The text explains how to choose temperatures that decrease the likelihood of a misleading outcome.

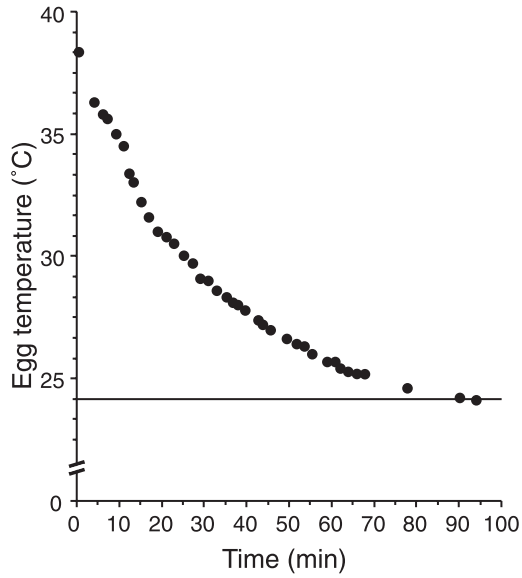

Fig. 5. Cooling curve for a 58-g unfertilized chicken egg (i.e., containing no embryo) placed at room temperature (~23°C). This curve approximates what would happen to the temperature of the egg if it contained a fully ectothermic embryo. If students want to test intermediate temperatures between 23 and 38°C, the curve can be used to estimate how long it should take an ectothermic egg and its contents to reach that temperature when the egg is removed from the 38°C incubator and placed at room temperature. Note that the experiment is performed on 16-day-old embryos, which may equilibrate to a new temperature more quickly than an unfertilized egg because *1*) they have a lower mass [bird eggs lose mass as the embryos develop (31)] and *2*) the more highly developed circulatory system in the embryo can distribute heat more rapidly (42).

poultry specialists generally agree that the LCT of the "comfort zone" (common parlance for the TNZ) in chickens is ~32°C at hatching and ~23°C 4 wk later, by which time the birds have increased substantially in both body mass and insulation (38). At least some students will know that it is common to keep newly hatched chicks under heat lamps for the first few days of life and will use this fact in their reasoning about how low the LCT might be in a late-stage embryo if it were endothermic. An additional general principle that students can discuss is that the LCT is typically lower (and the TNZ wider) as animals increase in size or become cold adapted (5, 7). For a 15- to 20-g bird (the approximate mass of the embryo between 16 and 18 days of incubation) descended from a warm-zone species and raised commercially in warm conditions, one would not expect to find a LCT as low as 23°C (see also Fig. 1).

EQUILIBRATION AT DESIRED TEST TEMPERATURES. The egg cooling curve (Fig. 5) provides approximate times required for an unfertilized chicken egg, which contains no embryo, to reach a particular internal temperature when it is removed from a 38°C incubator and placed at room temperature (~23°C). According to this curve, an egg undergoing Newtonian cooling should reach an internal temperature of 23°C within 90 min and 30°C within 25 min. Note that if the eggs were placed in a 30°C environment to cool, the internal temperature would take >25 min to reach 30°C because the temperature differential between the center of the egg and the surrounding air would be smaller; thus, to speed up cooling from 38°C to temperatures higher than room temperature, place the eggs at room temperature for the time indicated on the cooling curve and then place them in an incubator at the desired temperature. Because eggs

containing living embryos tend to cool more quickly than unfertilized eggs (37), the cooling rates shown in Fig. 5 are likely to be overestimates, especially for eggs weighing <58 g.

To enhance the effectiveness of the student-centered experiment design process, students are provided with the egg cooling curve only when they ask for it. Remind students that using the cooling curve to design the experiment does not imply that the embryos are ectotherms and thus does not "give the results away;" rather, it allows students to design an experiment that will work for both endotherms and ectotherms.

Respirometry. It is recommended that the F_E be within the range of 20.00–20.90%. Keeping values in this range will prevent any stress due to low oxygen availability while ensuring that oxygen content will be sufficiently different from ambient to be accurately measurable. Decreasing the size of the respirometer or increasing the incubation time in the respirometer will decrease F_E, whereas increasing the size of and/or decreasing the egg's time in the respirometer will have the opposite effect. Ideally, incubation times and chamber volumes should be chosen so that the same chamber size and incubation time produce usable results at both temperatures. To minimize the influence of any transient effects of handling on the embryos, it is recommended that the eggs remain in the respirometer for a minimum of 10 min. Note that some oxygen analyzers require up to 24 h of equilibration after being turned on before they will provide accurate measurements. A sample airflow circuit is shown in Fig. 6. Consult the manufacturer's instructions for the analyzer warm-up times, calibration instructions, and details of airflow circuitry.

Calculations. For calculations of $\dot{V}O_2$, students will find it very helpful to use a spreadsheet or other calculating tool that has been preprogrammed to make their calculations as soon as they have entered their measurements. This will hasten the process of collecting class data and, in the case of experiment *design 1*, assigning eggs to treatment groups so that the experiment can be completed within the 3-h laboratory period. Students using hand calculators frequently make errors; mistakes of this kind can be especially critical if erroneous calculations are used to divide the eggs into balanced treatment groups, as in experiment *design 1*.

Eggs. Fertilized chicken eggs of a specified age can be purchased from a commercial supplier. Although 15- to 16-day-old embryos are recommended because the $\dot{V}O_2$ differentials at the recommended test temperatures are large, students have obtained data with clear results from embryos that were mistakenly sent to us by the vendor at 6 days rather than 16 days after fertilization. Thus, a wide range of embryo ages appears to be suitable for this experiment.

At the end of the experiment, eggs can be frozen and disposed of according to your institution's guidelines. Small cracks that develop in the eggs during the experiment can be sealed with veterinary-grade cyanoacrylate adhesive (Vetbond or Nexaband), which differs in composition from and has lower toxicity than over-the-counter adhesives such as Krazy Glue (30).

Embryo masses. Instructors may want the students to dissect and weigh their embryos so that the masses used in the calculations of mass-specific rates are empirically determined. If they want to do so, embryos can be killed by placing the eggs in a jar containing halothane vapors (4). We generally do not ask students to dissect the embryos because we reuse them in later laboratory sections to reduce the total numbers of eggs required and because a single 3-h laboratory period is already very full without this extra step. In this case, use the information shown in Table 1 for calculations.

Regulations. Current federal animal use guidelines do not govern vertebrate embryos. Before beginning this experiment, however, you should consult your Institutional Animal Care

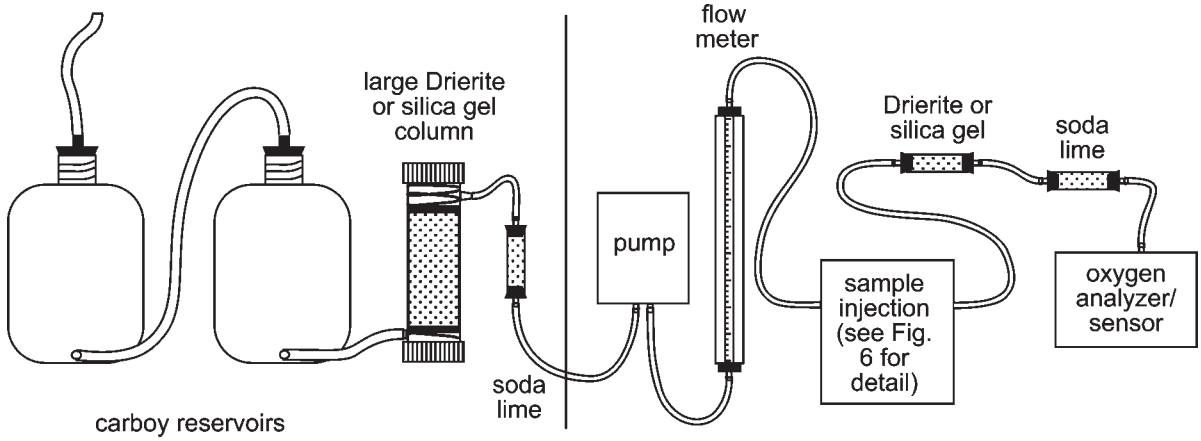

Fig. 6. Airflow circuit for oxygen analysis of air samples (components are not to scale). Air flows from the *left* to the *right*, ending in an in-line oxygen analyzer or sensor. Items shown to the *left* of the vertical dashed line are optional. Empty 5-gallon carboys serve as reservoirs to dampen small local fluctuations in the oxygen content of the source air. Use large-diameter vacuum tubing (thick walls prevent collapse) to connect the carboys. Smaller-diameter tubing (standard wall thickness) can be used for the remainder of the circuit. Water vapor and carbon dioxide must be removed from the air before the sample reaches the oxygen analyzer. A large column containing Drierite (W. A. Hammond) or silica gel desiccant and a small column containing soda lime remove water vapor and carbon dioxide, respectively, from the air stream, thus preserving the working life of the smaller Drierite/silica and soda lime columns downstream of the sample injection; this arrangement allows students to work for prolonged periods without having to change the contents of any of the columns. Small columns can be *1*) purchased ready-made, *2*) made from a 10-ml syringe covered by a one-hole stopper, or *3*) made from wide-bore glass or acrylic tubing with a one-hole stopper in each end. In all cases, a thin layer of cotton at the bottom and top of the tube or syringe barrel is necessary to keep dust from column contents from entering the oxygen analyzer. Indicating Drierite/silica and soda lime are easiest to use because they change color when exhausted; the desiccant properties of Drierite and silica gel can be regenerated by drying (follow manufacturer's instructions carefully to minimize loss of color). The location of the pump and flowmeter in the circuit may be different for your system or if the optional components of the circuit have been omitted; consult the manufacturer's instructions.

and Use Committee or other research ethics committee for policies at your institution. Hatching the eggs is permissible only with an approved animal use protocol, as stipulated in the *Guide for the Care and Use of Laboratory Animals* (17). Eggs of all native species of vertebrates are protected by law and may not be collected without the applicable federal and state research permits.

Background information on avian incubation and embryo development. The stage at which birds become fully endothermic depends on whether birds are altricial (helpless, featherless, and sightless at hatching) or precocial (ambulatory, sighted, and nearly or completely endothermic at hatching) (40). As is common in ground-dwelling birds, chickens are precocial, but their thermoregulatory capacity is not fully developed until several days after they hatch, which takes place on *day 21* of incubation. If challenged with small ($\leq$3°C) reductions in ambient temperature, an 18-day chicken embryo responds not by increasing $\dot{V}O_2$ but by failing to decrease $\dot{V}O_2$, and, by 19 days, embryos respond with a small, transient increase in respiration rate (for a review, see Ref. 42). At this stage, the ability of the embryo to thermoregulate fully seems to be limited by two factors. First, $\dot{V}O_2$ is constrained by the ability of oxygen to diffuse through the eggshell. Evidence that embryos are operating under an oxygen limitation comes from the observation that internal pipping (when the chick's beak breaks into the air cell inside the egg at around *day 19*) and exposure to an artificial high-oxygen atmosphere result in an increase in $\dot{V}O_2$. Second, the metabolic capacity of the embryo to produce heat is limited, even in experiments where the oxygen availability is experimentally increased. As a consequence of low heat production, the embryo temperature begins to drop at $T_a < 38$°C. Arrhenius (Q_{10}) effects then cause the metabolic rate to drop, thus further decreasing heat production capacity and further lowering embryo temperature. Thus, even embryos with some thermogenic capacity would appear ectothermic by the major criterion used in this laboratory exercise: a positive relation between $\dot{V}O_2$ and T_a. However, at the age (~16 days) and temperatures (23 and 38°C) suggested for this experiment, the embryos should be fully ectothermic.

During incubation in many species, the incubating parent(s) may leave the eggs unattended for varying periods of time (17) to search for food or fend off predators. An incubating parent energetically challenged by a continuous lack of food due to low temperatures, bad weather, or lack of prey may even abandon the eggs so that it can search farther from its nest for food; if conditions improve before too long, the parent may be able to resume incubation with good hatching success, especially if the interruption in incubation has occurred during the less vulnerable early stages of incubation (13). In some species, the eggs are not incubated until the entire clutch has been laid, with the result that the eggs hatch synchronously (13). Thus, it is normal for avian eggs in nature to experience periods of cooling. However, the Arrhenius relation reminds us that although low temperatures may not kill a developing embryo, we would expect developmental processes to slow as eggs cool. The temperature below which developmental progress becomes unmeasurable [the "physiological zero temperature" (13, 17)] is close to 23°C, substantially higher than some of the temperatures from which embryos have been known to recover (35). Eggs kept for long periods of time at temperatures above the physiological zero temperature but below the optimum incubation temperature may develop abnormally. Embryos are much more capable, however, of surviving the state of nearly suspended animation at temperatures below the physiological zero temperature (13). For example, fertilized chicken eggs may be kept at 11–13°C for several weeks without apparent harm to the embryo (13). Nevertheless, it is generally to an incubating parent's advantage to keep egg temperature close to the optimum incubation temperature once incubation has begun.

Because students are measuring rates of gas exchange, they will be curious about how oxygen and carbon dioxide are exchanged across the eggshell (see Ref. 31 for further details). Under a dissecting microscope or with a hand lens, students can see pores in the shell, through which three important gasses pass: oxygen (inward), carbon dioxide (outward), and water vapor (outward). Because they are focusing on the growth of the embryo, students will initially be surprised to learn that the mass of the egg decreases steadily during incubation (31). This decrease in mass is due to the steady loss of water vapor throughout incubation, which would proceed at a lethally high pace if the incubator were not humidified (13). This general trade off between water loss and gas exchange is a central problem for plants, in which open stomata facilitate the gas exchange (primarily carbon dioxide uptake) necessary for photosynthesis but hasten desiccation by permitting increased water loss. In a discussion of gas exchange by avian embryos, it is also worth mentioning that the moist membranes under the eggshell are similar to other gas exchange organs such as gills, lungs, and amphibian skin. If sufficient oxygen is present, all that is required for gas exchange to take place at a physiologically useful rate is a relatively large, thin, and moist exchange surface and a blood supply that carries oxygen and carbon dioxide convectively between the exchange surface and tissues.

Equipment: Descriptions and Alternatives

Incubators. A humidified incubator at 38°C is needed to house the eggs when they are not in use for the experiment. If this incubator is also large enough to hold the respirometers during test incubations, it is the only incubator required. Room temperature is completely adequate for the second test temperature, although students will recognize that using two incubators of similar type for the two temperature treatments results in a better-controlled experiment. Temperature-regulated water baths, which are relatively small, inexpensive, and commonly available, work well as treatment incubators; weights, such as large stones, pieces of brick, or flexible plastic bags filled with sand, can be placed on the lid of the respirometer to keep it submerged in the water bath during incubation.

Respirometers. Respirometers may be of several different designs. The minimum requirements are that *1*) the chamber must be air tight; *2*) if air samples are removed from the respirometer for measurement by an oxygen analyzer, they must be under neutral or positive pressure when the sampling device is disconnected from the respirometer; and *3*) removed samples must be of sufficient volume for the oxygen analyzer to make accurate measurements.

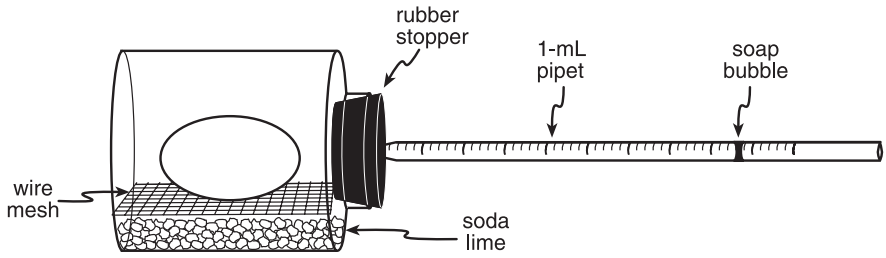

Fig. 7. A simple manometry chamber for direct measurement of $\dot{V}O_2$. See the text for an explanation.

JAR-TYPE RESPIROMETERS FROM WHICH AIR SAMPLES ARE WITHDRAWN. A jar-type respirometer with a single port and a two-way stopcock is inexpensive and easy to make (Fig. 2), and disposable plastic syringes can be used for air sample collection. Directions for their use are detailed above in *Instructions for Students: Measurements and Calculations*. Three-way stopcocks are not recommended because students frequently make mistakes when using them, leading to the loss or contamination of air samples.

SYRINGE-TYPE RESPIROMETERS FROM WHICH AIR SAMPLES ARE EXPELLED. Large syringes can serve as respirometers. At the end of the respirometry incubation period, the air sample to be analyzed can be expelled into a sample syringe by pressing on the plunger of the respirometer, but care must be taken not to damage the egg in the process. For insects or very small eggs, commercially available plastic syringes (e.g., 60 ml) can be used as respirometers. Larger syringe-type respirometers, modeled after those used by Vleck and Kenagy (39), can be constructed from large-diameter acrylic tubing; note, however, that manufacturing syringe-type respirometers requires a skilled fabricator, specialized materials and tools, and regular maintenance.

Manometry chamber. A simple manometry chamber can be made by turning a jar on its side and closing the opening with a one-hole stopper into which the tip of a 1-ml pipet has been inserted (Fig. 7). A false floor for the chamber can be fashioned from wire mesh to separate the egg or animal from soda lime on the bottom of the chamber. As the animal respires, the exhaled carbon dioxide is absorbed by the soda lime. Thus, changes in the volume of the gas space in the chamber, recorded by the movement of a soap bubble in the pipet, are due to the removal of oxygen by the animal in the chamber. This apparatus requires no oxygen analyzer, is extremely cheap and easy to make, and, because it so graphically makes the point that respiring animals consume oxygen, is especially suitable for beginning physiology students. A colleague uses such an apparatus to demonstrate the effect of thyroid hormones on the metabolic rates of rats to fourth graders. This design can also be scaled down for use with very small animals; as an undergraduate, one of us (S. M. Hiebert) used a miniature version of this apparatus to measure $\dot{V}O_2$ values by an individual *Drosophila*. Where extremely small chamber volumes are needed, a concentrated solution of KOH can be substituted as a carbon dioxide absorbant, but care must be taken to prevent this caustic solution from coming into contacting with either the students or the animals.

The volume of oxygen consumed (V; in ml) (read directly from the pipet) is converted to $\dot{V}O_2$ at STPD by the following equation:

$$\dot{V}O_2(\text{in ml/h}) = V\left(\frac{273}{T_a}\right)\left(\frac{P_b}{760}\right)\left(\frac{60}{t}\right) \quad (5)$$

where all abbreviations are as described in *Instructions for Students:* Measurements and *Calculations, IV. Calculating* $\dot{V}O_2$.

To ensure success, check the air tightness of all fittings and use a dilute solution of soap and water for the soap bubble; wet the inside of the pipet before the animal is placed into the chamber so that the bubble does not break as it moves toward the stopper. After the animal has been placed in the chamber, make a bubble by squeezing the dilute soap solution (e.g., from children's bubble toy) or create bubbles in dishwashing liquid by opening the cap and rapidly squeezing to create bubbles above the liquid. Use an eyedropper or fingers to transfer a bubble (or bubbles) to the horizontal pipet.

Students should record the position of the soap bubble at frequent intervals so that if the soap bubble bursts prematurely, they will still have data to analyze. Note that a warm animal (endotherm or warm ectotherm) will heat the air inside the chamber at first, causing the volume inside the chamber to expand and the bubble to move outward. Once the temperature has equilibrated, the bubble will start to move in the direction of the stopper as a consequence of oxygen consumption. Asking students to plot the position of the soap bubble beginning immediately after a warm animal has been placed inside the chamber is a good way to find out whether they are

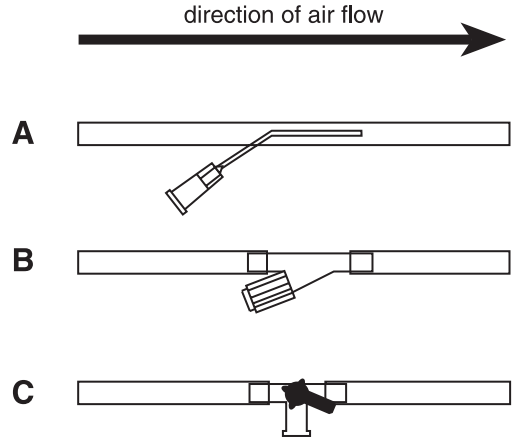

Fig. 8. Three possible arrangements for air sample injection ports, all of which are compatible with the standard male Luer fitting of the sample syringe. *A*: a bent, blunt-end 18-gauge needle is inserted directly through the wall of the flexible plastic tubing. *B*: a needleless valve opens when a syringe is connected to the fitting and seals automatically when it is removed. *C*: a three-way stopcock can also be used, but this requires student practice because it is easy for students to set the lever incorrectly and thus inadvertently inject a sample into room air rather than into the analyzer air stream.

observing the apparatus carefully and recording their data accurately! Instructors may want to ask students to identify potential shortcomings of this method; however, the effect is sufficiently robust to allow students to draw clear conclusions from the relative $\dot{V}O_2$ values at the suggested temperatures.

The design shown in Fig. 7 works best when the entire apparatus can be placed inside an incubator. If a water bath is used to warm or cool the respirometry chamber, or for better control of the effects of changing ambient air pressure on the manometric apparatus, a U-shaped manometer tube filled with fluid can be used to monitor the $\dot{V}O_2$ in the chamber, as illustrated in *box 5.4* of Ref. 21 and as described in Ref. 37. For student use, a calibrated buret containing water can be inserted into the stopper in place of the syringe containing pure oxygen. At the end of the measurement period, water is released from the buret until the manometer fluid level returns to the position marked at the beginning of the trial. The volume of water required is the difference between the initial and final readings on the buret, and this value is then substituted for V in *Eq. 5*. Flexible tubing (attached to the tip of the buret that protrudes through the stopper into the respirometer) can be used to direct water away from the egg and desiccant as it is released into the chamber.

Syringes for air samples. Each student or group will need a syringe for removing air samples from any jar- or syringe-type respirometer. For classroom use, plastic disposable syringes are preferable as long as students understand the potential problem of creating a low-pressure air space in the syringe when an air sample is removed; unless students follow the instructions carefully, they may cause room air to be sucked into, and thus contaminate, the air sample. Glass syringes, when held horizontal, do not have this problem because of the nearly frictionless fit between the plunger and barrel. When an air sample is expelled into a glass syringe, air pressure alone pushes outward on the plunger. However, glass syringes require extra care in handling, break more easily, and are more expensive to replace. Students will need to practice using a glass syringe before they use it in an experiment.

Syringe caps for friction-fit or Luer-type syringe tips can be purchased. However, students keen to create an airtight seal tend to strip the threads from Luer-type syringe caps in fairly short order. We prefer to make our own airtight syringe caps by cutting off the needle (flush with the plastic base) of a 27-gauge or smaller (i.e., higher gauge) disposable syringe needle. Cutting the needle crimps the needle opening shut, and the plastic base of the needle has a Luer fitting that conveniently screws into or slides onto the syringe tip.

Oxygen analyzers and sensors. An oxygen analyzer or sensor is needed for jar- and syringe-type respirometers. Air is pulled through the sensor by a pump that runs continuously (Fig. 5). An air sample is injected into the continuously flowing air stream (follow instructions provided with the oxygen analyzer; see Fig. 8 for sample injection ports). As the sample air is pulled through the analyzer, the leading and trailing edges of the bolus mix with the room air in the sample line. Thus, the concentration of oxygen drops until the middle of the sample has entered the oxygen analyzer and remains low as this region of undiluted sample passes through the analyzer; thereafter, the concentration again rises until the sample has completely cleared the analyzer. The lowest concentration registered by the analyzer is an entirely serviceable measure of F_E for the purposes of this experiment.

The flow rate of sample air through the oxygen analyzer should be low enough and the sample large enough so that the lowest concentration can be read accurately. Test your system by injecting samples of several volumes (e.g., 10, 20, and 30 ml) into the air stream; if, for example, a 30-ml sample gives a lower F_E than a 20-ml sample of the same mixture, then the flow rate is too high (or the sample volume is too small). Adjust the sample size and/or flow rate until increasing the sample volume does not result in decreasing F_E readings. At the final setting, the oxygen analyzer output should pause at F_E, as it measures the interior of the sample bolus, before returning to F_I. Integrating the area under the curve of oxygen concentrations can be a more accurate method for determining F_E with small gas samples, but this method requires a continuous recording of analyzer output as well as additional calculations. Because the metabolic effect is so large at the suggested temperatures, however, the outcome of the experiment is robust to the small measurement errors that may result from the suggested protocol.

Barometers, hygrometers, and thermometers. A barometer, hygrometer, and thermometer should be available in the room where the experiment is taking place to measure P_b, RH, and T_a values, respectively.

Selected Articles for Students and Instructors

1. **Rahn H, Ar A, Paganelli CV.** How bird eggs breathe. *Sci Am* 240: 46–55, 1979.
2. **Vleck CM, Vleck D, Hoyt D.** Patterns of metabolism and growth in avian embryos. *Am Zool* 20: 405–416, 1980.
3. **Whittow GC, Tazawa H.** The early development of thermoregulation in birds. *Physiol Zool* 64: 1371–1390, 1991.

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