Pulmonary and cutaneous $\text{O}_2$ gas exchange: a student laboratory exercise in the frog

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Tattersall GJ, Currie S, LeBlanc DM. Pulmonary and cutaneous $\text{O}_2$ gas exchange: a student laboratory exercise in the frog. Adv Physiol Educ 37: 97–105, 2013; doi:10.1152/advan.00087.2012.—Gas exchange in animals is ultimately diffusion based, generally occurring across dedicated respiratory organs. In many aquatic amphibians, however, multiple modes of gas exchange exist, allowing for the partitioning of $\text{O}_2$ uptake and $\text{CO}_2$ excretion between respiratory organs with different efficiencies. For example, due to the physical properties of $\text{O}_2$ being vastly different between air and water phases, the lung and skin play disproportionately important roles in $\text{O}_2$ uptake.

Many aquatic frogs are renowned for their cutaneous gas exchange capacity, where often the majority of $\text{CO}_2$ is excreted across the skin. Furthermore, the roles of these gas exchange organs change with the animal’s behavior. Under diving conditions, most of the frog’s gas exchange needs must be met by the skin. In this article, we describe an interactive undergraduate laboratory that allows a class of students to share equipment while assessing pulmonary and cutaneous respiration in frogs provided with an air/water choice and under enforced dive conditions. Concepts explored in this laboratory exercise include animal energetics, diving reflex, pulmonary and cutaneous gas exchange processes, diffusion-based gas flux, and $\text{O}_2$ debt.

THE OBJECTIVES OF THIS LABORATORY are to compare the relative importance of two respiratory surfaces (i.e., the lungs and skin) in $\text{O}_2$ uptake, to evaluate whether cutaneous respiration is sufficient to fuel metabolism or whether an “$\text{O}_2$ debt” is incurred during enforced diving, and whether such a debt is repaid by preferential use of aerial or aquatic gas exchange. In this laboratory, students establish, by simple observation, the natural diving pattern of the frog. In addition, using closed-system respirometry, they measure the frog’s $\text{O}_2$ uptake from air (i.e., via the lungs) and from water (i.e., via the skin) during normal diving sequences, during enforced submergence, and during recovery. These measurements are then used to assess the normal proportion of total gas exchange attributed to cutaneous gas exchange, whether cutaneous respiration can be upregulated during a dive, and whether the postdive $\text{O}_2$ debt is preferentially resolved by pulmonary gas exchange.

Background

In the wild, many ranid frogs (such as bullfrogs, leopard frogs, and green frogs) spend much of their time underwater, diving frequently to swim or to avoid predation (3, 5, 7, 35, 38). Some frogs, like those belonging to the family Pipidae (e.g., *Xenopus laevis* and *Pipa pipa*) are entirely aquatic, behaving more like air-breathing fish in the amount of time spent at the surface and may go for prolonged periods without breathing air. Pulmonary ventilation in frogs is an intermittent process, with single breaths being separated in time by periods of breathholding (4, 6). Normal breathholding periods (i.e., between the lung ventilations in a breathing series) can be short at rest or more prolonged, such as when the animal is diving. Indeed, the frog is better adapted to a diving lifestyle than other diving vertebrates because, in addition to its lungs for air breathing, it has the ability to exchange $\text{O}_2$ and $\text{CO}_2$ through its well-vascularized skin. Accumulated $\text{CO}_2$ incurred during a dive can be lost across the skin, and, to some extent, $\text{O}_2$ uptake can occur continuously across the skin to supplement $\text{O}_2$ needs during the dive (4, 5, 10).

Although numerous other ectothermic animals, including fish and some reptiles, will also exchange respiratory gases across their skin, amphibians are exceptional in this regard, capable of obtaining routinely anywhere from 20% to 85% of their $\text{O}_2$ needs from passive diffusion across their skin. In contrast to pulmonary ventilation, cutaneous gas exchange occurs continuously and operates in both air and water (14, 15). Although primarily passive, for animals with low rates of metabolism, this form of gas exchange is more than sufficient. During hibernation in aquatic frogs, cutaneous gas exchange is the only mode of $\text{O}_2$ uptake available to them, but is still fully capable of meeting these needs with scope for increase (34, 36, 37). In some amphibians, such as lungless salamanders, nearly all of their $\text{O}_2$ originates from diffusive cutaneous gas exchange, with some minor contributions from diffusion within the buccal cavity. Indeed, the evolution of lunglessness in amphibians has occurred numerous times (1, 28), and one of the most diverse groups of salamanders comprises those that are primarily cutaneous skin breathers.

This cutaneous gas exchange in amphibians is facilitated by a branch of the pulmonary artery. In amphibians, the arch leaving the heart that supplies blood to the lung is the pulmonary–cutaneous artery (Fig. 1), which diverts mainly deoxygenated blood from the heart to the lung and skin. Interestingly, in amphibians, despite the presence of a three-chambered heart, separation of oxygenated and deoxygenated blood is remarkably efficient (39). Downstream from the heart, the pulmocutaneous artery diverges into the pulmonary artery (which supplies the lungs) and the cutaneous artery (which supplies the skin). These pulmonary and cutaneous branches receive nervous signals to the muscular sphincter (smooth muscle), which can alter the relative amount of blood flowing to the lungs or to the skin depending on gas exchange requirements (Fig. 1). During lung ventilation, the sphincter on the pulmonary artery is dilated, whereas, simultaneously, the sphincter on the cutaneous artery is constricted (8, 30, 42). The reverse occurs...
during cutaneous ventilation, which happens when an amphibian is diving, allowing deoxygenated blood to flow to the gas exchange organ best suited for O₂ uptake under specific conditions.

In addition to blood flow patterns, cutaneous and pulmonary gas exchange differ with respect to the effectiveness of diffusion. In general, blood flow to the lungs and skin is matched to the relative effectiveness of the respective respiratory organs (8). Based on thickness differences and the relative distance of capillaries to the respiratory medium, these different respiratory surfaces exhibit differing degrees of efficiency. The distance that respiratory gases must diffuse across the skin is as high as 18–60 μm, one to two orders of magnitude larger than the diffusion distance within the lung epithelia, which places an upper limit on the rate of gas diffusion across the skin (33). Nevertheless, diffusion capacity is known to be regulated in bullfrogs through the recruitment of underperfused capillaries under hypoxic conditions, due to an increase in the effective surface area for gas exchange (27). One further challenge with cutaneous respiration is that the lack of ventilatory mixing of the surrounding water causes stationary boundary layers to form over the skin, which increases the total distance across which O₂ must diffuse. This contributes to the O₂ diffusion limitation of amphibian skin (13, 26). One consequence of this diffusion limitation is that this sets an upper limit to the rate of O₂ exchange (25). When metabolic requirements rise or total gas exchange is compromised, such as occurs during an enforced dive, O₂ needs cannot be met through the skin alone. Therefore, during a prolonged enforced dive, an O₂ deficit develops (11), after which an apparent O₂ debt can be measured. This temporary elevation in total O₂ uptake results from the replenishment of O₂ stores as well as the biochemical processes involved in the recovery from the metabolic lactic acidosis.

The process of gas exchange was formally studied by August Krogh in the early 1900s (18, 24), where he determined the diffusion constants for numerous gases relevant to respiratory physiology. Demonstrating gas exchange in this manner, however, is technically quite difficult to achieve in an undergraduate laboratory. Luckily, amphibians offer a convenient and naturally evolved means by which this can be achieved using a few simple components, including an O₂ electrode.

Fig. 1. Sketch (A) and diagrammatic depiction (B) of the anuran heart and its major vessels (42). The “typical” anuran amphibian heart consists of two atria and one ventricle along with the distinct arches that lead to the systemic and pulmocutaneous circulation. The pulmocutaneous artery is shown, branching into the pulmonary and cutaneous arteries. The pulmonary artery delivers deoxygenated blood to the lungs, and the cutaneous artery delivers deoxygenated blood to the skin. The pulmonary artery receives parasympathetic innervation via the vagus nerve, where stimulation causes muscles in the arterial wall to contract, which reduces flow through the pulmonary artery, while facilitating blood flow to the cutaneous artery. Despite some mixing within the ventricle, the spiral valve helps to separate the different blood supplies, such that oxygenated blood goes primarily to systemic circuits and deoxygenated blood goes primarily to the pulmocutaneous circuit.
under a thousand dollars, a laboratory can be set up that demonstrates the basic principles of gas exchange partitioning. We have used this laboratory in third- and fourth-year animal physiology (environmental or comparative) courses since 1995, when it was originally designed and put into use at the University of Cambridge by Dr. Robert Boutilier. Since then, we have also implemented this laboratory successfully at Brock University and Mount Allison University using different species of frogs. The laboratory is easily accomplished in <3 h and allows for the demonstration of increases in gas exchange, O2 deficit formation, and the partitioning of gas exchange to different respiratory organs. As such, it is suitable for demonstrations of gas exchange in an animal that naturally engages in bimodal breathing. Finally, it is suitable for students interested in physiological ecology as well as for laboratories covering more complicated processes, such as ventilation-perfusion relationships (20). This laboratory can dovetail well with training exercises in experimental design (22), the strong inference method (21), or as part of a series of laboratories in comparative physiology exploring osmoregulation (2), activity metabolism (29), and hypoxia metabolism (12). As it does not involve euthanizing or otherwise harming the animals, it also conforms well to ethical justifications involving the use of animals in the teaching laboratory.

General Hypothesis

To help guide students regarding the reason for this laboratory and to link their experimental results back to lecture material in comparative animal physiology, we have found the following general hypothesis helpful to develop the theme for this laboratory:

O2 uptake is a diffusion-based process, taking place across respiratory surfaces that are in contact with the respiratory medium.

Predictions and Expected Outcomes

Depending on whether students are required to form their own hypotheses and experimental design, instructors may want to inform the students of the following expected outcomes arising from the published literature and the hypothesis described above:

1. Regulation of O2 uptake across gas exchange organs depends on the efficiency of the gas exchange surface.

2. The relative and absolute contributions of cutaneous O2 uptake increase when pulmonary O2 uptake decreases, especially during a dive, when pulmonary O2 uptake is minimal.

3. Cutaneous O2 uptake is insufficient to maintain total O2 requirements at predive levels.

4. Frogs incur an O2 debt during a dive and repay the O2 deficit by spending more time at the surface and exhibiting elevated pulmonary O2 uptake during the postdive recovery period.

MATERIALS AND METHODS

Animals

We have used four species of frog in this laboratory, the common frog (Rana temporaria), the Northern leopard frog (Lithobates pipiens, formerly Rana pipiens), the bullfrog (Lithobates catesbeianus, formerly Rana catesbeiana), and the African Clawed frog (Xenopus laevis). The choice of species depends on availability and geographic location. We recommend choosing a small- to medium-sized frog (30–100 g) and matching this size with a respirometer volume of no more than 10 times the mass (i.e., 300–1,000 ml). The use of bufonids or frogs with thick skin and relatively low rates of cutaneous gas exchange are not recommended. All procedures conformed with local and national animal care guidelines and were approved by the Animal Care and Use Committees of Brock University (AUPP no. 11-07-01) and Mount Allison University (AUPP no. 09-25R). The experiments described herein do not require euthanizing or harming animals, and adult frogs can be returned unharmed to other animal care protocols or adopted out.

Experimental Setup

In addition to 1 frog/student group, the following equipment is required:

- Glass jar (respirometer/dive tank)
- Magnetic stirrer
- Rubber stopper
- Two 3-ml syringes
- Two two-way stopcocks
- Polyethylene cannula tubing
- Stopwatch
- Two O2 electrodes (shared equipment for the entire laboratory)
- Two O2 meters (shared equipment for the entire laboratory)
- Weigh balance capable of measuring up to 1,500 g

O2 Electrode Use and Calibration

We use a polarographic O2 electrode system, which includes an electrode and meter (OM-4, Microelectrodes) to measure PO2. These electrodes work effectively for both water and gas phase samples. The electrode system was modified such that the electrode is housed within a temperature-controlled device along with an injection port allowing a small sample (<0.5 ml) of gas or water to reach the tip of the electrode. This can be built by a glass blower or technical machine shop or purchased from various companies that manufacture water-jacketed electrode holders (microelectrodes.com, Hansatech Instruments). We generally use two electrodes to speed up the measurements to accommodate more students as well as to have one electrode for measuring water samples and one electrode for measuring air samples. Although this laboratory could be conducted with O2 electrodes inserted directly into the respirometer, we generally use only one set of electrodes per laboratory as students will withdraw their samples into syringes. Samples are slowly injected such that the fluid or gas surrounding the tip of the electrode is completely changed over, thereby washing out previous samples. The polarographic O2 electrode can be calibrated with a sodium bisulfite solution, which extracts all dissolved O2 or a nitrogen gas supply to obtain the zero PO2 and with water-saturated room air to obtain the calibration for the gain adjustment. The water-saturated room air calibration (i.e., the gain or span setting on most polarographic meters) is set to a PO2 value with the contribution of water vapor removed using the following equation:

\[
PO2 = 0.2095 \times (Pb - PH2O)
\]

where 0.2095 is the fractional amount of O2 in atmospheric air, barometric pressure (Pb) is nominally 101.325 mmHg at sea level (typically ~99.325–100.658 kPa at Brock University), and water vapor pressure at 100% saturation (PH2O) is 2.338 kPa at room temperature (i.e., 20°C). The water vapor in water-saturated air at a given temperature can be estimated from the following empirically derived equation (41):

\[
PH2O = 0.1332e^{\left(\frac{18.55 - 3.8552}{T} - 214.690 \frac{T}{T^2}\right)}
\]

where T is temperature (in K). Therefore, a typical starting PO2 measurement (i.e., PO2 of water-saturated room air or room air-saturated water) would be estimated to be 20.30 kPa. Students will be
measuring a decline in $P_{O_2}$ during their experiments and therefore should expect $P_{O_2}$ values to be lower than 20.30 kPa for values taken after the 20- and 40-min marks.

Closed-System Respirometry

We use a closed-system respirometric technique to assess $O_2$ uptake (Fig. 2). By measuring the change in $P_{O_2}$ in a system (air or water) over time, one can determine the rate at which $O_2$ is being consumed by an animal from the two different respiratory media. If the area of contact between the air and water phases in a two-phase system is small (we use cylinders that are similar diameter to the animal), then it can be assumed that exchange of $O_2$ between the two phases is negligible because diffusion is slow. $O_2$ consumption from each phase (i.e., water vs. air) can be calculated separately; consumption from the air space corresponds to pulmonary gas exchange, whereas consumption from the water phase represents cutaneous gas exchange. Since amphibian tissue is much less sensitive to the build up of $CO_2$, it is not necessary to “scrub” the $CO_2$ from this closed respirometry system. The respirometer has two syringe ports available to attach sample syringes for $P_{O_2}$ measurements. One port is further connected to a thin piece of cannula tubing, which serves as a means to sample from the water, whereas the remaining port samples from the air space. These ports are connected to two-way stopcocks to ensure that the sample ports can be closed.

Overall Procedures

The frog should be placed in the experimental chamber with adequate water in advance of this laboratory exercise so as to enable it to habituate to its new surroundings. Students will need to determine the mass of the frog and the volume of the water within which the frog will be submersed. We use gravimetric assumptions (density of water or frog $\sim 1$ g/ml) and assess all of the volumes by weighing the full respirometer, the partly filled respirometer, the partly filled respirometer with the frog, and the empty respirometer. To proceed with the calculations after the experiments, students will need to determine the frog volume, water volumes during all three experiments (experiments 1–3), and air volumes during experiments 1 and 3. A digital scale can be used for mass determinations and frog mass converted to volume assuming a density of 1 g/ml. Continuously aerated water should also be available to students so that they can supply their frogs with freshly aerated water immediately before they commence their measurements. We generally aerate the water for several hours in advance of the laboratory. Throughout all experiments, students should make quantitative observations on the frog’s diving behavior, including the frequency and duration of its dives, its general activity levels during its dives (i.e., the time it spends moving about vs. sitting quietly), and the amount of time it spends submerged versus at the water’s surface. It is recommended that students use a stopwatch to note the time at which a dive begins and dive ends or simply to monitor the frogs over 30-s epochs and note whether the frogs exhibit greater movement and diving or surfacing behavior. If they assess the behavior appropriately, then quantitative comparisons can be made between all 20-min periods. We also ask students to observe the degree and location of improved vascularization or “pinkness” of the skin when the frog dives.

Experiments

Experiment 1: predive gas exchange at rest. During the first 40 min of this exercise, students first monitor $O_2$ consumption from the aerial and water phases while the frog behaves normally. Students are instructed to record individual dive times and activity levels of frogs throughout the experiment. The magnetic stirrer should be turned on so that water is continually stirred over the frog’s skin. Students sample the air and water to measure $P_{O_2}$ at 0, 20, and 40 min. They do this by drawing and measuring the air sample first and the water sample second, using the appropriate stopcocks and syringes. When withdrawing samples, students should draw them into the syringes and inject back into the respirometer two to three times to ensure a properly mixed sample. After taking an air sample, students should immediately replace the volume of air withdrawn with an equivalent volume of air. This action may introduce some error to the measurements but is important for the maintenance of a constant pressure. For each water sample, 1 ml of water should be drawn and discarded because of syringe dead space, and a subsequent “real” sample (2 ml) should be taken for analysis. Students should then immediately
replace each 2-ml sample with 2 ml of water from the larger syringe attached to the bung.

**Experiment 2: gas exchange during a dive.** During this 40-min experiment, the respirometer is filled entirely so that there is no air space, causing the frog to undergo an enforced dive. Enforced dives are quite natural for these animals (i.e., when they are trapped beneath ice in a pond or when they are forced to stay submerged because of an aerial threat). After the frog is submersed, all other measurements are as conducted in experiment 1. Students should note the behavior of the animal during the enforced dive, but they will not be able to record “dive times” since the animal is continuously submerged. Overall activity levels are instead best assessed throughout this experiment.

**Experiment 3: postdive gas exchange.** During this final 40-min period, recovery from the dive will be examined. The water from the respirometer should be emptied quickly and replaced with freshly aerated water up to the level used in experiment 1. The respirometer should be sealed and operated in the same configuration as during experiment 1. Once again, the frog’s dive times and activity levels should be recorded, and water and air samples should be taken for PO2 measurements at 0, 20, and 40 min.

Upon Completion of Experiments

Once all experiments have been completed, the volumes of the air and water phases in the respirometer should be measured as well as the mass of the frog (if not determined before the experiment). Depending on how volume was assessed, respirometer water volumes may need to be corrected for the volume that the frog displaces before the rates of O2 consumption are calculated throughout all three experiments.

Calculations of O2 Consumption

The molar amount of O2 (in µmol/l O2) in any closed system (air or water) is given by the following equation:

\[ [O_2] = (P_{O_2} \times \beta_{O_2}) \times V \]

where PO2 is measured in kPa, βO2 is the solubility (or capacitance) coefficient for O2 in the medium (air or water) at a particular temperature (in µmol O2·l\(^{-1}\)·kPa\(^{-1}\); Table 1), and V is the volume (in liters) of the medium (air or water). O2 consumption measurements in the literature are often reported as either VO2 or MO2; the different symbols used (V vs. M) relate to whether volume or molar concentrations are being measured, and do not over the top refers to the fact that these measurements are rate-based measurements with units of volume or mass per unit time. We prefer to deal with molar amounts of O2, therefore, to appropriately relate to the nature of gas diffusion. Therefore, to explain this to students, we provide them with an introduction to Henry’s law and a table of O2 solubility coefficients (β; in µmol·l\(^{-1}\)·kPa\(^{-1}\)) for them to convert PO2 measurements to molar concentrations of O2. Using the PO2 measurements collected for air and water samples from all the experiments and across all 20-min measurement intervals, O2 exchange rates for pulmonary and cutaneous sites (i.e., MO2skin + MO2lungs) can be calculated using the following general formula:

\[ MO_2 = \frac{\Delta PO_2 \times \beta_{O_2} \times V}{t \times m} \]

where MO2 is measured in µmol O2·g\(^{-1}\)·h\(^{-1}\), PO2 is measured in kPa; \(\Delta PO_2\) is the difference in PO2 between sample periods [i.e., (PO2(minute 20) – PO2(minute 20) or PO2(minute 20) – PO2(minute 40))], βO2 is the solubility or capacitance coefficient to convert gas pressure into gas concentration for the representative medium at the experimental temperature (in µmol O2·l\(^{-1}\)·kPa\(^{-1}\)), V is the volume (in liters) of the phase where the measurement was made (water volume or air volume, which is dependent on the experiment), t is the difference in time (in h; e.g., 20/60 min = 0.33333 h), and m is the frog’s body mass (in g). We sometimes convert time to hours since the numeric values are not as small and are easier to visualize. Total MO2 for any given 20-min interval is simply the sum of cutaneous plus pulmonary O2 uptake.

Sample Problems

Students with limited mathematical experience or those seeking reassurance that they have interpreted procedures correctly can be assisted by the following conceptual and quantitative problems.

**Question 1.** How much more O2 is carried in a liter of air versus a liter of water at 30°C?

**ANSWER.** Consult the table for the solubility/capacitance coefficients for water and air. The ratio can be calculated as follows:

\[ \text{Ratio} = \frac{\beta_{\text{Air}}}{\beta_{\text{Water}}} = 34.3 \]

**Question 2.** In the experiment you just conducted, assuming a frog weighing 100 g is sitting in 700 ml of water and the PO2 of the water declines from 20.26 to 18.93 kPa in a 20-min period. What would be the O2 consumption (in µmol O2·g\(^{-1}\)·min\(^{-1}\))?

**ANSWER.** O2 consumption can be calculated as follows:

\[ MO_2 = \frac{\Delta PO_2 \times \beta_{O_2} \times V}{t \times m} = \frac{(20.26 - 18.93) \times 12.52 \times 0.7}{20 \times 100} = 0.00582 \mu\text{mol O}_2 \cdot \text{g}^{-1} \cdot \text{min}^{-1} \]

For graphical purposes, it may be more intuitive to work with numbers that are not so small, so it is equally appropriate to report these values per hour by multiplying by 60:

\[ MO_2 = 0.00582 \mu\text{mol O}_2 \cdot \text{g}^{-1} \cdot \text{min}^{-1} \times 60 \text{ min} \cdot \text{h}^{-1} = 0.349 \mu\text{mol O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1} \]

**Question 3.** You measure the gas exchange in a resting X. laevis and determine the following: skin gas exchange is 0.34 µmol·g\(^{-1}\)·h\(^{-1}\) and lung gas exchange is 1.8 µmol·g\(^{-1}\)·h\(^{-1}\). What percentage of total gas exchange occurs across the skin?

**ANSWER.** The percent skin gas exchange can be calculated as follows:

\[ \text{Percent skin gas exchange} = \frac{\text{Skin gas exchange}}{\text{Skin gas exchange + lung gas exchange}} \times 100 \]

\[ = \frac{0.34}{0.34 + 1.8} \times 100 = 16\% \]

Table 1. β values, or solubilities, for water and air

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>βwater, µmol·l(^{-1})·kPa(^{-1})</th>
<th>βair, µmol·l(^{-1})·kPa(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>13.67</td>
<td>410.3</td>
</tr>
<tr>
<td>21</td>
<td>13.42</td>
<td>408.9</td>
</tr>
<tr>
<td>22</td>
<td>13.18</td>
<td>407.5</td>
</tr>
<tr>
<td>23</td>
<td>12.95</td>
<td>406.2</td>
</tr>
<tr>
<td>24</td>
<td>12.74</td>
<td>404.8</td>
</tr>
<tr>
<td>25</td>
<td>12.52</td>
<td>403.5</td>
</tr>
<tr>
<td>26</td>
<td>12.32</td>
<td>402.1</td>
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<tr>
<td>27</td>
<td>12.12</td>
<td>400.8</td>
</tr>
<tr>
<td>28</td>
<td>11.93</td>
<td>399.4</td>
</tr>
<tr>
<td>29</td>
<td>11.75</td>
<td>398.1</td>
</tr>
<tr>
<td>30</td>
<td>11.58</td>
<td>396.8</td>
</tr>
</tbody>
</table>

\(\beta\), O2 capacitance coefficient. βair is derived from the ideal gas law (PV = nRT), where n/VP = 1/RT and P is pressure, V is volume, n is the amount of gas, R is the gas constant, and T is temperature.
RESULTS AND DISCUSSION

Experimental Results

In adult *X. laevis*, cutaneous gas exchange comprises between 10% and 20% of total gas exchange (see Table 2 for a comparison with other amphian species) and is capable of rising during a dive, but not to levels sufficient to sustain the frog’s entire metabolic requirements (Figs. 3 and 4). Furthermore, cutaneous respiration rarely constitutes the only means by which frogs obtain their O\(_2\). *X. laevis* are fully capable of diving for 40 min, although most will incur an O\(_2\) debt during the dive and repay this during the first 20 min of their postive recovery. The fact that the cutaneous O\(_2\) uptake shows only a limited capacity to rise should be used by students to conclude that their frogs incurring an O\(_2\) debt. The predominant rise in pulmonary O\(_2\) uptake during the recovery period may also be used to conclude that the bulk of adjustments in O\(_2\) requirement in *X. laevis* are accommodated by the lungs (Fig. 4).

One advantage of this laboratory is that each student group obtains slightly different results depending on their individual frog. Since behavioral changes contribute highly to metabolic requirements, each group is forced to interpret their results in light of their own experimental data. Some frogs are extremely quiescent throughout all measurements and exhibit very small

Table 2. Rates of cutaneous O\(_2\) uptake rates from numerous salamanders (Order: Caudata) and frogs (Order: Anura)

<table>
<thead>
<tr>
<th>Species</th>
<th>Mass, g</th>
<th>Skin M(_{O_2}) (\mu\text{mol}\text{g}^{-1}\text{min}^{-1})</th>
<th>Percentage of total M(_{O_2})</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ambystoma tigrinum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>120</td>
<td>0.0238</td>
<td>28.8</td>
<td>Whitford WG, Sherman RE. Aerial and aquatic respiration in Axolotl and transformed <em>Ambystoma tigrinum</em>. Herpetologica 24: 233–237, 1968</td>
</tr>
<tr>
<td><em>Amphiuma means</em></td>
<td></td>
<td></td>
<td></td>
<td>Guimond RW, Hutchison VH. Aerial and aquatic respiration in the congo eel <em>Amphiuma means means</em> (Garden). Resp Physiol 20: 147–159, 1974</td>
</tr>
<tr>
<td><em>Cryptobranchus alleganiensis</em></td>
<td>423</td>
<td>0.0212</td>
<td>92.2</td>
<td>Guimond RW, Hutchison VH. Aquatic respiration: an unusual strategy in the hellbender <em>Cryptobranchus alleganiensis</em> (Daudin). Science 182: 1263–1265, 1973</td>
</tr>
<tr>
<td><em>Notophthalmus viridescens</em></td>
<td>1.5</td>
<td>0.0673</td>
<td>88.5</td>
<td>Wakeman JM, Ulstch GR. The effects of dissolved O(_2) and CO(_2) on metabolism and gas-exchange partitioning in aquatic salamanders. Physiol Zool 48: 348–359, 1975</td>
</tr>
<tr>
<td><em>Siren lacertina</em></td>
<td>458</td>
<td>0.00347</td>
<td>23.9</td>
<td>Guimond RW, Hutchison VH. Trimodal gas exchange in the large aquatic salamander, <em>Siren lacertina</em> (Linnaeus). Comp Biochem Physiol 46A: 249–268, 1973</td>
</tr>
<tr>
<td><em>Taricha torosa</em></td>
<td>12.5</td>
<td>0.0298</td>
<td>47.1</td>
<td>Wakeman JM, Ulstch GR. The effects of dissolved O(_2) and CO(_2) on metabolism and gas-exchange partitioning in aquatic salamanders. Physiol Zool 48: 348–359, 1975</td>
</tr>
<tr>
<td><em>Lithobates berlandieri</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>3.62</td>
<td>0.119</td>
<td>61.5</td>
<td>Burggren WW, Feder ME, Pinder AW. Temperature and the balance between aerial and aquatic respiration in larvae of <em>Rana berlandieri</em> and <em>Rana catesbeiana</em>. Physiol Zool 56: 263–273, 1983</td>
</tr>
<tr>
<td><em>Lithobates catesbeianus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>5.79</td>
<td>0.0446</td>
<td>54.5</td>
<td>Burggren WW, Feder ME, Pinder AW. Temperature and the balance between aerial and aquatic respiration in larvae of <em>Rana berlandieri</em> and <em>Rana catesbeiana</em>. Physiol Zool 56: 263–273, 1983</td>
</tr>
<tr>
<td>Water-breathing larvae</td>
<td>4.5</td>
<td>0.0567</td>
<td>57.6</td>
<td>Burggren WW, West NH. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog <em>Rana catesbeiana</em>. Resp Physiol 47: 151–164, 1982</td>
</tr>
<tr>
<td>Bimodal larvae</td>
<td>5.3</td>
<td>0.0767</td>
<td>66.7</td>
<td>Burggren WW, West NH. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog <em>Rana catesbeiana</em>. Resp Physiol 47: 151–164, 1982</td>
</tr>
<tr>
<td>Postmetamorphic</td>
<td>4.3</td>
<td>0.0117</td>
<td>11.7</td>
<td>Burggren WW, West NH. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog <em>Rana catesbeiana</em>. Resp Physiol 47: 151–164, 1982</td>
</tr>
<tr>
<td>Adult</td>
<td>228.2</td>
<td>0.00333</td>
<td>18.2</td>
<td>Burggren WW, West NH. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog <em>Rana catesbeiana</em>. Resp Physiol 47: 151–164, 1982</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>28.39</td>
<td>0.0169</td>
<td>40.2</td>
<td>Hutchison VH, Miller K. Aerobic and anaerobic contributions to sustained activity in <em>Xenopus laevis</em>. Resp Physiol 38: 93–103, 1979</td>
</tr>
<tr>
<td></td>
<td>106</td>
<td>0.00582</td>
<td>19.2</td>
<td>Present study</td>
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M\(_{O_2}\), molar O\(_2\) consumption.
changes in metabolic rate and also appear to have little O$_2$ debt to repay. Equally helpful, however, is providing students with the class data and allowing them to explore statistical tests to compare the treatments and to bolster any conclusions they arrive at regarding dive capacity and redistribution.

Problems Routinely Encountered by Students

The primary problem we have encountered is with how students misuse the mathematical formulae, which is usually because they do not use the appropriate units. Some students do not use the correct $\beta$O$_2$, the correct volume, or the correct change in PO$_2$. It is helpful to provide sample calculations in the laboratory and require that students perform calculations while in the laboratory to have them checked. We also have students submit both their raw PO$_2$ data and calculations so that the class data we provide to students are appropriately determined.

In some experiments, frogs may not breathe air at all during the first 20 min of the predive experiment. In some ways, this is a very helpful result as it demonstrates that frogs will voluntarily dive for extended periods of time. However, for an individual group, this can be frustrating, since they do require a control measurement against which to compare their diving measurements. We remind the students that they are measuring O$_2$ uptake with two 20-min intervals, so they should have at least one period of time to compare measurements. Indeed, sometimes, even over a 20-min period of time, some student groups do not demonstrate an appreciable decline in PO$_2$. This is often seen in the air sample and may be due to confusion over how to use the two-way stopcock or failure to ensure that the respirometer is properly sealed.

Due to the sharing of O$_2$ electrodes, and the simultaneous start of experiments, competition for the use of the O$_2$ electrode may occur. We ensure that students take their gas samples and keep the syringes sealed with the use of additional stopcocks. Instructors are also usually responsible for taking the O$_2$ measurements, since polarographic O$_2$ electrodes have a slow response time and patience is required to inject samples correctly. We often connect the O$_2$ electrode to a computerized data-acquisition system so that any injection pressure artifacts can be monitored as an exponential decay before the actual PO$_2$ value is reached.

Polarographic O$_2$ electrodes are notoriously prone to drift, which we have found can be rectified by providing precise temperature control ($\pm 0.1^\circ$C) to the electrode housing and an appropriate calibration gas source. To achieve this, we use a circulating water bath set to 25°C and place a jar of water inside, which is continuously aerated, providing access to a constant calibration source. Calibrations are always obtained in

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Fig. 3. Top: sample class data (means ± SD; $n = 18$) from X. laevis (average mass ± SD: 106 ± 21 g) showing cutaneous (skin), pulmonary (lung), and total O$_2$ uptake over 20-min intervals across three experimental regimes: predive, dive, and postdive recovery. Bottom: plot showing the percent time (means ± SD) that frogs spent underwater or active during the same experimental periods. M$\dot{O}_2$, molar amount of O$_2$ consumption.

Fig. 4. Sample class data demonstrating the contributions of pulmonary (lung) and cutaneous (skin) O$_2$ uptake to total O$_2$ uptake in X. laevis. The strong correlation between pulmonary and total O$_2$ uptake underlies the importance of the lung to responding to the variable O$_2$ demand requirements of the frog, whereas the nearly constant cutaneous O$_2$ uptake values support the notion that cutaneous gas exchange is less capable of reacting to the needs of the frog. Dotted line isopleths indicate the proportion of total O$_2$ uptake.
the same manner as when measuring samples by injecting the calibration water into the electrode housing. Equally importantly is to maintain a large reservoir of dechlorinated tap water at the same temperature that is continuously aerated. This reservoir will serve as the student’s source of aerated water for the experiments and, if properly aerated, students might even forgo taking an initial PO₂ measurement if time is critical.

On some occasions, relatively small changes in air PO₂ may occur. This is where we find that calibrating the electrode properly and often is crucial. Depending on the electrode, it may not be possible to obtain better than ±0.13 kPa consistency in measurements. Some simple solutions to this issue could involve increasing the time frogs are allowed to remain in the closed system or to decrease the volume of the air space to augment the decline in PO₂ that will occur when O₂ is removed by the frog.

Questions to Encourage Students in Their Analysis

The following questions serve as useful lessons to understand the frog’s behavior and physiology:

- In the “natural” condition, when the animal is diving voluntarily (i.e., during the first 40 min), what percentage of the total MO₂ is taken up separately by the lungs and the skin?
- Can the animal maintain this total MO₂ using only the skin during the enforced dive? If it cannot, what do you suspect might be happening?
- Is there evidence of repayment of an O₂ debt during recovery from the enforced dive?
- Is the relative importance of O₂ uptake by the lungs and by the skin during recovery from an enforced dive the same as when the frog was diving voluntarily?
- What scope for change is there for cutaneous respiration?
- What physiological and/or behavioral changes allowed the frog to alter the relative importance of cutaneous respiration?

Finally, we encourage students to compare the measurements of the total metabolic rate in their frogs to an equivalently sized endotherm, such as a mouse. This comparison sheds light on the metabolic differences between ectotherms and endotherms and serves as a reminder for how energetic requirements of endotherms are vastly different from ectotherms. Sometimes, mammalian values are expressed as VO₂ values, typically at a standardized temperature and pressure, so students may choose to convert their total MO₂ to VO₂. Since 1 mol of gas occupies 22.4 liters at 0°C and standard pressure (101.325 kPa), the conversion from moles to liters can be achieved by multiplying MO₂ by 22.4. If the students size match their values to compare an ectotherm with an endotherm, they should obtain values substantially lower (at least one order of magnitude) than those observed for an endotherm, such as a mouse. This comparison reflects the overall lower dependence on cutaneous usage of cutaneous gas exchange and altered diving behavior. Interestingly, frogs may still attempt to breathe from the air space, since O₂ sensors in the upper airways are not known to exist. Nevertheless, the lowered lung O₂ levels resulting from breathing air should reduce the duration of time spent underwater and increase the reliance on cutaneous O₂ uptake.

Reduced cutaneous gas exchange. By reducing PO₂ within the water, a reverse gradient for O₂ can be created, and students could examine the possibility of the skin acting as a potential site for O₂ loss rather than O₂ gain. If frogs were provided with an air space at normoxic levels but hypoxic water at levels significantly below normal venous O₂ levels [5.3–6.7 kPa (39)], students should be able to monitor a rise in O₂ in the water rather than a decline. Since the frogs cannot escape from the water, there will always be some potential for this to occur. The value of this particular exercise is twofold. First, it would demonstrate the passive nature of O₂ diffusion. Second, it would be a potentially ecologically relevant exercise since aquatic habitats are very often hypoxic, whereas the aerial environment where most amphibians inhabit is not.

Comparison of the effect of size on the importance of cutaneous gas exchange. Body size is an important factor in cutaneous gas exchange in amphibians (23). Aside from a few exceptionally large amphibians with extremely low metabolic rates (9, 40) where skin breathing has evolved secondarily as the primary site of gas exchange (17), one would expect cutaneous gas exchange to be less important with increases in body size. Indeed, respiratory capillary density scales negatively with body mass, supporting the overall lower dependence on cutaneous breathing in many amphibians (32). Ranid and pipid frogs show considerable size ranges, and, thus, an additional experiment for some advanced students to pursue would be assessing the influence of body size on the reliance on cutaneous respiration. The adjustment to the protocol would involve developing new dive tanks custom geared for the size of the frog. Our rule of thumb has been to use a total respirometer volume that is not greater than 10 times the volume of the frog and a water volume that occupies ~70–80% of the total respirometer volume. These values can be adjusted with preliminary experiments designed to obtain optimal changes in PO₂ levels in the water and air over 20- or 30-min periods of time.

Confounding role of gas exchange between water and air. In some studies of respiratory partitioning in bimodal breathers, mineral oil has been used to minimize the potential diffusion of O₂ from the aerial space to the water, due to an apparent lower O₂ diffusion coefficient (31). Alternatively, some studies have placed a diaphragm around an animal’s neck to allow for complete separation of the head from the body (19); one disadvantage of the latter approach is that it prevents sponta-
neous, normal behaviors. We have not observed any reason to use an agent like mineral oil, although as a point of discussion, students should be encouraged to examine potential improvements to their experimental design. Students may wish to explore the potential for diffusion to be occurring between the water phase and air phase over the course of their experiments by conducting blank respirometer trials, where instead of aerated water, hypoxic water could be used. If the air space starts at an elevated O2 level and the water starts at a low O2 level, then during the following 20 and 40 min, if diffusion between the two media is occurring, the O2 levels in each system should converge.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: G.J.T. and S.C. conceived and designed research; G.J.T., S.C., and D.M.L. generated figures; G.J.T., S.C., and D.M.L. interpreted results of experiments; G.J.T., S.C., and D.M.L. drafted manuscript; G.J.T., S.C., and D.M.L. edited and revised manuscript; G.J.T., S.C., and D.M.L. approved final version of manuscript.

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