Measuring the Oxygen Consumption of *Plethodon hubrichti* and *P. cinereus* to Find the Metabolic Cause of Range Limitation of *P. hubrichti*:

The Closed Respirometer and the Oxygen Probe.

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Abstract

The Peaks of Otter area, a mountainous region of the central piedmont of Virginia, varies in elevation from 152 m to 1280 m. *Plethodon cinereus* is a salamander that is found at all elevations in this area. *Plethodon hubrichti* is a salamander that is found only at elevations above 600 m. It has been suggested that metabolism is the cause for the range limitation of *P. hubrichti* at lower elevations. The metabolism of salamanders can be determined by measuring oxygen consumption. Two apparati were evaluated to measure the oxygen consumption of these two salamander species at different temperatures to test that hypothesis. An oxygen probe designed for oxygen measurements in aqueous solutions was tested first, and the second apparatus tested was a closed respirometer. After many trials the oxygen probe was rejected due to technical problems. The closed respirometer apparatus was modified several times during repeated trials. The goal was to obtain a dependable apparatus that yielded consistent results. A metabolic difference between the two salamander species could not be determined although several trials were performed.
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*Plethodon cinereus*, also Eastern Redbacked Salamander, is found from the highest peaks in the Peaks of Otter area to elevations below 600 m (Fig. 1). *Plethodon hubrichti*, also known as the Peaks of Otter Salamander, has a very limited distribution and is found only above elevations of 600 m in the Blue Ridge Mountains in central Virginia (Kramer, Reichenbach, Hayslett & Sattler, 1993) (Fig. 2). The limited distribution of *P. hubrichti* has been known for some time, but the reason is still not known.

*Figure 1*. The distribution of the Eastern Redbacked Salamander or *P. cinereus* (ARMI, 2002b).
This restriction is probably not due to *P. cinereus* overtaking and impinging upon *P. hubrichti*. A contact zone study was done by Wicknick in 1995 (cited in Aasen & Reichenbach, 2004), where she counted the number of salamanders of each of the two species in an isolated allopatric site (only one species found) and an overlapping sympatric site (both species found). These sites were revisited by Aasen and Reichenbach (2004) in 2003 and the number of animals of each species was counted again using the same methods as Wicknick. The results showed that there was no significant change in the salamander population of the two species at these sites which suggested that the contact zone between these two species was most likely static.

Environmental temperature (which varies at different elevations) has also been investigated as a range-limiter in salamanders. Each species of salamander has an ideal temperature range at which it survives best. Below or above that temperature range the animals lose function and eventually die. The upper limit of a salamander’s temperature
is called the critical thermal maximum (CTM), and has been defined as “value that is the arithmetic mean of the collective thermal points at which locomotor activity becomes disorganized and the animals lose their ability to escape from conditions that will promptly lead to their death” (Hutchison, 1961, Summary, para. 1). Hutchison (1951) was one of the first to conduct experiments to determine the critical thermal maxima of different species and subspecies of salamanders. Hutchison wanted to see if there was a relationship between the CTM and the topographical niche that salamanders inhabited. The results showed that the salamander species and subspecies tested had different CTMs and that there was a correlation between the CTM and the habitat and range that these salamanders occupied. Hairston (1951) also investigated the distribution of several salamander species and subspecies and found that they were limited in their vertical ranges. Hairston determined that the limited distribution was due in some cases to interspecies competition and competitive resistance and in some cases to the local climate. Ozak and Wiens (2006) came to the same conclusion based on their study of the distribution and isolation of North American salamanders. They found that the local climate caused the isolation and fragmentation of some populations. The salamanders were limited to certain locations because they were incapable of adapting to higher temperatures and drier conditions. Spotila’s (1972) research provided the proof that different species of salamanders indeed preferred different temperature ranges.

Environmental temperature (which varies with altitude) is thought to affect a salamander’s hydration state. Lungless salamander species perform gas exchange through their cutaneous and buccopharyngeal surfaces (Full, Anderson, Finnerty & Feder, 1988; Howard, 2003). The high permeability of their skin, though, allows water to be easily lost
from their body (Feder, 1983). Water loss that is unchecked can cause dehydration and death. When salamanders become dehydrated their body fluids become more concentrated and the water absorption rate through the skin increases (at a rate dependent on the soil moisture tension). When the soil moisture tension is lower than the osmotic pressure of the salamander’s body fluids, the salamander absorbs water. Since water loss cannot be controlled directly, the salamanders have to adjust their behavior to keep a balance. They can detect the humidity in the air and make changes accordingly. For example, salamanders are active at night when the humidity is high and the vapor pressure deficit (VPD) is low to decrease the dehydration rate. After foraging, the salamander will burrow into the soil to rehydrate (Spotila, 1972). Spotila conducted dehydration and humidity experiments to study how temperature affected the water loss of salamanders and how that influenced their distribution. He found that at higher temperatures the VPD increased, which caused the dehydration rate of the salamanders to increase. The dehydration rate was also affected by the size of the salamander; larger animals dehydrated at a slower rate. In the humidity experiments, salamanders were placed in a tube with varying humidity. The salamanders always migrated to the highest humidity to prevent water loss. Spotila’s results showed that the geographical niche a salamander inhabited had to enable it to keep its water balance and was therefore restrictive. Salamanders were restricted in elevation, because at higher elevation the temperature is lower and the humidity is higher. For example, *P. jordani* could tolerate a wide range of temperature as well as relative humidity (RH) and could therefore adapt to a wide range of elevations. *P. glutinosus*, on the other hand, had a low tolerance to temperature and was restricted to live at higher altitudes with a water source nearby.
Environmental temperature is thought to affect a salamander’s body condition. Research shows that salamanders are limited to habitats with favorable temperatures to maintain body condition. It is important for salamanders to maintain a positive energy balance and to do this they must take in more energy than they expend. Favorable temperature and moisture influences the time salamanders can spend foraging to obtain food for energy, which in turn affects the reproduction and size of salamanders. The frequency that females lay eggs depends on excess energy, which means the females are more productive in favorable conditions where they can obtain enough energy (Spotila, 1972). Reproduction also depends on the size of the female. The larger the mass of a female, the more eggs it produces (Finkler & Cullum, 2002). The growth and size of a salamander depends on the amount of time spent actively above soil obtaining energy. If the conditions are not favorable the animal spends more time in the soil without eating until the conditions are favorable (Feder, 1983). Furthermore, the shorter the growing season of a salamander the smaller in size the salamander will be. In turn, smaller animals dehydrate faster than larger animals and larger animals can forage for longer periods of time to obtain food (Spotila 1972). Hutchison (1961) found that salamanders, acclimated at room temperature, that were transferred to lower temperatures gained weight and those that were transferred to higher temperatures lost weight. He reasoned that the higher metabolic rate at higher temperatures could cause the weight loss in the salamanders.

Metabolic rate (as judged by oxygen consumption) may be a range-limiter in salamanders. Different factors influence the oxygen consumption of salamanders. Wood and Orr (1967) found a direct correlation between the size and oxygen consumption of
the salamanders (heavier animals had a higher oxygen consumption rate). Full suggested that the larger the salamander, the more limited it is in obtaining sufficient oxygen (1986); his experiments reinforced this (Full et al., 1988). The reason for this is the smaller surface area to volume ratio of the larger animals (Hillman, Shoemaker, Putnam & Withers, 1978). There was also a difference in the oxygen consumption between males and females. Males had lower oxygen consumption rates than either gravid (those carrying eggs) or nongravid females (Finkler, Sugalski & Claussen, 2003) and gravid females had the highest oxygen consumption rate (Finkler & Cullum, 2002). Norris, Grandy, and Davis (1963) tried to determine if oxygen consumption rates restricted salamanders in their distribution. They found that the oxygen consumption of salamanders increased at higher temperatures. Furthermore Norris and his colleagues suggested that some salamanders were more tolerant of higher temperatures than others, because the peak oxygen intake of the salamanders varied. Some salamanders had their peak oxygen intake at 30 °C, some at 25 °C, and some at 20 °C. The salamander’s heat tolerance correlated with the local climate of its habitat. This study showed that the oxygen consumption of salamanders was directly related to their metabolism. At higher temperatures, the destruction of enzymes can exceed the transformation of substrates resulting in two salamander species having different temperature tolerance but living in the same habitat due to difference in their metabolic machinery (Norris et al.).

Reichenbach and Brophy (2007) suggested that *P. hubrichti* is restricted in its distribution to elevations above 600 m due to their intolerance of the higher temperature and lower humidity at lower elevations. This hypothesis only accounted for the allopatric sites ranging in elevation between 518 m and 991 m. At the sympatric sites they suggest
that *P. hubrichti* is restricted due to competition with *P. cinereus*. Their study showed that the density of the *P. hubrichti* population decreased as elevation decreased. The density ranged from 0.059 / m² at the highest elevation and 0.002 / m² at the lowest elevation. They also counted the number of eggs per female and found that this number depended on the two factors: elevation and mass of the female. A decline in elevation and the lower mass of the female resulted in the decrease in number of eggs per female. The average number of eggs ranged from 1 at the lowest elevation site to 9.4 at the highest elevation and peaked at 10.2 at the 991 m site. These results correlate with the density data collected. Reichenbach and Brophy also determined the surface temperatures of the different sites using ibuttons® and found a direct correlation to elevation. The data they collected showed that the temperature decreased by 1 ºC for every 239 m the elevation increased (Fig. 3). The CTM of *P hubrichti* and *P. cinereus* was determined as well to see if there was a difference between the two species. The results showed that the average CTM of *P. hubrichti* was 33 ºC and 34 ºC for *P. cinereus*. This shows that *P. cinereus* can better acclimate to the increase in temperature at lower elevations than *P. hubrichti* and hence is not limited to higher elevations. Reichenbach and Brophy suggested that the intolerance of *P. hubrichti* to adapt to higher temperatures was due to a higher metabolic rate. This would explain why at lower elevations, the number of eggs per female decreases since there is less energy available. This also explains why the population density decreases with elevation since females have fewer eggs.
The graph in Fig. 3 was replicated from a powerpoint and not from a published source; therefore the citation is personal communication.
develop a reliable apparatus to quantify oxygen consumption (and therefore metabolic rate) of salamanders. Two methods, a closed respirometer (also called a closed manometric respirometer) and an oxygen electrode, were tested to find which method was the best for these measurements. Once chosen, the apparatus could be used to measure the SMR of both salamander species at 10 °C, 15 °C, 20 °C, and 25 °C. The results of the two species could be compared to see if the average SMR of *P. hubrichti* was higher than that of *P. cinereus*. This would reinforce the idea that *P. hubrichti* cannot survive at lower elevations due to higher temperatures.

**Method**

**Subjects**

*Obtaining and Maintaining Salamanders*

*P. hubrichti* and *P. cinereus* are both in the lungless plethodontidae family, the amphibian class, and urodela order (Allaby, 1999). The Peaks of Otter Salamander has been labeled as a species of special concern in the state of Virginia due to its limited distribution. This salamander can reach a length of 8.1 cm to 12.2 cm. The Redbacked Salamander is slightly shorter and can reach a length of 5 cm to 10 cm. The two salamander species can easily be identified by their skin color. The belly and sides of the Peaks of Otter Salamander are dark gray or black and its back is black or brown with brassy spots (Fig. 4) (Mitchell & Wicknick, 2000-2008). The Redbacked Salamander is found as two different morphs. Both morphs have white and black spots on their belly, but the red-backed morph has a dark body and a red strip that starts behind its head and extends to the tip of the tail (Fig. 5). The lead-backed morph is a little harder to identify because it lacks the red strip (Fig. 6) (Howard, 2003).
Figure 4. The Peaks of Otter Salamander (Mitchell & Wicknick, 2008).

Figure 5. The red-backed morph of the Redbacked Salamander (ARMI, 2002a).

Figure 6. The lead-backed morph of the Redbacked Salamander (Howard, 2003).

© 2005 John White was cited as the photographer in the web article of Mitchell and Wicknick (2008).
The salamanders were collected on September 15, 2007 at the elevation site of 975 m near Apple Orchard Mountain. This was done by turning over rocks and logs, collecting the salamanders, and storing them in zip lock bags. The rocks and logs were put back in their original location to avoid destroying the habitat. The salamanders were then taken out of the bags and sorted. Only mature males and females were kept and separated by species. They were placed in larger, labeled zip lock bags with soil from the collection site. Squirt bottles containing dechlorinated water were used to drench the soil in order to keep the salamanders moist and hydrated. The zip lock bags were then stored in coolers during transportation to keep the salamanders cool. The animals that were not used were returned to the collection site immediately after the selection and placed back under the rocks. The salamanders selected for the experiments were taken out of the coolers and stored in plastic containers that were weighed down with rocks so they could not escape. Soil was put into the containers and drenched regularly with dechlorinated water to keep it moist. The containers were stored in refrigerators at about 3 ºC. Several weeks before the experiments were conducted several salamanders of each species were put in an environmental chamber (Conviron CMP 3244), a device that simulates a desired environment, and acclimated at 20 ºC.

**Experimental Conditions of Salamanders**

*The effect of season and age on metabolism.* The time of year that salamanders were collected and the age of salamanders influences their metabolism. To eliminate any metabolic differences due to season, the salamanders were all collected at the same time during the fall. The influence of age on the metabolism of salamanders was taken into
account by selecting only the mature (able to reproduce) salamanders to be used in the experiments for this study (Hutchison, 1961).

*The effect of size (weight) on metabolism.* Body weight influences the metabolism of salamanders (Ryan & Hopkins, 2000) and especially when comparing two different groups of animals the weight of the animals needs to be taken into account with metabolic calculations (Feder 1976). To account for the weight, the salamanders (males and females) were weighed before the experiments were conducted. The animals were not weighed after the experiment, because previous research has shown that the mass of the animals did not change more than a few milligrams during experiments, some of which lasted up to six hours (Lamprecht, Matuschka & Schaarschmidt, 1990). Using their weight, the amount of oxygen consumed was determined per gram of the salamander.

*The effect of sex and reproductive condition on metabolism.* Research demonstrated that males have a lower metabolic rate than females because the production of eggs requires more energy than the production of sperm. Other research investigated the metabolic differences between gravid and nongravid females (Finkler et al., 2003). Gravid females were shown to expend more energy and have a higher metabolic rate than nongravid females (Finkler & Cullum, 2002). Another difference in the metabolism between males and females found was the metabolism to size ratio. The metabolism of males increases with mass whereas the metabolism of females stays constant with increasing mass. This is due to larger females producing more eggs than smaller females therefore expending the same amount of energy as smaller females (Ryan & Hopkins, 2000). A mixed population of females and males of both species was used for the experiments in this study to get an average of the metabolic rate of both species. It was
not determined if the salamanders used in the experiments were nongravid or gravid females. The salamanders were taken randomly in regard to sex or gravid state to get an average.

_The effect of food on metabolism._ When measuring the oxygen consumption of an animal, the food substances being metabolized by that animal (if any) should be taken into account. Food present in the gastrointestinal tract increases the rate of metabolism because different food substances cause different amounts of heat to be liberated (per unit volume of oxygen consumed) when they are oxidized (Hill et al., 2004). Ryan and Hopkins (2000) stated in their research that salamanders should be fasted for at least 48 hours prior to conducting any metabolism experiments. This allows their gut to empty. The animals used in this study were given no food a few weeks prior to running the experiments. They were not endangered by this fasting period since they were held at cold temperatures to keep their metabolic rate low.

_The effect of physical activity and photoperiod on metabolism._ Physical activity also plays an important role in metabolism because it increases the metabolic rate (Full, 1986). During the experiments in this study locomotion did not play a role in the metabolism of the salamanders, because they typically remained stationary except for small spontaneous movements of the head. It has been demonstrated that photoperiod affects the metabolism of salamanders. During light periods salamanders had a higher metabolic rate than during dark periods (Wood & Orr, 1969). The experiments for this study were conducted in constant darkness so differences in photoperiod would not affect the metabolic rate of the salamanders.
The effect of stress and temperature on metabolism. To minimize stress, which also plays a significant role in the metabolism of salamanders, the salamanders were acclimated to 20 ºC for at least one week prior to conducting the experiments. An acclimation period of one week has been suggested in literature when conducting metabolic experiments on salamanders (Feder, 1976). The salamanders were also placed into the working chamber for at least one hour before the experiment was conducted to minimize stress levels. Temperature plays an important role in metabolism of salamanders (Norris et al., 1963); therefore the temperature during the experiments must be held constant with a maximum temperature flux of 1 ºC (Hutchison, 1961). Each animal was used only once for an experiment to make sure the measurements were independent.

Materials

Oxygen Electrode

Background information. Leland C. Clark developed the first oxygen electrode. An oxygen electrode consists of two metallic electrodes, an anode and a cathode, that are covered by electrolyte fluid and a thin organic membrane that reacts with oxygen. A specific negative voltage runs to the cathode which reduces only oxygen and no other compound. The diffusion of oxygen across the membrane depends on the partial pressure of the oxygen which produces a current equal to the oxygen amount in the liquid being measured (Mitchell, 2006). When viewed and drawn under a microscope, the wire, the electrode at the very tip of the probe, and the Teflon membrane covering the tip can be seen (Fig. 7). The actual oxygen content of a liquid sample is calculated by taking into account the cathode current, temperature of the sample, temperature of the membrane,
pressure, and salinity (Mitchell, 2006). The probe is so sensitive that a temperature change of 1 °C can influence the reading by 6 % (Falck, 1997). Therefore, the probe has to be equilibrated with the temperature of the liquid being measured and the solution must be stirred constantly during an experiment (Mitchell, 2006).

*Figure 7.* Drawing of the lower part of the oxygen probe as seen under a bright field microscope at scanning power. The electrode A) was at the very tip of the probe. A rubber ring B) held the Teflon membrane C) in place, which covered the tip of the probe. The wire D) carried the current. The probe was filled with electrolyte fluid E). The walls of the lower part of the probe were made of glass F).

*The apparatus setup.* An ISE-730 oxygen electrode, which is derived from the Clark electrode, and a DO2-100 Current-to-voltage adapter from iWorx were used for the experiments. The probe contained a platinum cathode and a silver anode with a voltage of
-0.80 V across them. The DO2-100 device amplified the output current of the probe so that it could be recorded by a data acquisition unit. The amplification was 10 mV per nanoampere of current received from the probe. The apparatus was set up by connecting the computer via an USB cable to the iWorx data acquisition unit. A DIN8-maleDIN8 cable was used to connect the data acquisition unit to the output connection on the amplifier. The oxygen probe was then connected to the input connection, which is the BNC connector on the amplifier (iWorx, 2006).

The oxygen probe. There were four oxygen electrodes which were all used in the experiments. The oxygen probes were prepared by transferring the electrolyte fluid, a 3.0 M potassium chloride solution, into the tip using a plastic transfer pipette. The solution was dripped along the edge so no air bubbles would get trapped in the tip of the probe. The tip was then screwed back on the probe loosely so that the electrode did not extend through the membrane. The probe was soaked in distilled water after use.

Calibration experiments. The oxygen probes had to be calibrated with a deoxygenated solution and an oxygenated solution before use in experiments to set the minimum and maximum values (Falck, 1997). The measurement of oxygen content (S) in deionized water (100 % saturated) at 26 °C, 760 mm Hg atmospheric pressure, and 21 % oxygen in air should be $S = 252 \mu\text{MM O}_2$ (iWorx, 2006). The first calibration experiments were done in liquid solutions. For the deoxygenated solution, 1.3 g of sodium dithionite was added to 0.5 L of distilled water to make a 15 mM / L solution. Diet Coke, that was acclimated to room temperature and then used right out of the can, was used as the deoxygenated solution in some calibration experiments instead of the 15 mM / L solution. Deionized (DI) water was used as the oxygenated solution in the
calibration experiments. The experiments were conducted at room temperature although the temperature fluctuated one to two degrees (Celsius) during the experiments. The atmospheric pressure was measured during the experiments with an aneroid barometer and found to fluctuate about 0.01 to 0.02 mm Hg during the experiments.

The solution to be measured was put into a 250 mL beaker which was then placed on a magnetic stirrer (Thermolyne Cimarec® 2). A medium sized stirring rod was placed in the beaker and the magnetic stirrer was turned on. The speed was adjusted so that the stirring rod rotated quickly and evenly. The temperature of the solution was equalized to the room temperature. The prepared probe was adjusted to the temperature of the liquid being measured by placing it in the solution for about half an hour prior to conducting the experiment. The temperature of the liquid was monitored throughout the experiment with a thermometer and found to fluctuate one to two degrees (Celsius). When placing the probe into the solution it was submerged as far as possible without touching the stirring rod. For the calibration experiments, a value was taken only after the voltage reached a stable line. The value of the deionized water was used as the maximum value and labeled “Saturated DI water”. The 0 % calibration solution was prepared using sodium dithionite or coke. These were the minimum values and labeled “No Oxygen”. The probes were left in the solutions anywhere from 30 minutes to 1 hour.

The probes did not measure a big voltage difference between the two calibration solutions. Several trials were conducted, but the results were not consistent between experiments. A representative of the iWorx company, who had used the probes in liquid experiments, was contacted, in person, and asked for advice about the proper use of the probe and the equipment. The probes were sent to the company that made them to check
for any damage that could cause these errors. They were returned with the explanation that the tips of the oxygen probes are not to be screwed on too tightly. The electrode at the tip of the probe should not protrude through the membrane. The membrane could get stretched or even pierced, resulting in erroneous results. Electrical interference was observed in the recordings as well, but could not be eliminated.

 Calibration experiments were then done using gases to see if the probes would measure the oxygen concentration in gas as well as in liquid. The % oxygen versus the voltage the probes measured could be graphed. If the relationship between 100 % oxygen and 0 % oxygen proved to be a straight line, then a third point of reference could be determined such as the oxygen concentration of air, which is known to be 20 %. This would establish a reliable standard line that could be used in the metabolic rate experiments on the salamanders to determine the % oxygen they consume. The difference in oxygen concentration at the beginning of the experiment and at the end would give a value that could be inserted into that line to get an accurate measurement of the % oxygen the salamander consumed. Pure oxygen gas was used as the 100 % calibrating gas and helium and carbon dioxide gas were used as the 0 % calibrating gas. The gases were in tanks and the probes were flushed with the gas until a measurement was taken. To determine if the measurements in gas and liquid were consistent, the oxygen concentration of the calibration liquids was measured right after the measurements of the calibrating gases.

*Closed Respirometer*

*Background information.* A device that measures gas exchange is called a respirometer. There are two types of respirometers-- the closed respirometer and the open
respirometer. Both are typically used to measure the oxygen consumption of animals to
determine their metabolic rate. The open respirometer allows air to flow through a
chamber containing the animal whose oxygen consumption is being measured. An
oxygen meter can detect the oxygen concentration of the air before it enters the chamber
and after it leaves the chamber via an electrochemical or paramagnetic cell. The oxygen
consumption can be calculated by dividing the amount of air flow through the chamber
over the unit of time and the difference in the oxygen concentration before and after the
experiment. The open respirometer requires more costly equipment than the closed
respirometer. However, the open respirometer has the advantage in that one knows the
rate of oxygen consumption of an animal at all times during the experiment (Hill et al.,
2004).

The closed respirometer consists of two chambers that are identical in size and
content (Hill et al., 2004). In literature they are usually referred to as the working
chamber that contains the animal, and the reference chamber (Lamprecht et al., 1990).
The two chambers are connected by a manometer. A manometer is a U shaped tube that
is filled with a colored fluid, which measures the pressure changes of gas in the
chambers. A syringe is inserted into the working chamber and is used to adjust any
pressure changes. The system is closed to exclude the influence of factors in the
environment on the volume of gas inside the apparatus. If the environmental temperature
or pressure changes, the gas volume inside the closed respirometer is not affected since
both chambers are in a closed system. Therefore the barometric pressure does not have to
be measured throughout the experiment (Finkler et al., 2003). As the animal in the
working chamber breathes in oxygen and breathes out carbon dioxide, a substance inside
the chamber absorbs the carbon dioxide. The carbon dioxide-absorbing substance is placed in both chambers and the atmospheric pressure and temperature is kept constant so nothing but flux in oxygen influences the movement of the manometric fluid. This causes the pressure to change in the working chamber due to the oxygen consumption of the animal since only the carbon dioxide is being absorbed. There is no gas pressure change in the other chamber so the manometric fluid adjusts and shifts. The syringe is used to insert air back into the chamber and to readjust the manometric fluid to its initial position. At the end of the experiment, the amount of oxygen injected with the syringe into the chamber is equal to the amount of oxygen consumed by the animal. The oxygen consumption is measured over the time the experiment was conducted (Hill et al., 2004). The experiments in this study were conducted using a closed respirometer as described by Hill, Wyse, and Anderson (2004).

Experimental apparatus. The first respirometer (Fig. 8) was assembled using a 50 mL Erlenmeyer flask with a test tube containing 17 potassium hydroxide (KOH) pellets, some paper towel, as well as about 10 mL deionized water inside. The animal was placed inside the flask last. A size 6 stopper with two holes was placed on the flask. A 1 / 10 mL pipette was heated over a Bunsen burner and bent on one end at a 90 ° angle. The bent pipette was placed in one hole of the stopper and some dye was inserted into the open end of the bent pipette. A shortened piece of pipette was inserted into the other hole in the stopper. A rubber tube about 4 cm long was attached to the piece of pipette and a clamp was fastened to the end of the rubber tube. A second identical apparatus was set up as a control, only without the animal. The apparatus was very sensitive to even the slightest change in temperature and pressure. That made it almost impossible to work on the
chamber without causing the dye to be drawn into the flask or pushed out before the experiment even started. The oxygen consumption of the salamander was hard to detect due to sudden movement of the dye caused by environmental factors. Even with a control set up, it was hard to determine what the displacement of the manometric fluid was caused by. Another problem was that the experiments with this apparatus could only be run for only short periods of time. The bent pipette was not very long and the experiment had to be stopped when the dye reached the end of the bent pipette, before it was drawn into the chamber. If a syringe was inserted into a third hole in the stopper, the manometric fluid could be readjusted and the experiment could run for longer periods of time. However, that required handling of the apparatus which caused an increase in temperature and erroneous movement of the dye. The apparatus was discarded due to these problems.
Figure 8. Drawing of the first respirometer apparatus assembled. It consisted of a 50mL Erlenmeyer flask A), a number 6 stopper with two holes B), a bent 1/10mL pipette C), and a clamp D) fastened to a piece of rubber tubing E). A glass tube F) containing KOH pellets G) was placed inside the flask along with the salamander H), a piece of paper towel I), and deionized water J). A drop of methylene blue dye was inserted into the tip of the pipette K).

The second apparatus (Fig. 9) was a closed respirometer that was assembled using two 300 mL Erlenmeyer flasks. The flask size was reduced to 150 mL and then to 50 mL due to the small amount of oxygen the salamanders consume. The larger the flasks were, the longer the experiment had to be run before the oxygen consumption of the salamander could be detected. Two size six stoppers with two holes in each were used to seal off the two flasks. A 1 / 10 mL pipette was heated in the middle over a Bunsen burner and shaped into a U-form. The U shaped pipette (manometer) was then filled with diluted
methylene blue dye. This dye clung to the walls of the pipette so that it took more
pressure initially to move the dye to overcome the hydrogen bonds. Phenol red, which
contains alcohol, was used in some trials, the expectation was that it would adhere less to
the walls since it was less viscous than the methylene blue dye. The phenol red was
diluted to different concentrations with water and each dilution was used in experiments
and compared.
Figure 9. Drawing of the second closed respirometer apparatus assembled. It consisted of two 50mL Erlenmeyer flasks A), two number 6 stoppers with two holes each B), a 12cc syringe C), and a short piece of rubber tubing D). A glass tube E) was filled with 2g of KOH pellets F) and placed inside each flask. 10mL of deionized water I) was placed inside each flask as well along with 10cm x 10cm of paper towel G). The salamander H) was placed inside the working chamber I), but not inside of the reference chamber II). A bent 1/10mL pipette J) was placed between the flasks and connected to the flasks with rubber tubing K). Rubber tubing M) was also used so a clamp N) could be attached to close off the system. Methylene blue dye L) was inserted into the U-shaped pipette.
To make the manometer, two pieces of rubber tubing were connected from either end of the U-shaped bent pipette (see drawing in Fig.9). The other ends of the tubing were connected to short pieces of pipette. The pipette pieces were about 8 cm in length and had the rubber tubes attached. They were inserted into the hole in the rubber stopper of each flask so that the manometer was located between the two flasks. A 12 cc syringe was inserted into the second hole of the working chamber. Another broken pipette piece with a 4 cm long rubber tube attached was inserted into the second hole of the reference (without the animal) chamber. A clamp was attached at the end of the short rubber tube. The rubber tube fitted snugly on the pipette and the connection points were sealed with silicone lubricant to eliminate any air leaks. A small, thin, glass test tube was filled with 16 to 17 KOH pellets that weighed 2 g to absorb the carbon dioxide. These tubes were placed inside of both flasks. The amount of KOH was later increased to 3 g to ensure there was enough KOH to react with the carbon dioxide during longer experiments. The glass tubes were long and extended into the neck of the flask so the salamander could not get to the opening. KOH would be lethal if salamanders came in contact with it. It strongly irritates their skin, inhibits their gas exchange, and results in their death. A piece of 10 cm x 10 cm paper towel was placed into each of the flasks along with 10 mL of DI water to keep the salamanders from dehydrating. Due to temperature fluctuations between 1 °C to 2 °C during experiments, the flasks were submerged in 600 mL beakers filled with water that was adjusted to room temperature. The flasks were held under water with stoppers. A control was set up that was an exact replica of this apparatus, except that the working chamber contained no salamander.
The final apparatus (Fig. 10) was a modification of the second closed respirometer and was the one that was used in the recorded experiments. This apparatus was made with two 75 mL polycarbonate vials. Clear, vinyl tubing was chosen because it formed a tighter fit with the pipette than the tubing used for the second apparatus. Instead of connecting the end of the tubes to short pipettes they were directly inserted into the holes in the size 6 stoppers, thereby eliminating two connections in the apparatus. The vinyl tubing was also directly inserted into the second hole of the reference chamber with a clamp attached, eliminating another connection present in the second respirometer apparatus. By removing these connections, possible air leaks at these places were eliminated. The tip of the 12 cc syringe was inserted into a very short piece of vinyl tubing which was then inserted into the second hole of the working chamber. The fit was very tight so that no air could leak through. All connections were sealed off using silicon lubricant. A glass tube was filled with 3 g of KOH pellets and placed inside the flasks. Instead of using the thin test tubes used in the second apparatus, shorter, thicker, and wider glass tubes were used. This allowed for more exposure of the KOH pellets to the air in the chamber. The opening of these glass tubes was covered with elastic mesh that was held in place by a rubber band to keep the salamanders from getting in contact with the KOH. A piece of 8 cm x 8 cm paper towel, and 7.0 mL of DI water was placed inside both the polycarbonate vials. The vials were submerged in 600 mL beakers filled with water. Instead of using stoppers to hold the tubes in the water, the test tubes were held down with rubber bands so less water would be displaced.
Figure 10. Drawing of the final closed respirometer apparatus assembled. It contained two 75mL polycarbonate viles A), two number 6 stoppers with two holes each B), and a 12cc syringe C). A glass tube containing 3g of KOH pellets covered by elastic mesh that is held in place by rubber band D) was placed inside each of the viles. A 8cm x 8cm paper towel and 7.0 mL of DI water E) were placed in each of the viles as well. A U-shaped 1/10 mL pipette F) containing a few drops of phenol red dye G) was attached to the viles via vinyl tubing H). A clamp I) was attached to a piece of vinyl tubing to close off the apparatus.

**Experimental Procedure**

The experiments were conducted at 20 °C, because Reichenbach and Brophy (2007) determined that a difference in the CTM of the two salamander species could be seen at that temperature. Therefore, a metabolic difference should be seen at that
temperature as well. Time did not allow for metabolic experiments at different temperatures. The 600 mL beakers were filled with water and placed into the environmental chamber at 20 °C with a thermometer for the temperature to adjust. The two polycarbonate vials and their components were assembled including the KOH in the tubes, the paper towel, and the DI water. The already acclimated salamander was removed from its container in the environmental chamber, weighed, and placed into the working chamber. This eliminated any stress on the salamander during the experiment since it had time to adjust to the new environment. The rest of the apparatus was put together. To check for any leaks, a certain amount of air was inserted into the apparatus using the syringe to see if the manometric fluid shifted accordingly. If there was a leak, the manometric fluid would not displace the same volume of air inserted with the syringe. Once it was determined that the apparatus was air tight, the syringe plunger was placed in a position that left room for adjustments of the displacement of manometric fluid in both ways. The whole apparatus was submerged in the water-filled beakers that were equilibrated to the temperature of the environmental chamber. This buffered any temperature change inside the environmental chamber when working on the apparatus or readjusting the syringe. During an equilibration period of at least one hour, the clamp was not closed so that the whole apparatus could adjust to the experimental temperature. Every connection was greased again to make sure there were no leaks. The clamp was then closed and the pressure inside the apparatus was allowed to equilibrate for another 30 minutes or longer. This crucial step was to prevent any pressure difference in the chamber that was not due to the oxygen consumption of the salamander to affect the movement of the manometric fluid (Feder, 1976; Summerson, 1939; Hillmann et al.,
After the manometric fluid was adjusted and the pressure was equilibrated in both chambers, the syringe was used to adjust the manometric fluid so that it was level in both arms. Then the initial volume of air inside the syringe was recorded, along with the time at the start of the experiment. This was done on the control apparatus as well. The manometric fluid was not allowed to be displaced more than 2 mm before it was adjusted to its original position by inserting or removing air from the syringe. After a few hours the volume left in the syringe was recorded along with the time at the end of the experiment.

The total amount of air inserted with the syringe was determined from the recordings at the beginning and end of the experiment. This volume was equal to the volume of oxygen consumed by the salamander. To calculate the oxygen consumption of the salamander, any changes in the air volume of the control (should be zero) was subtracted or added to the volume of air inserted with the syringe in the main apparatus. This would take into account any changes due to factors other than the oxygen consumption of the salamander. The resulting volume in mL was then divided by the time in hours that the experiment was conducted and the weight of the salamander in grams (mL/hr x g).

Results

*Calibration Experiments with the Oxygen Probe*

*Calibrations in Liquid Solutions*

When measuring the oxygen content in the 0% solution, using the oxygen probes, a stable base line at 0 V was reached. When measuring the oxygen content of 100% solution the voltage jumped up by 2 V and then rapidly declined back down to 0 V. Two
of the probes measured 4.987 V for the deoxygenated solution and 4.62 V and 4.789 V for the oxygenated solution. The difference between these maximum and minimum values was very small (-0.028 V and -0.198 V). In some experiments the probe read a higher voltage for the deoxygenated solution than the saturated solution. The oxygen concentration in regular tap water was measured as 4.987 V. In the analysis mode, which zooms in on the recorded voltage, a response to electrical interference could be seen. It produced voltage variations not due to the oxygen concentration in the solutions. The electrical interference produced a consistent pattern of small voltage cycles. Even when tapping with the foot on the floor or just moving a hand a sudden increase in voltage could be seen that was larger than the voltage difference between the two calibration solutions. Further trials using all four probes could not determine a consistent value for a maximum and minimum value.

*Calibrations in Gas*

When measuring the voltage of pure oxygen gas the probes reached their maximum value at 4.987 V in half of the experiments conducted (5 out of 10). The values for the other 5 experiments were 0.1 V, 1.4 V, 1.4 V, 1.6 V, and 1.9 V. The calibrations with helium and carbon dioxide gases measured voltages around 0 V (+/- 0.03 V) in 7 out of 8 experiments. The measurements of the calibration liquids done using the same probe right after the measurements of the calibration gases were inconsistent. When the probes measured 0 V for the helium (He) and carbon dioxide (CO₂) gas and 4.987 V for the oxygen gas (O₂), the measurements in the liquid were still erroneous. There was no significant difference in voltage between the calibrating solutions. Repeated experiments had no consistent results.
Closed Respirometer

First Apparatus

No reliable data could be obtained from experiments using this apparatus.

Second Apparatus

Data obtained with this apparatus was inconsistent. The oxygen consumption rates measured ranged from 0.031 mL / hr x g to 0.798 mL / hr x g. The large difference shows that these measurements were erroneous. Twelve trials were run and produced the following oxygen consumption rates in mL / hr x g: 0.238, 0.063, 0.798, 0.046, 0.105, 0.119, 0.121, 0.239, 0.14, 0.06, 0.031, and 0.069 respectively.

Final Apparatus

The oxygen consumption data collected for the Redbacked salamanders was 0.076 mL / hr x g and 0.098 mL / hr x g. The oxygen consumption data collected for the Peaks of Otter salamanders was 0.085 mL / hr x g, 0.094 mL / hr x g, and 0.055 mL / hr x g.

Discussion

Experimental Conditions of Animals

When selecting the sample groups of the two salamander species used in the experiments, the sex and gravid or nongravid state of the salamanders were not taken into account. A more accurate average can be determined if a certain number of males, and nongravid and gravid females of each species is used. Measurements of a randomly mixed group do not give a true average due to the metabolic difference of female and male salamanders (Finkler et al., 2003). If more males than females were present in the sample group of one species and more females than males in the other group, the
metabolic rate could be different due to the gender and not due to differences between the species only. All the other factors that influence the metabolism of salamanders including season, size, age, food, activity, photoperiod, and stress were taken into account. The experiments were conducted on only a two to three salamanders of each species, but to get a true average at least 20 animals of each species should be used. Salamanders at different sites should also be compared in metabolic rate experiments. This would help determine the difference between the two species at different elevations.

*Oxygen Electrode*

The oxygen electrode seemed, initially, to offer several advantages. It seemed more practical in measuring oxygen consumption than the closed respirometer because it can be run without any monitoring throughout the whole experiment. This set up also required less work than the closed respirometer apparatus. Furthermore, the data can be analyzed, graphed and calculated by the computer. However, the oxygen probe is more expensive than the materials needed for the closed respirometer. Although the oxygen probe is designed to measure oxygen concentration of liquid solutions, measuring the oxygen concentration of water in the chamber containing the salamander is indirectly measuring oxygen consumption. The probe can also measure oxygen concentration of moist air, which is more direct in measuring the salamander’s oxygen consumption.

*Experiments in liquid.* When calibrating the oxygen probes in liquid solutions, the measurements were inconsistent and inaccurate. A fundamental problem was the absence of a significant voltage change with two different oxygen concentrations in the calibrating solutions. This could have been due to problems with the actual electrode. The difference in voltage between the deoxygenated and saturated solutions was very small.
In some experiments regular tap water measured 4.987 V, which was higher than the voltage of the maximum calibrating solution (100 % or saturated solution). A company technician suggested that the probe could have had a damaged membrane due to the electrode tip protruding too far into the membrane. This was corrected (if the membrane was not already punctured) by unscrewing the tip of the probe. The results, however, were not any different after this correction. A damaged platinum electrode or an air bubble located in the probe have been noted by the company that manufactured the probes as causing problems (Rank Brothers Ltd, 2002). The probes were checked for air bubbles before the experiments. They could be detected by looking closely at the tip of the probe. The iWorx company returned the probes after checking them and reported that the platinum electrodes were not damaged. No explanation for the erroneous measurements was found and no causes could be determined. The signals from electrical interference (Rank Brothers Ltd., 2002) posed a problem, because the interference could not be eliminated. Sometimes the interference was strong and sometimes it was insignificant not due to any change in procedure or setup, which was kept consistent.

*Experiments in gas.* When calibrating the oxygen probes in gas, the measurements were inconsistent and no reliable standard line could be established. When flushing the probe with pure oxygen gas the line reached the probe’s maximum at 4.987 V and could not go any higher. Since it was not known how high the Voltage actually would be at 100 % oxygen, no standard line could be established. No reason for the failure of the probes to reach the maximum every time they were flushed with pure oxygen gas could be determined. The procedure was consistent for every experiment conducted. When measuring the oxygen concentration of the calibration liquids immediately after the
calibration gases, the probes produced the same erroneous results as before. When measuring the same solution twice with the same probe the results were different as well. When measuring the solution with one probe first and then another probe the results were still inconsistent. It was finally determined that the probes were unreliable and could not be used to measure the oxygen consumption of the salamanders.

Closed Respirometer

The advantage of the closed respirometer is that it can be assembled with standard equipment that can be easily and cheaply purchased. During experiments the apparatus has to be closely monitored, because the manometric fluid has to be readjusted. The fluid is allowed to shift only about 2 mm in the tube before it has to be moved back to its original position. Therefore, an experiment is very time consuming when using this apparatus.

First apparatus. The first respirometer was not useful for measuring the oxygen consumption of salamanders. It had only one chamber; therefore, the atmospheric pressure and temperature change influenced the measurements. In fact, this apparatus was so sensitive to atmospheric temperature and pressure changes that it was not successful in attaining any reliable results. The oxygen consumption of the salamanders was so small that the experiments had to be run for a few hours to get any measurable results. The bent pipette was not long enough in this apparatus to run experiments for a long period of time. After a few trials the apparatus was regarded as unusable.

Second apparatus. The second respirometer proved to be very time consuming to set up since the apparatus had to be made airtight. There were many connections where the air could leak out and the rubber tubing fit on the pipette snugly, but not tight enough.
The flasks were so large that the experiments had to be run for three to four hours to get measurable results. The flasks were held under water by pinning the flasks down with rubber stoppers, but that method was inefficient since the rubber stoppers displaced a lot of water. This defeated the purpose of submerging them in the water, which was to compensate for any temperature changes in the environment. Measurements using this apparatus were often inaccurate, because any pressure changes in the chambers after the system was closed and the experiment had started were not always detectable. Sometimes the control moved more than the main apparatus due to pressure adjustments after the system was closed. There were some consistent results obtained using this apparatus. Adjustments had to be made to make the apparatus more reliable.

**Final apparatus.** The final apparatus was a modified form of the second apparatus. For this apparatus plastic centrifuge tubes were used, because of their small size. Smaller vinyl tubing was used as well, because it formed a tighter fit around the pipette. Another major improvement included the elimination of unnecessary connections, thereby minimizing possible air leaks. The flasks were held in the water by rubber bands stretched around the beakers. This helped to retain as much water volume around the centrifuge tubes as possible. The phenol red that was used as manometric fluid proved not to be significantly better than the diluted methylene blue dye. Both adhered to the glass pipette. A different fluid, that is less adhesive to the walls but has a large surface tension so it sticks together, would be ideal for use in future experiments using this apparatus. This apparatus yielded results that were consistent. The results for both species measured with this apparatus averaged an oxygen consumption rate of 0.071 mL/hr x g with a standard deviation of 0.043 mL/hr x g.
Conclusion

The inconclusive results of our experiments failed to support the hypothesis that *P. cinereus* has a lower metabolism at higher temperatures than *P. hubrichti* (Reichenbach & Brophy, 2007). The results suggest that the metabolism of *P. hubrichti* and *P. cinereus* is not significantly different at 20 °C, since there was no significant difference in the metabolic rate between the two species. However, most time was spent building a functional apparatus, so only a few animals of each species were tested at 20 °C and not at multiple temperatures (ranging from 10 to 25 °C) as initially intended. Therefore the results were not conclusive. It was determined that the closed respirometer is a better method for measuring the oxygen consumption of *P. cinereus* and *P. hubrichti* than the oxygen probe. It was more reliable and provided consistent results. If a metabolic difference can be determined between the two salamander species, it would explain the difference in their distribution. If *P. hubrichti* had a higher metabolic rate at higher temperatures than *P. cinereus* its distribution would be limited to higher elevations (above 600 m).
References


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Appendix

Figure Legends

Figure 1. The distribution of the Red-backed salamander or *P. cinereus* (ARMI, 2002b).

Figure 2. The distribution of the Peaks of Otter salamander or *P. hubrichti* (ARMI, 2002a).

Figure 3. Temperature vs. elevation of the study sites in the Peaks of Otter area (Reichenbach & Brophy, 2007, personal communication).

Figure 4. The Peaks of Otter Salamander (Mitchell & Wicknick, 2008).

Figure 5. The red-backed morph of the Redback Salamander (ARMI, 2002a).

Figure 6. The lead-backed morph of the Redback Salamander (Howard, 2003).

Figure 7. Drawing of the lower part of the oxygen probe as seen under a bright field microscope at scanning power. The electrode A) was at the very tip of the probe. A rubber ring B) held the Teflon membrane C) in place, which covered the tip of the probe. The wire D) carried the current. The probe was filled with electrolyte fluid E). The walls of the lower part of the probe were made of glass F).

Figure 8. Drawing of the first respirometer apparatus assembled. It consisted of a 50mL Erlenmeyer flask A), a number 6 stopper with two holes B), a bent 1/10mL pipette C), and a clamp D) fastened to a piece of rubber tubing E). A glass tube F) containing KOH pellets G) was placed inside the flask along with the salamander H), a piece of paper towel I), and deionized water J). A drop of methylene blue dye was inserted into the tip of the pipette K).

Figure 9. Drawing of the second closed respirometer apparatus assembled. It consisted of two 50mL Erlenmeyer flasks A), two number 6 stoppers with two holes each B), a 12cc
syringe C), and a short piece of rubber tubing D). A glass tube E) was filled with 2g of 
KOH pellets F) and placed inside each flask. 10mL of deionized water I) was placed 
inside each flask as well along with 10cm x 10cm of paper towel G). The salamander H) 
was placed inside the working chamber I), but not inside of the reference chamber II). A 
bent 1/10mL pipette J) was placed between the flasks and connected to the flasks with 
rubber tubing K). Rubber tubing M) was also used so a clamp N) could be attached to 
close off the system. Methylene blue dye L) was inserted into the U-shaped pipette.

**Figure 10.** Drawing of the final closed respirometer apparatus assembled. It contained 
two 75mL polycarbonate viles A), two number 6 stoppers with two holes each B), and a 
12cc syringe C). A glass tube containing 3g of KOH pellets covered by elastic mesh that 
is held in place by rubber band D) was placed inside each of the viles. A 8cm x 8cm 
paper towel and 7.0 mL of DI water E) were placed in each of the viles as well. A U-
shaped 1/10 mL pipette F) containing a few drops of phenol red dye G) was attached to 
the viles via vinyl tubing H). A clamp I) was attached to a piece of vinyl tubing to close 
off the apparatus.