Chapter 14

Effects of salinity on metabolic rate in black mollies

Christopher Beck[1], Lawrence Blumer[2] and Travis Brown[2]

[1]Department of Biology
Emory University
Atlanta, GA 30322
cbeck@biology.emory.edu

[2]Department of Biology
Morehouse College
Atlanta, GA 30314
lblumer@morehouse.edu

Chris Beck earned his Bachelors degree from the College of William and Mary, and his PhD from the University of Georgia at the Institute of Ecology. He is currently a Lecturer at Emory University and Adjunct Assistant Professor at Morehouse College. At Emory he teaches Ecology, Evolution, and Introductory Biology. His research interests are in the areas of behavioral and evolutionary ecology. He and Larry Blumer are collaborators on behavioral neurobiology studies on mate choice in fishes and sex ratio effects on insect parental investment.

Larry Blumer earned his graduate and undergraduate degrees from the University of Michigan, and he is currently Associate Professor of Biology at Morehouse College. His teaching interests are in the areas of Ecology, Environmental Biology, and Evolution and Behavior. His research interests are in the same fields and include recent studies on the neurobiology of mate choice and the neuroendocrine correlates of social stress in fishes.

Travis Brown received his Bachelor of Science degree from Morehouse College in 2002 and conducted the first protocol tests for this study as an undergraduate research project. He is currently a graduate student at the University of California at Berkeley where he is the recipient of a Packard Foundation scholarship. His graduate studies are focusing on environmental endocrinology and animal behavior.

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Contents

Introduction

The objectives of this study are to evaluate the metabolic rate of a fish by measuring direct oxygen consumption, and to evaluate the metabolic rate pattern of an intertidal fish in response to a range of salinity. Fishes acclimated to a single salinity are tested over a range of salinities to evaluate their metabolic rates. We have used this laboratory in our upper-level undergraduate ecology courses (at both Morehouse College and Emory University) for several years now. These classes were all single sections of 18 students or less. At first, we used wild caught sailfin mollies (*Poecilia latipinna*). However, recently we have begun using black mollies, which are an aquarium strain of the same species, because they are more readily available. Any small fish that can tolerate a range of salinities would be appropriate for this exercise. Some participants in the workshop suggested doing a comparison of osmoregulators and osmoconformers. This would be an interesting extension of the current study. However, we have not tested any osmoconformers. Another possibility for expanding this study would be examining the cost of osmoregulation in fishes of one species acclimated to different salinities.

Originally, to measure metabolic rate, we measured a change in oxygen concentration in a sealed flask of water over time. Because the fish was in the flask throughout the experiment, only minimal stirring could be used. We call this the "minimal stirring protocol." The best probes for this method are the no-stir probes that come with YSI Model 95 field dissolved oxygen meters. Unfortunately, YSI has decided to discontinue this model. We have since developed a new protocol for probes that require a high rate of stirring. We call this the "high stirring protocol." This protocol uses initial and final oxygen concentrations to determine metabolic rate. The minimal stirring protocol gives a more accurate estimate of metabolic rate, because multiple measurements of oxygen concentration are made. However, students can generally only make measurements on two fishes, using the minimal stirring protocol, during a typical 3-hour laboratory period. In contrast, with

the high stirring protocol, students can make measurements on five fishes. Additional important considerations related to each protocol are discussed in the Notes for the Instructor section.

Materials

For each group (minimal stirring protocol):

- dissolved oxygen meter – one per group, YSI 95 DO meter (or similar) with no-stir probe
- water or air-driven magnetic stirrer – one per group, Fisher 14-511-210 (regular stirrers generate heat and thus affect metabolic rate measurements)
- stirring bar (8x13mm) – one per group, Fisher 14-511-62, or glass flea (Appendix A)
- 125-mL Erlenmeyer flask – minimum one per group
- 100-mL or larger graduated cylinder – one per group
- small dip net – one per group
- black mollies – two per group
- 100-mL plastic beaker – two per group
- stop watch – one per group

For each group (high stirring protocol):

- dissolved oxygen meter – one per group, field DO meter or BOD meter
- magnetic stirrer, any type – one per group
- stirring bar (9.5x51mm) – one per group
- 125-mL Erlenmeyer flask (if using field DO meter) – 10 per group
- No. 5 rubber stoppers (if using field DO meter) – five per group
- 60-mL BOD bottles (if using BOD meter) – 10 per group
- 100-mL graduated cylinder or larger – one per group
- small dip net – one per group
- black mollies – five per group
- 100-mL plastic beaker – five per group
- stopwatches – five per group

For the entire laboratory:

- *Spirulina* flake fish food
- electronic balance – sensitivity to 0.1 g
- small container for weighing fish
- two 10-gallon aquarium tanks
- Coral Life aquarium salts, 25-gallon size
- five 5-gallon buckets for saltwater solutions
- water treatment chemicals – Novaqua and Amquel (3-oz size)
- Parafilm
- computer with Excel or statistical package to carry out linear regressions
- salinity meter, YSI Model 85 measures both DO and salinity

Notes for the Instructor

We generally purchase fish from the pet store several days before the laboratory period. This way, the fish get acclimated to the laboratory environment, and any individuals in poor health can be removed. If possible, we try to use individuals of the same sex. The number of fish that each group uses depends on the length of the laboratory period and which protocol for measuring metabolic rate is used. In a 3-hour laboratory period, each group can take measurements on two different fish using the minimal stirring probes, or five different fish using high stirring rate probes. The fish are NOT fed for 24 hours before the beginning of the experiment to make sure that they are post-absorptive.

To speed the laboratory along, we generally mix the different concentration saltwater solutions before the beginning of the laboratory. Also, we warm up the DO meters. Some meters take at least 20 minutes to warm up and stabilize. We have students run trials at salinities between freshwater (0ppt) and full seawater (30ppt), but higher concentrations could be used.

We generally put students in groups of two or three for this exercise. Larger groups often result in students without something to do. After each trial, we have one student in each group enter the data and carry out any necessary analyses, while the other students in the group set up the next trial.

Minimal stirring protocol

In this protocol, metabolic rate is estimated as the slope of the regression of absolute oxygen concentration versus time. Students measure oxygen concentration at 1-minute intervals during a 10-minute trial. The volume of water in the flask (125-mL Erlenmeyer) is used to convert oxygen concentration measurements in mg/L to total amount of oxygen (mg) for the regression analysis.

Several considerations are important if accurate measurements are going to be made. First, there needs to be a good seal between the oxygen probe and the neck of the flask. Flasks of the same type by the same manufacturer vary in the width of the neck. For some, the probe will stick in the neck and seal. For other flasks, the probe will go all the way to the bottom of the flask. Try to find flasks in which the probe will stick in the neck. If this isn't possible, you can wrap Parafilm around the probe so that it does stick in the flask neck. Second, the stirring needs to be slow enough as to not batter the fish, but consistent so that an oxygen gradient is not established in the flask. The best way to insure this is to find the correct speed for the stirrer and location for the flask on the stirrer before a fish is added to the flask. After the proper speed and location are found, the stirrer should be left running and the position of the flask marked with tape on the stirrer. Third, to get accurate dissolved oxygen readings, one would need to recalibrate the DO meter for each new salinity. However, this step is NOT essential for the metabolic rate measurement, because metabolic rate is measured by evaluating the rate of change in oxygen concentration. Finally, occasionally students will get erratic readings from the DO meter. A bad membrane on the probe is generally the culprit. The problem is usually fixed by just changing the membrane.

High stirring protocol

In this protocol, metabolic rate is estimated as difference in initial and final absolute oxygen concentration divided by time. Students measure oxygen concentration at the beginning and end of a 20-minute trial. The volume of water in the flask (125-mL Erlenmeyer) is used to convert oxygen concentration measurements in mg/L to total amount of oxygen in mg.

Because a high rate of stirring is necessary for most dissolved oxygen and BOD probes, the initial and final oxygen concentrations need to be determined without the fish in the experimental flask. As a result, there are several considerations that are necessary for this protocol to work. First, the initial dissolved oxygen measurement is made before the fish is placed in the flask. However, the probe will displace a large amount of water. So, the flask needs to be refilled with water after the fish is added and before the flask is sealed with a rubber stopper. There should be no headspace air bubble when sealing the flask. If a headspace of air is left in the flask, the oxygen from the air will dissolve into the water as the oxygen concentration in the water decreases. Second, to get the final dissolved oxygen measurement, the fish needs to be removed from the flask. In our testing, the best way to do this is to slowly pour (decant) the water into another 125-mL Erlenmeyer flask, leaving the fish at the bottom of the flask. The fish can then be dumped into a beaker of water. Then, it is important to slowly pour the water back into the original flask. The reason for this is that because of variation among flasks, the dissolved oxygen probe will go into the flasks at different depths. How close the probe is to the stir bar can affect the dissolved oxygen measurement. By using the original flask, both the initial and final measurements are made with the probe the same distance from the stirrer. The final two points are mentioned above for the minimal stirring protocol. To reiterate, to get accurate dissolved oxygen readings, one would need to recalibrate the DO meter for each new salinity. However, this step is NOT essential for the metabolic rate measurement, because metabolic rate is the rate of change in oxygen concentration. Occasionally students will get erratic readings

from the DO meter. A bad membrane on the probe is generally the culprit. The problem is usually fixed by just changing the membrane.

If you decide to use a BOD probe rather than a field probe, there are a few different considerations. First, use 60-mL BOD bottles, rather than the standard 250-mL bottles. With the larger bottles, the change in oxygen concentration is not sufficient in a short time period to get an accurate estimate of metabolic rate. Second, when using 60-mL BOD bottles, you can reduce the time between the initial and final measurements to 10 minutes. However, by reducing this time, students can generally only run three trials simultaneously, rather than five trials with the field probe and associated 20-minute sampling period. Third, with BOD bottles, the stoppers may displace more water than the probe. If so, allow the excess water to go back into the bottle when the stopper is removed at the end of the trial. It will not change the dissolved oxygen concentration much. If the excess water does not go back in the bottle, there may be a headspace bubble at the top of the BOD bottle, and the water in the bottle will be reoxygenated when it is stirred while getting the final measurement.

Student Outline

Objectives

1. Evaluate metabolic rate of a fish by measuring direct oxygen consumption.
2. Evaluate the metabolic rate pattern of an intertidal fish to a range of salinity.

Introduction

Aquatic organisms either conform to their osmotic environment or actively regulate internal water and solute levels to maintain their water balance. For regulators living in freshwater, their body fluids are hyperosmotic with respect to the environment. As a result, they tend to absorb water and lose solutes. To maintain their water balance, freshwater regulators produce large amounts of dilute urine and actively retain and take up solutes. In contrast, organisms in saltwater habitats are hypoosmotic with respect to their environment. Therefore, they tend to lose water and absorb solutes. Saltwater regulators will drink to increase body water levels, but must actively secrete solutes to maintain their water balance.

Among both freshwater and saltwater regulators, the active transport of solutes against the osmotic gradient is energetically expensive. As a result, as the osmotic stress on an individual increases, we would expect to see an increase in metabolic rate. However, under extreme osmotic stress, individuals cannot maintain their water balance, and physiological systems shut down, leading to a decrease in metabolic rate. Therefore, for freshwater organisms, we would expect an increase in metabolic rate from low to moderate salinities, and then a decrease in metabolic rate at high salinities. In contrast, in saltwater organisms, we would expect to see depressed metabolic rates at salinities both lower and higher than those found in their natural environment.

Although most aquatic organisms have physiological systems adapted to either freshwater or saltwater habitats, some species living in tidal marshes and streams experience daily fluctuations in salinity. Therefore, they must be able to rapidly adjust to changes in their osmotic environment. These adjustments may be very similar to the thermal acclimation that occurs seasonally in many

animals (Ricklefs, 2000). We would expect intertidal species to have a much wider range of salinity tolerance, and expect that these species only exhibit changes in metabolic rate at the extremes of a range in salinities. Experiments on two species of fish with wide salinity tolerances, milkfish, *Chanos chanos,* and sheepshead minnows, *Cyprinodon variegatus*, found that these species can acclimate to a wide range of salinities (Haney and Nordlie, 1997; Swanson, 1998). However, even if these fishes were allowed to acclimate to extreme osmotic environments, they still exhibited decreased metabolic rates in those environments. Although these experiments demonstrate the physiological responses of euryhaline fish acclimated to a wide range of salinities, fishes in tidal streams and marshes must respond to rapid changes in salinity. Another study on sheepshead minnows examined the effect on metabolic rate of increases and decreases in salinity from the salinity at which fishes were acclimated (Haney *et al.,* 1999). Within the normal range of salinities experienced by sheepshead minnows (2-40 ppt), changes in salinity did not result in significant changes in metabolic rate. In contrast, when fishes were acclimated to extreme osmotic environments (0, 50, and 60 ppt), changes in salinities within the normal range resulted in an increase in metabolic rate. In addition, changes in salinities from the normal range to extreme osmotic environments led to a decrease in metabolic rate. Both results suggest that metabolic rates are depressed with respect to normal in extreme osmotic environments. Sailfin mollies, *Poecilia latipinna*, are found in both freshwater and saltwater habitats along the Atlantic and Gulf coasts of the southeastern United States. Some populations are found in the same tidal streams as sheepshead minnows. Black mollies are an inbred variety of sailfin mollies (an aquarium hobby fish) that are adapted to freshwater. The purpose of this experiment is to determine whether black mollies exhibit a metabolic response to changes in salinity.

Hypotheses and Prediction

H_o: Null Hypothesis: no effect of treatment

Prediction: metabolic rate will remain constant and will not change at different salinities

H_1: Increased demands for osmoregulation require active transport and therefore an increase in metabolic rate

Prediction: metabolic rate will increase as salinity increases

H_2: Osmoregulation has limits that may be exceeded at extreme salinities

Prediction: high salinity will result in a decrease in metabolism due to the failure to osmoregulate

Methods

Study population

The fish for this experiment are purchased from a pet store, and were maintained in freshwater. The fish are maintained in our laboratory at 27° C at ambient daylight cycle. We feed the fish a *Spirulina* (B-G bacteria) flake food once daily. The fish will not be fed for 24 hours before the experiment to make sure that they are post absorptive (*i.e,* they are not digesting food, which increases metabolic rate).

Experimental design

You will work in pairs or groups of three for this study. Each group will carry out a set of metabolic rate measurements for two to five fishes. The results from all groups will be pooled for analysis. The metabolic rate of each fish will be determined at five different salinities, measured in parts-per-thousand of solutes (0, 5, 10, 20, and 30 ppt). Since the fish are acclimated to 0 ppt, you should test them at this salinity first. You will simulate a tidal change by increasing the salinity of the experimental environment to 5 ppt, and then sequentially to 10, 20, and 30 ppt.

Initial setup

Two steps must be completed before you can begin making your metabolic rate measurements. First, the oxygen meter must be warmed up and then calibrated. To warm up the probe, place it in a 125-mL Erlenmeyer flask filled with tap water and a magnetic stir bar. With the oxygen meter on and the magnetic stirrer running, let the oxygen probe warm up for 20 minutes. After the probe has warmed up, calibrate it following the directions attached to the probe. The second step (which can be done while the probe is warming up) is for each group to mix salts in water to prepare one of the experimental salinities. For a 5-gallon bucket, add 5 mL (1 capful) each of NovAqua and Amquel to condition the water. Then add sufficient salt to achieve the desired salinity. You will need to stir the water to get the salt to dissolve completely and the salinity of the water to be homogeneous. Use approximately 4 g of crystalline salt per part-per-thousand per gallon. Test the salinity with a salinity meter and adjust it appropriately by adding more salt or freshwater.

Metabolic measurements (Minimal stirring protocol)

You will determine metabolic rate by recording the change in oxygen concentration in a sealed system in one-minute intervals over a 10-minute time span. The metabolic rate is the slope of the regression between oxygen concentration (corrected for water volume) and time. For each metabolic rate determination, follow the procedure outlined below:

1. Fill a 125-mL Erlenmeyer flask with water from the bucket of the appropriate test salinity.
2. Add the magnetic stir bar and fish.
3. Insert the oxygen probe into the Erlenmeyer flask to create a sealed system. This will cause some water to spill out. If needed, wrap the probe and top of the flask with Parafilm. Start the stir bar spinning slowly by adjusting the water flow of the magnetic stirrer.
4. Allow the fish to adjust to the experimental setup for 5 minutes. The fish may be active at first, which will increase the metabolic rate.
5. After the 5-minute adjustment period, take an initial oxygen concentration measurement (Table 1).
6. Record the oxygen concentration every minute for the next 10 minutes (Table 1).
7. When you finish recording the oxygen concentrations, record the temperature of the water from the readout on the oxygen meter and measure the amount of water in the flask by carefully pouring the water through a net (to catch the fish and stirring bar) into a graduated cylinder. You will use the volume to convert the oxygen concentration measurements from mg/L to mg of oxygen in the flask. Place the fish in a beaker of 0 ppt water until beginning the next trial.

8. You are now finished with the metabolic rate measurement and the procedure can be repeated at the next salinity. Between trials, always return the fish to a beaker of 0 ppt water.

Table 1. Data Table for Oxygen Concentration (mg/L) and Temperature at a given Salinity. Prepare a separate table for each salinity tested.

	Elapsed Time (minutes)											Temperature
	0	1	2	3	4	5	6	7	8	9	10	°C
Fish 1												
Fish 2												

Metabolic measurements (High stirring protocol)

You will determine metabolic rate by recording the change in oxygen concentration in a sealed system in 20-minute time span. The metabolic rate is the change in oxygen concentration (corrected for water volume) divided by time. For each metabolic rate determination, follow the procedure outlined below:

1. Fill a 125-mL Erlenmeyer test flask with water from the bucket of the appropriate test salinity.
2. Add the magnetic stir bar.
3. Insert the oxygen probe into the test flask to create a sealed system. This will cause some water to spill out. If needed, wrap the probe and top of the flask with Parafilm. Start the stir bar spinning quickly.
4. After the dissolved oxygen reading stabilizes, record the initial oxygen concentration (Table 2).
5. Remove the stir bar, add the fish, top off the flask with water of the appropriate salinity, and seal the flask with a rubber stopper.
6. Leave fish undisturbed for 20 minutes. In the mean time, begin trials on additional fish (steps 1- 6). You should be able to start four more trials before the first trial is complete.
7. After 20 minutes, remove the stopper and slowly pour the water from the test flask into another 125-mL Erlenmeyer flask. *IT IS ESSENTIAL THAT YOU POUR THE WATER SLOWLY SO THAT ONLY NEGLIGIBLE AMOUNTS OF OXYGEN ARE ADDED TO THE WATER DURING THIS PROCESS.* After pouring out all of the water, the fish should be at the bottom of the flask. Pour the fish into a beaker of fresh water. Now, slowly pour the water from the Erlenmeyer flask back into the original test flask. Add the magnetic stir bar and insert the oxygen probe into the test flask to create a sealed system again. Start the stir bar spinning quickly. After the dissolved oxygen reading stabilizes, record the final oxygen concentration and the temperature of the water from the readout on the oxygen meter (Table 2).

Table 2. Data Table for Oxygen Concentration (mg/L) and Temperature at a given Salinity. Prepare a separate table for each salinity tested.

	Initial [Oxygen]	Final [Oxygen]	Temperature °C
Fish 1			
Fish 2			
Fish 3			
Fish 4			
Fish 5			

8. Repeat step 7 for the remaining fish. You are now finished with the metabolic rate measurement and the procedure can be repeated at the next salinity.
9. When you finish measuring the metabolic rate at all salinities, you need to determine the volume of the experimental test flasks. For each flask, fill the flask with water, add the experimental fish, and seal the flask with the rubber stopper. Then, remove the stopper and pour the water through a net (to catch the fish) into a graduated cylinder. Record the volume. You will use the volume to convert the oxygen concentration measurements from mg/L to mg of oxygen in the flask.

Calculating metabolic rate (Minimal stirring protocol)

To determine the metabolic rate, convert your oxygen concentration measurements to the total amount of oxygen in mg by multiplying by the water volume in liters. Then, run a linear regression with amount of oxygen as the dependent variable and time as the independent variable. The slope of the line will be the metabolic rate in mg oxygen per minute.

Calculating metabolic rate (Minimal stirring protocol)

To determine the metabolic rate, convert your oxygen concentration measurements to the total amount of oxygen in mg by multiplying by the water volume in liters. Then, take the difference between the initial and final measurements and divide by the total time.

Data Analysis

Body size measurements

In most organisms, metabolic rate is positively correlated with body mass. Bigger fish have higher metabolic rates. As a result, we need to determine the body mass of the experimental fish. At the end of the metabolic rate measurements, measure the mass of each fish by placing it in a pre-weighed container of water.

Statistical analysis

Before you do a comparison of average metabolic rates under the different salinity regimes, you need to determine if there are effects of either temperature or body size on metabolic rate. The relationship between body size and metabolic rate is generally exponential. So, you should do a natural log transformation of body size before you examine the relationship. You can examine the relationship between either body size or temperature and metabolic rate using simple linear regression. If you are interested in the simultaneous effects of body size and temperature, you can

use a multiple linear regression in which both body size and temperature are independent variables.

If either body size or temperature were significantly related to metabolic rate, you will need to use an analysis of covariance (ANCOVA) to determine the independent effect of salinity on metabolic rate. If neither body size nor temperature is significantly related to metabolic rate, then you can use an analysis of variance (ANOVA) to evaluate the effect of salinity on metabolic rate.

Acknowledgments

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Appendix A

Making Glass Flea Stirring Bars

Small stirring bar fleas can easily be made from paper clips and flint glass Pasteur pipettes.

Materials

- metal paper clips, cut in one-half inch lengths
- Pasteur pipettes, flint glass (one for each flea to be made)
- Bunsen burner
- long-nose pliers
- safety glasses

Wear safety glasses throughout the process of cutting the paper clips and heating glass.

Start by heating the narrow end of a glass pipette, while rotating it between your fingers. The flame will become yellow-orange (sodium flame) and as the glass becomes hot, the tip will close and become rounded. Remove from the flame and set aside to cool.

Once the pipette has cooled, gently drop a piece of paper clip into the wide end of the pipette so it lodges in the sealed end of the pipette.

Hold the sealed end of the pipette with long-nose pliers and the wide end with your fingers. Place the pipette in the Bunsen burner flame just past the end of the paper clip (toward the open end of the pipette). Rotate the pipette while heating and pull the open end of the pipette away from the pliers once the glass is molten. Continue to heat the end of the flea held by the pliers until the glass end is rounded. Set aside to cool.

With practice, you can make a large number of these very small stirring bars in a very short period of time. I would like to acknowledge Professor Hiroshi Ikuma who taught me (LB) how to make glass fleas when I coordinated an introductory biology laboratory course under his supervision at the University of Michigan.

Appendix B

Using Microsoft Excel for Statistical Analyses

Before you can use Excel for statistics, the Analysis ToolPak must be installed. If it is installed already, there should be a "Data Analysis" option in the tools menu. If not, to install the ToolPak, go to "Add Ins" under the tools menu and select "Analysis ToolPak" and "Analysis ToolPak – VBA." Now, you are set to do data analysis with Excel.

For the minimal stir protocol, students will need to run simple linear regressions to determine the metabolic rate. To run the regression, first enter the time data in one column and total amount of oxygen measurements (*i.e.,* oxygen concentration corrected for volume) in another column. Next, select Tools → Data Analysis → Regression. Total oxygen data is the Y Range, and time is the X range. If you want to see a plot of the data, select the "Line Fit Plots" box. Then, click OK. Excel will now create a new worksheet with the results of the regression. The most important parameter is the slope of the regression model. The slope is the coefficient of the X variable in the lower table. The slope should be negative because the fishes are consuming oxygen. However, use the absolute value as a measure of metabolic rate.

After metabolic rates have been calculated, students should carry out an analysis of variance (ANOVA) to determine whether there was a difference in metabolic rates among the salinities. Strictly speaking, because the same fish was evaluated at each salinity, a repeated-measures ANOVA should be used. However, Excel does not support this analysis and it can be difficult for students to interpret. Therefore, we calculate an independent-events ANOVA. To perform an ANOVA in Excel, the metabolic rate for each salinity needs to be arranged in columns. Select Tools → Data Analysis → ANOVA: Single Factor. Highlight all of your data including column titles, if you have them. Select "Grouped by Column." If you have column titles, select "Labels in First Row." Then click OK. Excel will then create an ANOVA table in a new worksheet. It will also report means and variances for each group. In the ANOVA table, P values ≤ 0.05 suggest a significant effect of salinity on metabolic rate.

To determine the effect of body size or temperature on metabolic rate, you can use the same method outlined above for simple linear regression. For body size, the data should probably be log-transformed first (*i.e.,* take the log of the data and use it rather than the raw data). If either temperature or body size has a significant effect on metabolic rate, an analysis of covariance (ANCOVA), rather than an ANOVA, should be used to test for differences in metabolic rates among salinities with temperature or body size as the covariate. ANCOVAs cannot be carried out in Excel.

Another option for data analysis is VassarStats. This is a free web-based program available at http://faculty.vassar.edu/lowry/VassarStats.html.