Chapter 11

Using Crustaceans to Illustrate the Principles of Osmoregulation, Acid-Base Balance, and Respiratory Physiology

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Introduction

The following exercises, which employ the crustacean model, were designed to strengthen students' understanding of osmoregulation (Exercise 1) and acid/base balance (Exercise 2), familiarize them with experimental design, and emphasize the importance of careful quantitative measurements. The intention was to develop exercises that could be performed with a variety of marine or freshwater crustaceans so that substitutions could be made if availability of any species became a problem. Students have successfully run the experiments with the marine crabs *Carcinus*, *Callinectes*, and *Cancer*, and with the fresh water crayfish *Procambarus* and *Pacifastacus*.

The exercises are intended for third- and fourth-year students, catering to those planning a career in physiology or other zoological or health-related professions. Since most of our laboratories in comparative physiology are of the project nature, we allow students two to three laboratory periods (3 hours each) to complete an experiment. The first week is spent in familiarization with equipment, techniques, and preparation of animals, as well as review of experimental design. In the second week, students, working in pairs, begin the collection of data. At the end of the data collection period, which may require two laboratory periods, data from all groups are complied and handed back to the students for analysis. Students are held responsible for statistical analysis of the data, and are required to submit a laboratory report where emphasis is placed upon data interpretation and discussion relative to the literature. Students are encouraged to be critical in their analysis of the data and the experimental design.

The preparation time for either of the following two experiments is approximately one full day if reagents and animal preparation as well as physical set-up of the exercise are taken into account.

Exercise 1: Osmoregulation

Materials

Equipment and supplies for each pair of students:

Glassware:

Test tubes and marbles, 8 ml (10)
Test tubes, 25 ml (14)
Beaker, 250 ml (1)
Beakers, 100 ml (4)
Tissue homogenizer, (1)
Volumetric flasks, 100 ml (8)
Graduated pipets, 1 ml (5)
Graduated pipets, 10 ml (2)
Graduated pipets, 5 ml (2)
Graduated pipets, 2 ml (2)
Reagents:

Ethanol, 60%, 50 ml
Citrate buffer, 50 ml
Ninhydrin solution, 50 ml
Ethanol, 95%, 50 ml
Phenol-alcohol solution, 50 ml
Na nitroprusside, 0.5%, 50 ml
Oxidizing solution, 50 ml

Other supplies:

Plastic syringes with 22 g needles, 1 ml (4)
Plastic conical centrifuge tubes, 15 ml (4)
Plastic mouse cages or similar containers (for use as animal chambers) capable of holding a crustacean and sea water (200 ml/10 g body weight) (4)
Pipetting bulb or pi-pump (1)
Grease pencil or marker and tape (1)
Vortex mixer (1)
Wash bottle with distilled H_2O (1)
Bunsen burner (1)
Ring stand and wire gauze (1)
Disposable gloves (2 pairs)
Graduated cylinder, 1-liter (1)
Microcentrifuge tubes, 1.5 ml (8)
Spectrophotometer cuvets (2)
Weighing paper (8 sheets) or aluminum weighing dishes

Common instruments:

Spectrophotometers capable of reading %T or A at 570 and 640 nm
Osmometer and standards
Chloridometer
Flame photometer
Drying oven
Centrifuges
Microcentrifuge tubes, 1.5 ml

Chemicals needed in preparation of various reagents:

Trisodium citrate
Ethanol (100%)
Phenol
NaOH
Hypochlorite (bleach)
L-ascorbic acid
Methyl-cellosolve (ethylene glycol monomethyl ether)

Glycine
Ammonium chloride
Sodium nitroprusside

Notes for the Instructor

Acclimation to Different Salinities

Three weeks prior to the date of the experiment divide the animals into four groups and begin stepwise acclimation of three of the groups to 75%, 50%, and 30% sea water (see Appendix A for details). Maintain the fourth group as a control in 100% sea water. Each group of animals should remain at the appropriate salinity for at least 3 days prior to use.

If you wish to test the salinity tolerance of freshwater crustaceans (crayfish), we have found that *Procambarus* will tolerate up to 30% sea water and *Pacifastacus* up to 75%. Acclimation times are the same as those suggested for marine crustaceans (Appendix A).

We prepare our own sea water by reconstituting sea salts (Aquarium Systems, Mentor, OH 44060). If you plan to prepare sea water in this fashion, you will need 2–3 days to allow the salts to completely dissolve. Also, since the animals are confined to a recirculating sea water system, it is necessary to install a biological filter to prevent the build-up of nitrogenous waste products. Under gravel filters or activated charcoal filters work well and are readily available.

Reagents

Some of the reagents used in the ammonia and amino acid assays are caustic or noxious, therefore appropriate precautions must be taken to reduce the possibility of contact with skin. I have noted which reagents should be handled with caution in Appendix B, where you will find specific directions on preparing the solutions for this exercise. We use dispensers to deliver these solutions, thereby reducing the chance of harmful contact and also reducing the amount of reagent wasted.

Ammonia Excretion

Students will need to know the approximate weights of the animals used in the measurement of ammonia excretion in order to maintain the correct water:body weight ratio during the flux period. We recommend 200 ml of water/10 g of body weight. This ratio will ensure that the ammonia concentration in the water during the flux period (30 minutes) does not exceed or fall short of the measurable concentration range of the ammonia assay.

Hemolymph Sampling

In this experiment, it does not matter whether the sample is obtained from a pre-branchial or post-branchial site. The details regarding post-branchial sampling are given in Appendix C. Pre-branchial hemolymph samples can be drawn with less work from any of the leg bases. Insert the syringe needle about 3–4 mm through the soft membrane at the joint between the protopodite and the endopodite. Carefully draw back on the syringe plunger to obtain the necessary volume. If no hemolymph enters the syringe, try a different location and a different angle. Sampling usually requires one or two practice tries before samples can be removed with relative ease. I allow students to practice on a few animals held in reserve.

Tissue Sampling

There is nothing difficult about tissue sampling. We prefer to use a claw (cheliped) since the animal will autotomize the appendage and regenerate a new one with time, also the animal does not have to be sacrificed in the quest for soft tissue. To remove the claw, pinch just distal to the plane of autotomy using a pair of hemostats or small pliers. When performed correctly, the claw will be released with no blood loss.

Since muscle tissue is used for amino acid analysis, it is mandatory that students wear disposable gloves to prevent contamination of the sample with amino acids from their skin. In the larger crabs (*Callinectes*) and crayfish, one claw will supply enough tissue for both amino acid and tissue water analyses.

After cracking open the claw the muscle is removed by careful scraping of the inside surface of the exoskeleton. Do not include the cartilage-like apodeme in the sample.

Tissue Amino Acid Analysis

Of the two colorimetric assays performed in this experiment, the amino acid analysis is more difficult. There are more steps involved and more chances for contamination of glassware with amino acid from other sources (such as fingers). The reagents are also more costly and dangerous than those used in the ammonia assay. Nevertheless, the results of the assay can be quite dramatic and physiologically noteworthy.

In step 5 of Part F, students are asked to consult the instructor regarding the appropriate volume of supernatant to use in the assay. We have found that 0.1 ml is appropriate for *Callinectes* and *Carcinus*. We have not performed the assay on muscle samples from freshwater animals.

Measurement of Serum Ion Concentration and Osmotic Strength

Sodium concentration can be measured using ion specific electrodes, flame photometry or atomic absorption spectrophotometry. Of these methods, flame photometry is the easiest to perform and is as accurate as the other methods. Although the techniques may vary from one flame photometer to the next, they all are based upon comparison of the flame emission intensity of the sample with that of a series of standards.

Chloride concentration can be measured with a variety of chemical titration methods (Oser, 1965), or with ion specific electrodes. The simplest and most accurate method, however, involves use of a coulometric chloride titrator (e.g., Buchler or Radiometer).

The most accurate means of determining osmotic strength is the use of a freezing point or vapor pressure osmometer. With most of these instruments, a small sample (200 μl or less) is needed. In some cases, however, even 200 μl is difficult to obtain, especially if serum is used. There is a much less expensive piece of apparatus that can be easily constructed which will allow you to measure the osmotic strength of small quantities (40 μl) of fluid. The apparatus and the technique are described in Appendix E.

Student Outline: Osmoregulation

Purpose: To study the osmoregulatory abilities of euryhaline animals.

Introduction

Aquatic animals vary widely in regard to tolerance to changes in environmental salinity. Those which can tolerate a wide variation in salinity are referred to as *euryhaline*, while those which are intolerant to changes in salinity are called *stenohaline*.

In addition, animals are characterized by their ability, or lack of ability, to regulate their body fluid ionic composition and osmotic strength. Osmoregulators maintain body fluid osmotic strength within narrow limits, while osmoconformers allow body fluid osmolarity to vary directly with that of the environment. Osmoconformity is usually the result of passive influx or efflux of monovalent ions and water across the permeable portions of the exoskeleton or epithelium. Osmoregulation is more complex, involving active uptake or active excretion of ions, and regulation of intracellular amino acid concentration.

In most cases, the marine stenohaline crustaceans are osmoconformers, inhabiting an environment which is subject to only minute fluctuations in salinity. However, many of the marine euryhaline species, those that migrate between ocean and estuary, can osmoregulate to some extent, in spite of the hyposmotic environment they encounter near freshwater. Since their hemolymph has a higher solute concentration than the environment, they are hyperosmotic regulators in brackish or fresh water. Upon return to the sea, however, many euryhaline crustaceans become osmoconformers as they resume osmotic equilibrium with sea water.

Several species of intertidal and land crabs are very good osmoregulators. Upon dry land or mud flats, many are capable of maintaining fairly constant blood osmolarity in the face of desiccation. They also maintain a blood osmolarity in sea water which is hyposmotic to the marine environment.

Crayfish are also good osmoregulators. They maintain their blood hyperosmotic to fresh water and can regulate blood solute concentration in spite of increasing salinity (up to 50% sea water). However, they rarely have contact with brackish water since most inhabit only freshwater lakes or streams.

The physiological processes associated with crustacean osmoregulation include the regulation of sodium and chloride transport across the gill and the regulation of tissue fluid amino acid concentration. If, as is the case for most euryhaline species, the hemolymph osmotic concentration changes during migration from one environment to another, there is readjustment of the intracellular fluid solute content to prevent tissue swelling or dehydration. Amino acids play an important role in this respect. For example, reduction of intracellular amino acids aids in maintenance of the osmotic ratio of tissue fluid to hemolymph when euryhaline crabs enter estuaries.

Sodium transport across the epithelium of the gill is increased to facilitate net influx of sodium in dilute media, a process which is necessary to maintain blood osmotic strength above that of the environment. For every sodium ion that enters the blood, one cation must be moved in order to maintain electrical neutrality, perhaps through the efflux of H^+ or ammonium (NH_4^+), a product of amino acid degradation. Thus, the decrease in the free amino acid pool may not only facilitate the maintenance of isotonicity between the intracellular and extracellular fluid compartments, but also provide, through deamination, a valuable counter ion, in the form of NH_4^+, for cation exchange with sodium.

In this exercise, you will study the ability of a marine decapod crustacean to regulate hemolymph Na^+, Cl^- and osmotic concentration in the face of dilution of the aquatic medium. Regulation of tissue amino acid concentration and ammonium excretion will also be considered in this investigation. Our

objective is to correlate changes in ammonium excretion and tissue amino acid regulation with regulation of hemolymph Na^+, Cl^- and osmolality.

    Three weeks prior to the experiment, the animals were divided into four groups. Stepwise adaptation of three of the groups to various concentrations of dilute sea water was initiated, while the fourth group was maintained as a control in 100% sea water.

Procedure

Part A: Ammonium Excretion

1. Label the four animal chambers at your lab bench according to the sea water concentrations used in this exercise. Using a graduated cylinder, add a volume of the appropriate sea water to each chamber at a ratio of 200 ml/10 g of body weight (consult the instructor concerning the weight of the animals you will use).

2. Pipet 10 ml of sea water from each of the four sea water stocks and place in appropriately labelled 25-ml test tubes. Mark these tubes "pre-exposure" and seal with parafilm.

3. At 2-minute intervals, select one animal from each of the four sea water concentrations and place in the appropriately labelled beaker of the same salinity. Note the time of entry for each animal.

4. At the end of 30 minutes, draw 10 ml of water from the first beaker, place in an appropriately labelled 25-ml test tube and seal the top with parafilm. If sampling occurs at times other than 30 minutes, record the total exposure time. Repeat with each of the remaining three beakers. Label each tube "post-exposure".

5. As soon as you have collected all four 10-ml "post-exposure" water samples, begin the ammonia analysis of these samples, plus the four "pre-exposure" samples, a blank and standards as outlined under Determination of Ammonium Excretion (Part B).

Part B: Determination of Ammonia Excretion (Nitroprusside Method)

1. Prepare a set of NH_4^+ standards (10, 25, 50, and 75 microequivalents NH_4^+/liter) from a stock $NH_4^+Cl^-$ solution (1 mmol/liter). Add 10 ml of each standard to appropriately labelled 25-ml test tubes. Add 10 ml of distilled water to a tube labelled "blank".

2. To all test tubes containing pre- or post-exposure samples, the standards and the blank, add the following reagents in the order listed, mixing the tubes thoroughly between additions (use a vortex mixer):
    (a) 0.4 ml of phenol-alcohol solution
    (b) 0.4 ml of nitroprusside solution
    (c) 1.0 ml of freshly made oxidizing solution (alkaline sodium citrate-hypochlorite)

3. Seal the tubes with parafilm and set aside in the dark for 1.5 hours to allow color development (colors will range from pale to dark blue).

4. Set the wavelength of the spectrophotometer to 640 nm. Turn on the power and allow the machine to warm up for 5–10 minutes.
5. Transfer the contents of the tube labelled "blank" to a cuvet.

6. Insert the cuvet containing the blank into the sample compartment and adjust the meter to read 100% transmittance (zero absorbance).

7. Transfer the 10 μmol/liter NH_4^+ standard to a cuvet. Remove the blank and insert the standard into the sample compartment. Read the percent transmittance or absorbance. Repeat with each of the remaining three standards, rinsing the cuvet between standards.

8. Transfer the water samples to the cuvet, rinsing the cuvet between samples. Read the percent transmittance (or absorbance).

9. Convert the readings for the standards and samples from percent transmittance to absorbance.

10. Determine a regression of absorbance vs. concentration for the standards, or plot the absorbance readings of the standards vs. concentration. (The instructor will demonstrate how to perform the regression if you are interested).

11. Determine the concentration of NH_4^+ in the water samples using the regression equation, or the standard curve.

12. Calculate the rate of NH_4^+ excretion (μmol/g/hour) using the following equation:
    *Live weight in grams from step 1, Part D.

$$\mu mol\ NH_4^+\ excreted\ =\ volume\ of\ media\ in\ liters\ x\ ([\ NH_4^+]\ post\ -\ [\ NH_4^+]\ pre\ -\ exposure)$$

$$Rate\ of\ excretion\ =\ \mu mol/g/hour\ =$$

$$\mu mol\ NH_4^+\ excreted\ \ x\ \frac{1}{(live\ weight\ in\ g)*}\ \ x\ \frac{1}{(time\ of\ exposure\ in\ hours)}$$

Part C: Sampling Hemolymph

1. Ask the instructor to demonstrate the method of drawing a hemolymph sample, then, using a 1-ml syringe and 22-g needle obtain 300–400 μl of hemolymph from each of the four animals used in the measurement of ammonium excretion.

2. Immediately inject the hemolymph sample into a 1.5-ml microcentrifuge tube. The sample may begin to clot within 1–2 minutes. To obtain serum, break up the clot by aspirating the sample back and forth several times with the needle and syringe used to draw the sample. Once the sample has liquified, label the centrifuge tube and spin for 3 minutes in the microcentrifuge.

3. Carefully remove the serum from the centrifuged hemolymph sample using a Pasteur pipet and transfer to a clean microcentrifuge tube. Seal the tube, label and store in the freezer. Serum osmolality, and Na^+ and Cl^- concentrations will be determined using these samples (consult instructor).

Part D: Sampling Muscle Tissue

1. Weigh each of the four animals to the nearest gram and record the weight.

2. Obtain eight aluminum weighing dishes (two per each sea water concentration) and label each with your initials, section number, and the appropriate sea water concentration. Weigh each dish to the nearest milligram and record the weight.

3. Before proceeding with the next step, put on a pair of disposable gloves (to prevent transfer of amino acids from your hands to the tissue).

4. Remove the chelipeds (claws) at the plane of autonomy (the instructor will demonstrate). Crack open the exoskeleton and carefully remove the muscle tissue. Blot the tissue, divide it in half and place each half in an appropriately-labelled weighing dish. Discard the exoskeleton.

5. Weigh each tissue and dish to the nearest milligram.

6. Place one set of tissues from each of the four sea water concentrations in the drying oven overnight, then weigh the dish and dried tissue to the nearest milligram.

7. If time permits, proceed with the tissue amino acid analysis (Part F) using the remaining set of muscle tissues, or place each in a storage vial and freeze for future amino acid analysis.

Part E: Determining the Percent Tissue Water

1. Calculate the tissue wet weight by subtracting the weight of the dish from the total weight of the dish and wet tissue.

2. Calculate the tissue dry weight by subtracting the weight of the dish from the total weight of the dish and dry tissue.

3. Percent tissue water = [(wet weight - dry weight) × 100%] ÷ wet weight.

Part F: Preparation of Tissue for Amino Acid Analysis

1. For the amino acid assay, you will need the muscle tissue samples from Part D. If you are performing the analysis on the same day that the tissues are removed from the animal, transfer each sample from its weighing dish to a homogenizer and add 1 ml of distilled water for each 25 mg of tissue. If the samples have been frozen, thaw them and then add to the homogenizer along with the appropriate amount of water. (*Note:* Make sure you transfer all of the tissue sample, otherwise the weights will be incorrect in the final calculations of amino acid concentration.)

2. Homogenize the sample thoroughly while keeping the mixture cool by surrounding the homogenizer with ice.

3. Empty the homogenized sample into a centrifuge tube, rinsing back and forth several times to transfer all of the homogenate.

4. Centrifuge for 10 minutes at 3000–4000 rpm.
5. Remove 0.1 ml of supernatant and add it to 0.4 ml of 95% ethanol in a microcentrifuge tube. Mix thoroughly to deproteinize the sample and centrifuge for 5 minutes at 3000 rpm. (*Note:* The amount of supernatant may vary depending on the animal. Consult your instructor.)

6. Remove 0.1 ml of supernatant and add to a clean 8-ml glass test tube. Evaporate the ethanol by placing the tube in boiling water.

7. Reconstitute the residue by adding 1 ml of water to the tube. Mix thoroughly. Measure the amino acid concentration of this sample by carrying out the steps in Part G.

Part G: Determination of Free Amino Acid Concentration (Clark, 1973)

1. Prepare a set of glycine standards (25, 50, 100, and 200 µmol/liter) from a 1 mmol/liter glycine stock solution.

2. Add 1 ml of each standard to an appropriately labelled 8-ml test tube. Also add 1 ml of distilled water to a tube labelled "blank".

3. To all tubes containing 1 ml of standard, or the tissue extract from step 7 of Part F or the blank, add 0.5 ml of citrate buffer. Mix thoroughly.

4. Add 1.2 ml of ninhydrin solution to each tube and mix thoroughly.

5. Cover each tube with a clean marble. *Caution:* Do not touch the marble with your bare fingers. Use a glove to avoid contamination from ninhydrin-positive substances on your hands.

6. Place the covered tubes in a boiling water bath for 40 minutes.

7. Remove the tubes and cool in water for 5 minutes.

8. Add 3 ml of 60% ethanol.

9. Read the absorbance of the standards and tissue extracts against the blank at 570 nm. Determine the amino acid concentration of the tissue extracts from a plot of the glycine standards vs. absorbance. The colors should range from pale to dark purple depending on amino acid concentration.

10. Calculate the free amino acid concentration per kg of tissue water using the following formula:

$$Tissue\ free\ amino\ acids\ (mmol/kg\ tissue\ water) =$$
$$(value\ from\ standard\ curve) \times (dilution\ factor)$$

$$dilution\ factor\ =\ 50^* \times \frac{A+(B \times C)}{B \times C} \times \frac{1\ mmol/liter}{1000\ \mu mol/liter}$$

where A = ml of water in step 1, Part D
       B = (% tissue water) ÷ 100
       C = tissue wet weight (in grams) × 1 ml/g
       * This value (i.e., 50) will vary depending on the volume used in step 5 of Part F.

Exercise 2: Acid-base Balance

Introduction

There are two acid-base experiments which students have a choice of performing in our comparative physiology course, one dealing with the response to forced exercise and the other with air emersion. Both experiments are outlined in the following pages, and although intended for use in senior level physiology courses, they may be modified for use in junior courses.

The Henderson-Hasselbalch equation and "Davenport diagram" (Davenport, 1974) are used to illustrate the relationship between pH, total CO_2, and PCO_2. Students are given examples of various acid-base disturbances and compensatory mechanisms in aquatic and terrestrial animals. Exercise and emersion are used in the laboratory to demonstrate the development of metabolic and/or acute respiratory acidosis, and the ventilatory compensation for the acidosis.

Materials

Equipment and supplies for each pair of students:

Glassware:

Test tubes, 8 ml (14)
Graduated pipets, 5 ml (2)
Graduated pipets, 1 ml (2)
Beakers, 100 ml (2)

Reagents:
Perchloric acid (cold), 8% (50 ml)
NAD-LDH-glycine buffer mixture
  (Sigma Lactic Acid Kit; make up
    immediately before use) (20 ml)

Other supplies:

Plastic syringes with 22-g needles, 1 ml (5)
Plastic microfuge tubes, 1.5 ml (5)
Plastic mouse cages or similar containers capable of
  holding a crab and 2 liters of water (2)
Pi-pump or pipet bulb (1)
Vortex mixer (1)
Grease pencil or marker and tape (1)
Wash bottle with distilled water (1)
Spectrophotometer cuvets (2)
Ice bucket and ice (1)
Test tube racks (2)

Common items:

Spectrophotometers capable of reading at 340 nm
Centrifuge
Water bath at 37°C
Sigma Lactic Acid kits (one kit performs 45–60 assays; #826-UV, Sigma Chemical Co., P.O. Box
    14508, St. Louis, MO 63178)
Micropipettors (100 μl, 250 μl, 400 μl) and tips

Apparatus for pH measurement:

Acid-base analyzer (e.g., Radiometer or Cameron Instruments)

Cooling coil and water bath (for cooling acid-base electrodes below ambient temperature)
PE 50
Saturated KCl
Dilute bleach (for cleaning electrodes)
Distilled water (for cleaning electrodes)
Precision buffers (pH 7.4 and 6.9)

Apparatus for total CO_2 measurements:

Cameron chamber with PCO_2 electrode and readout
Water bath set at 37°C
Stir plate and stir bar
Vacuum line or aspirator for emptying chamber
0.01 N HCl (1 liter)
Large syringe labelled "0.01 N HCl"
$NaHCO_3$, 15 mmol/liter (1 liter) [1.26 g $NaHCO_3$/liter = 15 mmol/liter]
Hamilton syringes, 50 µl (2; label one "HCO_3^- std" and the other "sample")
CO_2 cylinder, 5% [balance air or N_2]
CO_2 cylinder, 1% [balance air or N_2]
Calibrate the PCO_2 electrode (at 37°C) to 20 torr with 1% CO_2 and 100 torr with 5% CO_2

Apparatus for attaching electrodes and recording heart and scaph rates:

Dental drill
Dental dam
Crazy glue
Polythermalese wire (or other insulated 34 gauge wire)
PE 160 tubing
Impedance convertors and chart recorders

Notes for the Instructor

Order enough large crabs or crayfish so that each pair of students has one animal. The animals should be large (100 g or more) because a number of blood samples will be drawn over a fairly short period of time. Two to three days prior to use, prepare each animal for hemolymph sampling from the pericardial sinus as described in Appendix C. It is best to drill a hole on either side of the heart so that samples may be drawn alternately from either site. If you wish to follow the changes in ventilation rate and/or cardiac rate during the experiment, follow the directions in Appendix D for placement of recording electrodes around the gill bailers and heart.

Basically, the two experiments described in the next few pages involve measurement of hemolymph pH, total CO_2, and lactate concentration. PCO_2 is calculated from measured values of pH and total CO_2, using the Henderson-Hasselbalch equation. If ventilation and heart rate are to be recorded, records must be obtained prior to drawing the hemolymph samples.

Twenty-four hours prior to the laboratory, place the crabs or crayfish in individual plastic rate cages or other suitable containers of equal size. If the sides of the containers are transparent, cover them with black plastic (garbage bags will do) to decrease the chance the animals will be disturbed. It is also best to cover the tops of the containers for the same reason. If you plan to record ventilation and/or heart rate, drape the leads of the recording electrodes over the side of the container.

Before the laboratory begins, demonstrate the technique of hemolymph sampling as outlined in Appendix C. Make sure each pair of students has enough syringes and needles for all hemolymph samples. Microcentrifuge tubes containing 400 μl of 8% perchloric acid should be on ice at each station.

Since accurate measurement of pH and total CO_2 are imperative, it is best that you make the pH measurements yourself and assign students the task of measuring total CO_2. We use a Radiometer pHM72 or G 297/G2 pH electrode thermostatted to the ambient temperature of the animals (Cameron Instruments Blood Gas Analyzers are also available). Total CO_2 measurements are made with the Cameron technique (Cameron, 1971). Details regarding the construction and set-up of the Cameron chamber are given in Appendix F.

Hemolymph samples should be drawn at the intervals indicated below:

Exercise experiment:	*Emersion experiment:*
pre-exercise	pre-emersion
immediately after exercise	immediately after re-immersion
60 minutes post-exercise	60 minutes post re-immersion
120 minutes post-exercise	120 minutes post re-immersion
180 minutes post-exercise	180 minutes post re-immersion

At least 250 μl of sample must be obtained for measurement of pH, total CO_2, and lactate concentration. Stagger the starting times for each of the student pairs so that you will avoid a backlog of samples for determination of total CO_2 and pH.

Student Outline: Acid-Base Balance

Regulation of body fluid acid-base status is a process fundamental to maintenance of life in all animals. Most animals maintain body fluid pH between 7 and 8, slightly alkaline with respect to the pH of neutrality of water. Almost all of the cellular biochemical reactions are affected by variation in pH; therefore, regulation of acid-base status is important. An animal's acid-base status can be determined by monitoring blood pH, total CO_2 (blood HCO_3^- and CO_3^{-2} concentration), and PCO_2 (partial pressure of CO_2 in blood).

PCO_2 is determined by the rate of cellular CO_2 production and the efficiency of gas exchange across the respiratory surface (lungs or gills). In most animals PCO_2 is closely monitored and adjusted by variation in ventilation. The relationship between pH and PCO_2 is defined by the Henderson-Hasselbalch equation:

$$pH = pK + \log\left(\frac{[\ HCO_3^- + CO_3^= \ ]}{\alpha \ x \ PCO_2}\right)$$

where α is the CO_2 solubility coefficient (mmol/liter/mm Hg).

Increase in PCO_2 causes a rise in $[H^+]$ and, therefore, a decrease in pH. Decrease in PCO_2 has the opposite effect. Acid-base disturbances which are caused by variation in PCO_2 are referred to as respiratory acidosis or alkalosis. An increase in PCO_2 causes a respiratory acidosis, while a decrease in PCO_2 results in a respiratory alkalosis.

Metabolic acid-base disturbances can occur as a result of the addition of a "fixed" acid or base to blood. Fixed acids, such as lactic acid, cannot be removed by the respiratory system; they must be metabolized or excreted. Increases in fixed acid concentration in the blood result in metabolic acidosis, while the development of a metabolic alkalosis is due to the increase in fixed base. Although both disturbances result in little change in PCO_2, total CO_2 concentration is significantly affected: total CO_2 decreases in a metabolic acidosis and increases in a metabolic alkalosis.

In exercise, both a metabolic and a respiratory acidosis are common because of increased cellular metabolism resulting in a significant elevation in CO_2 and lactic acid production. Compensation for the acidosis includes a rapid reduction in PCO_2 through hyperventilation, the metabolism of lactate (which is a slower process), and an increase in blood carbonate ($HCO_3^- + CO_3^-$) concentration to return acid-base status to normal.

Air emersion for 2–4 hours also results in a respiratory acidosis, and, in some crustaceans, development of a metabolic acidosis also occurs. Because most crustaceans are water-breathers, exposure to air causes the gills to collapse and, therefore, makes it difficult for the animal to exchange CO_2 for oxygen. Consequently, CO_2 increases in the circulation and the oxygen content decreases. The animal may reduce its metabolic rate as a means of coping with the stress, or resort to increased anaerobic metabolism, thus producing lactic acid. As in exercise, the compensation for the acidosis is mainly via increased ventilation upon return to the aquatic environment (i.e., re-immersion).

You have a choice of performing either experiment, that is, exercise or emersion, as outlined on the following pages. Since repetitive blood sampling is necessary, careful use of the syringe and needle is a must to avoid damage to the heart.

Exercise Experiment

Part A: Pre-Exercise

1. When all is ready, record the gill bailer and heart rates for 2–3 minutes using the previously implanted impedance electrodes.

2. While the recordings are being made, fill the dead space for a 2-ml syringe and 22-g needle with crustacean Ringer's solution (or seawater if applicable).

3. After the recordings are made, carefully uncover the container. Gently hold the animal in place under water with one hand and insert the tip of the syringe needle about 0.5 cm into the pericardial sinus. Slowly and carefully withdraw at least 0.25 ml (250 µl) of hemolymph. Hemolymph is more viscous and opaque than water. Check the syringe contents to make sure you indeed have drawn hemolymph.

4. Immediately transfer 100 µl of hemolymph into a microcentrifuge tube containing 400 µl of ice cold 8% perchloric acid. Thoroughly mix the hemolymph and acid, then place the microcentrifuge tube on ice. *Note:* The sample must remain in contact with the cold perchloric acid for at least 5 minutes to ensure complete deproteinization.

5. Hand the syringe containing the remaining hemolymph sample to the instructor for pH determination. You will also measure the total CO_2 concentration of the sample (refer to section on Analytical Procedures).

Part B: Exercise

1. Upon completion of the pre-exercise sampling, begin prodding the animal into continual movement for 15–20 minutes. If you are using crayfish, try coaxing the animal to perform tail flips. By the end of the exercise period most animals will be unresponsive to stimulation.

2. Immediately upon completion of the exercise period, record the gill bailer and heart rates and draw a hemolymph sample (250 μl). Dispense 100 μl of hemolymph into cold perchloric acid as performed in the pre-exercise period. Determine pH and total CO_2 using the remaining hemolymph sample.

Part C: Post-Exercise

1. Record the ventilation and heart rates, and remove a sample of hemolymph (250 μl) at the post-exercise times recommended by the instructor.

2. Dispense 100 μl of hemolymph into perchloric acid. Determine pH and total CO_2 using the remaining hemolymph sample.

Air Emersion Experiment

Part A: Pre-Emersion

1. While the animal remains submerged and undisturbed in a covered container, record the ventilation rate (both left and right gill bailers if possible) and heart rate using the previously-implanted impedance electrodes. Record for 2–3 minutes.

2. Following these recordings, draw at least 250 μl of hemolymph (as described in Appendix C). Transfer 100 μl to cold perchloric acid, mix thoroughly and store on ice for at least 5 minutes.

3. Measure pH and total CO_2 as soon as possible using the remainder of the sample (the instructor will measure pH; you will measure total CO_2 according to the directions in the section Analytical Procedures).

Part B: Emersion

Carefully drain the container, or transfer the animal to a dry container. Disturb the animal as little as possible for the next 2–4 hours.

Part C: Re-Immersion

1. At the end of the emersion period (2–4 hours), record heart rate and ventilation rate (if possible). Draw 250 μl of hemolymph for pH, total CO_2 and lactate determination, as before. (*Note:* It may not be possible to record gill bailer movements while the animal is out of water using the impedance technique.)

2. Re-immerse the animal in water and record the ventilation and heart rates every 10–15 minutes for the first hour after re-immersion.

3. At the end of 1, 2, and 24 hours post re-immersion, record ventilation and heart rates and then draw 250 μl of hemolymph for pH, total CO_2, and lactate determination.

Analytical Procedures

Part A: Determination of Lactate Concentration in Hemolymph

One of the most accurate and easy methods of lactate determination is performed using a Sigma Lactic Acid Kit. This is an ultraviolet spectrophotometric method based on the following reaction:

$$\text{lactate}^- \longrightarrow \text{pyruvate}^-$$

$$\text{NAD}^+ \longrightarrow \text{NADH}$$
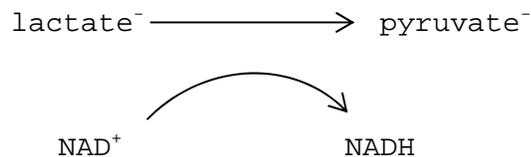
in the presence of lactate dehydrogenase (LDH) and glycine buffer. A spectrophotometer capable of reading absorbance at 340 nm must be available.

1. Centrifuge all microcentrifuge tubes containing hemolymph and perchloric acid for 10 minutes at 3000 rpm. The perchloric acid will deproteinize the hemolymph; therefore, at the end of centrifugation a white precipitate will form at the bottom of the tube and the supernatant will contain the lactate present in the hemolymph.

2. Label a set of clean test tubes, one tube for each hemolymph sample. Also, label one tube "blank".

3. Prepare the NAD-LDH-glycine mixture according to the direction in the Sigma Lactic Acid Kit. Add 2.8 ml of this mixture to each test tube.

4. To the tube marked "blank", add 0.2 ml of 8% perchloric acid. Mix thoroughly.

5. Transfer 0.2 ml of supernatant from each centrifuge tube to the appropriately labelled test tube and mix thoroughly.

6. Incubate the samples and the blank for 30 minutes at 37°C.

7. Set the wavelength of a spectrophotometer to 340 nm. Insert the blank and set the machine to read zero absorbance. Read the absorbance of all samples and convert absorbance to concentration (mmol/liter) using a standard curve prepared in the manner outlined in the directions included in the Sigma Lactic Acid Kit.

Part B: Determination of Hemolymph pH

An accurate pH meter and sensitive microelectrode are mandatory for measurement of hemolymph pH. Temperature is set by circulating water through the jacket from a thermostatically-controlled water bath. Calibration is performed with a pair of precision buffers which bracket the normal range of hemolymph pH values for crustaceans. The electrode requires a minimum of 50 μl of sample. The equilibration time at 15–20°C is about 2–3 minutes. During that length of time, the hemolymph may clot in the electrode, especially if it has been sitting in the syringe for very long. To reduce the chances of clotting, store the syringes on ice. If clotting does occur in the electrode, the clot can be removed by carefully running a length of nylon monofilament through the electrode capillary.

Part C: Determination of Hemolymph Total CO_2 (The Cameron Technique)

Total CO_2 can be measured with a technique developed by Cameron (1971) which is based upon the principle that all forms of CO_2 (dissolved CO_2, carbonate, bicarbonate, and protein-bound CO_2) are converted into the soluble form when acidified. The increase in CO_2 tension (PCO_2), upon addition of the sample to the acid, is measured and compared with the change in CO_2 tension obtained with addition of a known amount of a bicarbonate standard, as described in detail later on.

A CO_2-sensitive electrode which is fitted into a temperature-regulated cuvet is used to measure changes in the CO_2 tension. The CO_2 electrode is initially calibrated with two gases so that 1% and 5% CO_2 gas mixtures read 20 and 100 torr, respectively. A small magnetic stir bar is placed in the cuvet to ensure continual flow of sample past the electrode, and the cuvet is filled with 0.01 N HCl. Standardization with a 15 mmol/liter $NaHCO_3$ solution is performed prior to determination of the hemolymph sample total CO_2.

Procedure for Cameron Technique

1. Fill the cuvet of the Cameron chamber with 0.01 N HCl at 37°C. Make sure no air bubbles are trapped in the cuvet.

2. Using a 50 μl Hamilton syringe labelled "standard", inject about 20 μl of 15 mmol/liter $NaHCO_3$ solution into the cuvet to bring the PCO_2 of the acid solution up to about 20 torr.

3. Carefully measure out 10 μl of the 15 mmol/liter bicarbonate standard, note the PCO_2 of the cuvet, and inject the standard. Wait 3 minutes, then read the PCO_2 again. Record the change in PCO_2 (ΔPCO_2).

4. Carefully transfer 40 μl of hemolymph to the syringe labelled "sample". Note the PCO_2 and then inject the hemolymph into the cuvet. Wait 3 minutes then read the PCO_2 again. Record the change in PCO_2 (ΔPCO_2).

5. Add another 10 μl of bicarbonate standard to the cuvet and measure the change in PCO_2 as previously done in step 4.

To Calculate the Hemolymph Total CO_2

Average the PCO_2 for the standards which bracket each hemolymph sample and then use the following formula to calculate hemolymph total CO_2:

$$Total\ CO_2\ (mmol/liter) =$$

$$\Delta PCO_2\ hemolymph\ x\ \frac{15\ mmol/liter}{average\ \Delta PCO_2\ standard} - \frac{10\ \mu l}{40\ \mu l}$$

where: 10 μl = volume of standard injected
         40 μl = volume of hemolymph injected
         15 mmol/liter = concentration of bicarbonate standard

Part D: Calculation of Hemolymph PCO$_2$

Hemolymph PCO$_2$ can be calculated using the Henderson-Hasselbalch equation and a set of alignment nomograms for determination of CO$_2$ solubility (Truchot, 1976).

1. Henderson-Hasselbalch equation:

$$pH = pK + \log\left(\frac{[\; HCO_3^- + CO_3^= \;]}{\alpha \; x \; PCO_2}\right)$$

where *pK* and α (solubility coefficient) are derived from Truchot's nomographs.

2. If [HCO$_3^-$ + CO$_3^=$] = Total CO$_2$ - (α × PCO$_2$), then the above equation may be rewritten as:

$$pH = pK + \log\left(\frac{TCO_2 - (\alpha \; x \; PCO_2)}{\alpha \; x \; PCO_2}\right)$$

where TCO$_2$ = total CO$_2$ concentration.

3. Rearranging the above equation:

$$PCO_2 = \frac{TCO_2}{\alpha \; x \; [1 + antilog(\; pH - pK \;)]}$$

Literature Cited

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Davenport, H. W. 1974. The abc of acid-base chemistry. University of Chicago Press, 124 pages. [ISBN 0-226-13705-8].

Gross, W. J. 1954. Osmotic responses in the sipunculid *Dendrostomum zostericolum*. Journal of Experimental Biology, 31:402–423.

McDonald, D. G., B. R. McMahon, and C. M. Wood. 1979. An analysis of acid-base disturbances in the hemolymph following strenuous activity in dungeness crab, *Cancer magister*. Journal of Experimental Biology, 79:47–58.

Oser, B. L. 1965. Hawk's physiological chemistry. Fourteenth edition. McGraw-Hill, New York, 1472 pages. [pages 1108–1112]

Truchot, J. P. 1976. Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L.): Carbon dioxide solubility coefficient and carbonic acid dissociation constants. Journal of Experimental Biology, 64:45–57.

APPENDIX A
Animals

Supply houses which sell certain species of crayfish and crabs:

Crayfish
Procambarus sp.

- Atchafalaya Biological Supply Co., Raceland, LA 70394
- Carolina Biological Supply Co., 2700 York Rd., Burlington, NC 27215
- Marinus Biological Supply Co., 1400 W. 7th St., Long Beach, CA 90813

Pacifastacus leniusculus

- College Biological Supply, Bothwell, WA 98011

Crabs: Callinectes sapidus

- Gulf Specimens Co. Inc., P.O. Box 237, Panacea, FL 32346

Carinus maenas

- Marine Biological Lab Supply Department, Woods Hole, MA 02543
- Ocean Resources, Peak's Island, ME 04108

To facilitate acclimation to the various salinities used in Exercise 1 (iono- and osmoregulation), animals should be on hand no later than 3 weeks prior to the scheduled experiment. It is best to allow the animals to recover from the stress imposed by transportation by placing them in clean, well-aerated water for a few days. Make sure the water temperature is compatible with the species. *Callinectes* sp., *Procambarus* sp., and *Pacifastacus* sp. survive quite well at 20°C, but *Carcinus* sp. and most of the *Cancer* sp. crabs require temperatures ranging from 10–15°C.

After the animals have recovered from trauma of shipment, divide them into four groups (i.e., one group for each salinity). If you are using marine species, maintain one group in 100% (i.e., 30–35 parts per thousand) seawater. Place the other three groups in 75% seawater for at least 5 days. Following acclimation to 75% seawater, transfer two groups to 50% and maintain them at this salinity for at least 5 days. Finally, remove one of the groups acclimated to 50% and place these animals in 30% seawater again for 5 days.

Feed the animals small pieces of smelt or dry cat food every other day throughout the acclimation process. Stop feeding 2 days prior to the experiment.

If you plan to acclimate fresh water crayfish to dilute seawater, follow the same schedule but do not attempt to acclimate the animals to salinities greater than 75% seawater. Most crayfish, with the exception of *Pacifastacus* sp. which is euryhaline, cannot tolerate such drastic changes in salinity.

Unless you are in an area where seawater is abundant, you will probably resort to holding animals in a recirculating water system. Because of the build-up of nitrogenous wastes, such a system can be a problem. However, the problem can be overcome if proper biological filters are in place prior to the arrival of the animals. Since our seawater supply is very limited, we have constructed a number of aquaria equipped with home-made undergravel filters through which the water is recirculated. The aquaria are actually plastic laundry tubs (50 cm × 50 cm × 30 cm deep) and the filters are constructed from plastic "egg-crate" grid covered with plastic window screening. A 1" layer of fine gravel (crushed limestone for marine systems or granite for freshwater systems) placed on top of the screening serves as the filter bed and substrate for denitrifying bacteria. An air lift system, consisting of a plastic pipe and air stone, is set into the filter bed to aerate the water and provide circulation through the filter. It will take time for the denitrifying bacteria to become well established in the filter bed. Therefore, I recommend setting up the system a few weeks before the animals arrive or if time is a factor, replace half the water every third day for the first week or two after the animals have arrived. You may wish to monitor nitrate and NH_4^+ levels during this period.

APPENDIX B

Reagents for Exercise 1

Reagents for Ammonium Analysis

1. *Primary standard:* 1 mmol/liter NH_4Cl = 0.0535 g/liter in distilled water. Make dilutions of the primary standard for 75, 50, 25, and 10 μmol/liter.

2.* *Sodium nitroprusside, 0.5%:* 0.5 g of Na nitroferricyanide (nitroprusside) per 100 ml of distilled water. Store in a dark bottle.

3.* *Phenol-alcohol solution:* 10 g phenol per 100 ml of 95% ethanol.

4.* *Oxidizing solution (make fresh daily):* (10 parts alkaline sodium citrate [200 g trisodium citrate and 20 g NaOH]) and (2.5 parts of hypochlorite [laundry bleach]). *Note:* Commercial grade laundry bleach will do; however make sure the bleach is fresh.

Reagents for Free Amino Acid Analysis

1. *Citrate buffer, pH 4.8:* 42 g citric acid and 16 g NaOH. Dilute to 1 liter with distilled water.

2. *Ninhydrin stock:* 11.5 g ninhydrin and 100 ml of methyl cellosolve (ethylene glycolmonomethyl ether).

3.* *Ninhydrin solution (make fresh daily):* 10 ml ninhydrin stock and 4 ml of 1% ascorbic acid. Make up to 120 ml with methyl cellosolve.

4. *1% ascorbic acid:* 1 g ascorbic acid in 100 ml of distilled water.

5. *Glycine standard:* 1 mmol/liter glycine = 0.075 g/liter in distilled water. Dilute to give 200, 100, 50, and 25 μmol/liter standards.

* Due to the caustic, toxic, or light-sensitive nature of these reagents it is best to place them in dispensers.

APPENDIX C
Hemolymph Sampling

Hemolymph sampling is usually somewhat traumatic to an animal and can be even more traumatic to the novice drawing the sample. There is, however, a method of hemolymph sampling from the pericardial sinus surrounding the heart which is quite easy and less traumatic to all parties concerned (McDonald et al., 1979). The materials required are a variable-speed drill, medium gauge latex dental dam, and cyanoacrylate glue. The dental dam can be purchased through a dental supply company and the cyanoacrylate glue ("Krazy glue") is obtainable from a variety of sources. Although a dental drill is much easier to operate, a variable-speed hand drill with a flexible shaft will suffice.

A few days prior to hemolymph sampling, a hole is drilled through the dorsal carapace just lateral to the area overlying the heart. The animal must be restrained during this procedure, and it is also wise to place bands around the claws. Make the hole about 1 mm in diameter and about 5 mm lateral to the midline. It should be deep enough (2–3 mm) to expose the epidermis overlying the pericardial sinus. Do not drill through the epidermis or you may destroy the heart beneath. Once the hole has been drilled, dry the exoskeleton with tissue paper and then place a ring of cyanoacrylate glue around the hole. Cut a 1-cm^2 piece of dental latex and place it over the hole. Gently press down on the latex to seal the hole. Mark the hole with nonwater-soluble ink (a felt tip pen works well) and return the animal to water. Allow 48 hours for recovery from the surgery.

To sample the hemolymph, attach a 22-gauge needle to a 1-ml glass or plastic syringe. Fill the dead space of the syringe and needle with crustacean Ringer's solution or seawater. Carefully insert about 0.5 cm of the needle through the latex and into the hole overlying the epidermis. Slowly draw back on the plunger of the syringe. Hemolymph should enter the syringe quite easily with very little suction. If it is difficult to draw a sample, do not continue. Remove the needle, refill the deadspace and try again.

Be very careful that you do not impale the heart by inserting the needle too far. To prevent this from happening, we mark the needle at 0.5 cm or place a piece of PE tubing around the needle shank so that only 0.5 cm is exposed. With a little practice, you can obtain a hemolymph sample without disturbing the animal at all.

APPENDIX D
Measurement of Ventilation and Heart Rates

Ventilation and heart rates are recorded using a pair of electrodes and a device for measuring impedance. The Model 2991 Impedance Convertor (UFI, 495 Embarcadero, Morro Bay, CA 93442) is such a device. When connected to a pen recorder, a clear signal of gill bailer movement or heart contraction is easily obtainable with the impedance convertor. Recording electrodes are made from 34 AWG polythermaleze wire (Belden Corp., Chicago, IL 60644), which is a thin copper wire insulated with lacquer. Before attaching the wire to the animal, the lacquer insulation is carefully scraped or burned off the terminal 0.5 cm of the wire. Likewise, the other end of the wire, which is attached to the impedance convertor, is also cleaned of its insulation.

Each event (heart rate or gill bailer rate) must be recorded with a pair of electrodes. It is best to use about 2 feet of wire for each electrode of the pair in order to have enough slack to reach the impedance convertor from the animal in its container. To reduce chance of damage to the wire and to prevent the animal from becoming entangled, the electrode wire should be fed into a length of PE 160 tubing. Identification of the electrode pairs at either end of the tubing can be made by tagging each electrode wire with enamel paint, or by attaching a small piece of tape. This procedure should be done prior to attachment of the electrode pairs to the animal.

Before recording gill bailer or heart rates, holes must be drilled in the animal's carapace for placement of the electrodes. Figures 11.1 and 11.2 illustrate the correct location of the holes. To record gill bailer rates, one electrode is hooked beneath the branchiostegite as indicated in Figure 11.1, and the other electrode is placed in a small hole drilled into the branchiostegite just above the gill bailer. The electrodes are held in place by small patches of dental dam glued to the branchiostegite with cyanoacrylate glue. Note from Figure 11.2 that the electrodes for recording heart rate are inserted into small holes in the carapace on either side of the heart and held in place with dental dam.
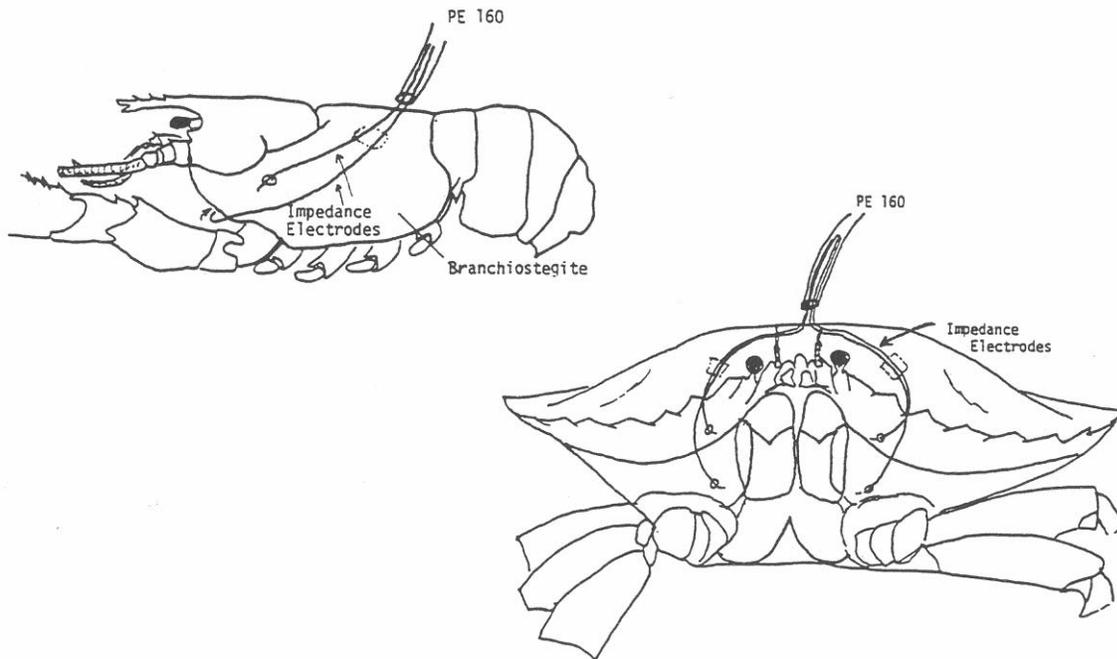

Figure 11.1. Location of impedance electrode placement for recording gill bailer movement of a lobster or crayfish (*top*, side view) and a crab (*bottom*, frontal view).
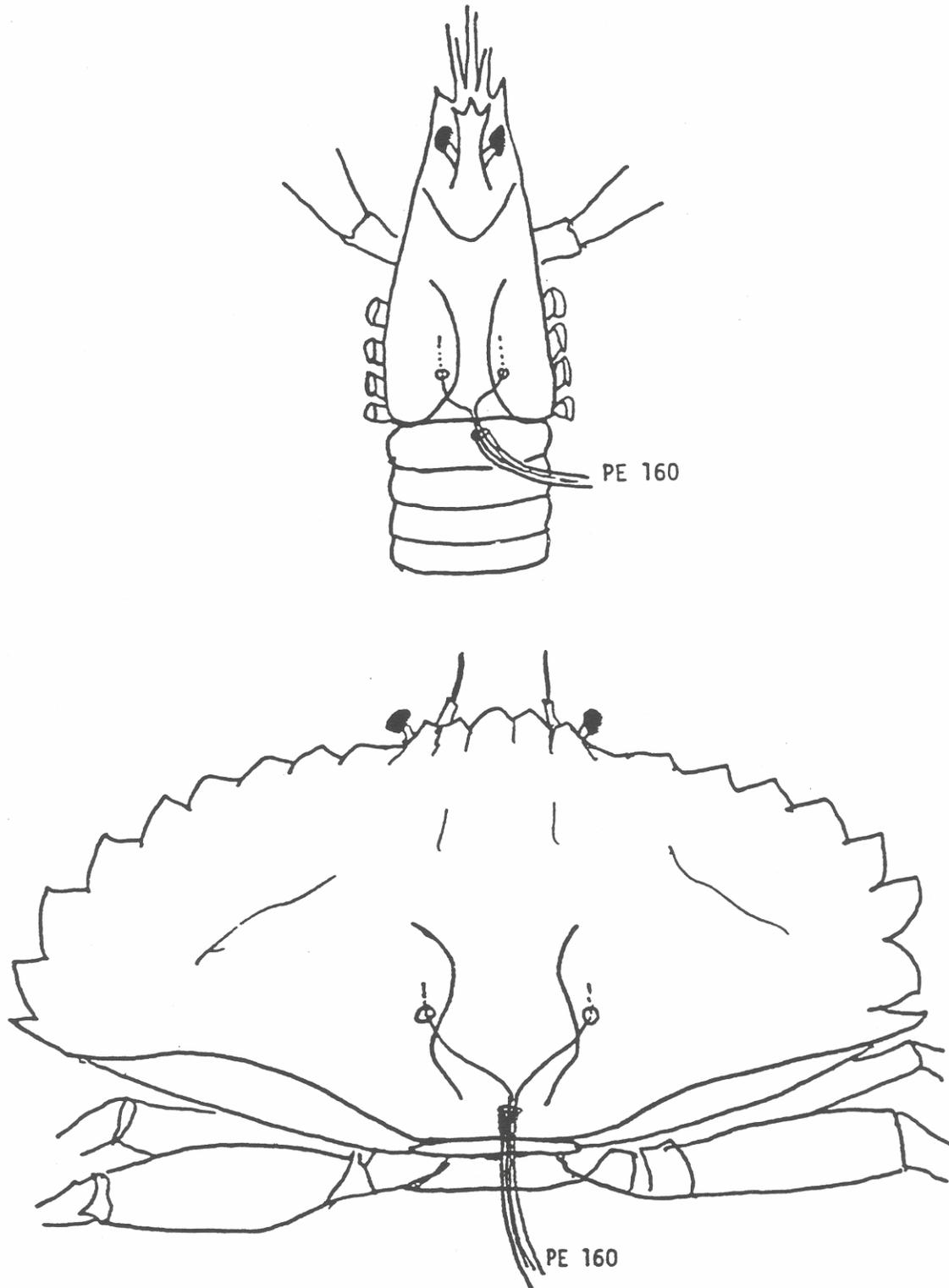
Figure 11.2. Location of impedance electrodes for recording heart rate of a lobster or crayfish (*top*, dorsal view) and a crab (*bottom*, dorsal view).

APPENDIX E
Measurement of Osmotic Pressure (Osmolal Concentration)

The osmol concentration of a fluid can be determined without directly measuring the freezing point (or melting point). This ingenious method was described by Gross (1954). Briefly, test solutions are drawn into capillary tubes, frozen, and their time to melt observed under polarizing filters placed at 90° angles. Standards of known NaCl concentrations are prepared in the same manner so their time of melting may be compared to that of the unknowns. The time required for the melting of known NaCl solutions is plotted against the concentrations of solution. From the standard curve, the osmotic concentration of unknowns can be determined in terms of the standards.

The apparatus is illustrated in Figure 11.3. Light is reflected by means of a mirror upward through a polarizing filter. Only light directed in a single plane is allowed to pass through the filter and enter an alcohol bath. The bath (ethanol at -4 to -6°C) holds a rack of capillary tubes containing frozen samples of the known and unknown solutions. Ice crystals in the sample rotate the polarized light at such an angle that light rays pass through the top polaroid filter (placed at a 90° angle to the bottom filter). The tubes when viewed through the top filter appear as bright columns of fluorescent light against a dark background. As the alcohol bath warms, the tubes melt and the light gradually rotates to its original plane. Since the top filter is at a right angle to the polarized light the rays are blocked and the bright fluorescent tubes grow dimmer. The tubes containing the most concentrated solutions melt first. If time to melt for subsequent tubes is recorded, a standard curve may be plotted as "time to melt" versus concentration of the standards. Osmol concentrations of the unknowns are found from this plot.

Materials

Glass capillary tubes, container of 75 mm × 1.5 mm (O.D.)
Dry ice and 100% ethanol mixture (-70°C)
Ethanol, 25% (-4°C)
Light source (100 watt incandescent blub or fluorescent lamp)
Pyrex glass casserole dish (75 mm deep × 120 mm wide × 220 mm long)
Plastic rack (90 mm × 75 mm) capable of holding 12 capillary tubes
Glass mixing rod
Polarizing filters, each 100 mm square (2)
Viewing box (20 cm high × 15 cm wide × 25 cm long) with shelf to support glass dish and lower
    polarized filter
Mirror
Stop clock

Procedure

1. Prepare a set of NaCl standards of the osmolalities listed in Table 11.1.

2. For each of the five standards, a distilled water blank, and each of the samples, obtain a glass capillary tube. Fill each tube about half full with the appropriate fluid.

3. Center the fluid in the tube (Figure 11.3) and seal both ends with putty, making sure there is an air space between the putty and fluid at either end.

4. Mount the tubes in the plastic tube holder, keeping a record of the position and contents of each tube.
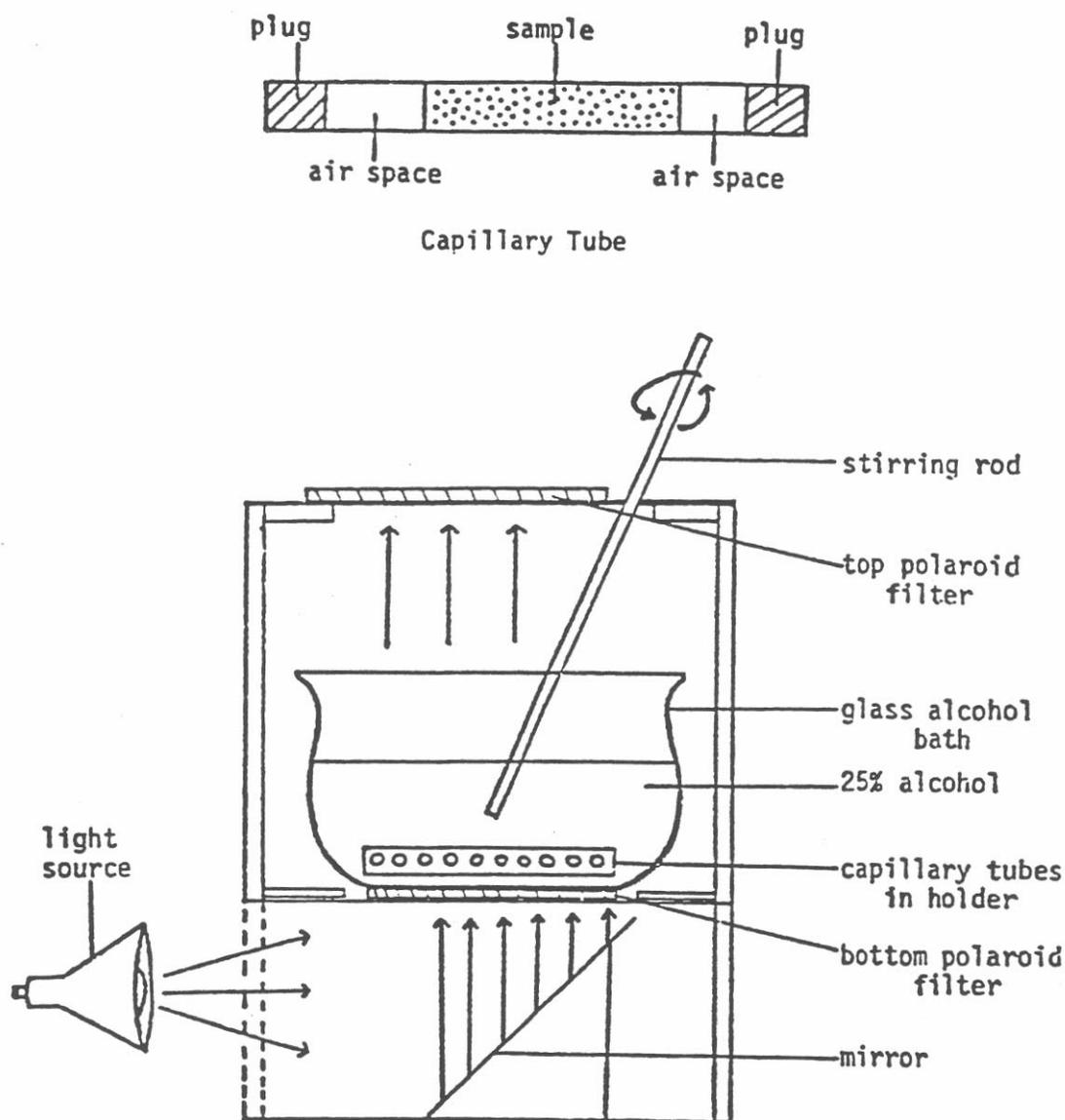
Figure 11.3. Apparatus for determination of osmotic concentration of fluid by measurement of time to melt (Gross, 1954).

5. To snap-freeze the samples, quickly dip the holder containing the capillary tubes in and out of a dry ice/100% ethanol mixture at -70°C. *Note:* The tubes may break if you leave the rack in the dry ice/ethanol mixture for more than 2 seconds.

6. Immediately transfer the holder containing the frozen samples to a dish of 25% ethanol at -4°C.

Table 11.1. Preparation of osmolal standards.

Osmolality mOs/kg H_2O	NaCl g/kg H_2O
100	3.089
300	9.457
500	15.930
750	24.100
1000	32.230

7. Slide the dish into the viewing box, close the box and place the upper polarizing filter over the viewing hole in the top of the box. The upper filter must be at 90° to the polarity of the lower filter. Close the box and center the light source so that light is reflected up into the dish.

8. Continually stir the bath with a glass rod while observing the tubes. Note the fluorescence of the frozen samples.

9. Start the stopclock when one of the tubes has reached a point where approximately 80% of the sample has melted, as indicated by a decrease in fluorescence. Record the position of this tube. *Note:* Do not wait for the tube contents to melt completely because of the possibility that pure water crystals may have formed during freezing.

10. Continue to observe the tubes, recording the position of each tube and the time to reach 80% melt.

11. For *each run*, plot the osmolal concentration of the standards vs. time to melt and, by extrapolation from the standard curve, determine the osmolal concentration of the samples. The standards *must* be included in each run as the time to melt will vary from run to run. There is no need to fill new capillaries with fresh standard. As long as the tubes are properly sealed, the standards may be used over and over again.

Construction of the Viewing Box

Our viewing boxes are made of 0.25" plywood cut to the dimensions stated above. A 4"-diameter hole cut in the top allows you to view the rack of tubes through a polarizing filter placed over the hole. In addition, 0.5"-diameter holes cut into the top allow access for the stirring rod.

The center shelf should also be 0.25" plywood or hardboard. A hole 4" in diameter should be cut to allow light to reflect up through a polarizing filter attached to the bottom of the shelf.

Construct the box so that you may open one end to insert the glass dish or remove the shelf. Our boxes contain a removable panel which simply slides into place.

You will need to drill a 2.5"-diameter hole near the bottom of one side to allow light to enter the box from your light source. The light source may be a desk lamp or microscope lamp. A mirror placed near the back of the box should be adjusted to reflect light upward through the lower polarizing filter.

APPENDIX F
Cameron Chamber

The Cameron chamber (Cameron, 1971) can be constructed from acrylic plastic or glass, using the dimensions given in Figure 11.4.   The electrode jacket shown in the figure is designed to fit a Radiometer (type E5036) PCO_2 electrode which is held in place by a cap threaded to fit the portion of the electrode jacket which extends from the side of the chamber.   Water is circulated through the chamber from an external water bath at 37°C. The cuvet is filled with 0.01 N HCl, and a small stir bar placed in the bottom of the cuvet ensures continual mixing of the blood sample with the acid solution when the chamber is placed on a magnetic stir plate. Following measurement, the sample and used acid solution are aspirated from the chamber and fresh acid solution is added.

Calibration of the electrode requires 1% and 5% CO_2 gas mixtures.   This procedure as well as details regarding standardization (15 mM HCO_3^-) and sample measurement are described in Exercise 2.
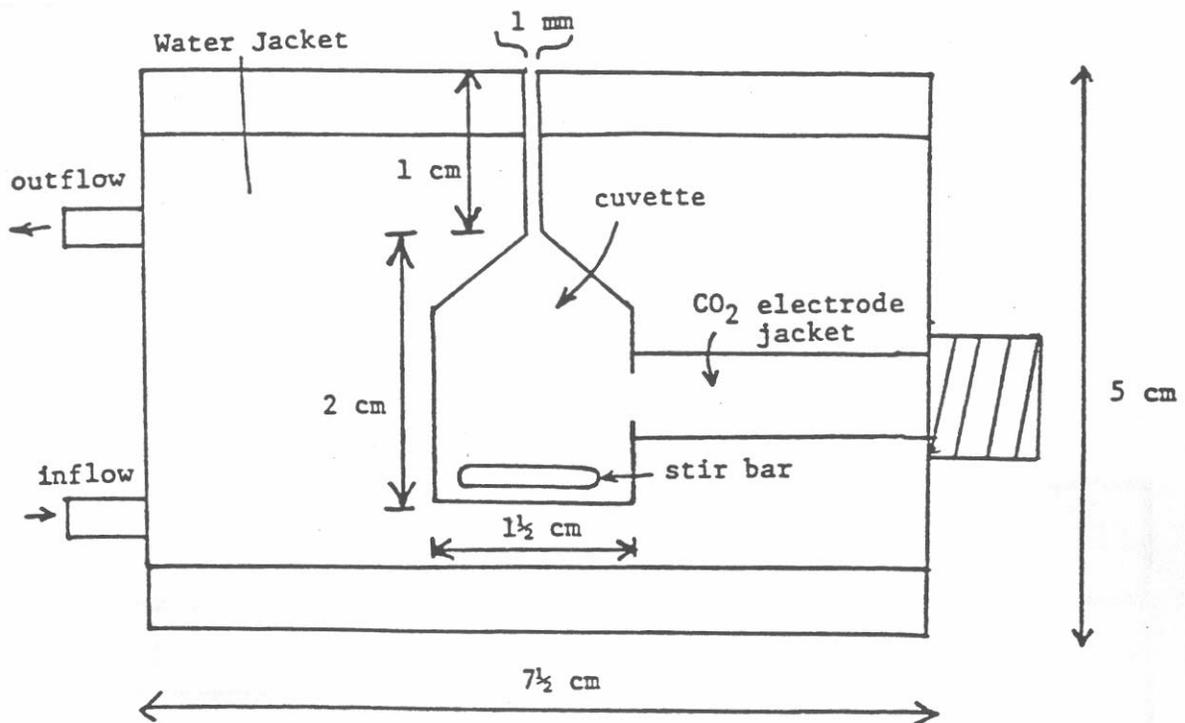

Figure 11.4. Side view of the Cameron chamber showing dimensions of the sample cuvet and water jacket (Cameron, 1971).

APPENDIX G

Samples of Class Data for Exercises 1 and 2

Exercise 1

Table 11.2. Sample class data for *Callinectes sapidus* (blue crab).

Parameter	Sea water			
	100%	75%	50%	30%
Hemolymph Na$^+$ (mmol/liter)	473	387	302	300
Hemolymph Cl$^-$ (mmol/liter)	467	422	329	311
Hemolymph osmolality (mOs/kg H$_2$O)	995	810	718	636
Tissue free amino acids (mmol/kg tissue H$_2$O)	474	356	313	293
Tissue H$_2$O (%)	78.2	80.5	81.6	80.3
NH$_4^+$ excretion (μmol/hour/g)	0.414	0.571	1.371	1.195

Table 11.3. Sample class data for *Carcinus maenas* (green rock crab).

Parameter	Sea water			
	100%	75%	50%	30%
Hemolymph Na$^+$ (mmol/liter)	491	430	343	292
Hemolymph Cl$^-$ (mmol/liter)	506	441	335	295
Hemolymph osmolality (mOs/kg H$_2$O)	995	878	660	560
Tissue free amino acids (mmol/kg tissue H$_2$O)	285	323	250	154
Tissue H$_2$O (%)	76.9	79.2	78.3	77.8
NH$_4^+$ excretion (μmol/hour/g)	0.079	0.299	0.384	0.568

Exercise 2

Table 11.4. Sample data for forced exercise experiment using *Procambarus* sp.

	pH	Total CO$_2$ (mmol/liter)	Lactate (mmol/liter)	PCO$_2$ torr	Ventilation rate (beats/minute)
Before exercise	7.670	7.6	0.2	3.7	110
Immediate post-exercise	7.445	6.7	7.0	5.4	227
30 minutes post-exercise	7.460	4.4	3.5	3.4	159
60 minutes post-exercise	7.470	1.3	2.5	1.0	121
90 minutes post-exercise	7.495	1.9	1.0	1.4	111

APPENDIX H
Additional References

Exercise 1

Gerard, J. F. and R. Gilles. 1972. The free amino acid pool in *Callinectes sapidus* (Rathburn) tissues and its role in the intracellular osmotic regulation. Journal of Experimental Marine Biology, 10:125–136.

Gilles, R. 1979. Mechanisms of osmoregulation in animals. John Wiley and Sons, New York, 667 pages. [ISBN 0-471-99648-3]

Mangum, C. P., and D. Towle. 1977. Physiological adaptation to unstable environments. American Scientist, 65:67–75.

Exercise 2

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