Chapter 7

The Good and the Bad: Symbiotic Organisms From Selected Hosts

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Gayle Pittman Noblet received a BS degree in Biology from Oklahoma Panhandle State University and a PhD in Biology from Rice University. She is a Professor of Zoology in the Department of Biological Sciences at Clemson University where she teaches upper-level undergraduate and graduate courses in parasitology and protozoology. She also has taught invertebrate biology. Although her research interests are quite diverse, most projects completed in her laboratory have involved protozoan parasite/host systems. One parasite/host system of primary interest during Gayle's tenure at Clemson has been the avian malaria parasite *Leucocytozoon smithi* that causes a serious disease (leucocytozoonosis) in turkeys and is vectored by *Simulium* black flies. Another major research focus involved the use of limnological techniques to examine the distribution in aquatic ecosystems of potentially pathogenic, small free-living amoebae (e.g., *Naegleria fowleri* which causes fatal primary amoebic meningoencephalitis in humans). In conjunction with Dr. Sharon Patton at the University of Tennessee School of Veterinary Medicine and staff at the Clemson University Livestock-Poultry Diagnostic Laboratory, a study of seroprevalence of the protozoan parasite *Toxoplasma gondii* in South Carolina domestic and feral swine was completed, with analyses and recommendations relative to potential impact on swine production and human health in South Carolina. In addition to her research with protozoan parasites, a study involving examination of the distribution of snail vectors for the blood fluke *Schistosoma mansoni* on the Caribbean island of Dominica was completed in collaboration with Dr. Ray Damian at the University of Georgia.

More recently, Gayle has become interested in parasite communities in wildlife as potential sources of infection to humans (zoonosis) and as potential bioindicators of environmental toxicant pollution. Much of the earlier toxicant work was done in

conjunction with faculty in the Department of Environmental Toxicology at Clemson University, with one PhD student completing an examination of the potential utilization of amphibians and reptiles and their parasite communities as bioindicators of environmental contamination by polychlorinated biphenyl toxicants. Currently, one PhD student is examining the effect of a naturally occurring toxicant (mycotoxin) on the immune response of laboratory mice experimentally infected with the protozoan parasite *Trypanosoma cruzi*. Additional interests with zoonotic parasites of wildlife (primarily *Trypanosoma cruzi)* are being pursued in conjunction with staff in the South Carolina Department of Natural Resources.

Michael J. Yabsley received a BS degree in Biological Sciences from Clemson University in 1997 and a MS degree in Zoology in August 2000. During his graduate studies at Clemson, Michael served as a teaching assistant for laboratories in parasitology, protozoology, invertebrate biology, developmental biology, and human anatomy and physiology. While an undergraduate, Michael completed research studying the effects of copper exposure on emergence of cercariae of a trematode, *Posthodiplostomum minimum*, from experimentally infected *Physella* snails. His M.S. research concerned an investigation of the prevalence of *Trypanosoma cruzi* in raccoons as reservoir hosts throughout the state of South Carolina and adjacent areas of Georgia. *Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, is of zoonotic importance, as it occurs naturally in a wide variety of vertebrate hosts and is vectored by reduviid bugs.

Michael currently is pursuing a PhD in Veterinary Parasitology in the Department of Medical Microbiology and Parasitology at the University of Georgia in collaboration with the Southeastern Cooperative Wildlife Disease Study unit in Athens, Georgia. His research involves the study of host-parasite interactions and epidemiology of *Ehrlichia chaffeensis* and the HGE agent (an *Ehrlichia* sp. closely related to *E. phagocytophila*), the causative agents of human monocytic ehrlichiosis (HME) and granulocytic ehrlichiosis (HGE), respectively. While conducting research, Michael also will serve as a teaching assistant for epidemiology and veterinary parasitology.

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Introduction

Nothing stimulates student interest more than observation of living material, especially in the case of one organism living on or in another organism. With that in mind, this exercise was designed to expose students to the complex and exciting science of symbiology and to the ever-present existence of symbiotes in the environment around them. Although no phyla of organisms lack representative species living in symbiosis, either as hosts or as symbiotes, the study of such organisms and their relationship is largely neglected in most curricula. To share our enthusiasm for and appreciation of the study of symbiotic organisms (including, but not just exclusively those of medical and veterinary importance), we developed this laboratory exercise to demonstrate living symbiotes that are easily accessible in one's own environment. Please

understand that the individual sections of this exercise have been developed and used by the senior author over the past 28 years as part of the laboratory experience for her senior undergraduate/graduate level Medical and Veterinary Parasitology Laboratory at Clemson University, during which time graduate students like Michael have made significant contributions. Although this exercise was completed as a 3-hour ABLE workshop, the sections of the exercise are designed for use as individual exercises, or in a variety of combinations, to introduce and to illustrate symbiosis dynamically to students at any level. Further, our hope is that such exercises might stimulate students to recognize/identify and then examine other common symbiotic relationships and organisms that occur in their surrounding environment.

Materials

For 20 students working in pairs:

General supplies:
Newspapers to cover lab benches
10-15 dissecting pans
Appropriate numbers and sizes of dishes – deep disposable Petri plates, large and small finger bowls, Stender dishes – for placement of organs to be removed from host organisms (e.g., stomach, small intestine, large intestine, liver, lungs of frog or gills of fish, heart, bladder, spleen)
10-15 scalpels or razor blades
10-15 sharp scissors
15-20 dissecting needles
1-2 boxes of microscope slides and cover slips
1 container of Vaseline
Pasteur pipettes and bulbs
Disposable gloves – 1 box of each size
Lab coats or disposable aprons
5-10 dissecting scopes and lights
5-10 compound microscopes
5-10 3, 5 or 10 mL syringes
Biohazard or heavy duty plastic bag for animal carcasses

Termite flagellates:
20-30 termites and insect saline

Rumen ciliates:
Access to a fistulated cow or a veterinarian willing to "tube" a cow to collect rumen fluid

Trypanosomes in *Drosophila*:
20-30 wild-caught *Drosophila* and insect saline

Avian malarial parasites:
1-2 wild-caught pigeons
Lancets, cotton, and ethanol or isopropyl (rubbing) alcohol to sterilize skin
Blood staining rack and blood staining kit or methanol and Giemsa stain
Commercially prepared blood smears of *Haemoproteus columbae*

Fish dissection:
10-12 wild-caught fish
Normal saline for cold-blooded vertebrates
Fish food, 1-2 aquaria or buckets, air stones, and aquarium pumps

Frog dissection:
10-12 frogs – wild-caught or purchased from a biological supply company
Normal saline for cold-blooded vertebrates

Please note – Recipes for reagents and a list of sources for materials and host animals are included in Appendices A & B.

Notes for the Instructor

Discussion of the complexities of symbiosis is interesting in itself; however, observation of living symbiotes from or in naturally occurring hosts provides students with an experience that can be presented in no other way. The real challenge to the instructor is the prep for any of the individual demonstrations or exercises, which includes selection of the exercise(s) to be completed and host organism(s) to be collected. Since collection of appropriate host animals is essential to the success of any of these laboratory exercises, we provide a brief description of the source of host animals as routinely used in our laboratory; alternate sources are included as available.

Termites (as host to intestinal flagellates) are easily obtained from pieces of a rotten log lying on the ground in moist areas of woods. Or for those of you from the windswept grasslands or plains area of the United States, as described for Keith County, Nebraska, by John Janovy, Jr. (1978), a cow's fecal pat (manure) or "cow-pie" may be a good source of termite hosts. If you are unable to work with termites, an alternate source of flagellates would be wood roaches (*Cryptocercus*) as described by Cleveland et al. in 1934. If you are not inclined to "wander in the woods" or "across the plains," then termite flagellates also are available to purchase from biological supply companies such as Carolina Biological. With such purchases, some restrictions may apply. For example, residents of Canada must apply for a Canadian Department of Agriculture permit to receive this material, while residents of Hawaii, Tennessee, and Vermont must apply for state permits.

Another fascinating example of mutualism is illustrated by examination of the bizarre ciliates that occur in the rumen of cattle. Rumen fluid can be obtained from a fistulated cow if one is available at a nearby experimental station or university or if a veterinarian friend is willing to "tube" a cow (aspirate or siphon fluid) to collect rumen fluid. An alternate source of rumen fluid would be a nearby abattoir (slaughterhouse) or a deer-check station from a hunter-killed

deer. Considering the mode of transmission involves cows chewing their cud and then licking and grooming their young, something as simple as examination of cow saliva may reveal some of these mutualistic ciliates. But certainly seasonal variations and exposure to air and extremes in temperature are important factors dictating whether these ciliates could be observed alive in saliva.

To illustrate commensalism, wild-caught *Drosophila* fruit flies may be dissected. Fruit flies can be collected by placing a piece of fruit (e.g., banana peel) in a wide-mouth jar in a cool shaded place outdoors for 1-2 days depending on the season. Observations indicate that ~10% of wild-caught fruit flies are infected with promastigote forms of *Leptomonas* flagellates. Dissections (as described in the Student Outline) are quick and simple, so success would be expected if students working in pairs dissect 10-15 fruit flies. **PLEASE NOTE** – Fruit flies in culture, as available from a biological supply company, most likely will **NOT** be infected and are not a good choice for use in this exercise.

Another example of commensalism can be demonstrated by preparation and examination of a stained blood smear from a wild bird. Although somewhat closely related to *Plasmodium* species that are the causative agents of malaria in humans, many haemoproteids in birds (e.g., *Haemoproteus columbae* in pigeons) appear to cause no disease and consequently may be considered commensals. In any case, pigeons are easily obtained at night from known roost sites, and students enjoy learning to make blood smears. Although pigeons tend to be somewhat wary of live traps, success can be achieved with patience and proper pre-baiting of traps. Since the wing veins of a pigeon tend to be very small and collapse quickly, bleeding of a leg vein is preferred, as a single drop of blood is needed for the making of a smeared slide and several slides can be made from a single prick of a leg vein. For demonstration purposes and as a supplement to this exercise, stained permanent mounts of *Haemoproteus* can be purchased from a biological supply company.

Both fish and frogs (including tadpoles) are excellent sources of symbiotic species. Fish can easily be obtained from nearby ponds or lakes by use of a cane pole, fishhook, weight (sinker), and bait (earthworm). Although any wild-caught fish will serve the purpose, commonly used small fish are sunfish, perch and crappie. Catfish and white suckers also have been used. On occasion, bass have been obtained alive from bass fishermen at a marina. Bluegill sunfish are available for purchase from biological supply companies; however; we recommend the use of wild-caught fish as we have no prior experience with purchased fish and some parasites (e.g., monogeneans on gills) are lost very quickly by fish in captivity.

Frogs and tadpoles commonly are obtained from lagoons or water traps on golf courses, but also should be available from small ponds or lakes. Collection methods for frogs may include dip netting, hand collecting at night with a flashlight or headlamp, or use of a Gees minnow trap (Memphis Net and Twine, Memphis, Tennessee). Frogs also may be purchased from a biological supply company, as these suppliers apparently use wild-caught frogs as their source. Permanent mounts of many species of both protozoan and helminth symbiotes of fish and frogs (as described in the Student Outline) can be purchased from a biological supply company and may serve as an important supplement to this exercise.

The last section of this exercise was designed primarily as an instructor's demonstration to illustrate the complete life cycle of the rat tapeworm, *Hymenolepis diminuta*, rather than as a student exercise in itself. However, with reference to the presentation by Brant and Hanelt (1999) at last year's ABLE Workshop/Conference, one can see that many creative uses can be made of *H. diminuta* and it's life cycle stages. The primary objective in our presentation during the 2000 ABLE Workshop/Conference was to stimulate interest in tapeworm biology and to stimulate discussion about the potential effects of environmental or nutritional conditions on the intimate relationship between a symbiote (tapeworm) and its host (rat) (see Student Outline). Although the life cycle of *H. diminuta* can be maintained in a research/teaching environment, purchase of *H. diminuta* eggs to infect *Tenebrio* beetles and/or *Tenebrio* beetles infected with *H. diminuta* larva (cysticercoids) to infect rat hosts is probably preferable for teaching purposes.

The exercises as described in the Student Outline do not present any great technical difficulties; however, organization is the key to success. All solutions should be prepared in advance and clearly labeled. General supplies as listed in the Materials section should be placed in the laboratory at workstations for use by an appropriate number of students. Use of Vaseline-ringed cover slips will facilitate observation of living material for a more extended period of time. To prepare a Vaseline-ringed cover slip, wipe a thin film of Vaseline on the outer edge of the palm of your hand, gently scrape each side edge of a cover slip across the Vaseline film to form a thin rim, and then place the cover slip (Vaseline side down) over a drop of fluid and press down gently to seal. Depending on your school guidelines for animal care and use, a person designated to euthanize animals should begin that process in such a way as to ensure a smooth transition from the instructor's introductory lecture to the actual hands-on student dissection of host animals and observation of living symbiotes.

Having the greatest respect for living creatures, we as instructors try to convey that respect to our students, while at the same time emphasizing the profound value of the use of animals in research and teaching. Immeasurable advances in scientific and medical knowledge have improved human and animal health, have led to the alleviation of pain and suffering, and have saved countless lives. At Clemson University, the Institutional Program for Animal Care and Use is administered by two veterinarians and a 12-member Animal Research Committee. Animals are maintained in accordance with all animal welfare regulations and federal guidelines to ensure humane care. All of Clemson University animal research facilities and programs have received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). For additional information concerning AAALAC and the AAALAC Position Statement, please refer to http://aaalac.org/NewFiles/position.htm.

Student Outline

Symbiosis has been defined as the interaction of organisms in which one organism lives with, in, or on another species of organism. The intimacy of this relationship can vary greatly, from the complex sharing of physiological mechanisms (e.g., adult tapeworm in the small intestine of a vertebrate host) to the more simple living together of species (e.g., the wide variety of invertebrates found on a marine floating dock or piling).

The three main categories of symbiosis are mutualism, commensalism, and parasitism. **Mutualism** is a symbiotic relationship in which both host and symbiote benefit. One of the earliest defined examples of mutualism was the association of algae and fungi known as lichens. Numerous other examples of mutualism can be found throughout the animal kingdom, such as termite flagellates and the lesser-known rumen ciliates. Termites supply the environment (in particular, the low oxygen tension) and food (comminuted wood) required by the flagellates and, in turn, the protozoans degrade the wood to useable food products for the termites. Similarly, ruminants (e.g., cows) host a variety of bacteria and protozoans that degrade cellulose to compounds such as fatty acids which then are utilized as nutrients by the host. The symbiotic relationship is obligate for these rather bizarre looking ciliates in the rumen of cattle, whereas cattle can flourish in their absence as long as symbiotic bacteria are present. Both termites and rumen fluid may be available for examination and observation of the diversity of these mutualistic protozoans.

Commensalism occurs when one organism (commensal) receives benefit from the association, while the other (host) is not affected (neither benefited nor harmed). A large number of symbiotes are considered commensals, primarily as a result of a lack of a clear understanding of the intimacy of the relationship. However, many other symbiotes may be designated as parasites largely as a result of "guilt by association" due to close taxonomic relationship with known pathogens. To illustrate commensalism, *Drosophila* fruit flies will be dissected to observe flagellates living in the gut of the insect host. Alternatively, a pigeon will be available to make a thin blood smear to be stained and examined under oil immersion for the gametocyte stage of *Haemoproteus columbae* within the cytoplasm of host nucleated-red blood cells.

When one organism (parasite) receives benefit at the expense of the other (host), the relationship is **parasitism**. Examples of parasites are the well-known malarial parasites (*Plasmodium* spp.) and common dog and cat roundworms (*Toxocara* spp.). In general, parasites cause some type of pathology or nutritional drain on their hosts, but successful parasites have adapted to the environment provided by the host in such as way as not to kill the host. Fish and frogs represent two host types that are excellent models for studying parasite diversity. Fish serve not only as definitive hosts for several species of parasites, but also as intermediate hosts for others. Commonly, nematodes and acanthocephalans are found in the intestines of fish, with monogeneans on gills and intermediate forms of both trematodes and acanthocephalans in the liver. Frogs are excellent hosts for the observation of a variety of protozoan types in the cloaca and worms (trematodes and nematodes) in lungs, body cavity and digestive tract. Either fish or frog hosts (or possibly both) will be available for necropsy during this laboratory for observation of live symbiotes.

As with most areas of biology, the above categories are very arbitrary, since they are based on the current knowledge of the apparent degree of integration and interaction between two organisms. Many symbiotes, which are considered to be commensals, may become parasites if host conditions are compromised. For example, the common rat tapeworm, *Hymenolepis diminuta*, is a commensal that resides in the small intestine of laboratory rats, absorbing excess nutrients not needed by the host. However, if the rat becomes stressed (e.g., by starvation, crowding, pregnancy of females), then the presence of the tapeworms may become a burden to the rat, with the relationship of symbiote with host reverting to that of parasitism. To facilitate your understanding of this complex host-parasite relationship, all stages of the life cycle of the rat tapeworm will be demonstrated during this laboratory (from eggs passed in rat feces through developmental stages in the beetle intermediate host to infection of the small intestine of the rat definitive host with adult cestodes).

Mutualism
Termite Flagellates
The relationship between termites and their flagellates is a mutualistic one. While the termites supply the environment and food required by the flagellates, the latter ingest wood particles (by phagocytosis at the posterior end of the cell) that are enzymatically degraded into materials that then can be used by the termites.

Select an active termite from the container provided. Place the termite on a microscope slide and separate the last abdominal segment from the body, being careful not to damage the gut. To do this, hold the last abdominal segment firmly against the microscope slide with a dissecting needle, grasp the thorax with forceps, and pull the body away from the tip of the abdomen. This procedure should leave the gut attached to the last abdominal segment. Tear the enlarged hindgut apart in a drop of insect saline to liberate the protozoans. Be sure to tease the gut mucosa completely to dislodge flagellates present there. Remove the remnants of the gut and abdomen, stir the remaining material, cover it with a Vaseline-ringed cover slip, and examine the specimen under a compound microscope.

Three species of the termite *Zootermopsis* share a characteristic flagellate fauna, the common species of which are shown in Figure 7.1. This fauna includes the very large hypermastigids in the genus *Trichonympha*. *Pseudotrypanosoma giganteum* is a moderate-sized flagellate with a well-developed undulating membrane. *Streblomastix strix* is smaller, elongate, and has a marked spiral thickening. In addition to these three conspicuous genera, there are one or more species of small flagellates, almost always including *Tricercomitus termopsidis*, and less frequently *Hexamastix termopsidis* or other species.

For the purpose of this exercise, it is not necessary to distinguish between the abundant species of *Trichonympha* that are fairly similar in their biology. Nor is it necessary to distinguish the various small flagellates that may be grouped as "small trichomonads." Thus, the four types of flagellates to be identified from *Zootermopsis* are: *Trichonympha* spp., *Pseudotrypanosoma giganteum*, *Streblomastix strix,* and the small trichomonads.

Examine enough specimens of each so that you can identify them quickly and without difficulty. Do all appear to ingest wood particles? If not, which ones do? Examine two or three termites to estimate the usual numbers of each type of flagellate.

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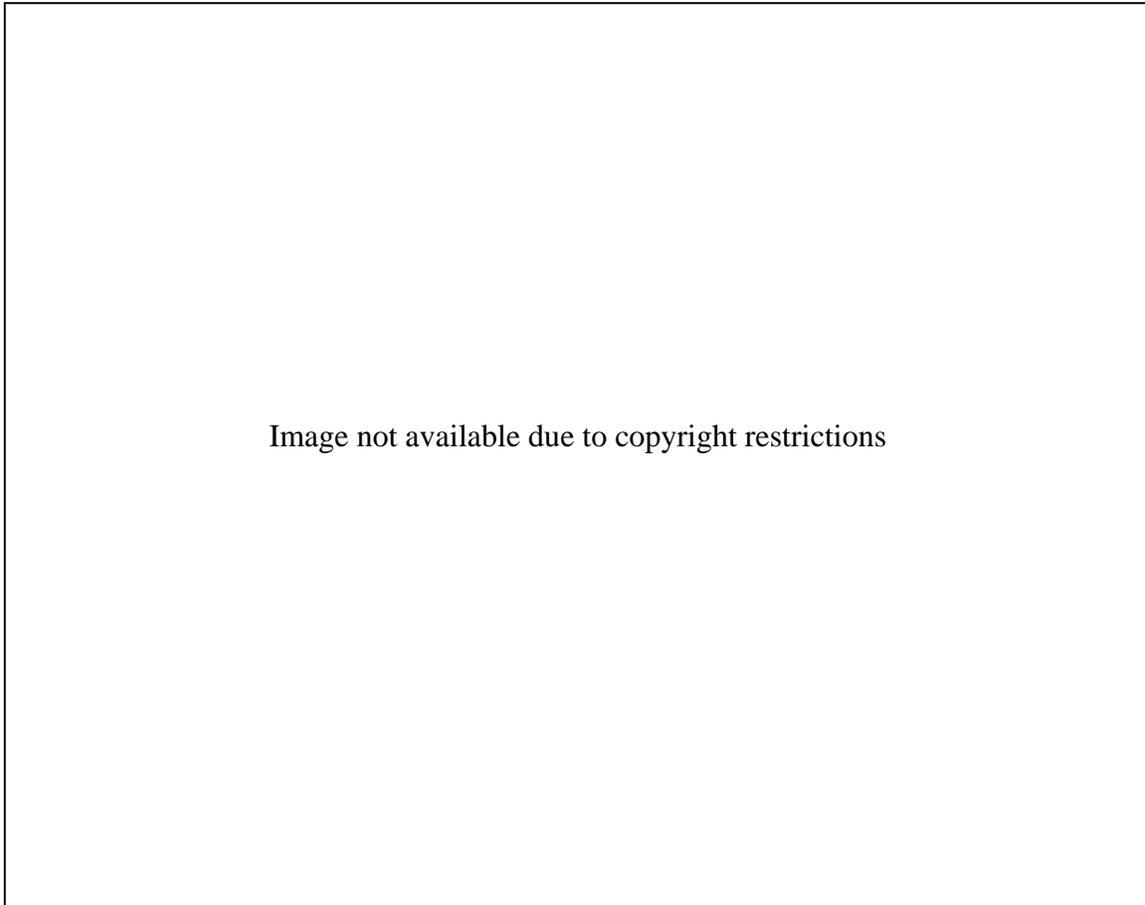
Figure 7.1. Flagellates of *Zootermopsis* spp. A, *Streblomastix strix* x1030; B, *Tricercomitus termopsidis* x665; C, *Holomastigotes elongatum* x700; D, *Trichonympha agilis* x410; E, *T. campanula*, x150; F, *Holomastigotoides hartmanni* x250; G, *Holomastigotoides tusitala*; H and I, *Pseudotrichomonas keilini* x2200; J, *Hexamastix termopsidis* x2670; K, *Pentatrichomonoides scroa* x1500; L, *Pseudotrypanosoma giganteum* x435; M, *Barbulanympha ufalula* x210. From Kudo, R.R. 1966. Protozoology. 5th ed. Reprinted courtesy of Charles C. Thomas, Publisher, Ltd., Springfield, Illinois.

Rumen Ciliates

Rumen ciliates have been known to exist since the mid-19th century due to the fact that they are much larger than bacteria and can be seen with the aid of a microscope. Information on classification, morphology, and occurrence of rumen ciliates has been available for many years, with data on nutrition, metabolism and related subjects of more recent origin. A tremendous number and amazing variety of ciliates swarm in the rumen and reticulum of ruminants, and a few species occur in the large intestine. Many are holotrichs, with cilia over the whole body, but the most bizarre ones are ophryoscolecids or entodiniomorphs. No attempt will be made during

this lab to differentiate all the species, but major genera should be noted. Please note that none of these symbiotic ciliates form cysts. Young ruminants acquire the protozoans when the mother licks and grooms them and they swallow some of the saliva and digesta. Infections also are acquired when young animals feed on hay and grass contaminated with the saliva of older, infected animals.

Examine a drop of fluid from the rumen of a fistulated cow. While referring to the diagrams shown in Figure 7.2, find and identify holotrichous species in the genera *Isotricha* and *Dasytricha*. In addition, observe entodiniomorph ciliates (Order Entodiniomorphida) in the genera *Entodinium*, *Epidinium*, and *Ophryoscolex*. These entodiniomorphs lack abundant simple somatic cilia which have been replaced by compound ciliary organelles known as membranelles arranged as zones of cilia. The pellicle is firm, sometimes drawn out into spines. All members of this order belong to the family Ophryoscolecidae, in which the ciliary tufts are retractable and are limited principally to the oral and adoral areas plus one metoral (anterodorsal) group. There may also be skeletal plates. All are symbiotic in the gastrointestinal tract of ruminant herbivores, including anthropoid apes.

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Figure 7.2. Ciliates of ruminants. **A.** *Buetschlia parva*. x1,090. **B.** *Isotricha prostoma*. x320. **C.** *Isotricha intestinalis*. x640. **D.** *Dasytricha ruminantium*. x420. **E.** *Ophryoscolex caudatum*. x425. **F**. *Entodinium bursa*. x640. **G**. *Entodinium minimum*. x640. **H**. *Entodinium caudatum*. x640. **I**. *Entodinium bicarinatum*. x640. **J**. *Entodinium furca*. x640. **K**. *Epidinium ecaudatum*. x425. From Becker and Talbott. 1927. Reprinted courtesy of Iowa State College Journal of Science, Iowa State University Press, Ames, Iowa.

Commensalism

Living Trypanosomes in an Invertebrate Host

Protozoans known as hemoflagellates are a group of polymorphic flagellates, some of which cause dreaded diseases of both economic and medical importance (e.g., African trypanosomiasis or sleeping sickness and American trypanosomiasis or Chagas disease). For purposes of this exercise, we will examine invertebrate hosts (wild-caught *Drosophila* fruit flies) for live promastigote forms of the genus *Leptomonas*. An infection rate of ~10% is expected in wild populations of *Drosophila*.

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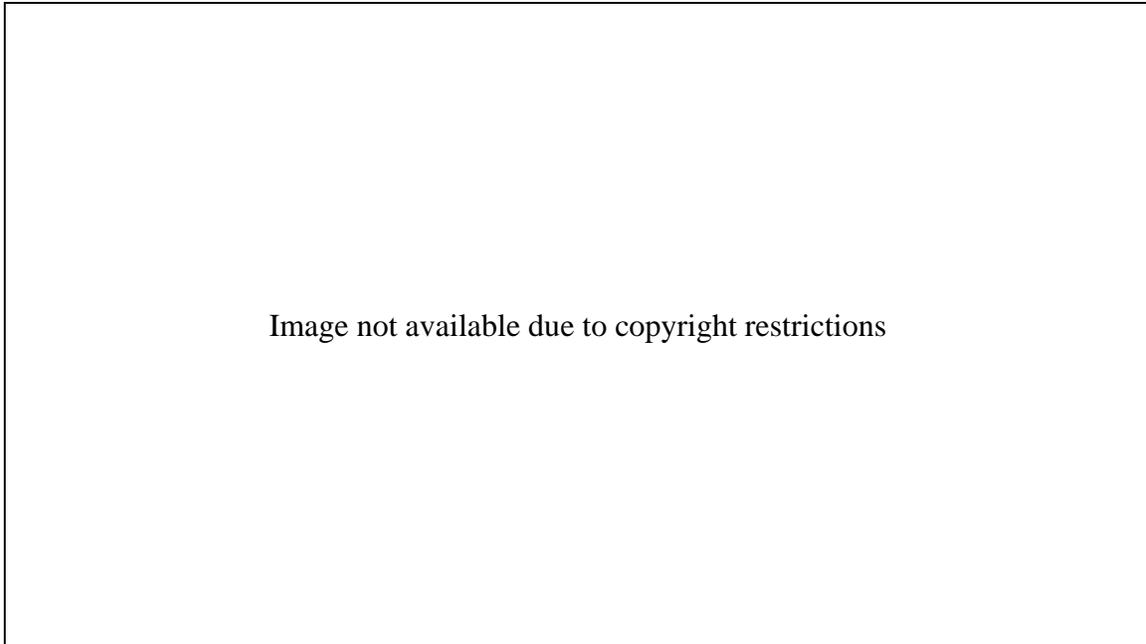
Figure 7.3. Promastigote, as observed from the salivary glands of *Drosophila*. From Dailey, M. D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. 6th ed. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Place the vial containing the adult *Drosophila* fruit flies in a freezer **just until** flies become immotile. Place a single fly on a clean microscope slide (clean a microscope slide by rinsing in 95% alcohol and wiping with a clean cloth or Kimwipes; then handle the slide only by its edges to avoid leaving finger marks). For the dissection, place one dissecting needle near the end of the abdomen, another in the head, and then pull forward to remove the head; the gut and salivary glands should remain attached to the head if the dissection is clean. Add a drop of insect saline and use the two dissecting needles to tease apart the salivary glands (or tease the entire head, including the salivary glands apart in the drop of insect saline). Add a cover slip and examine the specimen immediately with reduced illumination under a compound microscope. The characteristic whip-like movement of these relatively large and very active flagellates can be observed easily under x400 magnification.

If a permanent mount is desired, carefully remove the cover slip. With a Pasteur pipette, use one drop of insect saline to rinse the under surface of the cover slip, being careful to position the cover slip so that the drop will fall onto the moistened area of the microscope slide just examined. Allow the slide to air dry completely. Place the microscope slide on a staining rack. Fix by flooding the slide with methanol for l-3 minutes. Stain the slide with Giemsa stain for 30 minutes. Rinse the slide with water and return it to the staining rack to dry. When dry, the microscope slide should be scanned with the high-dry objective of a compound microscope. When a stained area with flagellates is located, examine the area with the oil-immersion objective (x1,000).

Avian Malarial Parasites

Haemoproteids are symbiotes of mammals, birds, and reptiles. The family is characterized by having all phases of schizogony in fixed tissue cells (endothelial cells of the lungs) and only gametocytes appearing in circulating erythrocytes. Insect vectors, insofar as known, are pupiparous hippoboscid flies and ceratopogonid midges.

Haemoproteus columbae is a common parasite of columbriforme birds (pigeons), especially in warm climates where the hippoboscid vectors flourish. Gametocytes, the only form in red blood cells, are sausage-shaped and bent at each end so as to partially surround the nucleus of the host cell. The cytoplasm of the parasite contains pigment (hemozoin) derived from the breakdown of hemoglobin in erythrocytes. The host cell may become enlarged and its nucleus displaced laterally as the gametocytes attain full size. Microgametocytes are 11.9 to 15.3 μm long by 2.5 to 4.3 μm in diameter with a large oval nucleus, 2.8 by 5.8 μm, that stains light red; the cytoplasm is light blue. Macrogametocytes are l3.7 to l7.l μm long by 2.3 to 2.8 μm in diameter with a small, almost spherical nucleus 2.l by 2.3 μm in size. Both the nucleus and cytoplasm stain darker than in the microgametocyte.

Examine prepared slides of *H. columbae* gametocytes on demonstration. In addition, make a thin blood smear from an infected wild pigeon. Clean two slides by rinsing in 95% alcohol and wiping with a clean cloth or Kimwipe. Handle the slides only by their edges to avoid leaving finger marks. Place a small drop of fresh blood on the end of one slide, place the slide at a 45° angle to the other slide, allowing the drop of blood to spread across the end of the slanted slide, and then push the slanted slide forward over the length of the other slide. Place the smeared slide on a staining rack and allow the slide to air dry (a matter of a few seconds if the smear is thin enough). Fix by flooding the slide with methanol for l-3 minutes. Stain with Giemsa for 30 minutes. Rinse the slide with water and scan with high-dry objective. Then observe gametocytes under oil-immersion.

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Figure 7.4. Mature C-shaped gametocyte of *Haemoproteus* spp. partially surrounding the dark nucleus of an avian red blood cell. From Dailey, M. D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. 6th ed. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Parasitism
Dissection of Fish and Frogs

Before beginning any dissection, make certain that you have the required materials (i.e., dissecting scope, dissecting kit, wax-bottom pan or similar low container, appropriate sized finger bowls or Petri dishes, medicine droppers or Pasteur pipettes, several slides and cover slips, paper towels or Kimwipes, and the proper concentration of physiological saline). With respect to the last item, the following salines are recommended:

fish... 0.6% NaCl
amphibians and reptiles... 0.7% NaCl

Host animals to be dissected should be freshly euthanized. (Placement in MS-222 for anesthetization followed by euthanasia with decapitation of fish and reptiles. Intraperitoneal injection of MS-222 or sodium pentobarbital for amphibians.) Animals (especially warm-blooded hosts) should be necropsied as soon after death as possible since some species of worms migrate upon death of the host, with the result that their normal location becomes uncertain. Others undergo changes in the dead host. If the necropsy must be delayed, animals should be kept in a refrigerator until that time. Your instructor will advise you about the disposal of the carcass following necropsy. Under no circumstances, however, should remains be left in the laboratory sinks or trashcans.

Fish Parasites

When examining fish, the entire outer surface should be searched carefully, especially the oral region, the gills, and opercula, and the fins. The body surface, gills and fins may bear ectoparasites such as monogeneans, larvae of freshwater clams (glochidia), leeches, and fish lice (parasitic crustaceans). The outer flesh and sub-integumental tissues of the fish frequently harbor a variety of encysted juvenile flukes and cestodes, including such common ones as blackspot, yellow grubs, and tapeworm plerocercoids. Species of certain encysted flukes

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(metacercariae) may give the entire surface of the fish a salt-and-pepper appearance, consequently the name "black spot." If available, examine preserved specimens of fish with "black spot" on demonstration.

Following external examination of a fish, make an incision through the mid-ventral body wall, cutting around the anus and urogenital opening. Before removing the viscera, look for parasites that may be free in the body cavity, beneath the peritoneum, or in the mesenteries, and examine the liver surface for spots or nodules (cysts) that might be due to parasites. Encysted flukes can be distinguished by their thin hyaline appearance, through which the larva is often obvious. A cyst that is more or less opaque, nonhyaline, may be that of a tapeworm larva. These cysts more closely resemble host tissue and can be distinguished from those of trematodes by the presence of numerous, glasslike granules known as calcareous "corpuscles" or bodies. Cysts too small to open with dissecting needles should be pressed between slides and examined under the low power of the compound microscope to determine the contents, if possible. Microsporidian cysts and myxozoan cysts may be mistaken for eggs of flukes or tapeworms unless carefully observed for the presence of polar filaments, which may be expressed from within minute pyriform spores by pressure on the cover glass. When examining fish, cut away one side to more fully expose the organs. Then remove the internal organs, one at a time, and place each organ in a separate dish of physiological saline. Place a temporary label giving relevant data with each dish.

Remove the entire digestive tract from the esophagus to the posterior portion. With scissors, separate stomach, small intestine, and large intestine. Depending upon size, place parts in dishes with normal saline and open each one separately. For small specimens, Syracuse watch glasses may be best, whereas larger ones may require dishes of different sizes. Using an appropriate size syringe (without needle) filled with saline and inserted into one end of the small intestine, flush the intestinal contents into a finger bowl. Examine washings by transferring a few drops of the saline mixture to a slide, covering with a Vaseline-ringed cover glass, and observing under low power objective of a compound microscope for movement. To continue with the dissection, carefully cut open the small intestine by inserting sharp scissors into one end. Use a slight lifting motion when cutting to avoid damage to any remaining parasites. Tease the lining to remove parasites that adhere to it and put the sample into Stender dishes or larger vessels. Repeat as appropriate with other organs. To dislodge specimens buried in the villi or other lining, you may need to allow open organs to remain in saline for an hour or longer and then scrape the lining with a scalpel. If the opened organs are placed in the refrigerator for several hours, more parasites may go into the saline solution during that time. If the saline solution becomes cloudy, allow the suspension to sit for a few minutes and then carefully decant the fluid and add fresh saline.

Small amounts of feces from the lower part of the large intestine may be mounted on a slide, covered with a cover slip, and examined with the compound microscope for eggs of parasites.
Carefully examine the contents of the gall bladder, liver, swim bladder, and urinary bladder, remembering that parasites may occur in any part of the body. Occasionally acanthocephalan and cestode larvae are found free or encysted in the coelomic cavity and its

mesenteries. The brain, lens, and eye chambers often possess larval strigeoid flukes of the genus *Diplostomulum*.

When a parasite is removed, call it to the attention of the instructor.

Protozoan Parasites

      Ichthyophthirius multifiliis is a protozoan ciliate (Phylum Ciliophora) commonly known as the causative agent of "ick" among tropical and cultured fish. Adult trophozoites (trophonts) are as long as 1 mm and possess a mouth (cytostome) typical of this group of ciliates. The macronucleus is a large horseshoe-shaped body that encircles the tiny micronucleus. Each of several contractile vacuoles has its own micropore in the pellicle, and a permanent cytopyge (cell anus) is located at the posterior end of the cell. Mature trophozoites form pustules in the skin of their fish hosts and are set free to swim feebly about when the pustules rupture, finally settling on the bottom of their environment or on vegetation. Within an hour, the ciliate secretes a thick, gelatinous cyst about itself and begins a series of transverse fissions, producing up to 1000 infective cells (tomites or swarmers) that can survive ~96 hours without a host. Apparently, the swarmers burrow into the fish's skin with their pointed anterior end to become trophozoites within three days, ingesting debris of host cells and forming a pustule that reaches over 1 mm in diameter. Examine fresh or stained slide preparations as available.

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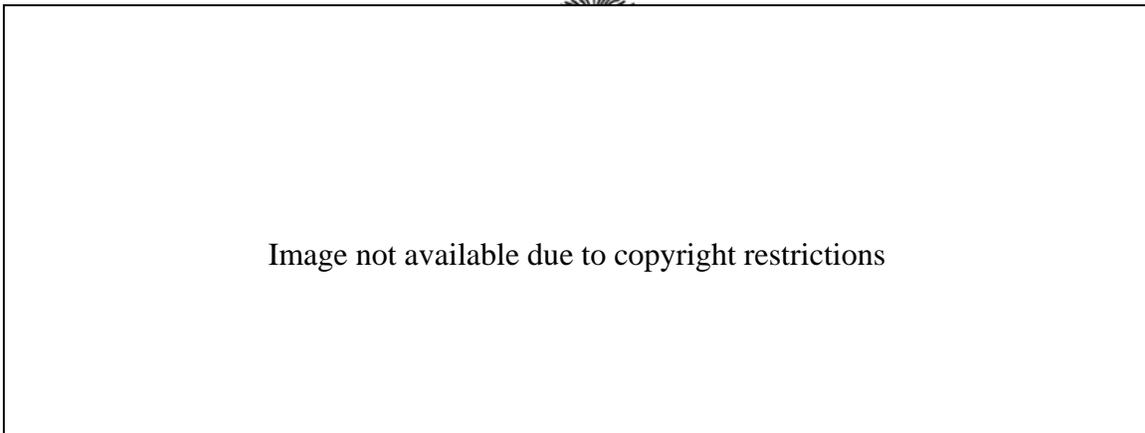
Figure 7.5. *Ichthyophthirius multifiliis* trophont from a pustule in the skin of a fish host. From Marquardt, W. C., R. S. Demaree, and R. B. Grieve. 2000. Parasitology and vector biology. Second edition. Reprinted courtesy of Academic Press, Orlando, Florida.

Gill Parasites – Monogeneans

      Most monogeneans (Phylum Platyhelminthes) are ectoparasites of fishes and amphibians, living attached to the skin and gills; however, a few species are known to occur in the urinary bladder and mouth of amphibians (e.g., *Sphyranura oligorchis* on the gills of the perennibranchiate mudpuppy *Necturus maculatus*) and reptiles where they find the necessary moisture in these hosts which may leave the water. One unusual species occurs in the orbit of the hippopotamus.

The posterior end of monogeneans bears a highly characteristic organ, the opisthaptor. The opisthaptor may extend for a considerable distance anteriorly along the trunk of the worm or may be confined to the posterior extremity. It may be sharply delineated from the body or may be merely a broad continuation of it. Opisthaptors develop into one of two types during ontogeny. One basic type of opisthaptor occurring in the Subclass Monopisthocotylea consists of a single unit that may be subdivided into shallow loculi and usually develops directly from the larval haptor. One, two, or three pairs of large anchors are usually present, along with many tiny marginal hooklets. The second basic type, occurring in the Subclass Polyopisthocotylea, is complex, commonly subdivided with suckers, clamps or anchor complexes. Marginal hooklets are usually absent, and the larval haptor is absent or reduced to pad-supporting terminal anchors.

Gyrodactylus spp. (Subclass Monopisthocotylea; Family Gyrodactylidae)

Species of this genus are parasitic on gills of freshwater and marine bony fishes. The small body is elongate. The opisthaptor is without divisions and bears a pair of large anchors and l6 marginal hooklets. A single pair of lobelike head glands forms the prohaptor at the anterior end of the worm. The subterminal mouth is followed by a globular pharynx and a short esophagus that divides into a pair of intestinal ceca that terminate near the posterior end of the body.

The testis is a small median body lying between or behind the intestinal ceca, and the cirrus is armed with spines at its opening. The genital pore is submedian behind the pharynx. The ovary is post testicular and medial, vitellaria form 2 lobes surrounding the ends of the ceca, and a fully developed embryo is present in the uterus.

When born, the precocious larvae (subadults) appear much like their parents, attaching to the gills of their hosts and growing directly without metamorphic changes into adults. When fully developed, the larva contains a less developed larva in its uterus; before birth a second larva appears in the uterus of the first, a third inside the second, and even a fourth inside the third. The exact mechanism of this unique embryogenesis is not known, but it is considered to be a type of sequential polyembryony with as many as four individuals resulting from a single fertilized egg. Since only a day or so is required for a worm to mature after birth and give birth to another worm already developing within it, massive infection can build up quickly. Not having a free swimming larval oncomiracidium for dispersal purposes, *Gyrodactylus* must depend on direct transmission of adult (or subadult) from one host to another.

Dactylogyrus vastator (Subclass Monopisthocotylea; Family Dactylogyridae)

These monogeneans occur commonly on the gills of the carp and are especially detrimental to young fish in crowded ponds. Adults are up to l.2 mm long by 0.3 mm wide. Two prominent head lobes (head organs), each with glands, and two pairs of eyespots are located anterior to the pharynx. The opisthaptor bears one pair of large anchors with bifurcate roots united by a single rod-shaped bar and seven pairs of small marginal hooklets. There is a single, oval testis located slightly posterior to the ovary. The small oval ovary is situated near the midbody, and the vaginal opening is toward the right margin of the body, slightly preequatorial.
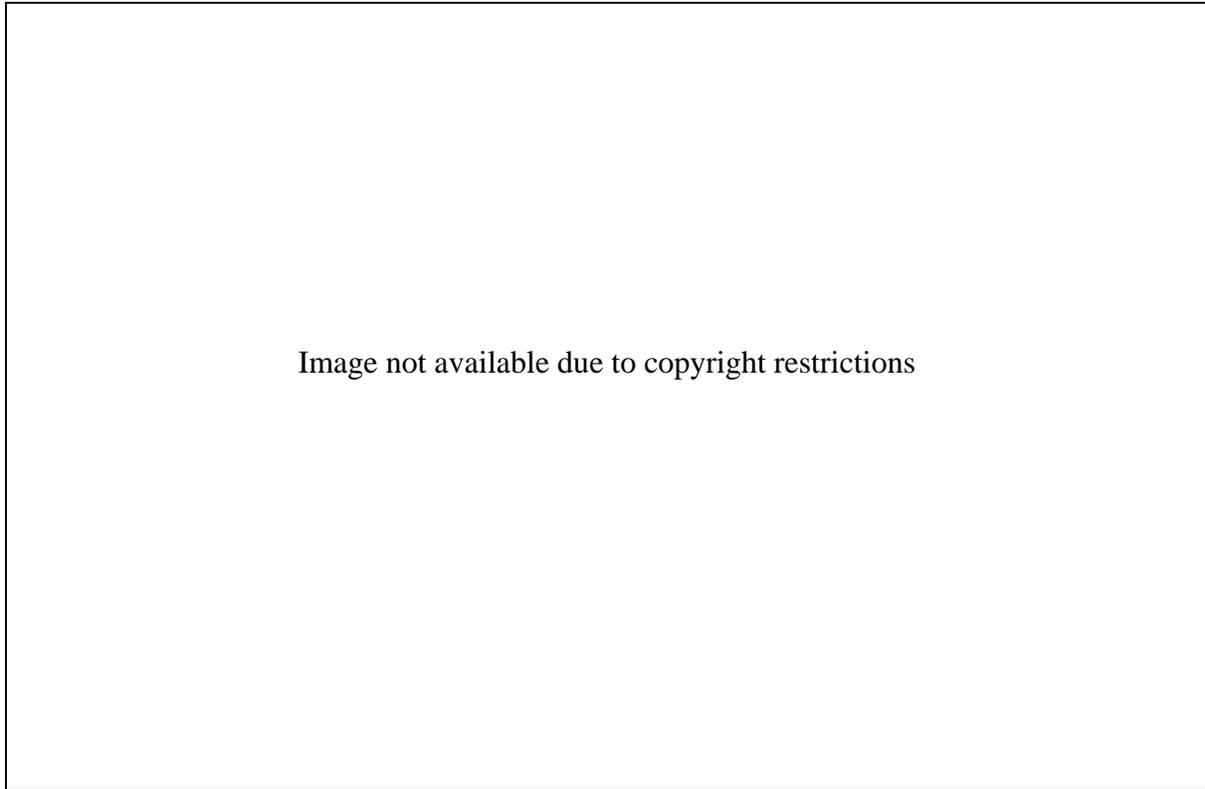
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Figure 7.6. From Dailey, M.D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. 6th ed. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Liver Parasites – Larval Trematodes and Larval Acanthocephalans

     Posthodiplostomum minimum is a strigeid digenetic trematode (Phylum Platyhelminthes), the adult form of which is characterized by a somewhat cup-shaped forebody (with the digestive tract and an oral sucker, an acetabulum and a tribocytic organ for attachment) and a hindbody that is more cylindrical (containing the reproductive organs). The juvenile worm (metacercaria), also known as a "white grub," is found commonly encysted in tissues of bluegills and ~100 other species of fish intermediate hosts in North America. The strigeid juvenile encysts in all visceral organs (especially the liver) of fish except the testis and often is present in great numbers. Although the most common definitive host for *P. minimum* in the wild is the great blue heron (*Ardea herodias*), a number of other birds are suitable experimental hosts (including 1-3 day old unfed chicks). Snail-first intermediate hosts consist of several species of the genus *Physella*. Although usually not pathogenic, *P. minimum* has been implicated a few times in fish mortality or deformity (e.g., a case report of more than 2,000 metacercariae in one fish only 6 cm long).

     Carefully examine the liver of a bluegill sunfish (*Lepomis macrochirus*) for encysted juvenile trematodes and acanthocephalans. Under view of a dissecting scope, carefully tease encysted larvae from the excised liver of a fish host into a small Stender or Petri dish. Larval acanthocephalans (Phylum Acanthocephala) can be distinguished from juvenile trematodes by the prominent spines on the invaginated proboscis of the juvenile (cystacanth).

Intestinal Parasites – Adult Acanthocephalans and Cestodes

       Leptorhynchoides thecatus is a species of spiny-headed worm (Phylum Acanthocephala) that parasitizes the small intestine of a variety of fish throughout the United States, but which occurs primarily in several species of freshwater bass. The life cycle of this species (see Figure 7.7) includes only two hosts – fish definitive hosts and amphipod (*Hyalalla azteca*) intermediate hosts. Eggs released from adult female worms are fully developed when passed in host feces. Upon ingestion of eggs by the intermediate host, acanthors (embryos with a spined aclid organ at the anterior end) hatch and burrow through the intestinal wall to develop through acanthella to the infective cystacanth stage (juvenile with invaginated proboscis) in the hemocoel of the amphipod ~1 month postinfection. The cycle is completed when an infected amphipod is eaten by an appropriate fish host. Larvae freed in the stomach of the fish enter the pyloric ceca where they mature in 4-8 weeks. The proboscis of adult worms characteristically is armed with 12 longitudinal rows of hooks, each row including 12-13 hooks. Each hook is surrounded throughout much of its length by an ensheathing collar.

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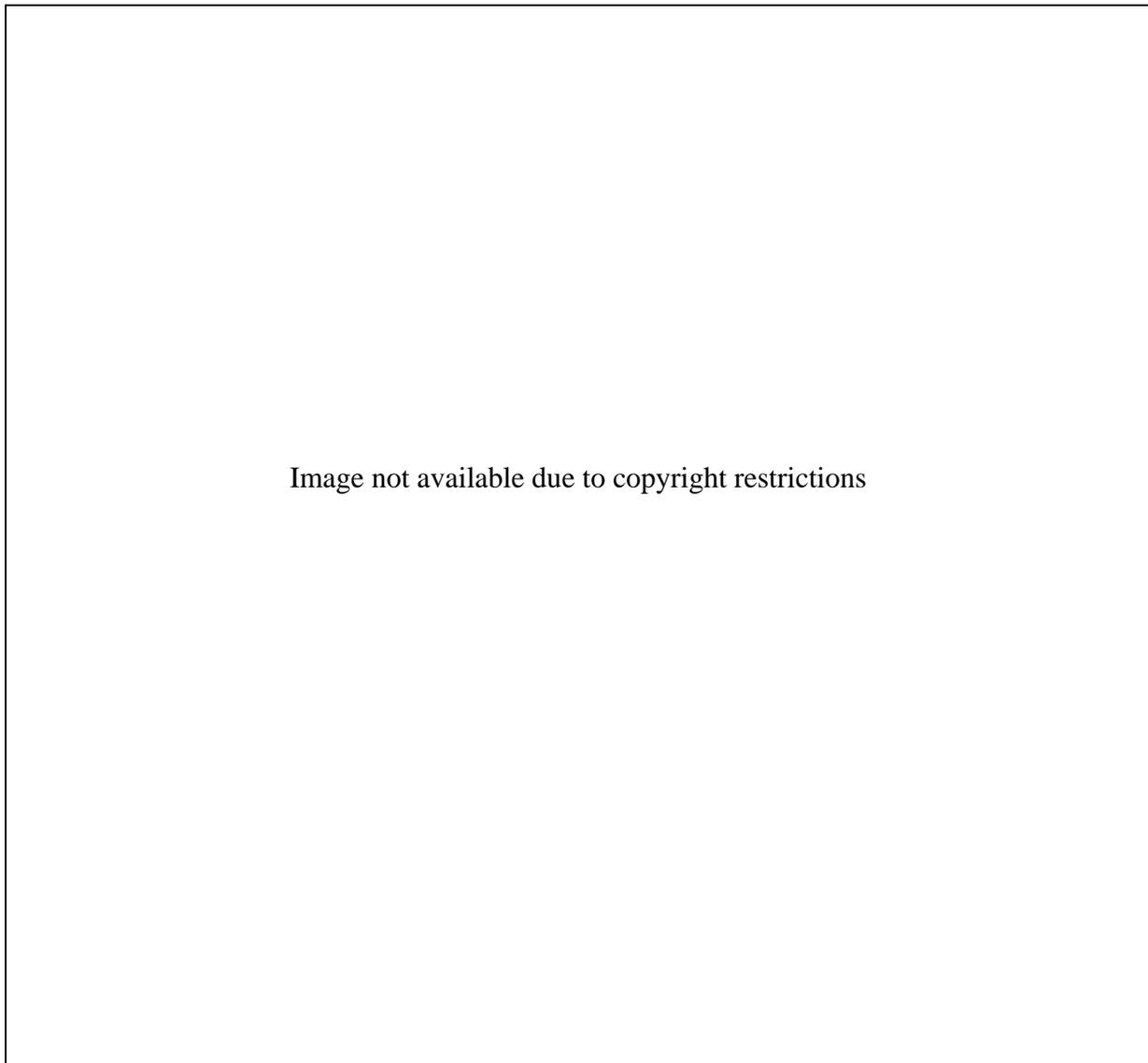
Figure 7.7. Life cycle of *Leptorhynchoides thecatus*. A, egg containing acanthor; B, acanthor in gut of amphipod; C, acanthella and D, cystacanth in hemocoel of amphipod; E, adult in gut of fish. Numbers 1 and 2 indicate intermediate and final hosts, respectively. From Dailey, M.D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. Sixth edition. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Proteocephalus ambloplitis, commonly known as the bass tapeworm, has an extensive range throughout North America due to inadvertent use of infected fish in stocking ponds and lakes. In addition to adult tapeworms (Phylum Platyhelminthes) in the intestine of fish, juvenile cestodes (plerocercoids) may occur in the coelom and viscera, especially the gonads and liver. The scolex of the adult worm has four muscular cup like suckers (acetabula). The strobila is made up of a chain of units (proglottids), each of which contains a complete set of both male and female reproductive organs. The life cycle of this proteocephalid cestode (see Figure 7.8)

includes a copepod as an intermediate host. Fish ingesting an infected copepod may then serve as a paratenic host (with pleroceroids in the body cavity) or the final definitive host (with adult cestodes in the intestine).
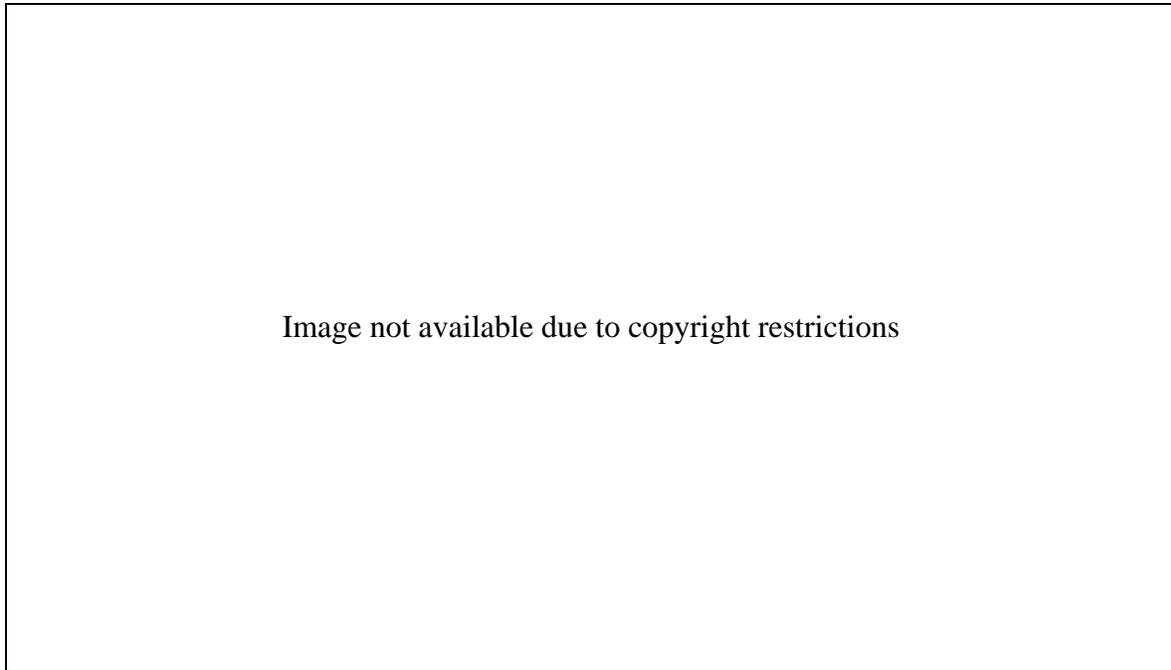
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Figure 7.8. Life cycle of *Proteocephalus ambioplitis*. A, egg containing oncosphere; B, oncosphere in gut of copepod; C, plerocercoid 1 in hemocoel of copepod; D and E, plerocercoid 2 in body cavity of fish; F, adult cestode in intestine of fish. From Dailey, M.D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. Sixth edition. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Frog Parasites

Frogs, toads, and salamanders are often excellent sources of a number of kinds of parasites. Places to look include the mouth cavity (also in Eustachian tubes), intestine, urinary bladder, lungs, and coelomic cavity. Larvae also may encyst between the skin and musculature. The colon of frogs and toads is a good place to find assorted protozoans, including opalinids and ciliates. Prior to anesthetization of a frog, select appropriate sized dishes for each of the organs to be removed from the frog; fill each dish with amphibian saline. Anesthetize a frog by placing it in MS-222; then inject the anesthetized frog with 0.3% MS-222 or sodium pentobarbital (120 mg/kg IP). Cut through the ventral body wall of the euthanized frog to expose the internal organs. Refer to the text below and Figures 9-13 for diagrams of some of the more common frog parasites. For the diagrams in composite Figure 7.11, label letters for these species are included in brackets in the text.

Parasites of the Digestive Tract

With scissors, remove the digestive tract from the body cavity of the frog; at the posterior end of the frog, pull the large intestine craniad with forceps and cut it loose as close to the pelvis as possible. Detach the small intestine from the stomach and large intestine, and place each in a dish containing a few mL of amphibian saline. Before cutting open the small intestine, flush it out using a small syringe filled with amphibian saline. Then insert sharp scissors into one end of the intestine and, while pulling up, carefully cut open the small intestine and then the stomach and large intestine. Mix the contents from each section of the digestive tract with saline in the individual dishes and transfer a few drops of the saline mixture to a slide, seal the sample with a Vaseline-ringed cover glass, and observe the sample under the low power objective of a compound microscope for movement. Then examine the lining of each section of the digestive tract for the presence of adherent or intracellular symbiotic protozoans, roundworms, and flatworms.

Several species of protozoan symbiotes are commonly found in the frog digestive tract. A small, ovoid flagellate with a sharply pointed end, an undulating membrane and three anterior flagella actively lashing about is *Tritrichomonas batrachorum* [B]. One or more large ciliated protozoans are commonly present. Opalinids (*Opalina* spp. – ciliated, but not a true ciliate) will appear more flattened in cross section, while the true ciliates *Nyctotherus cordiformis* [D] and *Balantidium entozoon* [C] are more ovoid and possess a conspicuous cytostomal groove and a large macronucleus. A more detailed description of the life history and morphology for the most commonly encountered symbiotes of frogs is included in the following text, with a listing of additional species and diagrams near the end (Figure 7.11).

Species of the genus *Opalina* have an oval, flattened body with longitudinal rows of cilia-like organelles, whereas *Cepedea* species are more circular in cross-section [E]. As is typical for opalinids, both lack a mouth, cytopyge, and contractile vacuoles, and the cell body contains many nuclei of the same kind. Fully developed individuals may exceed 300 μm in length and appear as opalescent bodies to the naked eye. Reproduction is by asexual and sexual means. Asexual reproduction during the summer, fall, and winter is by plasmotomy in which the multinucleate body divides several times with cytoplasmic division occurring independent of nuclear division. Sexual reproduction is by the formation of gametes that fuse to form a zygote that eventually produces the trophozoite stage (see Figure 7.9 for life cycle diagram).

Accelerated asexual division in the recta of frogs in the spring (their host's breeding season) produces numerous small individuals with a few nuclei that encyst. These encysted forms are voided with the host's feces. When swallowed by young tadpoles, the small, uninucleate forms released from the cysts function as gametocytes by dividing repeatedly eventually to form minute, fusiform gametes. Two gametes fuse to produce a zygote that develops into a new trophozoite or vegetative stage. Examine stained specimens on microscope slides provided and prepare living specimens in physiological saline as described for the dissection in the preceding sections.

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Figure 7.9. Life cycle of *Opalina ranarum*, showing synchronization of the cyst-producing phase of the reproductive cycle with the sexual cycle of the amphibian host. From Smyth. J. D. and M. M. Smyth. 1980. Frogs as Host-Parasite Systems I. Reprinted courtesy of Macmillan Press Ltd, Basingstoke, Hampshire, United Kingdom.

Nyctotherus cordiformis is a large heterotrichous ciliate parasite found in the colon of amphibians [D]. Trophozoites are laterally compressed, ovoid to kidney-shaped, with the cytostome on one side. The anterior half of the cell contains a massive macronucleus, with a small micronucleus nearby. Study living specimens obtained from the dissection of a tadpole or frog. How does the frog host become infected with this symbiote? Remember – frogs and tadpoles are an excellent source of a number of kinds of symbiotic protozoans and helminthes.

Balantidium entozoon [C] is another ciliate that also may be present in the dissection of the tadpole or frog. In addition, be on the lookout for flagellates in the genus *Trichomonas* and the symbiotic amoeba *Entamoeba invadens*.

Carefully examine the contents of the intestine for the presence of small trematodes. Amphistome digeneans characteristically have a thick fleshy body and a large powerful posterior sucker (acetabulum). Two genera that commonly occur in the rectum/cloaca of frogs are *Diplodiscus* and *Megalodiscus*. Please note – any encysted juveniles (metacercariae) observed in the skin of the frog host are probably those of *M. temperatus*. With reference to the diagrams below, what characteristic(s) may be used to distinguish these two trematodes?

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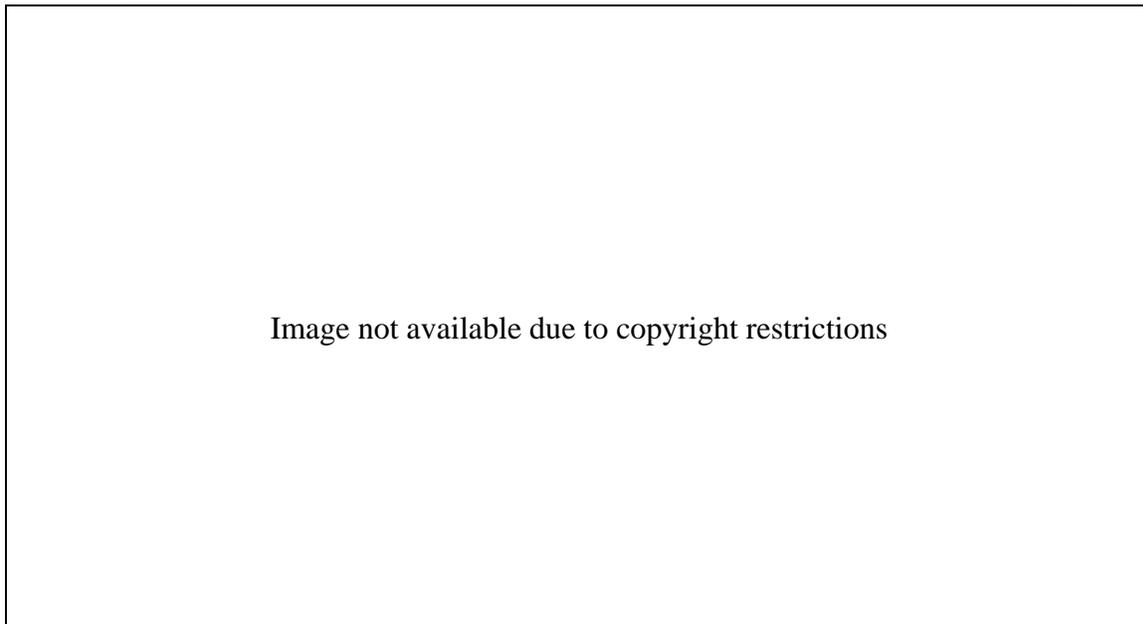
Figure 7.10. A, *Diplodiscus subclavatus*. From Smyth. J.D. and M.M. Smyth. 1980. Frogs as Host-Parasite Systems I. Reprinted courtesy of Macmillan Press Ltd, Basingstoke, Hampshire, United Kingdom; B, *Megalodiscus temperatus*. o, ovary. From Ulmer, M.J. 1970. Studies on the helminth fauna of Iowa I: Trematodes of amphibians. American Midland Naturalist 83:38-64. Reprinted courtesy of the American Midland Naturalist, University of Notre Dame Press, Notre Dame, Indiana.

Distome digeneans which may be encountered include the following: *Cephalogonimus amphiumae* [H] in the small intestine; *Glypthelmins quieta* [F] in the duodenum and adjacent intestine; *Loxogenes arcanum* [G] in the bile duct and adjacent region; and *Halipegus* sp. in the Eustachian tube and oral cavity. Other adult worms in the small intestine include a tapeworm *Cylindrotaenia americana* [I], an acanthocephalan *Acanthocephalus ranae* [J], and nematodes *Oswaldocruzia pipiens* [L] and *Aplectana americana* [K].

Nematode larvae and eggs, as well as eggs of digeneans, for species of adult worms present in lungs (e.g., *Rhabdias ranae*) may also be present. Nematode eggs can be readily distinguished by the color, shape, and presence of larvae within the eggs.

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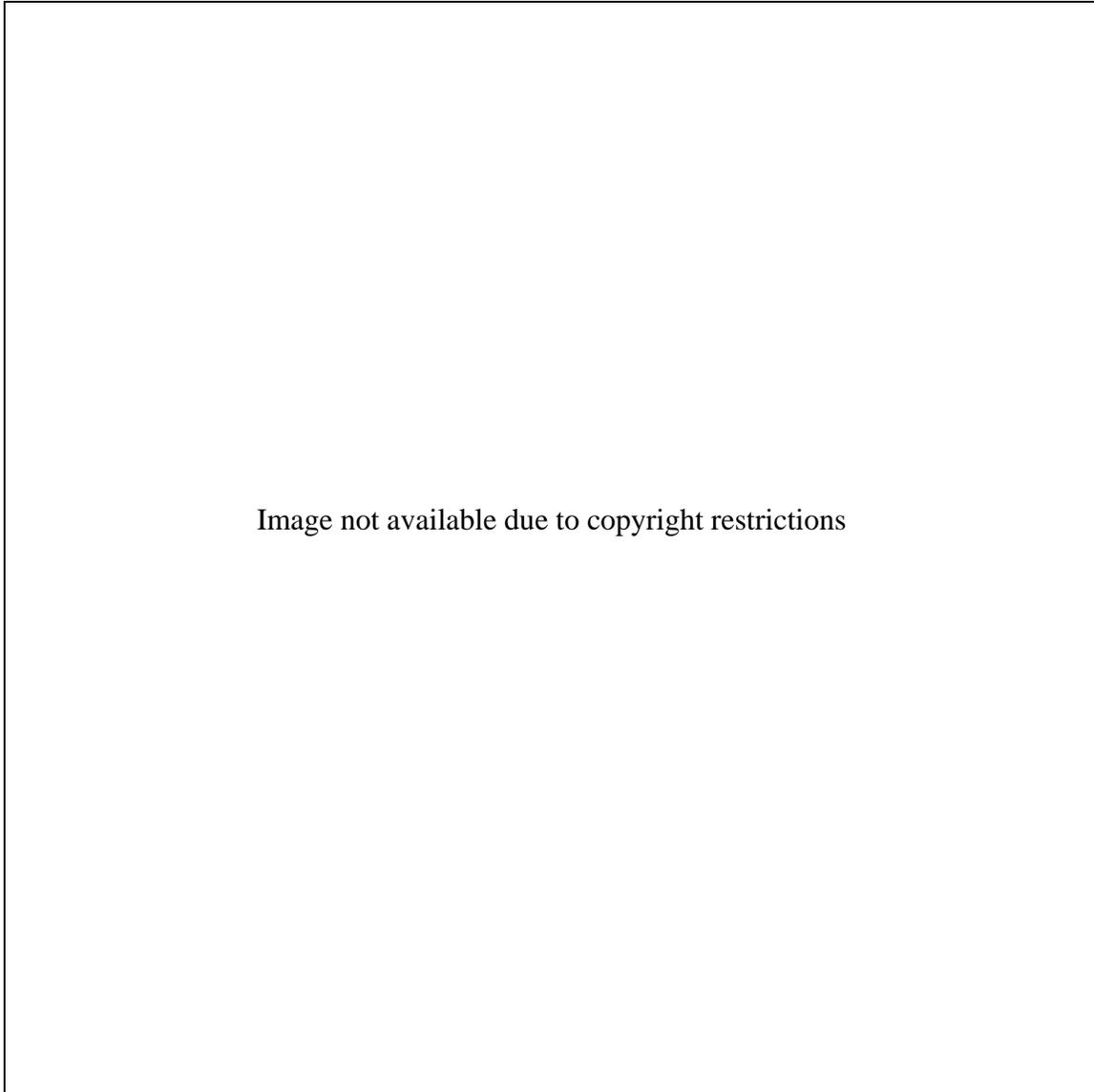
Figure 7.11. A, *Trypanosoma rotatorium*; B, *Tritrichomonas batrachorum*; C, *Balantidium entozoon*; D, *Nyctotherus cordiformis*; E, *Cepedea* sp., entire specimen and cross section; F, *Glypthelmins quieta*; G, *Loxogenes arcanum*; H, *Cephalogonimus amphiumae*; I, *Cylindrotaenia americana*; J, *Acanthocephalus ranae*; K, *Aplectana americana*, male; L, *Oswaldocruzia pipiens*, male; M, *Rhabdias ranae*, anterior end and embryonated egg; N, *Foleyella americana*, female. From Dailey, M.D. 1996. Meyer, Olsen & Schmidt's Essentials of Parasitology. Sixth edition. Reprinted with the permission of The McGraw-Hill Companies, New York, New York.

Parasites of the Lung

Carefully remove the lungs by cutting them at their point of attachment to the larynx. Place them in a dish containing warm Ringer's solution and examine for dark bodies. Tease open the lungs and examine them carefully for movements of parasitic worms. Among the species of trematodes commonly found in the frog lung is a relatively large digenean called *Haematoloechus*. A nematode commonly found is *Rhabdias ranae* [M]; if present, larvae and eggs will probably be present in the intestine. If removed to a slide in distilled water, both forms will discharge eggs in enormous numbers. In the case of *R. ranae*, the eggs may hatch, releasing small, writhing larvae. Mount the parasites on a slide, add a drop of water, and examine them for details of internal anatomy.

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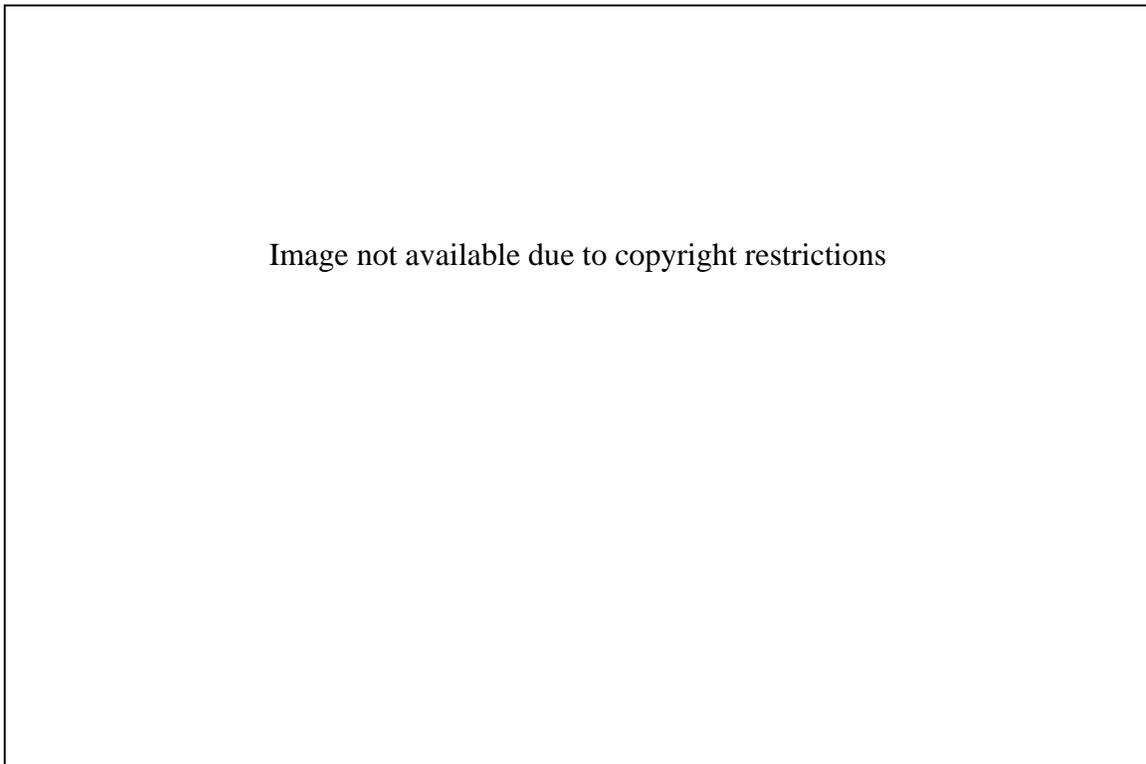
Figure 7.12. A, *Haematoloechus longiplexus*; B, *H. similiplexus*. From Ulmer, M.J. 1970. Studies on the helminth fauna of Iowa I: Trematodes of amphibians. American Midland Naturalist 83:38-64. Reprinted courtesy of the American Midland Naturalist, University of Notre Dame Press, Notre Dame, Indiana.

Parasites of the Bladder

Cut open the urinary bladder and examine for the presence of parasites. At least six species of trematodes have been reported from the frog bladder. Two of the more common trematodes are *Gorgodera* and *Gorgoderina*. Both are characterized by a much-enlarged ventral sucker (acetabulum) that is used by the worm to adhere firmly to the bladder wall. These genera can be distinguished by the number of testes: *Gorgoderina* with two testes and *Gorgodera* with testes divided into several round or irregularly shaped bodies (usually nine) arranged in two

longitudinal rows of five and four. A monogenean, *Polystoma nearcticum*, with six muscular suckers and a pair of large hooks on the opisthaptor, occurs in the urinary bladder of the tree frog (*Hyla versicolor*).

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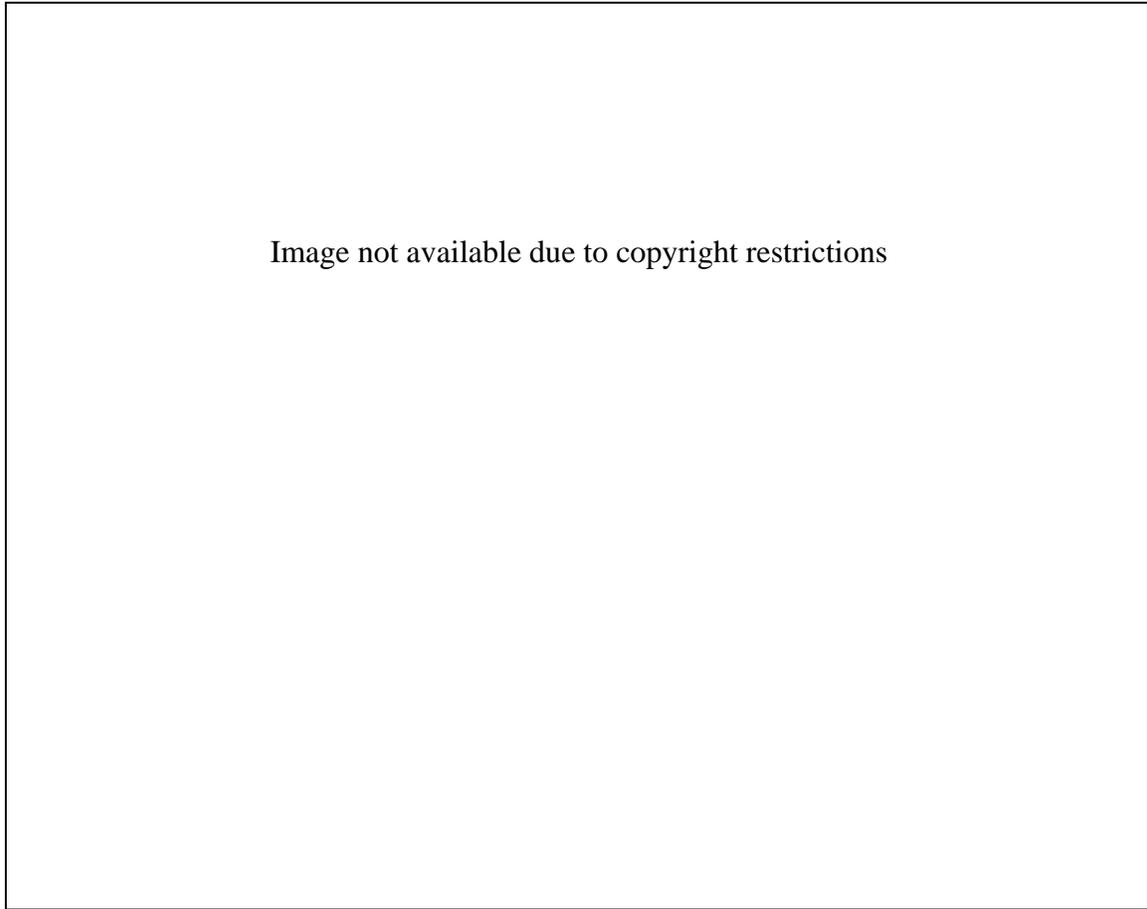
Figure 7.13. A, *Gorgoderina simplex*; B, *Gorgoderina attenuata*; C, *Gorgodera amplicava*. o, ovary; sv, seminal vesicle; t, testis; v, vitellaria. From Ulmer, M.J. 1970. Studies on the helminth fauna of Iowa I: Trematodes of amphibians. American Midland Naturalist 83:38-64. Reprinted courtesy of the American Midland Naturalist, University of Notre Dame Press, Notre Dame, Indiana.

Other Parasites
        Carefully examine all other regions of the frog body for the presence of parasites. Pay particular attention to the lining of the body cavity, mesenteries, mouth, and surfaces of internal organs. Identify and list the kinds of parasites found in any of these areas – roundworms, flukes, tapeworms, or protozoa. Any nematode found in the subcutaneous connective and muscular tissues is probably *Foleyella americana* [N].

Upon examination of a blood smear, one may find *Trypanosoma rotatorium* [A]. Although seldom present in great numbers, this hemoflagellate may more likely be seen in blood smeared from kidney or liver, rather than in blood from peripheral circulation.

Potential Effect of Environmental/Nutritional Conditions on the Intimate Relationship Between Symbiote and Host

Demonstration of the life cycle of the rat tapeworm Hymenolepis diminuta
The complete life cycle of the rat tapeworm, *Hymenolepis diminuta*, will be demonstrated to facilitate discussion of how a symbiotic relationship may change in response to factors that affect the host. *Hymenolepis diminuta* is a cosmopolitan parasite of rats that requires an insect intermediate host (commonly a beetle). Under favorable environmental and nutritional conditions for the host, *H. diminuta* is considered to be a commensal, subsisting off excess nutrients in the small intestine of the host. However, if the rat host becomes stressed by environmental/physiological conditions (e.g., crowding, starvation, or pregnancy of females), the symbiotic relationship between symbiote and host may revert to that of parasitism (Insler and Roberts, 1976).

For demonstration of living adult tapeworms, euthanization and dissection of one infected rat (*Rattus rattus*) is required. To facilitate such a demonstration, tapeworm eggs passed in rat feces are used annually to infect beetles. Larval tapeworms that develop in beetles then are used to infect young rats to maintain the parasite for another year.

A rat infected with larval tapeworms (cysticercoids) by your instructor six weeks prior to this lab will be euthanized and made available to you for dissection. While wearing gloves, place the euthanized rat in a dissecting pan, with ventral body surface facing up. Using forceps to pull the skin up, make a longitudinal incision through the body wall and muscle layers from the pelvis to the neck region. **Caution** – Be sure to make the cut deep enough to include the muscle layers (not just the skin) so that the internal organs are exposed. Also, be careful to use sharp scissors and a slight lifting motion when cutting to avoid damage to the underlying tissues and organs. Identify the stomach and make an incision immediately below it and then another incision above the large intestine to allow removal of the entire small intestine to a large Stender dish/finger bowl containing a small amount of normal saline (0.85% NaCl solution). Adult tapeworms will be visible through the intestinal wall as areas of white mass in contrast to the dark fecal material. Using a 30-50 mL syringe (without needle) filled with warm normal saline and inserted into one end of the small intestine, flush the intestinal contents into a large finger bowl. Transfer living tapeworms from the washings to a small finger bowl with clean, warm saline for further observation under a dissecting scope. For a dramatic observation of the motility of the scolex and acetabula (suckers), use a razor blade or sharp scissors to remove the tiny anterior end (~0.5 inch) of one worm; place this scolex in a couple of drops of saline on a slide under a Vaseline-ringed cover slip and examine it under a compound microscope.

Larval tapeworms (cysticercoids) may be collected by decapitating and removing the posterior tip of the abdomen of an infected beetle intermediate host and then forcing water through the beetle with a Pasteur pipette. Cysticercoids, seen in the washings as tiny white dots

by the naked eye, then can be viewed under the dissecting scope, collected by Pasteur pipette, and placed on a microscope slide in a drop of water under a cover slip for examination with a compound microscope. For observation of tapeworm eggs, soak a couple of fresh fecal pellets collected from an infected rat in water for several hours to make the sample. Then place a drop of the fecal suspension on a microscope slide with a cover slip and examine the sample under a compound microscope. Note – Addition of a drop or two of water to the microscope slide for dilution may be required if the suspension is too thick.

Advantages of this host/parasite system include the following: The complete life cycle can be maintained under laboratory conditions; this cestode has been used extensively as a model system for biochemical/physiological/immunological studies; and this cestode is host specific for rats (i.e., completion of development does not occur in animals other than rats) and consequently does not pose a health threat to humans.

Maintenance of Hymenolepis diminuta *stock*

Fresh feces are collected from rats that have been infected with *Hymenolepis diminuta* for at least 17 days. Feces soaked in water for several hours to disperse the fecal pellets are then passed through a kitchen strainer with cheesecloth to remove hair and large particles. This suspension is centrifuged for ten minutes at 2,000 RPM, the supernatant fluid discarded, and the pellet resuspended in a saturated sodium chloride or zinc sulfate solution. Saturation is assured by the addition of a few grams of the same dry salt to each centrifuge bottle. Following centrifugation as before, the tapeworm eggs are aspirated from the surface of the supernatant. The salt is removed by placing the eggs directly into 2,000 mL of tap water and allowing them to settle to the bottom (~30 minutes). The water then is decanted carefully and fresh water added. Four changes of water are used in this way.

Tapeworm eggs are collected by filtration using a Buchner funnel (with #1 Whatman paper) attached to a heavy-walled side-arm flask under suction. Eggs are carefully scraped from the surface of the filter paper and mixed with apple scrapings (applesauce) to form a thick paste on a small piece of filter paper. The paper with paste is placed in a plastic box (4 x 6 x 1 1/2 inches) containing beetles (the mealworm, *Tenebrio molitor*) starved for 2-3 days. The filter paper is trimmed so that a minimum of moist paper is exposed to attract beetles to the eggs. After about 24 hours, the uneaten mixture of eggs and applesauce is removed and the floor of the box is covered with a wheat germ/flour/oatmeal mixture. At 25˚C, the resulting larval tapeworms (cysticercoids) are infective to rats by the twelfth day after infection.

Cysticercoids are collected by decapitating and removing the posterior tip of the abdomen of an infected beetle and then forcing water through the beetle with a Pasteur pipette. The cysticercoids are counted and the desired number taken up in a minimum of water (about 0.5 mL) in a thick-walled glass pipette. The pipette then is placed into the mouth of the hand-held rat (with a short extension of the pipette in the throat of the animal with neck extended), the water containing cysticercoids discharged, and the head of the animal stroked until all fluid is swallowed.

Infected rats may be maintained for approximately one year. During that time period, feces dropped onto trays may be collected for use in experimental infection of beetle intermediate hosts.
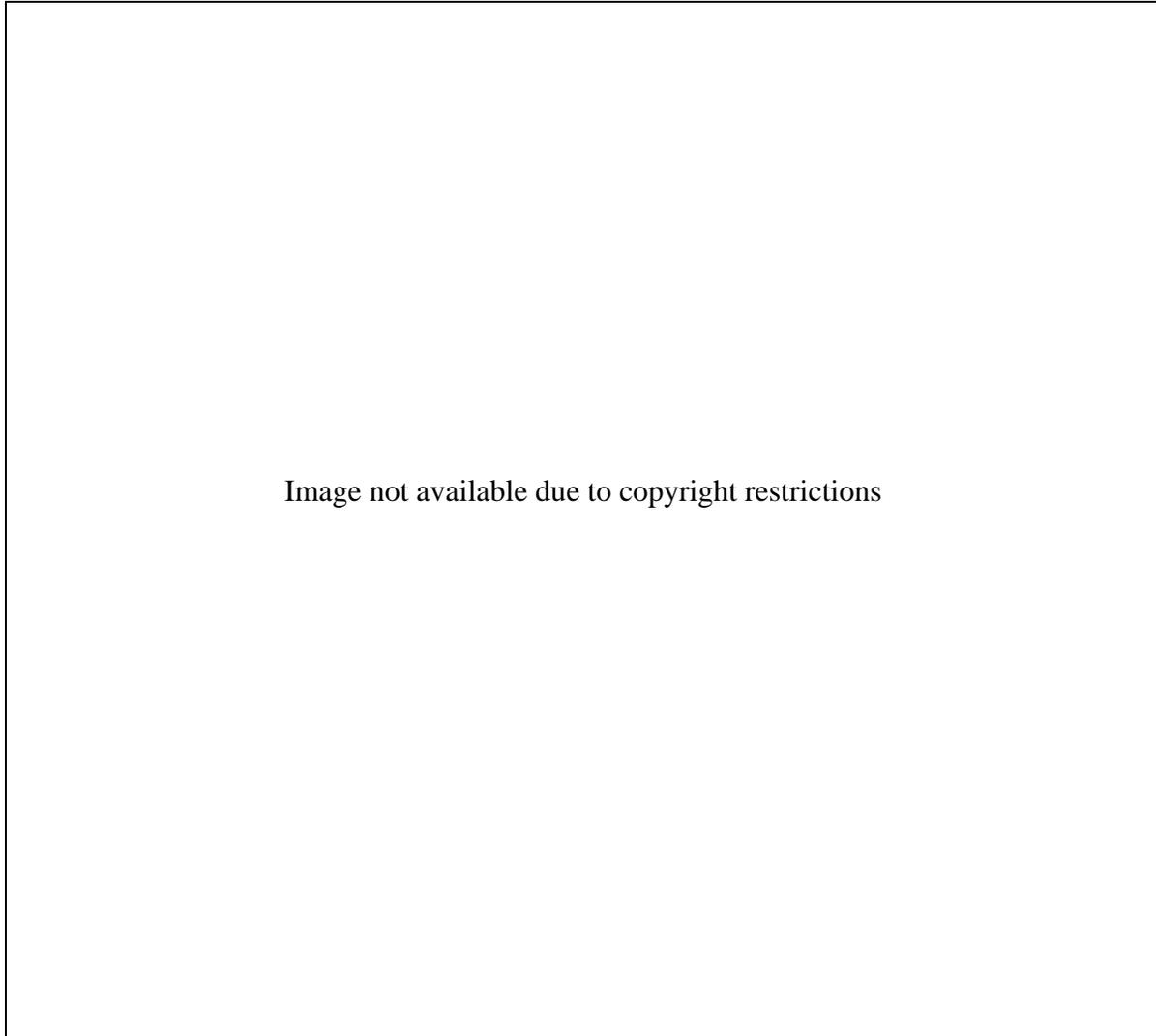
Image not available due to copyright restrictions

Figure 7.14. Life cycle of *Hymenolepis diminuta*. From Read, C. P. 1972. Animal Parasitism. Reprinted with the permission of Prentice-Hall, Inc., Englewood, New Jersey.

Acknowledgments

Literature Cited

Brant, S. V., and B. Hanelt. 1999. Quantitative investigation of the crowding effect of *Hymenolepis diminuta* in *Rattus norvegicus*. Pages 339-355, *in* Tested studies for laboratory teaching (S.J. Karcher, Editor). Proceedings of the Twenty-first Workshop/Conference of the Association for Biology Laboratory Education (ABLE), June 1-5, 1999, The University of Nebraska-Lincoln, Lincoln, Nebraska.

Insler, G. D., and L. S. Roberts. 1976. *Hymenolepis diminuta*: Lack of pathogenicity in the healthy rat host. Experimental Parasitology, 39:351-357.

Janovy, John, Jr. 1978. Keith County Journal. St. Martin's Press, New York, New York, 210 pages.

Cleveland, D. R., S. R. Hall, E. P. Sanders, and J. Collier. 1934. The wood-feeding roach *Cryptocercus*, its protozoa and the symbiosis between protozoa and roach. Memoirs of the American Academy of Arts and Sciences, 17:185-342.

Resource/Reference Books

Cable, R. M. 1977. An illustrated laboratory manual of parasitology. Fifth edition. Burgess Publishing Company, Minneapolis, Minnesota, 275 pages.

Dailey, M. D. 1996. Meyer, Olsen & Schmidt's essentials of parasitology. Sixth edition. Wm. C. Brown Publishers, Dubuque, Iowa, 289 pages.

Dogiel, V. A., G. K. Petrushevski, and Y. I. Polyanski (editors). 1970. Parasitology of fishes. T.F.H. Publications, Inc., Ltd., Neptune City, New Jersey, 384 pages.

Elkan, E. and H. Reichenbach-Klinke. 1974. Color atlas of the diseases of fishes, amphibians and reptiles. T.F.H. Publications, Inc., Neptune City, New Jersey, 256 pages.

Hoffman, G. L. 1967. Parasites of North American freshwater fishes. University of California Press, Berkeley, California. 486 pages.

Jahn, T. L., E. C. Bovee, and F. F. Jahn. 1979. How to know the protozoa. Second edition. Wm. C. Brown Company Publishers, Dubuque, Iowa, 279 pages.

Kudo, R. R. 1966. Protozoology. Fifth edition. Charles C. Thomas, Springfield, Illinois, 1174 pages.

Lee, J. J., S. H. Hutner, and E. C. Bovee (editors). 1985. An illustrated guide to the protozoa. The Society of Protozoologists, Lawrence, Kansas, 629 pages.

Levine, Norman D. 1973. Protozoan parasites of domestic animals and of man. Burgess Publishing Company, Minneapolis, Minnesota, 406 pages.

Marquardt, W. C., R. S. Demaree, and R. B. Grieve. 2000. Parasitology and vector biology. Second edition. Academic Press, San Diego, California, 702 pages.

Olsen, O. W. 1974. Animal parasites. Their life cycles and ecology. University Park Press, Baltimore, Maryland, 562 pages.

Pritchard, M.H. and G. O. W. Kruse. 1982. The collection and preservation of animal parasites. University of Nebraska Press, Lincoln, Nebraska, 141 pages.

Read, C. P. 1972. Animal parasitism. Prentice-Hall, Inc., Englewood, New Jersey, 182 pages.

Roberts, L. S. and J. Janovy, Jr. 2000. Gerald D. Schmidt & Larry S. Roberts' foundations of parasitology. Sixth edition. The McGraw-Hill Companies, Inc., Boston, Massachusetts, 670 pages.

Schmidt, G. D. 1970. How to know the tapeworms. Wm. C. Brown Company Publishers, Dubuque, Iowa, 266 pages.

Schell, S. C. 1985. Handbook of trematodes of North America North of Mexico. University Press of Idaho, Moscow, Idaho, 263 pages.

Smyth. J. D. and M. M. Smyth. 1980. Frogs as host-parasite systems I. The MacMillan Press, Ltd., London, England, 112 pages.

Woo, P. T. K. 1995. Fish diseases and disorders. Volume1. Protozoan and metazoan infections. Cab International, Wallingford, United Kingdom, 808 pages.

APPENDIX A – Reagents and Solutions

10% Buffered Neutral Formalin

Na_2HPO_4.. 0.7625 g
NaH_2PO_4.. 0.01875 g
Formalin...100.0 mL
Distilled H_2O...900.0 mL

Giemsa Stain

Giemsa's powder.. 1.0 g
Glycerine (glycerol)...66.0 mL
Methyl alcohol (methanol), absolute...................................... 66.0 mL

To the alcohol-glycerine, add glass beads (3-5mM) and the dry powder. Allow the alcohol-glycerine to penetrate the dye for a few minutes and then rotate the flask for ~2 minutes. This mixture is agitated about every 30 minutes until the procedure has been repeated 6 times. When possible, the stain should be made up early in the day so that the final shaking will be completed before the end of the working day. When prepared in this way, the stain is ready for immediate use. Store in tightly stoppered bottle. The stock solution is diluted just before use with distilled water or dilute buffer solution. A suggested formula is the following:

Giemsa stock solution... 1.0 mL
Distilled water or phosphate buffer (~0.1 M, pH 6.5).................. 20.0 mL

Or use a commercially available kit (e.g., Hema 3 Staining System as available from Fisher Scientific)

Glycerol Alcohol

Ethanol, 70%...90.0 mL
Glycerol... 10.0 mL
Mix and store in a well-stopper bottle.

Glycerin-Jelly

Granulated gelatin, purified...8.0 g
Distilled H_2O..52.0 mL
Glycerin, pure... 50.0 mL
Phenol.. 0.1 gm

Soak gelatin in water for at least 60 minutes. Then dissolve gelatin by heating in a warm water bath (65-75° C). Filter the mixture through several layers of cheesecloth or fine gauze previously moistened with hot water. Finally, dissolve phenol in the glycerin and add very gradually to the gelatin while stirring. Stir until the mixture is homogenous while heating the mixture in the water bath for ~30 minutes. Store in tightly closed, dark glass containers to prevent evaporation

of water and to retard discoloration. Refrigerate until needed; immerse the container in a water bath maintained at ~50 C immediately prior to use.

Insect Saline

Sodium chloride...9.0 g
Potassium chloride...0.2 g
Calcium chloride ($CaCl_2 \cdot H_2O$)..0.27 g
Dextrose..4.0 g

Dissolve in distilled water. Add sufficient sodium bicarbonate to bring the pH to 7.l2. Bring total volume to 1000 mL with water.

0.3% Solution of MS-222

3-aminobenzoic acid ethyl ester... 3.0 g
Distilled H_2O... 1000.0 mL
To be buffered with Trizma Base or sodium bicarbonate (baking soda) to pH 7.4.

Normal Saline for Cold-Blooded Vertebrates

Sodium chloride (for amphibians).. 7.0 g
Sodium chloride (for fish)... 6.0 g
Distilled H_2O... 1000.0 mL

Ringer's Solution for Cold-Blooded Vertebrates

Sodium chloride... 8.0 g
Sodium bicarbonate.. 0.2 g
Potassium chloride... 0.2 g
Calcium chloride (anhydrous).. 0.2 g
Dextrose (optional).. 1.0 g
Distilled H_2O... 1000.0 mL

Zinc Sulfate Solution

Zinc sulfate.. 330.0 g
Warm water... 1000.0 mL

Dissolve zinc sulfate in water. Adjust to a specific gravity of 1.18.

APPENDIX B – Sources for Specimens and Supplies

Carolina Biological Supply Company, P.O. Box 6010, Burlington, NC 227215, 1-800-334-5551, www.carolina.com.

Fisher Science Education, 485 South Frontage Road, Burr Ridge, IL 60521, 1-800-955-1177, FAX 1-800-955-0740, e-mail info@fisheredu.com, web site www.fisheredu.com.

Fisher Scientific, US Headquarter, Pittsburgh, PA, 1-800-766-7000, FAX 1-800-926-1166, web site www.fishersci.com.

Memphis Net and Twine Co., Inc., P.O. Box 80331, Memphis, TN 38108-0331, 1-888-674-7638, FAX 901-458-1601, e-mail memnet@memphisnet.net.

Ward's Biology, 5100 West Henrietta Rd., P.O Box 92912, Rochester, NY 14692-9012, 1-800-962-2660, FAX 1-800-635-8439, e-mail customerservice@wardsci.com, web site www.wardsci.com.