CHAPTER 8

Parasitology

SECTION 1 - General Parasitology
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**SECTION 2 - Detection of Whirling Disease (*Myxobolus cerebralis*)
by Pepsin-Trypsin Digest**
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**SECTION 3 - Diagnosis of *Bothriocephalus acheilognathi*
(Asian Tapeworm) in Wild Fish Populations**
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Section 1 – General Parasitology

I. Introduction

The purpose of this chapter is to provide guidance in the detection and identification of the numerous parasitic organisms that infect a broad range of fish species in natural populations.

A. TARGETED PARASITES

Myxobolus cerebralis, the myxozoan responsible for Whirling Disease is the only targeted parasite of the Survey, however it is important to document the occurrence and severity of infection for all parasitic organisms encountered in natural fish populations. Information regarding presence, and more importantly pathology associated with parasites, will broaden current understanding of the life history and distribution of these organisms in their natural environment and in wild fish populations. This data will be included in the WFS Database as supplemental information.

B. PATHOGENS OF REGIONAL IMPORTANCE (PRI)

Fish Health biologists have also identified two parasitic organisms, Ceratomyxa shasta and Bothriocephalus acheilognathi as Pathogens of Regional Importance (PRI). Information is provided for detection of Bothriocephalus acheilognathi (Asian Tapeworm) as new information has surfaced since the last edition of the AFS-FHS Blue Book (2004). For detection and PCR corroboration of Ceratomyxa shasta, refer to Chapter 9 – Corroborative Testing of Parasites by PCR.

Refer to Woo (1995) and Hoffman (1999) for excellent reviews on fish morphology, life cycles, control, taxonomy, and presumptive identifications. More general discussions on fish parasites can be found in Post (1987), Kent (1992), Stoskopf (1993) and Lasee (1995). Methods for general necropsy and preparation of samples for study can be found in Luna (1968), Cable (1977), Humason (1979), Brown and Gratzek (1980), Frimeth (1994) and Dailey (1996). General references for pathological effects of parasites include Ribelin and Migaki (1975), Roberts (1978), Ferguson (1989) and Woo (1995). Keep in mind that necropsies for parasites should follow collection of viral and bacterial samples.

C. MAJOR GROUPS OF FISH PARASITES - (Modified from Hoffman 1999)

Fungi: usually filamentous, nonseptate; Ichthyophonus often occurs as spheres.

Protozoa: commonly referred to as single-celled animals, including amoebas, ciliates, flagellates and sporozoans.

Monogenea: flukes with flattened body; posterior attachment organ (haptor) which bears hooks or clamps; lack true suckers; attach on exterior body of fish host (some exceptions) and exhibit simple life cycles with no intermediate hosts.

Trematoda (Digenetic): flukes with flattened body; oral and ventral suckers (some exceptions); and exhibit complex life cycles involving multiple hosts.

Cestoidea (Tapeworms): worms with flattened segmented body (usually); and head (scolex) usually bears suckers, hooks or suctorial grooves, occasionally no organs of attachment.

Nematoda (Roundworms): thin, elongate worms with cylindrical body covered by a rigid cuticle; one or both ends attenuated; and no organs of attachment.

Acanthocephala (Spiny-headed worms): body cylindrical, sometimes slightly flattened; and spectacular hook-bearing eversible proboscis present.

Crustacea (parasitic): external parasites, may be louse-like (Argulus); worm-like (Lernaea); or grub-like (Salmincola, Ergasilus).

Hirudinea (Leeches): external parasites; some dorsoventrally flattened; others more cylindrical; and body segmented, with anterior and posterior suckers.

Glochidia: larval freshwater clams encapsulated in fins and gills; resemble minature clams with shells; and some armed with hooks.

II.   Key to Major Taxa of Adult Parasites of Fishes

(modified from Frimeth 1994 - Does not apply to larval Cestoda, Digenea or Nematoda)
1.   Individual organism microscopic (except *Ichthyophthirius*) and single-celled, but may be multinuclear..Protozoa
     Individual organism usually visible without a microscope and multicellular ....
     2
2.   Body worm-like.. 3
     Body not worm-like... 8
3.   Body dorsoventrally flattened, not round in cross-section 4
     Body not dorsoventrally flattened, round in cross-section 7
4.   Body with segmentation or distinct external annulations or rings 5
     Body without segmentation or distinct external annulations or rings 6
5.   Anterior attachment organ present which may include hooks and muscular suckers, posterior attachment organ not present; gut absent .... **Cestoda**
     Anterior and posterior attachment organs present with well defined posterior sucker, gut present .... **Hirudinea**
6.   Posterior attachment organ present which usually includes hooks (hamuli) and marginal hooks; external parasites ....**Monogenea**
     Posterior attachment organ not present, possesses circumoral and ventral suckers except for blood dwelling species; internal parasites .... **Trematoda**
7.   Anterior spined proboscis present ....**Acanthocephala**
     Anterior spined proboscis not present .... **Nematoda**
8.   Body in form of two hinged shells (valves); organism encysted on surface of gills or fins .... **Glochidia**
     Body not in form of two hinged valves, possesses appendages for attachment to host surface; organism not encysted, external parasite...**Crustacea**

III. Some North American Fish Parasites Listed by Location in Host

(Modified from Hoffman 1999)

A. EGGS
Fungi: Saprolegnia and relatives.
Protozoan: Carchesium (on walleye and trout eggs), Epistylis reported from catfish eggs, *Pleistophora variae* in golden shiner eggs, *P. sulci* in *Polyodon spathula* eggs, and *Thelohania baueri* in *Pungitius pungitius* eggs, former Soviet Union.

B. BARBELS
Protozoa: *Henneguya* sp. in *Ictalurus nebulosus*, *Ichthyophthirius* occassionally
Trematoda: *Gyrodactylus* spp.

C. SKIN AND FIN SURFACES
Fungi: External fungi, *Saprolegnia* and relatives, *Exophiala pisciphila*
Protozoa: Ectoparasitic protozoa (*Ambiphrya, Amphileptus, Bodomonas, Chilodonella, Colponema, Cyclochaeta, Epistylis, Ichthyoboda, Ichthyophthirius, Oodinium, Trichodina, Trichophrya*), Microsporidea, Myxosporidea, *Myxobolus squamalis* (in scales of salmonids)
Monogenea: *Gyrodactylus*, usually not other monogeneans
Trematoda: Metacercariae of many species, including *Neascus* (black spot)
Nematoda: Undescribed larval nematode in skin nodules of ictalurids (eastern half of USA)
Crustacea: *Argulus, Lernaea, Ergasilus, Salmincola*.

D. NARES
Protozoa: *Apiosoma* sp., Europe, *Amphileptus, Chilodenella, Myxobolus, Tetrahymena, Trichodina, Trichodinella*
Monogenea: *Aplodiscus nasalis* in *Hypentellium etowanum*, *Cleidodiscus monticelli, Pellucidhaptor catostomi, P. nasalis, P. pricei*
Nematoda: *Philometra* in bluegills and largemouth black bass
Copepoda: *Ergasilus megaceros* in fallfish and catfish, *Ergasilus rhinos* in centrarchids, *Gamidactylus, Gaminispatulus, Gaminispinus, Lernaea, Paragasilus, Salmincola*

E. GILLS
Fungi: *Dermocystidium*
Protozoa: *Ambiphrya, Amphileptus, Bodomonas, Chilodonella, Cryptobia, Dermocystidium, Epistylis, Ichthyoboda* (Costia), *Ichthyophthirius*, Microspora, Myxosporea, *Piscinoodinium, Trichodina, Trichophrya*
Monogenea: *Gyrodactylus, Dactylogyrus, Cleidodiscus*, and many other species
Trematoda: Many metacercariae, ova becoming miracidia of *Sanguinicola*
Copepoda: *Achtheres, Argulus, Lernaea, Ergasilus, Salmincola, Lepeophtheirus*

F. MOUTH
Protozoa: *Apiosoma*, Europe, Myxosporea

Trematoda: *Leuceruthrus*
Nematoda: *Philometra nodulosa* in suckers and buffalo fishes
Copepoda: *Lernaea cyprinacea*, *Salmincola* (*S. lotae* in burbot)

A. BLOOD
Protozoa: *Trypanosoma* (*Cryptobia*), *Trypanoplasma* free, *Babesiosoma*, *Dactylosoma*, *Haemogregaerina* in red blood cells, rarely *Kudoa*, *Sphaerospora* trophozoites (Csaba bodies) free in carp and other cyprinids, Europe
Trematoda: *Sanguinicola* in blood vessels, including gill vessels
Nematoda: *Philometra sanguinea* in caudal fin blood vessels of goldfish, *P. obturans* in gill vessels of pike, former Soviet Union

H. ESOPHAGUS
Trematoda: *Azygia*, *Cotylaspis*, *Derogenes*, *Halipegus*, *Proterometra*

I. STOMACH
Protozoa: *Schizamoeba* spp.
Monogenea: *Enterogyrus* spp. (freshwater, tropical)*, Enterogyrus* sp. in foregut of *Pomacanthus paru*
Trematoda: *Allocreadium*, *Aponeurus*, *Azygia*, *Caecincola*, *Centrovarium*, *Derogenes*, *Genolinea*, *Hemiurus*, *Leuceruthrus*
Nematoda: *Haplonema*

J. INTESTINE AND PYLORIC CECA
Protozoa: *Hexamita*, *Schizamoeba*, *Eimeria*.
Trematoda: *Allocreadium*, *Crepidostomum*, *Lissorchis*, *Alloglossidium*, additional adults of many species
Cestoda: *Proteocephalus*, *Bothriocephalus*, *Eubothrium*, additional adults of many species.
Nematoda: *Contracaecum*, *Camallanus*, *Spinitectus*, additional adults of many species.
Acanthocephala: *Neoechinorhynchus*, *Echinorhynchus*, *Pomphorhynchus*, additional adults of many species.

K. SWIM BLADDER
Trematoda: *Acetodextra* in *Ictalurus punctatus*
Nematoda: *Cystidicola* spp. in salmonids, *Huffmanella huffmanella* in *Lepomis cyanellus*

L. BODY CAVITY: MESENTERIES, LIVER, SPLEEN
Fungi: *Ochroconis humicola* in coho salmon; *O. tshawytscha* in chinook salmon, *Phoma herbarum* in northwest salmonids
Protozoa: Many Myxosporea, rarely Microsporea, *Goussia* spp.
Trematoda: Many metacercarial species including *Ornithodiplostomum*, white grub (*Posthodiplostomum*), adult *Paurorhynchus* (bucephalid), adult *Acetodextra* in catfish

Cestoda: Larval *Diphyllobothrium*, *Haplobothrium*, *Ligula*, *Proteocephalus*, *Schistocephalus*, *Triaenophorus*.
Acanthocephala: Larvae of *Echinorhynchus salmonis*, *Leptorhynchoides thecatus*, *Pomphorhynchus bulbocolli*
Nematoda: Adult *Philonema*, many larval species
Copepoda: Rarely *Lernaea* in small fishes

M. GALL BLADDER
Protozoa: Myxosporea, *Hexamita*?
Trematoda: *Crepidostomum cooperi*, *C. farionis*, *Derogenes* sp., *Plagioporus sinitsini*, *Prosthenhystera* sp., *Pseudochaetosoma*, Europe
Cestoda: *Eubothrium salvelini*, larval Dilepidae (armed pleurocercoids)
Nematoda: *Rhabdochona* sp., occasionally *Capillaria catostomi*

N. HEPATIC BILE DUCT
Trematoda: *Phyllodistomum* spp.

O. KIDNEYS
Fungi: *Ichthyophonus hoferi* in many fishes, *Ochroconis* spp. in West Coast coho and chinook salmon
Protozoa: Myxosporea in tubules, sometimes interstitial, sometimes cysts
Trematoda: Metacercariae of *Nanophyetus salmincola*, *Posthodiplostomum minimum centrarchi*, probably others, adult *Phyllodistomum* in renal tubules and ureters

P. URINARY BLADDER
Protozoa: Myxosporea, *Vauchomia* (trichodinid) in *Esox* spp.
Monogenea: *Acolpenteron*.
Trematoda: *Phyllodistomum*.

Q. OVARIES
Protozoa: *Henneguya oviperda* in *Esox lucius*, Europe, *Pleistophora ovariae* in golden shiners, *Thelohania baueri* in *Gasterosteus aculeatus* ova, former Soviet Union
Trematoda: *Acetodextra ameiuri*
Nematoda: *Philonema* spp. in salmonids
Cestoda: *Proteocephalus*

R. TESTES
Protozoa: *Hexamita*
Cestoda: *Proteocephalus*

S. EYES
Protozoa: *Henneguya episclera* in *Lepomis gibbosus*, *H. zikaweiensis* in *Carassius auratus*, China, *Myxobolus corneus* in cornea of *Lepomis macrochirus*, *M. hoffmani* in sclera of *Pimephales promelas*
Digenea: *Diplostomum spathaceum* in lens, *Diplostomulum scheuringi* in vitreous humor and other species of *Diplostomulum*

Nematoda: *Philometroides* sp. in eye orbit of southeastern centrarchids

T. CARTILAGE
Protozoa: *Henneguya brachyura* in fin ray of *Notropis* spp., *H. schizura* in sclera of *Esox lucius*, *Henneguya* sp. in branchial arch of *Pomoxis* spp., *M. cartilaginis* in centrarchids, *Myxobolus cerebralis* in salmonids, *M. hoffmani* in sclera of eye of *Pimephales promelas*, *M. scleropercae* in cartilaginous sclera of eye of perch and additional species

U. NERVOUS SYSTEM
Fungi: *Ichthyophonus hoferi* in brain
Protozoa: *Mesencephalicus* in brain of *Cyprinus carpio* (Europe), *Myxobolus cerebralis* affects CNS (although parasite is in cartilage), *M. hendricksoni* in brain of *Pimephales promelas*, *M. arcticus*, *M. kisutchi*, *M. neurobius* in CNS of salmonids
Monogenea: Some species in lateral line pits
Trematoda: *Diplostomulum*, *Euhaplorchis*, *Ornithodiplostomulum*, *Parastictodora*, metacercariae on brain, *Psilostomum* metacercariae in lateral line canal.

V. MUSCLE AND CONNECTIVE TISSUE
Protozoa: *Myxobolus insidiosus* in muscle of cutthroat trout, chinook and coho salmon, *Heterosporis* in muscle of yellow perch, and many Myxosporea and Microsporea
Trematoda: Many metacercarial species including yellow grub (*Clinostomum*), and black spot (*Neascus* spp.)
Cestoda: Larval *Diphyllobothrium*, *Triaenophorus*
Acanthocephala: larval forms
Nematoda: larval *Eustrongylides*

IV. General Methods
(Brown 1980; Frimeth 1994; Daley 1996; Hoffman 1999)

Following collection, fish should be held in well-aerated containers at the appropriate temperature. Care should be taken to handle fish humanely and euthanize with approved anesthetic prior to examination. After euthanasia, fish can be examined in the field (if microscopes are available) or transported, on ice, to the laboratory.

A. NECROPSY PROCEDURES

1. Examine skin, fin and gills for larger parasites that can be seen with the naked eye (e.g., *Ichthyophthirius*, larger monogeneans, leeches, etc.). If the fish is small, it can be placed into a petri dish with water or normal physiological saline and examined using a dissecting microscope. The fins and gills can be removed from larger fish and examined similarly. If a microscope is not available, a hand held lens can be used for external examinations

2. Prepare a mucus wet mount by scraping the dorsolateral surface of the fish with the dull side of a scalpel blade. Transfer the mucus to a clean microscope slide, add a drop of saline and cover with a cover slip. Remove several fins and prepare a wetmount. Examine preparations for smaller external protozoa using 100 and 450X magnification of a compound microscope.

3. Remove the operculum of the fish with a scissor. If the fish is small, remove the entire gill arch and transfer to a slide. Add saline and cover slip. With larger fish, it is necessary to remove the bony arch before preparing the gill wetmount or use a few filament tips removed with a scissor. Examine the gills with 100X of a compound microscope looking for external protozoans, cysts (which may be *Ichthyophthirius*, trematode metacercariae, Microsporea or Myxosporea) and monogenetic flukes.

4. Open the fish. Examine the body cavity for encysted parasites. Remove a small amount of blood from the heart. Dilute 1:1 with saline and examine at 100X for *Trypanoplasma* and *Trypansoma*. Blood smears can also be prepared at this time and later stained for blood sporozoa.

5. Remove the viscera *in toto* from fish. For small fish this should be done in saline with the use of a dissecting microscope. Tease apart the organs with fine forceps or dissecting needles. Remove a drop or two of fluids and mucus from the intestinal tract and transfer to a slide. Add a coverslip and examine for protozoa (*Hexamita*, *Schizamoeba* and *Eimeria*). Open the intestinal tract the entire length and examine with a dissecting microscope for helminths. Examination of the intestinal tract may also be facilitated by compressing a longer section of the intestine between two glass slides and examining with a

compound microscope.

Individual organs of larger fish can be removed, transferred to saline in a petri dish and examined for larval parasites. Squashes of kidney, liver, spleen and gonads can be prepared. Remove the swim bladder, being careful not to deflate it. Examine with a dissecting microscope for nematodes and trematodes. Stretch out the entire intestinal tract and cut open longitudinally with a small fine point dissecting scissors (subdivide the tract if it is too large).

The tract may be flushed with saline and gut contents examined separately from the tract wall. Keep in mind, that most parasites will be small and transparent.

6. Carefully remove the gall bladder with fine forceps, and examine for trematodes. Prepare a wetmount and examine for sporozoans and ciliates. Likewise, remove the urinary bladder and examine for *Phyllodistomum* and sporozoans.

7. Remove each eye using a forceps and scissor. Place into a petri dish with saline. Use low power of a dissecting microscope to observe any movement of digenetic unencysted larval flukes (*Diplostomulum*). Cut open the eye and examine the lens for the eye fluke *Diplostomum spathaceum*.

8. Cut the head lengthwise and remove the brain. Squash some of the brain onto a slide, add a drop of saline and coverslip. Examine for Myxosporea and trematode metacercariae (e.g., *Ornithodisplostomum*).

9. The musculature can be examined by slicing the epaxial musculature at regular intervals and looking for larval worms, sometime encysted, and Microsporea and Myxosporidea cysts. Some can only be seen with higher magnification.

10. Concentration methods for myxosporidans are sometimes required, such as for detection of *Myxobolus cerebralis --see* Section 2: Detection of Whirling Disease (*Myxobolus cerebralis*) by Pepsin-Trypsin Digest (PTD) (Modified from Dr. Rich Holt, Oregon Department of Fish and Wildlife, 1987 and excerpted from the Alaska Department of Fish and Game, Fish Pathology Section Laboratory Manual, Meyers, ed. 1997.)

    See also Whirling Disease Foundation-AFS Standard Protocols for Whirling Disease Research (version 1.0 - May 2001) and Markiw and Wolf (1974) for digestion techniques, and O'Grodnick (1975) for the plankton centrifuge method.

B. FIXATION OF PARASITES

The most commonly used fixative for preserving and storing parasites include alcohol-formol-acetic (AFA or Davidson's Fixative), Bouin's, formalin and glycerine alcohol (Humason 1979). Formalin is probably the most commonly used and preferred fixative. Preservation in cold fixatives is not recommended because most parasites will contract and make identifications difficult or impossible.

Prior to fixation, worms should be thoroughly washed in saline and cleansed of mucus.
1. Protozoa
    a. For Myxosporea: cut out the cyst with enough adjacent tissue and place in 10% formalin.
    b. For trophozoites of motile forms: place as many protozoa as possible on a clean microscope slide, add one drop of PVA-AFA (polyvinyl alcohol-acetic acid formalin alcohol) fixative adhesive, mix, spread over slide and allow to dry. Also, protozoans can be transferred to a vial of 10% formalin (keep in mind they will usually shrink).

2. Monogeneans/Trematodes
    Trematodes should be transferred to a small glass petri or stender dish. Remove excess saline or water. Heat 10% formalin or AFA to 85-90°C (begins to steam but not boil) in a fume hood. Add hot fixative to dish containing trematodes. For thicker worms, flatten under a coverslip and flood with warm fixative. For monogenea, drop infected gills into 10% formalin. Larger monogenes can be removed and fixed under light coverslip pressure.

3. Cestodes
    Procedures are similar to that of trematodes; kill in 80°C water or formalin and store in buffered 10% formalin.

4. Nematodes
    Kill in warm (80°C) glycerine alcohol (1 part glycerine:3 parts 95% ethanol) and transfer to cold glycerine alcohol for storage.

5. Acanthocephala
    For acanthocephalans it is necessary to evert the proboscis prior to fixation. Place worms in distilled water and refrigerate overnight. Transfer to warm 10% formalin or AFA.

6. Leeches
    Fix in warm 10% formalin, or if very thick, flatten between two slides and flood with 10% formalin.

7. Copepods

Remove copepods, if possible, and drop into glycerin alcohol or 70% ethanol. If not easily detached, cut out a small piece of tissue containing parasite and place in 10% formalin or 70% ethanol.

C. PREPARATION OF PARASITE WHOLE MOUNTS

Only two whole mount techniques will be presented here: Semichon's Acetocarmine Technique for monogoneans, trematodes, cestodes and acanthocephalans and Glycerin Jelly Technique for whole mounts of small nematodes and microcrustacea. More comprehensive staining and whole mount procedures can be found in parasitology and histology laboratory manuals (Luna 1968; Cable 1977; Humason 1979; Dailey 1996).

Staining should be done using glass dishes with ground covers (stender preferred). Screw-cap vials may also be used. Always use at least four times the volume of reagent to the bulk of specimen. Containers should always be covered, except when changing solutions or observing specimens. Care must be taken when transferring specimens from one liquid to another. Several methods can be used: 1) pour off the first liquid and then add second liquid immediately (never allow the specimens to dry out); 2) if the specimens are small and delicate, transfer with a bulbed pipette using a dissecting microscope or 3) most specimens can be successfully transferred to a second liquid by lifting them gently with a camel hair brush. A small slip of paper labeled in pencil can be used to identify specimens through staining process.

The fixative should be thoroughly removed from specimens prior to staining. Wash out formalin in distilled water and AFA in 70% alcohol.

1. Semichon's Acetocarmine Staining Technique (AFA Fixative preferred)
    To stain, cover specimens with Semichon's aceto-carmine stain and allow to stand overnight.
    a.   Remove stain and save (it can be reused). Wash off excess stain in two changes of 70% ethanol (5-10 minutes for each change).
    b.   Destain using 70% acid alcohol. Observe specimens using a dissecting microscope and destain until parenchyma and muscles are nearly free from stain (light pink) while internal organs are well stained (red).
    c.   Stop destaining by replacing or pouring off acid alcohol with two changes of basic alcohol (5-10 minutes each change).
    d.   Dehydrate in 95% ethanol for 15 minutes.
    e.   Counterstain with dilute fast green in 95% ethanol. Observe specimens with dissecting microscope using care not to over stain (until very light bluish green).
    f.   Dehydrate in two changes of 100% ethanol for 10-15 minutes each.
    g.   Clear in methyl salicylate (or xylene replacement) for at least 10 minutes. Specimens are ready for mounting when they sink.
    h.   After clearing is complete, transfer specimen to a microscope slide using a clean camel hair brush. Center specimen and orient correctly (ventral side up and anterior end towards bottom of slide).
    i.   Add a coverslip and add sufficient mounting medium (Permount™) to fill space

under the coverslip (try to avoid introducing any bubbles). As the mount dries, additional Permount may need to be added.

j.   When completely dry, excess Permount may be cleaned from the slide using a razor blade.

k.   Label slides and store in a horizontal position.

2.   <u>Glycerin Jelly Technique</u> (Double coverslip method, Figure 1)

a.   Transfer specimens to glycerin alcohol if they were not fixed in hot glycerin alcohol.

b.   Place specimens in stender staining dishes containing glycerin alcohol. Cover dish with filter paper or a Kimwipe to allow slow evaporation of the alcohol in the glycerin alcohol. The specimens are ready to mount when all the alcohol has evaporated and there is only glycerin jelly remaining in the dish. This may take one to several days.

c.   Heat glycerin jelly mounting medium in a water bath until it liquifies. Do not allow jelly to exceed 60°C as it will caramelize and become brown. Glycerin jelly will solidify at room temperature, so it will be necessary to use a warmed pipette to transfer the molten glycerin jelly to your coverslip.

d.   Add a small drop of liquified glycerin jelly to the center of a large coverslip (22 or 25 mm diameter). Use a camel-hair brush to transfer specimen to the drop of jelly. Place the coverslip on a slide warming table to prevent the jelly from hardening.

e.   Add a smaller diameter coverslip (18 mm) to cover the specimen. The glycerin jelly should completely fill the space covered by the smaller coverslip. If not, add a small drop of glycerin jelly.

f.   Allow the glycerin jelly mount to harden by removing preparation from warming table.

g.   Clean off any excess glycerin jelly from around the rim of the larger coverslip using a razor blade and a cotton applicator stick dipped in 70% ethanol.

h.   Place a drop of Permount onto the center of a clean microscope slide. Center the mount with the larger coverslip up on top of the drop of Permount. Add sufficient Permount to the slide to fill the remaining space created by the overhanging larger coverslip. Allow Permount to harden.

i.   Label slide.
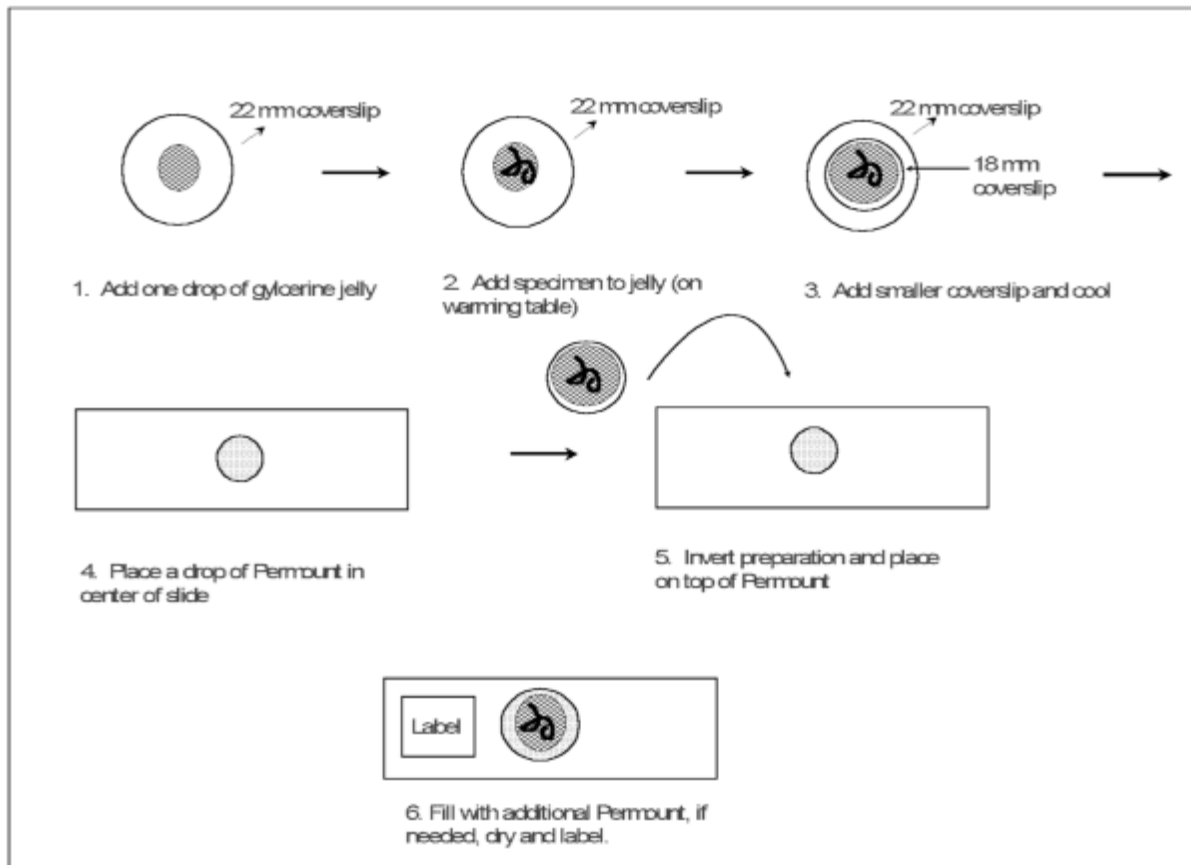
Figure 1. **Double coverslip method for Glycerine Jelly Mounts.**

V. Keys for Identification of Parasites

Taxonomic keys for presumptive identifications of parasites can be found in Hoffman (1999). In most cases, definitive identifications will require more extensive review of primary and secondary literature. When in doubt, samples (slides or preserved) can be sent to a parasitologist for confirmatory identification.

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Appendix 8.A – Reagents and Solutions

Acid Alcohol
      Ethanol, 70%   98 mL
      Hydrochloric acid   2 mL

AFA (Alcohol-formol-acetic) Fixative
      Ethanol, 85%   85 mL
      Formalin   10 mL
      Acetic acid, glacial   5 mL

Glycerine Alcohol
      Ethanol, 70%   95 mL
      Glycerine   5 mL

Alkaline (Basic) Alcohol
      Ethanol, 70%   500 mL
      Ammonium hydroxide, concentrated   0.5 mL

Bouin's (picro-formol-acetic) Fixative
      Picric acid, saturated aqueous solution   75 mL
      Formalin, commercial   25 mL
      Acetic acid, glacial   5 mL
Specimen's are usually left in it for about 24 hours before being transferred to 70% ethanol.

Fast Green, Stock
      Fast green, powdered   0.2 gm
      Ethanol, 95%   100 mL

Formalin
Commercial formalin is treated as 100% (although it is a 40% solution of formaldehyde gas in water). Thus 5% formalin, which is 2% formaldehyde, contains
      Water, distilled   95 mL
      Formalin   5 mL

Glycerine Jelly
Soak 7 gm of granulated gelatin in 40 mL of distilled water for 30 minutes. Then melt in a warm water bath and filter through several layers of cheesecloth previously moistened with hot water. Dissolve 1 gm phenol in 50 mL of glycerin and add to the gelatin. Stir until the mixture is homogeneous.

Saline Solution
      Sodium chloride   7 gm
      Water, distilled   1000 mL

Semichon's Acetocarmine
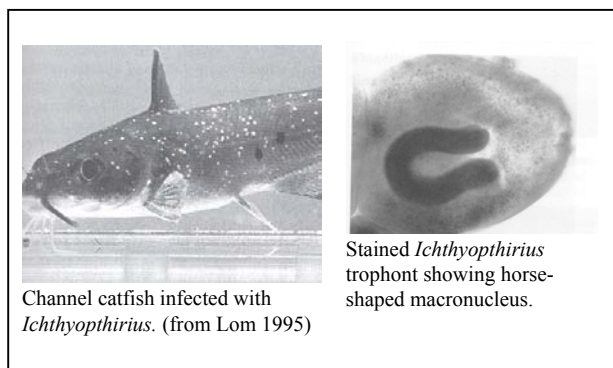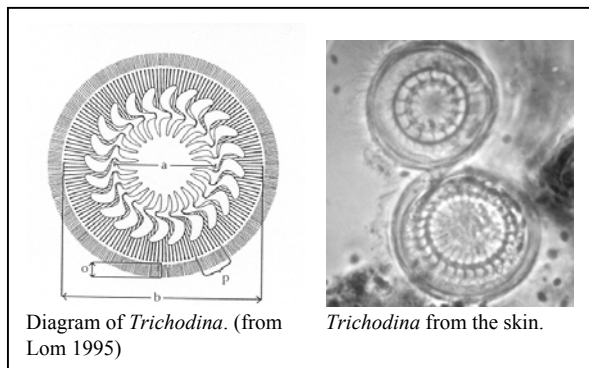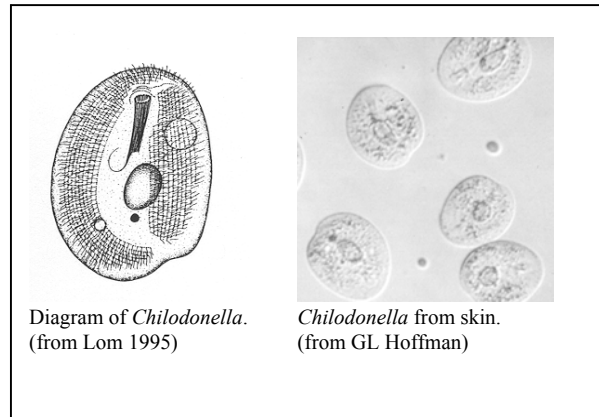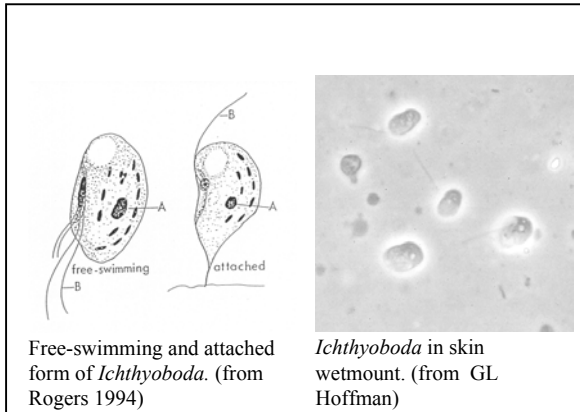
        Acetic acid, glacial   100 mL

        Water, distilled   100 mL

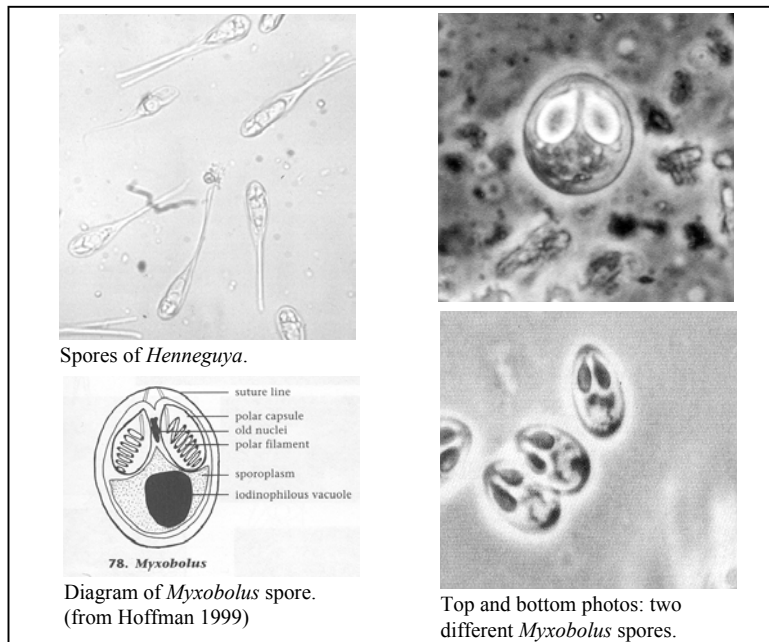   Carmine "in excess"   1.5 gm (about)

Mix distilled water and acetic acid in an Erlenmeyer flask and add carmine. The objective is to prepare a saturated solution of carmine; however, do not add more than will go into solution. Heat in boiling water bath for 15 minutes, then cool flask in cold water and filter the contents. This stock stain should be diluted with approximately two parts of 70% ethanol before use.

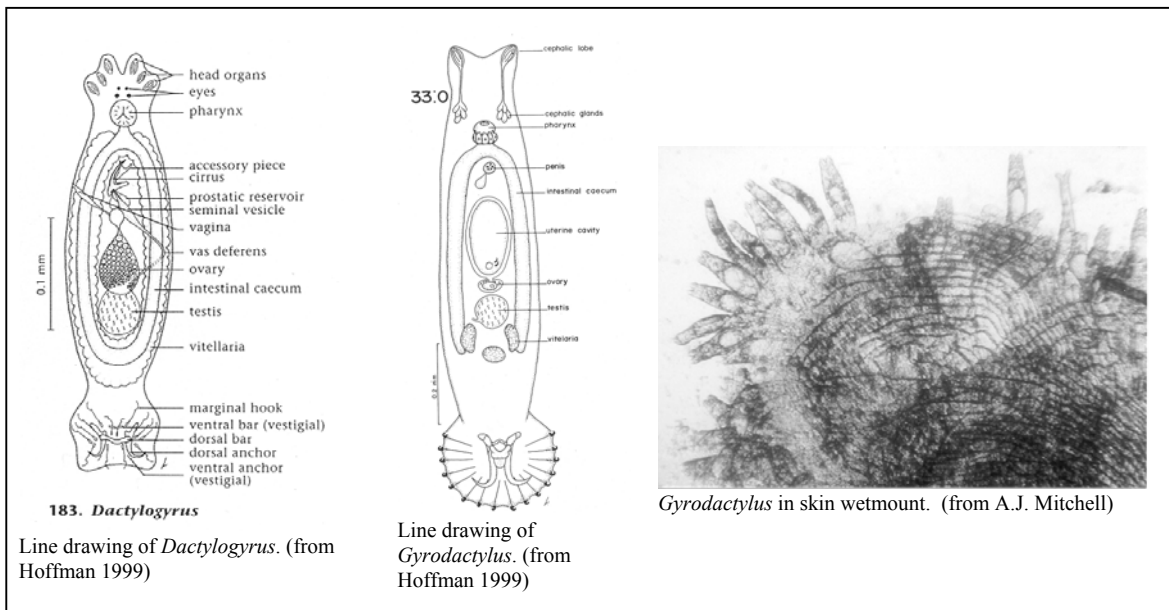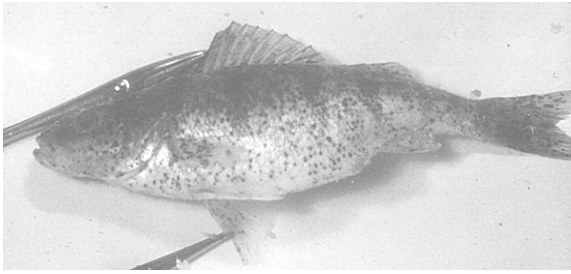# Appendix 8.B

EXTERNAL PROTOZOANS

Free-swimming and attached form of *Ichthyoboda*. (from Rogers 1994)

Ichthyoboda in skin wetmount. (from GL Hoffman)

Diagram of *Chilodonella*. (from Lom 1995)

Chilodonella from skin. (from GL Hoffman)

Diagram of *Trichodina*. (from Lom 1995)

Trichodina from the skin.

Channel catfish infected with *Ichthyopthirius*. (from Lom 1995)

Stained *Ichthyopthirius* trophont showing horse-shaped macronucleus.

MYXOSPOREANS

Spores of *Henneguya*.

Diagram of *Myxobolus* spore. (from Hoffman 1999)

suture line
polar capsule
old nuclei
polar filament
sporoplasm
iodinophilous vacuole

78. *Myxobolus*

Top and bottom photos: two different *Myxobolus* spores.

MICROSPORANS

Microspora spore. (from Dykova 1995)

Spores of *Glugea*. (from Woo 1995)

Heterosporis. Top photo: large white cysts in musculature o f yellow perch (infected fish above, uninfected below). Bottom photo: spores and vesicles. (from D.R. Sutherland)

MONOGENES

183. *Dactylogyrus*

Line drawing of *Dactylogyrus*. (from Hoffman 1999)

Line drawing of *Gyrodactylus*. (from Hoffman 1999)

Gyrodactylus in skin wetmount. (from A.J. Mitchell)

DIGENETIC TREMATODES

Yellow perch infected with metacercariae of black grubs (*Neascus*). (from Michigan DNR)

Metacercarial cysts of white grub (*Posthodiplostomum*).
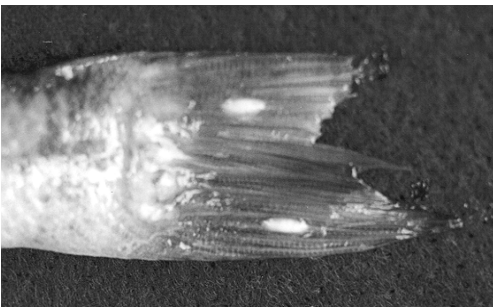
Yellow grub (*Clinostomum*). Top photo: metacercarial cysts on caudal fin. Photo on right: acetocarmine stained wholemount (w.m.).
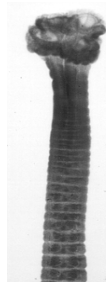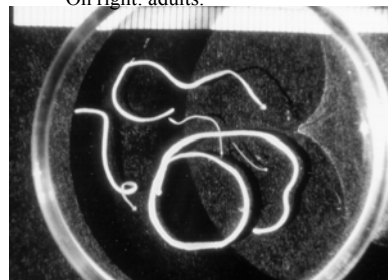

Adult *Allocreadium* (stained w.m.).

Adult *Alloglossium* (stained w.m.).
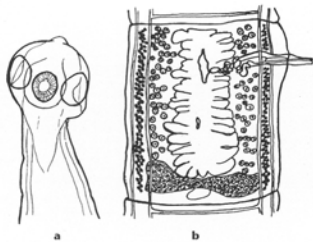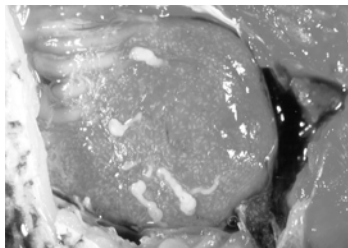
Proteocephalus or Bass tapeworm. Top photo: larval or metacestodes on the liver of bass. Bottom: diagram of the adult, scolex and proglottid. (from Hoffman 1999)
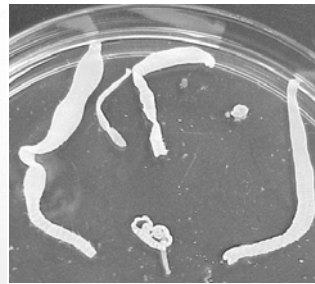
Corallobothrium (Catfish tapeworm) adults. On left: stained wholemount (note fleshy appendages surrounding suckers). On right: adults.

Bothriocephalus acheilognathi or Asian Tapeworm. Note arrow-shaped scolices.

NEMATODES

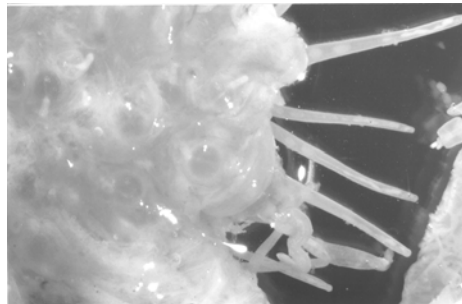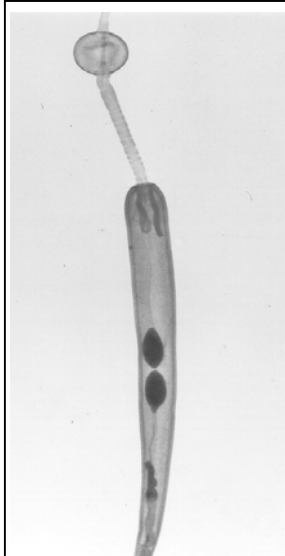*Cystidicola* (swim bladder nematode) from a bloater chub.
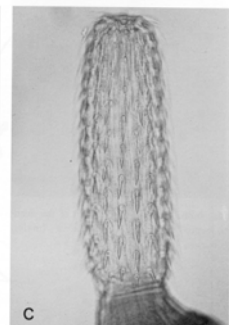
Capillaria from intestine of lake trout.

Philometra in fascia of cheeks of a white sucker. (from Ribelin and Migaki 1975)

ACANTHOCEPHALANS

Pomphorhynchus bulbocolli. Left: wholemount, acetocarmine stained (note large bulb below proboscis). Top: Live worms, *in situ*. Bottom: Attached worms with bulb protruding through intestinal wall.

Metechinorhynchus salmonis. A: adults, *in situ*. B: wholemount, acetocarmine stained. C: proboscis armed with spines.
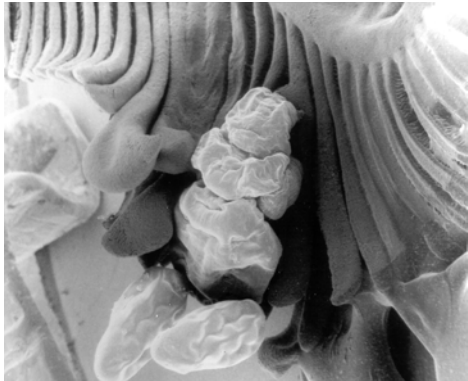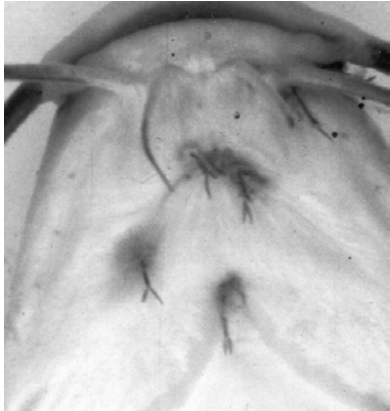
The fish louse, *Argulus* (acetocarmine stained).

Ergasilus from the gills of a deepwater sculpin.

Scanning electron micrograph of *Salmincola*, the gill maggot.

The anchor worm, *Lernaea*. Top: adults attached to ventral surface of a channel catfish. Note egg sacs of copepods.   Right: Stained wholemount preparation.

Section 2 – Detection of Whirling Disease (*Myxobolus cerebralis*) by Pepsin-Trypsin Digest (PTD)

The following literature and laboratory protocols were reviewed and incorporated here to proffer a single protocol for detection of *Myxobolus cerebralis* by enzyme digest:

> - Markiw, M.E. and K. Wolf. 1974a. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements-sequential enzymatic digestions and purification by differential centrifugation. Journal of the Fisheries Research Board of Canada 31:15-20.

> -Lorz, H.V., and Amandi, A. 1994. Whirling Diseases of Salmonids, VI: 1-7 in Thoesen, J. C. (Ed.), Suggested procedures for the detection and identification of certain finfish and shellfish pathogens (4th Edition). Fish Health Section, American Fisheries Society, Bethesda, MD.

> - Dr. Rich Holt, Oregon Department of Fish and Wildlife, 1987, modified by Hauck and Landin and excerpted from Meyers, Editor, 1997. Alaska Department of Fish and Game - Fish Pathology Section Laboratory Manual.

> - Stanek, D., 1998. Pepsin/Trypsin Digest for Myxobolus cerebralis (Whirling Disease). unpublished laboratory protocol. Washington Animal Disease Diagnostic Laboratory, Pullman, WA.

> -Parasite Detection and Assessment of Disease - Standard Protocols for Whirling Disease Research. Standardized Protocols Advisory Committee. Whirling Disease Foundation and the Fish Health Section of the American Fisheries Society. Draft Version 1.0, May 2001.

Many of these protocols are similar in the fundamental steps used to digest and concentrate spores obtained from cranial tissues (defleshing the heads, pH of enzyme reagents, concentration of spores by centrifugation, etc.). The majority of differences between various protocols relate to: specific equipment used for defleshing and/or grinding cartilage, small differences in incubation period and temperature depending on fish size, and the inclusion or omission of a sucrose gradient to further separate spores from bone and other background debris. The WDF-AFS Standard Protocol also includes procedures for counting and quantifying spores.

I.   Sample Collection

Samples are collected for preliminary testing of *Myxobolus cerebralis* by the Pepsin-Trypsin Digest method. If samples test positive for Myxobolus spores, additional corroborative testing will be performed on the digested spore preparation by Mc-PCR or the archived half of the head, either by histology or Mc-PCR. The first step is to divide individual fish tissues for Pepsin-Trypsin Digest (PTD) and Archive (ARC) sample sets. Caution should be used in sample collection with forethought to the corroborative method to be used and to prevent cross-contamination of Mc-DNA. Use separate tools for each 5-pool of head tissue or disinfect tools with 10% chlorine (followed by a diH$_2$0 rinse) between sample pools.

A. FOR SMALL FISH – LESS THAN 6 INCHES
1. Remove head including gill arches, using a sterile scalpel for each set of 5 heads. Cut the heads longitudinally from the snout to the operculum.
2. Place one half of the 5 heads in a whirl-pak™ bag labeled with fish ID and "PTD".
3. Place the second set of 5 halved heads in another whirl-pak bag labeled with fish ID and "ARC" for corroborative testing by histology or Mc-PCR.
4. Keep samples cold or on ice during collection. Place both sets of samples in a –20 or -70°C freezer upon return to the laboratory.

B. FOR LARGER FISH – GREATER THAN 6 INCHES
It may be necessary to excise the target tissue and then halve the material into the separate bags labeled for PTD and ARC.
1. Use a large biopsy punch (12-18mm for fish 6-12 inches) or a coring tool for adult fish.
2. Remove the tissue punch, or core, and cut it in half using a scalpel.
3. Place one half of tissue from 5 heads in a whirl-pak bag labeled with fish ID and "PTD".
4. Place the second set of 5 halved tissues in another whirl-pak bag labeled with fish ID and "ARC" for corroborative testing by histology or Mc-PCR.
5. Keep the PTD samples cold and the Archive samples frozen. Place the archive in a –70C freezer upon return to the laboratory.

C. HISTOLOGY
If histology will be used for corroborative testing, follow the above procedure except place the second set of halved head tissue in Davidson's or Bouin's fixative and label as Archive. Tissues are transferred to alcohol at 24 hours, however heads do not need to be processed unless a positive PTD test occurs.

II.   Preliminary Detection Procedure - Pepsin-Trypsin Digest (PTD)

SOLUTIONS: **0.5% Pepsin**:   To 1 L water add 5.0 g pepsin and 5 mL concentrated hydrochloric acid.

0.5% Trypsin (in Rinaldini's Solution):
Make a 1-L solution of distilled water containing

NaCl (sodium chloride)		8.0 g
KCl (potassium chloride)		0.2 g
$HOC(COONa)(CH_2 COONa)_2 \cdot 2H_2O$ (sodium citrate)		1.0 g
NaH_2PO_4 (sodium phosphate)		0.05 g
$NaHCO_3$ (sodium bicarbonate)		1.0 g
Glucose		1.0 g
Phenol Red (0.5%)		1.0 mL
Trypsin		5.0 g

A. Thaw the frozen samples overnight in the refrigerator. Frozen samples can also be thawed the day of the assay, but will require more time to process in step B.

B. To deflesh the soft tissue, soak the heads in 60°C water for 35 minutes (smaller fish) and up to 60 minutes (larger fish).

C. Remove soft tissue from bone and cartilage by rinsing heads in a fine mesh strainer, using water pressure and a rubber spatula to dislodge all soft tissue (skin, eyes, lower jaw, muscle). Discard all wash solution and soft tissues into 10% chlorine solution.

    Note: For Larger heads, it may be easier to dissect bone and cartilage directly from the soft tissue using forceps.

D. Collect all bone, cartilage and gill elements in tarred aluminum foil, a tarred electric blender, or a tarred beaker. Record the weight of the sample on the datasheet.

    Note: This is a good stopping point for the assay if unable to complete in one day. Store the tissue preparations in the refrigerator overnight, or frozen for long-term storage. Continue the assay the next day by allowing the samples to reach room temperature before proceeding with next step.

E. Add 20 mL of pepsin solution per gram of tissue. Blend in electric blender for 2-3 minutes. (If the blender cannot hold the entire volume of pepsin needed, add a small volume, blend the sample, then add the remainder of pepsin to the sample after it is transferred to a beaker in step F below).

F. Transfer the tissue and pepsin into a beaker and stir for at least 30 minutes at 37°C.

G. Pour the digest into a pre-weighed 50 mL conical tube. Centrifuge at 1200 x g for 10 minutes. Discard supernatant into 10% concentrated bleach.

H. Weigh the 50 mL tube and tissue, subtract tube weight and record tissue weight on the datasheet.

    Optional: Check digest material for spores using a wet mount. If negative, proceed to next step.

I. Add 20 mL trypsin solution per gram of tissue in the centrifuge tube. Shake tube, and then pour back into original beaker used in step F.

J. Adjust pH to 8.5 with 1 N NaOH. Digest at room temperature for minimum of 30 minutes. (Be sure to sanitize pH probe after each use).

K. Pour digested sample through cheesecloth or non-adsorbent disposable filter (mesh size approximately 200um). Retain filtered fluid. Discard cheesecloth or filters in biobag and autoclave.

L. Centrifuge fluid in original tube (step G) at 1200 x g for 10 minutes.

M. Re-suspend pellet in 0.5-1mL of sterile diH$_2$0, or PBS. If samples cannot be examined immediately, freeze the preparations to prevent growth of bacteria and/or fungi.

Note: Formalin inhibits the detection of Mc by PCR. Therefore DO NOT USE FORMALIN as a preservative if PCR will be utilized for corroborative testing of the digest material.

Researchers have also found better extraction of DNA when PTD spore preparations are stored in water, or frozen when compared to samples stored in ethanol (personal communication, John Wood, Pisces Molecular, LLC.)

Therefore, if corroborative testing of digest samples will be performed with Mc-PCR, samples can be frozen (-20°C or –70°C). Thaw samples and process the digest material through the DNA extraction step for Mc-PCR (see Chapter 9 – Step D for DNA Extraction from PTD preparations). Freezing will not affect the quality or quantity of DNA provided samples are not frozen and thawed repeatedly.

N. Thoroughly mix the sample and load a hemacytometer using a pasteur pipet. Examine the pellet suspension for spores, using the 40x objective.

O. A minimum of 150 fields should be examined at 200x magnification. Observe for spores with appropriate morphology (slightly ovoid, lenticular in lateral view, 2 polar capsules with a sporoplasm, a clearly visible suture line with no sutural ridge, no valve extensions or processes, 8-12μm in size).

P. Optional: Calculate spores per mL using the following equations:

Cells per mL   = the average count per each square **x** dilution factor **x** 10^4

Total cell number = cells per mL x the original volume of fluid from which
                                    cell sample was removed

III. Identification of *Myxobolus cerebralis*

Myxobolus cerebralis can easily be confused with its congeners in the genus *Myxobolus*; therefore, the following should facilitate identification. In wet mount and, in some cases, stained preparations from digested specimens, it is difficult to distinguish these species. A combination of several species may be detected in one sample.

Myxobolus cerebralis: ovoidal front, lenticular profile; 2 pyriform polar capsules at anterior end. Sporoplasm without iodinophilous vacuole; therefore, no stain is taken up by the sporoplasm and the entire spore stains the same color using Lugols iodine. Vacuole staining is not a very accurate method due to variation within the species. Some spores have unusual processes but generally are 2/3 the size of *Myxobolus kisutchi*. *M. cerebralis* is about 8-10 μm and is histozoic in cartilage/bone tissue, primarily in the head, but also in the spinal column. Capsules are about 2/5 of spore length. Common hosts of *M. cerebralis* include *Oncorhynchus nerka, O. clarki, O. mykiss, O. aquabonita, Salmo salar, Salvelinus fontinalis*, and *O. tshawytscha*. Refractory hosts include *O. kisutch* and *S. trutta, Salvelinus namaycush* and splake.

Myxobolus kisutchi: Ovoidal with 2 polar capsules at anterior end. Sporoplasm with iodinophilous vacuole. Sometimes with posterior prolongation of shell. It is about 7-8.5 μm and is histozoic in or adjacent to nervous tissues. Hosts include *O. kisutch, O. tshawytscha*, and *O. mykiss*. *Myxobolus insidiosus* spores are pyriform or tear-drop shaped and the long axis is longer than *M. kisutchi*. The iodinophilous vacuole stains dark orange if the spore is young and the storage area has not been used up. Giemsa stains vacuoles very well. Skeletal muscle is infected and white patches on the skin have been associated with heavy infections.

Other species of Myxosporidia that may confuse diagnosis of *M. cerebralis* in salmonids include:

Myxosoma dermatobia: in *O. kisutch*: spores 8-10 μm in size. In skin under epithelium or scales. Narrow ends of polar capsules widely apart. Produces ulcers.

Myxosoma squamalis: in *O. kisutch, O. mykiss*. Found in scale pockets. Scales are raised, giving the appearance of warts. Fixed spores are 8-9 μm in diameter, uniform and have equal polar capsules with a narrow ridge paralleling either side of suture ridge.

Myxobolus neurobius: in *O. kisutch, O. nerka, Thymallus arcticus, S. trutta, S. alpinus, Salmo salar*. In spinal cord, brain, and nerves. Fixed spores pyriform 8 x 10-12 μm. Polar capsules occupy less than half of the spore length. Fresh spores are larger (8-14 μm).

Myxobolus arcticus: in central nervous system of *O. kisutch, O. nerka, S. malma, S. neiva* (Russian char)*, T. arcticus, S. alpinis,* and *Coregonus clupeaformis.* Fresh spores are large, 7.5 x 14-16 μm, with elongated polar capsules.

M. insidiosus: in muscle of *O. clarki, O. tshawytscha,* and *O. kisutch* of Western U. S. Fresh spores are similar in size and shape to *M. arcticus* (9-11 x 12-17 μm).

IV. Corroborative Testing for *Myxobolus Cerebralis*

PCR technique may be superior to other detection and corroborative methods due to the specificity and extreme sensitivity of this molecular tool. The Survey offers an excellent opportunity to utilize the PCR method and evaluate this tool alongside the standard detection methods, such as PTD, and confirmatory methods such as histology.

A.   **POLYMERASE CHAIN REACTION (PCR) TESTING OF ARCHIVE TISSUE**

To utilize PCR as a corroborative method, heads are halved during the sample collection process. One set of halved heads is used for preliminary testing for Mc spores. The second set of halved heads is archived for corroborative testing by PCR (frozen) or histology (preserved in fixative). If the Digest sample set tests positive for Myxobolus spores, the corresponding samples from the frozen archive set can be used to confirm the identification of the spores as *Myxobolus cerebralis* by PCR. Drs. Ron Hedrick and Karl Andree recommend using the archived head tissue for PCR corroborative testing rather than the digested spore material.

B.   **PCR OF PEPSIN-TRYPSIN DIGEST (PTD) SPORE PREPARATIONS**

An alternative method for corroborative testing is to utilize the spore preparation obtained from the Pepsin Trypsin Digest method (Baldwin 2002). The PTD spore preparation must be centrifuged to concentrate the spore material, then heated in a microwave oven to disrupt the spores and release the Mc DNA. See Chapter 9 - Corroborative Testing of Parasites by PCR, for procedures for each method.

C.   **CONFIRMATORY IDENTIFICATION BY HISTOLOGY**

Confirmatory diagnosis can be accomplished by histology: Fresh or frozen heads are fixed in Bouin's or Davidson's. These fixatives are preferred over 10% neutral buffered formalin because the acetic acid assists in decalcification. Decalcify as specified below, then paraffin embed, section and stain (May-Gruenwald Giemsa or Toluidine blue). Scan cartilage tissues at 40-400x magnification. The presence of spores in cartilage confirms diagnosis.

Decalcification Procedure for Detection of Whirling Disease

1. Purpose

    Tissue sectioning of large fish heads for the presence of *Myxobolus cerebralis* spores can be facilitated by chemical decalcification of fixed bone/cartilage and of frozen samples which are later placed into a fixative. Only heads or wedges from fish over 8" should be decalcified. Wedges from fish 6-8" in length are adequately decalcified in Bouin's or Davidson's fixative.

2. Equipment/supplies

    Lerner D-Calcifier solution (Hydrochloric acid, polyvinylpyrrolidone)
    Dissecting needles
    pH paper
    Forceps
    Beakers
    Pipets
    Magnetic stirring rods
    Stirring plate
    Graduated cylinders
    Watch glasses
    0.1 N NaOH
    Scalpels
    5% Ammonium Oxalate solution $C_2H_2O_4 \bullet (NH_3)$

3. Procedure

    a. A 2½ - x 1½- x ¼ cm section of the skull (Bouin's fixed) is removed with scalpel and forceps from an area encompassing the otolith/auditory canal. The section is rinsed, weighed and placed in a cassette. The size of the section may vary according to the size of cassettes, molds, etc., that are available in the lab.
    b. The cassette is placed in a beaker with a magnetic stirring rod and sufficient D-calcifier to provide a ratio of 20 mL D-calcifier to 1 g of tissue sample. Cassettes from the same case number or lot of fish may be pooled into one beaker. Care must be taken not to overload the beaker with cassettes, or the stirring rod will not function. Stirring assists in decreasing the decalcification time by increasing the permeation of the specimen with the solution. Cassettes should be labeled with a marker that will withstand acidic solutions, or identity of the specimens will be lost. The beakers should be covered to reduce evaporation and for safety reasons.
    c. The covered beakers are placed onto a stirring plate for 4-16 hours at room temperature. The time depends on the thickness of the specimen and the amount of bone/cartilage present. If laboratory personnel cannot be present to monitor this process during these hours, then the cassettes must be removed from the solution, rinsed with distilled water, and submerged in a beaker of distilled water

until the next day when the digestion procedure can be resumed with fresh decalcifier.

    d.   The degree of remaining calcification is tested by a combination of physical and chemical testing during the decalcification process.

4.   Physical test

A dissecting needle is pressed against the bone/cartilage area to test its softness. Puncturing is not suggested since this alters the integrity of the specimen. This physical test will give some indication of the progress of the digestion and should be performed approximately 3 hours after the initiation of digestion and periodically thereafter.

5.   Chemical test

Decalcification is complete when the chemical test cannot detect any calcium in the decalcification solution.

    a.   This test requires removal of 5 mL of the decalcifying solution from the beaker of specimens after 3 hours of decalcification, and also every hour thereafter; and placing that amount into a small beaker with a piece of pH paper.

    b.   The solution is neutralized (pH 7.0) with 0.1 N sodium hydroxide as indicated by the pH paper which is immediately removed with forceps.

    c.   1 mL of ammonium oxalate solution is added to the neutralized solution and mixed. This solution is allowed to stand for at least 15 minutes to determine if a precipitate forms. The precipitate is calcium oxalate and indicates that the decalcification is incomplete. Decalcification should be continued until no calcium oxalate precipitate can be detected.

    d.   After decalcification is complete, the specimens are rinsed in distilled water and loaded into a tissue processor.

6.   Discussion

This procedure was developed for the histological examination of large heads that were fixed or frozen/fixed. A 10% nitric acid solution was found to be essentially equal to the D-calcifier in decalcification time and effectiveness, but required a larger ratio of solution per gram of specimen (50:1). The D-calcifier was selected as the agent of choice due to its commercial availability. Other commercial products, especially those that do not require overnight monitoring of the decalcification process, and formic acid may also be suitable but have not been tested.

Giemsa stains of decalcified tissue sections are not as intense as normally fixed samples, but *Myxobolus cerebralis* spores are still evident. Specimens have also been "partially" decalcified before loading them into the tissue processor and these

specimens, although a little more difficult to section, appeared to retain better staining properties.

V. Bibliography

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Section 3 -   Diagnosis of *Bothriocephalus acheilognathi* (Asian Tapeworm) in Wild Fish Populations

I. Introduction

Various tapeworms can be found in the gastrointestinal tract of fish. Of particular concern is the segmented Asian tapeworm, *Bothriocephalus acheilognathi* (Yamaguti, 1934) which was introduced into the United States from Asian fish imports. This tapeworm's preferred infection site is the stomach and can exceed 50 cm, but most infections are made up of large numbers (more than 100) smaller worms. A review of procedures to evaluate fish for this species can be found in the AFS-FHS Blue Book (Mitchell 1994). Mitchell reported the tapeworm in cyprinids, poecilids, silurids, percides, and centrarchids. Since that publication, confirmed infections have been documented in other Cyprinidae and Poeciliidae: humpback chub, *Gila cypha*; bonytail chub, *Gila elegans*; and Yaqui topminnow, *Poeciliopsis occidentalis yaqui* (Landye and McCasland 1997). It has been found commonly in young of the year common carp, *Cyprinus carpio* and adult western mosquito fish, *Gambusia affinis*. The genus *Bothriocephalus* found parasitic in American freshwater fish was revised by Scholz (1997). Dove *et al.* (1997) reported the tapeworm in common carp, mosquito fish, *Gambusia holbrooki* and western carp gudgeon, *Hypseleotris klunzingeri* in Australia.

II. Sampling Methods

If a stereomicroscope is available, gastrointestinal tracts can be examined in the field after viral, bacterial, and other parasitic samples are obtained.   It is best to remove the gastrointestinal tract and place in physiological saline in a plastic bag. Transport samples on ice to the laboratory. The examination for tapeworms should be within 48 hours of euthanasia, but viable Asian tapeworms have been seen after five days of refrigeration. In heavily infected fish, a few Asian tapeworms will dislodge from the stomach during sample collection.

A.   **FISH LESS THAN ~ 15 cm** (with relatively thin walled internal tissue)
1.   Multiple gastrointestinal tracts (GI) can be laid on a piece of plexiglass or glass and then another piece of the same material can be laid on top of the tissues (Mitchell 1994).

2.   Using binding clips, the pieces of glass/plastic can be compressed and viewed utilizing a stereo microscope. If possible Asian tapeworms are found, they can be extracted from the preparation for further viewing under the stereomicroscope.

B.   **Fish larger than 15 cm**
1.   Remove the entire GI tract and place in saline solution contained in a large petri dish. In extremely large fish the tract can be subdivided into several dishes, but the stomach should be examined first.

2.   Stretch out the GI tract in the dish and cut longitudinally and examine the contents with a stereo microscope.

3.   Most Asian tapeworms will be found in the gastric section of the tract, but examination of the remaining sections and pyloric caecae might reveal segments of the tapeworm. Usually, this condition is found in fish species that are accidental hosts. Obviously with this type of examination other types of tapeworms, nematodes, and trematodes might be encountered.

C. QUICK FREEZING METHOD OF PRESERVATION
An alternate method for maintaining samples for a later examination is to utilize the quick – freezing method of preservation. Samples can stored at -20°C until examined. To "super cool" alcohol, place 95-100% Ethyl alcohol in 0.5 L to 1 L polyethylene bottles and place in a cooler containing dry ice. Alcohol can be also cooled by placing alcohol bottles in -80°C ultra cold freezer. Chill at least 2-3 hours before use.

1.   Fish less than approximately 15 cm:
   a.   Make a small incision in the abdominal cavity of the fish, be careful not to cut the GI tract.
   b.   Place the fish into a deep dissecting pan.
   c.   Pour super-cooled alcohol over the fish (wear protective gloves, safety glasses, and apron).   Alcohol can be reused by using a large funnel to pour alcohol from the pan back to bottle. Chill before using on another fish.
   d.   Store fish in a plastic bag on dry ice or in -20°C freezer.

2.   Fish larger than 15 cm:
   a.   Remove the GI tract from fish and place into a deep dissecting pan.
   b.   Pour super-cooled alcohol over the tract (wear protective gloves, safety glasses, and apron).
   c.   Store frozen GI tracts in a plastic bag on dry ice or in -20°C freezers.

III. Presumptive Diagnosis

Live Asian tapeworms are segmented and can be recognized by their arrowhead or pit viper shape of the scolex. This shape appears due to the posterior part of the scolex being wider than the first few proglottids of the tapeworm. If only dead or preserved samples of the worm are available, this shape can be hard to determine, but usually the pit viper shape is present in a few of the specimens.   The tapeworm, which is found in the stomach or upper gastrointestinal tract, has two bothria that are deep, elongated sucking grooves on the scolex and has no neck. The scolex is wider than the first few proglottids of the worm.

IV. Corroborative Diagnosis

Several other genera of tapeworms have similar scolices to Asian tapeworms (Mitchell 1994). After eliminating the nonsegmented tapeworms and those with suckers on the scolex, *Eubothrium*, *Bathybothrium,* and *Marsipometra* species remain. If a neck (a nonsegmented area which is at least twice as long than the first segment) is present, these latter three species can be

eliminated. Not all *Eubothrium* sp. have a neck, but they do have a dorsal or ventral furrow. *Bothriocephalus acheilognathi* can be distinguished from other bothriocephalids by the presence of incision-like openings in the bothria (Scholz 1977). Thus an Asian tapeworm, *B. acheilognathi* will have no neck, no furrow, no suckers, but will have bothria. An excellent review of the Asian tapeworm is presented in Hoffman (1999). For a complete understanding of the systematics of *Bothriocephalus* in American fishes, Scholz (1997) should be reviewed. When in doubt, samples of the tapeworm preserved in alcohol can be sent to a parasitologist for corroborative identification.

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