R. E. Clopton

This work supported in part by National Science Foundation grant NSF9705179.

Index

General Working Rules for Hotel Intestine

While it is my general philosophy that students learn the most when they are allowed to teach themselves and pursue their own interests, any laboratory requires a certain amount of order and structure if progress is to be made. Since our funding from NSF depends on our performance from year to year, these rules are particularly important. I don't ask much, but I when I do ask, I mean it.

Lab Rules

1. Break no federal, state, or local law. If you do, tell me so that I can do my best to look out for your interests, the lab's interests, and the reputation of Cedar Point. If (god forbid) the situation should arise, DO call me from jail 24 hrs, 7 days week: I will come bail you out.
2. Be careful when driving in the Sandhills. The roads can be treacherous. Other drivers are often your biggest problem.
3. If you have medical problems, let me know: we will get you to a doctor. If need be, we will get you to a hospital. Your health is important: we will find a way to get you the care you need.
4. When you leave CPBS, let someone know where you are going so that we know where to start looking for you if you don't return.
5. Keep track of your equipment and keep it in good working order. DO NOT borrow equipment from other scientists without asking first - it pisses them off. (Trust me on this one: I KNOW when someone else has been using my forceps or microscope).
6. Eat well and drink plenty of water. This is a harsh environment and it takes some time for your body to adjust.
7. Help each other out as needed: this is a team effort.
8. Plan on putting in 20-40 hours each week. More time is expected if you are not taking classes or as insect population spikes occur.
9. Keep your laboratory space in reasonable order. You don't have to be a clean-freak, but science doesn't happen very quickly in a pig-sty.
10. Take good notes. Don't depend on your memory: it quickly becomes over-crowded.
11. If you use the last of something, make up new or let me know so that all of the materials and solutions are there when people are ready to use them.
12. There will be a weekly laboratory meeting, the time to be set on June 7 after the general camp meeting. Don't miss meetings. We will eat breakfast together each day to make schedule and effort adjustments as needed.
13. We are funded by the National Science Foundation; thus, we are here on the tax-payers dollar. Make it worth their investment. Remember that all of the data, specimens, and notebooks you collect belong to the United States government and to my laboratory. You will be required to turn them in when you leave the project.

Lab Advice

1. Ask. If you don't understand, a protocol doesn't work for you, or if you don't know how to do something: ask someone. We'll teach you. Everyone has to learn sometime.
2. Interact with the other students and researchers on the Station. You can learn more from them than you will ever learn from me. The exposure is important: so make an effort to meet people, make yourself easy to get along with, talk to people, eat dinner with someone new a couple of times each week.
3. Take the time to go to the beach or to town. Have fun . . . we are here to work, but science is a way of life, not a job. Learn to take advantage of the good things that come as a result of being a field biologist.
4. If something goes wrong, if something goes right, if you have a problem, if you need to talk to someone: come see me. You wouldn't be here if I didn't like you already. If you can't talk to me, talk to Mary: she's good people.
5. Whomever is doing the most important/demanding task chooses the music. Don't abuse each other. (Bob is likely to go stark raving mad if you make him listen to country music all day long.)

6. In my own lab space, I'm always doing the most demanding task, so I always choose the tunes.
7. If you can think of a better way to do something, suggest it: we are all learning all the time.
8. Somewhere, sometime, somehow, something will go wrong. When something goes wrong in our lab, our main concern is "What do we do to get ourselves back on track?" Our success depends upon our ability to get things done no matter what problems we encounter. You will make mistakes. I will make mistakes. Let the rest of us know so that we can get the problems fixed. We do not care "whose fault it is": guilt is for god and small children. Blame never paid the piper. Do learn from your mistakes, though: Good judgment comes from experience: experience comes from bad judgment.
9. Remember: you represent my lab, Peru State College, and the National Science Foundation. Your work and personal carriage contribute to reputations all along that spectrum. I have a lot of faith in each of you. Make us all proud and famous.

CHECKLIST FOR TAXA RECOVERED

The following specimens/data MUST be collected for each gregarine taxon we work on this summer. Use one checklist for each taxon. I have a set of master checklists and you should update my lists once each week so that I can assign extra help where it is needed.

Gregarine Taxon:_____

Host Taxon:_____

Host Voucher Number: _____

Host Specimens

1. **Pinned or Vialed Museum/ Voucher Specimens**
   We need a minimum of five per host species. seven to ten is much better.
   Voucher specimens: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
2. **Genetic Samples**
   At least one vial of host tissue, preferably muscle tissue, fixed in 95% EtOH
   Genetic specimens: ① ② ③
3. **Host intestines fixed for histology**
   We need a minimum of five per host species. Fix and store in Bouin's
   Voucher specimens: ① ② ③ ④ ⑤ ⑥ ⑦

Gregarine Permanent Slide Mounts

1. **Semichon's Carmine stained smears**
   We need a minimum of 10 per host species. More is better.
   Required minimum: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
2. **Hematoxylin & Eosin stained smears**
   We need a minimum of 10 per host species. More is better.
   Required minimum: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
3. **Semichon's Carmine stained gamonts**
   We need a minimum of 10 per host species. More is better. These may be in # 1, above, or you may fix them in vials with AFA for staining in the fall
   Required minimum: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
   Fixed in vials: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
4. **Hematoxylin & Eosin stained gamonts**
   We need a minimum of 10 per host species. More is better. These may be in # 2, above, or you may fix them in vials with Bouin's for staining in the fall
   Required minimum: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
   Fixed in vials: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩

Gametocysts/Oocysts

1. **Dry, in water, or fixed**
   We need a minimum of 10 per host species.
   Required minimum: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩
2. **Gametocysts for developmental histology**
   Fixed samples, at least one each day, until mature. 5 mature gametocysts in Bouin's.
   Samples fixed: ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩

Photographs/Video

1. At least 30 minutes of video _____(RRK will sign off)
2. Photographs for publications _____(REC will sign off)

Notes

PROJECT SUMMARY

RUI Microbiological Survey & Inventory: Eugregarinida (Protista: Apicomplexa) parasitizing selected mandibulate arthropods of the Nebraska Sandhills.

This project supports integrated faculty and undergraduate research through a study of biodiversity among gregarine (Protista: Apicomplexa) parasites of selected mandibulate arthropods (insects, millipedes, centipedes, and macrocrustaceans). The survey is significant because it focuses on the gregarines, numerically prevalent and taxonomically diverse but scientifically enigmatic group of eukaryotic microbes parasitizing the intestines of arthropods, an invertebrate group of ecological, agricultural, and medical importance. The project will sample 12 habitat types in the Sandhills of western Nebraska, survey an estimated 125 arthropod species representing 46 families, and recover an estimated 148-200 gregarine species from 12 families. The survey targets a microbiological group that is parasitic (the most common trophic habit on earth) in mandibulate arthropods (numerically and taxonomically more prevalent than any other known group of organisms on earth). Thus the project focuses on an understudied group with profound scientific implications: the most common parasites of earth's most common animals. The project will produce host and parasite specimen collections, preserved tissue specimens for future biochemical and genetic analysis, and taxonomic descriptions and identification documents in both printed and electronic forms. Electronic descriptions, digital images, hypertext dichotomous keys, and a complete project database will be made available through the project's WWW site at Peru State College (http://www.peru.edu/gregarina). Over a three year period, the project will provide research opportunities, field research experience, and expert systematic training for up to 12 undergraduate students.

No comparable survey has been conducted for a microbiological group in North America and no museum collection of New World gregarines currently exists. Thus, this project will provide the seminal collections, databases, and survey methods for future taxonomic and survey work on the Nearctic gregarines. The descriptions, databases, and specimen collection produced by this project will define the normal scope and procedure for future studies of gregarine systematics, ecology, and evolution as well as community structure and population regulatory studies of their arthropod hosts.

(Excerpted from NSF Grant Proposal 9705179)

FIXATION AND STAINING PROTOCOLS

Field Fixation

General Procedures and Notes

Five different fixation reagents are presented below: AFA (Alcohol-Formalin-Acetic Acid), Bouin's Fluid (Aqueous), Brasil & Duboscq's Fluid (Alcoholic Bouin's), Hollande's Fluid and Schaudinn's Fluid. All are excellent fixatives. Schaudinn's is best for iron hematoxylin staining procedures and preserves nuclear features with minimal artifact. AFA is best for acid carmine staining, but it can produce some small amount of nuclear distortion. On the other hand, AFA is the least toxic and poses the fewest disposal problems. Hollande's Fluid is an excellent protist fixative and is best for protargol staining. It's application for other stain protocols has not been fully investigated. In general, I stick to AFA or Bouin's. AFA in the field, Bouin's for material that will be sectioned at a later date.

Wet smears for microscope slides: All fixatives are used in a similar manner. Prepare a thin smear of host intestine (with gregarines) on a clean coverslip and drop them face down into a petri-dish of fixative. The slips should float on the surface with no air bubbles. This holds the specimens against the glass until they are affixed through fixation of gregarine and host protiens. In each case, allow the slips to fix for at least 5 minutes, unless the stain protocol specifies a different fixation time. Rinse excess AFA or Brasil & Duboscq's Fluid from specimens with 70% EtOH. Rinse AFA fixed specimen's for 5 minutes; Bouin's fixed specimens until all traces of yellow are removed. After fixation in Schaudinn's, Bouin's, or Hollande's Fluid specimens must be dehydrated through a graded alcohol series: 30% (optional) 50%, 70% (3 minutes each). Remove excess mercury from Schaudinn-fixed specimens with two washes in Lugol's Iodine working solution (2 washes, 5 minutes each). Specimens fixed in Bouin's, or Hollande's Fluid should be washed in 70% EtOH until all traces of fixative color are removed, or at least until the fixatives no longer discolor the EtOH. After fixation gregarines may be stained and processed as permanent mounts. A similar technique may be used with microscope slides rather than coverslips EXCEPT that slides won't float. They are simply immersed in the fixative.

Entire fixation for gamonts or host intestines: Bouin's, Brasil & Duboscq's Fluid, and AFA are all suitable fixatives for this technique (in that order of preference). All fixatives are used in a similar manner. Specimens or host intestines are immersed in a vial of fixative (the volume of fixative should be at least 10 times the volume of tissue). Fixation should proceed for at least 30 minutes, but specimens can be stored in fixative for several years without damage. Rinse excess fixative from specimens with 70% EtOH. Rinse AFA fixed specimen's for 5-30 minutes, depending upon size (at least 3 changes of 3 minutes each); Bouin's or Brasil & Duboscq's fixed specimens should be rinsed until all traces of yellow are removed. (A few drops of lithium carbonate can be used to increase the solubility of the picric acid and speed up the rinse, but this technique often affects the staining properties of the tissue.) *Bouin's fixed specimens should be dehydrated through a graded alcohol series: 30% (optional) 50%, 70% (3 minutes each).* After fixation gregarines may be stored in alcohol or stained and processed as permanent mounts.

Intestines should be straightened by prefixation. Flood one half of a slide with fixative and drag the intestine through the fixative. The intestine will straighten as it is dragged across the glass and through the fix. Postfix in vial as usual.

Reagents

AFA (Alcohol Formalin-Acetic Acid Fixative)

Formalin (40% aqueous formaldehyde)	60 ml
95% Ethanol	500 ml
Glacial Acetic Acid	40 ml
Distilled water	400 ml

Mix all reagents and use without dilution. AFA keeps well (up to one year). The formation of a cloudy precipitate or the distinct smell of formic acid indicates that the solution should be discarded soon and replaced with a fresh stock.

Brasil & Duboscq's Fluid (Alcoholic Bouin's Fixative)

Picric acid (saturated spirits)	75 ml
Formalin (40% aqueous formaldehyde)	25 ml
Glacial Acetic Acid	5 ml

Saturated spirits of picric acid are prepared by adding picric acid crystals to 70% ethanol until no more will dissolve. Some precipitate should remain as evidence of saturation. There is some explosive hazard with picric acid. This hazard is reduced by storing the acid under 70% EtOH, also providing a ready saturated solution. Stores well for several months.

Schaudinn's Fluid

Mercuric Chloride (sat. aq.)	66 ml
95% Ethanol	33 ml
Glacial Acetic Acid	5 ml

Prepare a saturated aqueous solution of Mercury Bichloride with distilled water and store in a glass bottle in a cool, dark place. Some precipitate should remain as evidence of saturation. Schaudinn'd Fluid does not stor for more than a few days; however, the mercuric chloride and ethanol may be mixed as a stock solution that will keep for several months. Add 5 ml of acetic acid to 100 ml of stock solution to prepare a working solution for use within 48 hours.

Lugol's Iodine

Iodine (resublimed)	2 gm
Potassium Iodide	3 gm
95% ethanol	100 ml

Store in a cool, dark place. Keeps forever. Used to remove mercury from fixed material to avoid precipitation in specimen. Add to 70% ethanol to form a straw-colored working solution. (Extra Lugol's in the working solution doesn't hurt.) Add more Lugol's stock to the working solution and the color disappears.

Bouin's Fluid

(Aqueous Bouin's Fluid Fixative)

Picric Acid, saturated aqueous	75 ml
Formalin	25 ml
Acetic Acid, glacial	5 ml

This mixture keeps well, but some persist in making the solution up immediately before use.

Hollande's Fluid

Picric Acid, wet crystal	4 g
Cupric acetate	2.5 g
Distilled water	100 ml
Formalin	10 ml
Acetic Acid, glacial	1.5 - 5 ml

Dissolve cupric acetate in water, followed by the picric acid. Add the formalin and acetic acid after solution. After fixation, transfer by graded series to 70% EtOH and continue repeated, gentle fluid changes until picric acid and cupric acetate are removed. Stores well.

Field Procedure for Semichon's Acid Carmine Staining

Procedure

Primary Fixation. Fix specimens or tissues and affix them to slides or coverslips. AFA is the preferred fixative. Note that Semichon's is itself a fixative and may be used to simultaneously fix and stain wet smears.

Fixative Rinse. Rinse out excess fixative in 70% Ethanol for 3 minutes

Stain. Stain in Semichon's Acid Carmine for 2 minutes. Large gregarines may need to stain for up to 5 minutes.

Stain Rinse. Rinse out excess stain in 70% Ethanol for 3 minutes

Primary Destain. Destain in 1% Acid Alcohol. Time varies from 5 seconds to 5 minutes depending on species and size. Larger species may be destained in 5% Acid Alcohol.

Secondary Destain. Remove excess background stain by dipping in 5% Acid Alcohol (1-2 seconds).

Neutralization. Rinse in Alkaline Alcohol for 3 minutes to neutralize destain acid.

Dehydration. Dehydrate in a graded ethanol series: 95% (5 minutes); 100% (3 changes, 3-5 minutes each).

Clearing. Clear in a graded xylene or toluene series: 50% xylene/50% Absolute alcohol (5 minutes); 100% xylene (3 changes, 3-5 minutes each).

Mounting. Mount in Damar Balsam (Damar Xylene).

Reagents

Semichon's Acid Carmine Stain

Glacial Acetic Acid	250 ml
Distilled water	250 ml
Carmine Alum Lake	per vid (ca. 5 gm)
70% Ethanol	ca. 500 ml

Combine water and acid in a flask and add carmine until no more will go into solution. Bring contents of the flak to ca. 95 C over a boiling water bath, but DO NOT BOIL. Cool and filter. (A double drip coffee filter works well.) Measure stain volume and add an equal volume of 70% EtOH. Store away from excessive heat and light. Keeps for years. Discard stock stain when stained cytoplasm begins to appear muddy.

1% Acid Alcohol Destain

Hydrochloric Acid	1 ml
70% Ethanol	100 ml

Primary destain.

5% Acid Alcohol Destain

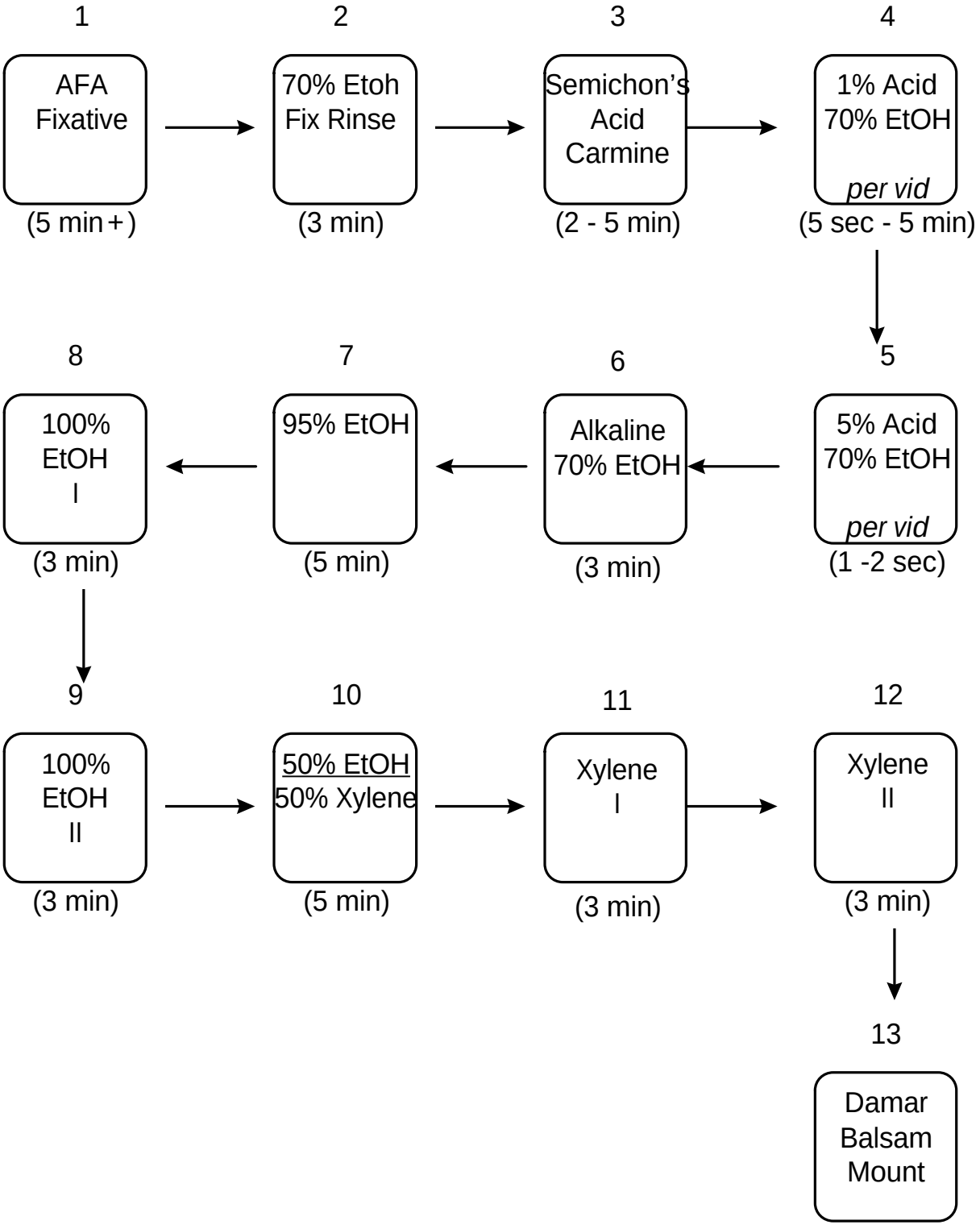
Hydrochloric Acid	5 ml
70% Ethanol	100 ml

Secondary destain. Also used for very large gregarines.

Alkaline Alcohol

A saturated solution of Sodium Bicarbonate in 70% Ethanol. Dilute 1:1 with 70% EtOH before use.

Flow Chart for Preparation of Permanent Specimens using Semichon's Acetocarmine



Field Procedure for Harris Hematoxylin & Eosin Staining

Procedure

Primary Fixation. Fix specimens or tissues and affix them to slides or coverslips. (AFA or Bouin's Fluid are preferable fixatives.) Rinse out excess fixative as appropriate (see *Fixation Protocols*).

Hydration. Hydrate by graded alcohol series 50%, 3 min; Water, 5 - 7 min.

Stain. Stain in Harris Hematoxylin for 15 minutes.

Stain Rinse. Remove excess stain by rinsing in running tap water for 3 min.

Destain and Differentiate. *This is the critical step that determines the quality of the final preparation.* Destain in 1% acid alcohol until the cytoplasm has only a faint stain but the sharp nuclear stain still remains.

Destain Wash. Briefly rinse in tap water to remove excess acid and halt destain.

Hematoxylin Bluing. Dip in ammonia water or lithium carbonate water until specimens are bright blue (3-5 dips).

Final Rinse. Rinse in running tap water for 10 - 20 min.

Eosin Staining

Alcohol Option

Eosin Counterstain. Stain with eosin for 15 sec - 2 min. Agitate gently before allowing specimens to stain in order to produce an even stain.

Dehydration. Dehydrate in a graded ethanol series: 50%, 70%, 95% (3 min each); 100% (3 changes, 3 min each). This step also removes excess eosin - timing in 95% EtOH depends upon the specimen.

Clearing. Clear in a graded xylene or toluene series: 50% xylene/50% Absolute alcohol (3 min); 100% xylene (3 changes, 3 min each).

Mounting. Mount in Damar Balsam (Damar Xylene).

Xylene Option

Dehydration. Dehydrate in a graded ethanol series: 50%, 70%, 95% (3 min each); 100% (3 changes, 3 min each).

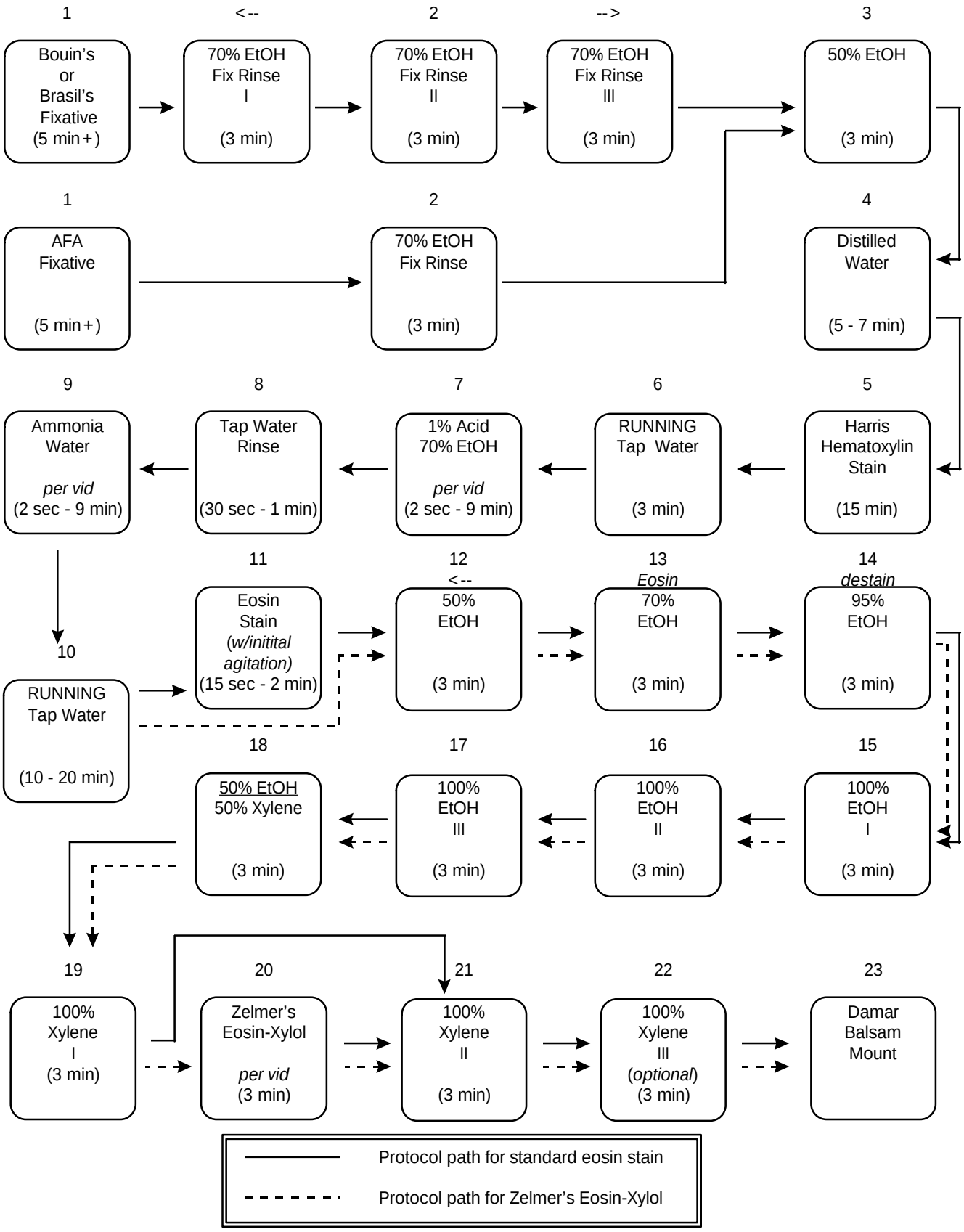
Clearing and Counterstaining. Clear in a graded xylene or toluene series: 50% xylene/50% Absolute alcohol (3 min); 100% xylene (3 changes, 3 min each). Use Zelmer's Eosin-Xylol in place of the first change of pure xylene.

Mounting. Mount in Damar Balsam (Damar Xylene).

Notes:

1. *This is an excellent staining procedure that produces satisfactory results regardless of the skill level of the technician as long as destaining is sufficient.*
2. *In many cases (especially sectioned material), the 50% EtOH rinses can be dropped.*
3. *For gregarines at least, Zelmer's Eosin-Xylol provides the best, most predictable stain. Use this method rather than the standard eosin stain before dehydration.*

Flow Chart for Preparation of Permanent Specimens using Harris Hematoxylin & Eosin



Reagents

Harris' Hematoxylin

Hematoxylin Crystals	2.5 g
100% EtOH	25 ml
Ammonium or Potassium Alum	50 g
Distilled Water	500 ml
Mercuric oxide (red)	1.25 g

❶ Dissolve the hematoxylin in the alcohol and ❷ the alum in the water with heat. ❸ Remove from heat and mix the two solutions. ❹ Bring to a boil as rapidly as possible (limiting this heat to *less* than 1 min and stir often). ❺ Remove from heat and add the mercuric oxide slowly. ❻ Reheat to a simmer until it becomes dark purple. ❼ Remove from heat immediately and plunge the vessel into a basin of cold water until cool.

The stain is ready to use as soon as it is cool. Add 2 - 4 ml of glacial acetic acid per 100 ml solution to increase precision as a nuclear stain. Filter before use.

1% Stock Alcoholic Eosin

Eosin Y, water soluble	1 g
Distilled Water	20 ml
Dissolve and add:	
Alcohol, 95%	80 ml

Dilute the alcoholic stock 1:3 with 80% EtOH to produce a Working Eosin Solution just before use. Add 0.5 ml glacial acetic acid to each 100 ml of stain and stir before use.

1% Acid Alcohol

Alcohol, 70%	500 ml
Hydrochloric Acid, concentrated	5 ml

Ammonia Water

Tap water	1000 ml
Ammonium hydroxide, 28%	2 - 3 ml

Saturated Lithium Carbonate

Lithium Carbonate	1 g
Distilled water	100 ml

Zelmer's Eosin-Xylol

Eosin Y, water soluble	1 g
Distilled water	100 ml
Hydrochloric Acid, concentrated	2.5 ml
Xylene	100 ml

Dissolve the eosin in the water. Add the hydrochloric acid and mix well. Allow the precipitate to settle overnight and decant the clear supernatant. Add Xylol to the precipitate and shake gently or stir with a glass rod; avoid an emulsion. During this process the free acid of eosin is dissolved in the xylene layer. Allow to form an organic - inorganic interface and decant the nearly colorless xylol extract which stains organic tissues on contact.
(Thanks to Derek Zelmer, Wake Forest University, for this trick.)

Field Procedure for Heidenhain's Iron Hematoxylin Staining

Procedure

Primary Fixation. Fix specimens or tissues and affix them to slides or coverslips. (AFA and Bouin's: suitable. Schaudinn's Fluid: preferable.) Rinse out excess fixative as appropriate (see *Fixation Protocols*).

Hydration. Hydrate by graded alcohol series (50%, 30%, 15%; 3 minutes each) and rinse 3 min in distilled water.

Mordant. Mordant in 2% aqueous iron alum for 45 minutes. Increasing the time to 2-3 hours will sharpen the staining or centromeres and spindle fibers, but no additional resolution is gained after 3 hours. Specimens may be allowed to mordant for up to 12 hours. *An iron alum solution may be re-used for mordanting, but never mordant in an iron alum solution that has been used to destain.*

Mordant Rinse. Wash out excess mordant be rinsing specimens in distilled water. (Sections, 15-30 seconds; smears, 1 minute) Excess washing will lead to slow and inferior staining.

Stain. Stain in 0.5% Hematoxylin stain solution for 2-3 hours at room temperature. If the entire specimen is black, reduce staining time or samples of variable thickness will destain at very different rates.

Stain Rinse. Remove excess stain by rinsing in distilled water for 5-10 minutes.

Destain and Differentiate. *This is the critical step that determines the quality of the final preparation.* Destain in 1-2% solution of iron alum or a 1-2% solution of ferric chloride (my preference) until clouds of stain are no longer given off. Continue until the cytoplasm has only a faint stain but the sharp nuclear stain still remains. If specimens become too lightly stained, let them destain completely and return to step 4. End destaining by transfer to distilled water. Aqueous picric acid (saturated) can also be used to destain. It works more slowly and produces a better stain, but it takes time to learn to judge the progress of the destain. There is also little remedy for removing too much stain.

Destain Wash. Rinse the samples for several minutes in tap water. Most tap water is alkaline enough to give the stain a blue-black tint. This color shift can be used to judge the extent of destaining. If additional destain is desired, return to destaining solution. When destaining is satisfactory wash out the destain in distilled water for ca. 30 min (4 water changes, 7 minutes each).

Hematoxylin Bluing. Blue the preparation by washing for 5 minutes in saturated lithium carbonate solution. Some tap water is alkaline enough to blue the stain and make this step redundant.

Dehydration. Dehydrate in a graded ethanol series: 25%, 50%, 70%,95% (3 minutes each); 100% (3 changes, 3-5 minutes each).

Clearing. Clear in a graded xylene or toluene series: 50% xylene/50% Absolute alcohol (5 minutes); 100% xylene (3 changes, 3-5 minutes each).

Mounting. Mount in Damar Balsam (Damar Xylene).

Notes: This is an excellent staining procedure, but it does require a practiced hand. Harris Hematoxylin and Eosin is much more predictable and should be used by inexperienced workers.

Reagents

0.5% Hematoxylin Working Stain

Alcoholic Hematoxylin Stock	5 ml
Distilled Water	95 ml
Potassium Permangante Oxidizer	per vid
Lithium carbonate (sat. aq.)	3 drops

Add Potassium Permanganate Oxidizer dropwise with constant stirring until the staining solution changes from a brown to the color of a deep port wine. (Oxidation is not required when a fully ripen stock is used.) Add the lithium carbonate after oxidation. When the stain begins to produce a muddy cytoplasmic stain, it has become over-ripe and must be replaced (1-2 days). Strength may be altered, but the 0.5% working solution seems to work best.

Potassium Permanganate Oxidizer

Potassium Permanganate	0.2 gm
Distilled water	100 ml

To 1 ml of the above solution add ddH_20 to make 100 ml. Stable for 24 Hours. Used to rapidly ripen the working hematoxylin staining solution for immediate use when no fully ripened stock solution is available.

2% Iron Alum Solution

4% Iron Alum Stock Solution	50 ml
Distilled Water	50 ml

2% Ferric Chloride Solution

4% Ferric Chloride Stock Solution	50 ml
Distilled Water	50 ml

Stock Solutions

4% Iron Alum Stock

Ferric ammonium sulfate (iron alum)	4 gm
Distilled water	100 ml

Use violet crystals of iron alum. Yellow crystals have been altered by light. Filter the solution before use. Strengths between 1.5% and 4% may be used, but a 2% solution for mordanting and a stronger (4%) solution for destaining seem to work well.

Alcoholic Hematoxylin stock solution

Hematoxylin, light crystals	10 gm
Absolute Ethanol	100 ml

Will keep for several years if stored in a cool, dark place. The stock is not actually ripe when made, but gives best results after 4-6 months. When no ripe stock is available, the working solution can be artificially ripened by adding 0.02% Potassium permanganate drop-wise to achieve a deep port-wine red color.

Lithium Carbonate Solution

Saturated aqueous solution.

4% Ferric Chloride Stock

Ferric Chloride	4 gm
Distilled water	100 ml

Field Procedure for Protargol Staining

When silver albumose is activated by metallic copper silver is selectively accumulated by certain structures, particularly the pellicular fibrils and the epimerite. This silver can be reduced by hydroquinone and then replaced or toned by gold to improve differentiation.

Procedure

Primary Fixation. Dissect intestines of infected hosts into physiological saline and prepare wet smears. Float-fix smears in Bouin's (Aqueous) or Hollande's fluid for 30-60 min (after initial fixation, complete fixation may be completed in a Columbia jar.

Rinse and Dehydrate. Rinse samples in water for 3 minutes and transfer by graded alcohol series (15%, 30%, 50%; 3 minutes each) to 70% EtOH. Change the 70% EtOH until it is no longer conspicuously discolored by picric acid or cupric acetate. Allow smears to harden in 70% EtOH for 30 - 45 min. Smears not to be impregnated immediately may be stored in 70% EtOH.

Potassium permanganate - Oxalic acid bleaching. Pass smears down a graded EtOH series (50%, 30%, 15%; 3 minutes each) to distilled water. Transfer smears to 0.25% aqueous Potassium permanganate for 5 - 10 min. Triple rinse in distilled water. The smears will now appear dark brown. Bleach the smears in 2.0 - 2.5% aqueous oxalic acid for 5 - 10 min to bleach out the brown color. Triple rinse in distilled water, 3 min each. (This step is variable across samples. Some ciliates require only 1-2 minutes oxidation and 3 min bleaching. Other workers have completed this step with dilute commercial bleach. In any case, this step will make the albumen layer of a poor preparation detatch.)

Protargol Impregnation. Place 0.5 g of bright copper (no. 18 wire coil or copper granules) in the bottom of a Columbia jar. If the copper is oxidized, flame and douse in 95% EtOH before use. Dissolve Protargol just before use by dusting on the surface of distilled water in a beaker. A 1.5% solution is required (0.15 g per 10 ml Columbia jar -- just a pinch will do). Move the smears to the activated Protargol for 18 - 24 hr at room temperature. *NB*: Carefully dust the protargol on the distilled water: protargol readily forms clumps that will not go into solution.

Reduction. Remove the smears from the protargol solution, rinse them briefly in distilled water (optional), and reduce them in Hydroquinone-Sodium sulfite developer. The smears will darken over 5 - 10 minutes, but it is not likely that they will be over-reduced, but watch closely. Remove the smears as soon as the albumen layer turns yellowish.

Rinse. Double-dip rinse in 3 changes of distilled water. Smears may now be toned with gold chloride, or photofixed. Gold toning is not required but gives a finer preparation.

Gold Toning. Transfer smears to 1% Gold Chloride for approximately 5 min or until the smears turn gray. Dip-rinse toned smears twice in distilled water and transfer to 2% aqueous oxalic acid for 5 - 10 min. Smears should become purplish to reddish-blue. Double-dip rinse in 3 changes of distilled water.

Photo Fixation. Transfer rinsed smears to a 5 - 10% aqueous solution of sodium thiosulfate. Photo fix for 1-3 minutes and transfer to distilled water rinse, 2-3 changes of 3-5 min each. Do not wash too long as the albumen layer is fragile at this point and likely to detatch from the coverslip.

Dehydration. Gradually dehydrate smears (30, 50, 70% EtOH; 3 min each, 95, 3 X 100% EtOH; 5 min each).

Clear and Mount. Clear in a graded xylene or toluene series (50%, 100%, 100%; 5-10 minutes each) and mount in Damar Balsam.

Reagents

Activated 1.5% Protargol (Strong Silver Protein)

Place 0.5 g of bright copper (no. 18 wire coil or copper granules) in the bottom of a Columbia jar. If the copper is oxidized, flame and douse in 95% EtOH before use. Dissolve Protargol just before use by dusting on the surface of distilled water in a beaker. Carefully dust the protargol on the distilled water but do not stir: protargol readily forms clumps that will not go into solution (rather like corn starch in warm water). A 1.5% solution is required (0.15 g per 10 ml Columbia jar -- just a pinch will do).

0.25% aqueous Potassium permanganate

0.25 g $KMnO_4$, ddH_20 to 100 ml. Stable for 24 hr.

2.0 - 2.5% aqueous oxalic acid

1 g Oxalic acid , H_20 to 50 ml. Stable for 24 hr.

Stock Solutions

0.5 g of bright copper (no. 18 wire coil, granules, or turnings, light)
1% Gold Chloride. (Commercial EM Grade).

Hydroquinone-Sodium sulfite developer

Sodium sulfite, anhydrous (Na_2SO_3)	5 g
Hydroquinone ($C_6H_6O_2$)	1 g
ddH_20	100 ml.

Dissolve the sodium sulfite completely before adding the hydroquinone. Stable for 7-10 days. May be used undilute or diluted 1:2--1:20 to avoid rapid development or one-sided development. Finer preparations are obtained using a conditioned developer made from one part fresh developing solution and one part ripe “brown” developer. It is best to prepare new devloping solution each week and mix it with retain ripened developer. Oxidation of the developer proceeds from clear through yellow to light brown (most effective) and later to dark brown (ineffective as a developer but suitable for ripening new developer.)

Photofixer

5 - 10% aqueous solution of sodium thiosulfate. 17.5 g sodium thiosulfate ($Na_2S_2O_3$ + 5 H_20), ddH_20 to 250 ml. Stable for years.

Permanent Mounting Media for Gregarines

Evaluation of Available Media

Three permanent mounting media are readily available: Canada Balsam, Piccolyte (e.g. Permount®), and Damar Balsam. Canada balsam has proven unsatisfactory for most gregarine work because it tends to collapse and distort specimens, even when used in a very dilute solution. Permount is somewhat more satisfactory, although it still produces some artifacts and the long term stability of this product, in my own mind, has not been demonstrated. Damar balsam is by far the superior mountant for gregarine work: it produces artifact-free mounts, does not readily bubble, has archival properties equal to those of Canada Balsam, is of an almost neutral pH, and it is relatively inexpensive. Unfortunately, no ready-to-use preparation of Damar Balsam is currently available although the materials are easily obtained. Preparation of Damar Balsam is outlined below.

Reagents

Damar Balsam

Damar Gum (lumps)	25 gm
Xylene	ca. 50 ml

Damar Gum is available as a raw resin collected from the damar pine along the Pacific Rim. Use only light color damar gum. (Dewaxed gum is heat damaged and unsuitable.) Choose lumps that a relatively free of dirt and debris. Spectrum Chemical Corp. is an excellent supplier. Dark colored lumps have been exposed to high heat and should be rejected. Cautiously heat the gum damar over a low heat until it melts. Add to the melted mass approximately 6-8 times it's own volume of xylene and stir to form a uniform solution. Filter through a double layer of drip coffee filters. Any fine precipitate that forms overnight should be removed by refiltering the mountant. Damar Balsam will thicken if allowed to evaporate--adjust consistency to your tastes. Specimens cleared in xylene can be mounted directly in Damar Balsam and will harden overnight on a 40 C slide warmer. The mountant, of course, keeps indefinitely.

I have had considerable luck in making Damar Balsam with less xylene than is prescribed above. Thus the mountant can be used the next day without waiting for it to thicken. However, heat must be very carefully controlled and the damar must be filtered when hot for this method to bring success.

Field Fixation for Scanning Electron Microscopy

Procedure

Primary Fixation. Dissect intestines of infected hosts into fixative (2.5% glutaraldehyde in 0.1M Na-cacodylate buffer - 0.1M sucrose pH 7.4). Free attached trophozoites and agitate to rinse gregarines. Placed in fresh fixative for 1 to 3 h. Oocysts may be placed directly into fresh fixative for 24-48 hr.

Rinse and Store. Rinse samples for 15 min in each of three 0.1M Na-cacodylate buffer without sucrose. Store samples under refrigeration in fresh 0.1M Na-cacodylate buffer without sucrose.

Secondary Fixation. Post-fix in 1% Osmium tetroxide prior to processing in laboratory. This is not a part of the field preservation process.

Reagents

0.1M Na-cacodylate buffer - 0.1M sucrose pH 7.4

25 ml 0.4 M Na-cacodylate stock, 4 ml 0.2 M HCl, ddH_20 to 100 ml. Adjust pH to 7.4 with HCl.

0.1M Na-cacodylate buffer without sucrose pH 7.4

25 ml 0.4 M Na-cacodylate stock, 25 ml 0.4 M Sucrose stock, 4 ml 0.2 M HCl, ddH_20 to 100 ml. Adjust pH to 7.4 with HCl.

2.5% glutaraldehyde in 0.1M Na-cacodylate buffer - 0.1M sucrose pH 7.4

1 ml 25% stock Gluteraldehyde, 0.1M Na-cacodylate buffer - 0.1M sucrose to 10 ml.
2.5 ml 25% stock Gluteraldehyde, 0.1M Na-cacodylate buffer - 0.1M sucrose to 25 ml.
5 ml 25% stock Gluteraldehyde, 0.1M Na-cacodylate buffer - 0.1M sucrose to 50 ml.
10 ml 25% stock Gluteraldehyde, 0.1M Na-cacodylate buffer - 0.1M sucrose to 100 ml.

Stock Solutions

25% Gluteraldehyde.

Commercial EM Grade.

0.4 M Na-cacodylate stock.

21.4 g Na-cacodylate in 250 ml dd H_20.

0.4 M Sucrose stock.

34.32 g Sucrose, ddH_20 to 250 ml.

0.2 M HCl stock.

20.8 ml 12 M HCL in , ddH_20 to 250 ml.

Miscellaneous Protocols

Fixation and Storage of Genetic Samples

Decontamination Wash. Rinse gregarines or host tissues in saline to remove extraneous proteins. Rinse at least three times with complete fluid replacement.

Fixation and storage. Fix and store in 95% EtOH in the dark.

Fixation and Storage of Insect Specimens

Hard-bodied Insects. Kill with ethyl-acetate or by freezing. Pin through the throax, placing the pin to the right of the midline (Fig 1). Adjust height with a pinning block and arrange appendages (Figs 2-3). Place temporary label on pin and allow to dry. Add permanent labels. Store in fumigated case. Odonates should be killed, placed in paper triangles (Fig. 4), soaked in acetone for 30 min-1 hr, allowed to dry, and placed in an odonate envelope with a 3 x 5 data card.

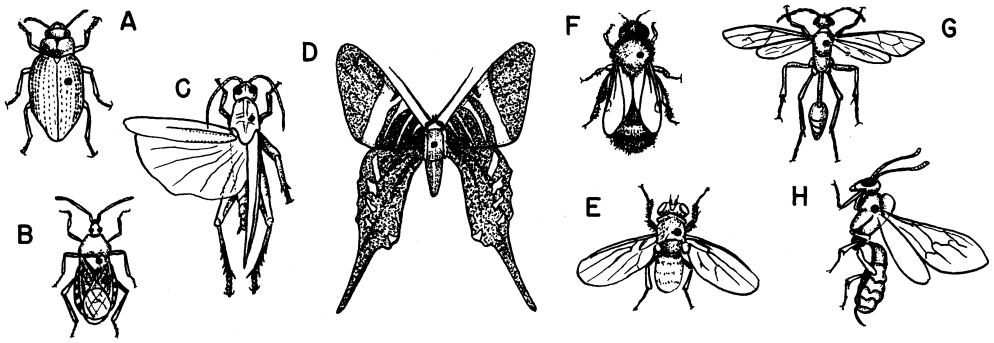


Figure 1. Pin placement for various insects.

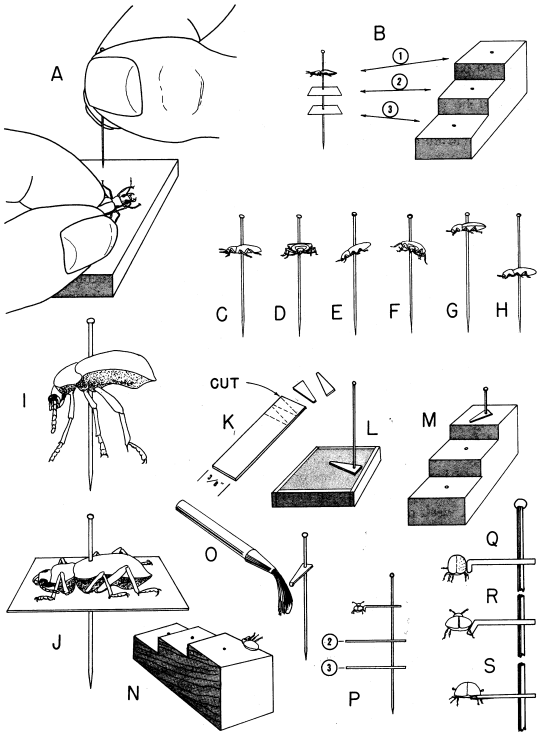


Figure 2. Pinning Techniques. A. Starting the pin with a pinning base. B. Use of a pinning block: (1) Specimen Level, (2) Locality label, (3) Collector's label. C-D. Correct method of pinning. E-H. Incorrect method of pinning. I-J. Propping dropping legs. K. Cutting points. L-M. Mounting point. N. Positioning specimen. O. Applying cement or glue. P. Finished specimens with label. Q-S. Bending point to match specimen.

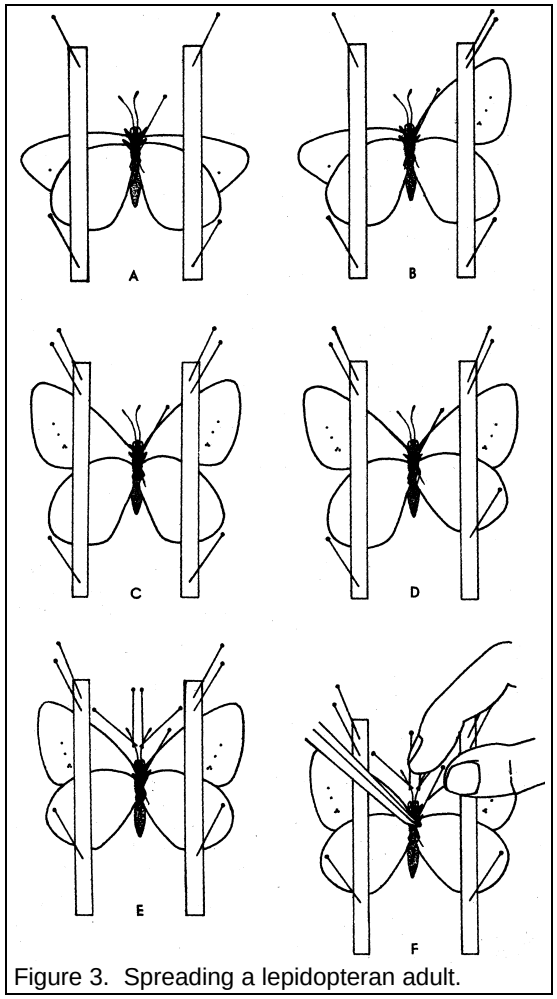


Figure 3. Spreading a lepidopteran adult.

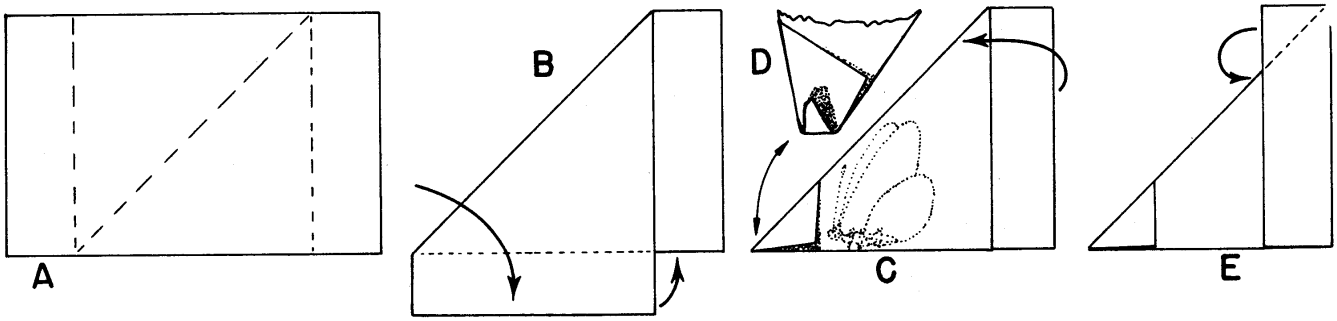


Figure 4. Folding a paper triangle for field storage and acetone processing of lepidopterans and odonates.

Soft-bodied Insects. Fix and store in 70% EtOH. Large specimens should be fixed overnight in AFA and then moved to 70% EtOH for storage. Place label in vial with specimen. (Fig 5).

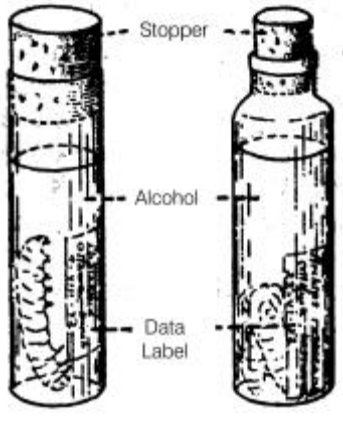


Figure 5. Alcohol or fixative storage and labeling of soft-bodied insects.

FIELD NOTEBOOKS AND DATA COLLECTION

Data collection has become increasingly problematic in science. Simply put, your data must not only be correct, it must be "legally defensible". You have been assigned notebooks to keep your data. Your notes are the property of the laboratory and you will be required to turn in your data and books when you leave the project. Please use the following format:

First Page

Save the first page to record your personal shorthand. Define all of your abbreviations on the first page. You may also want to keep locality coordinate data here for quick reference. You will want to keep your cyst storage data on the last few pages of your notebook.

The remainder of your notebook should be used in page pairs: a single line of data will extend from the left margin of the left page to the right margin of the right page. Each page is divided into six columns. Label and use the columns as follows:

Left Page

Host I.D. #: Host identification number. Each host animal you process is identified with it's own number. Our numbering system combines your initials and the year with a serial number. So, REC980001 = Richard E Clopton, 1998, first host of the season. RRK970081 = Robert R. Kula, 1997, 81st host of the season. This number is attached to EVERYTHING from this host. It goes on the slides, in the vials, on the specimens - this is the only thing that links collected specimens to data so, IT IS VITAL THAT YOU KEEP ORGANIZE YOUR DATA TO MATCH OUR SYSTEM.

Host Morph.: Host morphotype. You won't always *know* the species of your hosts right away - you'll have to work on and later confirm their identifications. But, you can give a group of organisms that you *think* are the same species your own code name. After you get the correct name, we can add the name as appropriate to the notebooks and the data base. Several examples are given in the table fragment below.

Host sp.: Host species. The generic name and specific epithet of the host.

Local: Collection locality. This is usually our own name for the local and is based on something we can identify on a USGS survey map or a Plat Map.

County: County and State (if not NE) where your host was collected.

Location: Map coordinates for the collecting site. We collect this data from the Global Positioning Satellite Network with a hand-held transceiver.

Host I.D. #	Host Morph.	Host sp.	Local	County	Location
REC970081	10 spot skimmer	*Libellula pulchella*	Nevens	Keith	N41.12.26.4 N101.25.04.2
RRK970061	Aeshnid. A	*Aeshna multicolor*	CPBS	Keith	N41.12.25.8 W101.36.56.8
REC970005	Amer Ruby Spot	*Hetaerina americana*	Cedar Creek	Keith	N41.11.01.9 W101.21.35.1
RRK970130	Big Opaca	*Eleodes*	CPBS	Keith	N41.12.25.8 W101.36.56.8
RRK970012	Caenidae	*Caenis*	Beckius	Keith	N41.12.25.8 W101.36.56.2
JP971001	Coccinellidae	*Coccinellidae 1*	CPBS	Keith	N41.12.25.8 W101.36.56.8
RRK970033	Cybister	*Cybister*	Nevens	Keith	N41.12.26.4 N101.25.04.2
RRK970159	Cyrtacanthacridinae A	*Melanoplus differentialis*	Peru Bottoms	Nemaha	N40.29.04.5., W95.41.53.7

Example of the left page of a data notebook

Right Page

Date: Collection date. Use a three letter code for month OR use roman numerals for the month. (June 6, 1998 = "Jun/6/98" or "VI/6/98" NOT 6/6/98) DO NOT USE THREE SETS OF DIGITS. (We can't legally defend such a date system.)

Sex/Stadium: Sex and developmental stage of host (adult, larvae, etc.)

Process: Processing of the specimen. Usually from the following options, note that each line may use more than 1 of the following codes:

Curate. Host was preserved as a permanent voucher specimen (either dried or in alcohol). For each host surveyed, we should have at least 5-10 specimens curated. The first specimen curated is usually called the reference specimen - if you are unsure of your identifications - check the host back against your reference specimen from the collection.

PM. Post mortem. The host was dissected for parasites. Indicate infected/uninfected (or +/-) in the *Notes* column.

SL. Slides were made. Indicate the number of slides in parentheses after the code (i.e. SL(3) = 3 slides made from this host intestine).

Genetic. Host and/or parasites fixed and stored for genetic analysis.

Histo. Host and/or parasites fixed for histology.

Cyst. Cysts were collected. Include cyst storage numbers in the notes column.

Host Ref. #: Host Reference Number. The first voucher specimen curated is usually called the reference specimen. This is used to validate host identifications and is deposited in the museum for future reference.

Notes: Use this column as you see fit. Indicate infection status as appropriate. Indicate really good or important slides as necessary. I usually write the slide codes in this column as well.

Date	Sex/Stadium	Process	Host Ref. #	Notes
26-Jun-97	adult	PM	REC970077	Not Infected
25-Jun-97	Adult	Curate	RRK970061	Reference Specimen
12-Jun-97	adult	Curate	REC97001	Reference Specimen
03-Aug-97	Adult	PM	REC9700137	Infected
16-Jun-97	Nymph	PM	RRK970001	Not Infected
17-Jun-97	Adult	PM	JP970011	Not Infected
17-Jun-97	Larve	PM	RRK970031	Infected-Troph.,Cysts
05-Sep-97	Male/Adult	PM	RRK970155	Infected-Slide

Example of the right page of a data notebook.

Specimen Labels

Specimen labels are the difference between a scientific specimen and a dead animal: one is priceless where the other is worthless.

Microscope Slides

Etch the Host ID # on the right end of the slide. If several slides come from a single host, serialize them with lower case letters (REC980001a, REC980001b, REC980001c, etc.). We will eventually put full slide labels on each slide. You should see me every Monday morning to get slide labels made for the previous week's slides. You should never have more than 2 weeks of unlabelled slides in your possession.

Left Label: Collection Data *(Locality data)*

Right Label: Deposition data

Xiphocephalus *ellisi* Clopton, 1998 ex. *Eleodes opacus* July 5, 1997 NE: Keith Co. CPBS	*NSF Project* *DEB-9705179* REC9700198
(Parasite genus) *(Parasite species epithet)* *(Parasite Author)* *(Host species)* *(Collection date)*	*(Copy exactly: NSF Identifier)* *(Copy exactly: Grant Number)* *(Host Identification Number)*

Whole Organisms in Vials

Write the Host ID # in pencil on a slip of index card and place it in the vial.

Histology Specimens

Write the Host ID # in pencil on a slip of index card and place it in the vial.

Genetic Specimens

Write the Host ID # in pencil on a slip of index card and place it in the vial. Indicate the source and type of tissue included (e.g. "host, muscle" or "gamont, whole")

Cysts

Write the Host ID # in permanent marker on the vial. Also record any other storage data in your notebook.

Hosts in Vials

Write the Host ID # in pencil on a slip of index card and place it in the vial.

Hosts on Pins

Write the Host ID # on a slip of index card and place it on the pin. We will eventually put full labels on each pin. Fully label all hosts within 2 weeks of collection. You should see me every Monday morning to get labels made for the previous week's specimens. Or, you can print your own by hand on 1 x 3 cm pieces of card stock using the following format:

Top label: Host/collecting data *Bottom label: Voucher label*

NE: Keith Co.; Cedar Point Station N41°12'36.4" W101°38'56.8" July 4 , 1997: R. E. Clopton, coll.	Clopton: NSF DEB-9705179 NSF PROJECT VOUCHER REC-9700198
(State, County, Locality) *(GPS Coordinates)* *(Collection date, Collector)*	*(Copy exactly: Grant Number)* *(Copy exactly: NSF Identifier)* *(Host Identification Number)*

Tips, Tricks, and Traps: Hard earned advice from previous researchers

1. Trick: Dip your cover slips in xylene before you make your mounts, the mountant spreads more easily.
2. Trick: Drain excess mountant by holding a new slide preparation vertically and touching the edge of the slide/slip to a paper towel.
3. Trick: Remove excess mountant with a heated razor blade after the slide has dried.
4. Trap: Arrange your slips or slides so that the specimens always face the same way (usually toward some mark on the container), otherwise, you will eventually mount a cover slip upside down and ruin the preparation.
5. Tip: Count on running a stain procedure once just to see how it works before you begin trying to collect data.
6. Tip: Always check out a set of slides you've just made under the microscope. Adjust your technique to correct any problems you see.
7. Trap: Any Sport's Bar in western Nebraska. You're just asking for a beating.
8. Tip: Some days if it weren't for bad luck you wouldn't have any luck at all. Rather than get too frustrated, check out what someone else is doing, do your laundry, go to the beach. Tomorrow will be better. On the other hand, some days you are golden. Keep at it as long as your luck holds and the organisms cooperate: there's plenty of time to sleep in the grave.
9. Tip: Red Hot Burritos. You can live on 'em if you have to.
10. Tip: You will make a reputation in life: take time to make it a good one.