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MANUAL FOR THE CULTURE OF SELECTED FRESHWATER INVERTEBRATES

Edited by
S.G. Lawrence

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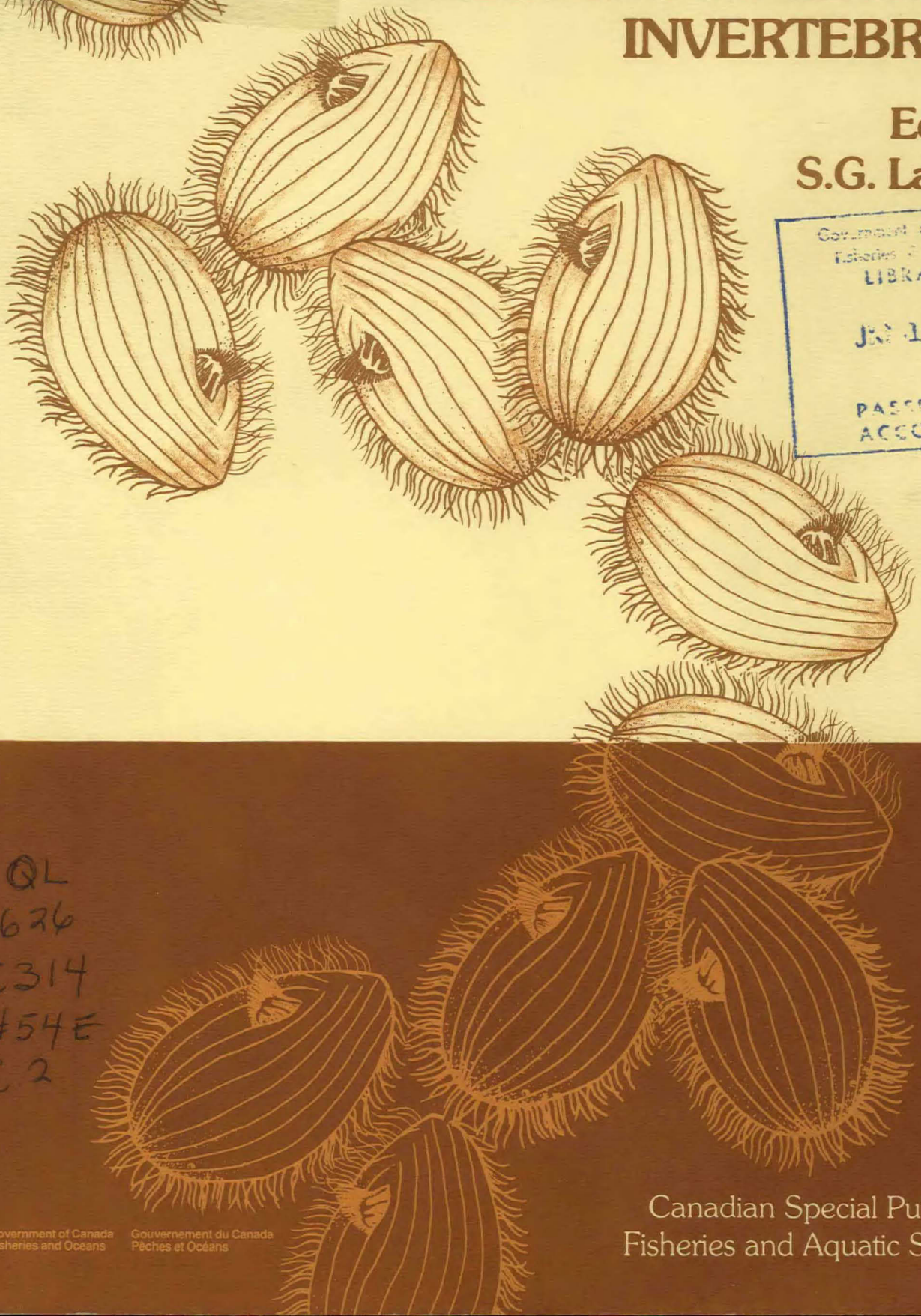

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MANUAL FOR THE CULTURE OF SELECTED FRESHWATER INVERTEBRATES

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ABSTRACT/RÉSUMÉ

Lawrence, S.G. (ed). 1981. Manual for the culture of selected freshwater invertebrates. Can. Spec. Publ. Fish. Aquat. Sci. 54: 169 p.

Selected freshwater invertebrates representative of various trophic levels are grown or held at the Freshwater Institute for purposes of studying basic physiological and nutritional relationships, effects of toxic materials on morphological and physiological phenomena and effects of continued laboratory culture on population dynamics, physiology and morphology. *Tetrahymena vorax*, *Helisoma trivolvis*, *Artemia* sp., *Daphnia magna*, *D. pulex*, *Hyalella azteca*, *Gammarus lacustris lacustris*, *Orconectes virilis*, *Chironomus tentans* and *Hexagenia rigida* have been cultured continuously for two or more generations.

Procedures for culturing organisms on various nutritional bases are outlined in detail so that organisms of known history, age, physiological condition and number can be produced for given experimental purposes on schedule. Techniques such as storage of partly developed eggs or over-wintering animals, artificial insemination of insect egg masses, washing procedures and production of given life phases specifically for use in toxicological studies are described.

Methods for collecting and transporting organisms from the field are outlined; specific environmental preferences, diseases and parasites, and geographic distribution are described and discussed. A list of pertinent literature on the culture, taxonomy, ecology and uses in toxicological research of each species is provided. Culture procedures which are in developmental stages are described for a number of invertebrates.

Key words: Zooplankton culture, benthos, experimental culture, bioassays, aquatic insects, crustacean culture, mollusk culture, protists, *Tetrahymena*, *Helisoma*, *Artemia*, *Daphnia*, *Hyalella*, *Gammarus*, *Orconectes*, *Chironomus*, *Hexagenia*.

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L'Institut des eaux douces abrite ou élève des invertébrés d'eau douce sélectionnés représentatifs de divers niveaux trophiques dans le but d'étudier les rapports physiologiques et alimentaires de base, les effets d'éléments toxiques sur des phénomènes morphologiques et physiologiques, et l'action d'une culture soutenue en laboratoire sur la dynamique des populations, la physiologie et la morphologie des organismes. C'est ainsi qu'ont été élevés sans interruption, depuis au moins deux générations, les groupes suivants: *Tetrahymena vorax*, *Helisoma trivolvis*, *Artemia* sp., *Daphnia magna*, *D. pulex*, *Hyalella azteca*, *Gammarus lacustris lacustris*, *Orconectes virilis*, *Chironomus tentans* et *Hexagenia rigida*.

Les modes de culture d'organismes à partir de divers aliments de base sont décrits en détail de façon que des organismes dont l'histoire, l'âge, l'état physiologique et le nombre sont connus puissent être produits à temps prévu à des fins d'expérimentation. Sont également décrites des techniques s'appliquant par exemple à l'entreposage d'oeufs incomplètement développés ou d'animaux en hibernation prolongée, à l'insémination artificielle de substances ovulaires d'insectes, ainsi que des methodes de lessivage et la production d'étapes d'existence données, à des fins spécifiques de recherche toxicologique.

On y trouvera encore la description de méthodes de collecte et de transport d'organismes tirés du milieu naturel, ainsi qu'une étude détaillée de milieux spécifiques choisis de préférences à d'autres, de maladies et de parasites, et de répartition géographique. Le manuel comprend une liste d'ouvrages pertinents sur l'élevage, la taxonomie, l'écologie et les usages de chacune des espèces dans la recherche toxicologique. Enfin, ces techniques d'élevage d'un certain nombre d'invertébrés, techniques qui n'ont pas encore franchi toutes les étapes de développement, y sont aussi traitées.

Mots-clés: élevage de zooplancton, benthos, milieux de culture expérimentale, titrage biologique, insectes aquatiques, élevage de crustacés, élevage de mollusques, protistes, *Tetrahymena*, *Helisoma*, *Artemia*, *Daphnia*, *Hyalella*, *Gammarus*, *Orconectes*, *Chironomus*, *Hexagenia*.

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INTRODUCTION

Laboratory assessment of the environmental impact of new chemicals on the biota of inland waters has been based in the past on very few available organisms (e.g. the study of nitrilotriacetic acid (NTA) in Canada in the early 1970's). Problems occurred not only in the lack of suitable test organisms which were of importance in local foodwebs, but also in the absence of a logical sequence of research and testing procedures for estimating the impact of new compounds on aquatic systems. In 1971, a committee headed by R.D. Hamilton of the Freshwater Institute recommended that an Aquatic Environment Protocol Program be implemented. The primary aims of the program were to establish a variety of aquatic organisms in culture, to select the species which showed desirable responses under stress conditions for intensive study and to unify the whole into practical test procedures. This manual is a summary of ongoing work on the acquisition and intensive study of selected invertebrate organisms suitable for experimental purposes. A compendium of methods used at the Freshwater Institute for toxicity tests has been compiled and edited by E. Scherer (Toxicity tests for freshwater organisms. 1979. Spec. Publ. Can. J. Aquat. Fish. Sci. 44) and can be used as a companion volume to this volume.

During the 1970's, the favored procedure in North America for assessing toxicity in freshwater was the LC_{50} test (the concentration of toxicant which causes 50% of the assayed organisms to die within a predetermined time, usually 48 or 96 h). The organism of choice was typically the rainbow trout fingerling. This test assumed great significance and still serves as a guide to legislators for setting limits on concentrations of toxicants entering waterways. However, in more recent impact assessments, the LC_{50} serves only as the first in a series of procedures which also assess sublethal effects such as reproductive impairment, change in growth rate or in behavior, interferences in enzyme formation and morphological change at the organ or cell level.

The rainbow trout represents only one trophic level in the aquatic ecosystem and while it is available in culture in North America, laboratory holding facilities are large and expensive to maintain. Other fish of importance in the waters of the Canadian Shield and the prairies are difficult or impossible to culture under laboratory conditions. When such organisms are used to assess effects of introduced chemicals, they are available for short periods of the year from natural sources and are usually subject to the stresses of transport and other handling procedures.

Invertebrates represent a variety of trophic levels (Table 1), and once culturing techniques are available, many organisms of prominence in various food webs can be produced in large numbers. As well, since invertebrate life cycles are usually short, a great deal of information about responses to stress conditions can be gathered in a short time and effects of stress on consecutive generations can be assessed. Laboratory culture of invertebrates then, is not seen as an end in itself, but as a tool used to produce large numbers of organisms of known history, life phase and physiological condition potentially useful in experiments on the toxicity to organisms of different trophic level of chemicals introduced into the aquatic environment.

Before any useful data can be obtained from organisms grown in the laboratory, several prerequisites must be satisfied. Conditions of culture must be

Table 1: Ecological characteristics

	Zoogeography			Basic Habitat, Feeding Type					Lakes				Rivers, Streams		
				Benthos			Plankton								
	2 or more continents	Widespread in North America	Localized in North America	Grazer	Omnivore	Detritvore	Herbivore	Omnivore	Oligotrophic	Eutrophic	Ponds	Sewage Lagoons	Silt Bed	Rock Bed	Salterns
Tetrahymena vorax		X					X				X				
Helisoma trivolvis		X		X					X	X	X	X	X		
Daphnia magna	X						X			X	X	X			
D. pulex	X						X			X					
Artemia sp.	X						X								X
Hyalella azteca		X		X	X				X	X	X	X	X		
Gammarus lacustris lacustris	X	X			X			X	X	X	X				
Orconectes virilis		X			X				X	X	X		X	X	
Chironomus tentans	X					X				X	X				
Hexagenia rigida			X	X		X			X	X			X		

reproducible. The chosen animal population should be capable of completing repeated life cycles under the control conditions provided. Adequate nutrition and water quality are fundamental to the production of disease-resistant, parasite-free animals which exhibit normal behavior. As well, basic environmental requirements and limits must be known so that time changes can be made in the laboratory environment concurrent with changes in life phase or in rate of growth.

Tables 1 and 2 demonstrate that there is no 'standard' invertebrate entirely suitable for use in all procedures. These organisms are representative of a wide variety of feeding types, freshwater habitats and reproductive regimes. Such factors must be considered in the selection of an organism for any specific purpose. As an example of how multiple toxic effects can be measured, we have shown in the chapter on *Tetrahymena* how several investigators have made use of organisms cultured under different conditions to assess a particular toxicant at concentrations producing lethal, sublethal and chronic behavioral effects on individuals and populations.

S.G. Lawrence
Winnipeg, 1981

Table 2: Life history characteristics

	Life Span (days)	Generation, 20°C (days)	Multiple Life Stages: Distinct	Multiple Life Stages: Similar	Reproductive Characteristics: Simple Division	Reproductive Characteristics: Obligately Sexual	Reproductive Characteristics: Total/Partial Parthenogenesis	Reproductive Characteristics: Hermaphrodite	Fecundity: Neonates/Brood or Egg Mass	Fecundity: Number Broods/ Female	Size Ranges: At Hatch, Length (mm)	Size Ranges: At Maturity, Length (mm)	
Tetrahymena vorax	(potential)	0.15	X		X		X		N/A	N/A	N/A	0.03-0.25	
Helisoma trivolvis (two subspecies)	>150	70		X				X	~25	~14	1-2	a) ♂ ≤ 10 b) ♂ ≥ 12	
Daphnia magna (Freshwater Institute Culture)	48	10		X			X		10	~11	1.0	2.3-6.0	
D. pulex (Freshwater Institute Culture)	35	6		X			X		6	10	no data	1.5-3.2	
Artemia sp.	50	9		X		X	some places		~100	11	0.3	10	
Hyalella azteca	variable	30		X		X			1-30	variable	~1.0	2-10	
Gammarus lacustris lacustris	variable	30		X		X			1-60	variable	~2.0	7-26	
Orconectes virilis	~1000	400		X		X			variable	variable	(rostral-carapace) 4.0	variable	
											First Larva	Final Larva	Adult
Chironomus tentans	variable	variable	X			X			1500-3000	1 or more	0.6-1.2	14-21	6.0
Hexagenia rigida	180-365	180-365	X				X		2000-4000	1	1.0	22-28	18-28

FORMAT

The first three sections of each chapter present information germane to the natural life history and ecology of the organism by means of references to existing literature. The succeeding sections detail the collection and transport of organisms, and describe culture methods in detail. The organisms used in these studies are in various stages of culture; moreover, they exhibit a wide range of habit with respect to life cycle. Thus it was not possible to write each chapter using exactly the same format. However, it is possible to include all pertinent information under general guidelines so that the reader can find certain types of information easily. The chapter format which follows is generally the same for all chapters but varies in detail in several ways. Sections 1-3 are universal; 4-8 are not. For example, *Tetrahymena vorax*, a ciliated protozoan, is cultured in Biologically Defined Culture on sterile broth and as a predator preying on a single organism; Sections 5 and 6 are used to describe these techniques. *Hexagenia rigida*, an insect with a more complex life cycle, can be grown using mass culture techniques on undefined media and certain stages can be effectively stored; these procedures are described in Sections 4, 5 and 7.

CHAPTER FORMAT

INTRODUCTION

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1.2 Toxicological precedents
1.3 Geographic distribution

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2.2 Life cycle
2.3 Ecological relationships
2.4 Behavior
2.5 Diseases and parasites

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BIOLOGICALLY DEFINED CULTURE

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CULTURES FOR TOXICITY TESTS

Tetrahymena vorax (Kidder, Lilly & Claff 1940) Kidder 1941

S.G. Lawrence, M.H. Holoka and A. Furutani

INTRODUCTION

1.1 General information

Tetrahymena (Hymenostomatida:Tetrahymenidae) is the most familiar of all ciliate genera (Corliss 1973a, b) and perhaps of all animal organisms biochemically, physiologically and genetically (Hill 1972, Elliott 1973). Nutritional requirements are known in chemical detail (Holz 1973, Kidder 1967, Kidder and Dewey 1951). The genus is widespread geographically, species having been found in all freshwater habitats examined (Corliss 1954, 1970, 1973b, Elliott 1970).

T. vorax was first described by Kidder et al. (1940). Corliss (1970, 1973b) described the ten accepted species of the genus and grouped them by means of complexes of structural, physiological and morphological characteristics. Shaw and Williams (1963) examined physiological characteristics of the ciliate cultured in the absence of all other organisms including bacteria (axenically). Williams (1964) studied division synchrony in *T. vorax* induced by temperature shock. Buhse (1966a, b) has studied transformations in the dimorphic life cycle of *T. vorax*.

Taub (1976) and Taub and McKenzie (1973) have used *T. vorax* to examine trophodynamics in freshwater microcosms. Lawrence (1974) and Lawrence and Holoka (1979) have grown *T. vorax* and *Chlamydomonas reinhardtii* in a two-stage chemostat system to study effects of toxicants on a simple food chain.

1.2 Toxicological precedents

There is little information available about effects of toxicants on *T. vorax*. *T. pyriformis*, because of the wealth of information on its nutrition, physiology, morphology and genetics (e.g. Hill 1972, Elliott 1973), has been the organism of choice. However, growth characteristics of the two organisms are similar and it is probable that much of the toxicological information pertinent to *T. pyriformis* is applicable to *T. vorax*.

Bergquist and Bovee (1976) found that 15 mg L^{-1} cadmium caused depression of growth rate in *T. pyriformis* grown and tested in organic medium. Carter and Cameron (1973) ascertained 96 h TL_m (a variation of the LC_{50} test) values of several heavy metals using *T. pyriformis* grown axenically in organic medium but washed prior to testing. The TL_m value for cadmium sulfate in distilled water was 0.84 mg L^{-1}. Lawrence and Holoka (unpublished data) found that the growth rate of *T. vorax* grown monoxenically (in the presence of one food organism) in a predator-prey system was depressed by exposure to 0.01 mg L^{-1} cadmium (as cadmium sulfate). Taub (1976) tested the effects of 0.0065 mg L^{-1} cadmium on a microcosm composed of an alga, a protozoan (*T. vorax*), a rotifer and three crustaceans. The population of one of the crustaceans decreased and that of the alga increased but there was no apparent effect on *T. vorax*.

Carter and Cameron (1973), Tingle et al. (1973), Thrasher and Adams (1972) and Bowles and Wolfson (1976) have worked with various mercuric compounds in order to quantify physiological and morphological effects on *T. pyriformis*. These data include TL_m values, assessments of effects on generation time, and an appraisal of effects on sulfhydryl groups located in or near the cell surface. Cooley et al. (1972, 1973), Geike and Parasher (1976), Schultz and

Dumont (1977) and Schultz et al. (1978a, b) have investigated the effects of organic compounds and mixtures such as Mirex, Aroclor and shale oil waters on physiological and morphological parameters in *T. pyriformis*.

1.3 Geographic distribution

The distribution of *T. vorax* is not as well known as some other species in this genus and indeed Corliss (1973b) remarked that its species complex has been far too little investigated in nature. So far as is known, *T. vorax* is found only in fresh water.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

Tetrahymena species are generally pyriform with 17-42 uniform ciliary rows which are composed of repeating units of cilium and basal body (kinetosome). The mouth or cytostome is small, close to the anterior end, and its axis is parallel to the body axis. Within the cytostome there is an undulating membrane and three membranellae (giving the genus its name) which serve to direct food into interior vacuoles. The macronucleus is ovoid, and micronuclei absent or few to numerous.

T. vorax is a member of a complex with *T. patula* and *T. paravorax* with whom it shares several characteristics. These species have a polymorphic life cycle (Williams 1961). The free swimming stage is dimorphic and, depending on the food source, has a small body with a small mouth opening (microstome) or a large body with a large mouth (macrostome). Both *T. patula* and *T. vorax* have a reproductive cyst stage and under some environmental conditions interconversions between microstome, macrostome and cyst occur freely, although binary fission can occur in either free swimming form without the intervention of the cyst stage. In the microstomous form of *T. vorax*, whether fed bacteria or cultured axenically, the posterior end is drawn out into a tail, a characteristic unique to the species (although Williams (1960) described a rare variant of *T. patula* as a tailed organism). *T. vorax* has 17 or 18-28 ciliary rows (kineties) depending on the strain. The macronucleus is placed centrally; micronuclei are absent in all extant strains.

The size of the microstome varies with culture conditions. The range is 31-115 µm (Kidder et al. 1940). Williams (1961) found that various strains of *T. vorax* V_2 cultured axenically were 77-108 µm in length. When fed on *C. reinhardtii* cultured in an inorganic medium containing vitamins in a two-stage chemostat, *T. vorax* microstomes were 115 µm long (Lawrence and Holoka, unpublished data). Macrostomes form when the organism is fed other ciliates (e.g. *T. pyriformis*) or becomes cannibalistic. The length, depending on the food source and on the strain used, ranges from 107-250 µm (Williams 1961, Kidder et al. 1940).

Williams (1964), Buhse (1966a, b, 1967), Buhse et al. (1970), Nicolette et al. (1971), Sherman et al. (1978) and Ryals et al. (1979) have studied the morphology and physiology of the transformations between microstome and macrostome forms fed on *T. pyriformis*, exposed to stomatin (the transforming component

produced by *T. pyriformis*) or, induced by heat shock.

2.2 Life cycle

Sexual reproduction does not occur in extant strains of *T. vorax* since it is amicronucleate. Asexual reproduction occurs through binary fisson. In *Tetrahymena*, a new mouth, which is inherited by the posterior sister cell (opisthe), is formed late in the cell cycle in the process of stomatogenesis. The old mouth is inherited by the anterior sister cell or proter. The site of stomatogenesis is the equatorial region posterior to the old mouth. The first sign of the process is the formation of a field of kinetosomes which eventually develops into the mouth by a gradual ordering of the field into a species-specific oral pattern, and formation of the mouth membranes in association with the pattern of kinetosomes. The division furrow forms anterior to the new mouth. The formation of a microstome from a macrostome is somewhat more complex but is a variation on the fundamental process (Buhse 1966b).

When macrostomes are formed from microstomes, oral replacement occurs. The new kinetosome field is laid down just posterior to the old mouth which is resorbed. The new mouth is larger than the old one. The complete division process does not take place. This process is described in Buhse (1966b) and in Buhse et al. (1970).

The division rate of *T. vorax* depends on environmental conditions, especially on food source and temperature of culture, and on the strain used (Table 3).

Table 3. Rates of division in *Tetrahymena vorax* under various culture conditions

Strain	Medium or food	T°C	h/division	References
D (original, now extinct)	*Aerobacter cloacae* (monoxenic)	25	6	Kidder et al. 1940
	Undefined organic (axenic)	25	8	Kidder et al. 1940
	Dead protozoans	?	5.5	Kidder et al. 1940
	Pasteurized yeast	25	10	Kidder et al. 1940
V_2D	Undefined organic (axenic)	20	9	Williams 1961
	Defined organic (axenic)	20	12.3	Shaw and Williams 1963
V_2M	Undefined organic (axenic)	20	16	Williams 1961
	Defined organic (axenic)	20	10.2	Shaw and Williams 1963
	Defined organic plus phospholipids (axenic)	20	8.5	Shaw and Williams 1963
V_2S	Undefined organic (axenic)	20	9	Williams 1961
	Defined organic (axenic)	20	11.9	Shaw and Williams 1963
	Defined organic plus phospholipids (axenic)	20	7.4	Shaw and Williams 1963
	Chlamydomonas reinhardtii nitrogen and light limited chemostat, no vitamins (monoxenic)	25	17	Taub and McKenzie 1973
Tur	Undefined organic (axenic)	27	3.0±0.18(SD)	Williams 1964
Freshwater Institute (FWI)	*A. aerogenes* in carbon limited chemostat (monoxenic)	24	3.7	A. Furutani(Freshwater Institute) unpublished data
	C. reinhardtii plus vitamins in nitrogen limited chemostat (monoxenic)	20	5.5-6.5	Lawrence et al. this work

2.3 Ecological relationships

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
27	Optimum for axenic culture of *T. vorax* Tur.	Williams 1964
18-20	Optimum for transformation from micro- to macrostome in strain V_2S.	Buhse 1966a
5	Cell death ensues in 12 h in strain V_2S.	Buhse 1966a
36	Cell death ensues in 30 min in strain V_2S.	Williams 1964
2. pH		
5.0-8.6	Extremes for growth in lab populations of *T. pyriformis*.	Prescott 1958
5.5-6.5	Optimum for transformation from micro- to macrostome in strain V_2S.	Buhse 1966a
<4.0, >9.0	No transformation occurs in strain V_2S.	Buhse 1966a

COLLECTION

3.1 Preferred habitats

T. vorax has been found in a freshwater pond near Woods Hole, Massachusetts, U.S.A. (Kidder et al. 1940) and in a roadside ditch near Winnipeg, Manitoba (the FWI strain, A. Furutani, unpublished data). This strain was initially isolated using a chemostat to which an aliquot of the sample was added. Subsequently, it was maintained axenically on YEP medium (Appendix B5) containing *Aerobacter aerogenes* (Section 5) and monoxenically in a defined medium containing *C. reinhardtii* (Section 6).

3.2 Collection procedures

Acid clean glassware (Appendix B1) should be used to collect Protozoa. The mesh size of nets should be < 20 µm. Collections can be made at the surface or deeper and the animals concentrated using nets or filters.

3.3 Transport

The container should be maintained at the ambient temperature so long as this is not > 25°C. An insulated cooler should be used if transport times are longer than 1-2 h.

3.4 Sorting and isolation

3.4.1 Equipment and materials

1. Dissecting microscope.
2. Glass dish (e.g. Petri plate).
3. Fine pipette (4 mm diameter tubing, bore ∿ 40 μm); Pasteur pipettes.
4. Small test tubes, (Appendix A2), plugged with cotton and sterilized.
5. Mouth siphon (Appendix A1).
6. Sterile Cerophyl medium (Appendix B2, 3) inoculated with *Aerobacter aerogenes*, *Pseudomonas ovalis* (Appendix B7) or bacteria associated with the ciliate 24 h prior to isolation procedure.

3.4.2 Isolation

1. Using Pasteur pipettes, fill the small test tubes with about 0.25 mL Cerophyl medium containing bacteria (bacterized medium).
2. Pour the sample into the glass dish and, using the dissecting microscope and fine pipette on a mouth siphon, select a ciliate and gently transfer it to the bacterized medium in the tube. Avoid extreme changes in pressure. Replace cotton plug.
3. Place the isolated ciliates in the dark or very dim light at 20-25°C for 3-4 d.

MASS CULTURE TECHNIQUES[1]

5.1 Small volume techniques

5.1.1 Equipment and materials (Glassware acid clean, Appendix B1)

1. 250 mL Erlenmeyer flasks, containing sterile 75 mL Cerophyl medium (Appendix B3) inoculated aseptically with *Areobacter aerogenes*, *Pseudomonas ovalis* (Appendix B7) or bacteria associated with the ciliates 24 h prior, plugged with cotton.

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[1] All procedures in Section 5 are to be carried out using aseptic techniques.

2. Pasteur pipettes, sterile.

3. Small test tubes containing ciliates as described in Section 3.4.2.

5.1.2 Culture initiation

1. Gently draw up the contents of a small tube using a Pasteur pipette. Avoid extreme changes in pressure.

2. Gently release the ciliates from the pipette into the medium in the Erlenmeyer flask, and plug the flask with cotton.

3. Keep at 20-25°C in the dark.

5.1.3 Culture maintenance

1. At 20°C, the ciliates should be transferred to freshly bacterized medium every week.

2. At 25°C, transfer every 3-4 d.

3. If cultures of *Tetrahymena* feeding on one species of bacterium are needed, clean the ciliates as in Section 6.1 and inoculate into medium as prepared in Section 5.1.1.1.

BIOLOGICALLY DEFINED CULTURE[2]

6.1 Axenic culture

6.1.1 Equipment and materials (Glassware acid cleaned, Appendix B1)

1. Glass agglutination slides (e.g. Klein slides) with 12 depressions, wrapped in aluminum foil and sterilized (1 slide per 2-3 ciliates).

2. Square plastic Petri plates, sterile (1 plate per agglutination slide).

3. 4 mm diameter pipettes with approximately 30 µm bore tip, cotton plugged, sterile (16 pipettes per slide).

4. Washing medium (NC medium, Appendix B6), 10-20 mL, sterile.

5. Small test tubes (Appendix A2) plugged with cotton, sterile (1 tube per washed ciliate).

6. Pasteur pipettes, plugged with cotton, sterile (1 pipette per 4 tubes).

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[2] All procedures in Section 6 are to be carried out using aseptic techniques.

7. YEP medium (Appendix B5) sterile, 10 mL per tube.

8. Mouth siphon (Appendix A1).

9. Dissecting microscope.

10. Glass dish (e.g. Petri plate), sterile.

11. Petri plates containing sterile nutrient agar or potato dextrose agar (1 plate each medium for each clean ciliate), Appendix B4.

12. Thioglycollate broth, sterile, 10 mL per tube (1 tube per ciliate produced), Appendix B4.

13. Ciliate culture from field sample or as in Section 5.1.

6.1.2 Washing procedure

1. Place an agglutination slide into a Petri plate aseptically by unwrapping the foil and sliding it into the partially opened plate. This combination is hereafter called the agglutination slide.

2. Half fill each agglutination slide depression with washing medium using a Pasteur pipette.

3. Pour organisms into the glass dish and place under the microscope.

4. Attach a fine pipette to the mouth siphon and draw up 1-3 ciliates.

5. Set the glass dish aside, and replace with an agglutination slide.

6. Gently release or blow the ciliates into the washing medium of a corner depression. (Mark the starting place and establish direction of transfer).

7. Repeat steps 4-6 (using a fresh pipette for each transfer) for each agglutination slide.

8. Every 10-15 min thereafter, transfer the ciliates to a fresh depression using a new pipette until the fourth depression in each agglutination slide is reached. Leave the ciliates at this point for 30 min to 1 h.

9. Every 10-15 min thereafter, transfer the ciliates to a fresh depression (using a fresh pipette for each transfer) until the eighth depression is reached. Leave the ciliates in this depression for 8-12 h.

10. Place about 0.25 mL YEP medium into the small, cotton plugged tubes using Pasteur pipettes.

11. Transfer the ciliates through depressions 9-12 at 10-15 min intervals using a fresh pipette for each transfer.

12. Transfer one ciliate into the YEP medium using a sterile small pipette.

13. Incubate in the dark or dim light at 20-25°C.

6.1.3 Contamination checks

1. Examine the tubes after 24 and 48 h and discard those with visible contamination.

2. After 48 h, inoculate a small amount of medium from visibly clean small tubes onto nutrient agar, potato dextrose agar and into thioglycollate broth. Incubate at 20-25°C for 5-7 d and check for growth.

3. 4-7 d after the end of the washing procedure, examine the small tubes to ensure that the protozoans have reproduced.

4. Transfer 50 or more ciliates from a single small tube to a larger tube containing 10 mL sterile YEP medium.

5. If initial contamination tests are negative the medium in the larger tubes should be tested for contamination 24-48 h after transfer of the ciliates.

6.1.4 Culture maintenance

1. Clean cultures of ciliates should be transferred by loop or pipette to fresh YEP medium at 2 week intervals if held at 18-20°C, at weekly intervals if held at 25°C.

2. Contamination tests should be repeated at least every 2-3 months.

6.2 Monoxenic culture

6.2.1 Stock cultures

6.2.1.1 Equipment and materials

1. Acid cleaned glassware throughout (Appendix B1).

2. Sterile NC medium (Appendix B6) contained as follows:

   a. 10 mL in 20 mL test tubes.

   b. 50 mL in 125 mL Erlenmeyer flasks OR 100 mL in 250 mL Erlenmeyer flasks.

3. 1 mL serological pipettes, sterile.

4. Stock culture *Chlamydomonas reinhardtii* (Appendix B8) in liquid medium.

5. Axenic culture *T. vorax* as generated in Section 6.1 or from other source.

6. Incubator maintained at 20°C, light intensity ∿200 $\mu E \cdot m^{-2} \cdot s^{-1}$.

7. Area or incubator maintained at 20°C in dim light, below 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ or in the dark.

8. Rotating shaker (optional).

9. Petri plates containing sterile nutrient agar or potato dextrose agar (Appendix B4).

6.2.1.2 Initial procedure

1. Using a pipette, withdraw 0.2 mL *C. reinhardtii* culture and place 0.1 mL on a nutrient agar plate and 0.1 mL on the potato dextrose agar plate. Incubate plates at room temperature (20-22°C) for 1 week. Examine for microbial growth.

2. After ascertaining the *C. reinhardtii* is free of other organisms, place 0.05-0.1 mL of the algae into a test tube containing NC medium. This is the newly established stock culture referred to below.

3. Withdraw 1.0 mL *C. reinhardtii* culture and place into a flask containing NC medium. This is the flask culture.

4. Incubate new stock cultures and flask cultures 48-96 h at ∿200 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures will grow more uniformly if they are agitated gently on a rotating shaker.

5. After incubation, use a pipette to withdraw 1 mL fluid from the axenic culture of *T. vorax* and transfer it into the algae flask culture as generated in step 3 above. Incubate in dim light below 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ or in darkness.

6. Withdraw 0.1 mL fluid from test tube culture of *C. reinhardtii* as established in step 2 above and transfer into a test tube containing NC medium. This is the continuation of the stock culture.

6.2.1.3 Maintenance procedures

1. Every 3-5 d, transfer 1.0 mL of the stock culture of *C. reinhardtii* to NC medium in flasks. This culture is to be used in step 2 following.

2. Every 3-5 d, transfer 1 mL of the algae-protozoan culture to a 48-96 h *C. reinhardtii* culture and incubate as in Section 6.2.1.2.5. This procedure establishes a serial monoxenic culture, gradually freed of organic medium.

3. Every 7-10 d (in synchrony with steps 1 and 2 above if desired), transfer 0.1 mL *C. reinhardtii* from the test tube culture as established in Section 6.2.1.2.2 into a test tube containing NC medium (this procedure establishes a serial stock culture).

4. Stock cultures of *C. reinhardtii* and algae-protozoan cultures should be checked for contamination at least every month or upon each transfer if desired as in Section 6.1.3.2.

6.2.2 Monoxenic culture in a two-stage chemostat

6.2.2.1 Equipment and materials

1. *C. reinhardtii* culture as established in Section 6.2.1.
2. Protozoan culture grown on *C. reinhardtii* as in Section 6.2.1.
3. Chemostat apparatus (Appendix A3), sterile.
4. Controlled environment room or bath.
5. Inoculation equipment: syringe, needle, capped 10-15 mL serum bottle, 5 mL pipettes, sterile.
6. Microscope (compound).
7. Counting chamber (e.g. Sedgwick-Rafter cell), Lugol's iodine (Appendix B9).
8. Drying oven.
9. Nucleopore filters (25 or 47 mm diameter), 0.22 µm pore, and filtering apparatus.
10. NC medium (nitrogen limited) (Appendix B6) in the reservoir.

6.2.2.2 Initial procedures

1. Pump NC medium (nitrogen limited) into the algal vessel to 2/3 chosen volume.
2. Pipette 8-10 mL *C. reinhardtii* culture into a serum bottle and cap.
3. Draw 3-5 mL algae into a syringe.
4. Inoculate into the algae vessel. Allow vessel to fill and overflow into the protozoan vessel over $\sim$ 24 h.
5. When the algal medium has become faintly green and has half-filled the protozoan vessel, repeat steps 2-4 using the protozoan culture and inoculating into the protozoan vessel.
6. At the end of a further 24 h, sample each vessel and count the cells using the counting chamber and microscope. If numbers are near 10^6 algae mL^{-1}, and 10^3- 10^4 protozoans mL^{-1}, set the pump to the desired working dilution rate.

6.2.2.3 Maintenance procedure

1. Check the dilution rate 3-4 times weekly by measuring the amount of effluent produced in a given time.

2. Count cells every 2 d, using 0.3 mL Lugol's iodine per 100 mL sample to fix cells.

3. Every 2-3 d, 35 mL culture taken directly from each vessel may be filtered and various analyses (e.g. nitrogen series, dry weight) made on filtrate and cells.

6.2.3 Production

1. *C. reinhardtii* will divide every 4-5 h at 20°C in NC medium if the medium is agitated by stirring or bubbling.

2. *T. vorax* will divide every 5.5-6.5 h at 20°C feeding on *C. reinhardtii* if the medium is agitated by stirring only. Production in a chemostat depends on dilution rate.

3. For every 10^6 algae mL^{-1}, 10^4 protozoa mL^{-1} are produced.

4. 10^6 *C. reinhardtii* = ∿0.045 mg dry weight (dried 24 h at 60°C).
   10^4 *T. vorax* = ∿0.023 mg dry weight (dried 24 h at 60°C).

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Helisoma trivolvis (Say)

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INTRODUCTION

1.1 General information

Helisoma trivolvis (Say) (Basommatophora:Planorbidae) is one of the most widely distributed snails in North America (van der Schalie and Berry 1973). It can be readily cultured in the laboratory (Krull 1937, van der Schalie and Berry 1973, Flannagan 1974, Eversole 1978)[3], has been used in the testing of potential pollutants (van der Schalie and Berry 1973, Flannagan, 1971, 1974). and in physiological studies (Kater 1974, Russell-Hunter and Eversole 1976, Wood 1976). Field studies relating to various aspects of the life history and ecology of this species have been conducted by Morris (1970), Boerger (1972, 1975) and Eversole (1978). *H. trivolvis* is resistant to polluted environments (Harman 1974). The ability of these snails to tolerate and bioaccumulate substances which are toxic to many other organisms means that they may play a crucial role in the transfer of such compounds in the food web. The genus *Helisoma* has been studied intensively because of its similarity to other planorbid snails such as *Biomphalaria* and *Bulinus*, intermediate hosts of the human blood fluke which causes schistosomiasis in humans (van der Schalie and Berry 1973). *H. trivolvis* itself can serve as an intermediate host for some parasites (see Section 2.5).

1.2 Toxicological precedents

Van der Schalie and Berry (1973) studied the effects of a wide range of temperatures, including sudden temperature changes, on the growth and reproduction of *H. trivolvis* and other snails. Flannagan (1971) investigated the effects of trisodium nitriloacetic acid (Na_3NTA) at different pH levels on survival of this species. Effects of Na_3NTA on mortality, growth and fecundity of this species over four generations has also been studied (Flannagan 1974, Flannagan and Cobb 1979). Its use as an indicator organism and its tolerance to various environmental conditions are discussed in Harman (1974).

1.3 Geographic distribution

H. trivolvis is distributed in North America from the eastern U.S.A. northwest to Alaska (Baker 1928). Two subspecies occur in the Canadian interior basin. *H. t. subcrenatum* (Carpenter) is distributed from California to the Yukon Territory and east to central North America. It is the subspecies which occurs on the prairies east of the Rocky Mountains. *H. t. trivolvis* (Say) is found throughout the boreal forest region in the Canadian interior basin east to the Maritime provinces (Clarke 1973).

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[3] Morris (1970) was not successful in rearing this species in the laboratory at 15-18°C or 22-25°C, 15 h daylength.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

"Ram's horn", the common name for *Helisoma*, is derived from the planospiral shape of the shell. Keys to, and descriptions of *H. trivolvis*, are given in Harman and Berg (1971) and Clarke (1973). *H. t. subcrenatum* is distinguished from *H. t. trivolvis* by its shorter axial length (= greater height). In mature specimens it is usually less than 10 mm, whereas the axial length of *H. t. trivolvis* is usually more than 12 mm and exceeds 10 in most populations (Clarke 1973). The anatomy of *H. trivolvis* is described in Baker (1945) and Abdel-Malek (1954).

2.2 Life cycle

These organisms are functionally hermaphroditic and normally both individuals become fertilized through reciprocal copulation. Eggs are laid in gelatinous capsules and a single snail lays numerous egg capsules over its life span. Development is direct and snails hatch as miniature adults complete with protective shells (Purchon 1968). In the laboratory at 23°C, the generation time (egg to egg) of *H. trivolvis* was 70 d (Flannagan 1974). The life span and life cycle of this species varies considerably in nature. Morris (1970), in a study in Alberta, Canada, estimated that snails could live up to 5 years with spawning beginning in the first year and continuing to the fifth. (The adult population usually consisted of individuals recruited the previous year.) Eversole (1978), in a study in upstate New York, U.S.A., found an annual life cycle and a 12-15 month life span for two of the three populations that he studied. This variation may be due to different environmental conditions, as well as differences between subspecies. Morris (1970) was probably studying *H. t. subcrenatum* and Eversole (1978) the subspecies *H. t. trivolvis*.

2.3 Ecological relationships

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
0-34	Extremes for field populations.	Boerger 1975
24-30	Range for growth and reproduction.	van der Schalie and Berry 1973
28	Optimum for growth and egg production.	van der Schalie and Berry 1973
30	Optimum for egg laying.	van der Schalie and Berry 1973
10	Initiation of spawning in field populations.	Morris 1970 Eversole 1978

Physical parameter	Associated factors or limits	References
2. pH		
7-9	Optimum for laboratory culture.	Flannagan and Cobb 1979
7.0-8.4	Extremes for field populations.	Harman and Berg 1971
3. Dissolved oxygen[4]		
0-15.1 mg L^{-1}	Extremes for field populations.	Harman and Berg 1971
4. CO_2		
0-43 mg L^{-1}	Extremes for field populations.	Harman and Berg 1971
5. Alkalinity (as $CaCO_3$)		
26-365 mg L^{-1}	Extremes for field populations.	Harman and Berg 1971

2.4 Behavior

Egg laying in *H. trivolvis* occurs mainly in spring and early summer (Morris 1970, Boerger 1972, Eversole 1978). Eversole (1978) observed a population which had an additional peak of egg laying in early fall. Eggs are deposited on a variety of substrates (e.g. stone and vegetation) in a gelatinous capsule under the surface of the water. Number of eggs per capsule varies considerably. Morris (1970) recorded a mean of 37 eggs per capsule (range 10-68), and Boerger (1975) a mean of 24.5 eggs per capsule (range 1-54) in field populations. Juvenile snails are frequently found on living vegetation whereas adults are often located on inorganic substrates (Harman and Berg 1971). The normal defense response of these organisms is to contract the foot into the shell. A prolonged response of this sort, especially if the snail is lying on the bottom of the aquarium, indicates the animal is in stress and may be dying, or that it is dead. Under anaerobic conditions where they are not able to come to the surface to breathe, the foot is extended maximally out of the shell and the organism becomes completely motionless (von Brand et al. 1950).

[4] Brief periods of low or zero oxygen levels can be withstood because these snails can come to the surface of the water and breathe atmospheric air. They are also able to respire anaerobically for short periods (von Brand et al. 1950). Adequate supplies of dissolved oxygen are required for egg development (Roney 1943).

2.5 Diseases and parasites

H. trivolvis serves as the intermediate host for the trematodes *Echinoparyphium flexum* (Morris 1970), *Cephalogonimus salamandrus* (Dronen and Lang 1974), *Alloglossidium macrobdellensis* (Corkum and Beckerdite 1975) and *Cyclocoelum brasilianum* (Taft 1975).

COLLECTION

3.1 Preferred habitats

H. trivolvis is found in habitats ranging from eutrophic marshes and ponds to oligotrophic lakes (Harman and Berg 1971). The most productive areas for collection are eutrophic, shallow bodies of water such as ponds, swamps and ditches.

3.2 Collection procedure

Snails can be hand-picked from rocks and submerged vegetation and from the under surface of the water on which they skim. They are also collected by sweeping a net through aquatic vegetation and sediments.

3.3 Transport

Snails are easily transported in plastic bags containing water from the collection site. If bags are tied, a large air space should be left to permit snails to come to the surface of the water to breathe. Transport should be in an insulated container to prevent large fluctuations in water temperature.

3.4 Sorting and isolation

Snails can be sorted by hand because of their relatively large size and protective shells.

MASS CULTURE TECHNIQUES

Methods described are those used to maintain the continuous stock cultures at the Freshwater Institute and are not necessarily optimal.

5.1 Stock culture techniques

5.1.1 Equipment and materials

1. Dechlorinated water (Appendix B18).
2. 8 L glass aquarium (~30 x 17 x 20 cm).
3. Air pump, air line tubing, small airstone, regulating check valves.

4. Tetra-Min B (Appendix B10).

5. Iceberg lettuce (food grade, available at local food markets).

6. Silica sand (60-100 grade), 3-4 g per aquarium.

7. $CaCO_3$, ~1 g per aquarium (necessary only if water is quite soft).

8. Ordinary room light (or small fluorescent light bank).

9. Substrates, e.g. small stones or plexiglass plates.

5.1.2 Procedures

1. Place 6-7 L dechlorinated water in the aquarium. Maintain temperature at 20-25°C on a 16 h light to 8 h dark (16L:8D) cycle.

2. Aerate gently but at a constant rate.

3. Add sand. This is ingested by snails and used in the trituration of food.

4. Add ~1 g $CaCO_3$ if the water is very soft. This will provide a source of calcium for shell construction and will buffer the water.

5. Add 10 snails per aquarium. This low density will encourage egg laying.

6. Provide food liberally but avoid depletion of dissolved oxygen. A recommended amount is 2 g Tetra-Min B and 1 g lettuce twice per week for 10 mature snails. Algae will become established within a short period and provide an additional food source.

7. Change water at least weekly and remove uneaten food. Replenish sand and $CaCO_3$.

8. Densities should be kept low (depending on size of the snails) to get the best egg production and growth. Adults may consume egg capsules before hatching occurs especially if densities are high or if the food supply is inadequate. If attached to removable substrates, eggs can be transferred to other aquaria.

5.1.3 Production

At 23°C, snails lived for at least 5 months and started egg laying 70 d after hatching. A mean of 350 eggs per snail (n=10) were laid during this period. Mortality in young snails was quite rare (Flannagan 1974). More rapid production would be expected at higher temperatures (see Section 2.3).

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Daphnia magna (Straus)

Daphnia pulex (Leydig) Richard

S.L. Leonhard and S.G. Lawrence

INTRODUCTION

1.1 General information

Daphnia (Cladocera:Daphnidae) is a genus which is among the dominant consumers of primary producers in freshwaters (Hebert 1978). It is found in oliogotrophic and eutrophic lakes, in ponds and in reservoirs where it forms a source of food for both invertebrate and vertebrate predators. Members of this genus are therefore central to many freshwater food webs. Other environmental factors being equal, larger forms are present in water bodies in which large predators do not occur, and smaller forms are generally found in lakes and ponds in which large fish predators are present. Hall (1964) described this phenomenon when he observed that some planktivorous fish fed extensively on large daphnids but not on smaller forms. Brooks and Dodson (1965) postulated the 'size-efficiency' hypothesis to explain how predation and other factors shape the community structures of both invertebrates and the secondary consumers which prey on them. Grygierek et al. (1966), Galbraith (1967), Dodson (1970), Sprules (1972), Hairston and Pastorok (1975), Hall et al. (1976), Lampert and Schober (1978) and O'Brien et al. (1979) have further investigated the relationship of size of *Daphnia* to the form of predation pressure it experiences.

Porter (1977) reviewed investigations into the various relationships among environmental conditions, algae and zooplankton in freshwater ecosystems. She pointed out that the most prominent zooplankters are cladocerans and copepods and that it is known that during periods of peak abundance such as late spring and summer, populations of these crustaceans are potentially able to graze over 100% of the daily phytoplankton production. In a study of the feeding activities of zooplankton in lakes, Haney (1971, 1973) showed that *Daphnia* and other cladocerans had higher filtering rates than grazing copepods and were responsible for $\sim$ 80% of the community grazing rate. Porter (1977) discussed whether grazers such as *Daphnia* control algal abundance and concluded they can within limits. If algae are palatable and in a size range amenable to being filtered by grazers and if community rates of grazing are $\geq$ algal generation times, then the phytoplankton population will not increase. Since large or toxic algae are rejected by cladocerans (Porter and Orcutt 1980) the relative abundance among algal species can be shifted by their selective grazing activities (K.G. Porter, University of Georgia, personal communication). In an investigation of another aspect of these relationships, Porter (1976) showed that algal cell growth and productivity may be enhanced by passage through the gut of *Daphnia* as intact, metabolizing cells. Coveney et al. (1977) showed that in a eutrophic lake, one species of *Daphnia* fed primarily on algal plankton, and another smaller form on bacteria, and so established that these herbivores transferred two sources of energy to secondary consumers.

The energy budgets, growth and reproductive efficiency of *Daphnia* have been studied by Richman (1958) and Slobodkin (1959). These investigations made it possible to better interpret various studies of trophodynamics which have established that both *D. magna* and *D. pulex* can be important in the transfer of energy from one trophic level to another in aquatic food webs. Rigler (1961), McMahon and Rigler (1963, 1965) and McMahon (1965) investigated the relationships among food concentration, age, temperature and filtering rate

in *D. magna*. Burns (1968, 1969) investigated the relationships between daphnid body size, temperature and size of particle filterd in both *D. magna* and *D. pulex*. Schindler (1968) showed that feeding, assimilation and respiration rates were related in *D. magna*. Berman and Richman (1974) showed that *D. pulex* is able to select food of a certain size and that filtering rates change with respect to particle size. Lampert (1977a, b, c) investigated carbon metabolism in *D. pulex* as related to physiological characteristics and to production efficiency. Hrbácková and Hrbácek (1978) studied the growth rate of neonates of *Daphnia* species at different natural food concentrations and Peterson et al. (1978) investigated the rates at which *D. pulex* filter bacteria and yeast from lake water. Buenning (1978) proposed a model which predicted growth in *D. magna* as a function of food concentration.

Daphnids have been cultured in various types of undefined media starting with Banta (1921) who grew them in hay infusion and diluted manure. Ivleva (1973) outlined methods for mass cultivation of *D. magna* and *D. pulex* using chemically and biologically undefined media and food sources. Provasoli et al. (1970), D'Agostino and Provasoli (1970) and Murphy (1970) solved problems attendant on growing daphnids in the absence of bacteria by evolving media containing vitamins and organic substrates as well as selected algal prey. Harvey (1972) grew *D. pulex* in semi-continuous culture through several generations. Lampert (1975, 1976) described a continuous flow system in which large numbers of organisms could be produced under controlled conditions and determined total production of *D. pulex* maintained in a chemostat with a green alga as the principal resource. Berge (1978) outlined a method to produce large numbers of *D. magna* specifically for toxicity tests.

1.2 Toxicological precedents

Daphnids have been used for decades as bioassay organisms in a variety of tests to determine the toxicity of a wide spectrum of substances.[5] Toxicity tests using daphnids include short term acute-lethal procedures and longer term, lifetime and multigeneration chronic tests used to investigate physiological or genetic perturbations.

Early methods for screening hazardous chemicals have been displaced in North America by a procedure for acute toxicity tests using fish, macroinvertebrates and amphibians recommended by the United States Environmental Protection Agency (Stephan 1975). The American Society for Testing and Materials Committee E35 on pesticides has prepared and updated a working document for the proposed standard practice for conducting toxicity tests with *D. magna* (R. Comotto, EPA, Wash., D.C., personal communication). Additional uses of daphnids for evaluating the enviornmental safety of materials appear in Adema (1978), Behie et al. (1977), Biesinger and Christensen (1972), Buikema et al. (1976), Duthie (1977), Kersting (1978), Kimerle et al. (1977), Leeuwangh (1978), Leonhard (1979), Malley et al. (1979), Rausina et al. (1978) and Stroganov et al. (1979).

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[5] We encourage readers to request a recent bibliography on *Daphnia* from: Dr. A. Buikema, Jr., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, U.S.A. 24061.

Are daphnids reliable indicators of toxicity? Canton and Adema (1978) showed that short term toxicity tests (LC_{50} 48 h) with three *Daphnia* species were reasonably reproducible. Westlake et al. (1978) recommended daphnids for superior sublethal bioassays. These organisms yielded the lowest LC_{50} (3.1%) for liquid oil effluent in a series of sublethal toxicity tests. In various tests with rainbow trout and flagfish, the EC_{50} for this effluent ranged from 10-50%.

1.3 Geographic distribution

D. magna and *D. pulex* are holarctic species. *D. magna* occurs chiefly in temporary ponds, small lakes and in sewage lagoons throughout most of North America. It is present in Eurasia from England and North Africa to China and Manchuria (Brooks 1959). *D. pulex* occurs in ponds and lakes throughout North America (except in the southeastern United States or Alaska), South America, Greenland and Europe.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

Brooks (1957) described the morphology of *Daphnia* and dealt with taxonomic difficulties raised by the polymorphic habit of various species.

2.1.1 *D. magna*

D. magna adult females are large (2.3-6 mm). The first instar mean length is 0.99 mm (Hutchinson 1967). In addition to this large size, two other characteristics are particularly helpful in distinguishing this species: 1) the deeply lobed margin of the postabdomen and 2) ridges on the head which run parallel to the mid dorsal line.

2.1.2 *D. pulex*

D. pulex adult females are medium to large (1.5-3.2 mm) (Brooks 1958, S.L. Leonhard, this work). The head is broadly rounded anteriorly and concave posteriorly. The spicules on the valves are confined to the posterior half of the ventral margin, and extend nearly to the head on the dorsal margin.

2.2 Life cycle

There are two distinct parts to the life cycle of *Daphnia* which can be explained best by the mode of reproduction.

2.2.1 Parthenogenesis

When food is plentiful and population density is low, females produce diploid parthenogenetic eggs which develop into female young. Eggs are deposited and carried in the brood chamber. They develop into completely independent neonates which eventually escape into the surrounding water. The

neonate continues to develop during 2-3 growth stages (instars) by molting and then passes through ∿ 5 more pre-reproductive instar stages (Anderson 1932, Anderson and Jenkins 1942) before the first clutch of eggs is produced. Each female can produce 17-20 clutches during its reproductive life (Anderson and Jenkins 1942). The mean clutch size is 12, usually reached in the eleventh or twelfth instar (Anderson and Jenkins 1942), and the maximum is about 60 (Green 1955).

2.2.2 Sexual reproduction

When populations are under stress (e.g. low oxygen levels, crowding, starvation or cooling temperatures) males are produced from diploid parthenogenetic eggs. When males appear, the females of the population start producing haploid eggs which require fertilization. Two eggs are extruded into the brood chamber, fertilized and enclosed by the ephippium, a thickening of the carapace. The entire case containing partially developed embryos is shed as a unit at the next molt. The embryos lie dormant until suitable conditions for development occur again. These embryos become females which produce diploid parthenogenetic eggs to complete the cycle.

2.3 Ecological relationships

The ecological limits for *D. pulex* vary from population to population. A list of limits and optima for *D. magna* is given below.

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
0-41	Extremes in laboratory culture.	Brown 1929
25	Optimum for laboratory culture.	Brown 1929
25	If conditions are not crowded, females will live for 40 d.	Anderson and Jenkins 1942
	Under crowded conditions females live for:	MacArthur and Baillie 1929
8	108±4 (SD)	
10	86±3	
18	45±0.5	
28	29±0.5 d.	
2. Dissolved oxygen		
Saturation - very low levels (0.6 mg L^{-1})	Hemoglobin can be produced so dissolved oxygen levels can be very low without death occurring.	Fox 1948, Fox et al. 1951

Physical parameter	Associated factors or limits	References
3. pH		
7.1-8.0	Laboratory culture.	Leonhard and Lawrence, unpublished data
4. Salinity	Can be adapted to 3-4% seawater.	El'tsina 1939
5. Ionic balance and osmotic shifts	Extremely sensitive to imbalances, particularly the major ions: Na, K, Ca, Mg.	Naumann 1934
	Survival of first instar larvae increases as osmotic pressure increases.	Stamper 1969

COLLECTION

N.B. Sections 3 and 5 are to be considered continuous if a large culture of *Daphnia* is to be set up. All equipment listed in both sections should be assembled prior to collection of field samples. Food should not be added to aquaria when animals are not present or severe oxygen depletion will result.

3.1 Preferred habitats

Large numbers of *D. magna* may be found in temporary ponds and in some sewage lagoons in summer. *D. magna* strains cultured at the Freshwater Institute include those collected from a local sewage lagoon, and another from a pothole lake in the Erickson, Manitoba area. *D. pulex* cultured at the Freshwater Institute was obtained from West Blue Lake, Manitoba.

3.2 Collection procedures

3.2.1 Equipment

1. 3-5 L glass or nalgene bottles.
2. 500 µm mesh dip net or zooplankton vertical sampler.
3. Insulated container to hold bottles.

3.2.2 Procedure

1. Half fill bottles with water from the collection site.

2. Sweep the dip net through the surface waters near the shore or take a 1-3 m vertical tow sample in deeper water.

3. Place sample in the container. Density of daphnids should not exceed 500 L^{-1}. The final requirement is 50 adult organisms to initiate the culture.

4. Place the bottles in the insulated container to maintain the temperature which should not exceed 25°C.

5. Sort within 6-8 h.

3.4 Sorting and isolation

3.4.1 Equipment and materials

1. Aquarium (10 or 25 L).

2. Dechlorinated water (Appendix B18) to fill aquarium.

3. Dissecting microscope.

4. Glass Petri dish.

5. Phytoplankton nets (230-950 µm mesh) set in frames (Appendix A4), sterile.

6. 2 L sterile dechlorinated water.

7. Pipettes (inside diameter of tip large enough to pick up adults, ∿ 1.5 mm) or eye dropper.

8. Aeration system (e.g. pump and air stone).

9. Aquarium as described in Section 5.2.

3.4.2 Washing and separation method

1. Place sample diluted 1:5 with dechlorinated water (brought to sample temperature) into the aquarium.

2. Aerate gently as in Section 5.2.2.2 to avoid 'floater daphnids', i.e. organisms trapped at the water surface because of air bubbles caught under the carapace or in the gut.

3. Allow water to reach 20-22°C over 24 h.

4. Sieve sample through the series of phytoplankton nets until the *Daphnia* adults are isolated from other species.

5. Flush the net with sterile dechlorinated water.

6. Place contents of nets in the glass dish containing dechlorinated water on the microscope stage.

7. Using the pipettes or eye droppers, transfer 50 daphnids to a wet net of same size and flush five more times with sterile dechlorinated water. Place in aquarium as described in Section 5.2.

MASS CULTURE TECHNIQUES

5.2 Large volume techniques (*D. magna*, *D. pulex*)

5.2.1 Equipment and materials

1. Aquarium and screen, aeration system (Appendix A5), 10 or 25 L.
2. Large pipettes (inside diameter of tip 1.5 mm) or small aquarium nets.
3. Standard food (Cerofood) (Appendix B13).
4. pH meter or indicator.
5. 1 mol L^{-1} NaOH, 1 mol L^{-1} HCl.
6. Dechlorinated water (Appendix B18).

5.2.2 Initial procedure

1. Fill aquarium to within 5-10 cm of top with dechlorinated water, mark level on the outside of the glass.
2. Place aeration system air stone in a back corner and establish air flow at $\sim$ 200 mL min^{-1} in a 25 L aquarium or $\sim$ 30 mL min^{-1} in a 10 L aquarium.
3. Add 0.1 mL standard food per 25 animals.
4. Determine pH. Adjust as needed to 7.1-8.0 with NaOH or HCl.
5. Add two adult daphnids L^{-1} using pipettes to select and transfer.
6. Place aquarium in ordinary room light.
7. Fit screen to aquarium top.

5.2.3 Maintenance procedure

1. Feed culture every second day (0.1 mL standard food for each 25 animals); adjust amount for increase in numbers.
2. Remove excess daphnids with aquarium nets. The population can be maintained without crowding at densities of 100 L^{-1}. Crowding causes males to be produced and ephippial eggs to be formed (see Section 7).

3. Replace evaporation losses with distilled water every week.

4. Every 2 months siphon the water to about 20 mm in depth, remove debris and bring up to volume with fresh dechlorinated water.

5.2.4 Production schedule

5.2.4.1 *D. magna* (±SD) (Leonhard and Lawrence, this work)

1. First clutch occurs 10±2 d after birth.

2. Second and succeeding clutches occur every 2-3 d.

3. Each female has an average of 10±2, (range 3-36) eggs per clutch and reproduces an average of 11±1 times. The life span is 48±9 d at 20-22°C.

5.2.4.2 *D. pulex*

Responses in *D. pulex* vary with the source of the organisms. Production of the culture at the Freshwater Institute is similar to that for *D. magna*. Shober and Lampert (1977) noted that well defined food concentrations reduced the variability in growth and production of young.

BIOLOGICALLY DEFINED CULTURE[6]

6.3 Dixenic culture (*D. magna*)

6.3.1 Axenization process (based on Provasoli et al. 1970)

6.3.1.1 Equipment and materials

1. DM medium (Appendix B14), sterile.

2. Vitamin mix (Appendix B15), sterile.

3. Antibiotic mix (Appendix B16), sterilized by passage through a 0.22 µm pore filter.

4. Depression plates (9 depressions, capacity of each ≥ 1 mL). Place in large Petri plates and sterilize.

5. Pipettes with openings 1.0-2.5 mm to admit gravid females without harm, sterile.

6. Transparent glass agglutination slides (Klein slides) with 9-12 depressions; sterilized and placed in sterile square plastic Petri plates.

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[6] All procedures in Section 6 are to be carried out using aseptic techniques.

7. Capillary tubes (75 mm long, 1.1-1.2 mm inside diameter), sterile (several to a test tube).

8. Clean Petri dish or bowl to fit on the base of a microscope, with small amount of DM medium.

9. Mouth siphon (Appendix A1).

10. Dissecting microscope.

11. 5 mL serological pipettes, sterile.

12. *Daphnia* culture containing gravid females.

13. Cultures *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* (as grown in *Tetrahymena vorax*, Section 6.2 and Appendix B8).

14. Standard test tubes, sterilized and capped.

6.3.1.2 Procedure

1. Add vitamin mixture to DM medium (Appendix B15, 16).

2. Follow procedure as outlined in *Tetrahymena vorax* 6.1.2.1-3, using antibiotic mix as the washing medium in the first six depressions of the depression plate, DM medium in the final three.

3. Attach a pipette to the mouth siphon, draw up a female in which young can be seen to move and place it into the Petri dish or bowl.

4. Observe the female at frequent intervals until neonates leave the brood pouch.

5. Pick up each neonate with a capillary tube and place in the first depression of the plate.

6. Follow procedure as outlined in *Tetrahymena vorax* 6.1.2.7-9, except daphnids need not be bathed overnight.

7. Place 2.5 mL of each algae culture into a sterile test tube.

8. Place a single washed neonate daphnid into each tube of mixed algae.

9. Maintain tubes at 20-22°C, normal room light conditions ($\sim$ 10 $\mu E \cdot m^{-2} \cdot s^{-1}$), for $\sim$ 10 h daily.

10. 48 h after cultures are initiated, start contamination tests using procedure outlined in *Tetrahymena vorax* 6.1.3 except incubate duplicate plates and tubes at 20° and 30°C.

6.3.1.3 Culture maintenance

1. In 7-10 d, the neonate matures and produces its first (primipari) clutch. The algae will be depleted so adult and young must be transferred by pouring or pipette to fresh dixenic algae culture (see 7, above).

2. The population is maintained by transfer every 5-7 d.

3. This system can be maintained in larger containers (e.g. 250 mL Erlenmeyer flasks). The amount of the medium should not exceed half the volume of the container.

STORAGE TECHNIQUES

7.1 Ephippia production

The sexual stage in the life cycle of daphnids (Section 2.2.2) is produced by a number of conditions such as temperature change, crowding, starvation and evaporation of medium.

7.1.1 Temperature change

1. Set up a culture as in Section 5.2.

2. Lower the temperature used for culture gradually, 2°C d^{-1} to 5°C.

3. If the temperature cannot be lowered to 5°, lower to 6-10°C, and use crowding, starvation, or evaporation of medium as an accompanying factor.

7.1.2 Crowding

1. Collect 2000 females (as produced in Section 5.2 or 8.3) which have neither reached the primiparous molt nor produced the first clutch (see Section 5.2.4.1) and place in 1 L medium.

2. Maintain culture at 20°C.

3. Ephippia are produced 2-4 d after primipari.

7.1.3 Starvation

1. Set up culture of females actively producing young (see Section 5). Lemcke and Lampert (1975) showed that fasting adult *D. pulex* utilize more than 67% of their fat and carbohydrates within 2-4 d. Starvation stress appears to be least for animals 2-2.5 mm in length.

2. Withhold food. Ephippia production starts in 3 d and ephippia are shed in 7 d.

7.1.4 Evaporation of medium

1. Set up culture of actively reproducing females (see Section 5).

2. Allow medium to evaporate slowly over 1 week; ephippia production ensues.

7.1.5 Collection of ephippia

7.1.5.1 Equipment and materials

1. Glass spatula or filter paper.

2. Beakers, 250 mL.

7.1.5.2 Procedure

1. Gently brush ephippia from sides of dry aquarium or skim from surface with spatula or filter paper.

2. Place in beakers; many thousand per beaker can be conveniently stored dry.

3. Dry 24-72 h at 20-22°C, cover.

4. Place ephippia in a dark cupboard or refrigerator.

7.1.6 Rehydration of ephippia

7.1.6.1 Equipment and materials

1. Vials set in racks (see Section 8).

2. Dechlorinated water (Appendix B18).

3. Pasteur pipettes with bulb.

7.1.6.2 Procedure

1. Place 20 mL dechlorinated water into each vial.

2. Place 10 ephippia into the water.

3. Hatch takes place within 24 h with 5-30% success rate.

4. Treat hatched daphnids as a first instar stage (see Section 8.2).

CULTURES FOR TOXICITY TESTS

8.1 Equipment needed

1. Parthenogenetically reproducing population as in Section 5.2

2. 50-125 glass vials, 25 x 95 mm.

3. Dechlorinated water (Appendix B18) or DM medium (Appendix B14).

4. Tray or rack to hold 10-30 vials.

5. 20°C incubator.

6. Dissecting microscope or hand lens (6x magnification).

7. Gridded Petri plate, 20 mL capacity.

8. Black plastic sheet to fit under Petri plate.

9. Beaker, 600-1000 mL.

10. Pasteur pipettes and bulb.

11. Standard food (Appendix B13).

8.2 Initial procedure

1. Allow water or medium to reach 20°C.

2. Place 20 mL water or medium into each of 25-30 vials, set into tray.

3. Place a portion of a reproducing population into a Petri dish or small beaker.

4. Using the pipette and bulb, transfer a single female, preferably bearing eggs, into each vial.

5. Add 0.01 mL standard food to each vial and 0.01 mL every other day thereafter.

6. Place vials at 20°C.

7. Change water every other day, prior to feeding.

8.3 Age-classed culture procedure

1. Set up 25-100 vials as in 8.2.

2. Examine vials for young each day.

3. If young are present, tip vial contents into the gridded Petri dish set on the black plastic.

4. Using a microscope or hand lens, remove young and place singly in vials or place young of the day into a 600-1000 mL beaker, 1/2-3/4 full of water or medium. Record the time of birth.

5. In vials, young are raised to adulthood. Young arising from these daphnids are used in toxicity tests.

6. In beakers, young of the day are raised to adulthood and used as

brood stock of known age and history.

8.4 Maintenance procedures

1. In vials, feed 0.01 mL standard food every other day, change water or medium before feeding. Maintain at 20°C or room temperature not to exceed 25°C.

2. In beakers, feed 0.01 mL standard food for each organism every other day. Maintain at 100 organisms L^{-1} at $\sim$ 20°C but do not exceed 25°C. Change water every other day prior to feeding.

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Artemia sp. L.

S.L. Leonhard

INTRODUCTION

1.1 General information

Artemia (Anostraca:Artemiidae), the brine shrimp, is a small primary consumer in highly saline lakes and salterns throughout the world. It is widely cultured as a food for fishes and macrocrustaceans. Depending on the source of the stock, reproduction is bisexual or parthenogenetic and viviparous young or hard-shelled resting eggs (cysts) may be produced. Dried cysts remain viable for several years and a culture may be initiated within 48 h after incubation in saline water. Hatching yields numerous nauplii of uniform age and known history which require minimal maintenance in the laboratory. The brine shrimp may therefore be an ideal test organism for bioassay.

Persoone and Sorgeloos (1972), Sorgeloos (1973) and Sorgeloos and Persoone (1975) have developed culture systems for *Artemia* and Provasoli and D'Agostino (1969) developed defined media. Littlepage and McGinley (1965) compiled a bibliography of *Artemia* that provides abstracts of historical references. The proceedings of the International Symposium on the Brine Shrimp (held in Corpus Christi, Texas, August 20-29, 1979) provide current information on morphology, genetics, radiobiology, physiology, biochemistry, molecular biology, ecology and culture (Persoone et al. in press).

1.2 Toxicological precedents

While toxicity data obtained using brine shrimp are not strictly applicable to freshwater systems, in the absence of any other data, TL_m values should provide an estimate of toxicity within an order of magnitude (Price et al. 1974). *Artemia* has been used as a screening organism for fungal toxins (Harwig and Scott 1971) and petrochemicals (Price et al. 1974), and in bioassays for insecticide residues (Michael et al. 1956) and oil dispersants (Zillioux et al. 1973). Effects of DDT and derivatives of non-persistent pesticides on reproductive performance of *Artemia* were examined by Grosch (1967, 1973) and Cunningham (1976) assessed effects on reproduction of dimilin, an insect growth regulator. Acclimation and tolerance of *Artemia* to copper salts were investigated by Saliba and Krzyz (1976).

1.3 Geographic distribution

Artemia is cosmopolitan, found usually in warm, dry regions on every continent. It occurs in bodies of water of almost any size which have no appreciable outflow and which have become unusually saline as a result of evaporation. In the U.S.A., the best known sources are the Great Salt Lake, Utah and salterns in San Francisco Bay, California. These localities represent environments in which sodium chloride is the principal salt. In Canada, Chaplin Lake, Saskatchewan is a reliable source of *Artemia*. In this location, sodium sulfate is the major salt.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

Lockhead (1941) outlined the general biology and morphology of *Artemia*. Sorgeloos (in press) described development from egg to adult.

Artemia is not a true shrimp. Adult females and males are present in about equal numbers and are approximately 12 mm long. They lack a carapace and have "phyllopodous" trunk limbs which beat constantly, a simple ladder-like nervous system, with segmentally arranged ganglia and a long tubular heart with paired ostia in nearly every trunk segment.

2.2 Life cycle

Sorgeloos (in press) described the life history of *Artemia*. Usually the life cycle begins with hydration of the inactive dry cysts (200-300 µm in diameter) in weakly saline water at 20°C. Within hours of hydration, the outer membranes of the cyst burst and the embryo appears, surrounded by the hatching membrane which gradually leaves the shell of the cyst, ruptures and frees the nauplius. Development is gradual with simple metamorphosis through about 15 molts. The digestive tract is functional at the third naupliar stage (3-4 d after hatching) at which time food must be available. From the tenth instar on, morphological changes take place leading to sexual differentiation. The adult animals (8-12 mm long at 6-9 d old) copulate and may produce 10-11 broods (Squire 1970, Grosch 1973) during the life span of the female which is ∿ 50 d (Grosch 1973). The average brood size is 134 (Squire 1970). Lockhead (1941) recorded the normal life span of both sexes at 4 months but average laboratory survival is 35-50 d. Females may produce two kinds of eggs following copulation: thin-shelled eggs in which development proceeds rapidly and which yield free-moving nauplii or thick-shelled eggs which contain late blastula stage embryos when laid and which may remain dormant for many months. Females from certain localities are parthenogenetic.

2.3 Ecological relationships

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
5-36	Larvae tolerance range in laboratory populations.	Karim 1974
	Tolerance of cyst for:	Lockhead 1941
190	1 h	
81	24 h.	
2. Salinity		
5-80%	Tolerance of larvae at 5-20°C.	Karim 1974

Physical parameter	Associated factors or limits	References
3. pH		
7.5-10.0	Optimum for laboratory cultures.	Lockhead 1941
5.0	Survival for 5 d.	Lockhead 1941
4. Dissolved oxygen (mg L^{-1})		
2-8	Extremes for laboratory populations.	Gilchrist, in Sorgeloos and Persoone 1975
3	Minimum concentration for cyst hatching.	Gilchrist, in Sorgeloos and Persoone 1975
5. Light		
2000 lx	Brief illumination of cysts after hydration needed. Duration and intensity depends on strain.	Sorgeloos and Persoone 1975

2.4 Behavior

Newly hatched *Artemia* are photophilic (Zillioux et al. 1973). The swimming appendages or phyllopoda move at 150-250 beat min^{-1} (Lockhead 1941) when the animal is healthy. This metachronal rhythm decreases when the organism is distressed. Lack of phyllopodal movement indicates death (Price et al. 1974).

COLLECTION

3.1 Preferred habitats

Saline lakes and salterns.

3.2 Collection procedures

1. Adults and resting eggs or cysts can be collected. The resting eggs can be gathered from the leeward side of evaporating brine pools. Cysts from Chaplin Lake, Saskatchewan may be collected from May to September by scooping plastic buckets along the shoreline. Aliquots are poured into standard enamel dissecting trays and rinsed with lake water. The standing lake water is decanted, the trays covered with aluminum foil or plastic wrap and shipped to the lab.

2. Adults and cysts are available commercially from many tropical fish supply stores.

HOLDING OR MAINTENANCE TECHNIQUES

4.1 Holding

The following procedures are outlined principally for *Artemia* from Chaplin Lake in which sodium sulfate is the major salt. Commercial sources of *Artemia* are usually collected from locations in which sodium chloride predominates; consequently, artificial medium components vary for each source.

Commercial cysts may be washed, dried and stored over $CaSO_4$ in a nitrogen atmosphere (Clegg 1974). Eggs on trays (Section 3.2.1) are placed at room temperature (20-25°C) in a dark cupboard, and stirred daily for 1 week. The cysts are then sieved to break up clumps and remove empty shells and packed into insect rearing vials which are sealed with silicon stoppers. These cysts may be stored at 10-25°C for up to 4 years.

MASS CULTURE TECHNIQUES

N.B. These methods are not suitable for mass generation of resting eggs (cysts).

5.1 Stock culture or age-classed animals for bioassay

5.1.1 Equipment needed

1. Insect rearing vials, 25 x 95 mm.
2. Gridded Petri plates with lids.
3. Inoculating cards (Appendix A6).
4. 5 mL beakers.
5. Spatula.
6. Artist's brush, small and fine.
7. Pipettes and bulbs.
8. Artificial saline water (ASW)[7] (Appendix B12), 100% and 50% strength.

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[7] This description applies to "sulfate" source *Artemia*; "chloride" cultures are reared in commercial synthetic seawater.

9. Standard food (Cerofood) (Appendix B13).

10. Dissecting microscope.

11. 5 µL Eppendorf syringe with disposable tips.

5.1.2 Initiation of culture

1. Fill glass insect rearing vials with 20 mL 50% ASW.

2. Using the spatula, tamp *Artemia* cysts into the well on the inoculating card (which accommodates about 1800). Brush away excess.

3. Pour contents of one card into each vial.

4. With vigorous aeration, 95% of the cysts will hatch in ∿ 26 h at 20°C in ordinary room light. Subsequent mortality is negligible.

5. The vial contents are poured into a gridded Petri plate and examined under a dissecting microscope at low power to facilitate identification of larval stages.

6. 5 larvae of equal age are transferred by pipette to a 5 mL beaker. Add 100% ASW to the Petri plate and beakers as required.

7. Gently flush the larvae with 100% ASW into an insect rearing vial. Bring volume to 20 mL with fresh 100% ASW medium.

5.1.3 Maintenance of cultures

Naupliar stages I-III, which are present 3-4 d after cysts are rehydrated, need not be fed. Thereafter, juveniles and adults which appear 6-9 d after hydration are fed 5 µL standard food per vial on alternate days following replacement of the medium. The stale medium is decanted to 1-3 mL and fresh 100% ASW to 20 mL is poured gently down one side of the vial.

5.2 Large volume techniques

This method is recommended for the continuous production of juvenile and adult *Artemia*.

5.2.1 Equipment and materials

1. 20 L aquarium (Appendix A5), screen and aeration system.

2. Artificial saline water 50% (ASW) (Appendix B12).

3. Standard food (Cerofood) (Appendix B13).

4. Pipettes and bulbs.

5.2.2 Initiation of culture

1. 0.75 g cysts (∿ 18,000) are inoculated into 20 L 50% ASW at 20°C

in continuous ordinary room light.

2. Aerate continuously but gently at one end of the aquarium only.

5.2.3 Maintenance of mass culture

1. Daily add 1.5 mL of standard food (Cerofood) to the aquarium.

2. Juveniles and adults (Section 2.2) are harvested by pipette and transferred to small beakers as required.

3. Debris is siphoned from the bottom of the aquarium every week.

4. The original volume of medium is restored weekly with deionized water.

5. Once a month decant the medium to $\sim$ 5 L and add 15 L 50% ASW to the aquarium.

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Hyalella azteca (Saussure)

B.G.E. de March

INTRODUCTION

1.1 General information

Hyalella azteca (Amphipoda:Talitridae) is a small crustacean which breeds in permanent lakes, ponds and streams throughout most of North America during the summer (Bousfield 1958). The ubiquity of this organism and the ease with which it can be cultured has made it a useful animal for experimental purposes.

1.2 Toxicological precedents

The use of *H. azteca* in toxicological experiments has been limited because of three problems: 1) inconsistency and large variation in results, 2) cannibalism while under stress and 3) lack of adequate mass culture techniques. However, some bioassays using *H. azteca* have been done. *H. azteca* does not appear to be either significantly more or less sensitive to toxicants than rainbow trout, *Salmo gairdneri* (Maciorowski 1975). Animals which are smaller due to age, culture conditions or heredity are significantly more sensitive than larger animals (de March 1979).

A bioassay should be done with animals of the same age, reared under the same culture conditions, and from the same source. Meeting these criteria should reduce the confidence interval of the LC_{50} values, therefore facilitating the comparison and characterization of toxicants and ensuring reproducibility of results. The control of reproduction with photoperiod and temperature (de March 1977), and the control of adult sizes with temperature (de March 1978) are mechanisms which can be used to produce homogeneous experimental organisms at any time.

1.3 Geographic distribution

H. azteca has been found in lakes from Guatemala ($\sim$ 16°N) (Strong 1972) and the Caribbean Islands ($\sim$ 12°N) (Bousfield 1973), to Inuvik, N.W.T. ($\sim$ 68°N) (P. Stewart, personal communication), and from the Atlantic to the Pacific, including coastal islands. The northern limits of its distribution, corresponding approximately to the tree line, are probably determined by temperature (de March 1978). The southern limits of its distribution may be determined by southern limits of North American waterfowl species distributions (Niethammer 1953, Rosine 1956, Maguire 1963), since amphipods can be carried in their feathers.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

Saussure (1858) first described the species and named it *Amphitoe aztecus*. It was more thoroughly described as *Hyalella dentata* by Smith (1874), as *H. knickerbockeri* by Weckel (1907), and as *H. azteca* by Stebbing (1906), Saunders (1933) and Shoemaker (1942). Bousfield (1958) gives an excellent description of *H. azteca*. The species is not easily confused with any other, so simple keys such as Pennak (1953) are sufficient for identification.

The genus *Hyalella* is the only freshwater representative of the superfamily Talitroides which generally consists of "beach flies" (Bulycheva 1957). This taxon contains animals considered to be primitive and of terrestrial ancestry (Bousfield 1973). Most members of the taxon including *H. azteca*, have the ability to "walk" over short stretches of dry land. *H. texana*, probably a close relative of *H. azteca*, occurs on the Edwards Plateau in Texas (Stevenson and Peden 1973). *H. pampeana* and *H. curvispina* are found in South American fresh waters (Cavalieri 1968). *H. azteca* shows some morphological, reproductive and behavioral variation within North America (Bousfield 1958, Strong 1972, 1973, Pennak and Rosine 1976, de March 1977, 1978). Genetic exchange probably takes place through transportation by waterfowl (Niethammer 1953, Rosine 1956, Maguire 1963).

Geisler (1944) gives an account of the morphology, mating behavior, sexual development and molting patterns of *H. azteca*. Adult males are larger than females and have larger second gnathopods. The second gnathopods of the female retain the juvenile form although they lengthen with growth of the animal. Eggs in the female can be seen both in the ovaries and in the brood pouch.

2.2 Life cycle

Reproduction is obligately sexual. The eggs and then live young are carried in the brood pouch in the abdomen of the female. At each mating, the female molts and releases the young from the previous mating. The young go through $\sim$ 8 molts to maturity. Mature adult females can produce 1-30 young at each molt; the number is predictably related to the size and source of the female (Strong 1972).

H. azteca populations in Manitoba initiate and terminate reproduction in response to photoperiod in the spring and fall respectively (de March 1977). In southern Manitoba, reproduction occurs $\sim$ 3 months after the spring equinox, usually in conjunction with temperatures > 20°C. The start of reproduction is delayed at colder temperatures. Some populations produce only one generation per year (Mathias 1971) but up to 5 generations are produced in warmer climates. Summer animals are relatively small and winter animals, large. In laboratory cultures, adult sizes of *H. azteca* were inversely proportional to early development temperatures (de March 1978). This relationship also most likely applies to natural populations. Mating stops around the fall equinox (de March 1977) but does not occur in the last brood. All adults except the last brood(s) produced die in the fall (Embody 1911, Cooper 1956, Strong 1972). The overwintering animals become large (Cooper 1965, Strong 1972, de March 1978) and contain relatively low quantities of lipid, chitin, and high quantities of carbohydrate compared to summer populations (Yurkowski and Tabachek 1977). All detectable activity by these amphipods stops at temperatures slightly < 10°C. Animals may be cooled to 0°C, or possibly less, during the winter (Pennak and Rosine 1976). In the spring, the adults produce large numbers of young quickly and synchronously. These adults may die when they have reproduced (Cooper 1965, Strong 1972), or reproduce several times before dying, as did animals transferred to this laboratory.

The annual cycle may differ geographically. Some populations, especially the more southern ones, may not respond to photoperiod as a cue for reproductive behavior (e.g. the Oregon population, Strong 1972). As well, the adult

size - developmental temperature response may be different at different latitudes (de March 1978). Lack of food or oxygen probably can prevent reproduction or growth when photoperiods and temperature are otherwise favorable (Wilder 1940).

H. azteca is considered to be a detritivore. In this laboratory, it was found that foods high in protein (e.g. Tetra-Min B) were ingested most quickly and left the tanks clean. Plant material may be ingested less efficiently. Hargrave (1970) found that both ingestion rate of material epiphytic on *Chara* (13.3-115 μg h^{-1}) and efficiency of assimilation (4.7-92%) were highly variable. Ingestion of bacteria and diatoms ranged from 25-30.5 μg h^{-1} and efficiency of assimilation from 60-82.5%. Bluegreen and green algae were less used as food, and were not assimilated as efficiently.

H. azteca is an important food source for fish, waterfowl, wading birds, salamanders and larger invertebrates (Jackson 1912, Titcomb 1930, Cooper 1965, Mathias 1971, Podesta and Holmes 1970a, b).

2.3 Ecological relationships

Biological parameter	Associated factors or limits	References
1. Hatching time (days)		
18-22	20-22°C	Bovée 1950
5-10	26-28°C	
26.5	15°C	Cooper 1965
12.7	20°C	
9.3	25°C	
8.5	23.6°C	Embody 1911
8.0	24.0°C	
# Days = 1/[(0.00864 x °C) - 0.759]		de March 1978
2. Number of instars		
Prereproductive: 5 to 8		Cooper 1965
Postreproductive: indefinite		Geisler 1944
3. First instar duration (days)		
9	17.3°C	Embody 1911
5	25.3°C	
40+	10°C	Cooper 1965
11	15°C	
5	20°C	
4	25°C	

Biological parameter	Associated factors or limits	References
3. First instar duration (Continued)		
8-12	16-18°C	Geisler 1944
4. Time to first mating (days)		
98	15°C	Cooper 1965
26	20°C	
33	25°C	
# Days = 1/[(0.00188 x °C) - 0.0130]		de March 1978
5. Adult molt period (days)		
a. Females		
18-20	20-22°C	Bovée 1950
7-8	26-28°C	
b. Males		
21-25	20-22°C	
8-10	26-28°C	
6. Growth of juveniles		
Age (days) / Size (mm)		
0 (birth) / 1.27	23°C	Embody 1911
10 / 1.63		
20 / 2.04		
40 / 3.2		
80 / 6.4		
7. Adult head length (mm)		
Oregon population:		
0.35-0.85	20-27°C	Strong 1972
Michigan population:		
0.48-0.70	15-25°C	Cooper 1965
Manitoba population:		
0.84±0.10 (SD) (males)	10°C during early development.	de March 1978
0.76±0.02 (females)		
0.57±0.07 (males)	23°C during early development.	
0.48±0.06 (females)		

Biological parameter	Associated factors or limits	References
8. Adult length (mm)		
2-7.5	25°C	Strong 1972
2-10	10-26°C	de March 1977

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
0-33	Natural temperature range.	Embody 1911 Bovée 1949 Sprague 1963
26-28	Optimum young per unit produced.	de March 1977, 1978
0-10	Resting temperatures: complete immobility, no feeding.	de March 1977, 1978
	Determines limits of distribution.	Bousfield 1958
10-18	Juveniles destined to be "large" adults are produced. Low reproductive rate. Long maturation time.	de March 1978
$\geq$ 20	Juveniles destined to be "small" adults are produced. High reproductive rate. Short maturation time.	de March 1978
33-37	Lethal value depends on population.	Bovée 1949 Sprague 1963
2. Light (Photoperiod = alternating number of hours of light and dark)		

Physical parameter	Associated factors or limits	References
2. Light (Continued)		
16L:8D or >16L	Breeding successful and continuous.	de March 1977
8L:16D or <8L	Reproductive resting stage.	de March 1977
12L:12D	Induces reproduction after lower daylengths and stops reproductive after longer daylengths. Will maintain resting stage indefinitely.	de March 1977
3. Light intensity[8]		
12 $\mu E \cdot m^{-2} \cdot s^{-1}$	Terminates reproduction at 20-30°C, at 16L:8D.	de March 1977
55 $\mu E \cdot m^{-2} \cdot s^{-1}$	Guarantees reproduction.	de March 1977
2200 $\mu E \cdot m^{-2} \cdot s^{-1}$	Maximum light intensity at water surface in natural waters in Manitoba.	J. Shearer, FWI, personal communication
10 $\mu E \cdot m^{-2} \cdot s^{-1}$	Average room light intensity.	
4. Oxygen		
Low O_2 tension	Survival in stagnant tanks.	de March unpublished data
0.7 mg L^{-1}	Survival 24 h LC_{50} at 20°C.	Sprague 1963
0.7 mg L^{-1}	Average O_2 death point.	Pennak and Rosine 1976
5. Ionic balance		
Ca >7 mg L^{-1}	Lowest concentration at which observed in the field.	de March unpublished data

[8] 1 $\mu E \cdot m^{-2} \cdot s^{-1}$ = 102.47 lx in the laboratory in which experiments were performed. Conversion may be different for different types of light. Light intensity and photoperiod probably act synergistically.

Physical parameter	Associated factors or limits	References
5. Ionic balance (Continued)		
Salinity 30 ppt	Will survive at this concentration if slowly acclimated.	U.T. Hammer, Univ. of Saskatchewan, personal communication; de March unpublished data
6. pH		
6-8	Optimum survival in bioassay.	de March 1979
4-5	Gradual mortality occurs.	de March 1979

2.4 Behavior

2.4.1 Non-sexual behavior

H. azteca remains hidden during the day but feeds. It prefers to be near, but hidden from, strong light (Holmes 1901, Phipps 1915). Animals appear to be very active at night. When lights are first turned on the laboratory, it takes several minutes for swimming activity to cease. Activity levels are probably also related to hunger and temperature.

Animals will often swim into a weak current, possibly an adaptation for living in streams. The author has seen animals swimming into a current in order to avoid prolonged exposure to a toxicant "plug" (e.g. laundry bleach) going through a rectangular tank.

2.4.2 Sexual behavior

Mating may be initiated at night when aimals are most active. *H. azteca* shows complex, ritualized mating patterns (Kruschwitz 1972, Strong 1973). The male mounts and typically 'rocks' the female and may be carried for some time. Actual copulation takes place after the female molts.

Strong (1973) showed that the proportion of the female molt cycle spent in amplexus (i.e. paired) varied among populations, and was negatively correlated with the intensity of predation by fish. Amplexus lasted 1-5 d at 22 and 25°C (Strong 1973). It may last several weeks at 10°C (de March, unpublished data) or may occur within one night at 26°C, and thus not be detected (de March 1977).

2.5 Disease and parasites

Hyalella azteca is the intermediate host for several parasites, but only one host-parasite relationship has been well studied (Bethel and Holmes 1973, 1977, Podesta and Holmes 1970a, b). Cystacanths of the acanthocephalan helminth

Corysoma constrictum Van Cleave 1918, which appear as a red inclusion in the body cavity, cause *H. azteca* to become photophilic (i.e. prefer well lit areas) and phototactic (i.e. swim into the light when disturbed). The amphipod is therefore more conspicuous, and more prone to being eaten by its predators. In southern Manitoba, *H. azteca* is usually infected in late fall, during the waterfowl migration, when eggs of the parasite are released in the feces of the birds and ingested by the amphipod. The cystacanths of the parasite are carried by the large, overwintering animals until spring. Waterfowl are reinfected by eating these conspicuous amphipods during the spring migration. The parasite disappears in the summer breeding population of *H. azteca*.

Bethel and Holmes (1973, 1977) showed that different parasites in *Gammarus lacustris* caused unique abnormal behavioral traits which made the amphipod more susceptible to being eaten by the definitive host. These same parasites live in *H. azteca*, where the behavioral changes induced by the parasites may be the same as in *G. lacustris*.

Two larval trematodes of fish occur in *H. azteca* (Hazen and Esch 1977), but effects on the amphipod were not described.

COLLECTION

3.1 Preferred habitats

H. azteca is most abundant in permanent warm eutrophic or mesotrophic lakes in which aquatic plants such as *Chara*, *Elodea*, *Myriophyllum*, *Sphagnum*, *Lemna* and *Utricularia* are common. It has been found in lakes, ponds, sloughs, marshes, rivers, streams, ditches and spring streams, but not always in high numbers (Bousfield 1958). Lakes which support populations are usually 20-30°C for most of the summer. The populations maintained at the Freshwater Institute were collected from prairie pothole lakes near Erickson, Manitoba (Sunde and Barica 1975). In this area, densities of > 10,000 animals m^{-2} are common. Algal mats under small dams are also often good collecting sites.

3.2 Collection procedures

Animals are collected most easily by washing aquatic plants over a 200-550 µm mesh net. A sufficient quantity of strained or filtered water ($\sim$ 30 L for the following instructions) should also be collected to start laboratory cultures.

3.3 Transport

The best transportation container is a large plastic bag containing 1 L water from the collection site, the remainder of the bag filled with air or oxygen. Up to 200 animals may be placed in the bag which should then be put into a larger container such as a box, barrel or cooler. The water temperature should not be allowed to change more than 5°C, or exceed 25°C.

3.4 Sorting and isolation

Described below are laboratory conditions which allow preliminary examination of the culture and do not promote reproduction or rapid aging.

3.4.1 Equipment and materials

1. Glass aquarium, ∿ 40 L capacity.
2. Small stone aerator, placed on an air line (an automobile fuel filter is necessary if the air contains impurities).
3. Fluorescent lights, two 30 W (optional).
4. Timer (optional).
5. Spawning mat (as used for tropical fish).
6. 30 L water from collection site (see Section 3.2).
7. Dechlorinated water (Appendix B18).
8. Tetra-Min B (Appendix B10).
9. Light meter (optional).

3.4.2 Preparation of aquarium

1. Place field-collected water into the aquarium. Heat or cool water to within 2-3°C of the temperature at which *H. azteca* are held.
2. Hang aerator in aquarium just above bottom; bubble gently.
3. Place light over aquarium so that intensity at the surface is 12 $\mu E \cdot m^{-2} \cdot s^{-1}$. Establish a 12L:12D photoperiod using the timer, or cover or enclose tank so that light intensity is no greater than 12 $\mu E \cdot m^{-2} \cdot s^{-1}$, or turn room lights off for at least 12 h each day.
4. Place spawning mat in the bottom of aquarium.

3.4.3 Transfer of animals collected to aquarium

1. Place up to 200 *H. azteca* into aquarium. N.B. Remove other species, particularly obvious predators or competitors (e.g. *Gammarus*, Coleoptera, Odonata, and voracious herbivores such as snails).
2. Allow the temperature to rise to no more than 20°C at the rate of 2°C d^{-1}. If overwintering animals were collected in cold water, and are to be maintained in the reproductive resting condition until experiment use, maintain low temperatures (<15°C).
3. Add 0.5 g Tetra-Min B. Do not add more food unless animals suddenly become active on the sediment or in the water column. The animals are photophobic and will hide in the spawning mats unless they are foraging for food.

4. Each day for one week, remove 20% of the field collected water, and replace with dechlorinated water.

MASS CULTURE TECHNIQUES

5.1 Stock culture (static system)

5.1.1 Equipment and materials

1. As listed in 3.4.1.1-8.
2. Fortrel or cotton batting, soaked in water overnight, or spawning mats.
3. Quartz sand of grain size smaller than animals of interest.
4. 10 mL serological pipette and bulb.
5. *H. azteca* population as established in 3.4.3 or other culture established in dechlorinated water.

5.1.2 Preparation of aquarium

1. As in 3.4.2.1-4.
2. Bring light level up to at least 55 $\mu E \cdot m^{-2} \cdot s^{-1}$ and establish a photoperiod of 16L:8D or >16L.
3. Place 5 mm layer quartz sand into aquarium.
4. Place fist sized piece of batting or mat into aquarium.
5. Mark level of dechlorinated water on outside of aquarium.

5.1.3 Initial procedure

1. Connect bulb to pointed end of pipette.
2. Transfer 50 adults to aquarium from *H. azteca* population as established in 3.4.3 using the pipette. A 1:1 sex ratio is established by chance only, since sexes are difficult to distinguish in non-breeding populations.
3. Add ~ 0.2 g Tetra-Min B.
4. Heat or cool water to 20-26°C, depending on type and number of animals required (see Section 2.3).

5.1.4 Maintenance procedure

1. Maintain population at 50 adults and 200-300 young by cropping using a pipette and bulb or a net. N.B. This number will be

reached in 3-4 weeks if mating takes place shortly after the population is established.

2. After the initial addition of food, add as much Tetra-Min B as required (*ad libitum*). 0.1-0.2 g 3 times a week is approximately the required amount. To prevent buildup of excess decaying food, it is suggested that animals be fed only when they become active on the sediment or in the water column.

3. Each week, remove 1/3 of the water present (not counting evaporation losses), and bring volume to the original level with dechlorinated water.

5.2 Large volume technique

5.2.1 Equipment and materials

1. Gridded continuous flow system (Appendix A10).

2. Dechlorinated water (Appendix B18).

3. Light meter.

4. Tetra-Min B (Appendix B10).

5. Stock culture of *H. azteca* as described in Sections 5.1.3 and 5.1.4.

6. 10 mL pipette with bulb.

7. Small, fine aquarium net.

5.2.2 Initial procedure

1. Adjust water temperature to that of the stock culture.

2. Establish water flow to a replacement rate of 4 h.

3. Establish light to a surface intensity of $\sim$ 55 $\mu E \cdot m^{-2} \cdot s^{-1}$ using the light meter.

4. Establish photoperiod of 16L:8D.

5. Transfer 50-100 adults into each culture tray using the pipette and bulb.

6. Add Tetra-Min B as in 5.1.4.2.

7. Adjust temperature to a constant value between 18° and 26°C at the rate of 2°C d^{-1}.

5.2.3 Maintenance procedure

1. Maintain light intensity, photoperiod, temperature and flow rate as established.

2. Remove unused food prior to feeding.

3. Add Tetra-Min B *ad libitum*.

4. Numbers should be maintained at 200 adults and young by cropping with the pipette and bulb or the small net.

5.2.4 Productivity of cultures

Once the cultures are established, the doubling time at 20°C will be < 2 weeks. Cooper (1965) estimated that the maximum possible turnover rate in a natural population was 4% d^{-1}. This can no doubt be exceeded in the laboratory if animals are not stressed.

STORAGE TECHNIQUES

7.5 Reproductive cycle manipulation

The determination of factors affecting the induction and termination of the reproductive resting stage was carried out in groups of animals of mixed age structure. For this reason, it is not known which size or age classes responded. It is probable that virgin females responded to the inductive stimulus most strongly and that older females did not respond for more than one reproductive season. Enough time should elapse between the termination and the induction of the reproductive resting stage to allow young to reach adult size, or to allow females to produce the desired number of young, whichever applies.

7.5.1 Induction of reproductive resting stage

7.5.1.1 Equipment and materials

1. Reproducing culture of *H. azteca* (Section 5.2) in a continuous flow system (Appendix A10).

2. Dechlorinated water (Appendix B18) in which dissolved oxygen level is near saturation.

3. Light meter.

4. Tetra-Min B (Appendix B10).

7.5.1.2 Initial procedure

1. Use only if large overwintering animals are desired: lower temperature to 16°C at 2°C d^{-1}. Leave for 1 month.

2. Establish photoperiod at 8L:16D.

3. Dim light to at least 12 $\mu E \cdot m^{-2} \cdot s^{-1}$.

7.5.1.3 Maintenance procedure

1. Wait for 2 weeks for last brood to be released.

2. Lower temperature at 2°C d^{-1} to a constant temperature between 0 and 16°C. Animals age less at lower temperatures; however, fluctuation is more stressful at low temperatures. 10°C is recommended for long-term storage. Animals can be stored for 6 months at this temperature.

3. Establish flow of dechlorinated water over culture tank at replacement rate of 10 h.

4. Add Tetra-Min B to culture *ad libitum* once weekly. Little may be required.

7.5.2 Termination of reproductive resting stage

7.5.2.1 Equipment and materials

1. Culture of resting *H. azteca* as established and maintained in Sections 3.4.3 or 7.5.1.2 and 7.5.1.3 above.

2. As described in Section 7.5.1.1.2-4 above.

7.5.2.2 Initial procedure

1. Change photoperiod to 16L:8D.

2. Raise surface light intensity to ∿ 55 $\mu E \cdot m^{-2} \cdot s^{-1}$ in increments of 10 $\mu E\ d^{-1}$.

3. Raise temperature of resting culture (see 7.5.1.3 above) to a constant value between 20° and 26°C, at 2°C d^{-1}.

4. Establish flow of dechlorinated water over culture tank at a replacement rate of 4 h.

7.5.2.3 Maintenance procedure

1. Maintain temperature, photoperiod and water flow as established.

2. Remove uneaten food prior to feeding.

3. Feed cultures Tetra-Min B *ad libitum* three times weekly; more food will be required with warm temperatures.

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Gammarus lacustris lacustris G.O. Sars

B.G.E. de March

INTRODUCTION

1.1 General information

Gammarus lacustris (Amphipoda:Gammaridae) is a common freshwater crustacean which lives in permanent lakes, ponds and small streams in northern temperature climates of North America and Eurasia. *G. lacustris* is important as fish food in many northern areas of the world. Consequently, most of the literature, both in North America and Eurasia, consists of production and biomass estimates in studies concerned mainly with fish. There are relatively few studies on the autecology of *G. lacustris*. However, in many ways, *G. lacustris* is typical of the genus and studies of many other fresh and brackish water species are applicable to it as well.

1.2 Toxicological precedents

The hardiness of *G. lacustris*, its convenient size and ubiquity have made it a popular bioassay organism for the last 20 years. It is recommended as a test species of the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). A variety of toxicants and stressors have been tested with this species (Nebeker and Gaufin 1964, Gaufin et al. 1965, Macek et al. 1976a, b, Sanders 1969, 1972, Smith 1973). It is generally hardy under control conditions, but exhibits a wide range of susceptibilities to different compounds. Gaufin et al. (1965) observed that many specimens found dead in test aqauria during bioassays were in the process of molting and suggested that this part of the life cycle was highly susceptible. They further describe *G. lacustris* as the "ideal test animal(s), adapting readily to all requirements of the bioassay laboratory". Other *Gammarus* species have also been used for bioassays (Rehwoldt et al. 1973, Arthur and Leonard 1970, Arthur and Eaton 1971, Judy 1979).

G. lacustris can be used in behavioral studies since it swims in the water column continuously when not sated by food. Swimming speeds, respiration rates, activity patterns, diurnal rhythms and avoidance patterns can be observed. I have monitored preference, avoidance and/or intoxication in response to different dissolved components of heavy metals in water in a preference-avoidance test system similar to that described by Maciorowski et al. (1977). *G. lacustris* appears to be capable of detecting low sublethal concentrations of some metals.

G. lacustris has potential as a food organism in fish toxicant bioaccumulation studies because of its importance in food chains. In addition, toxicants, just as parasites, may cause behavioral changes which influence the chance of *G. lacustris* being eaten (see Section 2.5).

1.3 Geographic distribution

G. lacustris is found in North America north to the Arctic coast, in Europe north to the Faeroe Islands and the Scandinavian countries and in Russia most likely to the northern coast. It is not found in Iceland, Greenland, or the Canadian arctic islands. Distribution data (Holsinger 1972) and temperature tolerance data (Sarviro 1977, Smith 1973) suggest that the southern limits of distribution may be defined by maximum mean summer temperatures near

20°C, and limited by maximum high temperatures near 30°C. In North America and Eurasia, it extends farthest south in the mountainous areas of New Mexico, California, Asia Minor and Italy.

These amphipods are conspicuous prey for small fish, and are possibly devoured to extinction in lakes with little cover. *G. lacustris* is more common in arctic lakes without large predatory fish than in lakes with such fish. I do not believe that water hardness limits its distribution (e.g. Titcomb 1930), since it occurs in arctic soft water lakes and is easily cultured in soft water in the laboratory. *G. lacustris* is dispersed on the feathers of waterfowl (Daborn 1976) and on the fur of small mammals (Peck 1975), as are other amphipods.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

G. lacustris is in many ways a typical gammarid, a group described by Bousfield (1973). North American freshwater and marine Gammaridae have undergone rapid recent evolution related to glaciation patterns and chance isolations. For this reason, the taxonomy of the family is complicated. Diagnostic features may be variable and species may differ by seemingly minor details. Speciation appears to have occurred primarily by adaptation to different environmental ranges. One of the best clues for identification is the source of the population and this knowedge, coupled with the excellent keys for freshwater species by Bousfield (1958) and Holsinger (1972), makes identification easy. There are nine freshwater species of *Gammarus* (Holsinger 1972) and 10 Atlantic coast species (Bousfield 1973).

There are several subspecies and races of *G. lacustris* in North America and Eurasia. *G. lacustris* G.O. Sars 1864 occurs in North America in the interior of the continent and in Europe. *G. lacustris limnaeus* S.I. Smith 1871 (previously *G. limnaeus*) occurs in the St. Lawrence River drainage basin and in Newfoundland (Bousfield 1958). The two species are interfertile (Hynes and Harper 1972), and diagnostic characteristics are variable (Holsinger 1972). Instructions in this culture manual are probably applicable to both subspecies.

2.2 Life cycle

Reproduction is obligately sexual. The females mate, carry eggs and then young in a brood pouch on the abdomen. The young are released, often over a period of time, when they reach a certain stage of maturity or when the female is "squeezed" by pairing with a male. Mature females produce up to 60 young per brood (de March, this work). The number is predictably related to the size of the female (Biette 1969, Menon 1966).

The number of broods produced in the summer depends on water temperatures. In Big Island Lake, Alberta, adults reproduced once in the summer and then died (Menon 1966). Near Erickson, Manitoba (50°30'N, 100°10'W), females produced either one or two broods each summer (J.A. Mathias, FWI, personal communication). Laboratory populations collected in the spring and held at 15°C have produced three broods in approximately 5 months. However, only ∿ 10% survived

to produce the third brood.

Most populations, regardless of life expectancy, commence pairing in winter or early spring, and produce young in the same spring or summer. Day-length of 12 h or less acts as a reproductive stimulus in adult-sized animals (de March, unpublished data). Adult-sized animals collected in late fall (October) and kept at laboratory temperatures between 10-20°C commenced reproductive behavior and produced young in several months, while those collected in mid-summer did not. Temperatures affected only the rate of reproduction, and did not act as a reproductive stimulus or deterrent (Smith 1973).

Egg incubation times were 4 and 2 weeks at water temperatures of 14.9°C and 22°C respectively in animals from Big Island Lake, Alberta (Menon 1966). Maturation time varied from 2 months to several years, depending on temperature and food. In colder climates, e.g. near Chesterfield Inlet, N.W.T., animals require at least 3 years to mature (de March, this work).

G. lacustris is an opportunistic species, eating whatever is available to it. It survives on a diet of algae in many arctic lakes (Moore 1977). Protein fish foods such as Tetra-Min B and trout pellets are eaten readily. In natural conditions, *G. lacustris* will often skeletonize dead fish in nets in several days or bite the hairs on swimmers' legs. *G. lacustris* can also be predatory and its presence influences the structure of the zooplankton community in lakes (Anderson and Raasveldt 1974). It may feed on chironomids in the winter (Menon 1966). I have seen *G. lacustris* eat live Trichoptera and minnows. Sparsely fed cultures always appear to be clean and healthy since the weakest animals are eaten, probably when they molt.

2.3 Ecological relationships

Biological parameter	Associated factors or limits	References
1. Egg production		
Number per female	Dependent on size of females.	Hynes and Harper 1972
Mean = 35.5 eggs	13 mm females; mid-summer, Credit River, Ontario.	Hynes and Harper 1972
Mean = 5 eggs	7 mm females; early August, Credit River, Ontario.	Hynes and Harper 1972
2. Juvenile production		
Number per female per molt	Dependent on size of females.	de March this work
Mean = 25	Field-collected near Auburn, New York, held in laboratory.	Embody 1911

Biological parameter	Associated factors or limits	References
2. Juvenile production (Continued)		
Maximum = 59	Field-collected near Sturgeon Lake, Minnesota and held in laboratory conditions.	Halligan and Eaton 1978
3. Hatching time		
∿ 1 month	15°C	de March this work
∿ 4 months	5°C	Menon 1966 de March this work
40-49 d	12°C	Hynes and Harper 1972
4. Maturation time and rate		
4-5 months 2.5-3 months	15-24°C 27°C	Smith 1973
∿ 3 months ∿ 4500 (°C x days)	15°C	de March this work
5. Period between molts (days)		
38 32 30 26	9°C 14°C 15.5°C 17°C	Embody 1911
6. Adult size (mm)		
Largest: 22.42 Southern distribution	Collected near Auburn, New York.	Embody 1911
Largest: 26.0 Northern distribution	Collected near Tuktoyyaktuk, N.W.T., Canada.	de March this work
Size of smallest breeding female		
9	Auburn, New York.	Embody 1911
7	Credit River, Ontario.	Hynes and Harper 1972

Biological parameter	Associated factors or limits	References
7. Growth		
Age (days) / Mean size (mm): 0 / 2.16; 5 / 2.30; 10 / 2.50; 20 / 3.06; 40 / 7.50; 80 / 8.16; 140 / 10.71; 180 / 11.73	Mean temperature, 9°C.	Embody 1911
8. Oxygen consumption (cc O_2 h^{-1} g^{-1})		
∿ 400	5°C, calm water	Roux 1972
∿ 600	10 " "	
∿ 1050	15 " "	
∿ 1600	20 " "	
∿ 1800	25 " "	
∿ 1500	27 " "	
∿ 700	5°C, turbulent water	
∿ 1000	10 " "	
∿ 1300	15 " "	
∿ 1450	20 " "	
∿ 1700	25 " "	
∿ 1500	27 " "	
∿ 1000	15°C, calm water and substrate.	
∿ 1050	15°C, calm water in glass.	
∿ 1300	15°C, turbulent water and substrate.	

Physical parameter	Associated factors or limits	References
1. Temperature (°C)[9]		
0-30	Natural temperature range.	Menon 1966 de March unpublished data
17-18	Optimum temperature for growth and development.	Ferrari and Bellavere 1977, Roux 1972, Sarviro 1977, Smith 1973
2. Oxygen		
None	Survives in completely anoxic lakes up to 4 months.	de March this work
3. pH		
4 and 11	Extensive mortality in laboratory cultures.	de March this work
4. Conductivity		
50 µS cm^{-1}	Survival and reproduction near Chesterfield Inlet, N.W.T.	de March this work
5000 µS cm^{-1}	Survial at Erickson, Manitoba.	

N.B. Although *G. lacustris* is capable of surviving and reproducing at many extremes, it is sensitive to changes. Culture conditions should be gradually imposed.

2.4 Behavior

2.4.1 General behavior

G. lacustris are most active in open water at night, and when foraging for food. If recently fed, they will often stay at the bottom of tanks. They will swim into a weak current for short distances (∿ 20 cm). Unlike *G. pseudolimnaeus*, they are not well adapted for survival in a current, since they cannot maintain their position in it.

[9] Relationships between reproduction, growth or size and temperature for other *Gammarus* should provide good estimates for *G. lacustris*, (e.g. Steele and Steele 1973, 1975a, b).

2.4.2 Sexual behavior

As many gammarids, *G. lacustris* spends a good part of its adult life paired. Pairs are easily separated, and animals find new mates readily. A mature female is paired approximately 3/4 of the time. In my preference-avoidance test system (Section 1.2), I have found that a pair has the same swimming pattern as a single animal, i.e. the male controls the direction of movement. Pairs often separate under the influence of a toxicant.

2.5 Diseases and parasites

G. lacustris is the intermediate host for several parasites. These parasites do not kill the animal, but modify its behavior so that it is more likely to be eaten by the definitive host. Infection is often cyclic, i.e. amphipods are infected by migrating birds in the fall, and birds are newly infected by amphipods in spring while migrating north. The summer amphipod populations do not harbor the parasite, since it cannot be transferred from parent to offspring.

G. lacustris infected with *Polymorphus minutus* (Acanthocephala) lack carotenoid pigments in the carapace and thus appear light blue rather than brown or grey (Hindsbo 1972). The blue amphipods are more conspicuous on a dark background and are more phototropic, and are thus more likely to be eaten by waterfowl. In my preference-avoidance studies with *G. lacustris*, blue amphipods appeared to swim faster than brown in a strongly lit test.

Polymorphus paradoxus make *G. lacustris* photophilic (i.e. light-loving) causing them to cling to surface material rather than swimming to the bottom when evading a predator (Bethel and Holmes 1973, 1974, 1977). They are then eaten, usually accidentally, by muskrats, mallards and beavers, the definitive hosts of the parasite. *Polymorphus marilis* makes amphipods photophilic, but does not alter their evasive behavior. Diving ducks, which eat at the surface of submerged vegetation, are the definitive hosts of this parasite. Because these parasites are common, care should be taken to avoid contamination when handling cultures of *G. lacustris*.

COLLECTION

3.1 Preferred habitats

G. lacustris is found in the largest numbers in permanent eutrophic or mesotrophic lakes which do not often exceed 20°C, and which provide cover for the animals. They are exceptionally common in winterkill prairie potholes (i.e. those in which winter dissolved oxygen levels are too low for fish to survive) which do not freeze to the bottom in winter. In the pothole lakes near Erickson, Manitoba, local densities of 10,000 m^{-2} have been observed (J.A. Mathias, FWI, personal communication). *G. lacustris* is also found in oligotrophic arctic lakes. I found densities near 30 m^{-2} near Chesterfield Inlet, N.W.T.

3.2 Collection procedures

The collection method will depend on the type of lake and the size of the population. In shallow potholes, collecting with a net near the edges of the lake in spring often yields high numbers. Trawls can be used to collect large numbers from the water column in mid-summer. Amphipods are also easily collected by washing aquatic plants over a 1-2 mm mesh net. In mid-winter they often live just under the ice, will surge through a hole and can be collected with a dip-net. Care must be taken not to expose the animals to temperatures < 0°C.

In deeper lakes *G. lacustris* can be caught in standard minnow traps baited with pieces of fish, but SCUBA or grabs may be required. I have used a water-tight underwater settling chamber, with netting separating the chamber from a suction tube connected to a bilge pump on a boat above the diver for collecting *G. lacustris*.

A method for collecting *G. lacustris* in bulk has been developed at the Freshwater Institute (J.A. Mathias, personal communication). Animals congregate on netting suspended in the water. When this netting is drawn into a boat, the amphipods swim downward and are picked up in a bag net towed beneath the boat. As many as 100 kg of animals in excellent condition can be collected in 1 h.

3.3 Transport

See *Hyalella azteca*, Section 3.3 but do not allow temperatures to rise to > 15°C.

3.4 Sorting and isolation

As described for *Hyalella azteca* with the following differences:

3.4.3.1 Competitors for food need be removed only if they are numerous. Most are not harmful to adult *G. lacustris*.

3.4.3.2 Allow the temperature to rise to no more than 15°C.

3.4.3.3 Feed 1 g Tetra-Min B (Appendix B10). Like *H. azteca*, this species becomes active in the water column when hungry. This trait can be used to estimate their need for food.

MASS CULTURE TECHNIQUES

As described in *Hyalella azteca* with the following differences:

5.1 Stock culture

5.1.1.2 Use spawning mats rather than batting.

5.1.1.3 Sand or gravel will do.

5.1.1.4 A 30 mL pipette with 8 mm mouth.

5.1.2.3 Sand or gravel will do.

5.1.2.4 Use spawning mats.

5.1.3.3 Add 1 g Tetra-Min B (Appendix B10).

5.1.3.4 Bring water to 10-15°C.

5.2 Large volume technique

5.2.1.1.6 A 30 mL pipette with 8 mm mouth.

5.2.1.2.7 Adjust temperature to < 20°C.

5.2.1.3.4 New cultures should be started if animals reproduce, since the young may be eaten if not separated from the adults.

STORAGE TECHNIQUES

G. lacustris probably can be stored under various conditions for long periods of time. We have reared animals from the juvenile stage to non-reproducing adults > 2 years old at 15°C, a relatively high temperature. In arctic lakes with long cold winters, animals may be 4 years old before reproducing. Animals can be maintained at high densities under very cold conditions, since their metabolism is low. Gonadal development may or may not occur under the described high density storage conditions, depending on the condition of animals stored and the light regime imposed (see Sections 7.5.1 and 7.5.2).

7.4 High density storage

7.4.1 Equipment and materials

1. Tub, plastic garbage can, or aquarium of 50-200 L capacity for use as the storage container.

2. Dechlorinated water (Appendix B18).

3. Aerator.

4. Spawning mats or any matrix (e.g. coarse netting) on which animals can maintain a hold.

5. A controlled environment room set at 1°C, or a cold water bath capable of maintaining 1°C in storage container.

6. Tetra-Min B (Appendix B10).

7.4.2 Initial procedure

1. Place animals and at least 1 L water per 100 animals into storage container.

2. Connect aerator and bubble water strongly (but not so strongly that animals cannot find footholds).

3. Let system cool to 1°C over one day.

4. An appropriate daylength may be imposed (see Sections 7.5.1 or 7.5.2).

7.4.3 Maintenance procedure

1. Maintain temperature.

2. Feed 1-2 g Tetra-Min B for each 1000 animals each week.

3. Remove 10% of water once per week and replace with water of the same temperature, or maintain a low flow-through rate (> 1 day) in the storage container.

7.5 Reproductive cycle manipulation

Adult-sized *G. lacustris* will not pair and reproduce until they have been exposed to short daylengths (< 12 h light), dim light (< 12 $\mu E \cdot m^{-2} \cdot s^{-1}$) or darkness. Once females start to reproduce, they apparently produce successive broods until death.

7.5.1 Induction of reproductive resting stage

1. Rear juveniles to adult sizes at a long daylength (16 h light) under bright light (> 55 $\mu E \cdot m^{-2} \cdot s^{-1}$) at any reasonable temperature.

or 2. Collect juveniles or adult-sized animals in the fall (before equinox), and rear under the same conditions as above.

7.5.2 Termination of reproductive resting stage

Expose adult-sized animals to short daylength (8 h light), and/or dim light (< 12 $\mu E \cdot m^{-2} \cdot s^{-1}$) or darkness at 15°C. Pairing should start in approximately 2 months. Termination may taker longer at lower temperatures.

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Orconectes virilis (Hagan)

S.L. Leonhard

INTRODUCTION

1.1 General information

Crayfish (Decapoda:Astacidae) are common benthic crustaceans. They are important in the diet of various reptiles, amphibians and fish (Neill 1951) and there is global interest in crayfish culture for human food (Huner and Avault 1974) and as bait. They are probably generalized feeders and forage for food at night (Crocker and Barr 1971). Population dynamics and production of *Orconectes* have been examined by Threinen (1958), Momot (1967), Weagle and Ozburn (1972) and Davies et al. (1977). Molting has been studied by Aiken (1967, 1968a, 1969a, b) and Stevenson (1974).

1.2 Toxicological precedents

The genus has been used extensively in field and laboratory bioassays. Both young-of-the-year and adults have been used in acute lethal dose determinations and to study bioaccumulation of toxicants in various tissues. Studies of the effects of fenitrothion (Leonhard 1974, McLeese 1976), mercury toxicity (Hamilton 1972, Doyle et al. 1976), industrial effluents (Maciorowski 1975) and low pH (Malley 1980) illustrate the range of toxicity tests in which *Orconectes* has been used. A method for using *O. virilis* as a test organism is outlined in Leonhard (1979).

1.3 Geographic distribution

Orconectes virilis lives under stones in both lakes and rivers (Crocker and Barr 1971). *O. virilis* is discontinuously distributed across North America from Maine to California, and northward from the Mississippi valley to Ontario, Manitoba, Saskatchewan and the Beaver River system in east-central Alberta (Aiken 1968b). Its type locality is Lake Superior (Riegel 1959).

GENERAL BIOLOGY

2.1 Morphology and taxonomy

The morphology of crayfish in Ontario, including *O. virilis*, is described by Crocker and Barr (1971). Information on the taxonomy of species is found in Hobbs (1976). *O. virilis* is identified by its lateral rostral spines, a narrow areola, and a straight medial margin on the dactyl (Fig. 1). This last feature and the two distal tubercles on the ventral margin on the articulation between dactyl and propodus distinguish *O. virilis* from *O. immunis*, a species with which it can be confused.

The female bears a seminal receptacle or annulus ventralis on the ventral thorax (Fig. 2A). This organ receives sperm from the male and retains it prior to fertilization. Its structure is a useful species identification character.

The copulatory stylets of the male (Fig. 2B) have three morphological forms: 1) a juvenile form present before sexual maturity, 2) a non-breeding adult form (second form) which appears each spring after the first molt of a

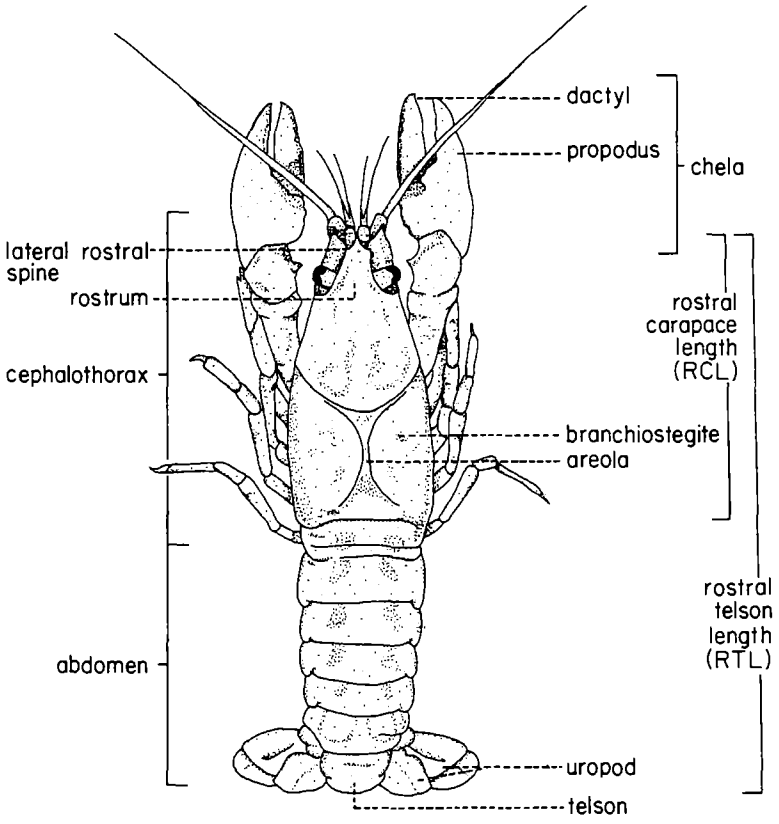

Fig. 1 Dorsal view of a mature (3 yr old class) *Orconectes virilis*

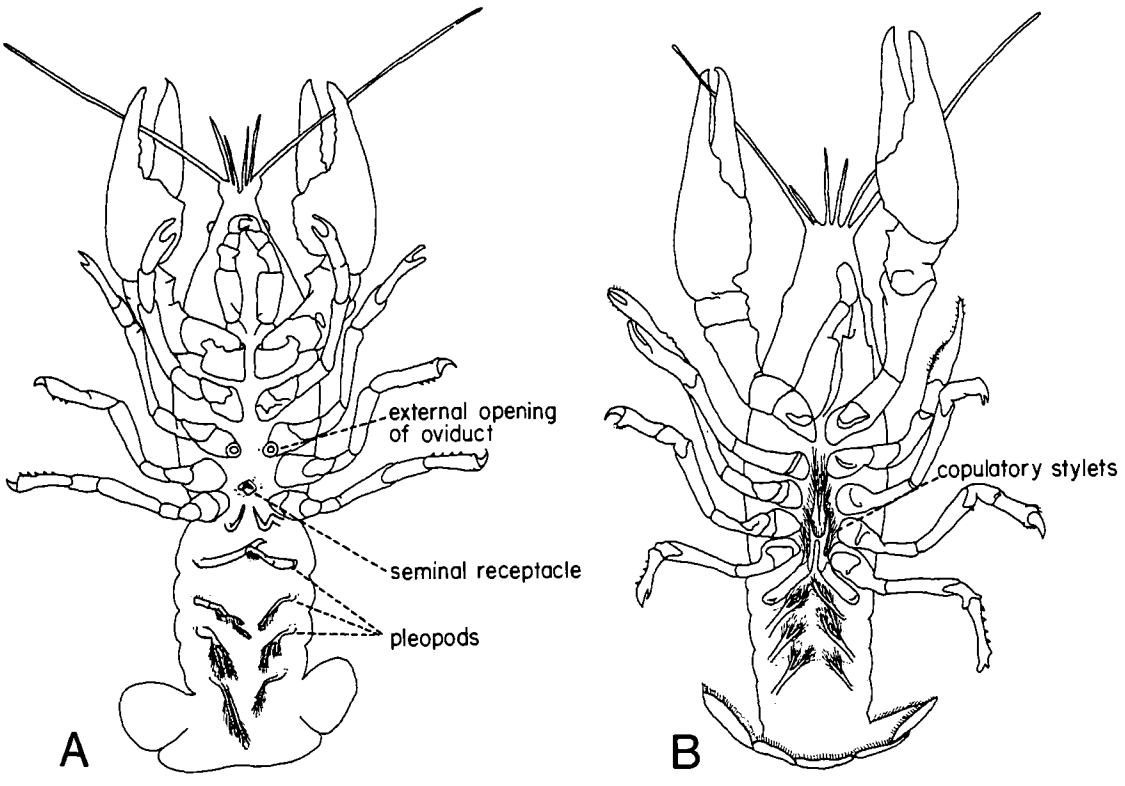

Fig. 2 Ventral view of mature *Orconectes virilis* A. Female B. Male

new growing season and 3) a breeding adult form (first form) which first appears at sexual maturity and then after the second molt of a growing season, usually during the summer. After yearling males become mature during the second summer of life, the sequence of molts is always the same; they change from reproductive to non-reproductive form in the spring and from non-reproductive to reproductive form in the summer (Momot 1967).

2.2 Life cycle

The life cycle is discussed in detail in Crocker and Barr (1971). Reproduction in crayfish is sexual. Mating takes place during the spring or fall. After the male has deposited sperm, the female carries it in the seminal receptacle (Fig. 2A) until the time of egg laying which usually occurs in the spring.

At oviposition, the female cleans her abdomen, lies on her back, fills the folded abdomen with glair (a translucent, water resistant, mucilagenous secretion), releases the spermatozoa, and extrudes the eggs. The female is said to be "in berry" when the eggs are in position. The number of eggs laid per female increases with size of the female (as measured by the length of the tail). The incubation period lasts 2-20 weeks, during which time the eggs are aerated by regular movements of the pleopods (Fig. 2A). The newly hatched crayfish are carried by the female until they reach the third instar when they are released and become free living. Size increases at each molt.

The molting cycle of *Orconectes* can be classified as follows (Stevenson 1974):

Stage A_1: begins when the crayfish molts; the molted animal is soft; lasts only a few hours.

A_2: the new carapace becomes leathery and secretion of postexuvial cuticle begins; lasts $\sim$ 1 d.

B : the carapace becomes brittle but the postorbital ridge and cervical groove can still be bent readily; lasts 1-3 d.

C_1: the postorbital ridge and cervical groove become rigid and changes in the pre-exuvial layers of the cuticle (e.g. phenolic tanning) are completed; lasts $\sim$ 2 d.

C_2: the carapace becomes rigid (except for the gastric region and areola); lasts $\sim$ 2 d.

C_3: the entire carapace is rigid; this stage merges with the next.

C_4: intermolt secretion of the cuticle is mostly complete, but secretion continues at a low rate; of variable duration.

D_0: beginning of premolt; epidermis starts separating from the cuticle at the edge of the uropods and telson; variable duration.

D_1: new setae develop at the edge of the uropods and telson; lasts $\sim$ 10 d.

D_2: secretion of new cuticle begins; lasts 1-2 d.

D_3: some of the old cuticle has been resorbed; entire body can be compressed readily; lasts ∿ 3 d.

D_4: the line of dehiscence opens; quickly merges with the next stage.

E : the animal is molting.

This last stage can be further subdivided (Aiken 1968a) as follows:

E_1: the thoracoabdominal intertergal membrane is distended and the posterior margin of the carapace is elevated slightly.

E_2: the carapace is thrown forward and the cephalic structures withdrawn.

E_3: the abdomen and related components are withdrawn.

E_4: the chelipeds and other walking legs are withdrawn.

The average life span of *O. virilis* is 20 months. The maximum reported life span is 3 years (Momot 1967) but Leonhard (unpublished data) has maintained animals in the laboratory for up to 4 years.

2.3 Ecological relationships

Physical parameter	Associated factors or limits	References
1. Temperature (°C)		
0-26	Tolerance range. Below 10°, locomotor activity falls off rapidly. At collection temperatures of 0.4 to 1.5°, animals are barely able to flex the abdomen. Egg laying is dependent on the elevation of water temperature to above 10° after overwintering.	Aiken, in Momot 1967
2. Dissolved oxygen (mg L^{-1})		
5-9	Optimum in laboratory studies.	Weagle and Ozburn 1972
3. pH		
5.75-9.0	Laboratory studies.	Malley 1980

Physical parameters	Associated factors or limits	References
4. Light	Complete ovarian maturation requires 4-5 months of low temperature and constant darkness.	Aiken 1969b
5. Calcium concentration (mg L^{-1})		
2.7-70	2.7 mg L^{-1} conc. from a Precambrian shield lake; 70 mg L^{-1} from a prairie river. Extremes unknown. Crayfish from soft waters tend to have lower Ca^{++} content than those from harder waters.	Malley and Leonhard unpublished data

2.5 Diseases and parasites

Descriptions and illustrations of abnormal conditions in crayfish are documented in Johnson (1977). Aberrant secondary sex characters, color anomalies, branchiobdellid infestation, microsporidial parasitism and bacterial lesions are discussed.

COLLECTION

3.1 Preferred habitats

Throughout much of its range, *O. virilis* inhabits stony beds of larger rivers, streams and lakes.

3.2 Collection procedures, Spring-Fall

3.2.1 Adults

1. Wade or dive with a large dip net. Place the net behind the crayfish and agitate the area in front of it.

2. Use a minnow trap baited with bacon, liver or fish. Place overnight in lakes or streams.

3.2.2 Young and adults

Trawl with a shrimp net baited with fish scraps.

3.2.3 Young-of-the-year

In a weeded area of the lake or stream, place a dip net (downstream in

running water) and kick the top layer of sediment gently forward.

3.3 Transport

3.3.1 Short distance (≤ 250 km)

3.3.1.1 Equipment and materials

1. Plastic bags (polyethylene, 30 pound weight), ∿ 40 L capacity.
2. Insulated container large enough to hold filled plastic bags.
3. Water obtained at collection site or dechlorinated water (Appendix B18).
4. Local water plants or aquarium filter wool.

3.3.1.2 Procedure (adults)

1. Place 20 L water into plastic bag.
2. Place 50-75 adults into water, close bag allowing 1/4 volume for air.
3. Place bag in insulated container for transport.

3.3.1.3 Procedure (young)

1. Fill bag to 1/8 capacity with water.
2. Place 200-500 young in alternate layers with local water plants (or filter wool) up to 3/4 capacity; close bag allowing a space for air.
3. Place bag in insulated container for transport.

3.3.2 Long distance transport (> 250 km)

3.3.2.1 Equipment and materials

1. See Section 3.3.1.1, 1-4.
2. Ice.
3. Paper towelling.
4. Thermometer.

3.3.2.2 Procedure

1. Bring water to 18°C or less.
2. Fill bag to 1/2 with water.

3. Place 50 adults or 100 young into bag.

4. Insert large pieces of filter wool to cover animals.

5. Close bag to form an air space on top 1/4.

6. Pack bag into insulated container.

7. Surround bag with ice and wet paper towelling.

3.4 Sorting and isolation

Young animals must be separated from adults to avoid cannibalism. Transport newly molted (soft-shelled) crayfish individually in closed 500 mL wide mouth plastic containers using water collected at the sampling site. It is preferable not to transfer newly molted animals.

3.4.1 Equipment and materials

1. 40 L glass aquarium for a static system or trough for a continuous flow system (Appendix A8).

2. 70 mm mesh dip net.

3. Polyvinylchloride (PVC) pipe, 70-120 mm long, 50-70 mm diameter, cut in half lengthwise.

4. Lights, fluorescent to provide $\sim$ 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the water surface.

5. Water from the collection site.

6. Tags (optional) (Appendix A9).

3.4.2 Preparation of aquarium or trough

1. Bring water to the temperature of the collection site.

2. Place 10 cm water in aquarium or trough.

3. Place PVC pipe in the aquarium or trough as burrows for each animal.

3.4.3 Sorting procedure

1. Adults: grasp carapace behind the first chelae or grasp both chelae at the carpal joint and lift; young: use dip net.

2. Tag if desired.

3. Place in aquarium or trough.

4. Feed as in 4.2.3.4.

4.2 Maintenance of field stocks

4.2.1 Equipment and materials

1. As in 3.4.1.1-4.

2. Dechlorinated water (Appendix B18) with ∿ 40-70 mg L^{-1} Ca^{++} (adjust with chalk stick or $CaCl_2$) or water from the collection site.

3. Timer switch to regulate photoperiod.

4. Flaked Tetra-Min B (Appendix B10) suspension (mix Tetra-Min B flakes 1:1 with distilled water) or extruded crayfish food (Appendix B17).

5. Supplemental food supply, i.e. daphnids, brine shrimp, fish scraps or dry dog food (ground beef-like).

6. Established culture or field population as in Section 3.4.

4.2.2 Preparation of aquarium or trough

1. Bring water to within 1-2°C of the water in which the animals are being held.

2. Static system (aquarium): Place 10 cm water and 1 PVC burrow for each animal into aquarium.

OR 3. Continuous flow system (trough): Establish flow of dechlorinated water at 200 mL min^{-1}; place 1 PVC burrow for each animal into the trough.

4.2.3 Initial procedure (for adults or young)

1. Place 1-50 individuals into the aquarium or 50-300 individuals into the trough. Loading density is 1 g crayfish mL^{-1} water daily.

2. Establish the photoperiod at 8L:16D for winter conditions; 16L:8D for summer-fall conditions.

3. Bring temperature to 12°C for winter or 18°C for summer-fall at a rate of 2°C d^{-1}.

4. Add 1 mL flaked Tetra-Min B suspension (or a 1 cm strand of extruded food) for each adult animal. Supplement with one of the following per adult: a) 20 live daphnids, b) 35 mm^3 frozen brine shrimp, c) 35 mm^3 frozen fish, d) 1 cm strand ground beef-like dog food.

5. Feed young 10% of their weight in extruded crayfish food.

4.2.4 Maintenance procedure

1. Maintain photoperiod and water temperature as established for summer-fall or winter collection. To change seasonal regimes, raise or lower temperature 2°C d^{-1}, and then change photoperiod 2 h d^{-1}.

2. Siphon uneaten food from aquarium or trough prior to feeding.

3. Feed weekly as in 4.2.3.

MASS CULTURE TECHNIQUES

Light and temperature regimes referred to are adjusted according to data obtained in a previous year period. From mid-October to mid-February, the cultures are in total darkness and are unfed and undisturbed to approximate normal winter conditions under ice. Light and temperature conditions are adjusted to seasonal variations on a daily basis for the rest of the year.

5.2 Large volume techniques

5.2.1 Equipment and materials

1. Holding tanks equipped for continuous flow of water; reservoir containing equipment for heating and cooling (Appendix A8).

2. Dechlorinated water (Appendix B18), Ca^{++} adjusted to 40-70 mg L^{-1}.

3. Extruded crayfish food (Appendix B17).

4. Fluorescent lighting.

5. Timer switch to maintain photoperiod.

6. Balance, weighing to nearest 0.1 g.

7. 20 weighed and tagged ovigerous females, and 20 weighed and tagged mating pairs, from a late spring or summer collection or from another established culture.

5.2.2 Adult culture

5.2.2.1 Initial procedure

1. Fill holding tanks with water from the reservoir, establish flow at 200 mL min^{-1}.

2. Place females and mating pairs in tanks.

3. Adjust light and temperature to simulate field collection site or the original culture.

4. Feed crayfish 1% of total body weight daily.

5.2.2.2 Maintenance procedure

1. Mid-February to mid-October.

   a) Adjust temperature and photoperiod to field measurements from previous year or from current field measurements (weekly intervals).

   b) Feed cultures daily at 1% of total body weight of crayfish.

   c) Maintain flow of reservoir water at 200 mL min^{-1}.

   d) Weigh each animal each week.

2. Mid-October to mid-February.

   a) Adjust temperature to winter lows over about 1 month.

   b) Cover tanks to exclude light.

   c) Cease feeding.

   d) Turn off water, but maintain as a static system. Provide 1 mL of water for each g of crayfish for each day of confinement.

5.2.2.3 Production

1. $\sim$ 40% of mating females and $\sim$ 60% of ovigerous females successfully produce young.

2. Each female bearing young produces an average of 101 young (range (measured in the field) 84-214).

5.2.3 Young-of-the-year culture

5.2.3.1 Equipment and materials

1. Dechlorinated water (Appendix B18), Ca^{++} adjusted to 40-70 mg L^{-1} or water from collection site.

2. Balance.

3. Aquarium or trough subdivided into compartments (Appendix A7) or 500 mL wide-mouthed containers.

4. 10-20 ovigerous females isolated in holding tank as in 5.2.2.1.1.

5. Extruded crayfish food (Appendix B17).

5.2.3.2 Initial procedure

1. Examine females frequently for hatchlings.

2. When it is evident that hatchlings will be released, prepare subdividied trough or aquarium by filling compartments 2/3 full of dechlorinated water or water from the collection site or place 250 mL water into wide-mouthed containers.

3. Weigh and measure rostral-carapace length (RCL) of each hatchling and place separately in a compartment or container.

5.2.3.3 Maintenance procedure

1. Examine each hatchling daily for weight, RCL and molt stage.

2. Add 10% of the hatchling weight in extruded crayfish food every 2 d.

3. When rostral-carapace length is 20 mm, organisms may be treated as in 5.2.2.2.

5.2.3.4 Growth

1. Year 0 crayfish (young-of-the-year).

   a) Number of molts: 7.

   b) Rostral-carapace length: 4 mm at birth; 17 mm at seventh molt.

2. Year 1 crayfish (yearling).

   a) Number of molts: female - 3; male - 4.

   b) Rostral-carapace lengths: 17 mm prior to first molt; 27 mm after third molt.

   c) R.L. France (Freshwater Institute, personal communication) reports that *O. virilis* from the Experimental Lakes Area, Ontario, have the following mean rostral-carapace length (mm)

Young	Stage I	Stage II	Stage III
12.2	19.9	23.5	27.3

   These animals are smaller than generally found in other locations.

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Chironomus tentans Fabricius

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INTRODUCTION

1.1 General information

Chironomus tentans Fab. (Diptera:Chironomidae) a non-biting midge, is a large fly whose bright red aquatic larval stage frequently inhabits eutrophic shallow water ponds and sloughs (Driver 1977) although it is also common in eutrophic lakes (Flannagan 1971). *C. tentans* can be important in the diet of young and adult surface feeding ducks (Driver et al. 1974) and the larvae and pupae can provide essential proteins for the egg-laying hens (Siegfried 1973). Other members of the genus are often extremely important as fish food (Hamilton 1972) and McLarney et al. (1977) demonstrated that larvae provide growth promoting supplements.

The biology of *C. tentans* facilitates its laboratory culture since the larvae are tolerant to a wide spectrum of conditions and adults mate even when confined. Sadler (1935) described its general biology and McLarney et al. (1974) described mass culture conditions used in fish food production. This paper incorporates many of the suggestions of Konstantinov (1958) for optimizing the culture of Chironomidae.

1.2 Toxicological precedents

The larvae of *C. tentans* have been used to assess the consequences of acute exposures to compounds such as detergents (Flannagan 1971), insecticides (Karnak and Collins 1974), lampricides (Kawatski et al. 1975) and heavy metals (Rathore et al. 1979). Studies of chronic toxic effects include assays on uptake and elimination of lampricides (Kawatski et al. 1974, Kawatski and Bittner 1975) and heavy metals (Hamilton 1972, Barica et al. 1973, Dodge and Theis 1970). Longer term multigeneration studies (Derr and Zabic 1972a, b, Townsend and Hamilton 1976, Macek et al. 1976a, b) have been valuable in assessing possible reproductive impairment. Sublethal responses such as changes in emergence success (Didiuk and Wright 1975, Giles et al. 1979, Wentsel et al. 1978b), growth rate (Wentsel et al. 1977b), avoidance behavior (Wentsel et al. 1977a), distribution in contaminated sediments (Wentsel and McIntosh 1977) and resistance to metal toxicity (Wentsel et al. 1978a) have been studied.

1.3 Geographic distribution

C. tentans has holarctic distribution and is locally common in the mid-continental areas of North America. Townes (1945) as well as Acton and Scudder (1971) have reviewed the distribution of the species.

GENERAL BIOLOGY

2.1 Morphology and taxonomy

The taxonomic position of North American Chironomidae was aligned with European forms by Hamilton et al. (1969). Saether (1971, 1980) clarified morphological terminology and placed *C. tentans* in the *Camptochironomus* subgenus (Saether 1977). Descriptions by Branch (1923), Sadler (1935) and Rempel

(1936) provide a general introduction to the features of the genus.

C. tentans has four life stages: egg, larva, pupa and imagine. Eggs are tan in color, elongated on the antero-posterior axis and 0.1-0.3 mm long. There are four larval instars. The first instar is 0.6-1.2 mm long and the head capsule is 0.1 mm wide. The head, three thoracic and nine abdominal segments, antennae and mandibles can be distinguished easily. Prothoracic and last abdominal parapods, four anal tubules and anal setae are present but ventral tubules (blood gills) are absent. Although hemoglobin production is initiated during the latter part of this stage, the larvae appear creamy white. The second instar is 2.0-4.5 mm long and the head capsule is 0.2-0.22 mm wide. Blood gills appear on the eighth abdominal segment. During this stage, the larvae become pink due to heightened hemoglobin production (Czeczuga 1961). The third larval instar is 6-12 mm long and the head capsule is 0.37-0.42 mm wide. The larva becomes characteristically bright red in color. The fourth instar is 12-24 mm long and the head capsule is 0.66-0.76 mm wide. In the fourth instar, the head capsule has a longitudinal black strip extending from the frontal to the clypeus.

The ultrastructure of larval integument has been described in Credland (1978). Credland (1975, 1976) and Wright (1975a, b, c) have also demonstrated how integument, anal tubules and blood gills affect osmotic regulation.

The pupae is black and the most obvious morphological changes are respiratory and locomotory in nature. Thoracic respiratory organs appear as tufts of white filaments just behind the head. The lamellar setae and caudal lobes are clearly developed to promote swimming movements. Wing sheaths, leg buds and the compound eyes are also prominent features.

In the adult (imagine) sexual dimorphism is striking. The male can easily be distinguished from the female both by its large plumose antennae and the paired genital claspers on the posterior tip of the abdomen. Palmen and Aho (1966) described adult morphology and characteristics of the female have been described by Saether (1977) and more generally by Wensler and Rempel (1962). The female adult weight is approximately twice that of the male; about 30% of the body weight is contributed by eggs.

2.2 Life cycle

2.2.1 Mating

Mating is initiated by the male; copulation takes approximately 20 s. Virgin females cooperate with the aggressive male but once mating occurs the female avoids further copulation by curling its abdomen down. Males are capable of mating with more than one female and frequently attempt copulation with other males. Mating behavior has been described by Hein and Schmulbach (1971, 1972).

2.2.2 Fertilization and oviposition

After copulation, spermatozoa are stored in paired spermatheca in the female where they are separated from the eggs until oviposition, 24-48 h after mating. Individual eggs are passed through the lateral oviducts into the common oviduct where fertilization takes place. Eggs are coated with a gelatinous

substance which is produced by the accessory gland and which consolidates individual eggs into the egg mass. The female initially anchors the anterior portion of the mass to her thorax and by maniupulating her abdomen, extrudes a ribbon of eggs. This ribbon consists of a gelatinous strand with eggs embedded at their posterior ends. After all the eggs have been extruded, the female drops the mass into the water. The gelatin is hydroscopic, and the mass quickly expands to a characteristic "C" shape (Sadler 1935). The anterior portion of the mass consists of a very sticky funnel-shaped gelatin coated wad which served as the oviduct plug and as the anchor for the egg mass in water. Takeda and Ohishi (1976) have demonstrated that it may contain a development hormone.

Females are non-selective with respect to oviposition site. Each female produces only one large egg mass but second smaller fragments frequently occur. Under crowded conditions, irregularly patterned masses occur, most likely because females are interrupted during oviposition. Virgin females do not lay eggs.

2.2.3 Embryogenesis

Because the chorion of the egg is clear, phases of embryonic development can be seen (Yajima 1960, 1964). Rates of embryogenesis are temperature dependant (see Section 2.3) and under optimal conditions, eggs isolated from a single mass develop synchronously. Eggs are the life stage least sensitive to toxicants (see Section 2.3) but Derr and Zabik (1972b) have demonstrated increased embryo sensitivity when exposure occurred during oogenesis.

2.2.4 Larval development

For a short period after hatching occurs, first instar larvae remain within the egg mass where they graze on materials adsorbed to the gelatin. Initially larvae are positively phototactic and unless the sediment contains enough dissolved oxygen and food resources, they migrate into the water column. In nature, wind and wave action often determine larval distribution (Davies 1976), but in laboratory culture, migrating first instars frequently become trapped in the water surface layer where they die. Rapid settling and immediate tube construction is critical for maximum production because larvae grow faster when associated with the sediment (Konstantinov 1958). To assure synchronous development and high production, it is imperative to provide adequate food and maintain high dissolved oxygen concentrations. Food additions must be carefully regulated because while too little causes migration into the water column, too much causes increased microbial activity and reduced oxygen concentrations. Both situations cause reductions in growth rates (Konstantinov 1971, Nagell 1978).

2.2.5 Metamorphosis, pupation and adult emergence

Metamorphosis occurs within the larval tube. The transformation is complete in 3 d. Initially, the prothoracic segments of the larva begin to swell, and feeding stops. The larval-pupal molt occurs and the pupa gradually darkens as hemoglobin is shunted to the gut and digested (Laufer and Poluhowich 1971, Schin et al. 1974). The pupa vacates the tube and swims on the sediment surface for $\sim$ 24 h. Small gas bubbles then evolve under the integument, made the pupa buoyant and help it rise. The case splits on the

dorsal side and the head and thorax are pushed through the opening. The wings are unfurled by a red fluid which is shunted through them. The abdomen is pulled from the case or exuvia, the adult defecates and then flies. The emergence process takes about 15 s.

2.2.6 Emergence patterns

Emergence is bimodal because males develop somewhat faster than females. At 20°C, under conditions described in Section 5, males begin to emerge on day 21 and females on about day 23. The sex ratio for normal populations is about 1.5 males to 1 female, although this ratio is variable according to environmental conditions. Adults weight 40% less than pupae and 60% less than late fourth instar larvae.

2.3 Ecological relationships

Physical parameter	Associated factors or limits		References
1. Temperature (°C)			
a. Embryonic development[a]	% hatch	time (h) (range)	Townsend this work
8	0[b]	-	
10	75±2 (SD)	264-480	
15	95±3	120-148	
20	99±1	55-57	
28	99±2	30-38	
35	75±13	28-34	
40	0[c]	-	
b. Larvae			
< 8	Development in fourth instar arrested.		Sadler 1935
0-35	Larvae found in the field.		Topping 1971, Brues 1928

[a] 3 replicates of 50 eggs in 20 mL dechlorinated water used for each temperature.

[b] development and bastokinesis occur.

[c] no development occurs, eggs rupture.

Physical parameter	Associated factors or limits	References
2. Dissolved oxygen ($mg\ L^{-1}$)		
a. Eggs[a]		
< 1	Embryogenesis terminated if incubation exceeds 48 h.	Townsend this work
1-12	Normal hatch.	
	[a] 3 replicates of 50 eggs incubated in BOD bottles containing dechlorinated water. Gradient achieved by gassing with O_2 or N_2.	
b. Larvae		
3	At 20°C, first instar larvae begin to migrate into the water column; fourth instars begin to undulate and to construct tubes away from the sediment.	Townsend this work
3. pH		
6.9-10.5	Extremes for field populations. High value from slough in Manitoba which is a collection site.	Curry 1962 Topping 1971 Townsend this work
5.5 and 6.5	Normal development in system described by Lillie and Klaverkamp (1977).	Townsend this work
4.5	60% reduction in emergence; females more sensitive than males. Sex ratio M:F = 2.5.	Townsend this work.
3.5	Eggs develop and hatch, but 100% mortality of first instars occurs within 1 h. 100% mortality in fourth instars.	Townsend this work

Physical parameters	Associated factors or limits	References
4. Photoperiods (light:dark)		
8L:16D	Development in fourth instar arrested.	Englemann and Shappirio 1965 Shilova and Zelentsov 1972
16L:8D	Development reinitiated in 65% of diapausing fourth instars.	Englemann and Shappirio 1965 Shilova and Zelentsov 1972
24 h light	Development normal.	Townsend this work
24 h dark	Larvae develop but do not emerge.	Townsend this work
5. Conductivity		
100-4000 $\mu S\ cm^{-1}$	Extremes for field populations.	Topping 1972
6. Desiccation	Larvae can lose 50% body water by weight and still recover when rehydrated.	Buck 1965
	Pupae are more resistant to desiccation than larvae.	Townsend this work

2.4 Behavior

See Section 2.2.

2.5 Diseases and parasites

Larvae are frequently parasitized by nematodes which cause intersex to occur (Rempel 1940). Hydracarina also occur on the ventral portion of the adult abdomen.

Hamilton and Saether (1971) and Hare and Carter (1976) correlated structural deformation with poor water quality.

In our laboratory cultures, both abdominal and wing vein ruptures occur. The former, most prominent in females, causes the egg mass to protrude laterally from the abdomen. Both types of rupture are lethal. On occasion, we have noted that an entire cohort (adults developed from a single egg mass) dies because the pupal case splits posteriorly and emerging adults drown.

COLLECTION

3.1 Preferred habitats

Fourth instar larvae are the easiest life stage to collect. They prefer sediments of shallow stagnant water which support emergent plants and rich organic matter. *C. tentans* is more likely to occur in semi-permanent than in permanent water bodies (Topping 1971, Driver 1977) because the species does not compete well when other members of the genus become established.

Large numbers of the larvae most frequently occur at < 2 m depth (Palmen and Aho 1966, Topping 1971). Palmen and Aho (1966) found large numbers of larvae in decomposing *Fucus*. Topping (1971) suggested that abundance of *C. tentans* was positively correlated with mud particles 0.59-1.9 mm in diameter. Slack (1967) found them in soft organic mud.

The larvae which initiated the breeding population maintained at the Freshwater Institute were obtained in June 1972 from a shallow Manitoba prairie slough where they were concentrated most heavily beside algal mats around the lakeward side of emergent plants.

3.2 Collection procedure

3.2.1 Equipment

1. 0.4 mm mesh net, mounted on steel frame.
2. Feather tip forceps.
3. Plastic bags (polyethylene, 30 pound weight), 15-20 L.
4. Insulated container large enough to hold filled plastic bags.
5. Ekman grab (Burton and Flannagan 1973) and boat.

3.2.2 Procedure

1. Place 10 L water obtained from the collection area in the plastic bag.
2. Rake the net so as to collect the top 10 cm of substrate or use an Ekman grab in deep water.
3. Sieve the sample by washing the net at the water surface.
4. Using forceps, sort larvae and place in the plastic bag. Handle gently, as skin abrasions will result in hemorrhage and death.
5. Place up to 500 larvae into 10 L water.
6. Place plastic bag in insulated cooler.

3.3 Transport

Aeration is not required for a transport time < 4 h if detritus is kept to a minimum. Usually animals are transported at collection temperature, but if this is > 20°C, care should be taken to provide sufficient oxygen.

3.4 Sorting and isolation

3.4.1 Equipment and materials

1. Aquarium with lids (Appendix A13).
2. Silica sand, 60-100 grade.
3. Tap water, dechlorinated by ultraviolet light (Appendix B18), pH $\sim$ 7.4, conductivity $\geq$ 500 $\mu S\ cm^{-1}$.
4. Air line, with 0.45 µm filter and an on-line oil filter.
5. Aerator (7.5 cm air stone).
6. Compressed air supply.
7. Fluorescent light supply (Appendix A13).
8. Tetra-Min B (Appendix B10).

3.4.2 Isolation procedure

1. Place 2 L sand in aquarium bottom, spread uniformly.
2. Add 8 L dechlorinated water.
3. Attach air stone to air line.
4. Adjust water temperature to collection temperature.
5. Place larvae in aquarium, up to 600 per aquarium.
6. Allow water to reach room temperature (20-22°C) over 12 h.
7. Maintain light cycle as 18 h light:6 h dark (18L:6D).
8. Add 3 g Tetra-Min B.
9. Adults will appear in 4-10 d.

HOLDING OR MAINTENANCE TECHNIQUES

4.2 Maintenance of field stocks

4.2.1 Equipment and materials

1. As in 3.4.1.1-8.
2. Aspirator bottle (Appendix A11).
3. Laying flask (Appendix A12).
4. Petri dish containing 15 mL dechlorinated water.
5. Feather tip forceps.
6. Electronic automatic monitoring system (optional; Townsend et al. 1975).
7. Adult male and female flies as produced in Section 3.4.

4.2.2 Isolation of eggs

1. Using the aspirator, gently withdraw 15 females. Under most situations at least equal numbers of males will be present in the aquarium and fertilization is assumed. If the sex ratio clearly favors females, withdraw animals of both sexes and to ensure that fertilization occurs, leave adults in the bottle for 15-20 min.
2. Transfer females to laying flask.
3. Egg masses will appear 24-48 h after fertilization. Field larvae were parasitized by white nematodes which frequently appeared along with the egg masses. By isolating the egg masses, these parasites were excluded from subsequent cultures.
4. Decant water and an egg mass from laying flask into a Petri dish.
5. Place the Petri dish in the aquarium such that the egg mass remains in the dish.
6. Observe the egg mass in 3 d to make sure eggs have hatched and first instar larvae have dispersed into the aquarium.

4.2.3 Maintenance of culture

1. Add 5 g Tetra-Min B to aquarium on the third or fourth day after the egg mass was added. Add 5 g per week subsequently.
2. Add dechlorinated water as needed to replace evaporation losses.

4.2.4 Cleaning procedures

Should the chironomid culture be contaminated with mites, nematodes (see Section 2.5), or heavy rotifer and microbial populations, eggs can be cleaned according to Townsend and Hamilton (1976). Note that while the eggs are surface sterilized by this procedure, no attempts are made to render the total system bacteria-free in the strict sense. Therefore, reasonable numbers of bacteria, protozoa and rotifers will appear. These do not appear to interfere with normal chironomid development.

4.2.5 Production

This culture will provide a regular supply of adults in low numbers with a minimum of management. The first generation will emerge synchronously but subsequently, all life stages will be present. If large numbers of adults are required, more food, added more frequently, must be furnished.

MASS CULTURE TECHNIQUES

5.2 Large volume technique (for production of synchronous cultures)

5.2.1 Equipment and materials

As in 4.2.1.

5.2.2 Collection and mating of imagines

As in 4.2.2.

5.2.3 Egg collection

1. As in 4.2.2.

2. Egg masses can be stored at 10°C if large numbers are required. Long term storage is not practical since even at 10°C eggs will hatch in 2-3 weeks.

5.2.4 Initiation of mass culture (see Section 4.2.2) Physical and chemical conditions:

1. Temperature: 20°C.

2. Conductivity: 400-800 $\mu S\ cm^{-1}$.

3. Dissolved oxygen: $\geq$ 90% saturation.

4. pH: 7.2-7.4.

5. Density: 1 egg cm^{-2} initially (if rearing container is built to specification given in Appendix A13 one egg mass per aquarium may by used).

6. Photoperiod: 18L:6D.

5.2.5 Maintenance procedure

1. The two most important factors which govern synchrony in the culture are food and dissolved oxygen concentration. It is important to adjust the feeding schedule to the needs of the population at any given time and to ensure high dissolved oxygen concentrations. The following schedule for addition of Tetra-Min B is suggested:

   Day 0: Add eggs.

   Day 1: 3 g Tetra-Min B.

   Days 3-12: 2 g every third day starting with day 3.

   Days 14-21: 3 g each day.

   Days 22-28: 2 g every second day starting with day 23.

2. Add dechlorinated water to replace evaporation losses as needed.

5.2.6 Production

These culture methods were designed to allow reproducible synchronous production of animals for bioassay purposes. Generally, the method optimizes production of biomass over time.

1. Hatching (egg to first instar), ± SD: 97±1% in 55-57 h.

2. Emergence pattern

   a) Males: start at day 21, peak at day 24.

   b) Females: start at day 23, peak at day 26.

3. Standard production for one egg mass under conditions outlined: (n=18), ± SD.

   a) % emergence: 49±16.

   b) Sex ratio (M:F): 1.4.

   c) Day peak emergence: 25.

   d) Average number emerging adults

      1) Male: 589±152
      2) Female: 417±162.

   e) Average weights (mg) individual organisms

	Wet weight	Dry (60°C, 24 h) weight
1) Late fourth instar		
i) Male	22.0	4.0
ii) Female	37.7	6.7

	Wet weight	Dry (60°C, 24 h) weight
2) Pupa		
i) Male	14.0	3.0
ii) Female	25.7	6.2
3) Adult		
i) Male	7.7	2.4
ii) Female	16.3	5.3

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Hexagenia rigida (McDunnough)

M.K. Friesen

INTRODUCTION

1.1 General information

The genus *Hexagenia* Walsh (Ephemeroptera:Ephemeridae) is widespread in North America (Edmunds et al. 1976). They are large mayflies, commonly found in lakes, rivers and streams with silty substrates (e.g. Lake Winnipeg and the Mississippi River). The nymphs are an important source of food for many species of fish (Neave 1932, Hoopes 1960) and subimagines (pre-adults) and imagines (adults) are consumed by fish, bats and birds. Members of the genus have been used as indicators of water quality (Fremling 1964a, 1970) and may play an important role in the removal of pollutants from a water body (Edmunds et al. 1976, but see Vallentyne 1952). Nymphs have been utilized as a commercial natural resource in Michigan where they are harvested and sold as fish bait (Hunt 1953). Mass emergences have occasionally disrupted traffic because roads have been blanketed by mayflies causing slippery conditions. Lights and radar equipment on boats have been rendered nonfunctional due to swarms of these insects (Fremling 1964b, 1968). Particles from the exuvia of subimagines can cause allergic reactions in humans (Perlman 1961).

Mayflies of this genus have been cultured in the laboratory (Spieth 1938, Fremling 1967, Thomforde and Fremling 1968, Friesen and Flannagan 1976). Fremling and Schoening (1973) describe a method for constructing artificial substrates so nymphs can be observed during laboratory studies. Field and laboratory investigations on *Hexagenia* include studies relating to various aspects of their biology (e.g. life history) and general ecology (Neave 1932, Hunt 1953, Fremling 1960, Carlander et al. 1967, Craven and Brown 1969, Walker and Burbanck 1973, Rutter and Wissing 1975, Mattice and Dye 1978, Flannagan 1979), population dynamics (Hudson and Swanson 1972, Horst and Marzolf 1975), bioenergetics and nutritional dynamics (Zimmerman et al. 1975, Zimmerman 1977), and oxygen consumption (Surber and Bessey 1974, Eriksen 1968). Field and laboratory investigations also include toxicity testing (Carlson 1966, Fremling 1970, 1975, Oseid and Smith 1975, Leonhard et al. 1980). Literature on the genus has been reviewed by Zimmerman (1977). Environmental requirements and pollution tolerance data have been compiled from the literature on *Hexagenia* by Hubbard and Peters (1978).

Field studies on *H. rigida*, including life history and population density information, have been conducted by Neave (1932) and Flannagan (1979). *H. rigida* is the only species in the genus in which parthenogenesis has been recorded (Friesen and Flannagan 1976). Effects of temperature and cold storage on hatching of fertilized eggs have been studied by Friesen et al. (1979).

1.2 Toxicological precedents

H. limbata, a species closely related to *H. rigida*, is one of the aquatic insects suggested as a bioassay test organism in Standard Methods for the Examination of Water and Wastewater (Anon. 1975). Nymphs of *H. rigida* have been used in acute 96 h bioassays with cadmium as toxicant (Leonhard et al. 1980). Eggs have been used to test the toxicity of saline groundwater (Giles et al. 1979) and methoxychlor (Friesen 1979a). Methods for using eggs in bioassays are outlined by Friesen (1979b).

1.3 Geographic distribution

H. rigida has been recorded from eastern and central North America (Burks 1953, Edmunds et al. 1976) and is largely confined to the upper part of the Mississippi drainage and the St. Lawrence drainage (Spieth 1941). It is widespread in Manitoba including the Winnipeg River (Ireland et al. 1973), the Red River (Friesen and Flannagan 1976), Lake Winnipeg (Neave 1932, Flannagan 1979) and Southern Indian Lake (D. Rosenberg, Freshwater Institute, personal communication). Although *H. rigida* is not as abundant in Lake Winnipeg as *H. limbata*, its relative abundance has increased from a ratio of 1:7 in 1927-1930 (Neave 1932) to a ratio of 1:4 in 1969 (Flannagan 1979).

GENERAL BIOLOGY

2.1 Morphology and taxonomy

Winged males are 18-24 mm long with a forewing span of 16-20 mm, while females are 18-28 mm long with a forewing span of 18-24 mm. Mature nymphs are 22-28 mm long, have well developed front legs and frontal processes, and strongly upcurved mandibular tusks (Burks 1953). Eggs are ovoid with approximate dimensions of 0.3 x 0.2 mm (Neave 1932). Keys to nymphs and adults can be found in Needham et al. (1935), Hamilton (1959), Burks (1953) and Edmunds et al. (1976)[10]. None of these keys give adequate characteristics to distinguish winged females and small nymphs from *H. limbata*, a species with which *H. rigida* is often found in nature. Identification of females is therefore usually based on associated last instar exuvia, or from reared offspring which can be identified once they reach a size of approximately 5-10 mm (J.F. Flannagan, Freshwater Institute, personal communication). Chorionic patterning on the egg (Neave 1932, Koss 1968) is not a good distinguishing characteristic, at least for the mayflies from the Red River near Winnipeg, Manitoba.

2.2 Life cycle

The longest period of the life cycle is the nymphal stage. Nymphs are aquatic and inhabit the sediment during their development. A determination of the number of instars of *Hexagenia* has not been made, but these organisms probably undergo numerous postembryonic molts. *Stenacron canadense*, a smaller mayfly, has been estimated to have 40-45 nymphal instars (Ide 1935). Prior to emergence, the nymph swims to the surface of the water, sheds its exuvium and emerges to the terrestrial subimaginal stage. The subimagine molts once more (to the imaginal stage), usually within 24 h if environmental conditions are suitable. Mouth parts of both stages are atrophied and animals cannot feed during this phase. The terrestrial phase normally lasts several days at most and is the period during which mating and egg laying occurs. Mating occurs during a complicated swarming and eggs are laid directly into the water. A female 2.2 cm long can produce 2,000-4,000 eggs (Friesen unpublished data). *H. rigida* are not known to mate in the laboratory but parthenogenetic development in eggs dissected from virgin females has been recorded in this species (Friesen and Flannagan 1976). The life cycle in nature varies from 2 years in

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[10] Genus identifications only.

the northern part of Lake Winnipeg to an alternating 14/22 month cycle in the southern part of Lake Winnipeg (Flannagan 1979). In the laboratory at approximately 20°C, the winged phase survives about 2-4 d, eggs hatch in about 2 weeks, and the nymphal stage lasts about 1 year (range of 6 months-2 years) (Friesen this work).

2.3 Ecological relationships

Work on *H. rigida* is limited, but information on other *Hexagenia* species is probably generally applicable (see Section 1.1). Effects of temperature on egg development time and viability have been studied by Friesen et al. (1979). Optimal hatch occurred at 28, 24 and 20°C, with first hatch ranging from day 8 at 28°C to day 14 at 20°C. The upper threshold limit for development is > 32°C and the lower limit is < 12°C.

2.4 Behavior

Nymphs are detritivores and live in burrows which they construct in the substrate. They will leave their burrows for extended periods when exposed to stressful conditions (e.g. low dissolved oxygen concentrations). Fertilized female imagines tend to be more photophilic than are males or unfertilized females, and thus can be selectively collected at lights (see Section 3.1.2).

2.5 Diseases and parasites

Trematode cysts (*Crepidostomum cornutum* Osborn) were found in some *H. rigida* nymphs collected from Lake Winnipeg (Neave 1932).

COLLECTION

Nymphs are available year round due to their long development period and overlapping cohorts, but collection of large numbers during a Manitoba winter is often not feasible. Eggs, once they have been laid, are very difficult to collect from the field since they are deposited into the water and settle directly to the substrate. Therefore, female imagines which probably have been fertilized are collected and eggs are extracted. Large quantities of eggs can be collected in the field when females, attracted to lights of an automobile, lay their eggs into a large container of water near the lights (Fremling 1967).

3.1 Preferred habitats

3.1.1 Nymphs

Nymphs may be found in lakes and rivers with silt substrates.

3.1.2 Fertilized imagines

During emergence periods, females can be found around lights (especially mercury arc lamps) (see Section 2.4) and on vegetation near lakes and rivers inhabited by nymphs. The best time for collection is late evening of the second day following a mass emergence provided weather is warm and dry. This allows time for both molting to the imaginal stage and mating to occur.

3.2 Collection procedure

3.2.1 Nymphs

3.2.1.1 Equipment and materials

1. Ekman grab (or other substrate sampler of adequate size).
2. Shallow pans, to hold mud sample.
3. Sieves (mesh size 0.3-3 mm, depending on size of nymph wanted).
4. Forceps.
5. Plastic bags (polyethylene, 30 pound weight, ∿ 45 x ∿ 90 cm).
6. Insulated container large enough to hold inflated plastic bags.

3.2.1.2 Procedure

Samples are taken with a dredge and washed through sieves immediately or put directly into bags to be sorted in the laboratory. Sorted nymphs are placed into bags with about 4 L of water from the collection site. This is adequate for transporting about 50 half-grown nymphs.

3.2.2 Fertilized imagines

3.2.2.1 Equipment and materials

1. Plastic bags (polyethylene, 30 pound weight, ∿ 45 x ∿ 90 cm), or other suitable container.
2. Standard 'butterfly' collecting net.
3. Insulated container large enough to hold inflated plastic bags.

3.2.2.2 Procedure

Animals can be collected by hand. Gently grasp them by the wings and place into an inflated bag. When sufficient numbers have been collected, the bags are tied leaving sufficient air to prevent animals from being crushed. Up to 50 animals can be placed into each bag. Animals can also be collected in areas which contain tall grasses using a sweep net.

3.3 Transport

3.3.1 Nymphs

Bags should be transported in an insulated container to prevent large fluctuations in temperature.

3.3.2 Fertilized imagines

Animals can be transported in the plastic bags. Over long time periods

(several hours) animals should be held in an insulated container so that temperature changes are minimal.

3.4 Sorting and isolation

3.4.1 Nymphs

Nymphs can be sorted by picking through the substrate with forceps or by sieving the substrate and then picking. Nymphs < 5 mm are difficult to isolate without injury.

3.4.2 Fertilized females

Fertilized females cannot be distinguished from nonfertilized females (but see Section 2.4). It is therefore important to make collections when conditions are favorable to mating (see Section 3.1.2).

HOLDING OR MAINTENANCE TECHNIQUES

4.1 Holding technique

4.1.1 Nymphs

See Section 5.1.1.

4.1.2 Fertilized imagines

Animals may be held in the bags in which they were transported. If they are not dissected immediately they will survive for at least 3 d if stored at 10°C.

4.1.3 Eggs (see also Section 5.1.2 for pathenogenetic and artificially inseminated eggs).

Eggs from females collected in the field are dissected out as in Section 5.1.2.3. They can be incubated at various temperatures or they may be held in cold storage (see Section 7.3).

4.1.3.1 Production

Success of hatch of eggs from females collected during peak emergence and mating may be >> 90% (Friesen et al. 1979).

MASS CULTURE TECHNIQUES

5.1 Stock culture

Continuous cultures at the Freshwater Institute were initiated by eggs extracted from imagines and from nymphs collected from the Red River, Manitoba.

A dioecious strain and a parthenogenetic strain are in culture at present, both in the third generation. The techniques described have been used to culture small or large numbers of organisms.

5.1.1 Nymphs

5.1.1.1 Equipment and materials

1. Dechlorinated water (Appendix B18).
2. Beakers or aquaria (size range: 1-20 L).
3. Silt from an area known to be inhabited by nymphs, autoclaved.
4. Air pump, aeration stone and airline tubing.
5. Pasteur pipette and bulb.
6. Tetra-Min B (Appendix B10).
7. Clear plastic wrapping material.
8. Aquarium lids made from screening.

5.1.1.2 Initial procedure

1. Place silt in an aquarium to a depth of $\sim$ 1 cm.
2. Fill the aquarium with dechlorinated water to a depth of $\geq$ 15 cm and mark the water level.
3. Aerate so that silt is not disturbed excessively and allow water to become saturated with oxygen.
4. Using the Pasteur pipette and bulb, gently transfer newly hatched nymphs to the aquarium at a density of $\sim$ 1-4 nymphs cm^{-2}.
5. Cover the aquarium with plastic wrap to minimize evaporation.
6. Place at 20-25°C.
7. Add Tetra-Min B at the rate of 0.1 g per 4 L aquarium twice per week.
8. The light cycle does not seem to affect growth and cultures may be held in continuous darkness. Cultures do best when light intensity is high enough so that an algal population is established in the aquarium. This probably provides an additional food source for the nymphs.

5.1.1.3 Maintenance procedure

1. Every 2-3 months, remove 30-50% of the water and replace with fresh dechlorinated water to the original level.

2. Water should be added every 2 weeks to compensate for evaporation between water changes.

3. Feed with Tetra-Min B as in Section 5.1.1.2.7 above.

4. About 6 months after establishing young nymphs in culture, prepare aquaria with silt $\sim$ 3 cm deep and with water to $\geq$ 15 cm and transfer nymphs to these containers to a density of $\sim$ 1 nymph per 5 cm^2.

5. Maintain as in steps 1-3 above.

6. When nymphs reach a size of $\sim$ 15 mm, cover with $\sim$ 1 mm mesh screening to which emerged animals can cling so that they will not fall into the water and drown.

7. Aquaria should be checked daily for emerged animals when wing pads of nymphs begin to darken.

5.1.1.4 Production

1. Up to 30% success has been achieved when eggs which were artificially fertilized, produced by parthenogenesis, or collected from the field were raised to adults.

2. Mortality of newly hatched nymphs may be high, but once they reach 5 mm in length, successful emergence of > 50% can be expected.

5.1.2 Eggs (see Section 4.1.3 for treatment of eggs collected in the field)

Eggs for pathenogenetic development are obtained from virgin female imagines or subimagines. Nymphs from which these females are reared may originate from field collections or from continuous laboratory cultures. Males and females in either winged stage may be used for artificial insemination. The method described for *H. limbata* where male genitalia are applied directly to the eggs (Neave 1932) does not work well for *H. rigida*; therefore a saline solution (Yeager 1939) is used (see Section 5.1.2.4).

5.1.2.1 Equipment and materials

1. Dechlorinated water (Appendix B18).

2. Petri dishes.

3. Forceps.

4. Scissors.

5. Yeager's saline solution (Appendix B11) (for artificial insemination).

6. Pasteur pipette and bulb (for artificial insemination).

7. Glass beaker covered with plastic wrap containing small piece cotton batting.

8. Subimagines or imagines (from field collection or as cultured in 5.1.1).

5.1.2.2 Preparation of subimagines or imagines

1. Place batting into beaker.

2. Place subimagines and imagines into the beaker, cover with plastic wrap. If parthenogenetic procedures are to be followed, virgin females must be used.

3. If organisms are not to be used within ∿ 3 h, keep containers at 10°C. About 3 h prior to dissection, transfer animals to the temperature at which eggs will be extracted.

5.1.2.3 Procedure for parthenogenetic eggs

1. Place ∿ 5 mL water into a Petri dish.

2. Obtain a virgin female from the holding beaker.

3. Using the scissors, sever the abdomen directly in front of the egg packets, i.e. where the abdomen joins the thorax.

4. Using thumb and forefinger on the rear of the abdomen, gently force eggs out of the opening and into the water.

5. Using forceps, gently tease eggs apart.

6. Add 10-15 mL water.

7. Cover dish and incubate (see Section 2.3).

5.1.2.4 Procedure for artificial insemination

1. Place two separate drops of Yeager's solution into a Petri dish.

2. Dissect out eggs into one drop as described above.

3. Dissect male genitalia into the second drop and tease apart using forceps and scissors. Remove large pieces of tissue.

4. Mix the two drops together gently.

5. Let stand for ∿ 10 min.

6. Add ∿ 15-20 mL water.

7. Cover dish and incubate (see Section 2.3).

5.1.2.5 Production

1. Parthenogenetic eggs

Eight of twelve females which were a second parthenogenetic generation in the laboratory produced offspring parthenogenetically. The percent hatching success ranged from < 1.0% to 17.5% (Table 4). The appreciable difference between percent hatch in the siblings (females 1 and 5, Table 4) indicates factors other than genetic ones have considerable influence on the successful development and hatch of these organisms. Stage of winged phase does not seem to affect percent hatch.

2. Artificially inseminated eggs

For the artificial insemination method, a set of eggs to which no sperm were added was incubated to estimate percent parthenogenetic development for each female. This value was then subtracted from percent hatch in the artificially inseminated eggs to estimate the success of fertilization. This may not be an accurate method, however, because some eggs in the 'fertilized' set may still develop parthenogenetically (Suomalainen 1962). For the sake of simplicity data from females which exhibited no parthenogenetic development was examined. There were 15 such females and the percent hatch in artificially inseminated eggs ranged from 0-46% with a mean of 25.6%. The sex ratio of emerged animals was 1:1 (Friesen this work).

Table 4. Percent hatch of parthenogenetically developed eggs from females of *Hexagenia rigida* in their second parthenogenetic generation in the laboratory.

Female	Stage	# eggs examined	% eggs hatched
1[a]	imagine	675	17.5
2	"	1139	5.4
3	"	2084	3.3
4	"	2648	2.4
5[a]	"	823	<1.0
6	"	1219	<1.0
7	"	>1000	0.0
8	"	>1000	0.0
9	"	>1000	0.0
10	subimagine	808	16.7
11	"	2602	1.5
12	"	>1000	<1.0

[a] siblings

STORAGE

7.3 Arrested development

Large numbers of viable eggs collected from the Winnipeg area are available only during the summer emergence. Therefore, two methods for prolonged cold storage of eggs have been developed so that the organisms are more generally available for toxicological and other studies (Friesen et al. 1979). In both methods, eggs in the middle stage of embryonic development are used because they withstand cold storage better than eggs just released from females or those in advanced stages of development. The direct transfer method, while simple, is less successful than the stepwise transfer method.

7.3.1 Equipment and materials

1. Dechlorinated water (Appendix B18).
2. Forceps.
3. Scissors.
4. Gridded Petri dishes.
5. Beaker, 1 L.
6. Constant temperature areas: Direct transfer method; 20 and 8°C. Stepwise transfer method; 20, 16, 12 and 8°C.
7. Hand counter.
8. Dissecting microscope, capable of magnifying up to 25x.

7.3.2 Initial procedures

1. Collect fertilized females (see Section 3.2.2).
2. Place 300 mL of water into the beaker.
3. Pool eggs from ∿ 20 females in the beaker following the dissection method in Section 5.1.2.3.
4. Mix eggs gently and thoroughly.
5. Fill Petri dishes with ∿ 15 mL water.
6. Using a Pasteur pipette, transfer eggs into dishes and cover. Each dish can hold at least 2500 eggs without affecting hatch as long as they are not clumped.

7.3.2.1 Direct transfer method

1. Incubate a control set of eggs at 20°C, monitor start of hatch and percent hatch over time to determine normal hatching response.

2. Incubate eggs destined for cold storage for 8.5 d at 20°C.

3. Transfer to 8°C.

4. Compensate for evaporation every 2 months using sterile water.

5. When required transfer directly to 20°C.

6. Expected response after return to 20°C

   a) Start of hatch: 6-8 d after transfer to 20°C.

   b) Success of hatch: no change up to 16 weeks of storage; hatch reduced to ∿ 70% of the control after 41 weeks.

7.3.2.2 Stepwise transfer method

1. Follow step 1 in Section 7.3.2.1.

2. Incubate eggs destined for cold storage for 6.5 d at 20°C.

3. Transfer them to 8°C in 4°C steps every 4 d, i.e. 4 d at 16°C and 4 d at 12°C.

4. Compensate for evaporation as in direct transfer method.

5. When required, transfer eggs to 20°C in 4°C steps every 7 d (i.e. 7 d at 12°C and 7 d at 16°C).

6. Expected response after return to 20°C

   a) Start of hatch: within 1 week after transfer to 20°C.

   b) Success of hatch: no change up to 16 weeks of storage; hatch reduced to ∿ 70% of the control after 52 weeks.

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NOTE added in press: the following publication contains information pertinent to the use of *Hexagenia* in toxicity tests.

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DEVELOPMENT LIST

The organisms in the development list have been in various stages of culture in the Freshwater Institute, but for various reasons are no longer being used. General information is provided for the worker interested in developing these culture methods further.

Table 5. General characteristics of animals in the development list.

Species	Habitat preference	Size	Feeding type	Reproductive type, Life span	Pertinent literature
Rotifers					
Keratella taurocephala Myers	Planktonic in oligotrophic somewhat acid lakes in temperate North America.	120 x 70 µm	Filters algae and bacteria	Parthenogenetic and sexual; ∿ 1 month	1, 3, 12, 15
Polyarthra vulgaris Carlin	Planktonic in oligotrophic to eutrophic lakes in temperate North America.	120 x 70 µm	Filters algae and probably bacteria	Parthenogenetic and sexual; ∿ 1 month	3, 12, 15
Insects					
Chaoborus sp.	Nearctic eutrophic and dystrophic lakes and ponds. Planktonic at night, benthic in daylight.	Fourth larval forms: 6-23 mm long	Opportunistic carnivore	Sexual; 1-2 years depending on species	5, 6, 8, 9, 10, 11, 14, 16, 17, 19, 21, 22
Paratanytarsus sp.	Common contaminant in Freshwater Institute water system.		Oetritivore	Parthenogenetic; 15 d at 20-22°C in lab culture	7, 18, 19, 20
Grensia praeterita (Walker)	Arctic lakes in North American tundra above tree line.	Adults: 250 mm long x up to 1.28 mm wide (at eyes)	Oetritivore	Sexual and probably parthenogenetic; ≤ 5 years	23
Pycnopsyche subfasciata (Say)	Clean fast flowing rivers under cobbles. Northeastern U.S., Ontario, Manitoba.	Egg mass: 10 mm (diam), Larvae: 18-20 mm long, Adults: 19-20 mm long	Larvae: omnivorous; Adults: adapted to feeding on liquids	Sexual; 0.5-1.0 in lab culture	2, 6, 13, 23

Table 6. Culture characteristics of animals in the development list.

Species	Life stage	Culture technique	Culture success
Keratella taurocephala and *Polyarthra vulgaris*	All stages	Place 10 animals in 100 ml water from collection site filtered through 40 µm mesh. Incubate at 15°C in the dark. Feed 1 mL of 72 h culture *Chlamydomonas reinhardtii* grown on DM medium (Appendix B8, 14) every 2-3 days.	Reproduction of several generations over 4 months.
Chaoborus sp.	Fourth larval stage	Place 1 larva in static dechlorinated water (Appendix B18), in a tube sized as necessary, at 20°C, pH 7.2. Light cycle 16L:8D at ~ 350 lx. Feed daily on zooplankton, benthic insect larvae, oligochaetes or other *Chaoborus* larvae. About one prey organism is taken per hour.	Reared to adults. Mating will not occur naturally in the laboratory.
	Eggs	Forced copulation techniques of McDaniel and Horsfall (1957), Wheeler (1962) or Wheeler and Jones (1963) used to obtain fertile eggs from adults. Culture in conditions described for fourth larval stage, above.	Eggs reared to adults. Oviposition of egg mass of 350-450 eggs occurs a few hours after fertilization. Embryonic development takes 37-50 h. Succession through four larval instars occurs in 60 d. Pupae overwinter. Adults live 6 d.
Paratanytarus sp.	Eggs	As described for *Chironomus tentans*.	Eggs reared to fecund adults. Egg masses contain 60±20 (SD) eggs.
Grensia praeterita	Larvae	Flow through culture as described for *Hyalella azteca*, at 5-15°C, pH 7.0 in soft water. Use gravel as substrate. Constant ordinary room light. Feed Tetra-Min B (Appendix B10).	Large larvae collected in fall reared to adults in 9 months.
Pycnopsyche subfasciata	All stages	Flow through tanks at 20°C, light cycle 16L:8D with cobbles simulating a natural situation. Egg masses should be maintained above, but touching the water line. Feed larvae mosses, decaying leaves and Tetra-Min B. Mature larvae should also be fed late instar *C. tentans* larvae.	Two generations reared in the laboratory.

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APPENDIX A

SPECIALIZED EQUIPMENT

1. Mouth siphon (for use with Protozoa and daphnids).

   a. Amber rubber tubing 30-40 cm long.

   b. Mouth piece: glass tube ∿ 7 cm long, with fire-polished ends.

   c. Pipette: tubing and pipettes are small for Protozoa (4 mm inside diameter (ID), 30 μm tip) and large for daphnids (500 mm ID, ∿ 1 mm tip).

2. Small test tubes (for handling Protozoa).

   a. Cut 5 mm ID soft glass tubing into 7.5 cm lengths. Acid clean (Appendix B1).

   b. Dry thoroughly, preferably in an oven.

   c. Using a bunsen burner, close one end of a tube. Turn the tube constantly to ensure even and complete closure. Cool.

   d. Using a pointed carbon rod, flare the open end slightly by heating the glass (which is kept constantly turning in the flame) and gently turning the tube on the carbon rod. Cool.

   These tubes may be plugged with cotton and sterilized. They are filled with medium aseptically using a syringe or Pasteur pipette. The diameter is small enough that capillary action keeps the medium in place when the tube is held horizontally for microscopic examination.

3. Two-stage chemostat apparatus (Fig. A1).

4. Nets for collection of small invertebrates.

   a. Cut a polypropylene jar (∿ 9 cm diameter) into 10 cm sections, and smooth the edges.

   b. Glue nitex netting of appropriate mesh size to one end with silicon glue.

   c. Let glue set for 24 h.

   This apparatus may be sterilized by autoclave.

5. Aquarium system for culturing daphnids (Fig. A2).

6. Inoculating card for *Artemia* cysts.

   a. Use a standard paper punch to make a hole in a Manila paper or a file card.

   b. Glue a piece of paper or card behind the hole so that a shallow well is formed. This well holds ∿ 1800 cysts when filled and levelled.

7. Apparatus for rearing *Orconectes* young (Fig. A3).

8. Apparatus for rearing crayfish (Fig. A4).

9. Marking procedures for *Orconectes virilis*.

Marks must identify individuals and, in general, be retained through the molt. A simple codon painted on the carapace with bright (preferably red) nail enamel will suffice for short-term identification. For longer studies, lasting one to two molts, a variety of tags are suitable:

a. Pleural clips produce a recognizable scar through three molts (Momot 1966).

b. Brands made with a soldering iron using a code of one to twelve dots on the carapace will mark up to 999 crayfish (Abrahamsson 1965).

c. An abdominal streamer tag, developed for marine decapods, remains intact without causing irritation or infection through at least one molt (Cummings and Reynolds 1978).

d. Injections of numbering-machine inks yield a persistent visible stain (Slack 1955, Black 1963).

e. Radioactive materials have been used in field studies of population movements (Merkle 1969).

f. Masses of crayfish could be marked by sprays of a fluorescent granular pigment but the tag is generally lost during molts (Brandt and Schreck 1975).

10. Gridded continuous flow culture system for *Hyalella azteca* (Figs. A5, 6, 7). W.A. Macdonald, Designer, Ecosystem Toxicology Section, Freshwater Institute.

This system was designed so organisms can be cultured under various light, temperature and flow conditions simultaneously in a small space. Fig. A5 shows the dimensions and format of the system. Fig. A6 shows how temperature is controlled and Fig. A7 shows how the water is distributed.

11. Aspirator bottle (Fig. A8).

Use gentle suction by mouth if animals are to supply egg masses. The aspirator can be attached to a pump if animals are collected to measure biomass or population numbers. The volume of the centrifuge tube is small enough so vacuum can be controlled easily.

12. Laying flask for collection of chironomid eggs.

a. Cut a 2 cm diameter hole at the base of a 1 L Erlenmeyer flask and stopper it with a cork.

b. Place a 12 x 4 cm plastic screen from the mouth of the flask to about 3 cm above the bottom.

c. Hold the screen in place with a cotton plug in the neck of the flask.

Collect fertilized females using the aspirator bottle (Fig. 8), deposit them through the neck of the flask, add about 3 cm dechlorinated water and replug. The screening provides a resting surface for the adults. It should not extend into the water since egg masses stick to it. The egg masses are decanted through the hole in the base without disturbing other gravid females.

13. Aquaria and stacking rack for culture of *Chironomus tentans* (Figs. A9, 10, 11).

Aquaria (Fig. A9) are constructed from seamed 0.6 cm (¼") plate glass sheets bonded with fungicide-free silicon cement. Two pieces of 28.8 x 1.25 cm (11.5 x 0.5") diameter glass tube are cemented either on the bottom plate or suspended from the two sides flush to the upper lip. One mm mesh plastic screen is glued to these struts; the enclosed area provides a bubbling zone that minimizes agitation to the remainder of the tank. Each bubble zone is equipped with a 7.5 cm air stone and 250 mL activated charcoal. Tygon tubing with an on-line Millipore Swinnex-25 filter holder and 0.45 µm pore-size filter is attached to the air stone. Each aquarium receives 2 L of silica sand and 8 L of water dechlorinated by ultraviolet light. The lid frame is constructed from urethane treated fir (Fig. 9). The plexiglass should be turned occasionally to avoid warping. Each end piece has an air vent (covered with nylon screen secured with Velcro) which provides a hole through which adults can be aspirated.

Six aquaria are stacked in a rack (76 cm wide x 64 cm deep x 1.9 m high) (Fig. A10). Trays slide out to faciliate maintenance. Each tray slides on inlaid wood strips and is equipped with metal handles. The sides of the rack have windows covered with opaque material which allows diffuse illumination.

A four unit light system (Fig. A11) is designed so that the two heat producing ballast units are placed well away from the rack. This allows photoperiod cycling with less than 0.5°C fluctuation. Normally four fixtures are clipped to each rack. The ballasts and timer are fixed to a 3 x 3 m piece of 1.25 cm (0.5") plywood. A pen temperature control which turns the lights off if the temperature rises > 1°C is placed on top of the rack.

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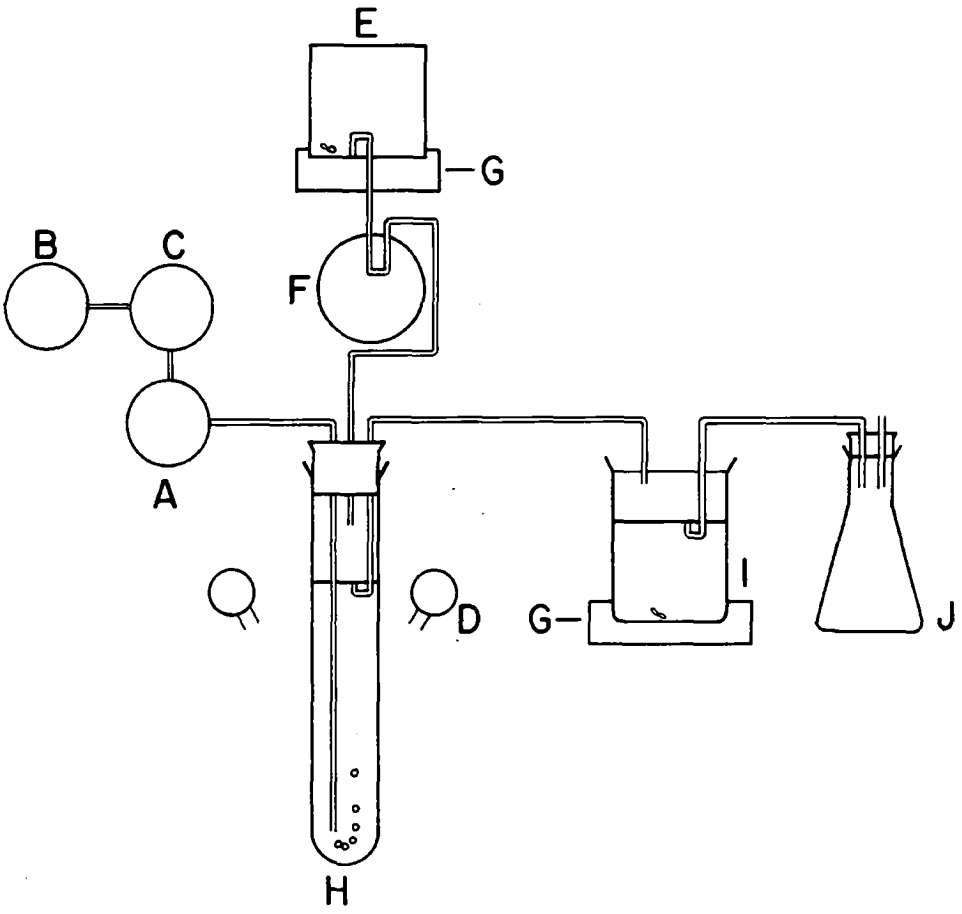


A. ACID AIR SCRUBBER

B. AIR PUMP

C. HUMIDIFIER

D. LIGHTS

E. RESERVOIR

F. PERISTALTIC PUMP

G. STIRRER

H. ALGAE CONTAINER

I. PROTOZOA CONTAINER

J. YIELD FLASK

Fig. A1 Two-stage chemostat apparatus for a algae-protozoan, prey-predator system

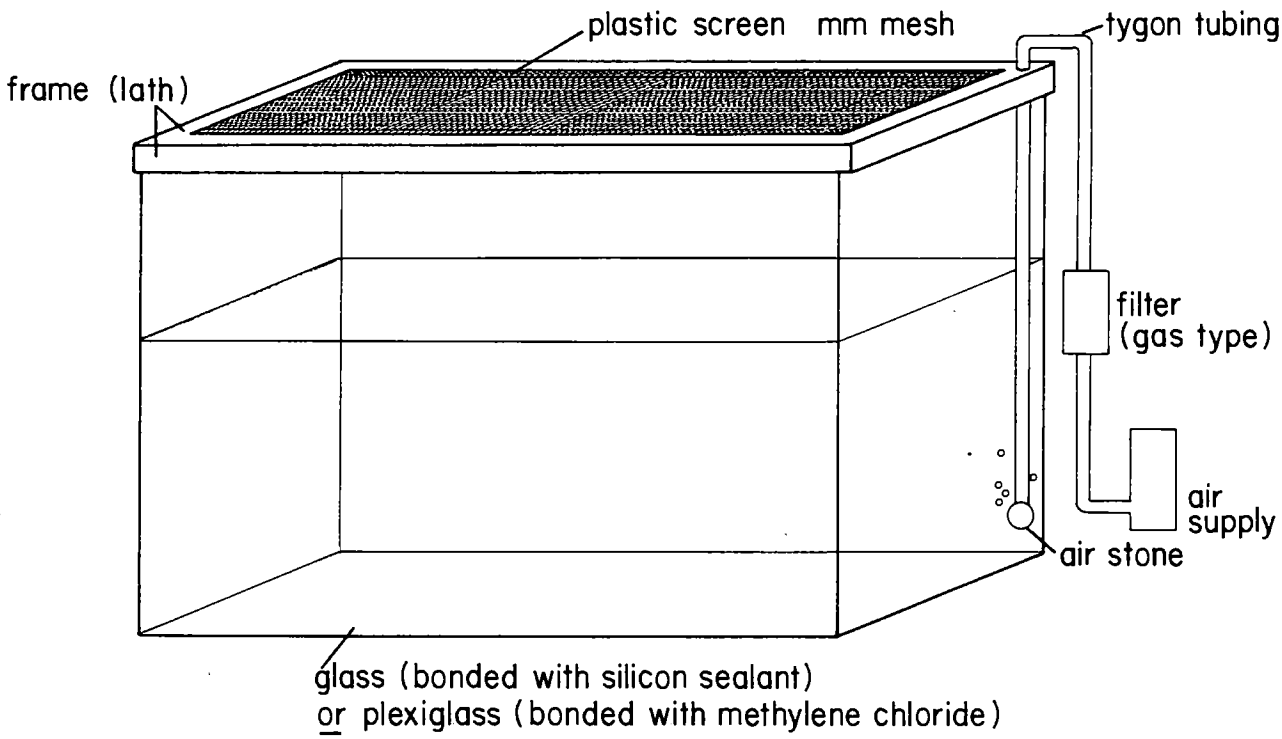


Fig. A2 Aquarium, screen and aeration apparatus for culturing daphnids

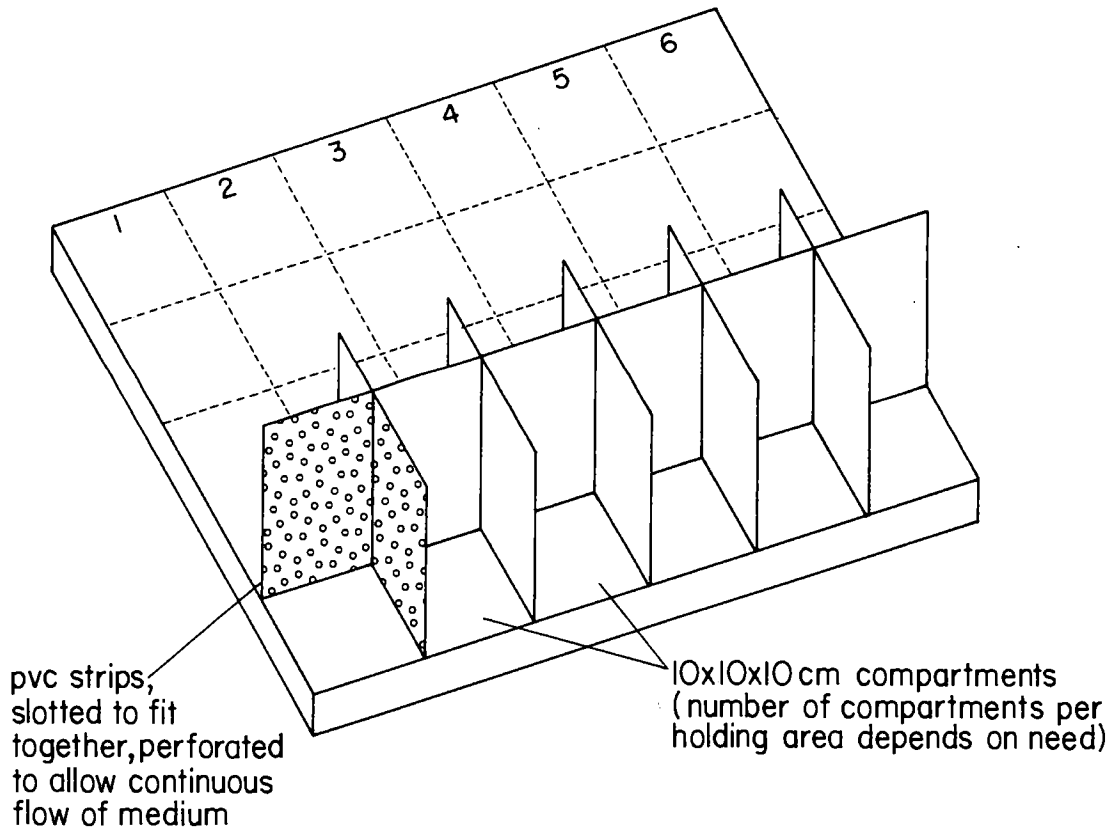
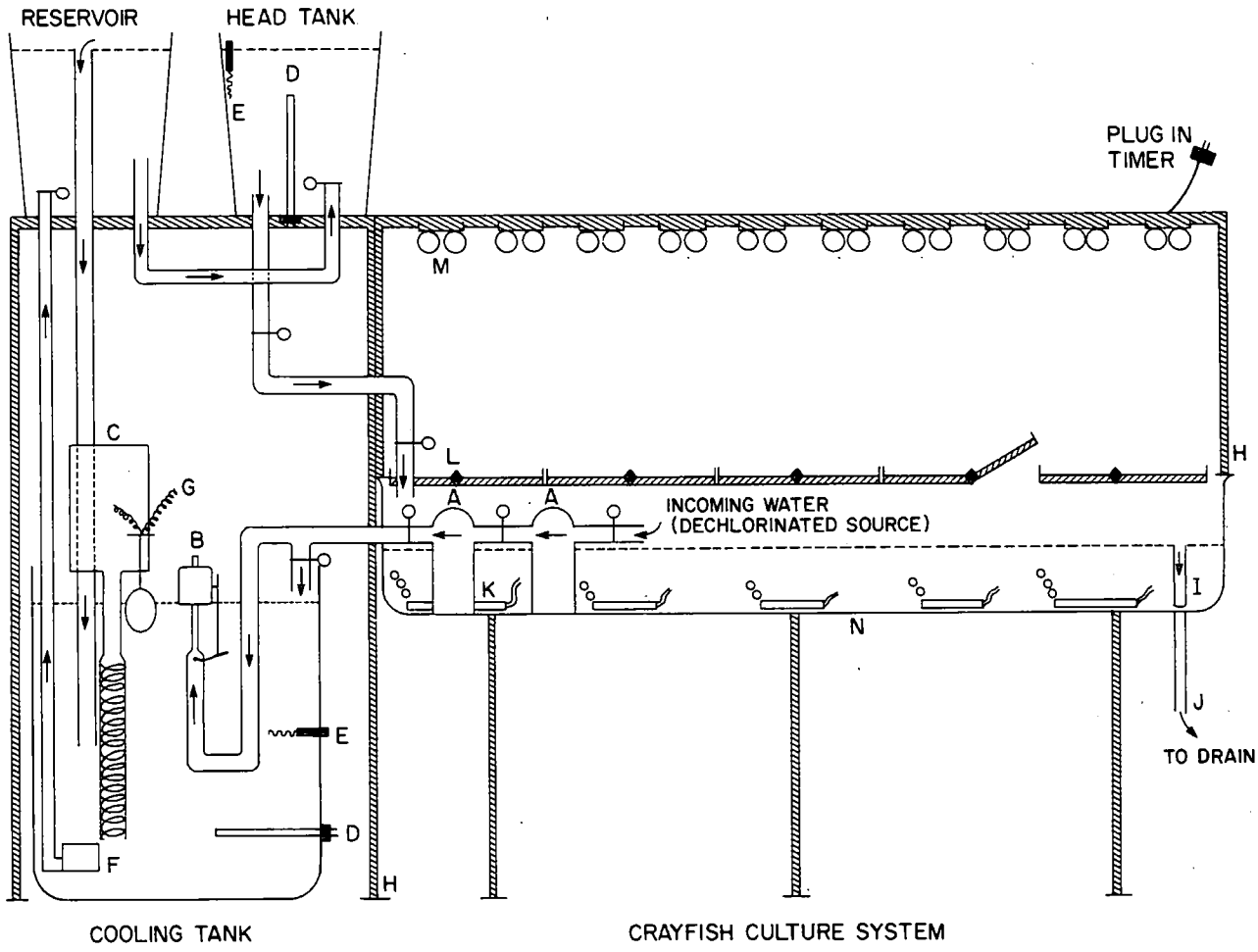


Fig. A3 Holding device for *Orconectes* young



A. FILTER (FOR PARTICULATE MATTER)

B. FLOAT VALVE

C. IMMERSION REFRIGERATOR (MIN-O-COOL ^{R})

D. IMMERSION HEATER

E. THERMISTOR PROBE

F. SUBMERSIBLE PUMP

G. SUBMERSIBLE SAFETY CLOSE

H. ANGLE IRON STAND

I. PVC STANDPIPE

J. FLEXIBLE TUBING

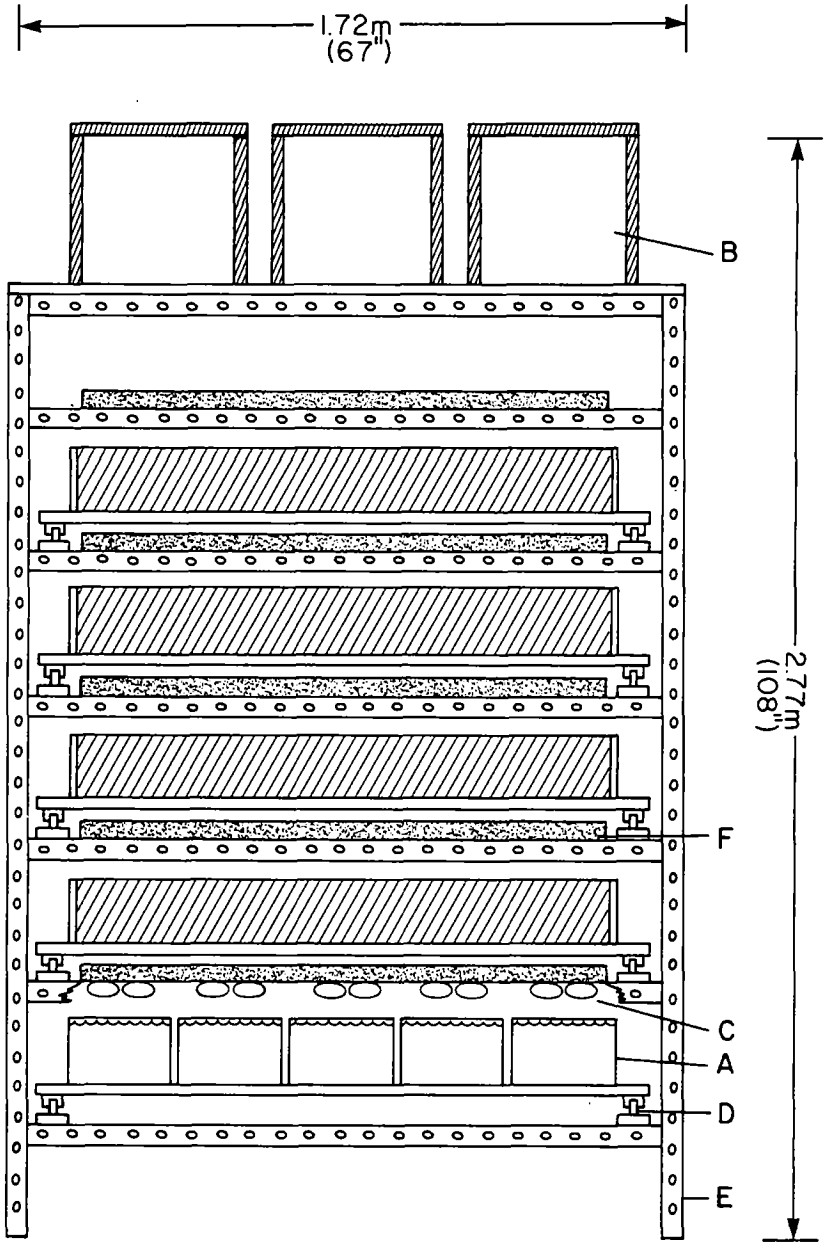
K. AIRSTONES

L. HINGED COVER

M. FLUORESCENT LIGHTS

N. FIBERGLASS TROUGH, 500 x 30 x 20.5 CM ON ANGLE IRON AND PLYWOOD FRAMES

Fig. A4 Reservoir, cooling and distribution system for use in the culture of *Orconectes*



A. EXPERIMENTAL TANKS (41 CM LONG X 20.5 CM WIDE X 15 CM HIGH)

B. HEAD TANKS

C. LIGHTS (SUPPORTS NOT SHOWN)

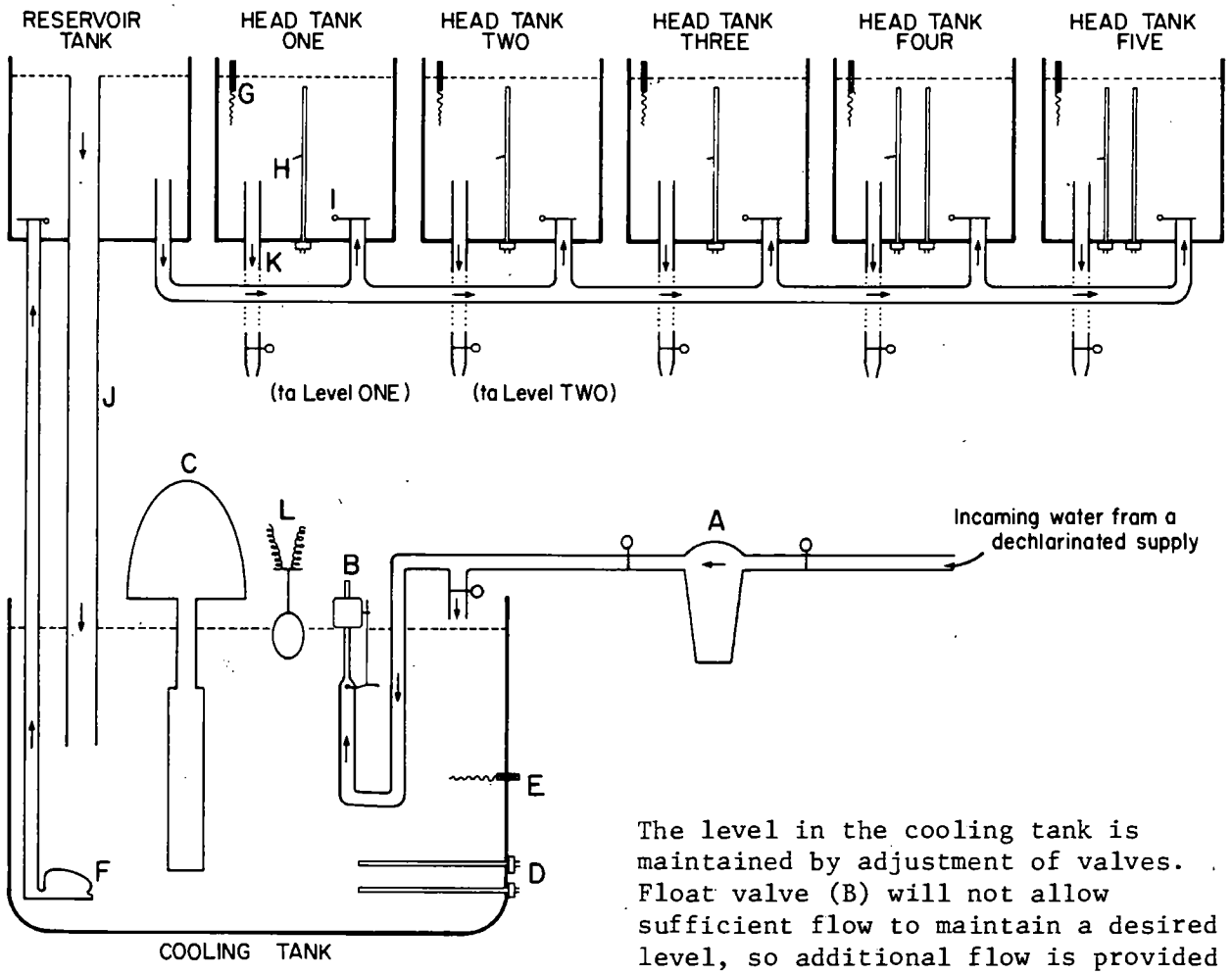
D. ROLLERS

E. SUPPORTS (ANGLE IRON)

F. LIGHT BALLAST (LIGHTS ARE ORDINARILY CONCEALED BY SUPPORTS)

Each level, which contains five tanks is mounted on rollers to expedite handling and examination of tanks. Two fluorescent bulbs are mounted over each tank and the amount of light reaching them is controlled by appropriate filters. 2.5 cm polystyrene sheeting is glued to the outside ends and sides of each tank for insulation.

Fig. A5 Gridded continuous flow system for culture of *Hyalella azteca* and *Gammarus lacustris lacustris*



A. FILTER (FOR PARTICULATE MATTER)

B. VALVES

C. SUBMERSIBLE REFRIGERATOR (MIN-O-COOL ^{R})

D. IMMERSION HEATER

E. TEMPERATURE SENSOR

F. IMMERSION PUMP

G. TEMPERATURE SENSOR

H. IMMERSION HEATER

I. CHECK VALVE

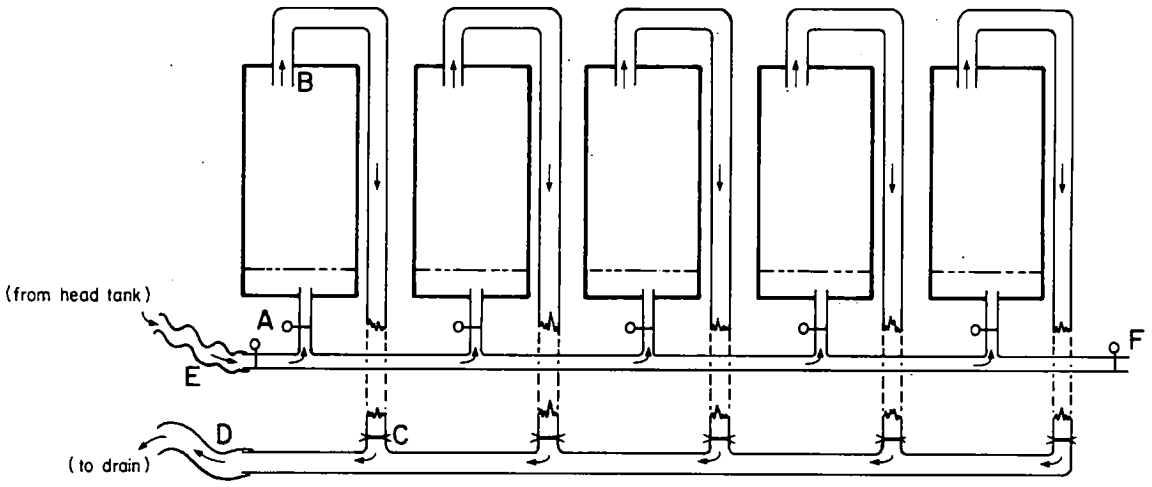
J. STAND PIPE AND DRAIN

K. OUTLET PLUMBING

L. OVERFLOW FLOAT AND SHUT-OFF SWITCH

The level in the cooling tank is maintained by adjustment of valves. Float valve (B) will not allow sufficient flow to maintain a desired level, so additional flow is provided by a preceding valve. Water is cooled by a submerged refrigerator (C) and maintained at a constant temperature by immersion heaters (D,H) controlled by thermistor units (E,G). Each head tank supplies water at a specific temperature through the outlet plumbing. Head tank outlets, which supply experimental tanks, are raised so that heaters will remain submerged if the water supply fails. Water is forced up to the reservoir tank by an immersion pump (F) and returns to the cooling tank through the reservoir standpipe and drain. Plumbing interconnects all head tanks with the reservoir tanks so that the water level is uniform. Check valves (I) prevent back-up of water from head tanks.

Fig. A6 Temperature control system and main water supply for culture of *Hyalella azteca* and *Gammarus lacustris lacustris*



A. INLET VALVES

B. EXPERIMENTAL TANKS

C. DRAIN COUPLING

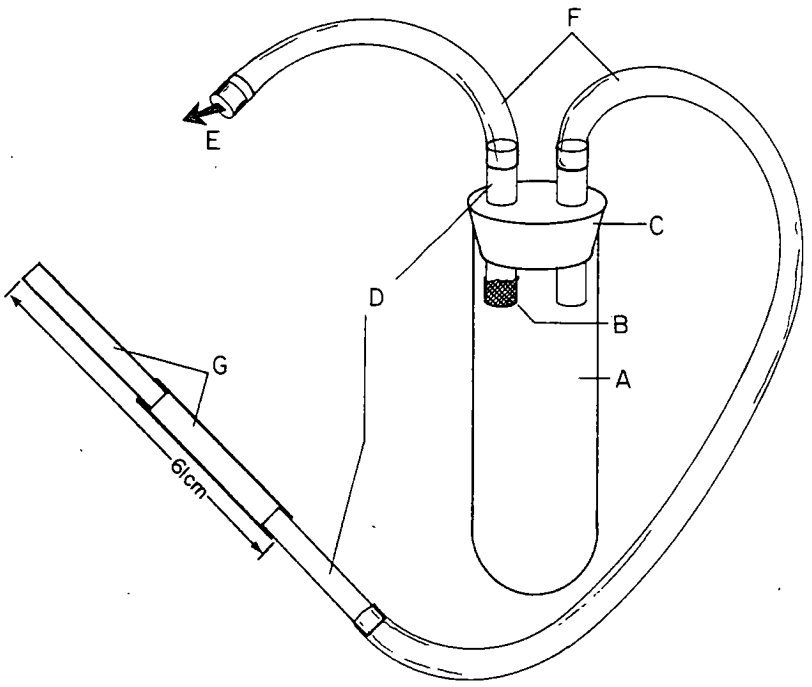
D. MAIN DRAIN

E. MAIN INLET

F. RELEASE VALVE

Arrangement of drains conserves working space and allows drain flow rate from each tank to be measured at the front of the system. Flow is adjusted at the inlet valves (A). A release valve (F) can be opened to increase supply line flow and reduce any temperature increase. The diagram shows one level of five tanks only.

Fig. A7 Experimental tank supply and drain system for culture of *Hyalella azteca* and *Gammarus lacustris lacustris*



A. CENTRIFUGE TUBE

B. NYLON SCREEN

C. RUBBER STOPPER

D. PVC TUBING

E. SUCTION, MOUTH OR PUMP

F. TYGON TUBING

G. COPPER TUBE EXTENDERS

Fig. A8 Aspirator bottle

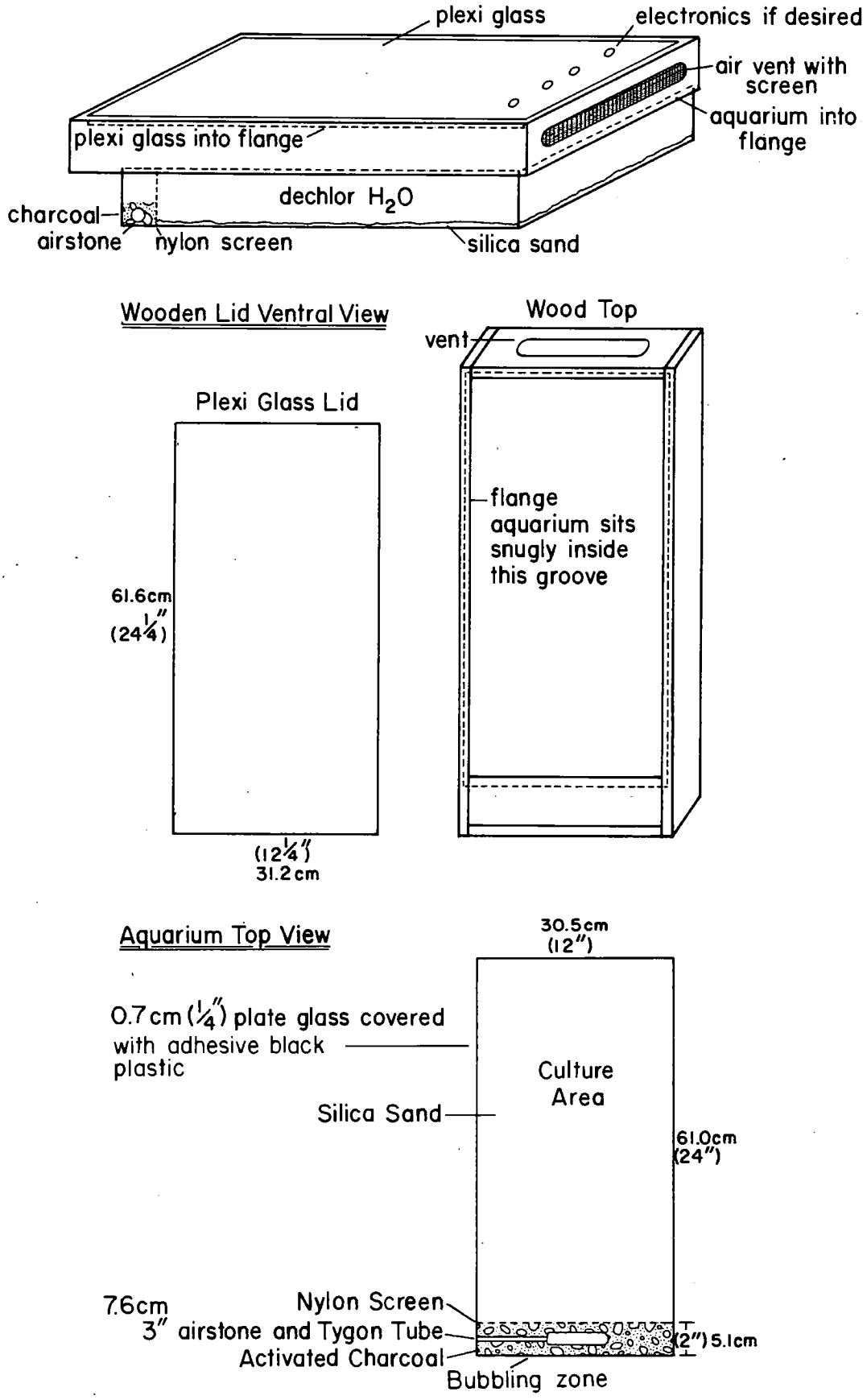
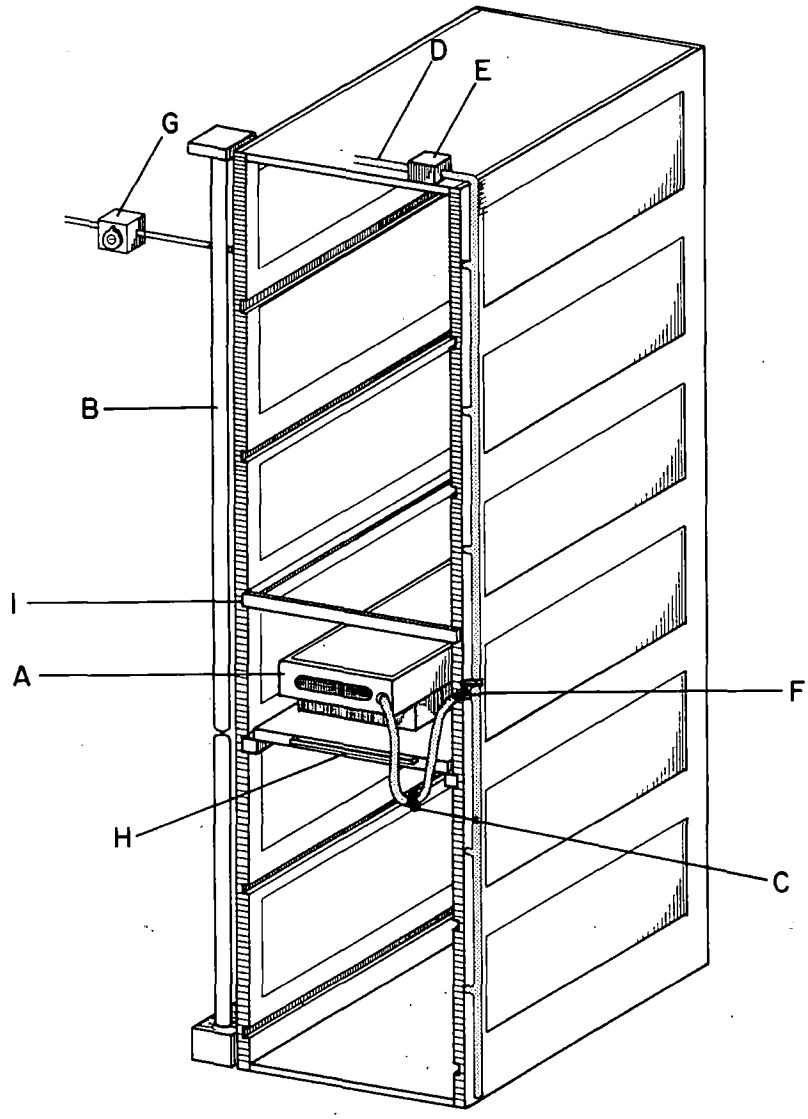


Fig. A9 Aquarium and lid for culture of *Chironomus tentans*



A. AQUARIUM

B. LIGHT BANK

C. FILTER

D. AIR LINE

E. GAS FILTER

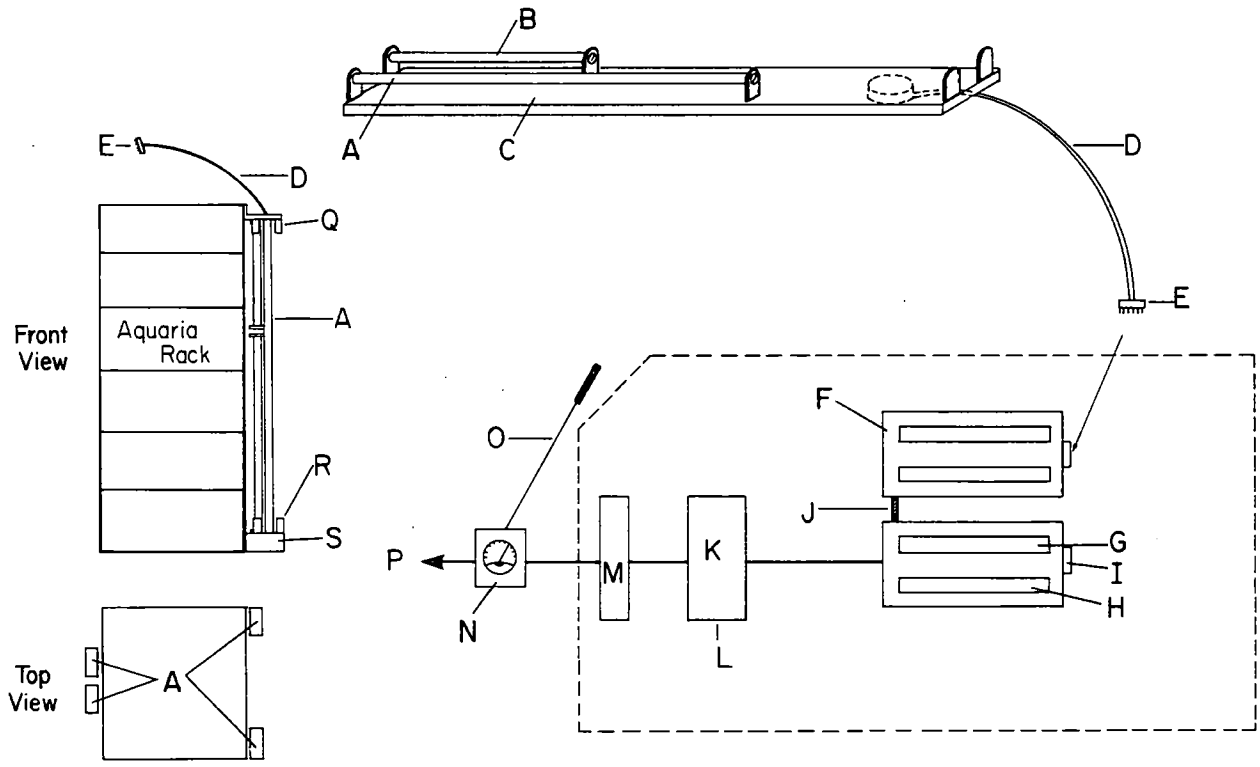
F. ON-OFF AIR VALVE

G. PHOTOPERIOD SWITCH

H. TRAY AND HANDLE

I. CRDSS BAR SUPPORT

Fig. A10 Rack for culture of *Chironomus tentans*



A. LIGHT FIXTURE: METAL CASE WITH LIGHTS STAGGERED TO AVOID DARK SPOTS

B. 40W T12 BULBS (TWO PER UNIT)

C. 20W T12 BULBS (TWO PER UNIT)

D. 12 CONDUCTOR CORD

E. 12 PIN MALE AMPHNEOL PLUG

F. BALLAST UNIT ONE, METAL CASE

G. 20W REMOTE START BALLASTS (TWO PER UNIT)

H. 40W REMOTE START BALLASTS (TWO PER UNIT)

I. 12 PIN AMPHNEOL FEMALE PLUG

J. BALLAST BOX JUNCTION

K. BALLAST UNIT TWO

L. 110V OUTLET

M. 40A SPST TORK TIMER

N. PEN TEMPERATURE CONTROL

O. TEMPERATURE SENSING PROBE

P. 110V SOURCE

Q. METAL CLIPS

R. RETAINING CLIPS

S. WOOD PLANK

Fig. A11 Light panel of aquaria rack for culture of *Chironomus tentans*

APPENDIX B

CULTURE MEDIA

1. Acid solution for cleaning glassware used to culture Protozoa and other organisms sensitive to surface active agents or heavy metals.

   Even new glassware is often coated with material detrimental to invertebrate organisms. Detergents, proteins (adsorbed onto glass surfaces) and traces of heavy metals often interfere with the culture of invertebrates.

   Procedure:

   a. Place enough distilled water to cover glassware in a large enamel pan or Nalgene container.

   b. Add concentrated HCl to ∿ 10% by volume.

      HCl is very volatile! Avoid breathing fumes!

   c. Immerse glassware; soak for 8-24 h.

   d. Rinse glassware in tap water 4-5 times, then in distilled water 3 times. Use glass distilled water if Protozoa are to be cultured.

2. Cerophyl (dehydrated cereal grass).

   Available from:

   Cerophyl Laboratories Inc.
   Kansas City, MO USA 64112

3. Cerophyl medium (protozoan culture medium).

Constituents	Quantity
Water (glass distilled or Super Q (Millipore))	∿ 1000 mL
Cerophyl (see 2 above)	1.5 g
0.1 mol L^{-1} NaH_2PO_4	0.3 mL
0.1 mol L^{-1} Na_2HPO_4	4.7 mL

   Place Cerophyl in ∿ 100 mL water in a small beaker and simmer for 1-5 min. Filter, discard powder. Dilute filtrate to 900 mL with water, add buffers, then water to make volume up to 1 L. Autoclave in tubes or flasks as needed.

4. Microbiological nutrient media.

   Available from:

   Difco Laboratories
   P.O. Box 1058 A
   Detroit, MI USA 48232

5. Yeast extract-peptone (YEP) medium for culturing *Tetrahymena vorax* axenically (see 4 above).

Yeast extract	0.25% (w/v)
Proteose peptone	1.0% (w/v)

   Sterilize by autoclave.

6. NC medium (based on Provasoli et al. 1970).

Constituents	Final concentration mmol L^{-1}	Final concentration µg L^{-1}
KCl	1.34	
$MgSO_4 \cdot 7H_2O$	0.32	
KH_2PO_4	0.88	
K_2HPO_4	0.68	
$NaSiO_3 \cdot 9H_2O$	0.14	
$NaNO_3$	0.584*	
$CaCl_2 \cdot 6H_2O$	0.1	
H_3BO_3	0.005	
$FeSO_4 \cdot 7H_2O$	0.002	
Thiamine-HCl		100
Ca-pantothenate		225
B_{12}		1

Use water twice distilled in glass or Super Q (Millipore) water as base. Add first six salts, adjust pH with 1 mol L^{-1} HCl to 6.7-7.0. Add rest of salts and vitamins. Autoclave at standard temperature and pressure.

---

* for NC-N limited medium (Chemostat): $NaNO_3$ is 0.146 mmol L^{-1}, and NaCl is 0.882 mmol L^{-1}.

7. *Pseudomonas ovalis, Aerobacter aerogenes.*

These bacteria may be obtained from:

The American Type Culture Collection
12301 Parklawn Drive
Rockville, Maryland, USA 20852

Both organisms may be grown on solid (nutrient agar), or in liquid (nutrient broth) media (see 4 above).

8. *Chlamydomonas reinhardtii, Scenedesmus quadricauda.*

These organisms may be obtained from:

Dr. R.C. Starr
The Culture Collection of Algae at the University of Texas
Department of Botany
University of Texas
Austin, Texas USA 78712

Culture: Aseptic technique required.

See *Tetrahymena vorax*, Section 6.2, dealing with growth of *C. reinhardtii*. Follow the same instructions for both algae but grow them separately.

9. Lugol's solution

Constituents	Amount
KI	10 g
I_2 (resublimed iodine)	5 g
Na acetate	5 g
Water (distilled)	70 mL

Dissolve KI in 20 mL H_2O, add iodine. When these constituents are in solution, add 50 mL H_2O and Na acetate.

10. Tetra-Min B — Tetra-Werke, West Germany
Distributed by Rolf C. Hagen Ltd.
3225 Sartelôn Street
Montreal, Quebec, CANADA
H4R 1E8

11. Yeager's solution

Constituents	Concentration (g L^{-1})
NaCl	10.93
KCl	1.57
$CaCl_2$	0.83
$MgCl_2$	0.17

Dissolve salts in distilled water, bring volume up to 1 L. Sterilize by filtration or $CaCl_2$ precipitates out.

12. Artificial Saline Water (ASW) for *Artemia*.

Saturated Na_2SO_4. The Freshwater Institute source is the salt from Chaplin Lake, Saskatchewan. Use about 250 g salt in 6 L deionized water.

13. Standard food (Cerofood).

1. Algal media (N.B. use a. or b.)

a. FW6 modified from Healey (1973). Stock solutions are all 100 mmol L^{-1}.

Constituents	mL L^{-1} medium
$NaNO_3$	30
KH_2PO_4	3
$MgSO_4$	4
$CaCl_2 \cdot 6H_2O$	2
$NaHCO_3$	10
Distilled water	950

Sterilize by autoclave if desired.

b. Bristol's solution (also known as Bold Basal Medium) (Nichols and Bold 1965).

Constituents	Stock solutions (g 400 mL^{-1})
$NaNO_3$	10
$CaCl_2$	1
$MgSO_4 \cdot 7H_2O$	3
K_2HPO_4	3
KH_2PO_4	7
NaCl	1

10 mL of each stock solution are added to 940 mL of glass-distilled water. To this is added a drop of 1% $FeCl_3$ solution.

2. 10 g trout crumbles, ground finely in a blender.

3. 0.5 g Cerophyl powder (see 2 above).

Mix trout crumbles and Cerophyl. Add to 150 mL algal medium and blend thoroughly. Strain through industrial grade white J cloth towelling 3 times. Reserve the solid residue (see 17 below). Store filtrate in refrigerator in sterile dilution bottles or sterile serum vials. Shake before using. Must be remade monthly.

14. DM medium.

Constituents	Final concentration	
	mg L^{-1}	µg L^{-1}
KCl	50	
$MgSO_4 \cdot 7H_2O$	40	
$CaCl_2 \cdot 6H_2O$	110	
K_2HPO_4	6	
KH_2PO_4	6	
$NaNO_3$	50	
$Na_2SiO_3 \cdot 9H_2O$	20	
Fe (as Cl)		500
Metal solution*	10 mL	

* 1 mL contains: 1000 µg Na_2 EDTA, 10 µg Fe (as Cl); 40 µg Mn (as Cl); 5 µg Zn (as SO_4); 1 µg Co (as SO_4); 200 µg B (as H_3BO_3).

DM medium may be sterilized by autoclaving. Add vitamin mix, B15 below, immediately before use.

15. Vitamin mix.

Constituents	Final concentration in DM medium ($\mu g\ L^{-1}$)
B_{12}	1
Thiamine-HCl	100
Calcium pantothenate	250

Sterilize by filtration through a 0.22 µm pore filter. Add aseptically to sterile DM medium. Do not store.

16. Antibiotic mix.

Constituents	Concentration ($\mu g\ mL^{-1}$)
Neomycin	10
Streptomycin	1500
Penicillin	6000
Tetracyline	400

Dissolve antibiotics in DM medium (see 14 above). Sterilize using a 22 µm pore size filter. Make fresh weekly.

17. Extruded crayfish food.

To standard food residue (see 13 above) (or to 10 mL standard food: 3 g beef extract), add enough commercial alginate food binders (e.g. Kelset or Keltrol; S-Scotia Marine Products, 8355 Aero Drive, San Diego, CA USA 92123) to make a paste. Cut the tip from a 50 mL disposable syringe. Pack the paste into the syringe, eliminate air gaps, and replace plunger. Firmly and evenly expel a strand of paste onto an aluminum foil tray. Do not exert pressure unevenly or strands will coil. Let strands dry overnight at room temperature. Break into 1 cm pieces and refrigerate in sealed plastic bags.

18. Characteristics of dechlorinated Winnipeg city water (Stainton et al. 1977)

	$mg\ L^{-1}$
Sodium	1.2-5.2
Potassium	1.2-1.7
Magnesium	5.8-7.2
Calcium	21.4-28.2
Iron	0.3-0.6
Zinc	0.4
Silicon (soluble, reactive)	1.3-1.5
Sulfate	4.6-7.7
Chloride	4.0-6.0
Ammonia-nitrogen	0.01-0.09
Nitrate-nitrogen	0.005-0.007
Phosphorus (soluble, reactive)	0.003-0.032

Additional water quality parameters follow:

pH	7.1-8.1
Conductivity	150-200 $\mu S\ cm^{-1}$
Dissolved inorganic carbon	1.5-1.9 $mmol\ L^{-1}$
Dissolved organic carbon	0.9 $mmol\ L^{-1}$
Hardness (as $CaCO_3$)	100 $mg\ L^{-1}$

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