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SYSTEMS CULTURE OF FRESHWATER SHELLFISH (BIVALVES)

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CONTENTS

ABSTRACT

ISOM and HUDSON (1982) reported a breakthrough in *in vitro* culture of parasitic freshwater mussel glochidia until transformation to juveniles. ISOM and HUDSON (1984b) reported that there is no physiological plasma basis, other than a possible immune response, for mussel-host specificity involving fish. All fish plasma tested from numerous species, including the common carp, *Cyprinus carpio* (Linn.), contains a non-specific component that initiates transformation of freshwater bivalve glochidia cultured *in vitro*. Successful rearing of juvenile mussels was also reported by HUDSON and ISOM (1984). Cost of *in vitro* culture and rearing of molluscs is nominal, and the technology could be easily adopted by aquaculturists.

As a result of these findings, there is now great potential for artificial culture of freshwater bivalves for conservation stocking purposes and as a source of protein for human consumption. Use of this approach should be considered to supplement the aquaculture of fish, especially cage culture. These new methods also offer the opportunity for genetic improvements and selective breeding of freswater bivalves that were formerly unavailable.

RESUMÉ

ISOM et HUDSON (1982) ont rendu compte d'une découverte sur la culture *in vitro* des métamorphoses glochidiales des moules parasites d'eau douce aux juvéniles. ISOM et HUDSON (1984) ont dit qu'il n'y a pas de base de plasma physiologique autre que la réponse d'immunité pour la specificité de moule hotêsse qui comprends le poisson. Toute la plasma du poisson mise a l'essai (beaucoup des espèces y compris la carpe *Cyprinus carpio* (Linn.)) contient un constituant non-specifique qui fait commencer une métamorphose des cultures glochidies d'eau douce *in vitro*. HUDSON et ISOM (1984) ont parlé aussi de l'élevage reussi des moule juvéniles. Le coût de la culture *in vitro* et de l'élevage des moules est nominal. La technologie pourrait être facilement transferrée aux aquaculturistes et aux autres.

Par suite de ces découvertes, il existe à present grand potentiel pour la culture artificielle des bivalves d'eau douce pour le but de la stockage pour la conservation et comme la source de la protéine pour la consommation humaine. L'usage de cette approche devrait être consideré en combinaison avec l'aquaculture du poisson, particulièrement dans la culture cagée. Ces méthodes nouvelles offrent aussi l'occasion pour les amélioration génétiques et pour l'élevage sélective des bivalves d'eau douce qui jadis était indisponibles.

IN VITRO TRANSFORMATION OF FRESHWATER MUSSEL (UNIONIDAE) GLOCHIDIA

The concept of artificial propagation of freshwater mussels in fish blood plasma originated with a simple experiment reported by LEFEVRE and CURTIS (1912), which failed. Subsequently, the use of artificial media for transformation of normally parasitic freshwater mussel glochidia was reported (ELLIS and ELLIS, 1926; ELLIS et al., 1930). However, glochidia used in the (1926) experiments were dissected out of fish gills, where they had been encysted for 18 to 96 hours. This undoubtedly contributed to their transformation. Several succeeding papers covered various aspects of the artifical mussel culture exerience, but neither the composition of the media nor the media nor the process was ever revealed or published.

The *in vitro* culture of parasitic mussel glochidia of North American species has been thoroughly discussed elsewhere (ISOM and HUDSON, 1982; ISOM and HUDSON, 1984). These reports give new methods for *in vitro* culture of mussel glochidia to juveniles without natural encystment in a fish host. HUDSON and ISOM (1984) reported success in rearing juvenile mussels that transformed *in vitro*. Juveniles transformed in fish were also reared by these methods. However, for the purpose of reporting

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below. This in depth description
to use the *in vitro* procedures. We
will greatly expand the understan

INITIATION OF *IN VITRO* CULTU

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PREPARATION OF UNIONID RING

1. Laboratory Equipment Needed

1.1 Sterile 1000 ml Erlenmey
1.2 Sterile 2000 ml Erlenmey
1.3 Top loading balance
1.4 Weighing "boats"
1.5 Spatula
1.6 Aluminum foil
1.7 Autoclave
1.8 Biological transfer hood

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the experiences and methods necessary for successful cultures, a more practical description is offered below. This in depth description is needed because of the problems encountered by those attempting to use the *in vitro* procedures. We believe this description and other published papers on *in vitro* culture will greatly expand the understanding of the techniques required to develop successful cultures.

INITIATION OF *IN VITRO* CULTURE

Gently and slowly pry open the shell of a female mussel. If the gills are enlarged and have swollen chambers, called "marsupia", the glochidia should be checked by puncturing a marsupium and removing some with a sterile Pasteur pipette. Place the glochidia on a slide with a drop of distilled water and examine them under a microcsope to see if they are mature. Mature glochidia will be free of their membrane encasements and should be closing an opening occasionally. Dead glochidia will have dispersed tissue and often have ciliates associated with or feeding on them. Viability can be checked by adding a drop of saline solution to the slide. Glochidia will close in response to the chloride. A 60 to 100% closure constitutes viability for culture purposes.

If the glochidia are mature, they may be removed by one of two methods. The first method allows survival of the parent mussel, which is highly desired when it is a rare species. The mussel is gently pried open, and a Pasteur pipette is used to puncture each gill chamber, or marsupium. The glochidia are aspirated into the pipette and placed in a 1000 ml beaker containing about 200 ml of distilled water. This is continued until enough glochidia are obtained to initiate cultures. If care is taken, mussels returned to their natural habitat can have a good chance of survival. For the second method, the mussel is opened fully, and the gills excised with scissors. The entire gill is cut longitudinally and swirled using forceps to release glochidia in a 1000 ml beaker containing distilled water.

After the glochidia have been dispersed in the distilled water, all gill tissue is picked out with a forceps. The glochidia are allowed to settle, and the water is poured off after swirling, leaving the glochidia in the beaker. Clean water (200 ml) is added, and the process repeated several times. Healthy glochidia typically have greater density than other glochidia and tissue, which allows rinsing of healthy glochidia to remove bacteria and debris.

A Pasteur pipette is used to add the glochidia to the tissue culture dishes (60 x 15 mm) filled with 2 ml of artificial medium, 1 ml fish blood plasma, and appropriate antibiotics. The glochidia should be drawn or aspirated into the pipette and allowed to settle toward the tip. Only one drop of glochidia suspension should be added to each culture dish to minimize conamination. This should yield a culture density of 200 to 1000 glochidia per dish. Shake the dish to disperse the glochidia, and place it in a CO_2 and air incubator at 24 °C. The incubator should have a continuous water supply in order to assure 100% humidity. Adjust the CO_2 flow to produce a pH of 7.2 to 7.4 in the medium. Alternative buffering systems can be used, such as HEPES.

PREPARATION OF UNIONID RINGERS PHYSIOLOGICAL SOLUTION

1. Laboratory Equipment Needed

1.1 Sterile 1000 ml Erlenmeyer flasks
1.2 Sterile 2000 ml Erlenmeyer flasks
1.3 Top loading balance
1.4 Weighing "boats"
1.5 Spatula
1.6 Aluminum foil
1.7 Autoclave
1.8 Biological transfer hood (HEPA filter type)

1.9 Vacuum and pressure pump for filtration (positive and negative)
1.10 Membrane filter apparatus
1.11 Sterile 100, 1000 ml graduated cylinders
1.12 0.45 and 0.20 µm membrane filters

2. Laboratory Methods

Unionid Ringers solution consists of two solutions, called A (100 X) and B, which are made separately to prevent precipitation.

Solution A (100 X) consists of the following chemicals: 1.2 g $CaCl_2$, 1.0 g $MgCl_2 \cdot 6\ H_2O$, which are added to 100 ml distilled or deionized water in a 1000 ml Erlenmeyer flask.

Solution B consists of the following chemicals: 1.53 g NaCl, 0.099 g KCl, 2.2 g $NaHCO_3$, which are placed in a 2000 ml Erlenmeyer flask to which is added 990 ml deionized water.

After A (100 X) and B solutions are made, cover each with alumium foil and autoclave for 15 minutes (1.05 kg/cm² pressure at 121 °C). When solutions have cooled to room temperature, combine by pouring 10 ml of solution A into 990 ml of B to make unionid Ringers. To minimize bacterial contamination, pass the solution through 0.45 µm filter (0.45 µm filters won't remove all bacteria). This may be supplemented by 0.20 µm or 0.22 µm filtration.

PREPARATION AND CONCENTRATION OF ANTIBIOTICS AND ANTIMYCOTIC

1. Laboratory Equipment

1.1 Sterile unionid Ringers solution
1.2 3 ml sterile syringes with 22 gauge needles
1.3 1 ml sterile insulin syringes with 22 gauge needles
1.4 Biological transfer hood
1.5 Sterile 100 ml graduated cylinder
1.6 Freezer (-20 °C desirable)

2. Preparation of the antibiotics, all under biological transfer hood, if possible

2.1 Carbenicillin: Add 4 ml unionid Ringers to a 1 g vial of carbenicillin to make the stock solution. Remove 0.2 ml stock solution and add it to 25 ml sterile unionid Ringers or sterile distilled water to make the diluted stock solution (2000 µg/ml). Add an appropriate amount of diluted stock to 3 ml of medium in culture dishes (Table 1).

2.2 Gentamicin sulfate: Available as 40,000 µg/ml stock solution. Remove 1 ml from stock vial of gentamicin sulfate and add to 39 ml unionid Ringers solution. Add appropriate amounts of this diluted stock solution to 3 ml of medium in culture dishes (Table 1).

2.3 Rifampin: Add 300 mg, usually available in capsule form, to 150 ml of sterile unionid Ringers or sterile distilled water to make a stock solution of 2000 µg/ml. Add an appropriate amount of diluted stock solution to 3 ml of medium in culture dishes (Table 1).

3. Preparation of amphotericin B

3.1 Add 10 ml of sterile unionid Ringers or sterile distilled water to 50 mg vial of active ingrediant to make a stock solution. Take 1 ml of stock (5 mg/ml) and add it to 9 ml of unionid Ringers solution (50 µg/ml; Table 2).

Table 1: Approximate final concent

	100	2(
Amount (in ml) of diluted stock to add to 3 ml of culture medium	0.05	0.

Table 2: Approximate final concent

	1 (1/5 X)
Amount (in ml) of diluted stock to add to 3 ml of culture medium	0.006

Table 3: Preferred final concentrat

Component
INORGANIC
$CaCl_2$
$MgCl_2 \cdot 6\ H_2O$
NaCl
KCl
$NaHCO_3$
ORGANIC
Essential amino acids
L-Arginine · HCl
L-Cystine
L-Glutamine
L-Histidine · HCl · H_2O
L-Isoleucine
L-Leucine
L-Lysine · HCl
L-Methionine
L-Phenylalanine
L-Threonine
L-Tryptophan
L-Tyrosine
L-Valine

Table 1: Approximate final concentration (µg/ml) of carbenicillin, gentamicin sulfate, and rifampin

	100	200	300	400	500	600	700	800	900	1000
Amount (in ml) of diluted stock to add to 3 ml of culture medium	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5

Table 2: Approximate final concentration (µg/ml) of amphotericin B

	1 (1/5X)	5 (1X)	10 (2X)	15 (3X)	20 (4X)	24 (5X)	30 (6X)
Amount (in ml) of diluted stock to add to 3 ml of culture medium	0.006	0.03	0.06	0.09	0.12	0.16	0.2

Table 3: Preferred final concentrations of culture medium components

Component	mg/l	Component	mg/l
INORGANIC		ORGANIC	
		Non-essential amino acids	
$CaCl_2$	120.0		
$MgCl_2 \cdot 6\ H_2O$	100.0	L-Alanine	8.9
NaCl	1530.0	L-Asparagine · H_2O	15.0
KCl	99.0	L-Aspartic acid	13.3
$NaHCO_3$	2200.0	L-Glutamic acid	14.7
		Glycine	7.5
ORGANIC		L-Ornithine	10.0
Essential amino acids		L-Proline	11.5
		L-Serine	10.5
L-Arginine · HCl	126.0	Taurine	31.0
L-Cystine	24.0		
L-Glutamine	292.0		
L-Histidine · HCl · H_2O	42.0	Vitamins	
L-Isoleucine	52.0		
L-Leucine	52.0	D-Ca pantothenate	1.0
L-Lysine · HCl	72.5	Choline chloride	1.0
L-Methionine	15.0	Folic acid	1.0
L-Phenylalanine	32.0	Inositol	2.0
L-Threonine	48.0	Nicotinamide	1.0
L-Tryptophan	10.0	Pyridoxal · HCl	1.0
L-Tyrosine	36.0	Riboflavin	0.1
L-Valine	46.0	Thiamin · HCl	1.0
		Others	
		Dextrose	100.0
		Phenol red	10.0

PREPARATION OF *IN VITRO* MEDIUM

1. Laboratory equipment needed

1.1 Sterile 0.1 ml pipettes
1.2 Sterile 1 ml pipettes
1.3 Sterile 5 ml pipettes
1.4 Sterile 10 ml pipettes
1.5 Sterile 25 ml pipettes
1.6 Biological transfer hood
1.7 Bunsen burner
1.8 pH meter

2. Preparation of complete medium

The complete medium made by adding all components to the basic unionid Ringers solution. All solutions are kept frozen or refrigerated until they are added individually under sterile conditions under a biological transfer hood when possible. Cotton plugged pipettes are sterilized for 15 to 20 minutes in an autoclave (1.05 kg/cm² at 121 °C) and flamed before using them to add the following:

Dextrose (20 % w/v)	5 ml, or 0.5 ml
Unionid Ringers	960 ml
Essential amino acids (50X; EAGLE, 1959)	20 ml
Non-essential amino acids (100X; EAGLE, 1959)	10 ml
Vitamins (100X)	10 ml
Phenol red (1 % solution) indicator	1 ml

Check the pH of 40 ml of complete medium; it should be 7.2 to 7.4. Add up to 3 ml of 1.25 M NaOH to the complete media to adjust the pH as desired.

If non-sterile mixtures are prepared, filtration must be used to complete sterilization (0.45 µm filter followed by 0.20 µm filter). Pressure filtration is preferred to vacuum filtration whenever possible. Stock culture medium is kept under refrigeration and should be replaced at least every three weeks.

Direct mixing of dry amino acids may be done to save costs. Prepare the concentrations as prescribed by EAGLE (1959). Preferred final concentrations of culture medium components are shown in Table 3.

STERILIZATION OF DISTILLED AND RIVER WATER

1.0 Laboratory equipment needed

1.1 2000 ml Erlenmeyer flasks (sterile)
1.2 Aluminum foil
1.3 Autoclave
1.4 Vacuum or pressure pump
1.5 Membrane filter apparatus
1.6 0.45 and 0.20 µm membrane filters

2. Laboratory procedures

2.1 Sterilize distilled water in a 2000 ml Erlenmeyer flask, cover top with aluminum foil, and autoclave 25 minutes at 1.05 kg/cm² pressure and 121 °C.

2.2 Sterilize river water in a 2000 ml Erlenmeyer flask, cover top with aluminum foil, and autoclave for 25 minutes. Ultraviolet light (UV) treatment or filtration using 0.45 µm filters followed by 0.20 µm filters may be used instead. Checking under the microscope is necessary to determine the proper intensity and timing for the UV light treatments.

COLLECTION AND PREPARATION

1. Laboratory equipment

1.1 10 - 12 ml syringes with
1.2 Sterile covered centrifug
1.3 Sterile heparin sodium (
1.4 Autoclave
1.5 Aluminum foil
1.6 Refrigerated high-speed
1.7 Vacuum pump
1.8 Pressure pump
1.9 Freezer
1.10 Biologicaltransfer hood (
1.11 Sterile 5 - 10 ml cotton
1.12 Sterile 500 ml Erlenmeye
1.13 Bunsen burner
1.14 Alcohol
1.15 Sterile forceps
1.16 Membrane filter apparat
1.17 0.45 and 0.20 µm membr

2. Preparation of equipment for t

2.1 Autoclave 10 ml syringes
121 °C. After syringes a
clotting. Heparinization i
barrel, allowing it to pa
atrace amount at the b
the following procedures
the top with foil and ar
refrigerated centrifuge is

3. Preparation of blood plasma

3.1 Fish blood is collected
mentioned above. Blood
and placed on ice for tr
at 1000 and 5000 r.p.m.
plasma is aspirated fron
Erlenmeyer flask or bott
day. Plasma is removed
hood. Coagulated protein
Plasma can be complete
ficult, time consuming, a

COLLECTION AND PREPARATION OF BLOOD PLASMA

1. Laboratory equipment

1.1 10 - 12 ml syringes with 18 gauge needles
1.2 Sterile covered centrifuge tubes for high speed centrifuge
1.3 Sterile heparin sodium (1000 USP units per ml)
1.4 Autoclave
1.5 Aluminum foil
1.6 Refrigerated high-speed centrifuge desirable
1.7 Vacuum pump
1.8 Pressure pump
1.9 Freezer
1.10 Biologicaltransfer hood (HEPA type)
1.11 Sterile 5 - 10 ml cotton plugged pipettes
1.12 Sterile 500 ml Erlenmeyer flask or bottle
1.13 Bunsen burner
1.14 Alcohol
1.15 Sterile forceps
1.16 Membrane filter apparatus
1.17 0.45 and 0.20 µm membrane filters

2. Preparation of equipment for the collection of blood

2.1 Autoclave 10 ml syringes with 18 gauge needles for 25 minutes at 1.05 kg/cm² pressure and 121 °C. After syringes are sterilized, they must be heparinized (1000 units per ml) to prevent clotting. Heparinization is accomplished by aspirating some heparin solution into the syringe barrel, allowing it to pass up the entire length, and depressing the plunger to remove all but atrace amount at the barrel end and in the needle. Prior to the collection of fish blood, the following procedures should be completed: centiguge tubes are wrapped individually at the top with foil and are autoclaved for 25 minutes at 1.05 kg/cm² pressure and 121 °C. The refrigerated centrifuge is set to reach a temperature of 10 °C standing and 4 °C running.

3. Preparation of blood plasma

3.1 Fish blood is collected by cardiac puncture or from the caudal artery using the syringes mentioned above. Blood is transferred from the syringes to the sterilized centrifuge tubes and placed on ice for transport. The centrifuge tubes are placed in the centrifuge and spun at 1000 and 5000 r.p.m. for 10 minutes. Centrifuge tubes are removed, and the top layer of plasma is aspirated from flamed 5 to 10 ml sterile plugged pipettes into a sterile 500 ml Erlenmeyer flask or bottle. The plasma is placed in the freezer at -20 °C until the following day. Plasma is removed from the freezer and allowed to thaw under the biological transfer hood. Coagulated proteins are discarded. Plasma is partially sterilized by 0.45 µm filtration. Plasma can be completely sterilized by 0.20 µm or 0.22 µm filtration; however, this is difficult, time consuming, and usually not done.

STERILITY TESTING

1. Laboratory equipment needed

1.1 Sterile 2000 ml Erlenmeyer flask
1.2 Aluminum foil
1.3 Top loading balance
1.4 Weighing "boats"
1.5 Spatula
1.6 Autoclave
1.7 Petri dishes
1.8 Bunsen burner
1.9 Innoculating loops
1.10 Refrigerator
1.11 Incubator
1.12 Biological transfer hood

2. Preparation of nutrient broth agar plates

Weigh 8 g of nutrient agar; pour it into a sterile 2000 ml Erlenmeyer flask. Dissolve it in 1000 ml of distilled water, and cover with alumium foil. Autoclave 20 minutes. Place petri dishes under biological transfer hood. Remove agar from autoclave. Cool to lukewarm temperature and pour into plates. Let agar solidify and then place inverted plates in a refrigerator.

3. Sterility testing

Place petri dishes under a biological transfer hood, and allow agar to reach room temperature. Flame innoculating loop until red, place into solution to be tested, and streak onto agar. Repeat this same procedure for each solution to be tested. Place plates in incubator at 24 °C for 24 hours. If bacterial colonies form or fungal growth occurs, the solution is contaminated and should be discarded. Solutions are streaked 24 to 48 hours prior to use, which allows detection of contaminants before cultures are endangered.

JUVENILE FRESHWATER MUSSEL CULTURE

HUDSON and ISOM (1984) discussed the laboratory rearing of juvenile mussels in detail. We believe that laboratory observations indicate juvenile mussels are subject to some predation if their food and water sources are not handled properly. In order to obviate this problem, we have adapted the procedures and system used for growing a supply of the hard clam, *Mercenaria* (CASTAGNA and KRAEUTER, 1981), in freshwater mussel culture. Basically, the procedures involved collecting and filtering river water through a 5 µm sack. Filtered water contains a diversity of phytoplankton which was allowed to grow for about 24 hours to a density of 100,000 or more cells/ml. When plankton yields were lower, we centrifuged filtered river water to obtain plankton, which was used as supplemental food.

Since our objective was to rear juvenile mussels to only 3 mm, which takes 50 to 60 days at 24 °C, prior to being transplanted in a natural environment, we used a static system and changed the water twice daily. Water containing the juveniles was filtered initially through nylon mesh screens with pores of 34 µm, and then through 75, 100, 202 µm screens as they grew larger.

We found that, as in mariculture (URBAN and LANGDON, 1984), the culture of freshwater mussels requires organic and inorganic particulate silt additions. Although the exact role of silt is unknown, it is thought to stimulate the filtration and absorption of soluble organic food, and to grind food to improve assimilation efficiency. The culture water with silt added should contain about 800 mg silt/l (HUDSON and ISOM, 1984).

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and McLARNEY (1984). The first
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CONCEPT FOR POLYCULTURE O

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We believe this system or commercial culture system should be developed for freshwater mussel aquaculture and conservation, possibly in combination with fish culture. Such systems will enhance survival of juvenile mussels released into their natural habitats or commercial systems for conservation or pearl culture.

Previous research on the culture of freshwater mussels as a food source has been described in papers, including VILLADOLID and DEL ROSARIO (1930), MILLER and McCLURE (1931), POST (1982), and McLARNEY (1984). The first three of these deal with the harvest and use of *Corbicula* as a human food resource throughout the range of the genus. POST (1982) evaluated freshwater mussels as a "new protein" source and concluded that they were moderately acceptable as food for humans and contained 17 amino acids, including the essential ones. In his book, McLARNEY (1984) briefly treats North American experiences with freshwater mussels as a food source. He also noted that "the use of freshwater clams in aquaculture remains an intriguing possibility".

SYSTEMS CULTURE OF FRESHWATER MUSSELS

Systems culture of freshwater mussels is defined here as beginning with the process of *in vitro* culture and continuing to rearing of these mussels to reproductive age for commercial or stocking purposes in managed waters. Mussels have been managed in containment (JONES, 1950). However, the technology required for systems culture of freshwater mussels has not been available until the development of *in vitro* culture and juvenile rearing systems (ISOM and HUDSON, 1982; ISOM and HUDSON, 1984; HUDSON and ISOM, 1984). In natural environments, only a few of the thousands of glochidia survive to transform to juveniles, and even fewer become adults. We can obtain in excess of 60% transformations *in vitro* and nearly 90% survival of juveniles. It is obvious that a systems approach would greatly enhance the potential for rearing freshwater mussels for any purpose. An economical approach is afforded by the opportunity for addition of such a system to a fish culture operation.

CONCEPT FOR POLYCULTURE OF FISH AND MUSSELS

Since freshwater mussels are mucoid filter feeders, they could be reared on the residue, bacteria, and planktonic algae associated with cage culture of fish. Association with cage culture of fish is suggested only as a first choice since there is a chance that the glochidia would parasitize the fish.

Conceptually, at least, the parasitic glochidia of caged mussels would fall to the benthic zone of the lake or pond and away from cages in which fish were being held. However, glochidiosis of the fish from excessive parasitism would be possible (MURPHY, 1942; MEYERS and MILLEMANN, 1977). Assuming mussels were reared in the natural benthic sediments, they should not interfere with but enhance culture of fish by removing excessive micro and soluble nutrients, bacteria, and algae. Another possibility would be to rear mussels downstream from fish culture systms in flowing water. A number of scenarios could be developed to take advantage of nutrient losses from fish culture to grow mussels and still obviate the parasitism problems. The extent to which mussels would improve water quality is unknown, but conceptually at least, they could be used to improve water qualtiy prior to recycling or release to the environment.

SWINGLE (1966), in an earlier FAO symposium, reported on the use of freshwater mussels as a means of increasing production of non-filter feeding fish in ponds from 314 kg/ha without mussels to 461 kg/ha with mussels. In addition, the mussels he harvested weighed 397 kg/ha with their shells removed.

FUTURE RESEARCH NEEDS

TVA sponsored an *in vitro* culture workshop on July 19 and 20, 1983, which was attended by two past presidents of the American Malacological Union and many others interested in malacology from the private sector, universities, and state and Federal Government agencies. A concluding session was held to generate ideas for future research. A number of problems have been resolved; the balance of research needs are enumerated below.

1. Bacterial control

While bacteria are not prohibitive to *in vitro* culture of mussels, they are a problem in some cultures and especially for summer spawning species. Sensitivity testing of antibiotics should be continued to help identify better bacteriological controls. We are confident in the controls that are now being used, but they could be improved. New antibiotics should be tested for efficacy as they become available.

2. Essential components of *in vitro* culture media

During the initial development of the *in vitro* media, it was decided to include all the amino acids that are found in all fish, thus leading to the development of a "universal" medium. The media (ISOM and HUDSON, 1982, 1984) proved adequate to transform species of the three subfamilies of the Unionidae. However, since the costs of media components are inexpensive, non-essential chemicals have not been identified. As noted above, we did determine that fish blood plasma contains an unidentified component that is essential to transformation. Deletion of non-essential elements could perhaps promote better or faster transformation and remove constituents which promote bacterial growth.

3. Mussel host identification

There are significant gaps in our knowledge of mussel-host relationships. Research should be conducted on the physiological relationships between mussels and their hosts. Their immune responses, as well as behavioral and ecological constraints on their relationships, should be determined. It has been shown that from a physiological perspective, numerous fish tested have a non-specific component that is essential for transformation of glochidia to juveniles (ISOM and HUDSON, 1984b). Rigorous laboratory or microcosm experiments should be initiated to determine the immunological response physiology of fish to mussel parasites. The current literature is very perplexing in that numerous reports have indicated host specificity, while the overwhelming bulk of the literature indicates non-specificity (HART and FULLER, 1974).

4. Freshwater mussel spawning in the laboratory

Artificial spawning of freshwater mussels has never been attempted, as far as we know. However, artificial spawning of mussels in the laboratory or under field conditions would greatly aid research and make glochidia available for fish host or stocking studies. Presently, locating mussels with mature glochidia is a difficult problem and severely limits the length of time in which studies can be conducted on each life stage.

We have obviated this problem to some extent by using *Anodonta imbecillis* Say, 1829, as a surrogate for developing culture and rearing procedures and refinements. Gravid specimens within a population of this species can be found about eleven months of the year in Alabama, USA. Only in January and February are they not available.

5. Breeding studies

Breeding or "crossing" of freshwa
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CONCLUSIONS

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REFERENCES

CASTAGNA, M.; KRAEUTER, J.N. 1981 rine Sci., Spec. Rep. (2

EAGLE, H.: Amino acid metaboli 1959

ELLIS, M.M.; ELLIS, M.D.: Grow 1926 solutions. Science **54** (16

ELLIS, M.M.; MERRICK, A.D.; E normal and adverse con 509-542.

GONZALES, O.N.; BERSAMIN, S. 1956 Lamarck) and oysters a

ISOM, B.G.; HUDSON, R.G.: In 1982 **96** (4): 147-151.

ISOM, B.G.; HUDSON, R.G.: Cul 1984a complex liquid growth

ISOM, B.G.; HUDSON, R.G.: Fre 1984b **70** (2): 318-319.

HUDSON, R.G.; ISOM, B.G.: Rea 1984 setting. The Nautilus 8

JONES, R.O.: Propagation of fres 1950

LEFEVRE, G.; CURTIS, W.C.: Stu 1912 sels. Bull. U.S. Bur. Fis

McLARNEY, W.: The Freshwater 1984 583 pp.

MEYERS, T.R.; MILLEMAN, R.E. 1977 mental infection with M **63** (4): 728-733.

MILLER, R.C.; McCLURE, F.A.: 1931 Canton. **10** (2-3): 307-

MURPHY, G.: Relationship of the 1942 **28**: 89-102.

5. Breeding studies

Breeding or "crossing" of freshwater mussels has never been done. Such studies would be valuable in resolving taxonomic problems with so-called ecomorphs, and supposedly closely allied species or subspecies. The use of *in vitro* culture and juvenile rearing procedures would greatly enhance the yield of such experiments since hundreds or even thousends of progeny could be obtained.

CONCLUSIONS

The preceding information provides for awareness and offers opportunity to develop systems for culturing freshwater bivalve molluscs that have been unavailable until now. Although our research was initiated for the purpose of conserving endangered or rare unionids, the application of findings to consumption and stocking purposes should be considered.

The harvest of unionids to obtain shell nuclei for cultured pearls, a combination of environmental factors, and multiple uses of water sources have greatly depleted mussel resources in the United States. A systems culture of unionids to support the pearl industry should be considered.

The greatest promise for systems culture of freshwater mussels is in combination with fish aquaculture. Mussels could be used to improve water quality, serve as food for human and animal consumption, or be recycled as fish food, thus reducing costs of fish culture.

REFERENCES

CASTAGNA, M.; KRAEUTER, J.N.: Manual for growing the hard clam Mercenaria. Virginia Inst. of Ma-
1981 rine Sci., Spec. Rep. (249): 110 pp.

EAGLE, H.: Amino acid metabolism in mammalian cell cultures. Science **130**: 432-437.
1959

ELLIS, M.M.; ELLIS, M.D.: Growth and transformation of parasitic glochidia in physiological nutrient
1926 solutions. Science **54** (1667): 579-580.

ELLIS, M.M.; MERRICK, A.D.; ELLIS, M.D.: The blood of North American Freshwater mussels under normal and adverse conditions. Bull of the Bur. of Fish. **56** (Bur. of Fish. Doc. No. 1097): 509-542. 1930

GONZALES, O.N.; BERSAMIN, S.V.: Tulya (Corbicula manilensis Philippi), Paros (Soletellina elongata
1956 Lamarck) and oysters as good sources of available calcium. Bur. of Fish., Manila, 6 pp.

ISOM, B.G.; HUDSON, R.G.: In vitro culture of parasitic freshwater mussel glochidia. The Nautilus.
1982 **96** (4): 147-151.

ISOM, B.G.; HUDSON, R.G.: Culture of freshwater mussel glochidia in an artificial habitat utilizing
1984a complex liquid growth media. United States Patent 4,449,480. 18 pp.

ISOM, B.G.; HUDSON, R.G.: Freshwater mussel and their fish hosts; physiological aspects. J. parasit.
1984b **70** (2): 318-319.

HUDSON, R.G.; ISOM, B.G.: Rearing juveniles of the freshwater mussels (Unionidae) in a laboratory
1984 setting. The Nautilus **84** (4): 129-135.

JONES, R.O.: Propagation of freshwater mussels. Progve. Fish. Cult. (January): 13-25.
1950

LEFEVRE, G.; CURTIS, W.C.: Studies on the reproduction and artificial propagation of freshwater mus-
1912 sels. Bull. U.S. Bur. Fish. **30**: 105-201.

McLARNEY, W.: The Freshwater Aquaculture Book. Point Roberts, Washington: Hartley and Marks, Inc.
1984 583 pp.

MEYERS, T.R.; MILLEMAN, R.E.: Glochidiosis of salmon fishes. I. Comparative susceptibility to experi-
1977 mental infection with Margaritifera margaritifera (L.) (Pelecypoda: Margaritanidae). J. parasit. **63** (4): 728-733.

MILLER, R.C.; McCLURE, F.A.: The freshwater clam industry of the Pearl River. Lingnan Science J.
1931 Canton. **10** (2-3): 307-332.

MURPHY, G.: Relationship of the freshwater mussel to trout in the Truckee River. Calif. Fish Game
1942 **28**: 89-102.

POST, A.R.: Evaluation of freshwater mussels (Megalonaias gigantea) as a new protein source. Diss., 1982 Univ. of Tenn., Knoxville. 164 pp.

SWINGLE, H.S.: Biological means of increasing productivity in ponds. FAO World Symposium on Warm- 1966 water Pond Fish Culture. FAO Fish Rep. **44** (4): 243-257.

VILLADOLID, D.V.; DEL ROSARIO, F.G.: Some studies on the biology of Tulla (Corbicula manilensis 1930 Philippi), a common food clam of Laguna de Bay and its tributaries. The Philipine Agriculturist **19** (6): 355-382.

URBAN, E.R. Jr.; LANGDON, C.J.: Reduction in costs of diets for the American oyster, Crassostrea 1984 virginica (Gmelin), by the use of non-algal supplements. Aquaculture **38**: 277-291.

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