RICHARD J. NEVES

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STUDY PLAN
NBS - Leetown Science Center
Research and Development Laboratory - Wellsboro

NUMBER: 821.01

TITLE: Development of culture techniques to produce juvenile freshwater mussels for research and restoration.

BACKGROUND/JUSTIFICATION:

The North American freshwater unionacean mussel fauna, the most diverse worldwide, has undergone serious decline over the past 100 years due to overharvest for buttons and pearls, habitat deterioration from channel dredging and dams, pollution from agriculture, mines and industry, and siltation from poor land use practices (Williams et al. 1993). Of the 297 recognized taxa within the families Margaritiferidae and Unionidae, only 70 (24%) are presently considered stable (Williams et al. 1993). Twenty one species (7%) are believed extinct, 58 (21% of remaining) are listed as federally threatened or endangered, and another 76 (28% of remaining) are specified as candidates for federal protection (USFWS 1991, 1994). In addition, several of the endangered species are considered functionally extinct, unable to reproduce in the wild due to loss of appropriate fish host or destruction of juvenile habitat (Bogan 1993).

Although the greatest decline in mussel fauna has occurred in the southeastern United States, particularly in the Mobil Bay, Tennessee River and Cumberland River drainages (Bogan 1993), decline of mussels in the north has intensified since the accidental introduction of zebra mussels into Lake St. Clair in 1988. Zebra mussels have now expanded throughout the Great Lakes, Mississippi River, and Hudson River drainages, attaching to and suffocating native unionids. Complete mortality has been reported among natives in as little as one year in Lake St. Clair (Nalepa 1994) and Western Lake Erie (Schloesser and Nalepa 1994). Many taxa currently considered stable are threatened with severe future declines as the zebra mussel continues to spread into large southern rivers and reservoirs. This situation led Williams et al. (1993) to conclude that "The high numbers of imperiled freshwater mussels in the U.S. and Canada...portend a trajectory toward an extinction crisis that, if unchecked, will severely impoverish one of our richest components of aquatic biodiversity."

The potential loss of freshwater mussels from the American landscape is not, however, simply an endangered species or biodiversity issue. The unionids often form a large portion of the zoobenthos in many rivers and lakes, and their activities can significantly alter ecosystem function (Vannote et al. 1980). As infaunal to semi-infaunal filter-feeders, mussels can exercise significant control over phytoplankton and suspended particulate dynamics. Strayer et al. (1994) estimated the impact of mussel filtration on suspended solids to roughly equal that of downstream flushing in the Hudson River. Mussel importance was thought to increase with rising flow rate due to downstream removal of the planktonic grazers.

The most significant contribution of unionids to aquatic ecosystems, however, may arise from their physical alteration of the benthic habitat, rather than with their role in the food chain. Living unionids and their spent shells provide large substrate particles in lakes and rivers with otherwise fine

grained sediments, thus altering hydrology and extending the range of organisms that depend on course-grained sediments (Strayer et al. 1994). This substrata often supports much of the macroinvertebrate productivity (Benke et al. 1984). Mussel beds are also used as spawning sites for fish (Pilto 1989), and Chatelain and Chabot (1983) reported lake trout in Petit lac des Cedres (southern Quebec) deposited eggs exclusively on accumulations of unionid shells, never on rock or rubble. Mussel burrowing activity alters sediment chemistry by increasing water content and oxygen, and homogenizing internal structures. This intensifies microbial activity, expands depth of organic decomposition reactions, and increases exchange of chemical solutes between sediments and water (Matisoff et al. 1985).

The progressive decline in the North American mussel fauna and the immediate threat posed by the zebra mussel have prompted conservation efforts among federal and state resource agencies. Restoration programs are directed towards determination of species distribution and status, documentation of causes of decline, identification and preservation of critical habitat, control of exotic species, establishment of refugia for critically imperilled populations, and reintroduction of extirpated populations. While some of these activities involve observation of wild animals in their natural environment, others require a supply of glochidia and metamorphosed juveniles for controlled experimentation and restocking. To date, these specimens have not been available, due to either a scarcity of the species or life stage in the wild, or to the expense of artificial propagation using natural fish hosts.

The culture of freshwater mussels in the absence of fish hosts was first proposed by Lefevre and Curtis (1912), who investigated the role of encystment in glochidia development. Although the authors concluded that glochidia obtained the majority of nutriment for metamorphosis from pieces of bitten gill or fin tissue, they based culture experiments on the belief that "the blood of the fish would offer the most favorable nutritive conditions for the development of the glochidia". Attempts to culture glochidia using the blood of fish and frogs, tissue extracts, and bouillon were unsuccessful.

Ellis and Ellis (1926) reported successful transformation of glochidia in physiological solutions containing inorganic salts, dextrose, and amino acids. The glochidia used in these experiments, however, had been dissected from the gills of hosts 18-36 hours after encystment. Later attempts to metamorphose glochidia obtained directly from the marsupia using inorganic salt solutions and shredded liver and spleen were unsuccessful (Ellis and Ellis 1927). Glochidia which had trapped pieces of liver upon closure were observed to digest the material within three days, then open to assumably seek more.

The parasitic process of glochidia was further studied by Arey (1932), who described newly released glochidia as having a simple mantle, provisional abductor muscle, shell, and a small undifferentiated mass of cells which became building blocks for future organs. At attachment, a large section of gill or fin tissue was bitten, usually filling the mantle completely. Tissue removed from the host contained epithelium, connective tissue, blood vessels and fin rays. Within 9 hours after attachment the bitten tissue was disassociated by extracellular digestion and phagocytotic absorption. As the cyst formed, the glochidia became isolated, with no special vascularization of the host tissue to facilitate nutrient passage. Soft tissue was dissolved by the second day, and digesta stored in vacuoles described as being similar to food vacuoles of protozoa. Tissue digestion was completed by day 11, during which time the glochidial mantle had been replaced, larval abductor destroyed and replaced

by two others, and organs formed. While the host tissue apparently formed the major source of nourishment during metamorphosis, the author speculated tissue fluids bathing the cyst may also contribute nutrients. These observations were later confirmed by Matteson (1948) who reported that the most successful glochidia were those that had bitten off a substantial piece of gill filament during attachment.

In an effort to replenish wild populations of declining mussels, the Tennessee Valley Authority (TVA) developed (Isom and Hudson 1982), then later patented (Isom and Hudson 1984) a method to propagate mussels in vitro. Glochidia were removed from ripe females by severing the gills, cleaned in deionized water, and transferred to tissue culture dishes in a laminar airflow cabinet. Each culture dish was supplied with 2 ml of "Complex Artificial Growth Media" containing free amino acids, glucose, vitamins, inorganic salts, antibiotics, and antimycotics, and 1 ml of pressure-filtered fish plasma. The glochidia were then cultured in an incubator under 5% carbon dioxide atmosphere at 23°C for 14-21 days. The carbon dioxide maintained pH at 7.2-7.4. Successful transformation was obtained for *Legumia recta* and *Fusconaia ebena*, but cultures were often contaminated with bacteria and protozoa.

Keller and Zam (1990) simplified the TVA culture methods by substituting commercial tissue culture media and horse serum for complex media and fish plasma. Transformation rates up to 96% were reported for *Anodonta (Utterbackia) imbecillis*, 40% for *Lampsilis teres*, and 20% for *Villosa lienosa*. Attempts to replace serum with other protein sources proved unsuccessful, possibly indicating the presence of a biologically-active compound in serum that is essential for glochidia metamorphosis. Further trials at Virginia Tech (Dick Neves, personal communication) indicate that present artificial medias can be used to reliably metamorphose *Utterbackia imbecillis* glochidia, but success with other species is low and inconsistent. *U. imbecillis*, however, along with *Strophitus undulatus* and *Obliquaria reflexa*, is a species reported to naturally metamorphose into juveniles without use of a fish host (Kat 1984). Artificial culture medias need to be developed which can predictably metamorphose numerous mussel species in large numbers before the technique of in vitro propagation can be applied to research and restoration efforts.

The goal of the proposed research is to develop methods to produce viable juvenile mussels on a predictable schedule using artificial culture medias for glochidia transformation. The alternate use of an intermediate fish host is not practical because at present less than 25% of all mussel species have even a single known host (Neves 1994). Host identification is a tedious process made difficult not only by the diversity of the fish fauna to test, but because a true host may be rejected through an immune response from prior infection of glochidia (Rueling 1919) or copepods (Coker et al. 1919). In addition, many declining species have too few reproductive specimens remaining to allow determination of an appropriate host, and artificial propagation may be their last option. This work responds to an immediate need of the FWS Endangered Species program (see attachments), and to 1994 NBS Directors Research Priorities i, ii, and viii by providing tools to allow linking of biological and physical factors, establishing causal relationships, and promoting biodiversity. The research will compliment ongoing NBS projects in refinement of techniques to metamorphose juveniles using fish hosts (Gainesville), development of holding techniques for adults mussels in refugia (Leetown), and improvement of aquaculture methods for post-metamorphosed juveniles (Virginia Tech).

OBJECTIVES:

1) Develop a captive brood stock population from which glochidia can be obtained year-round from the same mussel species.

2) Determine what nutrients glochidia obtain from the host during encystment.

3) Develop an artificial culture medium suitable for metamorphosing a wide variety of mussel species.

4) Determine if Strophitus placentas can be used to metamorphose other mussel species.

HYPOTHESES:

H_o1: The reproductive cycle of *Elliptio complanata* can be controlled by manipulating photoperiod and temperature without negative impact on fecundity or glochidia viability.

H_o2: The majority of nutrition obtained by glochidia from the fish host is derived from tissue bitten during attachment, with minor contribution from plasma or blood.

H_o3: The delivery form of artificial media is more important than the actual nutritional content in determining glochidia survival.

H_o4: The placentas of *Strophitus undulatus*, or an artificial copy, can be used to metamorphose other mussel species.

PROCEDURES:

Captive Brood Stock:

Approximately 480 mature *Elliptio complanata* will be obtained from Pine Creek, Tioga County, Pennsylvania during the winter months when natural temperature and photoperiod are at a seasonal low. The mussels will be transported to NRDL-Wellsboro, individually measured for weight and length, inscribed with an identification number using a rotary engraver, and divided into groups of 60. Each group will be randomly assigned to one of eight 475 l round fiberglass culture tanks containing 8 cm of sand/gravel substrate, a center drainpipe, and a polystyrene cover. Six tanks will have water temperature control within the range of 0.5°C and 30°C using internal 1/4 HP chillers and an externally heated water supply. Two tanks will have temperature control between 10°C (ambient well water) and 30°C. Light will be supplied to each tank using two incandescent bulbs controlled by an electronic timer.

Four environmental treatments will be randomly assigned to duplicate tanks of mussels. The control treatment will have a temperature/photoperiod regime mimicking natural conditions for Pine Creek, in which temperature ranges from 0.5°C and 22°C and photoperiod from 9.5 hours to 15 hours of

light seasonally (Figure 1). It is anticipated that under this regime males will release sperm between April and June on rising photoperiod/temperature, and glochidia released from females 3-4 weeks after fertilization (Matteson 1948). Mid-winter conditions will be prolonged for 6 and 12 weeks for the second and third treatments, with an anticipated equal delay in spawning time. This cycle will be repeated for three successive years, thus spreading spawning evenly over each 12 month period thereafter (Figure 2). A fourth treatment will be similar to the control group, except the lowest temperature attained will be 10°C during the winter. This is designed to determine if chilling of water below ambient is necessary to control mussel reproduction at Wellsboro. Mussels will be fed daily with phytoplankton obtained from a concrete pond at a rate adjusted to that which produces visible algal settling and/or mussel pseudofecal output.

All visible mussels will be counted on a weekly basis to determine burrowing activity, and movement recorded to assess aggregational behavior. Spawning events will be recorded as they occur, and reproductive condition monitored during the spawning season by examination of females for charged marsupia with reversing pliers or a veterinary endoscope. Reproductive activity of the captive population will be compared with representative samples of wild animals from the collection site. Non-destructive methods of inducing glochidia release such as low oxygen, handling, elevated temperature or carbon dioxide, and presence of host fish will be tested on ripe females during the trial. Fecundity will be measured by estimating total discharge number of glochidia (if possible), and glochidia viability determined using a saline stress test.

Nutrition from Host:

Studies to determine the nutritional contribution of host tissue and body fluids to the development of encysted glochidia will utilize hookless glochidia (*Elliptio complanata*) attached to gills, and hooked glochidia (*Alasmidonta undulata*) attached to fins of their respective hosts. Specific fish hosts for these two mussels will be identified using the methods of Neves et al. (1985). Yellow perch and banded killifish are known hosts for *Elliptio complanata*, but no host has been identified for *Alasmidonta undulata* (see Watters 1993).

To determine the initial nutrient gain from bitten tissue, sections of gill and fin margin will be removed from the host fish, freeze-dried, and analyzed for dry matter, protein, ash, amino acids, and lipid using standard techniques. Samples of newly released glochidia will be cleaned, weighed, and a subsample dried for compositional analysis. Fish will be infected with glochidia, and after 2 hours a subsample sacrificed by MS-222 overdose and frozen. Encysted glochidia will be dissected from the fish, weighed and dried for analysis. Assuming enough glochidia can be removed from the fish to obtain a reasonable estimate of tissue consumption, the difference in glochidia weight multiplied by percent tissue composition will reveal nutritional gain. If the yield of glochidia is small, the weight of bitten tissue will be estimated using volumetric measurements of the removed tissue section.

To estimate the role of nutrition gained from body fluids during encystment, fish will be infected, and live glochidia removed from cysts immediately after attachment and at several intervals between infection and metamorphosis. Interval length will be set after determination of average duration of parasitism. After dissection, glochidia will be transferred into culture chambers, propagated in the presence or absence of artificial media (see below), and developmental progress monitored.

Nutritional gain during encystment can also be estimated by injecting radio-labeled nutrients into the bloodstream of infected fish at intervals after glochidia attachment, then measuring label uptake by the mussels. Because this is beyond the physical capability of this lab, a separate work order will be prepared if this line of research is pursued.

Artificial Media:

Trials to develop artificial culture media will primarily utilize glochidia obtained from *Elliptio complanata* spawned (hopefully) from the captive population. Glochidia from other Atlantic slope species such as *Alasmidonta undulata*, *A. varicosa*, *A. marginata*, *Strophitus undulatus*, *Pyganodon cataracta*, and *Lasmigona subviridis* may also be used when gravid females become available from local populations. If successful medias are developed for these species, testing will be expanded to glochidia obtained from species outside the Atlantic slope drainage. Initial experiments will follow the procedures outlined in Keller and Zam (1990), but may likely be altered if progress is attained. The planned sequence of experiments is as follows:

a) Attempt to metamorphose Atlantic slope mussels using the medias developed by Keller and Zam (1990).

b) Expand testing of liquid medias to screen most commercially-available products.

c) Test media delivery form (liquid/gel) using the above formulations solidified with gelatin, agar, or alginates (Knauer et al. 1993).

d) Test supplementing liquid media with solid particulates such as minced gill, fin, muscle, liver, lipid liposomes (Hontoria et al. 1994), or protein microcapsules (López-Alvarado et al. 1994).

e) Test formulated "artificial gill" medias consisting of maroomerized spheres, extruded gelatinous noodles, or sterilized pressed surimi (i.e. artificial crab meat).

f) Test use of metamorphose-inducers identified for other mollusks such as epinephrine (Haws and DiMichele 1993), potassium (Campos et al. 1994), or the peptide GGR (Zimmer-Faust and Tamburri 1994).

g) Attempt to identify the compound(s) present in fish serum necessary for glochidia transformation by selected inclusion of suspect agents into serumless medias.

At the conclusion of each trial, glochidia transformation success (robustness) will be determined using a reference toxicant such as NaCl, $CdCl_2$, or $CuSO_4$ (Anne Keller, personal communication). If a successful media is developed, the viability of glochidia produced artificially will be compared with those produced from natural hosts.

Strophitus Placentas:

Strophitus undulatus has been identified as one of three mussel species that can metamorphose to

juveniles without use of a fish host (Kat 1984). Strophitus is unique in that the glochidia are embedded in short cylindrical gelatinous cords, often referred to as "placentas". Upon release from the female, some of the glochidia migrate to the placental surface and attach using a modified bysal thread, while others remain embedded within. Surface glochidia have been documented to parasitize fish (Baker 1928), whereas those remaining within the placenta are reported to metamorphose independently (Lefevre and Curtis 1912). If so, the placentas may act as either a nutritive source, a protective cyst, or both.

A series of trials will be conducted to characterize the nature of the placentas and if they can be useful in culturing other mussel species. Placentas will be collected, cleaned of debris and glochidia, dried, and analyzed for protein, lipid, ash, amino acids, and fatty acids using standard laboratory techniques. Fresh placentas will be screened for antibacterial, antifungal, and antiprotozoal activity using standard bacteriological protocols. Placentas will be added whole or minced to culture disks containing glochidia of other species to determine if they will be used as a nutritive source. If so, attempts will be made to manufacture artificial placentas in which newly released glochidia can be embedded.

DATA:

Captive Brood Stock:

Behavioral parameters to be measured include the number and proportion of the population fully buried in sediment and the number of paired animals within the population (defined as animals moving to within 5 cm of each other). Reproductive parameters to be measured include number of days from trial initiation to spawning, duration of the spawning season, number of spawning events, correlation of spawning events to photoperiod and temperature cycle, fecundity, percentage of population gravid, and viability of glochidia produced. The initial assumption is that all tanks contain equal numbers of males and females. Data will be evaluated using analysis of variance (SAS procedure PROC ANOVA or GLM) except correlations. Proportional measurements will be subject to arcsin transformation prior to analysis.

Nutrition from Host:

Host suitability will be determined by recording the presence/absence of attached glochidia, presence/absence of metamorphosis, average time until metamorphosis at standard temperature, total number of recovered juveniles, and percentage of viable juveniles. Tissue contribution to nutrition will be calculated as percentage of dry matter consumed relative to glochidia body weight and percentage proximate composition of consumed material.

Experiments to evaluate tissue fluid contribution will be designed as factorials of duration of encystment (e.g. none, 2 hr, 2 days, 8 days, full term) and subsequent culture media (e.g. water, water+antibiotics, water+antibiotics+serum). Response criteria will be percent survival, metamorphosis and viability, analyzed by least-squares after arcsin transformation of proportional data. Orthogonal polynomials will be used to partition time effects into single-degree-of-freedom contrasts to estimate response plane.

Artificial Media:

Data to be collected to evaluate effectiveness of media to propagate mussels include percent survival, days to transformation at standard temperature, percent transformation, and post-metamorphosis robustness. Experiments will be either simple random, block, or factorial designs, analyzed by standard ANOVA after arcsin transformation of proportional data. Treatment effects will be determined using a test of Least Significant Differences at the $P < 0.05$ level.

SCHEDULE:

FY95 Brood stock: collect mussels; set up environmental chambers; begin temperature/photoperiod manipulation; start behavioral observations; monitor reproduction; first spawn. Host: determine host species; analyze gill/fin tissue. Media: purchase/set up culture chambers; test liquid medias; test media form. Strophitus: set up culture tanks; collect animals.

FY96 Brood stock: continue temperature/photoperiod manipulation; behavioral observations; monitor reproduction; test spawn inducers; second spawn. Host: conduct tissue consumption experiments. Media: test addition of solid particulates; test metamorphosis-inducers. Strophitus: analyze placentas; determine metamorphosis rate; screen for antibiotic activity.

FY97 Brood stock: continue temperature/photoperiod manipulation; monitor reproduction; test spawn inducers; third spawn. Host: conduct body fluid contribution experiments. Media: test active serum compounds; test "artificial gill" medias. Strophitus: conduct culture experiments.

FY98 Finish all experimental work; manuscript preparation.

SAFETY/ANIMAL WELFARE:

No unusual hazardous procedures will be required. All field collections will be conducted by teams of at least two persons wearing safety vests and protective clothing while in deep, fast moving, or cold water. Maintenance of test animals will adhere to procedures outlined in "Protocol for the care and use of experimental fishes and other animals by the laboratories of the National Fisheries Research Center-Leetown".

COOPERATORS:

Rick Barrows, USFWS R-6, Bozeman Fish Technology Center, MT: formulation and production of solid culture medias.

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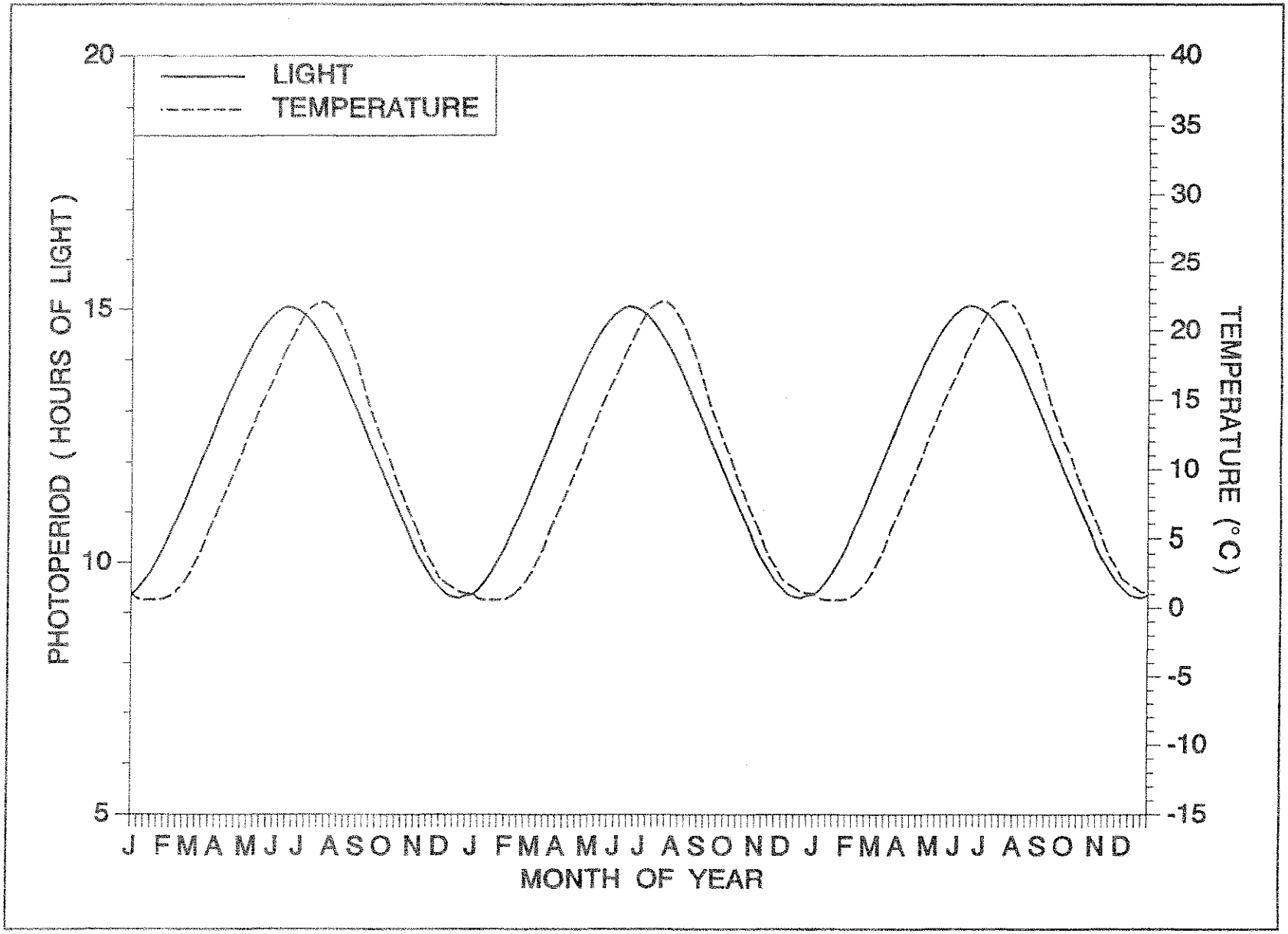

Figure 1. Seasonal fluctuation in temperature and light, Pine Creek, Tioga County, PA.

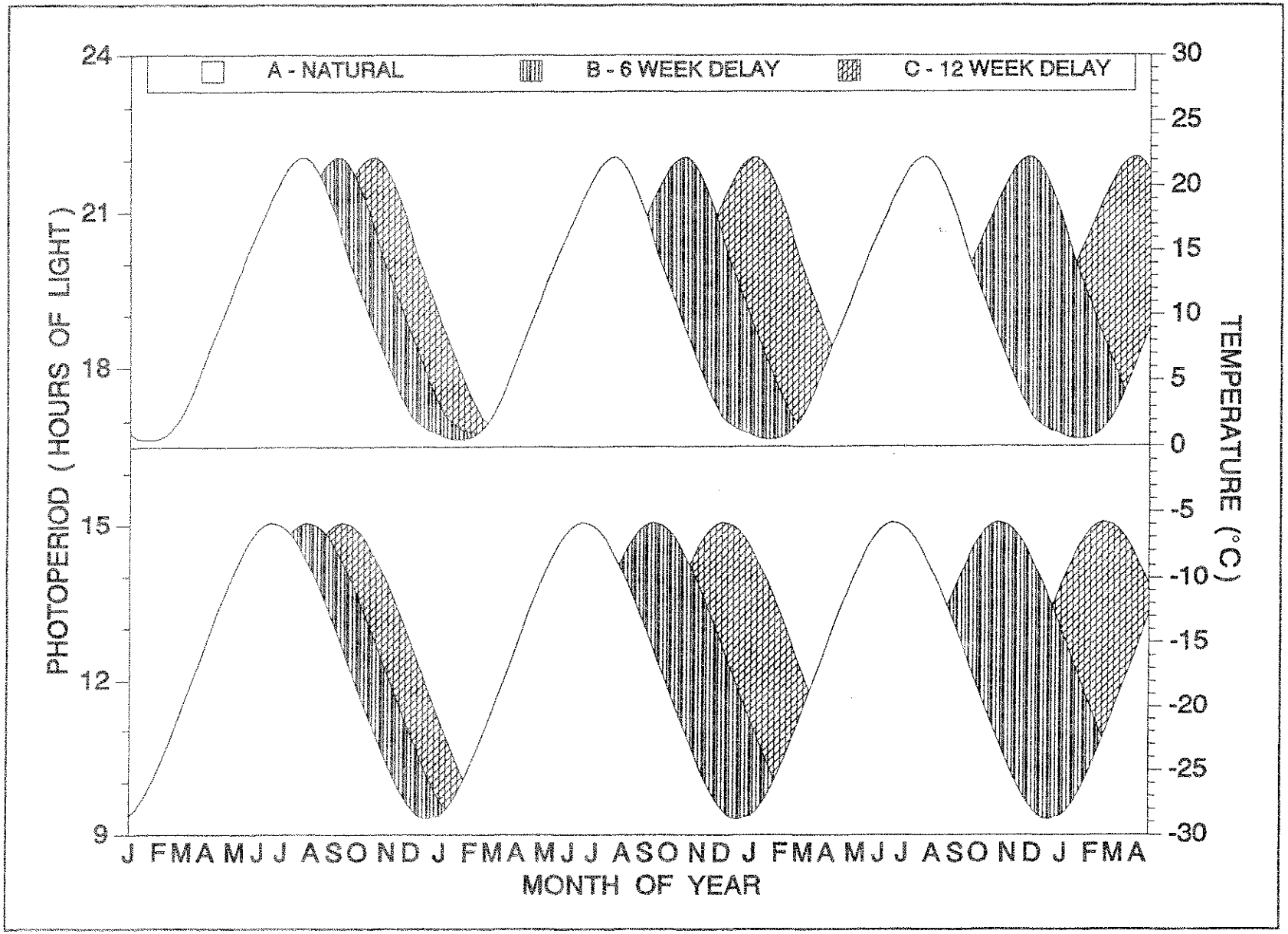

Figure 2. Temperature (top) and light (bottom) cycles to control freshwater mussel reproduction.

NATIONAL BIOLOGICAL SERVICE
Documentation of Information Need

DATE SUBMITTED: March 28, 1995 Form No. 1

BUREAU: U.S. FISH & WILDLIFE SERVICE, FWS

ORIGINATING OFFICE: Southwestern Virginia Field Office

BRIEF TITLE OF INFORMATION NEED:
Development of artificial media for transformation of mussel glochidia

TARGET FISCAL YEAR: 1996 NBS REGION: ?

DESCRIPTION OF INFORMATION NEED:
Artificial propagation technology is available for some species that have known fish hosts. However, host fish for most rare mussel species are unknown, & fish hosts cannot be identified because of sufficient numbers of individuals are not available for research. Artificial media for transformation of glochidia to juvenile mussels exits for some species. However, development of culture media for all rare species is critical to the FWS mandate to protect & recover mussels.

MANAGEMENT PROBLEM LEADING TO NEED:
No other wide-ranging, North American faunal group has suffered as dramatic a decline in this century as freshwater mussels. Habitat degradation is the primary cause for decline. Invasion of exotic zebra mussels now poses another serious threat to the Nation's mussel populations. The populations of many species have been so depleted that reproduction has ceased. For these species, artificial propagation is the only option that remains to secure their future.

BUREAU CONTACT: Roberta Hylton
703-623-1233

REGIONAL PRIORITY RANKING:
Not yet ranked, but should be a top priority since research findings could greatly benefit mussel species nationwide & since there is an FWS mandate to protect & recover freshwater mussels.

NATIONAL BIOLOGICAL SERVICE
Documentation of Information Need

DATE SUBMITTED: 3/22/95 Form No. 1

BUREAU: U.S. FISH AND WILDLIFE SERVICE, FWS

ORIGINATING OFFICE: Asheville Field Office

BRIEF TITLE OF INFORMATION NEED:
Development of artificial media for transformation of mussel glochidia

TARGET FISCAL YEAR: 1996 NBS REGION: S

DESCRIPTION OF INFORMATION NEED:
Artificial propagation technology is available for species that have known fish hosts. However, the host fish for many rare mussels is unknown, and insufficient individuals of these rare species are available for fish host research. An artificial media for transformation of mussel glochidia has been developed for some species. However, a culture media needs to be developed for all rare species if we hope to protect and recover these rare mussels.

MANAGEMENT PROBLEM LEADING TO NEED:
Freshwater mussels throughout North America are in rapid decline because of habitat distruction and now due to the impacts of zebra mussels. The populations of many species have been so depleted that reproduction has ceased. For these species, artificial propagation is the only option that remains to secure their future.

BUREAU CONTACT: Richard Biggins
PHONE: 704-665-1195

REGIONAL PRIORITY RANKING: ~~1 of 10~~ Paid regular yes