Artificial Propagation of Loach Minnow, *Rhinichthys cobitis*

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The loach minnow, *Rhinichthys cobitis* (Girard, 1856) is a small stream-dwelling fish of the family Cyprinidae. Historic habitat included the Gila River Basin of Arizona and New Mexico, and the San Pedro River Basin of Arizona and Sonora, México. The species was once considered locally abundant in the Gila River system above Phoenix, Arizona (Marsh, 1991), but today is restricted to scattered tributary populations in Arizona and New Mexico. Minckley (1973, 1987) describes data concerning historic and current distributions of this species for Arizona. Propst et al. (1988) describes distributions for New Mexico.

Loach minnow is listed as a threatened species under the authority of the Endangered Species Act, and is recognized as an imperiled species throughout its current range (Deacon et al., 1979; Johnson, 1987). It is listed by New Mexico as a threatened species and by Arizona as a species of special concern (AZGF, 1996).

This document is provided in fulfillment of an Intra-agency Agreement between the U.S. Fish and Wildlife Service and U.S. Bureau of Reclamation dated 03/23/98. This agreement provides for research directed towards the development of an artificial propagation protocol for loach minnow, Rhinichthys cobitis, *listed as threatened under the Endangered Species Act.*

Field collection, transport, hatchery receipt, retention, and development of controlled propagation techniques for this species was authorized under U.S. Fish and Wildlife Service Endangered Species Permit PRT-676811 as amended on 06/11/96. Written permission was provided by the Wildlife and Outdoor Recreation Division, White Mountain Apache Tribe, dated 06/10/96.

All data regarding the host population is considered proprietary and dissemination of these data is at the discretion of the White Mountain Apache Tribe. Consequently, this report deals only with post-collection research on development of artificial propagation techniques at the Alchesay Unit of the Alchesay-Williams Creek National Fish Hatchery Complex.

Disclaimer *The opinions and recommendations expressed in this report are those of the authors and do not necessarily reflect the views of the U.S. Fish and Wildlife Service, nor does the mention of trade names constitute endorsement or recommendation for use by the Federal Government.*

The loach minnow seldom exceeds 60 mm in total length, is elongate, and slightly flattened dorso-ventrally. Coloration is a mottled greenish-brown dorsally and on the sides. Ventral coloration is off-white. A distinguishing character differentiating loach minnow from other similar daces is the presence of two diffuse white spots at the base of the caudal fin. Breeding males exhibit intense red coloration of the lips, ventral surfaces, and bases of paired fins. Nuptial females exhibit similar coloration with red replaced by shades of yellow and yellowish-orange (Minckley, 1973).

Marsh (1991) outlined efforts required for successful recovery of the species. Primary objectives of this recovery plan included contingency planning and preliminary investigations for captive holding, propagation and rearing to include the following: 1) Determine wild stocks suitable for contribution to captive stocks. 2) Collect and transfer wild stocks to suitable facility. 3) Develop procedures and facilities for holding and maintaining. 4) Evaluate potential techniques for propagation. 5) Assess life-cycle requirements in hatchery environment. 6) Supply individuals as needed for reintroduction, research, public education, etc.

Hatchery propagation is an important and necessary part of conservation and recovery efforts directed towards imperiled species. This management tool has been proven effective in many recovery programs for birds, mammals, amphibians, and fishes. While artificial propagation may not be an immediate concern in recovery of loach minnow, it is important to develop the technology before it is needed to provide refuge for wild stocks. In the case of this species and others with relatively short life cycles, provision for artificial propagation is an integral part of maintaining this species outside of its natural habitat for any extended period of time.

Survival of loach minnow is dependent upon maintenance

of natural stream flows within its current range, limiting changes to riffle habitat including substrate composition, maintenance of macrophyte density and composition, and eliminating the threat of competition or predation by non-native species (Propst et al., 1991). A small, fragmented population of loach minnow occupies approximately 14 miles of stream located on the Ft. Apache Indian Reservation in Arizona. In late spring and early summer of 1996, conditions of below normal runoff with resulting low water levels, combined with hazardous fire conditions and heavy fuel loading within the watershed, prompted a response by the White Mountain Apache Tribe in cooperation with the Fish and Wildlife Service. It was determined that removal of a small percentage of loach minnow from the population for purposes of conserving genetic resources and supporting studies relative to artificial propagation was warranted (FWS, 1996).

On 06/11/96, biologists from the White Mountain Apache Tribe and Arizona Fisheries Resources Office collected 64 loach minnow from two isolated habitats within a single stream on the Ft. Apache Indian Reservation, Arizona. All loach minnow were collected using a 1.83 m x 2.44 m, 4.5 mm delta mesh seine. Twenty loach minnow were collected from Site A within a 50-meter reach of stream. Forty-four loach minnow were collected from Site B within a 25-meter reach of stream. Distance and stream morphology between populations suggested the possibility of genetic differentiation. Therefore, collections were kept separate. All 64 loach minnow were transported to the Alchesay Unit of Alchesay-Williams Creek National Fish Hatchery.

Materials and Methods

Loach minnow were transported to the Alchesay Unit of Alchesay Williams Creek NFH Complex (Alchesay) in two plastic ice chests containing 38 liters each of fresh water, 0.5% sodium chloride, 10 mg/l Furacin,® and 6 mg/l tricaine methane sulfonate (Finquil®). Sealed plastic bags containing ice were added to the transport media to reduce temperature during transport from a stream temperature of 25°C to a receiving hatchery temperature of 18°C upon arrival at Alchesay.

The wet lab at Alchesay, including receiving aquariums and closed recirculation system, was under construction at the time of loach minnow collection. Therefore, fish were temporarily received into two aluminum troughs 355 cm x 35 cm x 20 cm with a water depth of 11 cm. Receiving water chemistry matched transport protocol and temperature of 18°C. A supplemental oxygen supply was provided each tank using 180 cm sections of Micropore® tubing at an initial oxygen flow of 2.5 lpm for approximately one hour prior to receipt of fish. Upon introduction of loach minnow, oxygen flows were reduced to 1.0 lpm and fresh hatchery production water flow was initiated at approximately 70 l/hr (approximately 0.5 water exchanges per hour). After a period of two hours, supplemental oxygen was removed and inflow of hatchery production water was increased to 400 l/hr. Various shapes of 3.75 cm PVC pipe fittings were added to each trough to provide cover.

Approximately 250 ml of brine shrimp (*Artemia* sp.) nauplii solution was added to each trough twice daily consisting of 1.5 g cysts incubated to the first instar in one liter of 2.8% sodium chloride solution. Brine shrimp nauplii were supplemented with commercially prepared frozen bloodworms, krill, mosquito larvae, and adult brine shrimp fed three times daily. Nauplii were fed for a period of seven days, after which all feeding consisted of commercial preparations listed above. All feedings were recorded on a daily log.

Production water at Alchesay periodically contained significant amounts of silt, which continually settled onto the bottoms of holding troughs. To facilitate removal of silt and remove uneaten food, trough bottoms were vacuumed using 12 mm plastic tubing attached to a suction venturi pressurized by water flow from an adjacent indoor water faucet. The procedure was repeated twice daily.

On 07/17/96, the first mortality was recorded in the Site B loach minnow group. Pinetop Fish Health personnel diagnosed the cause as heavy, parasitic infestations of *Ichthyophythirius*, *Trichodina*, *Ichthyobodo*, and *Chilodonella*. At that time, diurnal water temperatures were fluctuating between 16.9°C and 19.3°C. Both troughs were afforded static bath treatments of formalin at 167 mg/l for a period of one hour using oxygen supplementation at 2 lpm diffused through 180 cm sections of Micropore® tubing. Treatments were repeated daily for a period of three days followed by one day of no treatment, then two more daily treatments for a total of five treatments between 07/17/96 and 07/22/96. All mortalities were sealed in plastic lock bags, labeled with date and collection site, then placed in an ultra-cold freezer at Alchesay at -80°C.

On 08/05/96, the wet lab was completed and all loach minnow were moved from aluminum holding troughs to the aquarium facility, comprised of six 75-liter aquariums. The system utilized two identical, self-contained filter packs that consisted of a 16 μm polyester mechanical filter, chemical filter containing activated carbon, an ultraviolet light sterilizer, and

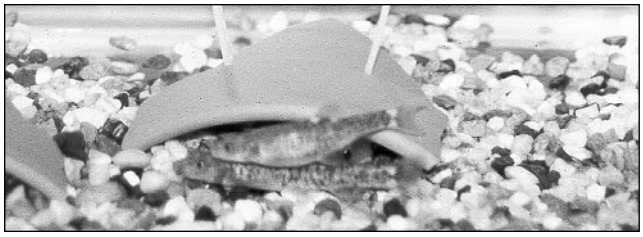

Fig. 1. Clay potsherds used for spawning habitat.

pump. Filter units were used separately, with one unit serving as a backup or to facilitate cleaning and maintenance of the filter packs. Water temperature was maintained by a single heat pump (Aquanetics™ Model AHP-5) connected to a 150-liter sump that served as a point of water aeration and biological filtration. The sump contained 0.28 cubic meters of 4 cm diameter circular plastic bacterial growth media (BioMax®) and a single 5 cm x 1 cm x 35 cm ceramic air diffuser, located under the plastic media, connected to a dual diaphragm electric air pump. Water flow of 6 lpm to each aquarium was controlled by a PVC ball valve and 4 to 20 lpm graduated flow meter. A 5-kilowatt propane-fueled standby generator and automatic power transfer switch was connected to the wet lab as a safeguard during electrical power failures.

Each aquarium contained natural colored gravel with a particle diameter that varied from approximately 2 to 8 mm. Gravel depth varied from 5 to 15 mm. Cover was provided by an assortment of 10 cm x 3.5 cm terra cotta clay pots inverted on the bottom of the aquarium (Fig. 1). Edges of the pots were broken in random patterns to provide access to the structures. Each pot was equipped with a nylon cable tie looped through two holes in the top, forming a loop to facilitate removal of the structures for cleaning and inspection of the undersurface for presence of egg masses.

Initially, aquariums were cleaned daily using the venturi-powered vacuum device described above. This system proved inadequate for a closed aquarium due to the significant amount of water removed during extended cleaning operations. In order to recycle water suctioned from system aquariums, a second plastic sump was added to the system. A Webster® model S-1 immersible pump was attached to the sump and connected to a 19 mm Amiad® in-line screen filter containing an 80 μm mesh removable screen cartridge. Approximately 4 m of flexible 12 mm plastic tubing was attached to the pump in order to reach all aquariums in the system. Following cleaning operations, the screen cartridge was removed and trapped particulates were flushed from the screen. The screen was disinfected with a mild chlorine solution and replaced in the filter housing.

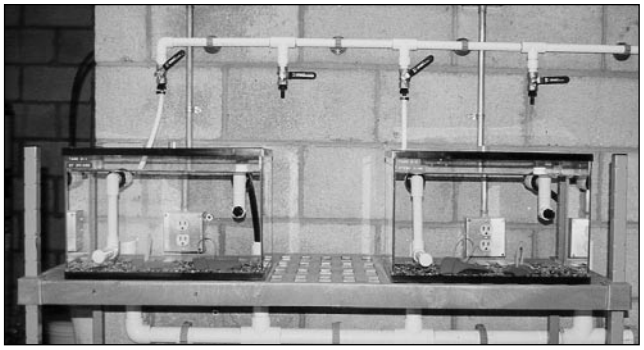

Fig. 2. 19-liter breeding aquariums.

At transfer of loach minnow from aluminum holding troughs to aquariums, 17 loach minnow from Site B remained from the initial collection of 44 fish. All 20 loach minnow from Site A survived initial holding. Four of six aquariums were used to facilitate the transfer of 37 loach minnow and were stocked at the following rates:

Aquarium #1 – 9 fish – Site B
Aquarium #2 – 8 fish – Site B
Aquarium #3 – 10 fish – Site A
Aquarium #4 – 10 fish – Site A

Temperature of production water in aluminum holding troughs and water in receiving aquariums was 18.7°C at time of transfer. Site B fish were heavily infested with *Ichthyophthirius* and exhibited approximately 5 to 30 epithelial cysts per individual. Site A fish were less severely infected and exhibited 0 to 10 cysts per individual.

Between the transfer date of 08/05/96 and 08/16/96, water temperature was lowered in gradual daily increments from 18.7°C to 13.3°C to lengthen the life cycle of the parasite. After 08/16/98, temperature was gradually increased and maintained at 15°C ± 1°C until 10/11/96. Feeding during this period consisted of commercial frozen preparations of brine shrimp, bloodworms, krill, and mosquito larvae fed alternately at a rate of approximately 0.40-0.66 g/fish/day. Food was shaved from frozen 2 cm cubes and introduced at the surface of each aquarium.

The wet lab was equipped with four dual 40-watt fluorescent bulb light fixtures and a 1.22 m x 1.22 m window with a south exposure. Use of artificial light was kept to a minimum throughout the study. The single window provided primary light and diurnal light cycles tracked natural conditions throughout the year.

On 10/16/98 aquarium temperatures were gradually lowered in synchronization with decreasing temperatures of the stream from which the loach minnow were collected.

Fig. 3. Stainless steel egg hatching sieves.

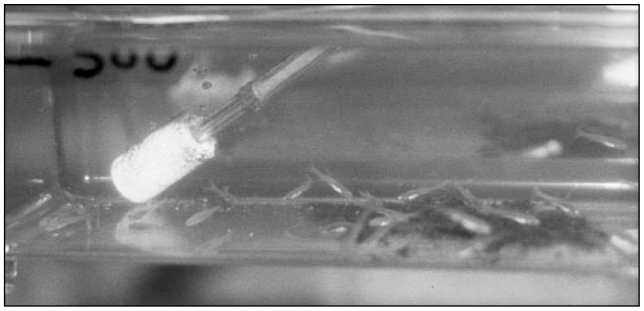

Fig. 4. 1000 ml nursery tank.

Stream temperatures were monitored weekly and aquarium temperatures adjusted accordingly. Water temperatures were lowered from 15°C on 10/11/96 to 7.2°C on 11/14/96, at which time native stream temperatures ranged diurnally from 6.9°C to 9.8°C. An aquarium temperature of 7.2°C was maintained from 11/14/98 to 03/19/97. In response to increasing spring stream temperatures, aquarium temperatures were incrementally increased from 7.2°C on 03/19/98 to 12.8°C on 04/30/97.

Beginning 04/03/98, normal feeding regimes described above were supplemented with live adult brine shrimp (*Artemia*), fed at a daily rate of approximately 200/aquarium/day. Brine shrimp were cultured in one liter clear acrylic plastic flasks (Hatch-Rite III® manufactured by Florida Aqua Farms, Inc.) in accordance with manufacturer's instructions. Light and heat required for hatching were provided by dual 20-watt fluorescent fixtures mounted in a modified laboratory gas ventilation hood. Live brine shrimp were fed daily for one week, then used as a periodical food supplement during the pre-spawn gamete maturation period. Beginning 04/22/97, dry food (TetraMin®) was offered at the surface in small, periodical feedings.

To facilitate spawning, two additional 19-liter aquariums (Fig. 2) were installed within the system. Setup was identical to larger aquariums and flow was adjusted and maintained at 1.5 lpm. Smaller aquariums were referenced as units 5A and 5B, while larger aquariums were referenced as units 20-1, 20-2, etc. Larger aquariums served as holding units for pre-spawn fish of both sexes. Smaller aquariums, which included two clay pot spawning structures, were each stocked with a single gravid female and two males displaying spawning coloration.

Prior to spawning, aquarium 20-1 was modified as an egg hatching unit (Fig. 3). Water inflow was routed to a 45 cm section of 19 mm PVC pipe located 10 cm below the water. The upper surface of the pipe was drilled with 3 mm holes on 12 mm centers throughout its length, producing a water upwelling effect. A single sheet of 6 mm rigid PVC was placed just above the water surface containing three circular cutouts in which three separate stainless steel screen sieves (Hubbard Scientific, Chippewa Falls, WI) were mounted and referenced as I-1, I-2, and I-3.

Treatment of loach minnow eggs for fungus infections (*Saprolegnia* sp.) followed several experimental protocols. These included immersion in an 800 mg/l formalin bath for 10 minutes; immersion in a 2.5 mg/l and 5.0 mg/l malachite green solutions for 15 minutes; and immersion in an 8.3 mg/l malachite green solution for 30 and 60 minutes.

Two styles of nursery tanks were used for rearing fry and fingerlings. Both were rectangular, clear acrylic units mounted inside of existing aquariums to maintain water temperatures. The first had a capacity of 2500 ml and were designated as N-1, N-2, etc. The second had a 1000 ml capacity and were designated as N-10, N-20, etc. (Fig. 4). Both were equipped with airstones and 3 mm polyethylene tubing that supplied air from a diaphragm air pump.

Initial fry food consisted of *Nannochloropsis oculata*, a golden-brown, single-celled microalgae (4-6 μm) received as an activated microalgae disk; the rotifer *Brachionus calyciflorus* (120-500 μm) received as dormant cysts; and the brine shrimp *Artemia* (first instar 400 μm) received as desiccated eggs. Rotifers were fed *Nannochloropsis* algae and a commercial food supplement (Roto-Rich®). Later instars of *Artemia* were produced by feeding *Nannochloropsis* algae and Roto-Rich.® All larval food organisms were obtained from Aqua Farms, Dade City, FL, and reared in accordance with instructions received from the vendor.

Results

Collection and Transport On 06/11/98, 64 loach minnow were collected from two stream sites on the Ft. Apache Indian Reservation and transported to Alchesay. Stress during collection was kept to a minimum through the use of 3 mm delta mesh seines rather than electro-fishing techniques.

Collection entailed disturbance of the cobble stream bottom by foot shuffling and seining immediately behind the disturbance. No mortality was experienced during the collection process.

Transport protocols followed those developed by the transport of native trout (*Oncorhynchus apache* and *O. gilae*) and are based on bioassays conducted at the Williams Creek National Fish Hatchery in trials using captive *O. apache*. Thousands of native trout have been successfully transported over a period of five years using these techniques. No mortality was experienced in transport of loach minnow for this study. All fish were received at Alchesay in excellent condition.

Holding Initially, loach minnow were received into aluminum troughs at Alchesay using hatchery production water. The source of the water was the North Fork of the White River, which contained significant populations of native and introduced fishes. Based on a history of the parasite *Ichthyophthirius* at Alchesay and its presence in the wild fish population, it was not unexpected that this disease would occur in loach minnow held in production water at this facility.

During the period between receipt of loach minnow on 06/11/96 and transfer of fish to the aquarium facility on 08/05/96, a total of 27 loach minnow succumbed to parasitic disease. It appears this species is very susceptible to parasites, particularly *Ichthyophthirius*, while held in captivity. Treatment with formalin at 167 mg/l for one hour was repeated on three consecutive days. This regime was repeated twice for a total of six treatments in seven days. There was no noticeable decrease in mortality as a result of this treatment. Interestingly, all mortality during the production water holding phase occurred in the Site B population. No mortality was experienced in loach minnow collected from Site A while fish were being held in hatchery production water.

On 08/05/98, all loach minnow were transferred from aluminum holding troughs to the Alchesay aquarium facility. This facility consisted of six 75-liter aquariums as a part of a closed recirculation system. Water used for initial filling was obtained from a domestic underground well. Almost all fish transferred exhibited numerous *Ichthyophthirius* epithelial cysts on body and fin surfaces. Infestations ranged from as many as 30 cysts per individual in the Site B fish, to a complete absence of the disease in some of the Site A fish. Following transfer to the aquarium system, two additional mortalities attributed to parasitic disease were experienced in the Site B fish. By 08/16/96, 11 days after transfer to the recirculation system, no epithelial cysts were visible on any loach minnow, and no further mortality attributable to parasitic disease was experienced in either group.

Several features of the aquarium system were apparent in actively stemming the incidence of parasitic disease. The ability to decrease water temperature is effective in extending the life cycle of many parasites, thereby reducing the number of infective stages of the parasite present in the system. Upper and lower temperature extremes may be effective in completely eliminating reproductive capabilities of *Ichthyophthirius*. Mechanical filtration (16 μm mesh) was capable of removing the 30-40 μm free-swimming, infective stage of "ich." Finally, the UV sterilizer rated at over 45,000 μwsec/cm^2 was capable of removing all bacteria, viruses, algae, fungi, and protozoa.

Feeding Adults Initial feeding of loach minnow adults with brine shrimp nauplii did not prove to be suitable for this life stage. While fingerling salmonids up to 50 mm actively feed on this small organism, no feeding behavior was observed in loach minnow adults in the presence of brine shrimp nauplii. Primary food items consumed by adult loach minnow in the wild are chironomid larvae and mayfly nymphs (Propst et al., 1988), which range in size from 1-5 mm. A suitable substitute for these wild foods was found in commercially prepared, frozen diets used in the aquarium industry. Foods readily accepted by loach minnow included adult brine shrimp, mosquito larvae, bloodworms, and krill. These foods were available in 1.75 cm^3 cubes that were individually "blister"-packaged, which simplified storage and use. Individual frozen cubes were shaved into ~0.5 mm sections and introduced at the surface of each aquarium. Through several weeks of trials, it was determined that approximately 0.40-0.60 g/fish/day seemed to provide a slight excess of food at 15°C. At temperatures below 15°C, slightly less food was fed, and at temperatures above 15°C, slightly more food was required. In order to provide varied nutrients, these four foods were each fed in sequence 3-4 times daily.

Adult loach minnow developed a "preference" for certain foods, judged by the relative aggressiveness of feeding behavior. Order of preference was bloodworms, mosquito larvae, brine shrimp, and krill. Feeding behavior in aquariums varied depending on the type of food introduced. As loach minnow were very cover-oriented, most time during daylight hours was spent under cover of the broken potsherds. When food was introduced, however, individuals rose to the surface and secured a large piece and immediately returned to cover where the portion of thawing food was consumed. After most large portions were eaten, fish would dart out of cover to secure smaller particles scattered on the aquarium bottom, always quickly returning to the security of cover.

Some dry foods were introduced periodically to measure

their acceptance by adult loach minnow. These included freeze-dried bloodworms, freeze-dried brine shrimp, and TetraMin® flake diet. Adult loach minnow appeared reluctant to accept dry diets in most cases. Steve Vives (pers. comm.) reports that all of his loach minnow were trained to feed on freeze-dried bloodworms, flakes, and small pellets of Bio Kiowa.® He was successful in introducing dry foods only after all fresh foods were withheld for several days. As the frozen diets used in this study proved readily available and simple to use, we did not pursue the exclusive use of dry diets for adult loach minnow.

Spawning The strategy used to solicit a breeding response in captive loach minnow was to pattern aquarium conditions with those occurring in the natural habitat. A large window present in the wet lab allowed sufficient sunlight to provide a natural light cycle. Beginning in early October, aquarium temperatures were incrementally lowered from 15°C to 7.2°C by mid-November. This temperature was maintained throughout the winter months until mid-March, when in response to increasing stream temperatures, aquarium temperatures were synchronously raised, incrementally from 7.2°C to 10°C on 04/11/97. At this temperature loach minnow exhibited an increase in feeding activity. Some females appeared to be gravid and males displayed a gradual increase of reddish hues on ventral surfaces.

At this time, two males and a single female from each site group were transferred to each of two 19-liter aquariums. The remaining loach minnow were left in site-specific 75-liter community aquariums. Inventory of adult loach minnow and distribution in study aquariums were as follows:

Aquarium	Site Group	No. of Adults
20-1	Empty	0
20-2	B	6
20-3	B	6
20-4	Empty	0
20-5	A	8
20-6	A	8
5-1	B	3
5-2	A	3

Between 04/11/97 and 04/26/97, aquarium temperatures were increased from 10°C to 12.2°C. Both males in Tank 5-2 displayed spawning behavior characterized by continuous movement, positioning alongside of the single female, and a shuttering movement indicating mock spawning, as no gametes appeared to be released. Males also paired alongside of each other and displayed the same spawning movements.

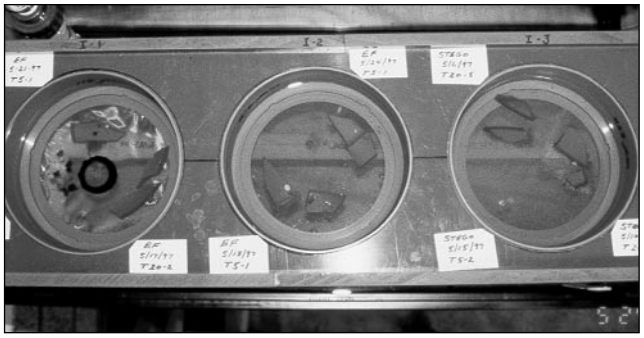

Fig. 5. Incubators containing egg masses attached to potsherds.

During this time, the female was also in a state of continuous movement, swimming from bottom to surface repeatedly, often wedging herself alongside the aquarium bottom edge for short periods of time. Corresponding with spawning behavior, male coloration intensified to brilliant hues of red on ventral surfaces, at the base of paired fins, and around the mouth. The abdomen of the female was significantly distended, indicating an advanced stage of gamete maturation.

On 04/27/98, at a temperature of 12.2°C, the female in Tank 5-2 appeared to have spawned judging by the reduced distention of her abdomen. Inspection of one of the clay potsherds revealed an egg mass attached to the shard were it contacted the gravel substrate. The translucent white egg mass was approximately 5 mm in diameter. Eggs, including some of the adhered gravel, were transferred to incubator I-3 mounted in tank 20-1.

Tanks 5-1 and 5-2 were exclusively used as breeding aquariums. Breeding strategy followed a pattern of rotating gravid females from community aquariums (20-2, 20-3, 20-5 and 20-6) to the smaller breeding tanks. No more than three fish were placed in each breeding tank at one time. Combinations of two males and one female, and two females and one male, were used alternately in both tanks. Successful spawns were produced with both combinations. Once females had spawned, spent females were returned to community aquariums and replaced with gravid females. Males were also rotated in and out of the breeding tanks. However, as none of the fish were marked, a precise use pattern for males was not documented.

Most egg masses were deposited on the undersurface of the potsherd, normally at the interface between the shard and the gravel substrate. Occasionally eggs were deposited entirely on the undersurface of the shard or entirely in the gravel substrate. On at least two occasions, single eggs or small egg masses were observed on the outside of the shards. As eggs were extremely adhesive, initially that portion of the shard with attached eggs was broken away, and along with attached

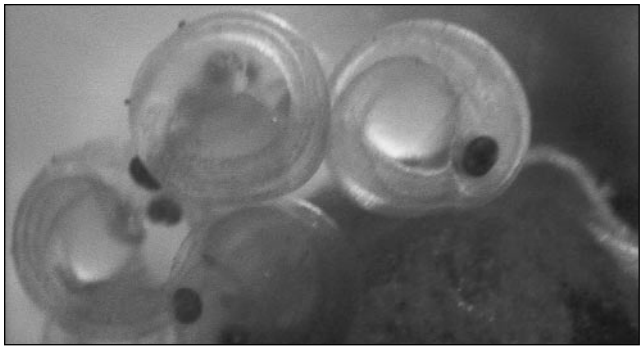

Fig. 6. Eyed eggs attached to gravel particle.

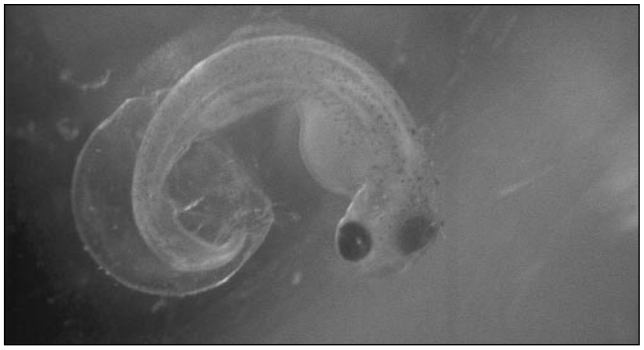

Fig. 7. Sack fry emerging from egg shell.

gravel, placed in the incubators (Fig. 5). It was later found that with extreme care, eggs masses could be removed from smooth surfaces without damage to the eggs. However, no suitable method was found to separate eggs from the gravel.

On many occasions, males were observed in attendance of egg masses prior to removal. Aggressive posturing of males was noted when eggs were disturbed during removal. This posturing consisted of a display of paired fins angled sharply downward accompanied by short, rapid movements forward in the direction of the disturbance.

Several egg masses were removed from community aquariums as not all gravid females could be accommodated in the two breeding tanks. On several occasions egg masses were left in place for a period of over 24 hours. Upon inspection of egg masses after this prolonged period, frequently only empty shell fragments were found adhering to shards. This may have been an indication of cannibalism or the result of mechanical damage caused by competition between males guarding the eggs masses.

Loach minnow spawns were collected from initial spawning on 04/27/97 at 12.2°C, to the final spawn on 06/19/97 at 17.2°C. Between these dates, temperatures were incrementally increased in synchronization with rising water temperatures in the donor stream. Field measurements and laboratory adjustments in temperature were made 2-3 times weekly. Table 1 represents a summary of all spawns noted during this study.

Egg Incubation and Hatching All incubation and hatching was conducted in a single 75-liter aquarium modified to produce an upwelling inflow to three hatching sieves. Diameter of eggs (Fig. 6) ranged from 1.3 to 1.8 mm. For 174 eggs observed from spawning to hatch, mean days to hatch at 13.9°C was 16.3; at 14.4°C, 16.5; at 15.0°C, 16.0; at 15.6°C, 14.2; and at 17.2°C, 12.1. Length of sack fry immediately after hatching ranged from 6.5 to 7.6 mm.

Fungus (*Saprolegnia* sp.) proved to be the greatest obstacle to successful egg incubation. It appeared that damaged or infertile eggs were often the source of the infection and fungus often spread from this initial source to fertile eggs within the affected egg mass. Egg masses least affected were those comprised of all fertile, normally developing eggs. Several chemical treatment regimes were used in an attempt to mitigate this disease. While no single treatment appeared to completely resolve the problem, a standing bath of malachite green at 8.4 mg/l for one hour seemed most effective. However, use of this concentration during the later stages of incubation appeared to induce a premature hatch.

Fry Development Fry hatched with a well-developed yolk sac approximately 2.0-2.5 mm in diameter. Posture was slightly curved ventrally (Fig. 7). As the fry matured, the curvature straightened and pigmentation in the head and dorsal regions appeared at approximately day 7 post-hatch (13.8°C). At approximately 10 days post-hatch, the lower jaw was developed and mobile; however, the buccal cavity and esophagus lacked sufficient development to allow feeding. At day 12, pectoral fins were completely formed and functional, gill opercula were developed and moving, and approximately 15% of the yolk sack was unabsorbed. On day 18 post-hatch, the yolk sack was completely absorbed, mouth parts and digestive tract appeared to be fully developed, and food was observed passing through the digestive tract (Fig. 8) and eliminated as feces.

Feeding of Fry and Sub-Adults Immediately after hatch, sack fry were moved to 2.5-liter and 1.0-liter closed plastic (nursery) tanks suspended in 75-liter aquariums in order to maintain system temperature. Glass bead air diffusers were provided to maintain oxygen levels. Water for the nursery tanks was provided directly from the supporting 75-liter aquariums.

Two main food sources were offered at initial feeding: *Nannochloropsis oculata*, a single-celled algae, and *Branchionus calyciflorus*, a protozoan rotifer. Both organisms were reared in 1.5-liter commercial rearing cones. Algae were reared to an

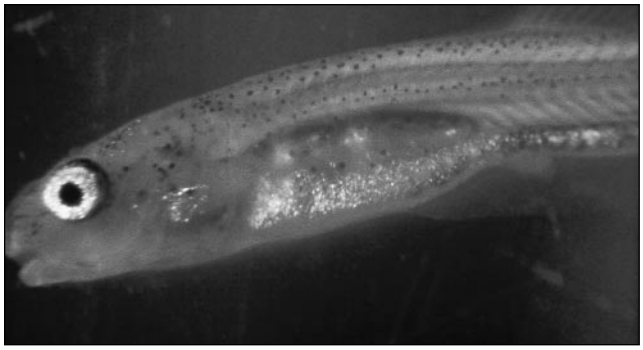

Fig. 8. Fry at initial feeding; note food in stomach and intestine.

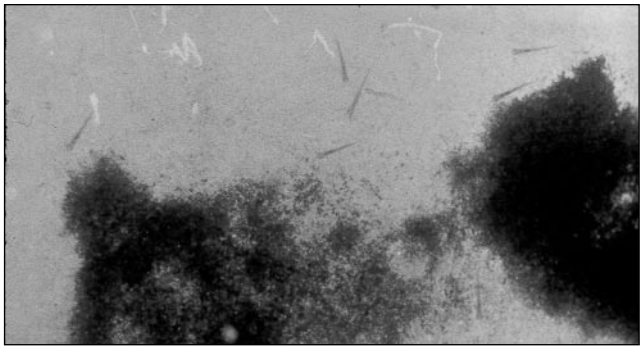

Fig. 9. Fry among algae mats on bottom of nursery aquarium.

optimum density of 8-15 million cells/ml and in turn were used to culture the herbivorous rotifers. Rotifer densities were maintained at 50-100/ml.

Initially, both feed organisms were offered to sack fry two days following hatch. Later study of larval development indicated that sack fry were physiologically unable to begin feeding until 9-18 days post-hatch at 13.8-17.2°C.

Algae and rotifers were first offered in 100 ml aliquots of stock-rearing solution for each organism, poured directly into nursery tanks twice daily. Prior to adding these solutions, an identical volume of tank water was removed and discarded. As the number of developing fry added to the nursery tanks increased, aliquots were increased to 250 ml. The number of sack fry stocked in 2.5-liter nursery tanks ranged from 14 to 54. Fry stocked in 1-liter tanks varied from 8 to 31. Periodically (2-3 day intervals), approximately 60% of the water contained in the nursery tanks was siphoned off and replaced with system water from the supporting aquarium. Water was siphoned off through a 53 μm sieve, and filtrate returned to the tank in order to preserve adult rotifers. Soon after initial feeding of algae, the single cells clumped together to form mats on the bottom of the tank. This structure proved valuable in providing preferred resting and feeding habitats for the fry (Fig. 9). In addition, these mats provided feeding areas for rotifers, which in turn were fed upon by the fry.

On 06/12/97, or approximately 30 days post-hatch for tank N-1, aliquots of algae and rotifers were increased to 500 ml for tanks N-1, N-2 and N-4. Total length of fry ranged from 9.0 to 10.5 mm. Between 06/12/97 and 06/20/97, five mortalities were recorded from tank N-1, the oldest fry. Suspecting that these fish may require a larger size food supply, newly hatched (first instar) *Artemia* nauplii (brine shrimp) were introduced in tanks N1-4. Brine shrimp rearing solution in approximately 250 ml aliquots was first passed through a 53 μm sieve and brine discarded. Brine shrimp were then washed from the sieve directly into the nursery tanks using a plastic pipette washer filled with water from the aquarium system. Fry in all tanks, including the youngest fry in N-3 (9-12 days post-hatch) aggressively fed on brine shrimp, indicating that mouth parts and esophagus were fully developed in this group by day 9 post-hatch. Development of fry in tank N-1, as reported above, was at a temperature of approximately 13.8°C. As the aquarium system temperatures were incrementally increased in synchronization with rising water temperatures in the donor stream, development of fry in N-3 was at approximately 15.5 to 17.2°C, indicating a marked acceleration of fry development with only a moderate increase in water temperature.

On 06/23/97, 39 days post-hatch for tank N-1, feeding in tanks N1-4 was discontinued with the exception of those algae present in the rotifer rearing solution. The initial feeding regime of algae and rotifers described for tanks N1-4 above was continued in 1-liter tanks N10-30 at a reduced rate to compensate for the smaller volume. Feeding of brine shrimp and rotifers in tanks N1-4 continued until 07/06/97 (52 days post-hatch for N-1). On this date the brine shrimp diet was supplemented with small portions of krill, shaved from frozen cubes, and introduced to tank N-1. Fry in this tank averaged 19 mm in total length. Several fry picked up the food and shook it aggressively, apparently ingesting the smaller particles. Larger particles were regurgitated.

Brine shrimp were first introduced to tanks N10-20 on 07/13/97, 23 days post-hatch. Fry seemed to easily ingest brine shrimp as long as the shrimp's maturation was not beyond the first instar. At this time temperatures fluctuated between 17.2 and 18.3°C. Fry in tanks N1-4 were fed brine shrimp exclusively in volumes of approximately 800 ml of rearing solution daily.

On 07/19/97, grower tanks were set up inside system aquariums 5-1 and 5-2, which were vacated at the end of the breeding cycle. Grower tanks, commercially produced as guppy breeding tanks, had a 1.0-liter capacity and a 0.5 mm mesh bottom that allowed water from the supporting **cont. on p. 11**

Table 1. Loach minnow spawn/hatch summary. LIP = left in place.

SPAWN DATE	SPAWN TANK	TANK TEMP °F	STRAIN (SITE)	NUMBER EGGS	INCUBAT. I.D.	HATCH DATE	HATCH NO.	N TANK I.D.	REMARKS
04/27/97	T5-2	54.0	A	?	I-3				04/30/97: Developing fungus. 05/05/97: Eggs discarded
05/02/97	T5-2	55.0	A	?	I-3				05/05/97: Discarded several fungused eggs. Remainder not developing. 05/06/97: Remaining 3 eggs dead; discarded.
05/03/97	T5-2	55.0	A	?	LIP				Eggs left in place. 05/06/97: Egg mass fungused; discarded.
05/03/97	T5-1	57.0	B	?	I-2	05/14/97 05/16/97 05/17/97 05/19/97 05/20/97 05/21/97 05/22/97 05/24/97	1 1 4 1 4 3 2 1	N-1 N-1 N-1 N-1 N-1 N-1 N-1 N-1	05/06/97: One fungused egg removed. Embryonic development observed. 05/09/97: First embryonic movement observed. 05/19/97: TL = 7.5-7.75 mm. Avg. egg size ~2.75 mm. 05/24/97: Pigmentation developing; lower jaw movement; larvae beginning to lay upright; mouth opening apparently not fully developed. 05/26/97: Pectoral fins developing; gill opercle movement; digestive tract not fully developed; ~15% yolk sac remains. 05/29/97: Larvae TL ~8.5-9.1 mm. 06/02/97: Larvae mouth and digestive tract functioning. Injesting food. 06/04/97: Sample TL = 12.0 mm. 06/17/97: 4 morts; TL ~9.0-10.5 mm.
05/04/97	T5-1	57.0	B	?	LIP 2 clumps				Eggs left in place; male tends to remain near. 05/05/97: Eggs disappeared.
05/04/97	T20-2	57.0	B	1	LIP				Egg left in place.
05/05/97	T-51	57.0	B	5	LIP				Eggs left in place attached singly on top of pot; male guarding? 05/09/97: Several fungused eggs removed from tank.
05/06/97	T20-5	58.0	A		I-3	05/24/97 05/25/97	3 4	N-2 N-2	05/24/97: TL ~6.5-6.9 mm.
05/09/97	T5-1	58.0	B						Several fungused egg masses removed.
05/10/97	T20-5	58.0	B		I-1	05/29/97 06/01/97	5 1	N-2	05/12/97: Removed several fungused eggs. Remaining eggs have embryonic development. 05/29/07: Hatch TL ~7.0 mm.
05/11/97	T20-2	58.0	B		LIP				Eggs left in place.
05/12/97	T20-5	58.0	A		LIP				05/15/97: Egg mass fungused; discarded.
05/13/97	T20-5	58.0	A		I-1				
05/15/97	T5-2	58.0	A		I-3	05/24/97 06/03/97	*2 1	N-2 N-2	05/19/97: Fungus attacking dead eggs; appears to be spreading to viable eggs. Formalin treatment at 200 ppm for 10 min. 05/21/97: Fungus problem continues; treated 200 ppm formalin for 20 min. 05/22/97: Treated with 2.5 ppm malachite for 15 min. 05/23/07: Treated with 5.0 ppm malachite for 15 min. *Note: Premature hatch; mechanical/chemical trauma. 05/31/97: Incubator temp. @ 59.0°F.
05/15/97	T20-3	58.0	B	1	I-3				05/19/97: Egg dead; discarded.

Table 1 (continued).

SPAWN DATE	SPAWN TANK	TANK TEMP °F	STRAIN (SITE)	NUMBER EGGS	INCUBAT. I.D.	HATCH DATE	HATCH NO.	N TANK I.D.	REMARKS
05/17/97	T20-2	58.0	B		I-1	06/01/97	2	N-4	06/01/97: Fungused egg mass with 2 viable eggs treated with 8.3 ppm malachite for 1 hr. Two viable eggs prematurely hatched following treatment. Dead eggs discarded.
05/18/97	T5-1	57.0	B	15	I-2	06/01/97 06/03/97 06/04/97 06/05/97	3 8 3 4	N-4 N-4 N-4 N-4	06/04/97: TL = 7.3, 7.5, and 7.6 mm.
05/21/97	T5-1	58.0	B		I-1	06/06/97 06/08/97	1 1	N-4 N-4	05/26/97: Fungus on egg mass; treat with 8.3 ppm malachite (a.m. & p.m.) for 0.5 hr. (both viable and dead eggs). Isolated in 300 ml beaker. 05/27/97: Repeat malachite treatment for 1 hr. 05/29/97: Two viable eggs remain in 300 ml beaker. 05/30/97: One viable egg remains.
05/24/97	T5-1	58.0	B		I-2	05/30/97	3	N-4	05/30/97: Two larvae prematurely hatch; eye pigmentation just developing.
05/24/97	T20-2	58.0	B	50	I-1	06/08/97 06/09/97 06/10/97 06/11/97	29 5 11 9	N-3 N-3 N-3 N-3	Male tended to continuously swim over egg mass as it was being removed from tank.
05/30/97	T5-1	60.0	B	12	LIP				06/04/97: Three egg masses fungused in tank substrate; discarded.
06/01/97	T5-1	59.0	B	1	I-2				06/04/97: Egg fungused; discarded.
06/01/97	T20-3	59.0	B	1	I-2				06/04/97: Egg fungused; discarded.
06/01/97	T20-3	59.0	B	1	LIP				Egg left in place.
06/01/97	T20-5	59.0	A	33	I-3	06/17/97 06/18/97	22 3	N-10 N-10	06/09/97: Five eggs fungused/dead; discarded.
06/02/97	T20-2	59.0	B		I-2	06/17/97 06/18/97 06/19/97 06/20/97	4 15 7 1	N-20 N-20 N-20 N-20	06/05/97: Fungus; treat 8.3 ppm malachite for 25 min. (one fungused egg + 2 clean eggs attached to same substrate). 06/13/97: Several egg masses treated for fungus with malachite at 8.3 ppm for 1 hr. 06/17/97: Discard 3 fungused eggs; treated 2 fungused egg masses with 8.3 ppm malachite for 1 hr.
06/03/97	T5-1	60.0	B	5	I-2	06/18/97	1	N-20	06/06/97: Discarded 3 eggs badly fungused; treated 2 remaining egg masses with 8.3 ppm malachite for 0.5 hr.
06/03/97	T20-2	60.0	B	35	I-2				
06/03/97	T20-5	60.0	A	6	I-3	06/17/97	3	N-10	06/05/97: Removed one fungused egg, treated remaining eggs with 8.3 ppm malachite for 2 min.
06/04/97	T5-1	60.0	B	5	I-2				
06/08/97	T20-5	59.0	A	6	I-3	06/20/97 06/22/97 06/23/97	1 1 1	N-10 N-10 N-10	
06/12/97	T20-3	59.0	B	1	LIP				6/13/97: Only broken shell remains.
06/13/97	T20-2	59.0	B	3	I-2				
06/19/97	T5-1	63.0	B	9	I-1	06/30/97 07/01/97 07/02/97	2 3 3	N-30 N-30 N-30	Eggs left in place overnight. Resulted in several shells plus 9 viable eggs the following day, which were transfered to I-1.

aquariums to circulate within the enclosures (Fig. 10), relieving personnel of the tedious task of frequent water changes required in all "N" tanks. A total of 11 advanced fry were transferred from N-1 to G-1, and 14 fry from N-2 to G-2, at approximately 60 days post-hatch. At this time, shaved sections of bloodworms, krill, and adult brine shrimp from frozen cubes were fed four times daily, supplemented with live brine shrimp and raised to advanced instars.

These feeding and rearing regimes were sustained in a continuous pattern that resulted in the gradual transfer of all advanced fry to grower tanks G1-7 by 08/15/97. At this time, feeding of live brine shrimp was discontinued and frozen diets of bloodworms, adult brine shrimp and krill were fed three times daily, supplemented with TetraMin® flakes and freeze-dried bloodworms twice daily. Mean total length of fish in G-1 was 25 mm at approximately 75 days post-hatch; fish in G-2, 14.5 mm at 68 days post-hatch; fish in G-3, 12.2 mm at 60 days post-hatch; and fish in G-4, 17.6 mm at 50 days post-hatch. The faster growth rate of fish in G-4 is attributed to an increased average rearing temperature and experience gained in rearing previous age groups that led to a more efficient progression through various food stages.

From grower tanks G1-7, fish were progressively transferred to 75-liter aquariums (Fig. 11) to accommodate increased growth. Once transferred to system aquariums, cover consisting of several clay potsherds scattered on the bottom was provided. In order to facilitate cleaning, no gravel was added to these aquariums.

A total of 179 loach minnow larvae were hatched from viable eggs incubated. From these larvae, rearing techniques described above resulted in the production of 164 sub-adults, 119 progeny from broodstock collected from Site B, and 45 progeny from broodstock collected from Site A. Survival rate from sack fry to sub-adult fingerlings was 92%.

Discussion

Most of the data presented were derived from a detailed daily log kept by Mr. Larry Wirtanen. Data entry was from 06/11/96, the date of initial receipt of loach minnow, to 08/20/97, at which time all sub-adults produced in this program were phased into a simple feeding regime involving commercially available frozen diets.

Not all observations recorded in the daily log were provided in this report. Mr. Wirtanen observed and recorded a significant amount of data concerning behavior, which was beyond the scope of the reporting requirements of this agreement. In addition, considerable detail was given to culture of algae, rotifers, and brine shrimp. These data were not presented in their entirety as techniques generally followed standard methods or instructions provided by aquaculture supply companies in association with the purchase of food organisms or culture equipment. Mr. Wirtanen also provided a broad scope of information gathered in association with a difficult struggle against the problem of fungus infection on eggs. These data are only briefly discussed due to lack of efficacy data for most treatment regimes attempted.

Fig. 10. Fry within screened grower cages.

The main goal of this research project was to develop and present a viable and repeatable protocol for the artificial propagation of loach minnow. Although the species presents a rather selective and specialized life history, it seemed to adapt well to modifications from its natural habitat. The species may not be a good candidate for extensive fish cultural techniques; however, these should not be ruled out in future investigations. Intensive laboratory techniques used in this study were patterned after successful methods used for other small, specialized fishes in order to ensure a relatively high probability of success. Mr. Steve Vives, Georgia Southern University, reports successful spawning of loach minnow at 23°C using a 16/8 hour light/dark cycle in an aquarium with water flow only provided by a power-head filtration system. Thus, one may assume that there are many uninvestigated and simplified techniques that could be applied to future attempts to culture this species.

Recommendations

Based on data collected at Alchesay for holding and culturing loach minnow from streams on the Ft. Apache Indian Reservation, the following protocols are suggested:

1) Loach minnow seem very susceptible to parasitic disease. Fish collected from the wild should be held in aquarium

Fig. 11. Sub-adult loach minnow in rearing aquarium.

systems equipped with mechanical filtration capable of removing particles ≥ 20 μm, and ultraviolet sterilization units rated at no less than 45,000 μwsec/cm^2 for the required system flow.

2) The artificial propagation facility should have a reliable temperature control system, aeration system, and a chemical or biological means of removing waste metabolites. To mitigate the effects of power failure, a backup power supply is recommended.
3) The facility should be equipped with sufficient natural lighting to reflect normal light/dark cycles, or a broad-spectrum artificial light system capable of simulating natural light cycles.
4) Adult loach minnow seem to adapt well to unnatural feeds. Commercially frozen feeds including brine shrimp, bloodworms, mosquito larvae, and krill are easy to store and prepare. Wild loach minnow readily accepted these diets. Data suggested that loach minnow might be successfully trained to accept artificial, dry diets. However, be prepared to withhold all natural feeds and subject fish to moderate periods of starvation in order to be successful.
5) Loach minnow in this study initiated spawning on 04/27/97 at a temperature of approximately 14°C after 3-4 months' exposure to temperatures of 7.2°C simulating winter water conditions. All aquariums were exposed to natural light cycles.
6) Inverted clay potsherds provided sufficient shelter and spawning strata for loach minnow. It is recommended that the potsherds be equipped with a plastic loop in order to provide for removal to assist in cleaning and inspection for eggs.
7) Pairings of two males to one female and one male to two females were both successful in producing fertile egg masses. Successful spawns were also produced in community aquariums containing 6-9 fish of mixed sexes. However, loss of several egg masses to cannibalism or mechanical damage by competing males in community aquariums gave preference to the former methods.
8) Screened-bottom egg incubators in an upwelling water flow regime proved adequate for hatching loach minnow eggs. Embryonic development and subsequent hatch are significantly effected by temperature. Variable incubation periods from 11 to 21 days were experienced at 13.8°C and 11-13 days at 17.2°C. Generally, longer incubation periods at cooler temperatures are preferred over shorter incubation periods at warmer temperatures to ensure proper embryonic development.
9) Unfortunately, a solution to problems associated with fungal disease on eggs was not developed. Treatment with formalin and malachite green at normal therapeutic levels for salmonids was not successful. This may be due to differences between adhesive and non-adhesive eggs. Some success in controlling fungus was seen with a standing bath of 8.3 mg/l malachite green for one hour. However, this concentration seemed to cause premature hatch in later stages of embryonic development. Suggestions for future research include the use of enzymes to remove the gelatinous coating that may promote or accelerate the infection. In addition, frequent prophylactic treatments using malachite green or a combination of malachite green and formalin may be effective. Caution should be exercised in handling and applying malachite green due to its potentially carcinogenic effects.
10) Newly hatched fry should be carefully observed during initial larval development in order to initiate first feeding in a timely manner. The period between hatch and sufficient larval development to allow feeding varies from as much as 18 days at 13.8°C, to as little as 12 days at 17.2°C. Watch the absorption of the yolk sac. It is better to initiate feeding too early than too late. Introduce food when the yolk sac has been reduced by approximately 90%.
11) *Nannochloropsis oculata*, a single-celled algae size 4-6 μm, combined with the rotifer *Brachionus calyciflorus*, size 120-500 μm, proved to be a satisfactory initial diet. These two organisms may be fed for 10-15 days post-hatch, at which time *Artemia* nauplii (first instar), size 400 μm, may be introduced. Reduced growth rates experienced in some lots of loach minnow fry may have been due to the failure to introduce brine shrimp at an early stage. Introduction of algae in sufficient quantities to form solid mats on the bottom of the nursery container seemed to be a significant asset in providing food and cover for fry. Rotifers and algae should continue to be fed until 30 days post-hatch,

at which time brine shrimp may be fed exclusively to 60 days post-hatch. After 60 days post-hatch, frozen diets may be introduced in reduced particle sizes. Note that these recommendations apply to fry reared at temperatures between 13.8 and 17.2°C. Feeding regimes should be accelerated at warmer temperatures and retarded at colder temperatures.

12) Much of the credit for the success experienced might be due to the meticulous efforts to maintain clean aquariums and nursery tanks during the course of this study. In a system using a large number of aquariums and the feeding of natural diets, we found the power siphon system of cleaning to be an invaluable aid in maintaining water quality and fish health.

Acknowledgments

In preparing a comprehensive report on artificial propagation research, from initial collection of laboratory specimens in June of 1996 to repatriation to the host population in January of 1998, it is not possible to mention all individuals involved in this project. Most of the credit for successful completion of this study goes to Larry Wirtanen, former Project Leader of the Alchesay-Williams Creek National Fish Hatchery Complex, who retired in January of 1998. Larry spent countless hours setting up the wet lab, feeding and observing specimens, maintaining detailed notes, and a multitude of other tasks to ensure survival and reproduction of this species. A significant part of this time included evenings and weekends, which is a demonstration of Larry's dedication. All photographs are credited to Mr. Wirtanen.

Bart Stegman, Biological Technician, is commended for his tireless effort in feeding and cleaning aquaria daily for almost two years. His attention to detail and maintenance of strict daily protocols was a significant factor in successful culture of this species. Credit for maintaining the wet lab on weekends and holidays goes to Anderson Quay and Jeff Cody of the Alchesay NFH permanent staff.

Stuart Leon, former Project Leader of the Arizona Fisheries Resources Office, was instrumental in initiating this study and providing guidance in compliance with environmental regulations and agency protocols. Stuart was assisted by his staff, including Leslie Ruiz, Daniel Parker, Tom Ensman, Mary Jo Stegman, and Lori Donovan.

This study would not have been possible without the cooperation of members of the Division of Wildlife and Outdoor Recreation, White Mountain Apache Tribe, including Director Hank Lavender, Assistant Director John Caid, and Fisheries Biologist Kelly Meyer.

The Pinetop Fish Health Center provided countless hours of assistance in monitoring the condition of captive broodstock, diagnosing disease, and providing recommendations for treatment. Special thanks go to Jerry Landye and Beth McCasland.

This project was not the first to investigate captive loach minnow. Previously, the Arizona Department of Game and Fish had awarded a two-year grant to Steve Vives, Georgia Southern University, for a similar investigation. Although Steve's research centered primarily on behavior, he has generously provided spawning data and suggestions for techniques we adopted for successful reproduction. Dave Propst and Paul Turner both have field experience in observing and collecting egg masses from wild populations, as well as observing spawning behavior in natural settings. Their suggestions and advice were instrumental in developing captive techniques. We are grateful to all those who assisted in this joint effort.

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