RELATIONSHIP BETWEEN FOOD TYPE AND GROWTH AND SURVIVAL OF LARVAL HYBRID DEVILS HOLE PUPFISH

by

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TABLE OF CONTENTS

LIST OF FIGURES……... 5

LIST OF TABLES……..……...……... 6

ABSTRACT…….…….. 7

INTRODUCTION…….. 8

PRESENT STUDY…… 15

REFERENCES…….. 18

APPENDIX A: RELATIONSHIP BETWEEN FOOD TYPE AND GROWTH AND SURVIVAL OF LARVAL HYBRID DEVILS HOLE PUPFISH……. 20

I. Abstract…….. 22

II. Introduction…….. 23

III. Methods……..……. 30

IV. Results…….……... 39

V. Discussion…….…….. 41

VI. References…….. 48

APPENDIX B: LABORATORY PROPAGATION OF HYBRID DEVILS HOLE PUPFISH…….…. 61

I. Abstract……..……. 63

II. Introduction…… 64

III. Methods…… 69

III. Results and Discussion……. 73

IV. References…….…..……. 77

LIST OF FIGURES

FIGURE 1.1. Containers used to propagate algal species collected from Devils Hole, Nevada..…….……..……..... 51

FIGURE 1.2. Nauplii collector schematic..……...…. 52

FIGURE 1.3. Percent occurrence of items in adult gut content analyses of Devils Hole pupfish, adapted from Minckley and Deacon (1975) and Blinn and Wilson (2005)… 53

FIGURE 1.4. Mean dissolved oxygen (mg/L after 24 h ± SEs) in experimental containers, including starting values and Devils Hole levels (Shepard et al. 2000)……..…. 54

FIGURE 1.5. Agitator and water bath containing treatment containers used in experiments testing adequacy of food types for hybrid Devils Hole pupfish larvae……. 55

FIGURE 1.6. Percent survival of hybrid Devils Hole pupfish larvae fed different food types through 14 d……...……..……..……..…… 56

FIGURE 1.7. Mean lifespan of hybrid Devils Hole pupfish larvae fed different food types (d ± SEs)…..…….…… 57

FIGURE 1.8. Mean total length (14-d means ± SEs) of hybrid Devils Hole pupfish larvae fed different food types, including mean starting length……...……... 58

FIGURE 2.1. Larval production of hybrid Devils Hole pupfish, by month, in laboratory aquaria, Tucson, Arizona, 2010-2011, with arrow indicating starting point of use of water changes…….……..…… 80

LIST OF TABLES

TABLE 1.1. Chi-square values for survival rate comparisons among food type treatments..…...……..…… 59

TABLE 1.2. Nutritional components of larval hybrid Devils Hole pupfish food types..…...……..…… 60

ABSTRACT

I examined growth and survival of larval hybrid Devils Hole pupfish, *Cyprinidon diabolis* x *Cyprinidon nevadensis mionectes,* fed different food types. Foods included Rio Grande Silvery Minnow flake food and prominent elements of the Devils Hole algal and invertebrate communities, including monospecific cultures and combinations of cyanobacteria Cyanophyta, green algae *Spirogyra spp.*, ostracods Ostracoda, amphipods *Hyallela azteca*, diatoms Bacillariophyta, and copepods Cyclopoida. I quantified survival, growth, and lifespan of larval hybrids among 14 food treatments. Larvae fed flake food had significantly higher survival and lifespan than those fed natural food types. Of the natural food types, larvae fed algae or cyanobacteria in monospecific cultures or in combination with invertebrates had the highest survival and lifespan. Pure invertebrate treatments yielded the lowest survival and lifespan. No significant difference in total length at 14 days was found among treatments. I also developed methods for laboratory propagation of hybrid Devils Hole pupfish and produced 500 hybrid pupfish larvae over a period of eight months by conducting a weekly 30% water change in parental aquaria lowering water temperature from 28°C to 23°C, then gradually raising it back to 28°C over 48 h. My methods for hybrid propagation employed temperatures suitable for Amargosa pupfish reproduction. Further information on the reproductive requirements of pure-strain Devils Hole pupfish is needed in order to modify my techniques for use with pure-strain Devils Hole pupfish.

INTRODUCTION

Devils Hole is a single limestone fissure within Death Valley National Park thought to have opened around 60,000 years ago (Riggs and Deacon 2004). It encompasses the entire range of one of the most imperiled vertebrate species known, the Devils Hole pupfish *Cyprinidon diabolis,* a relic of the last ice age. This pupfish species was stranded as receding glacial lakes moved across a drying and warming landscape (Lema and Nevitt 2004) and has survived in its current location—perhaps the smallest vertebrate distribution in the world—for 25,000 years (Moyle 1976).

The Devils Hole pupfish is unique from even its closest relatives in the Amargosa Valley. It is an iridescent blue, 2.5 cm-long fish that lacks pelvic fins. Its habitat is equally unique—the spring-fed Devils Hole remains a constant 33°C and contains approximately 2 ppm dissolved oxygen, providing a challenging environment even by pupfish standards (Shepard et al. 2000). At the water's surface, the dimensions are approximately 3.5 m x 22.0 m. Although water depth at Devils Hole is over 125 m (the actual depth is unknown), the fish only utilize the upper 30 m (Gustafson and Deacon 1998). Most of their time is spent on a limestone shelf 0.1 m under the water surface. This shelf is the primary area of biological production in Devils Hole, though it only receives full sunlight four months a year. The Devils Hole pupfish is thought to spawn almost exclusively on this platform (Blinn and Wilson 2005).

Following its 1967 listing as an endangered species (it was among the first species listed), the merit of protecting the Devils Hole pupfish received public scrutiny as

increasing agricultural and housing development demands in the area lowered the water level in Devils Hole, exposing, and therefore threatening, the algae-covered shelf on which the fish typically spawn (Andersen and Deacon 2001). In 1972, a lawsuit was brought against the developers and the state of Nevada, and after years of legal disputes, the U.S. Supreme Court upheld a lower court's ruling in its Cappaert v. United States (1976) decision to maintain a minimum water level in Devils Hole for the benefit of the pupfish (Deacon and Williams 1991). This decision protected the fish and also mandated that the water level be maintained so that the spawning shelf remained immersed.

Dramatic declines in the Devils Hole pupfish population have raised concerns over the future of the species. From the time monitoring began in 1972 until 1996, the population remained relatively constant, averaging just over 300 individuals. In 1997, the population began to decline, reaching an all-time low of 38 individuals in April 2006. More recently, a supplemental feeding program has likely helped raise the population to 104 individuals (Wilson 2011).

Many factors have been suggested as causes of the decline in Devils Hole pupfish population, one of which is reduction in suitable food. Researchers reported differences in the diet of Devils Hole pupfish from the 1970s—when pupfish abundance was high—to the 2000s—when pupfish abundance was low. Minckley and Deacon (1975) analyzed stomach contents of adult Devils Hole pupfish over 14 months, from 1967-1969. The primary element in the stomachs, by percent frequency observed, was inorganic particulate matter—predominantly travertine from the water surface and bottom substrate. *Spirogyra spp.*, the second most abundant element, though its presence

fluctuated drastically between seasons, was thought to be the feeding substrate from which invertebrates were taken (Minckley and Deacon 1975). Various diatoms also comprised a large portion of the diet. The remainder of the stomach contents consisted of amphipods *Hyallela azteca*, ostracods Ostracoda, protozoans, beetles Coleoptera, flatworms *Dugesia spp.*, and snails *Tryonia variegata.*

A similar study conducted by Wilson et al. (2001) showed both parallels and important differences with Minckley and Deacon's findings. Conducted from 1999-2001—several years after the marked decline in pupfish numbers—the study also showed a high occurrence of travertine in adult pupfish stomachs, likely the result of fish attempting to remove diatoms Bacillariophyta from the benthic surface. As in the earlier study (Minckley and Deacon 1975), diatoms were also an important component of pupfish diet. Beetles, flatworms, and snails were also small but important food sources, especially in the winter. Unlike the 1975 study, Wilson et al. (2001) detected few amphipods, and no ostracods, in stomach contents. Protozoans were not observed in Wilson et al.'s study. *Spirogyra*, which had been present in a large percentage of diet samples in the 1970s, was almost completely absent. By contrast, cyanobacteria, which were absent from the 1975 diet, comprised up to 19% of pupfish diets in the 2001 study.

Changes in Devils Hole pupfish diet may reflect changes in algae, cyanobacteria, and invertebrate populations in Devils Hole itself. Especially notable are a higher incidence of cyanobacteria and reductions in invertebrates such as ostracods with respect to the 1970s. Algal mats were deemed suitable substrate for invertebrates in Devils Hole (Minckley and Deacon 1975), yet in Wilson et al.'s (2001) study ostracods were only

observed in the deeper waters below the shelf. Cyanobacteria mats in Devils Hole also modify the environment of the shallow shelf in a way that is detrimental to survival of both fish eggs and larvae. During the 2002 census, divers noted a thick surface mat of cyanobacteria, causing the spawning shelf to become anoxic. Interstitial spaces in the gravel were filled with cyanobacteria, so Devils Hole pupfish laid eggs on top of the cyanobacteria mat, exposed to predation. That year the count of adult Devils Hole pupfish was much lower than average. The 2003 flood, however, removed a large portion of this mat from the shelf, and *Spirogyra* and diatoms were able to regain dominance (Riggs and Deacon 2004).

The positive response of Devils Hole pupfish numbers to supplemental feeding, changes in the algal and invertebrate communities between the 1970s when pupfish abundance was high, versus the 2000s when abundance was declining, and changes in pupfish diet between these two time periods suggest that food limitations may be in part responsible for the pupfish decline. Specifically, evidence suggests that the larval life stage is the limiting period. Census divers and scientists associated with the Devils Hole program have reported sighting larval pupfish on the limestone shelf and adult fish throughout the upper reaches of the water column. However, the few reports of fish between the larval and adult age classes, and the reduction in adults from previous years, suggest that many larvae are not surviving to adulthood. This pattern has been observed in both laboratory and natural settings (Deacon et al. 1995).

In addition to food supply, other factors may limit the larval life stage. Adult pupfish predation on eggs may limit the population in this system that does not allow

emigration, keeping the population below carrying capacity (Deacon et al. 1995). Considering the current population size, such processes only further imperil the species.

Recent, dramatic declines in the wild Devils Hole pupfish population prompted multiple attempts to develop reproducing captive populations in aquaria. The first attempt was conducted at Steinhart Aquarium in San Francisco from 1971-1973. Only one larva was produced during this time, and it survived only five months (Deacon et al. 1995). Baugh and Deacon (1983) also attempted to propagate pure-strain Devils Hole pupfish in aquaria at the University of Nevada-Las Vegas, were ultimately unsuccessful, and their study population perished. Later attempts were also conducted at Mandalay Bay Aquarium in Las Vegas but this population did not survive.

Refugia, small concrete pools stocked with small numbers of Devils Hole pupfish, were also established. These U.S. Fish and Wildlife Service (USFWS) refugia were established at School Springs and Point of Rocks on the USFWS Ash Meadows National Wildlife Refuge, and at Hoover Dam on Bureau of Reclamation land. The School Springs Refugium was lost due to pump failure, the fish at the Hoover Dam Refugium did not survive, and the Point of Rocks population was inadvertently hybridized with the Amargosa pupfish *Cyprinidon nevadensis mionectes,* a closely related species (Martin 2005). These hybrid pupfish were then transferred to USFWS Willow Beach National Fish Hatchery.

Specific concerns have arisen with attempts to propagate Devils Hole pupfish in captivity. Low Devils Hole pupfish egg viability has been repeatedly noted in both natural and laboratory settings. Deacon et al. (1995) observed hatch rates as low as 12%

and survival rates as low as 6% within Devils Hole, while in laboratory experiments, they observed only 4% hatching success, although 65% of eggs laid appeared to be transparent and viable. Among cage-held adults in Devils Hole, Deacon et al. (1995) note that only three offspring were produced per female per month from March through July, a period that encompasses the height of breeding. In Devils Hole they also observed a relationship between greater hatching success and larger diel fluctuation in oxygen saturation, which is the result of photosynthetic activities of algae on the spawning shelf.

The potential use of captive pupfish populations to buttress the wild Devils Hole pupfish population is complicated by the degree of genetic variation observed among captive populations, and between captive populations and the Devils Hole population. Wilcox and Martin (2006) examined genotypic plasticity among populations from Devils Hole, Point of Rocks Refugium, and School Springs Refugium, and found significant genetic divergence from the Devils Hole population in the two refugia populations, while Martin (2005) demonstrated significant genetic variation among multiple refugia, attributable to the founder effect commonly seen when a small number of individuals is used for captive breeding experiments. Loss of rare alleles likely contributed to the quick divergence of refugia populations from the Devils Hole population. Some of the alleles found in fish from Devils Hole were not present in refugia populations. Further, at Point of Rocks Refugium, where pure-strain Devils Hole pupfish were inadvertently hybridized with Amargosa pupfish, the genetic composition of the population shows little genetic contribution from Devils Hole pupfish, and demonstrates the comparably high fitness of Amargosa pupfish in captivity (Martin 2005).

As there are no remaining pure-strain pupfish outside of Devils Hole, the possibility of extinction has become quite real. Therefore, identifying factors limiting the population in Devils Hole is critical. Due to the marked change in food availability and composition in Devils Hole coincident with the population decline, examining differences in larval recruitment with each food regime under a laboratory setting is prudent. Currently, little information exists describing feeding habits of Devils Hole pupfish at the larval life stage. If an ideal food regime were found, it would facilitate laboratory propagation of the Devils Hole pupfish, provide important information for recovery of pupfish in Devils Hole, and offer principles that could be extended to recovery of other desert cyprinids. Additionally, previous lack of success rearing Devils Hole pupfish in captivity further complicates recovery efforts. Developing methods to propagate pure-strain Devils Hole pupfish in captivity is therefore imperative. My objectives were: (1) to test differences in larval hybrid Devils Hole pupfish growth, survival, and lifespan among Rio Grande Silvery Minnow flake food and combinations of the prominent elements of Devils Hole algal and invertebrate communities from both the 1970s—when pupfish were abundant—and the 2000s—when pupfish were scarce (i.e. *Spirogyra*, cyanobacteria, diatoms, amphipods, ostracods, and copepods); (2) to develop methods for culturing native Devils Hole invertebrates and algal species; and (3) to develop a reliable methodology for producing hybrid Devils Hole pupfish in a laboratory setting. Information herein may be of use to professionals working with a variety of pupfish species or species with limited distributions and rigid or hostile environments, such as those found in natural springs.

My experiments utilize hybrid Devils Hole pupfish obtained from the USFWS Willow Beach National Fish Hatchery. These hybrid pupfish were moved to Willow Beach from Point of Rocks Refugium. Though results from genetic testing confirm that this breeding stock is no longer pure, hybridized pupfish represent the only Devils Hole pupfish genetic material outside of Devils Hole. With the species far too imperiled to consider pure-strain pupfish for experimentation, these hybrids are the closest link to the Devils Hole pupfish, and therefore the best substitute for experimentation.

PRESENT STUDY

The methods, results, and conclusions of this study are presented in chapters to be submitted for publication appended to this thesis. The following is a summary of the most important findings.

I hypothesized that a change in plant communities from *Spirogyra*-dominant in the 1970s to cyanobacteria-dominant in the 2000s, and reductions in major invertebrates such as ostracods between these two periods were responsible for a reduction in pupfish numbers, and that the larval life stage was most affected. I studied the relationship between survival, lifespan, and growth and larval nutrition in hybrid Devils Hole pupfish, *Cyprinidon diabolis* x *Cyprinidon nevadensis mionectes* (Appendix A). As a result of the endangered status of pure-strain Devils Hole pupfish, hybrid pupfish were utilized in all experiments described herein. I tested Rio Grande Silvery Minnow flake food, as well as 13 treatments of natural Devils Hole food types in monospecific cultures or in

combination. These treatments included: (1) ostracods; (2) amphipods; (3) a concentrated, mixed culture of 30% diatoms, 30% rotifers, 20% ciliates, and 20% miscellaneous organisms henceforth referred to as "diatoms"; (4) ostracods+diatoms; (5) amphipods+diatoms; (6) cyanobacteria; (7) ostracods+cyanobacteria; (8) amphipods+cyanobacteria; (9) *Spirogyra*; (10) ostracods+*Spirogyra*; (11) amphipods+*Spirogyra*; (12) diatoms+*Spirogyra*; (13) diatoms+*Spirogyra*+copepods; and (14) Rio Grande Silvery Minnow flake food. All larvae used for this experiment were introduced into their treatment containers within 48 h of hatching and experiments lasted 14 d. There was a significant relationship between food type and both survival and lifespan. Larvae fed flake food had significantly higher survival and lifespan than those fed natural food types. Of the natural food types, larvae fed algae or cyanobacteria in monospecific cultures or in combination with invertebrates had the highest survival and lifespan. Pure invertebrate treatments yielded the lowest survival and lifespan. No significant difference between survival and lifespan of monospecific or combination algal and cyanobacteria treatments was identified. There was also no relationship between food type and total length at 14 d. Together, these data suggest that changes in dominant plant community type from *Spirogyra* to cyanobacteria and reductions in populations of invertebrates tested did not limit the larval life stage. Because the scope of this experiment includes only the larval life stage, it is possible that food type is limiting a different life stage or that density of each potential food species has changed over time such that overall food scarcity is the cause of the recent population decline. Therefore,

further investigations should be conducted to determine algal and invertebrate densities, and to provide a more complete survey of forage species in Devils Hole.

I also refined methods to propagate hybrid Devils Hole pupfish (Appendix B). Parental aquaria were maintained at 28°C. Feuerbacher (unpublished data) found significant differences in hybrid Devils Hole pupfish egg production among temperatures, and that 28°C results in the greatest number of eggs and highest rate of egg production. Only ten larvae were produced at static temperatures over a nine-month preliminary trial period. I altered my protocol and conducted a 30% water change twice weekly that lowered parental aquaria from 28°C to 23°C. Aquaria returned to 28°C over the course of 48 h with the use of aquarium heaters set to, and constantly maintained at, 28°C throughout the experiment. This methodology resulted in production of 500 hybrid Devils Hole pupfish larvae over eight months.

My results suggest that temperature manipulation may be a valuable tool in laboratory culture of hybrid Devils Hole pupfish. Because these hybrids contain predominantly Amargosa pupfish genetic material, the temperature used herein (28°C) is suitable for Amargosa pupfish reproduction while likely outside the range of pure-strain Devils Hole pupfish reproduction. Further information is needed about reproductive requirements, including temperature, of pure-strain Devils Hole pupfish to modify my methods for use with pure-strain Devils Hole pupfish.

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APPENDIX A

RELATIONSHIP BETWEEN FOOD TYPE AND GROWTH AND SURVIVAL OF LARVAL HYBRID DEVILS HOLE PUPFISH

RELATIONSHIP BETWEEN FOOD TYPE AND GROWTH AND SURVIVAL OF LARVAL HYBRID DEVILS HOLE PUPFISH

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ABSTRACT

I compared growth and survival rates (measured as survival and lifespan) of larval hybrid Devils Hole pupfish, *Cyprinidon diabolis* x *Cyprinidon nevadensis mionectes,* fed Rio Grande Silvery Minnow (RGSM) flake food and combinations of the prominent elements of the Devils Hole algal and invertebrate communities. These communities included green algae *Spirogyra spp.*, cyanobacteria Cyanophyta, diatoms Bacillariophyta, amphipods *Hyallela azteca*, ostracods Ostracoda, and copepods Cyclopoida. Larvae were introduced to one of 14 treatments within a day of hatching, and experiments were 14 d in duration, thus spanning the larval life stage. Following this period, survival, lifespan, and total length were measured and compared using a Chi-square test, and one-way ANOVA with Tukey and Scheffe post-hoc tests, respectively. Larvae fed RGSM flake food had significantly higher survival (85%) and mean lifespan (12.6 d) than those fed natural food types ($P < 0.001$). Of the natural food types, larvae fed algae or cyanobacteria in monospecific cultures or in combination with invertebrates had the highest survival (10 - 25%) and mean lifespan (8.6 - 9.7 d). Larvae fed only invertebrates did not survive the 14-d treatments. No significant difference between survival and lifespan of monospecific or combination algal/cyanobacteria treatments was identified. There was also no relationship between food type and total length at 14 d ($P = 0.934$). Together, these data suggest that changes in the dominant plant community type from *Spirogyra* (1970s) to cyanobacteria (2000s), and reductions in the populations of the invertebrates tested did not limit the larval life stage.

INTRODUCTION

Devils Hole is a single limestone fissure in Death Valley National Park thought to have opened around 60,000 years ago (Riggs and Deacon 2004). It encompasses the entire range of one of the most imperiled vertebrate species known. The Devils Hole pupfish *Cyprinidon diabolis* is a relic of the last ice age. It was stranded as receding glacial lakes moved across a drying and warming landscape (Lema and Nevitt 2004). The Devils Hole pupfish has survived in its current location—perhaps the smallest vertebrate distribution in the world—for 25,000 years (Moyle 1976).

The Devils Hole pupfish is unique from even its closest relatives in the Amargosa Valley. It is an iridescent blue, 2.5 cm-long fish that lacks pelvic fins. Its habitat is equally unique—the spring-fed Devils Hole remains a constant 33°C, and contains approximately 2 ppm dissolved oxygen, providing a challenging environment even by pupfish standards (Shepard et al. 2000). At the water's surface, the dimensions are approximately 3.5 m x 22.0 m. Though water depth at Devils Hole is over 125 m (the actual depth is unknown), the fish only utilize the upper 30 m (Gustafson and Deacon 1998). Most of their time is spent on a limestone shelf 0.1 m under the water surface. This shelf is the primary area of biological production in Devils Hole, though it only receives full sunlight four months a year (Blinn and Wilson 2005).

Public interest in this diminutive species has been considerable, and like other high profile, endangered terrestrial species such as California condor *Gymnogyps californianus* and giant panda *Ailuropoda melanoleuca,* protection of this fish has

received worldwide attention. The species was described in the 1930s, and in 1952, President Harry Truman declared Devils Hole a part of Death Valley National Monument under the American Antiquities Preservation Act to protect the pupfish and the unique structure of the fissure itself (Deacon and Williams 1991). Following its 1967 listing as an endangered species (it was among the first species listed), the merit of protecting the Devils Hole pupfish received public scrutiny as increasing agricultural and housing development demands in the area lowered the water level in Devils Hole, exposing, and therefore threatening, the algae-covered shelf on which the fish typically spawn (Andersen and Deacon 2001). In 1971, a lawsuit was brought against the developers and the state of Nevada. After years of legal disputes, the U.S. Supreme Court upheld a lower court's ruling in its Cappaert v. United States (1976) decision to maintain a minimum water level in Devils Hole to benefit the pupfish (Deacon and Williams 1991). This decision not only protected the fish, but also mandated that the water level be maintained so that the spawning shelf remained immersed.

Protection of the Devils Hole pupfish goes beyond the legal realm; a chain-link fence was erected around Devils Hole for both public safety and to protect the resource. Complete with motion-sensing cameras and topped with razor wire, the fence restricts access to all but the small research team in charge of research and monitoring in Devils Hole. In addition, a monitoring program was established that consists of visual counts conducted by divers and from the surface in both spring and fall.

The Devils Hole pupfish is thought to spawn almost exclusively on the shelf. Flooding in recent years jeopardized the fragile larval age class for a time, as rocks and

debris washed into Devils Hole, completely covering the shelf and cutting off this critical area of production. Government and volunteer crews cleared the shelf, restoring much of its former area (Lyons and Parker, unpublished report).

Devils Hole pupfish use a consort-pair breeding system in which the male follows a gravid female around the spawning shelf as the pair periodically drops to the substrate to spawn (Deacon et al. 1995). Devils Hole pupfish eggs are transparent, approximately 1 mm in diameter, and have an embryonic development period of one week. Newly hatched larvae are around 1.5 mm in length and remain in the interstitial spaces of the substrate until they reach 2-4 mm (Gustafson and Deacon 1998). Their size, when coupled with the lack of pigment, makes accurate larval surveys difficult (Deacon et al. 1995).

Dramatic declines in the Devils Hole pupfish population have raised concerns over the future of the species. From the time monitoring began in 1972 until 1996, the population remained relatively constant, averaging just over 300 individuals. In 1997, the population began to decline, reaching an all-time low of 38 individuals in April 2006. More recently, a supplemental feeding program likely helped raise the population to 104 individuals (Wilson 2011). The positive response of Devils Hole pupfish numbers to supplemental feeding, changes in the algal and invertebrate communities between the 1970s—when pupfish abundance was high—versus the 2000s—when abundance was declining—and changes in diet suggest that food limitations may be to blame for the decline in the pupfish population. Specifically, some evidence suggests that the larval life stage is the limiting period. Census divers and scientists associated with the Devils

Hole program have reported sighting larval pupfish on the limestone shelf, and adult fish throughout the upper reaches of the water column. However, the few reports of fish between larval and adult age classes, and the reduction in adults from previous years, suggest that many larval fish are not surviving to adulthood. This pattern has been observed in both laboratory and natural settings (Deacon et al. 1995). In addition to environmental factors, adult pupfish predation on eggs further acts to limit the population in this system that does not allow emigration, keeping the population below carrying capacity (Deacon et al. 1995). Considering the current population size, however, such processes only further imperil the species.

In addition to pupfish, Devils Hole contains a variety of invertebrates and algae. Shepard et al. (2000) examined samples collected between 1984 and 1998, and identified 44 genera and 77 species of algae in Devils Hole, including 20 genera of Cyanobacteria, 18 genera of Bacillariophyta, and 6 genera of Chlorophyta. Diatoms were the most diverse algal group and *Denticula elegans* was the dominant species. Together with cyanobacteria, diatoms currently compose over 90% of the current algal community; however, chlorophyta *Spirogyra spp.* provided the majority of the algal cover on the upper shelf until recent years. In addition to these algal species, four species of Diptera; a mollusk, *Tryonia variegata*; crustaceans (amphipods, *Hyalella azteca*; Ostracoda; and copepods, Cyclopoida); 2 coleopterans, *Stenelmis calidae* and *Neoclypeodytes cinctellus*; a flatworm, *Dugesia spp.*; and an oligochaete, *Dugesia dorotoccephala,* were present in Devils Hole (Wilson *et al*. 2001).

Over time, the food available to the Devils Hole pupfish has changed. Especially notable from the most recent surveys are a higher incidence of cyanobacteria and reductions in invertebrates such as ostracods with respect to the 1970s. Algal mats were deemed suitable substrate for many invertebrates living in Devils Hole (Minckley and Deacon 1975), yet no ostracods were observed on cyanobacteria mats in 2001 (Wilson et al. 2001). In fact, ostracods were only observed in the deeper waters below the shelf at this time. During the 2002 census, divers noted a thick surface mat of *Oscillatoria spp.* replacing *Spirogyra* and diatoms on the spawning shelf; however, a flood in 2003 removed this mat, and *Spirogyra* and diatoms regained dominance on the shelf (Riggs and Deacon 2004). Not only did the cyanobacteria mat in Devils Hole coincide with reduction in ostracod abundance, it also caused the spawning shelf to become anoxic, filling most interstitial spaces in the gravel with cyanobacteria and causing Devils Hole pupfish to lay eggs on top of the substrate, exposed to predation.

The diet of the Devils Hole pupfish reflects changes in available food that may be responsible for the recent population decline. Stomach content analysis of adult Devils Hole pupfish over a 14-month period from 1967-1969 found that the primary element in pupfish stomachs, by percent frequency observed, was inorganic particulate matter—predominantly travertine from the water surface, as well as bottom substrate (Minckley and Deacon 1975). *Spirogyra*, thought to be the feeding substrate from which amphipods, protozoa, and ostracods were taken, was the second most abundant element, although its abundance fluctuated drastically between seasons (Minckley and Deacon 1975). Various diatoms also made up a large portion of the diet. The remainder of the

stomach contents consisted of amphipods, ostracods, protozoans, beetles, flatworms, and snails.

A later study showed several parallels, as well as some important differences from the 1975 findings. Conducted from 1999-2001—several years after the marked decline in pupfish numbers—this study also showed both a high occurrence of travertine in pupfish stomachs, likely the result of fish attempting to remove diatoms from the substrate surface. Diatoms were also an important element at this time (Wilson et al. 2001). Beetles, flatworms, and snails were small but important food sources in 2001 as well, especially in winter when more easily digestible food types were in shorter supply. However, unlike the earlier study, in 2001 few amphipods, and no ostracods or protozoans, were detected in pupfish stomach contents. *Spirogyra*, which had been present in a large percentage of diet samples in 1975 study, was almost completely absent in 2001. By contrast, cyanobacteria, *Plectonema wollei* and *Chroococcus spp.*, which were absent from adult Devils Hole pupfish gut contents in the 1975 diet study, comprised up to 19% of pupfish diets in 2001 (Wilson et al. 2001).

Refugia, small concrete pools stocked with small numbers of Devils Hole pupfish, were established with little long-term success. These U.S. Fish and Wildlife Service (USFWS) refugia were established at School Springs and Point of Rocks on the USFWS Ash Meadows National Wildlife Refuge, and at Hoover Dam on Bureau of Reclamation land. However, School Springs Refugium was lost due to pump failure, the Point of Rocks Refugium population was inadvertently hybridized with the closely-related Amargosa pupfish *Cyprinidon nevadensis mionectes,* and the population in the Hoover

Dam Refugium did not survive. Significant genetic variation was found in all three refugia due to the founder effect commonly seen when a small number of individuals is used for captive breeding experiments (Martin 2005). In addition, as a result of hybridization at Point of Rocks Refugium, the genetic composition of hybrids shows little genetic contribution of Devils Hole pupfish, suggesting comparably high fitness of Amargosa pupfish in a captive setting (Martin 2005).

The experiments conducted herein utilize hybrid pupfish obtained from the U.S. Fish and Wildlife Service (USFWS) Willow Beach National Fish Hatchery. These hybrid pupfish were moved to Willow Beach from Point of Rocks Refugium. Though results from genetic testing confirm that this breeding stock is no longer pure, and that their genetic makeup is more heavily derived from Amargosa pupfish than Devils Hole pupfish, hybridized pupfish represent the only Devils Hole pupfish genetic material outside of Devils Hole. With the species too far imperiled to consider pure-strain pupfish for experimentation, these hybrids provide the closest link to Devils Hole pupfish, and, therefore, the best substitute for experimentation.

The possibility of extinction of Devils Hole pupfish has become quite real. Previous lack of success rearing Devils Hole pupfish in captivity further complicates recovery efforts. Due to marked change in food availability and composition in Devils Hole coincident with the population decline, examining differences in larval recruitment with each food regime under a laboratory setting is prudent. Currently, little information exists describing feeding habits of Devils Hole pupfish at the larval life stage. If an ideal food regime were found, it would facilitate laboratory propagation of Devils Hole

pupfish, provide important information for recovery of pupfish in Devils Hole, and offer principles that could be extended the recovery of other desert cyprinids. Methods used herein may be of use to professionals working with a variety of pupfish species, or species with limited distributions and rigid or hostile environments. Therefore, my objectives were: (1) to test differences in larval hybrid Devils Hole pupfish growth, survival, and lifespan among fish fed Rio Grande Silvery Minnow flake food and combinations of the prominent elements of the Devils Hole algal and invertebrate communities from both the 1970s—when pupfish were abundant—and the 2000s—when pupfish were scarce (i.e. *Spirogyra*, cyanobacteria, diatoms, amphipods, ostracods, and copepods); and (2) to develop methods for culturing native Devils Hole invertebrates and algal species.

METHODS

To identify which foods in Devils Hole provide highest survival and growth for larval hybrid pupfish, I compared fourteen treatments, including artificial food and individual food types found in Devils Hole, or a combination of these natural food types. These treatments included: (1) Rio Grande Silvery Minnow Starter 0511-m (formulated by the Fish Technology Center, Bozeman, Montana and produced by Silver Cup Fish Feeds, Tooele, Utah), which is currently being used to supplement natural foods available in Devils Hole; (2) representative elements of the community of algae and invertebrates from the 1970s assemblage, when the Devils Hole pupfish population size was larger;

and (3) representative elements of the community of algae, cyanobacteria, and invertebrates from the assemblage of 2001, when the Devils Hole pupfish population was in decline. Hybrid pupfish were used for all aspects of this experiment. I hypothesized that dominant elements of the 1970s food assemblage (*Spirogyra*, ostracods, and amphipods) would produce higher survival rates and more rapid growth in larval fish than dominant elements of the 2001 assemblage (cyanobacteria and diatoms), while Rio Grande Silvery Minnow flake food would outperform both natural assemblages.

Invertebrate, Algal, and Cyanobacteria Collection and Culture

In order to complete the algal, cyanobacteria, and invertebrate treatments, I first cultured species found in Devils Hole. With assistance from the National Park Service, I collected invertebrate, cyanobacteria, and algal samples from Devils Hole, using new equipment designated for use only in Devils Hole. I collected six 250-mL surface water samples in glass sample bottles from different regions of the spawning shelf. Using a turkey baster, I collected algal and cyanobacteria samples growing on a variety of substrate. The bulb of the turkey baster was compressed, the tip was placed one centimeter above the substrate, and the bulb was released to suction water, algae, cyanobacteria, and substrate into the turkey baster. Samples were then poured into 250-mL glass bottles. These samples included parts of the *Spirogyra* and cyanobacteria mats that form on the spawning shelf and water's surface. From these samples, I used a Zeiss Stemi® 2000 dissecting microscope (Carl Zeiss, Göttingen, Germany) at 6.5X magnification to manually extract *Spirogyra*, cyanobacteria, amphipods, ostracods, and

copepods for cultures of these food groups. I did not collect Coleoptera, as there were not sufficient numbers in the Devils Hole samples to sustain a viable captive population. These species are also too large to be considered an important larval food source.

I added Penicillin:streptomycin antibiotics to each sample at a concentration of 500 mg/L in order to remove cyanobacteria from *Spirogyra*, diatom, and invertebrate cultures (Hoff and Snell 1987). Because of the limited number of ostracods, copepods, and amphipods available in Devils Hole, these cultures were supplemented with additional organisms. Amphipods, cycloid copepods, and a morphologically similar ostracod species were obtained commercially to supplement the Devils Hole cultures (Sachs Systems Aquaculture, St. Augustine, Florida).

I used microbiological techniques (Hoff and Snell 1987) to isolate diatom and protozoan species. I applied water samples (100 μL) to TCBS agar (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) plates with one-milliliter pipettes, and distributed them over the agar surface with a stainless steel inoculation loop. From these spread plates, I used an inoculating loop to transfer colonies to a streak plate. I repeated this process until pure-strain colonies were formed (Hoff and Snell 1987). I inspected colonies under a Zeiss RA compound microscope (Carl Zeiss, Göttingen Germany) at 400X magnification to determine that a monospecific culture was found. I then separated these samples into 1 L glass culture bottles, which were brought to dissolved oxygen and temperature levels (measured using an Orion 4-Star ph/RDO Portable Multiparameter Meter [Thermo Fischer Scientific, Asheville, North Carolina] and Fisherbrand® Red-

Spirit Total Immersion Thermometer [Fisher Scientific, Hampton, New Hampshire]) similar to those of Devils Hole.

Having isolated the algal and invertebrate cultures necessary for the treatments, I propagated these cultures for use in feeding experiments by supplementing diatom and algae culture containers (Figure 1.1) with F/2 media (ProLine, Apopka, Florida) and providing them with full-spectrum Panasonic F40 40-W lighting (Panasonic Corporation, Secaucus, New Jersey) lighting installed into a MetaLux 122-cm Utility Fluorescent Light Strip (Cooper Lighting, Peachtree City, Georgia) and aeration from a Sweetwater aquarium air pump, vinyl air line tubing, and 2.5-cm x 1.25-cm Sweetwater silica air stones (Drs. Foster and Smith, Rhineland, Wisconsin). I fed invertebrates with native diatoms cultured in the laboratory once a sustainable population—defined as one that grew steadily without addition of more organisms—of each species was produced. I then split each sample into a second culture jar to protect against contamination and provide backup organisms.

I obtained a diverse array of invertebrate size classes through use of airlift vacuum nauplii collectors to provide larval pupfish with a full complement of sizes similar to those available in Devils Hole (Figure 1.2). I secured a 15-cm long, 4-cm diameter PVC section to an 18-cm long, 1.25-cm diameter PVC piece. I used a straight connector to secure a piece of 1000-μ nylon mesh (Sefar Incorporated, Depew, New York) to the bottom of the 4-cm piece. I attached a 6-cm section of 4-cm diameter PVC below this. Similarly, I attached a 250-μ nylon mesh (Sefar Incorporated, Depew, New York) piece via a second straight connector below the upper section. I drilled a 1.25-cm

hole in this connector, and attached the 1.25-cm diameter PVC piece to the 4-cm piece with clear plastic zip-ties and positioned it directly above this hole. I placed adult invertebrates in the upper section of this apparatus. A 2.5-cm x 1.25-cm air stone was placed down the smaller PVC tube and set to create a gentle boil. This created lift within the smaller PVC tube, drawing water from the bottom of the larger tube and thus creating gentle suction that aided in pulling invertebrate nauplii into the lower chamber where 250-μ nylon mesh prevented their escape. I maintained water levels below the top of the apparatus to prevent the escape of adult invertebrates.

I used rocks from the immediate area surrounding Devils Hole, including those that were removed from Devils Hole after recent floods, as a substrate for all culture aquaria. I established "living rock", i.e. rocks covered with live algal cultures, in the culture aquaria with either cyanobacteria or *Spirogyra* in pure single-species cultures as a reserve for culture containers and examined samples weekly under a Zeiss Stemi® 2000 dissecting microscope (Carl Zeiss, Göttingen Germany) at 6.5X magnification to determine whether they were still pure. In the event that they were not pure, I repeated the process of separation until a pure culture was derived.

Hybrid Pupfish Propagation

Larval hybrid Devils Hole pupfish were produced using the methods of Mapula (Appendix B). Hatching aquaria were examined daily for newly hatched larvae, and most larvae were collected within 1-2 d of hatching. Following collection, larvae were immediately stocked into experimental containers.

Experimental Design and Fish Stocking

Larvae were placed into individual experimental containers for the two-week duration of the experiment. Experimental containers consisted of 50 mL plastic centrifuge tubes. Forty larvae and 40 tubes (40 replicates) were used for each food type treatment. Treatments were as follows: (1) ostracods; (2) amphipods; (3) a concentrated, mixed culture of 30% diatoms, 30% rotifers, 20% ciliates, and 20% miscellaneous organisms henceforth referred to as "diatoms"; (4) ostracods+diatoms; (5) amphipods+diatoms; (6) cyanobacteria; (7) ostracods+cyanobacteria; (8) amphipods+cyanobacteria; (9) *Spirogyra*; (10) ostracods+*Spirogyra*; (11) amphipods+*Spirogyra*; (12) diatoms+*Spirogyra*; (13) diatoms+*Spirogyra*+copepods; and (14) Rio Grande Silvery Minnow flake food. Algae and cyanobacteria cultures begun with sources from Devils Hole, along with Devils Hole invertebrates supplemented with commercially obtained organisms were used for all larval feeding except flake food replicates. Native food types were selected for treatments based on potential availability to the Devils Hole pupfish larvae, as well as gut content analyses of larger fish conducted by Minckley and Deacon (1975) and Blinn and Wilson (2005). Only those species that could potentially be food for larval pupfish were used (Figure 1.3). Larval pupfish were held in this experiment for a period of 14 d, at which time they reached the fry stage, were visibly larger and much more active, and were released into a non-breeding population held in reserve.

To ensure proper temperature management and aeration of experimental containers, an agitator was constructed and placed on a 113-L water bath. The agitator

was constructed from a Dayton 60-rpm motor (Grainger Industrial Supply, Lake Forest, Illinois) affixed with a stainless steel arm that connected to a floating Lexan (SABIC Innovative Plastics, Pittsfield, Massachusetts) shelf with 3-cm holes pre-drilled for 80 containers. Water was added to the bath daily to ensure proper agitation and thus adequate aeration of the containers (Figure 1.4). Holes were drilled in the cap of each centrifuge tube to allow for greater airflow. To verify adequate oxygen saturation in experimental containers, an Orion 4-Star ph/RDO Portable Multiparameter Meter (Thermo Fischer Scientific, Asheville, North Carolina) was used and dissolved oxygen was measured randomly in containers of monospecific and combination algal and cyanobacteria treatments (n = 10 for each type). A 100-W titanium tube heater (Via Aqua, Singapore) was placed in the water bath, and the water temperature was set at 28°C to prevent temperature shock to larvae when they were transported from the hatching aquaria (Figure 1.5). I conducted these experiments at 28°C rather than 33°C because preliminary tests on hybrids resulted in 0% survival at the higher temperature.

One larval fish was added to each 50-mL tube, and each tube was randomly assigned to a treatment. Up to 80 tubes ran concurrently, as space in the agitator was limiting. All food types were fed daily in excess to larvae, and in combination treatments, each individual food type was fed in excess. Flake food was ground prior to application. Five milliliters of suspended diatom mixture was added by pipette each day to the appropriate containers. At least five of each appropriate invertebrate species were added to their respective containers daily. Only nauplii and small-sized organisms were added to accommodate for the small gape size of the larval pupfish. For *Spirogyra* and

cyanobacteria treatments, approximately one milliliter of *Spirogyra* or cyanobacteria was added to each container via pipette. Ninety-five percent water changes were conducted daily with well water treated with 125 mg/L Fish Sulfa Forte (Thomas Laboratories, Tolleson, Arizona) and allowed to aerate in one liter glass bottles for 24 h prior to use. For natural food type treatments, I routinely checked refuse water to ensure that live organisms remained after the 24-h period between feedings.

Larval survival was measured through two weeks, the length of the Devils Hole pupfish larval life stage, for comparison among treatments. Lifespan of each fish was recorded, and total length—measured to the nearest 0.5 mm—was recorded for each fish that survived through 14 d. Initial length was determined through random measurement of larvae (n = 50) and the difference in length before and after treatment was used as a measure of growth rate. The minute size of larval pupfish prohibited accurate individual weight measurement.

Nutritional Analysis

Nutritional analysis of native Devils Hole food types allowed me to document variations in food quality among treatments and the relationship between food quality and survival, lifespan, and growth of larval hybrid Devils Hole pupfish. Samples of *Spirogyra* and cyanobacteria (200 g each) were sent to Midwest Laboratories (Omaha, Nebraska) where proximate and fatty acid analyses were conducted. Midwest Laboratories used the methods of the Association of Official Agricultural Chemists International (AOAC 1995).

I was only able to maintain a small quantity (< 200 g) of diatoms and invertebrates during the experiment so I obtained approximate values for these organisms from available literature. Published nutritional analyses of species found in Devils Hole (Halver 2002; Hepher 1988) in the broader categories Chlorophyta, Cyanophyta, and Bacillariacaea, and for invertebrate species from the same genera as those found in Devils Hole, are reported herein. In addition, as discussed above, my "diatom" experimental treatments actually consisted of a mixture of diatoms, rotifers, and ciliates, so published values for rotifers (Halver 2002; Hepher 1988) are also reported below.

Statistical Analysis

Survival, average lifespan, and growth within each treatment were analyzed using SPSS 19 (International Business Machines, Armonk, New York). Survival was assigned a nominal value—either 1 (survived through 14 d) or 0 (did not survive through 14 d) was recorded for each replicate. A Chi-Square test was used to compare differences in survival among the 14 treatments. Treatments were then grouped into one of five categories: (1) flake food, (2) invertebrates, (3) *Spirogyra*, (4) cyanobacteria, and (5) diatoms. Mixed treatments were assigned to the category with the highest survival rate in monospecific treatments. Sample size was the same for each treatment (n = 40) except ostracod and amphipod treatments (n = 10). Subdivided Chi-square tests were run between all combinations of grouped treatments. Lifespan up to 14 d was recorded for each of the 500 larvae tested. One-way analysis of variance (ANOVA) with a Tukey post-hoc test was used to test for differences in lifespan among treatments. Total length

was only measured for those fish that survived through 14 d. Because different numbers of larvae survived each treatment, sample sizes were unequal, so one-way ANOVA with a Scheffe post-hoc test was used to test for differences in total length among treatments after 14 d. The Scheffe test evaluates trials containing unequal sample sizes with more power than the Tukey test (Ramsey and Schafer 2002). All statistics were evaluated at the $\alpha = 0.05$ significance level.

RESULTS

I found a statistically significant difference in mean survival rate among food treatments overall (Pearson Chi-square statistic=102.343, $P < 0.001$), indicating a significant difference between the mean survival of at least one of my 14 food treatments and the mean of means. Chi-square tests conducted between my five sub-groups—flake, invertebrate, *Spirogyra*, cyanobacteria, and diatoms (Table 1.1)—revealed significant differences in mean survival between sub-groups, with the exception of invertebrates vs. diatoms ($P = 0.087$), *Spirogyra* vs. cyanobacteria ($P = 0.307$), and *Spirogyra* vs. diatoms ($P = 0.070$), where no significant difference was found. Survival of larvae fed Rio Grande Silvery Minnow flake food treatment was 85%, followed by larvae fed algae or cyanobacteria in monospecific cultures or in combination with invertebrates (10-25%), while the monospecific invertebrate treatments—ostracods and amphipods—yielded 0% survival (Figure 1.6). Further, when these two invertebrates were added to any of the algal foods (i.e. ostracod+diatom, ostracod+*Spirogyra*, ostracod+cyanobacteria,

amphipod+diatom, aphipod+*Spirogyra*, and amphipod+cyanobacteria treatments), no improvement in survival over the monospecific algae treatments (i.e. diatoms, *Spirogyra*, and cyanobacteria) was noted.

A one-way ANOVA with a Tukey post-hoc test showed a significant difference ($P < 0.001$) in mean lifespan among all treatments. I found the flake food treatment to differ significantly from each of the 13 other treatments and to produce the longest lifespan—a mean of 12.6 d. No significant differences in mean lifespan (8.6 - 9.7 d) were observed among algal and cyanobacteria, and combination treatments. Addition of invertebrates to monospecific algal and cyanobacteria treatments did not increase larval lifespan over those fed monospecific algal types or cyanobacteria alone (Figure 1.7). The two monospecific invertebrate treatments (ostracods and amphipods) differed significantly from each of the 12 other treatments, but not one another, and produced the shortest mean lifespan (4.4 – 4.7 d).

A one-way ANOVA with Scheffe's post-hoc test indicated there were no significant differences ($P = 0.934$) in mean final larval length among treatments (5.7 - 5.9 mm). Larvae that survived through 14 d grew at the same rate (Figure 1.8).

Nutritional comparison of *Spirogyra* and cyanobacteria (Table 1.2) found differences in constituent content. Cyanobacteria had greater protein content, at 42.5% dry weight, than did *Spirogyra*, at 23.0% dry weight. Similarly, cyanobacteria had greater fat content, at 12.3% dry weight, than did *Spirogyra*, at 10.0% dry weight. *Spirogyra* had higher fatty acid content, at 0.14% omega-3 and 0.10% omega-6 dry

weight, than did cyanobacteria, at 0.02% omega-3 and 0.04% omega-6 dry weight (Table 1.2). Values derived from literature are available in Table 1.2.

DISCUSSION

Two important factors dictate survival and growth of Devils Hole pupfish. First, survival—measured here both by survival rate and lifespan—to adulthood requires access to adequate food. Second, the small size of pupfish larvae leaves them vulnerable to predation by adult pupfish, so rapid larval growth is important. In my experiment, survival of hybrid pupfish larvae (Figure 1.6) was highest when fed flake food, intermediate when fed algal species (i.e. *Spirogyra*, cyanobacteria, and diatoms) and algal species in combination with other food types, and lowest when fed monospecific invertebrates (i.e. ostracods and amphipods). Average lifespan by treatment mirrored the larval survival results. Lifespan was longest for the flake food treatment, intermediate for the algal and combination treatments, and shortest for the monospecific invertebrate treatments. In particular, no invertebrate treatment larvae survived past 6 d (Figure 1.7). Of larvae that survived, there was no significant difference in their average length at the end of the 14-d experimental period (Figure 1.8).

My data suggest that flake food was the best food source tested herein, for it produced the highest survival and longest lifespan of any treatment. An important question to consider is whether this reflects the higher percentage of key nutrients in flake food with respect to the other food types. Protein and lipids are particularly important

nutritive elements for larval fish (Webster and Lim 2002). Specifically, baitfish species of similar size and life history characteristics to Devils Hole pupfish require high levels of protein and fatty acids, especially those that are plant based, in their diets (Webster and Lim 2002). Further, while no specific information exists on the nutrient requirements of Devils Hole pupfish larvae, in general, fish have higher protein requirements at the larval stage than at any other life stage (Lovell 1998).

An analysis of Rio Grande Silvery Minnow flake food, in addition to analyses of Devils Hole *Spirogyra* and cyanobacteria and proximate values derived from several sources (Halver 2002; Hepher 1998) shows that key components of the Devils Hole assemblage do not differ greatly in nutritive value by dry weight from Rio Grande Silvery Minnow flake food and that no single nutritional component, except omega-3 fatty acids, is higher in flake food than the combination of native Devils Hole food types from either the 1970s or the 2000s (Table 1.2). Interestingly, rotifers—a component of "diatom" treatments—are particularly high in protein and lipid content and are unique because they are important food sources for invertebrates and may therefore represent a potential food type both for Devils Hole pupfish and for invertebrate species on which pupfish forage (Hertrampf and Piedad-Pascual 2000). However, "diatom" treatments had lower survival and shorter lifespan than Rio Grande Silvery Minnow flake food treatments.

Because protein and lipid content were not consistently higher in Rio Grande Silvery Minnow flake food than natural food types, I cannot conclude that differences in survival and lifespan between flake food and natural foods resulted from the lower nutritive value of natural foods. Therefore, other characteristics of the natural food types

may render them less suitable food sources for larval pupfish than flake food. My lifespan data are potentially revealing. In my experiment, I found that no fish in my invertebrate treatments survived longer than six days and survival of up to six days was likely due to the gradual absorption of the yolk sac as a food source (Huet 1986). Prior to the absorption of the yolk sac, I observed larvae with full stomachs in all of my treatments but the monospecific ostracod and amphipod treatments, indicating that larvae were unable to feed on the invertebrate food types. In contrast, average lifespan for monospecific algal and cyanobacteria treatments and combination algal treatments was approximately nine days, indicating that these larvae were feeding externally in addition to using the yolk sac. A large percentage of fish fed flake food survived the entire test period. Thus, a variable related to the larvae's ability to catch and/or digest the natural food types might explain the difference in survival and lifespan between flake food and natural food.

In contrast to the finely crushed flake food, larval pupfish must exert more effort to forage all of the invertebrate species used in this experiment. Foraging these foods poses several challenges—reaction time, transparency of prey items, and experience (Lee et al. 2005). However, larval fish of similar size to larval hybrid Devils Hole pupfish are able to feed on prey similar in size to the ostracods, amphipods, and copepods used in this experiment (Lee et al. 2005). Therefore, gape width is not likely limiting the larval hybrid pupfish's ability to forage effectively. Reaction times and swim speeds of larval hybrid Devils Hole pupfish have not been recorded, but as other fish of similar size are able to utilize these food sources, reaction time and speed are also not likely a limiting

factor in foraging ability (Lee et al. 2005). Thus, while gape width and reaction time for larval hybrid Devils Hole pupfish have not been measured, it is possible that another factor may be limiting ability to forage invertebrate species utilized in this experiment.

Constraints imposed by unique aspects of fish digestive systems may account for the greater survival and lifespan of larvae in the flake food treatment versus those in the natural food treatments. Most larval fish begin to feed before their digestive systems are fully developed. Many larval fish have only a foregut, midgut, and hindgut, rather than stomach and upper and lower intestines. Many of the support organs such as the pancreas, gallbladder, and liver, are immature. As a consequence, many viable plant cells or even invertebrates may pass undigested through larval digestive systems (Halver 2002). Digestibility may be crucial in dictating the greater survival and lifespan of larvae undergoing the flake food treatments versus larvae undergoing natural food treatments. Digestibility coefficients are not available for Devils Hole pupfish, but when formulating feed recipes, aquaculturists select feed constituents, in part, to provide the most easily assimilated product (Hepher 1998). While some of the natural food types may have ideal nutritive content, flake food may be assimilated much more easily and therefore translate into increased survival and lifespan (Halver 2002). The percent moisture of native food types may make it difficult for a larval fish to process adequate amounts of food to derive the same benefit. Thus, nutritive qualities intrinsic to Rio Grande Silvery Minnow flake food may not account for the greater survival and longer lifespan of larvae undergoing the flake food treatment than larvae undergoing natural food treatments. Instead, larval

pupfish may simply be less able to utilize the nutrients contained in the natural food I tested because they are less able to forage or digest these foods in sufficient quantities.

Given the dramatic decline in pupfish population in Devils Hole between the 1970s and 2000s, an understanding of the potential implications of my findings for larval survival and growth in natural settings is important. My results indicate no significant difference in larval survival, lifespan, or growth between algal and cyanobacteria treatments in my experiment. Thus, I found no significant difference in larval survival, lifespan, and growth when comparing dominant food types from the 1970s with dominant food types from the 2000s. I conclude that dominant elements of the 1970s community and the 2000s community are not significantly different in terms of food value for larvae. However, the overall *quantity* of food in Devils Hole may have changed over time. Unfortunately, no detailed information on food densities from the 1970s, when the pupfish population was relatively stable, is available for comparison with densities of the 2000s, when pupfish numbers have declined.

While the larvae in my natural food treatments had lower survival that those in my flake food treatments, larval survival of fish in my algae and cyanobacteria treatments may be comparable to the survival rate of fish in Devils Hole during the 1970s, the period during which Devils Hole pupfish were comparatively thriving. No information exists for survival of the Devils Hole pupfish at the larval age class in the wild, however evidence from 1968-1969 suggests that recruitment rate of juvenile Devils Hole pupfish was approximately 20% (James 1969). This rate is comparable to the larval survival rate that I observed in the laboratory during my algae treatments. Further, because pupfish

are an r-selective species, laying many eggs and providing little-to-no parental care after hatching, we may assume recruitment rate of larval pupfish to be lower than that of juvenile pupfish, as is found in many other fish species (Planka 1970). Consequently, survival rates seen for natural food types under laboratory conditions may have been high compared to those in Devils Hole, as the amount of available food type was not limiting, nor were factors such as predation and water quality.

My findings and the methodology used herein are necessarily limited. My experiment only examined the possibility of a food limitation to recruitment at the larval life stage—I did not take into account possible food limitations at any other stage in development, so food quality may be limiting older fish. I kept fish in relatively small (50 mL) containers filled to only 35 mL in order to allow for adequate aeration, limiting their movement and possibly affecting their ability to forage for invertebrates or microscopic food types. However, because fish were in close proximity to food sources in these containers, their ability to forage may have been enhanced.

I tested all major food types available to the Devils Hole pupfish, but it is possible that I missed a minor, yet important component of their diet. I did not test every conceivable combination of food types available in Devils Hole, nor did I test the entire 1970s community against the entire 2000s community. Rather, for this study I identified the most useful combinations of food types from both the 1970s and 2000s communities on the basis of size, availability, and analyses of adult pupfish diet. The list of native species present was similar for both time periods; however, relative dominance within the community differed over time. Because adequate data describing the relative proportions

of native food types in Devils Hole are only available from the late 1990s onward, I did not attempt to replicate the relative proportions of native food type densities in this study. While this might be possible for the 2000s community, it was not possible for the 1970s community, and consequently, my results would not necessarily be representative or beneficial to managers. I also did not examine differences between food types not relating to nutritional value. For example, Gustafson and Deacon (1998) noted that larval density was inversely correlated with *Spirogyra* density. Similarly, and to a greater extent, cyanobacteria can fill interstitial spaces in the substrate, creating anoxic conditions that may be damaging to eggs or larvae (Riggs and Deacon 2004).

Several potentially important areas for further research exist. Tests on feed suitability for different life stages would expand the scope of my research and help evaluate whether a food limitation exists at other stages of pupfish development. Further, cataloguing all Devils Hole flora and fauna with information on population levels and densities, especially as they vary by season, could be invaluable to researchers investigating flux in nutritive quality over time. Examinations of larval gut contents and feeding habits in Devils Hole itself would complement my results. Ultimately, it is only through a more comprehensive understanding of the ways in which Devils Hole is changing that better management techniques can be developed and implemented to respond to this dynamism.

In conclusion, my data suggest that there is a significant difference in larval hybrid pupfish survival and lifespan between Rio Grande Silvery Minnow flake food and natural food types, and that the artificial feed is a superior food source for larval

recruitment. I found no significant differences between algal and cyanobacteria treatments, suggesting that the 1970s Devils Hole community was not superior to the 2000s community, at least in terms of food value. My results also indicate that addition of native invertebrate species of all size classes to algal or cyanobacteria species does not affect the survival or growth of larval hybrid pupfish. For pupfish that survive through the larval stage, growth rate does not differ according to food type.

Building on this information, managers can begin to investigate other factors that may affect the recruitment rates of the Devils Hole pupfish. To run tests with other pupfish size classes or to further test larval feeding, the methods used herein may be utilized to propagate algal and invertebrate species from Devils Hole. These findings contribute to the body of knowledge used to manage Devils Hole pupfish, and may be applicable to the management of other pupfish species as well.

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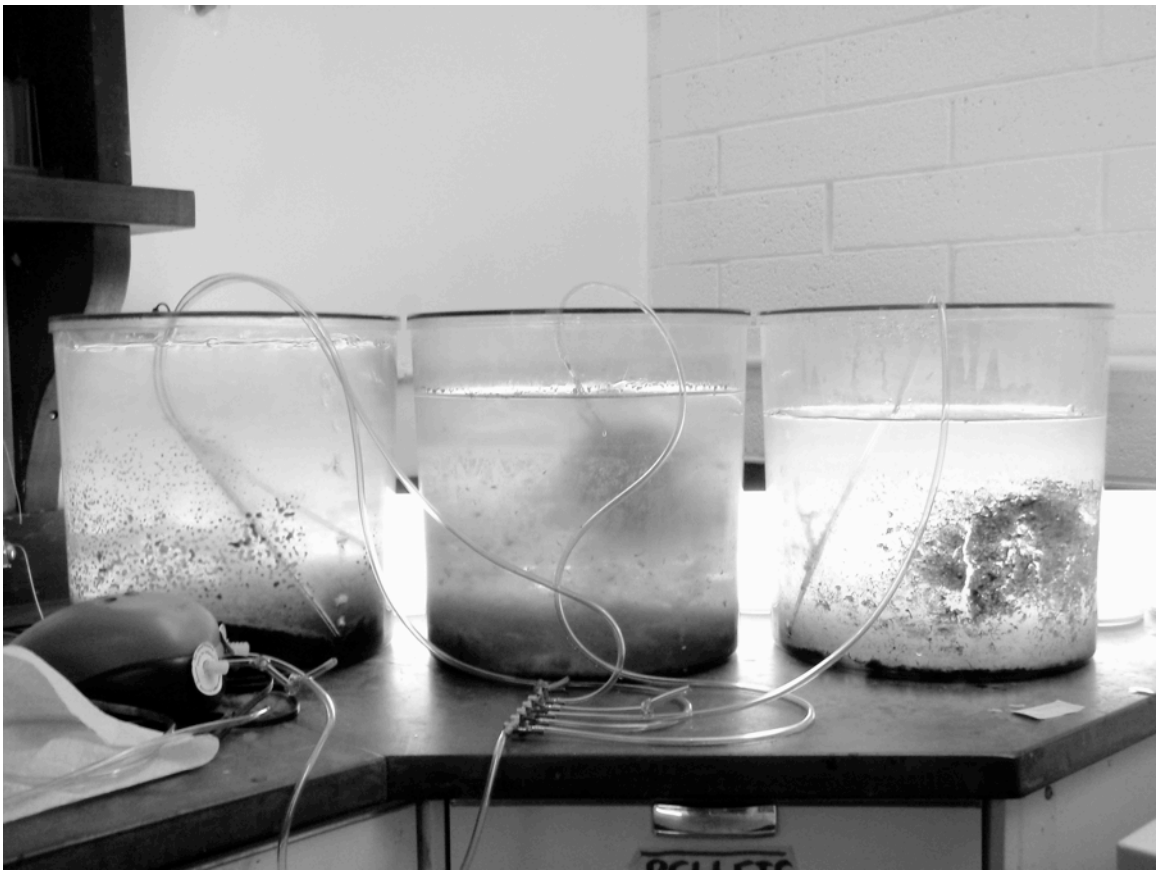

FIGURE 1.1: Containers used to propagate algal species collected from Devils Hole, Nevada.

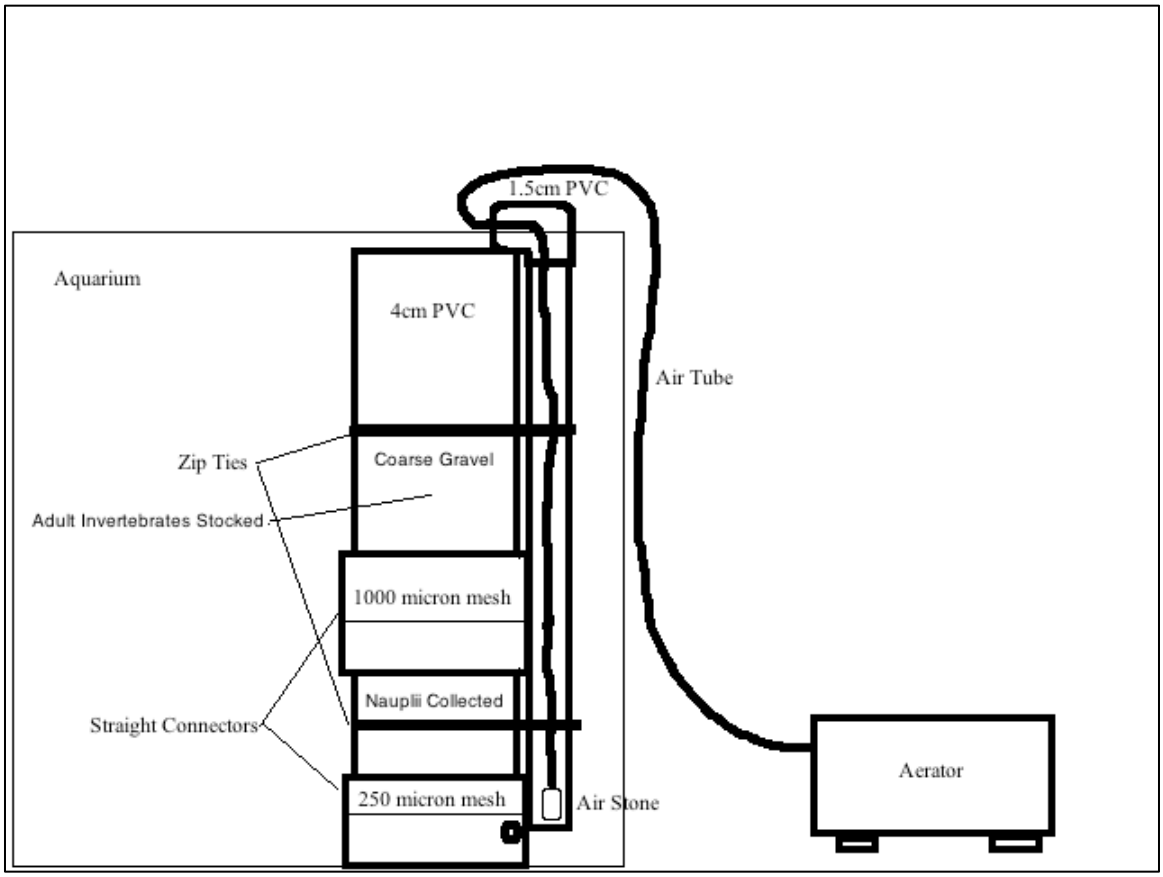

FIGURE 1.2: Nauplii collector schematic.

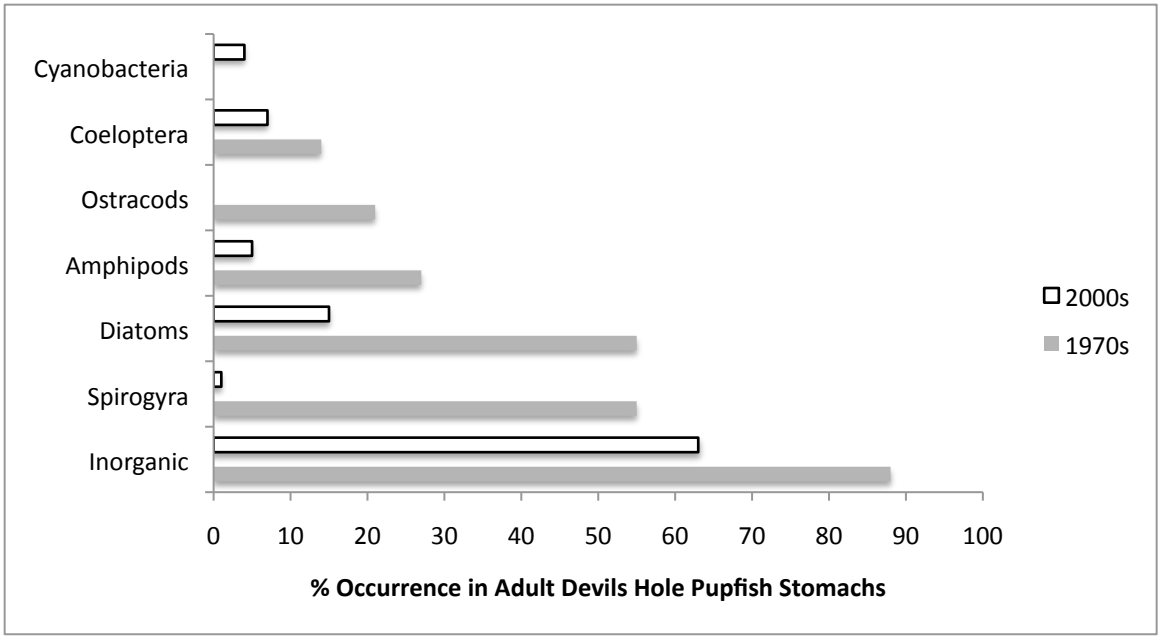

FIGURE 1.3: Percent occurrence of items in adult gut content analyses of Devils Hole pupfish, adapted from Minckley and Deacon (1975) and Blinn and Wilson (2005).

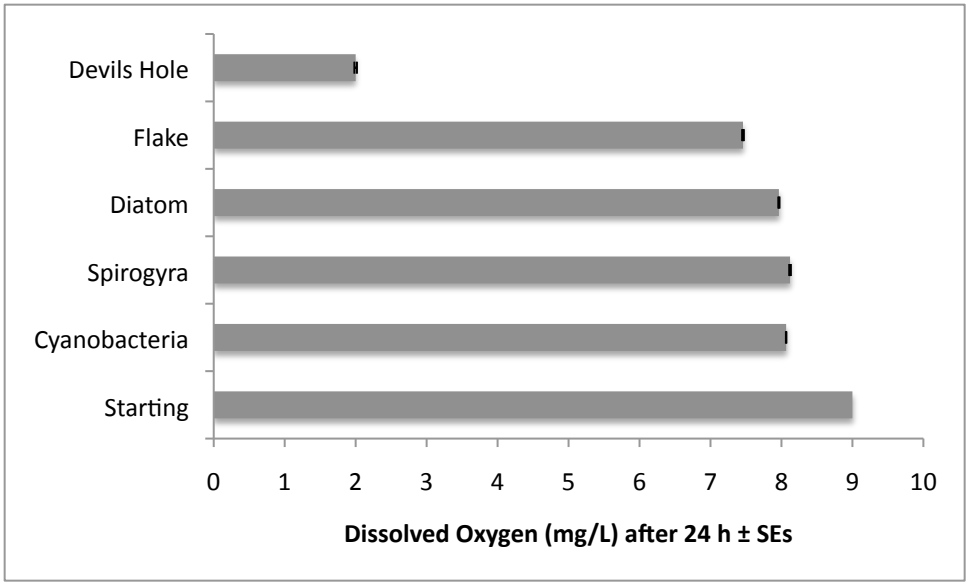

FIGURE 1.4: Mean dissolved oxygen (mg/L after 24 h ± SEs) in experimental containers, including starting values and Devils Hole levels (Shepard et al. 2000).

FIGURE 1.5: Agitator and water bath containing treatment containers used in experiments testing adequacy of food types for hybrid Devils Hole pupfish larvae.

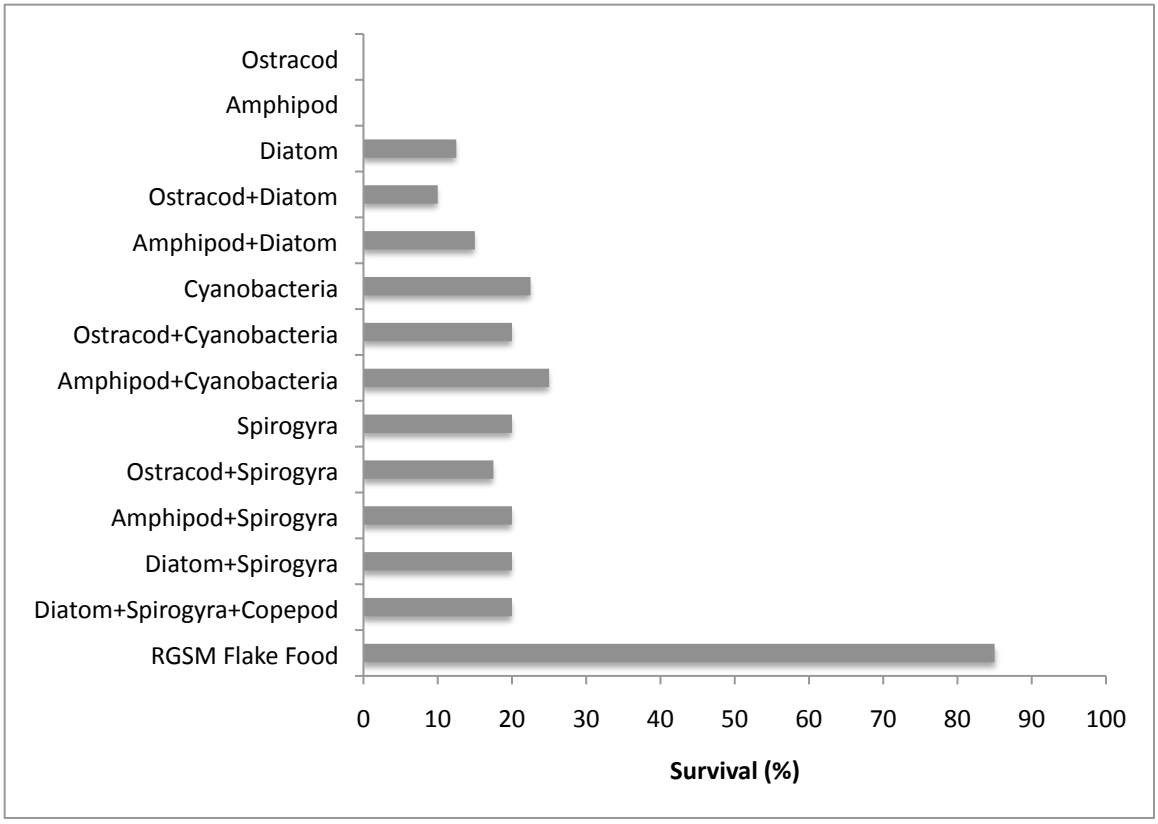

FIGURE 1.6: Percent survival of hybrid Devils Hole pupfish larvae fed different food types through 14 d.

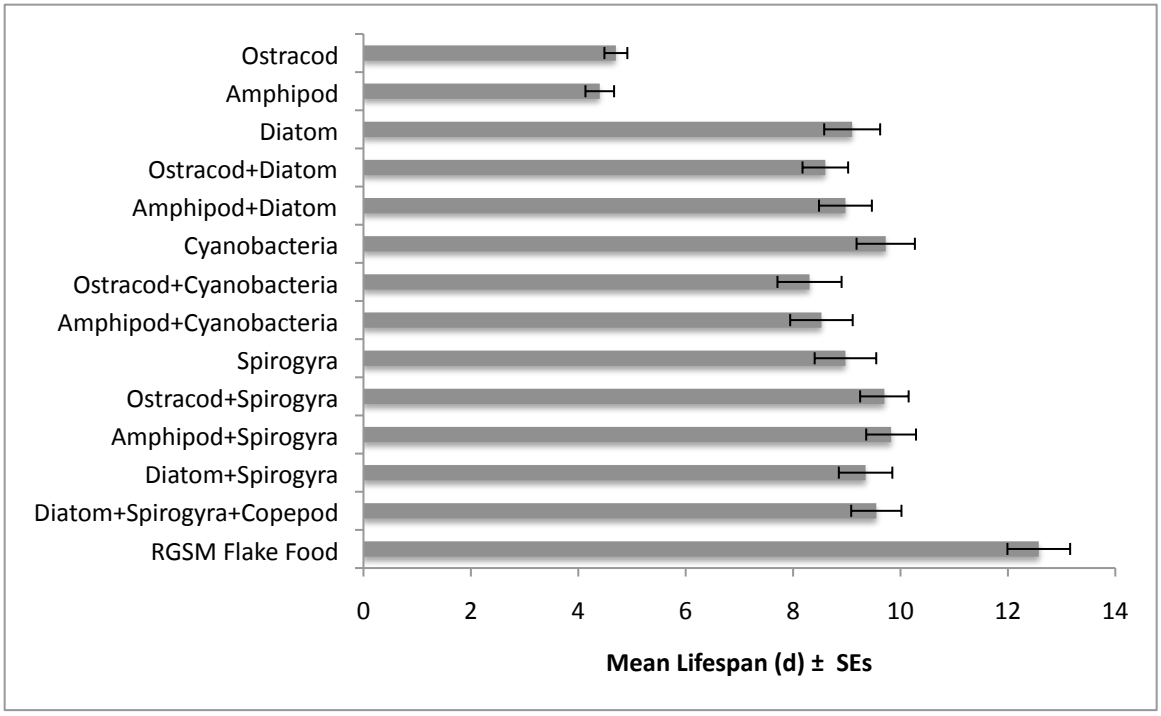

FIGURE 1.7: Mean lifespan of hybrid Devils Hole pupfish larvae fed different food types (d ± SEs).

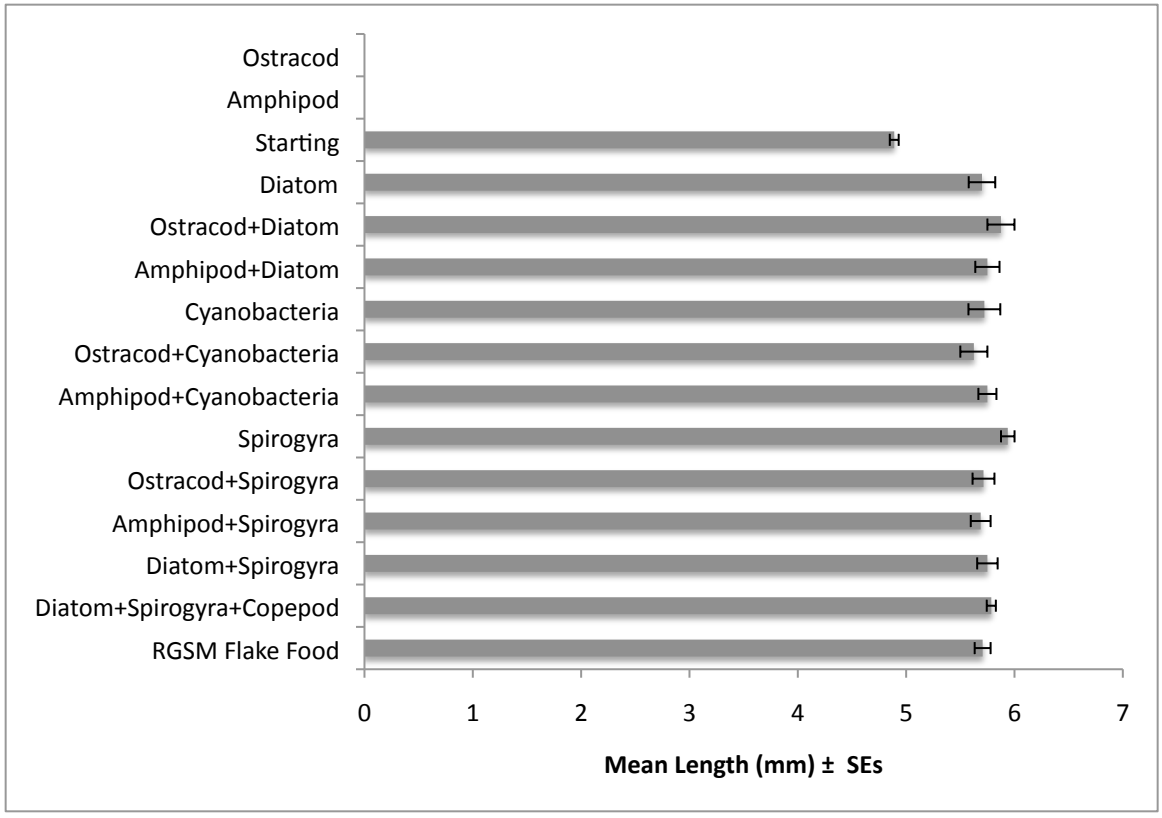

FIGURE 1.8: Mean total length (14-d means ± SEs) of hybrid Devils Hole pupfish larvae fed different food types, including mean starting length.

TABLE 1.1: Chi-square values for survival rate comparisons among food type treatments.

Comparison	*P*-value
Flake vs. Invertebrate	<0.001
Flake vs. *Spirogyra*	<0.001
Flake vs. Cyanobacteria	<0.001
Flake vs. Diatoms	<0.001
Invertebrate vs. *Spirogyra*	0.017
Invertebrate vs. Cyanobacteria	0.010
Invertebrate vs. Diatoms	0.087
Spirogyra vs. Cyanobacteria	0.307
Spirogyra vs. Diatoms	0.070
Cyanobacteria vs. Diatoms	0.030

TABLE 1.2: Nutritional components of larval hybrid Devils Hole pupfish food types.

	% Composition						
Food Type	Moisture	Protein	Lipid	Fiber	Ash	Omega-3	Omega-6
RGSM Flake Food[1]	7.66	45.20	16.60	0.83	7.87	2.03	0.08
Devils Hole *Spirogyra*[2]	93.24	23.00	10.00	<0.20	0.41	0.14	0.10
Devils Hole Cyanobacteria[2]	97.89	42.50	12.30	<0.20	19.30	0.02	0.04
Green Algae[3]	83.82	17.60 – 65.00	3.70	n/a	26.90	n/a	n/a
Cyanobacteria[3]	n/a	31.30	n/a	n/a	46.70	n/a	n/a
Diatoms[3]	n/a	30.70	9.90	n/a	38.30	n/a	n/a
Rotifers[3]	88.80	64.30	20.30	n/a	6.20	n/a	n/a
Invertebrates[3]	65.00 – 89.70	9.80 – 35.00	19.30 – 26.40	9.20 – 28.20	7.10 – 19.60	n/a	n/a

[1] Rio Grande Silvery Minnow flake food analysis provided by USFWS Fish Technology Center, Bozeman, Montana.
[2] Nutritional analysis conducted by Midwest Laboratories, Omaha, Nebraska.
[3] Nutritional values derived from Hepher (1988) and Halver (2002).

APPENDIX B

LABORATORY PROPAGATION OF HYBRID DEVILS HOLE PUPFISH

LABORATORY PROPAGATION OF HYBRID DEVILS HOLE PUPFISH

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ABSTRACT

I developed methods for reliable laboratory propagation of hybrid Devils Hole pupfish. Six spawning mops, constructed of yarn and tile, were placed in each of four parental aquaria, stocked with 24 adult fish each, for three days to attract adults and provide substrate for spawned eggs. A 30% water change was conducted on the same day that spawning mops were placed in the parental aquaria. After three days, spawning mops were transferred to six hatching aquaria held at 28°C temperature and provided constant aeration. All hatching aquaria were treated weekly with Fish Sulfa Forte and Rid Ich to prevent bacterial or fungal infection, and hatching aquaria were disinfected between hatchings. Aquaria were examined daily for newly hatched larvae, such that all larvae were collected within 1-2 d post-hatch. Only ten larvae were produced in parental aquaria held at static temperatures over first nine months of trials. Consequently, I found conducting a weekly 30% water change in parental aquaria, lowering water temperature from 28°C to 23°C then gradually raising it back to 28°C over 48 h produced 500 larvae over 8 months. It is possible that age distribution within parental aquaria may have also affected production rates over time. Adult hybrids may not have been old enough for peak production at the beginning of this experiment, thus examining the effects of age on egg production may be useful. My methods for hybrid propagation employed temperatures suitable for Amargosa pupfish reproduction. Further information on the reproductive requirements of pure-strain Devils Hole pupfish is needed in order to modify my techniques for use with pure-strain Devils Hole pupfish.

INTRODUCTION

Devils Hole is a single limestone fissure within Death Valley National Park that encompasses the entire range of one of the most imperiled vertebrate species known, the Devils Hole pupfish *Cyprinidon diabolis* (Lema and Nevitt 2004; Riggs and Deacon 2004). The spring-fed Devils Hole remains a constant 33°C and contains approximately 2 ppm dissolved oxygen, providing a challenging environment even by pupfish standards (Shepard et al. 2000). Though Devils Hole is more than 125-m deep (the actual depth is unknown), pupfish only utilize the upper 30 m (Gustafson and Deacon 1998).

The Devils Hole pupfish is unique from even its closest relatives in the Amargosa Valley. It is an iridescent blue, 2.5 cm-long fish that lacks pelvic fins. It has perhaps the smallest vertebrate distribution in the world (Moyle 1976). Devils Hole pupfish spend most of their time on a limestone shelf located just 0.1 m below the water surface. Although this shelf only receives full sunlight for four months of the year (Blinn and Wilson 2005), it is the primary area of biological production in Devils Hole. Pupfish are thought to spawn almost exclusively on the shelf.

Devils Hole pupfish use a consort-pair breeding system in which the male follows a gravid female around the spawning shelf as the pair periodically drops to the substrate to spawn (Deacon et al. 1995). Pupfish eggs are transparent and approximately 1 mm in diameter, with an embryonic development period of one week. Newly hatched larvae are approximately 1.5 mm in length and remain in the interstitial spaces of the substrate until

they reach 2-4 mm (Gustafson and Deacon 1998). Their size, when coupled with the lack of pigment, makes accurate larval surveys difficult (Deacon et al. 1995).

Recent, dramatic declines in the wild Devils Hole pupfish population prompted multiple attempts to develop reproducing captive populations in aquaria and refugia. The first attempt was conducted at Steinhart Aquarium in San Francisco from 1971-1973. Only one larva was produced during this time, and it survived only five months (Deacon et al. 1995). Baugh and Deacon (1983) also attempted to propagate pure-strain Devils Hole pupfish in aquaria at the University of Nevada-Las Vegas, but were ultimately unsuccessful, and their study population perished. Their observations of pupfish behavioral ecology and conclusions from their study are discussed more fully below. Later attempts were conducted at Mandalay Bay Aquarium in Las Vegas, and at the U.S. Fish and Wildlife Service (USFWS) Willow Beach National Fish Hatchery, but these fish did not survive. Refugia, small concrete pools stocked with small numbers of Devils Hole pupfish, were established by the USFWS at School Springs and Point of Rocks on the USFWS Ash Meadows National Wildlife Refuge, and at Hoover Dam on Bureau of Reclamation land. The School Springs Refugium was lost due to pump failure, the fish at the Hoover Dam Refugium did not survive, and the Point of Rocks population was inadvertently hybridized with the Amargosa pupfish *Cyprinidon nevadensis mionectes,* a closely related species (Martin 2005). These hybrid pupfish were then transferred to USFWS Willow Beach National Fish Hatchery and are the source of hybrid pupfish used for experiments conducted herein.

Attempts to maintain and propagate captive Devils Hole pupfish contributed to our understanding of pupfish behavioral ecology. In general, *Cyprinidon* species are noted for their morphological and behavioral plasticity, as the ability to adapt and vary behavior to shifting conditions within a small range is potentially crucial to survival (Baugh 1985). Predictably, Devils Hole pupfish behavior (in terms of survival, aggression, territoriality, and foraging) can vary dramatically between wild and captive conditions. In the wild, Devils Hole pupfish are less aggressive and territorial than other pupfish species, possibly because the high temperature within Devils Hole increases pupfish metabolic rates and forces them to spend more time foraging to compensate for energy expenditures (Baugh 1985). By manipulating aquarium conditions (water temperature, alkalinity, and dissolved oxygen) to match Devils Hole conditions, Baugh and Deacon (1983) achieved low mortality rates and noted aggression rates and activity levels comparable to that in Devils Hole. By contrast, in refugia where Devils Hole conditions are not maintained, pupfish are much more aggressive and territorial than their Devils Hole counterparts (Baugh 1985). Captive Devils Hole pupfish held at temperatures lower than those of Devils Hole exhibit aggression, foraging, and mating behaviors strikingly similar to wild Amargosa pupfish (Baugh 1985). It is primarily the males who exhibit an increase in aggressiveness and territoriality in captivity, and this increased investment in defending breeding territory results in greatly reduced foraging activity. Increased aggressiveness also poses a barrier to successful reproduction in captivity (Baugh 1985).

In addition to behavioral barriers to reproduction, biological and genetic barriers to reproduction may also be present, both in the wild and in captivity. A comparison of Devils Hole pupfish gonads to total body weight show that they are proportionally smaller than many fish species, though fertility issues such as gamete viability have not been closely examined (Taylor and Deacon 1991). Physical deformities within the species have also been documented. These deformities may reflect impacts of environmental conditions on pupfish development, or they may have resulted from the closed gene pool within Devils Hole that is the consequence of the lack of connectivity between Devils Hole and other systems (Taylor and Deacon 1991).

Low Devils Hole pupfish egg viability has been repeatedly noted in both natural and laboratory settings. Deacon et al. (1995) observed hatch rates as low as 12% and survival rates as low as 6% within Devils Hole, while in laboratory experiments, they observed only 4% hatching success, although 65% of eggs laid appeared to be transparent and viable. Among cage-held adults in Devils Hole, Deacon et al. (1995) noted average production of only three offspring per female per month from March through July, a period that includes what is thought to be the height of breeding. However, they did observe a relationship between greater hatching success and larger diel fluctuation in oxygen saturation, which is the result of photosynthetic activities of algae on the spawning shelf. Despite low Devils Hole pupfish egg viability, biannual monitoring indicates that the population within Devils Hole fluctuated between spring and fall censuses before the population began to decline in the 1990s (Wilson 2011). Thus, low

egg viability does not appear to be the factor impeding population increase in Devils Hole.

The potential use of captive pupfish populations to buttress the wild Devils Hole pupfish population is complicated by the degree of genetic variation observed among captive populations, and between captive populations and the Devils Hole population. Wilcox and Martin (2006) examined genotypic plasticity among populations from Devils Hole, Point of Rocks Refugium, and School Springs Refugium, and found significant genetic divergence from the Devils Hole population in the two refugia populations, while Martin (2005) demonstrated significant genetic variation in multiple refugia attributable to the founder effect commonly seen when a small number of individuals is used for captive breeding. Loss of rare alleles likely contributed to the quick divergence of refugia populations from the Devils Hole population. Some alleles found in wild Devils Hole pupfish were not in refugia populations (Martin 2005). Further, at Point of Rocks Refugium, where pure-strain Devils Hole pupfish were inadvertently hybridized with Amargosa pupfish, the genetic composition of the population shows little genetic contribution from Devils Hole pupfish, and instead demonstrates the comparably high fitness of Amargosa pupfish in this setting. Genetic variation through time has also been noted within refugia populations, particularly between 1998 and 2005 captive populations at Point of Rocks Refugium where hybridization occurred (Martin 2005).

In light of the behavioral and biological barriers to reproduction observed among wild and captive Devils Hole pupfish populations coupled with the absence of pure-strain pupfish outside of Devils Hole, the possibility of Devils Hole pupfish extinction has

become quite real. Successful captive propagation is therefore imperative. My objectives were: (1) to develop a reliable methodology for producing hybrid Devils Hole pupfish in a laboratory setting; (2) to quantify the total larval pupfish produced over the duration of an experiment, during which propagation techniques were refined; and (3) to discuss how captive propagation of hybrid Devils Hole pupfish is related to that of pure Devils Hole pupfish. My experiments utilized hybrid Devils Hole pupfish obtained from the USFWS Willow Beach National Fish Hatchery. These hybrid pupfish were moved to Willow Beach from Point of Rocks Refugium. Though results from genetic testing confirm that this breeding stock is no longer pure, and is, in fact > 50% Amargosa pupfish, it nevertheless contains the only Devils Hole pupfish genetic material outside of Devils Hole. With the species too far imperiled to consider pure-strain pupfish for experimentation, these hybrids are the closest link to the Devils Hole pupfish, and therefore the best substitute for experimentation. The methods used herein may be of use to professionals working with a variety of pupfish species, or species with limited distributions and rigid or hostile environments.

METHODS

Transfer of Hybrid Pupfish

Hybrid Devils Hole pupfish were first obtained from USFWS Willow Beach National Fish Hatchery, Arizona in the fall of 2008 and transported to the University of Arizona. Fish were transferred in 45-L plastic coolers and acclimated to new 492-L

aquaria according to the methods of Widmer et al. (2005). These aquaria were then preventatively treated with 0.125 mg/L Fish Sulfa Forte (Thomas Laboratories, Tolleson, Arizona), an antibiotic, and 0.25 mL/L of the antifungal Rid Ich (Kordon, LLC, Hayward, California).

Hybrid Pupfish Propagation

The F1 and F2 descendants of the transferred fish (all adults) comprised the parental generation for these trials. Fish were held in four 492-L parental aquaria maintained at 28°C and equipped with full-spectrum Panasonic F40 40-W lighting (Panasonic Corporation, Secaucus, New Jersey); 100-W titanium tube heaters (Via Aqua, Singapore); and Hydro Sponge V 125-g filters (Aquarium Technology Incorporated, Decatur, Georgia). Each parental aquarium was stocked with 24 adult hybrid Devils Hole pupfish, and numbers and sex ratios were maintained throughout the experiment. Photoperiod was set to 12 h: 12h.

The temperature setting of 28°C for spawning and hatching aquaria was identified through concurrent experiments conducted by Feuerbacher (unpublished data). These experiments tested hatching success of hybrid Devils Hole pupfish at six different temperatures ranging from 24-34°C and found that highest production was achieved at 28°C. I propagated pupfish from February 2010 to June 2011. From February 2010 to November 2010, I attempted propagation at a static temperature of 28°C. Thereafter, I conducted 30% once-weekly water changes using well water to drop the temperature of

each parental aquarium to 23°C. Following water changes, each aquarium was returned to 28°C by aquarium heaters over 48 h.

I fed adult fish daily, *ad libidum*, Rio Grande Silvery Minnow flake food and both frozen and live brine shrimp *Artemia spp.* Ostracods Ostracoda were also established in each parental aquarium. Spawning mops, constructed out of approximately 2 m of dark blue acrylic yarn, wound into a 10-cm diameter ball and attached to 10-cm white, glazed tiles with clear plastic zip-ties were placed into the parental aquaria. Twice weekly—on Mondays and Thursdays—I placed six spawning mops, equally spaced, in each parental aquarium (for a total of 24 spawning mops in all four parental aquaria used on each Monday and Thursday).

Twice weekly—also on Mondays and Thursdays—I transferred spawning mops previously placed in the parental aquaria to hatching aquaria. Spawning mops placed in parental aquaria on Monday were transferred to hatching aquaria on Thursday, while spawning mops placed in parental aquaria on Thursday were transferred to hatching aquaria on Monday. A total of six 38-L glass aquaria without filtration systems were employed to hatch eggs—three aquaria containing eight spawning mops each were used on Monday, and three aquaria containing eight spawning mops each were used on Thursday. Each hatching aquarium contained a 100W titanium tube heater (Via Aqua, Singapore) set to 28°C, a 30-cm x 2.5-cm flat air stone, and a ceramic-coated metal lid rack that held the tiles with attached spawning mops. Full spectrum, Panasonic F40 40-W lighting was installed into a MetaLux 122-cm Utility Fluorescent Light Strip (Cooper

Lighting, Peachtree City, Georgia) above each aquarium, and photoperiod was set to 12 h: 12 h.

When transferring spawning mops from parental to hatching aquaria, I gently removed each spawning mop and tile from the parental aquarium and placed it on the lid rack in a randomly selected hatching aquarium. Air stones in each hatching aquarium were set to provide a gentle roll of bubbles, resembling a slow boil, in order to mechanically deter fungal growth on eggs. Using a mortar and pestle, I crushed and added 250 mg of Fish Sulfa Forte to each hatching aquarium to deter bacterial growth; I added 5 mL of Amquel Plus (Kordon, LLC, Hayward, California) to regulate chlorine and nitrogen levels; and I added 5 mL Rid Ich (Kordon, LLC, Hayward, California) to deter fungal infection. Antibiotic treatments were applied only once to each batch of spawning mops. Eggs were allowed seven days to hatch in hatching aquaria, with two batches run concurrently. Spawning mops were then removed, rinsed with well water, boiled in well water for 15 minutes to sterilize yarn, and then placed back in parental aquaria. Hatching aquaria were disinfected between holding batches of spawning mops by filling each aquarium with a 10% bleach solution, allowing it to sit for 20 minutes, then thoroughly rinsing each aquarium with tap water.

Hatching aquaria were examined daily for newly hatched larvae. I shined a halogen flashlight into aquaria to detect the small, clear larvae against the black aquarium stand bottom. Larvae were collected daily using a 5-mL plastic pipette, because transport in mesh nets, even those designed for small age classes, proved too traumatic for pupfish larvae. It is unlikely that I detected every fish on the day of hatch, however daily

examinations increased likelihood that most larvae were obtained within 1-2 d of hatching. To quantify total larval production during this period, I recorded each larva captured.

I compared mean larval production per month for the time periods before and after the addition of water changes using a two-sample t-test in SPSS 19 (International Business Machines, Armonk, New York). Significance was evaluated at the $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

My methodology produced a total of ten larval hybrid Devils Hole pupfish at static 28°C temperatures (February 2010 to October 2010) and 500 larval hybrid Devils Hole pupfish when I regularly conducted water changes to lower the temperature from 28°C to 23°C and raised it back to 28°C over 48 h (November 2010 to June 2011; Figure 2.1). Water changes were correlated with significantly increased larval production ($P < 0.01$).

Proper temperature is critical to survival of fish eggs and larvae. Ovulation in some fish species slows as temperature increases, and eggs may be of lower quality at higher temperatures (Gillet 1991). Shrode and Gerking (1977) also found that oogenesis has a lower temperature tolerance than hatching range, indicating that this may be the most important stage to consider when seeking to increase production. They demonstrate that temperature at production and yolk diameter are inversely related in Amargosa

pupfish. Similar to eggs, larvae are sensitive to temperature. Atlantic cod *Gadus morhua* larvae are especially sensitive to temperature changes (Kjesbu et al 1996), and temperature during larval development impacts sex determination in Atlantic silverside *Menidia menidia* (Conover 1984).

Ambient water temperature can vary by season and with disturbance events. Events, such as heavy rainfall and flooding, have been observed to increase pupfish activity, including spawning, on the spawning shelf within Devils Hole (Wilson 2011).

Weekly water changes may have helped trigger increased larval hybrid Devils Hole pupfish production. Nine months of testing at 28°C static temperature yielded only ten larvae. For hybrid Devils Hole pupfish, conducting 30% water changes, bringing the temperature in parental aquaria from 28°C to 23°C, then raising it back to 28°C over 48 h, coincided with increased larval production (500 fish over the final eight months of my experiment). However, the addition of water changes was not applied randomly, and age distribution within the parental population may also have influenced larval production.

Similar frequency in water changes has not been required to produce hybrid Devils Hole pupfish in other trials. Feuerbacher (unpublished data) conducted a 20% water change followed by 14 d of acclimation to a static temperature. Following the acclimation period, eggs were successfully collected and hatched under these static temperatures for ten consecutive days. He found that total production of eggs and number of eggs per hybrid Devils Hole pupfish female per day were maximized at 28°C, that intermediate production rates occurred at 26°C and 30°C, and that production declined drastically as temperatures reached and exceeded 32°C.

There was little production following water changes in January and February 2011. This may be related to period of uncommonly low ambient temperatures during which time a series of pipes burst and it became difficult to maintain water temperatures in the laboratory.

Parental age may have also affected egg and larval production of hybrid Devils Hole pupfish in my trials. Feuerbacher's (unpublished data) egg production experiments were conducted using a parental population in which most fish were approximately 10 months old. At the beginning of my six-month larval production trials, parental age was bimodally distributed, with large numbers of fish older than 12 months or younger than six months of age. The high hatching success observed during spring 2011 may have been aided by the maturation of the younger age class such that they had become physiologically capable of producing more eggs.

Reproductive barriers, coupled with absence of pure-strain pupfish outside of Devils Hole, increase chances for extinction of Devils Hole pupfish. Successful captive propagation is therefore imperative. While hybrid Devils Hole pupfish were produced effectively using the above procedures, most interest centers on whether these procedures could produce pure-strain Devils Hole pupfish. This would be influenced by different reproductive requirements of Amargosa pupfish and Devils Hole pupfish. Amargosa pupfish reproduce within 25-31°C, with an ideal static temperature range for egg production of 24-32°C, and fluctuating temperatures of 32-28°C and 36-28°C giving highest egg production (Gerking and Lee 1983; Shrode and Gerking 1977). Reproductive tolerance range, defined as 50% hatch rate of optimal production, was 24-

30°C, which encompassed one-fifth of the Amargosa pupfish's normal activity temperature range and one-seventh of its critical thermal range (Gerking and Lee 1983).

Little information exists concerning temperatures optimal for pure Devils Hole pupfish production. Propagation studies in both aquaria and refugia; using temperatures, dissolved oxygen, and mineral contents at Devils Hole levels or with lower temperatures and higher dissolved oxygen, have failed to reliably produce pure-strain Devils Hole pupfish (Deacon et al. 1995). Black bass *Morone saxatilis* time spawning to coincide with fluctuations in water temperature (Secor and Houde 1995). Thus, spawning activity triggered by temperature manipulations in the laboratory may reflect adaptive strategies employed by Devils Hole pupfish in the wild based around infrequent disturbance events.

Because my experiments used hybrid pupfish, whose genetic makeup is primarily Amargosa pupfish, the optimum temperature (28°C) for reproduction of Amargosa pupfish (Gerking et al 1979; Gerking and Lee 1983; Shrode and Gerking 1977) was used as a baseline for determining appropriate temperatures for reproduction instead of those experienced by pure Devils Hole pupfish (32-34°C) . Because these production and hatching temperatures do not accurately reflect that found in Devils Hole, use of methods shown to reliably produce hybrid Devils Hole pupfish may not provide similar results for pure-strain Devils Hole pupfish. Further information about Devils Hole pupfish reproductive requirements is necessary before modifying these procedures for use with pure-strain Devils Hole pupfish.

Several potential areas remain for future research. Most importantly, determining differences in breeding requirements between hybrid and pure-strain Devils Hole pupfish

is necessary to modify these methods for use with pure-strain Devils Hole pupfish. These differences would inform how methods described herein should be altered for propagating pure-strain Devils Hole pupfish. Investigation of egg production and hatch rate under fluctuating, as opposed to static, temperature regimes may be useful for understanding production of pure-strain pupfish in this system where even minute diel fluctuations in temperature may provide critical relief from extreme temperatures.

In conclusion, I successfully produced a total of 500 hybrid Devils Hole pupfish larvae over an eight-month experimental period using my propagation methodology. Feuerbacher (unpublished data) found that the ideal static temperature for hybrid Devils Hole pupfish egg production is approximately 28°C. Intermediate production rates occur at 26°C and 30°C, while production declines drastically as temperatures reach and exceed 32°C. These findings may prove useful for the propagation of imperiled native fishes living in similarly harsh conditions. Further research on pure-strain Devils Hole pupfish is necessary, and techniques used herein could be used as a starting point in this endeavor.

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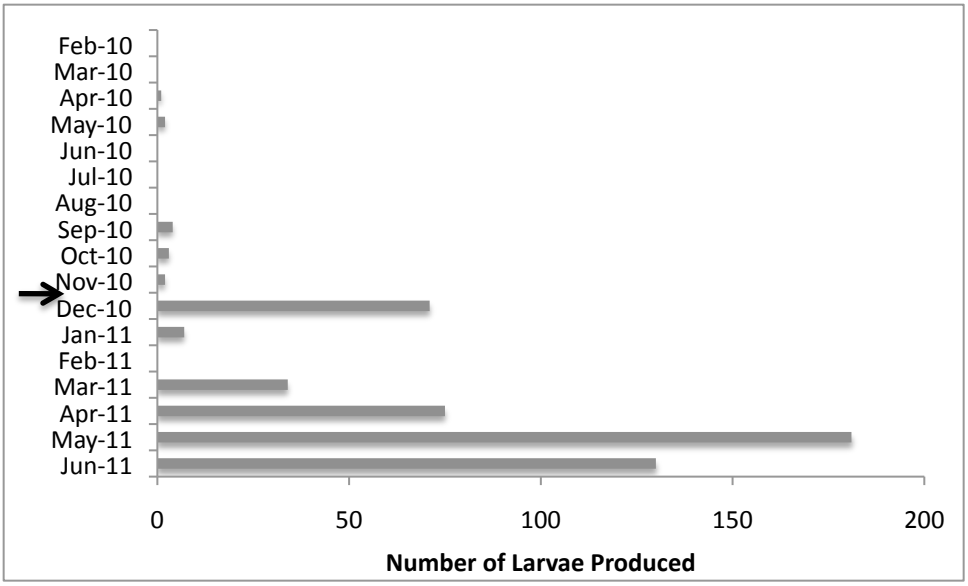

FIGURE 2.1: Larval production of hybrid Devils Hole pupfish, by month, in laboratory aquaria, Tucson, Arizona, 2010-2011, with arrow indicating starting point of use of water changes.