USE OF ULTRASONIC IMAGING TO EVALUATE EGG MATURATION OF HUMPBACK CHUB *GILA CYPHA*

by

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

_____ 05/09/2016

Scott Bonar, Ph.D. Date

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ABSTRACT

Humpback Chub *Gila cypha* are endangered cyprinids endemic to the Colorado River drainage and are adapted to live in fast currents of warm, turbid water. Although nine known aggregations of Humpback Chub currently exist in the main-stem Colorado River in the Grand Canyon, little is known about their reproduction. I hypothesized that Colorado River water temperatures below Glen Canyon Dam are too low due to hypolimnetic water releases from Lake Powell for female Humpback Chub to develop mature eggs for spawning.

Ultrasonic imaging, also called ultrasound, is an effective, non-lethal method used to determine sex and maturity of a variety of freshwater, anadromous, and marine fishes. However, many previous studies have been performed in laboratory environments. I developed a standardized method for ultrasonically scanning endangered Humpback Chub *Gila cypha* in remote locations within Grand Canyon, Arizona, USA. This method minimized stress to individual fish and took less than 1 min to perform. I was able to identify female fish with eggs based on two jpeg images and one 10 s video clip collected in the field. I also used ImageJ®, a National Institute of Health image processing program, to develop a brightness index to evaluate the maturity of eggs in female fish. I collected ultrasonic scans of captive, ripe Humpback Chub held at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) to determine that female fish were potentially ripe when a subsample of their eggs exhibited a brightness value within the 32-44 range. Although I was able to estimate egg maturity, I was not able to estimate egg mass of female fish.

I used ultrasound to evaluate reproductive condition of 751 Humpback Chub in Grand Canyon. I documented egg development in female fish from the main-stem Colorado River, Little Colorado River, Havasu Creek, and Shinumo Creek. Egg development in Humpback Chub varies by location and time of year. Potentially ripe (stage 3) female fish were found at all sample locations and dates except at Shinumo Creek in 2013 and 2014. Potentially ripe females were also detected in every main-stem aggregation except for Pumpkin Springs and in two locations outside of established aggregations.

Fisheries managers can use ultrasound to collect vital information about the reproductive status of fishes that cannot be killed and that are found in remote or rugged field locations. My findings indicate that female Humpback Chub are able to produce eggs throughout the main-stem Colorado River and that internal egg development and egg production likely do not limit recruitment. However, female fish may never experience the environmental triggers they need to spawn or may not experience conditions that would allow eggs and larvae to survive.

Effectiveness of Ultrasonic Imaging for Evaluating Presence and Maturity of Fish Eggs in Remote Locations

Abstract

Ultrasonic imaging, also called ultrasound, is an effective, non-lethal method used to determine sex and maturity of a variety of freshwater, anadromous, and marine fishes. However, most previous studies have been performed in laboratory environments. I developed a standardized method for ultrasonically scanning endangered Humpback Chub *Gila cypha* in remote locations within Grand Canyon, Arizona, USA. This method minimized stress to individual fish and took less than 1 min to perform. I was able to identify female fish with eggs based on two jpeg images and one 10 s video clip collected in the field. I also used ImageJ®, a National Institute of Health image processing program, to develop a brightness index to evaluate the maturity of eggs in female fish. I collected ultrasonic scans of captive, ripe Humpback Chub held at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) to determine that female fish were potentially ripe when a subsample of their eggs exhibited a brightness value within the 32-44 range. Although I was able to estimate egg maturity, I was not able to estimate egg mass of female fish. I successfully scanned 751 Humpback Chub in the field and collected jpeg images and video clips for each fish. Fisheries managers can use this non-invasive technique in remote or rugged field locations to collect vital information about the reproductive status of fishes that cannot be killed.

Introduction

Biologists must be able to accurately sex fish and determine their maturity to effectively manage their populations (Pope et al. 2010). Invasive methods such as blood samples, oocyte biopsies, and dissection of fish have traditionally been used to determine fish sex and gonad maturity (Blythe et al. 1994). However, these methods can be impractical, especially when working with valuable captive broodstock (Blythe et al. 1994) or with rare or endangered species (Bryan et al. 2007). Non-invasive methods, like the evaluation of external morphological differences between male and female fish, are often not accurate (Mattson 1991) which creates a need for the development of new methods to identify sex and reproductive status in fish.

Ultrasonic imaging, also known as ultrasound, was first used in medicine in the 1970s to examine the human heart (Novelo and Tiersch 2012). Fisheries researchers began using ultrasound in the 1980s to sex juvenile and mature Coho Salmon *Oncorhynchus kisutch* (Martin 1983) and Pacific Herring *Clupea pallasii* (Bonar et al. 1989). Over the past 30 years, ultrasound has been developed as an effective, non-lethal method to determine sex and maturity of a variety of freshwater, anadromous, and marine fishes (Novelo and Tiersch 2012).

Although ultrasound is an effective tool, its applications for field biologists have been limited. Most ultrasound research has occurred in controlled laboratory environments, and many studies have either physically or chemically restrained fish during ultrasonic scanning procedures (Novelo and Tiersch 2012). Although some studies have used portable ultrasound units in the field, little information on methods is available

for researchers or managers who wish to use this technique on fish in remote field locations (Evans 2003; Wildhaber et al. 2005).

Practical ultrasonic imaging techniques must be developed in order for fisheries managers to use this technology in remote locations. These locations include decks of marine fishing vessels, lakes and streams in the backcountry, rivers in deep canyons, and isolated aquaculture operations. One of the most remote locations in North America is the Colorado River as it flows through the Grand Canyon.

Using ultrasound in a remote field environment like the Grand Canyon presents many challenges. Air temperatures >40 °C, sand, and water all strain and can damage electronic equipment. Shade can be scarce, which makes discerning images on electronic screens difficult. Rough rides through the Colorado River's famous whitewater rapids can damage equipment. Opportunities to charge batteries are limited, and sampling trips can last from 10-18 d. Biologists have limited time within which they must capture fish and obtain information. These working conditions create a harsh environment for electronic equipment and a limited time frame for conducting ultrasound evaluations on fish.

My goal was to develop an ultrasonic imaging technique that could be used in remote field environments. My objectives were to:

1) Develop a standardized method to obtain ultrasonic images in remote field locations that minimizes stress to fish and is time efficient.
2) Develop evaluation techniques of ultrasound images to accurately identify female fish containing eggs and gauge maturity of eggs.

Methods

Development of Field Ultrasonic Scanning Procedure

I selected a remanufactured Sonosite M-Turbo-R ultrasound unit with a 13-6 MHz linear transducer to scan all fish (VetImaging, Irvine, California). This unit was selected because it is typically used by veterinarians, especially those who travel to farms to administer care to large animals. The transducer I used was designed to scan small mammals, birds, and reptiles, and can penetrate up to 4 cm deep when scanning. Both the unit and the transducer can withstand shock damage from being dropped about 1 m. The unit was not waterproof, so I transported it in a plastic foam-filled case (Pelican Custom, South Deerfield, Massachusetts). One battery would provide up to 2 h of continuous use.

I used Common Carp *Cyprinus carpio* to develop a standardized protocol to obtain ultrasonic image data from fish in the field. I used gill nets with 64-mm-stretch-measure mesh and set them overnight in Tremaine Lake, Arizona, on June 19, 2013, to collect 86 Common Carp. Captured fish were removed from gill nets and held in net pens until they were transported in oxygenated 94-L coolers to a greenhouse facility at the U.S. Forest Service Rocky Mountain Research Station, Southwest Forest Science Complex in Flagstaff, Arizona. Fish were held inside a greenhouse in a 1,000-L rectangular circulating tank system for 24 h before I inserted a unique PIT tag and ultrasonically scanned each fish. I then injected each fish ventrally between the pelvic fins with 0.5 mL of Ovaprim®, a synthetic hormone that induces spawning, and waited 1 week before ultrasonically scanning fish and dissecting them.

After 1 week, all fish were placed in 40-L tubs and euthanized with MS-222 (tricaine methane sulfonate) before I began taking measurements. I recorded TL (mm), SL (mm), FL (mm), and weight (g).

Before ultrasonically scanning a fish, I entered PIT tag numbers unique to each fish in the scanner. I also entered the location, date, and species. Finally, I set the scan type as musculoskeletal, and the scan depth to 2.6 cm. While the machine was saving this information, I externally photographed the left lateral side of each fish. I removed the fish from water, wet the end of the probe, and positioned the transducer probe parallel to the operculum. I then scanned the left lateral side of each fish twice, starting immediately posterior of the pectoral fin, moving toward the caudal fin, and ending at the anal opening or vent (Figure 1.1). The first full scan was saved as a 10 to 15 s video, depending on the fish TL. For the second full scan, I followed the same procedure, but saved two jpeg images per fish. One image was saved during the anterior half of the full scan, and the second image was saved during the posterior half of the full scan. I spent less than 60 s scanning each fish. The ultrasound machine automatically created a folder for each unique PIT tag number where all video files and jpeg images associated with that tag number were stored.

During the scanning procedure, I determined sex based on live images on the ultrasound machine. If I noticed an egg mass, I used calipers on the machine to measure and record the area (cm^2) of the mass. Immediately after scanning each fish, I externally sexed it and dissected it. I noted the sex of each fish and gonad weight (g). If the carp was a female, I measured the length (mm) and width (mm) of the ovaries. Finally, I measured the diameter (mm) of a subset of 30 eggs. This information was used to calibrate ImageJ® (National Institute of Health, Bethesda, Maryland), the software I used for analysis of egg maturity.

I also ultrasonically scanned Humpback Chub *Gila cypha* at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC, Dexter, New Mexico), a U.S. Fish and Wildlife Service hatchery that holds Humpback Chub broodstock and provides juveniles for stocking in Grand Canyon, to obtain reference ultrasound videos and images of ripe fish for comparison with fish scanned in the field. Hatchery specialists retrieved fish they believed to be adult females for all samples. I scanned Humpback Chub (N = 23) on April 22 and 23, 2014, when hatchery specialists were stripping eggs for production. These fish were held in an outdoor 405-m^2 pond. After I scanned fish on April 22, they were injected with a solution of carp pituitary gland hormone (2-3 mg per kg body weight) and saline (0.3%) to induce spawning. I waited 16 h before re-scanning these fish. I performed the full ultrasonic scanning method described above for a set of pre-spawn and post-spawn images for each fish. In 2015, I scanned 14 adult female Humpback Chub over a 3 month period. Fish were held in an outdoor 405-m^2 pond and were moved to a concrete outdoor raceway after I scanned them on February 26. I scanned these fish again on March 19, and on April 22 and 23 when hatchery specialists were spawning them. During the April 22-23 scanning, I used the same procedure from 2014 to obtain pre-spawn and post-spawn images.

Analysis of Ultrasonic Images

All images and clips were downloaded to a computer and evaluated using the software package ImageJ®. A fish was considered female if it had eggs, or indeterminate if eggs could not be identified.

I used ImageJ® to develop an egg brightness index to evaluate egg maturity and allow comparisons among samples from all field sites. I used a standardized reference point in the still image saved during the first half of the second full ultrasound scan. In images, I consistently found the white triangle created from ovary lining tissue at the anterior portion of the ovary. Immediately inside of the ovary lining triangle, where the egg mass was located, I selected an oval area between 39 x 29 pixels and 72 x 99 pixels (Figure 1.2). The pixel size of the selection depended on the degree of ovarian development of the fish. Next, I cropped the area within the selection circle. I then opened the Color Threshold dialog box and recorded the median pixel brightness for the selection.

I used ImageJ® to develop an egg mass index that would allow comparisons of the size of the egg mass detected in female Humpback Chub among field sites. I evaluated only females who were within a brightness range that indicated they could be potentially ripe. Both still images from the second full ultrasound scan were used to estimate egg mass. I used the polygon selection feature in ImageJ® to select the entire ovary visible in each image, and I recorded both perimeter and area of the selection for Common Carp. I used Microsoft Excel® to evaluate the relationships between the area estimated with the use of ImageJ® (pixels) and area estimated with the use of the ultrasound machine (cm^2), between the egg mass area estimated using ImageJ® (pixels) and ovary weight (g), between the ImageJ® estimation (pixels) and ovary area (cm^2), and between ImageJ® estimation (pixels) and gonadosomatic index values. Gonadosomatic index values were calculated following the methods of Strange 1996.

Testing Procedure in Field

Once I had developed these ultrasonic scanning procedures in the laboratory, I tested them during fish sampling trips in the Grand Canyon. I measured battery life, number of equipment malfunctions, total number of fish scanned, number of mortalities during scanning, and ease of machine transport. I measured battery life by noting the number of fish scanned before each battery lost power. I determined ease of transport by noting if the machine could be hiked into and out of Grand Canyon and transported by boat on river trips with no malfunctions.

Results

I used ultrasound to evaluate a total of 58 Common Carp. These fish ranged from 230-424 mm TL and included a mix of both ripe and non-ripe male and female fish. I correctly sexed 78% of Common Carp with ultrasound. Out of the 13 incorrectly sexed fish, two were females misidentified as non-females and four were ripe males misidentified as females.

All Humpback Chub scanned at SNARRC in April 2014 were correctly identified as females with eggs. I scanned the same 14 Humpback Chub in February, March, and April 2015 and correctly identified all fish as females with eggs. In order to confirm identification, eggs were stripped from these fish in April 2014 and April 2015.

The brightness index helped to evaluate egg maturity of Common Carp (Figure 1.3) and Humpback Chub (Figure 1.4). The three ripe female Common Carp had brightness values of 36, 43, and 47, and values for female Humpback Chub that released eggs consistently fell within the 32-44 range.

I found a relationship between the area (pixels) estimated with the use of ImageJ® and area estimated with the use of the ultrasound machine (cm^2) for Common Carp (Figure 1.5) I found no relationship between the egg mass area (pixels) estimated using ImageJ® and ovary weight (g) (Figure1.6), no relationship between egg mass area (pixels) estimated using ImageJ® estimation and ovary area (cm^2) (Figure 1.7), and no relationship between ImageJ® area estimation (pixels) and gonadosomatic index values (Figure 1.8).

Over three field seasons and a total of 162 d in the field, I successfully scanned 751 Humpback Chub in Grand Canyon. No fish were killed or injured during the procedure. On average, fish were handled < 60 s before release.

Two batteries, one in use and one spare, were sufficient to keep the ultrasound machine powered in the field. However, I had to purchase two replacement batteries over the course of the study. I was able to scan 50-60 fish with one battery before the ultrasound machine lost power. During a 10-d trip, I had to recharge one battery. During an 18-d trip, I had to recharge each battery once. Gas powered generators were used to recharge batteries during trips. If generators were not available, then I would have used a total of three fully charged batteries for a 10-d trip and four fully charged batteries for an 18-d trip.

The ultrasound machine was hiked into and out of field sites for seven sampling trips. The custom Pelican case was too bulky for backpacking, so the machine was wrapped in bubble wrap and stored in a dry bag inside of a backpack. The machine was stored in its case and flown by helicopter into and out of field sites for three sampling

trips either by internal load or in a sling load. For the remaining trips by boat, the machine was stored in its protective case inside of a waterproof box.

In April 2014, the ultrasound machine stopped working due to water damage to the motherboard. The cost of repair was US $3,000, and I used an identical machine loaned from Sonosite® during repairs. The water damage was caused by fish splashing water out of buckets they were held in before being processed. For the 2014 and 2015 main stem Colorado River sampling trips I changed where I kept the scanner in the work boats. Instead of keeping the machine beside me on the main seating bench, I kept the machine behind me so my body would intercept water from splashing fish. The ultrasound machine did not need any more repairs for the duration of this project.

Discussion

The technique I developed worked well for ultrasonically scanning Humpback Chub in Grand Canyon. It took < 1 min to process fish, and I was able to successfully scan 751 fish without causing significant delays in processing fish for other studies. No fish perished during or immediately after the scanning procedure. The machine performed well in air temperatures >40 °C and withstood exposure to sand. I was able to scan approximately 60 fish per battery. I prolonged battery life by scanning multiple fish at once instead of turning the machine off between individual fish scans.

I purchased the refurbished unit used in this study for US$15,490, the transducer for $6,000, and $395 per battery. Most ultrasound units used for fisheries research are less expensive and range from $2,000-$9,000; however, these units are either not portable or not durable. Units used in hospitals can cost more than $100,000 (Novelo and Tiersch

2012). The 13-6 MHz linear transducer I used is comparable to most other studies in which transducer frequency varied from 3.5 to 15 MHz (Novelo and Tiersch 2012).

I scanned live fish and did not physically restrain them (except for holding them), anesthetize them, or use water or gel as a medium. About 70% of fish ultrasound studies have reported using live fish, and 78% of studies either physically or chemically restrained fish in some way (Novelo and Tiersch 2012). Researchers submerged fish in order to use water as a medium in 63% of previous studies (Novelo and Tiersch 2012).

I scanned the left lateral side of each fish below the lateral line from the operculum to the anal vent. Flatfishes and Pacific herring were the only reported species scanned laterally, and they were scanned either anterior to the dorsal fin or around the gut region (Novelo and Tiersch 2012). The amount of time researchers spent scanning fish varied from less than 30 s to less than 10 min (Novelo and Tiersch 2012). I spent less than 60 s scanning each fish which is comparable to other studies that have used live fish.

I used ultrasonic images to correctly identify the sex of a large percentage of Common Carp (78%; $N = 58$). Misidentification can happen when gonads are too small or underdeveloped (Petochi et al. 2011) or when intersex characteristics are present in male and female fish (Martin-Robichaud and Rommens 2001). Accurate sex identification generally increases for large adult fish with mature gonads (Novelo and Tiersch 2012). Researchers have been able to accurately sex 95% of adult Striped Bass *Morone saxatilis*, 86% of adult Pallid Sturgeons *Scaphirhynchus albus*, 86% of mature and immature Shovelnose Sturgeon *Scaphirhynchus platorynchus*, and 78% of adult Baltic Cod *Gadus morhua* (Novelo and Tiersch 2012). However, for some species like Red Hind *Epinephelus guttatus*, gonads can be identified only immediately prior to

spawning, and for species like Neosho Madtom *Noturus placidus*, male gonads cannot be identified (Novelo and Tiersch 2012).

Due to time constraints and low screen visibility in the field, I was not able to evaluate live ultrasound images for egg maturity. Furthermore, when I saved images of egg masses and downloaded them to a computer, the images lost much of their resolution. I was unable to evaluate egg maturity directly, so I developed and used the brightness index to gauge egg maturity. The brightness index helped me quantify different stages of egg development I saw in the field; however, this index needs to be used in addition to visual evaluation of ultrasonic images for accurate evaluation of egg maturity. The most common error I experienced with the brightness index was unintentionally including non-egg tissue, especially some of the bright lining surrounding the egg mass, in the subsample of eggs I evaluated. The inclusion of this tissue caused the brightness index value to be much higher that it was without the tissue included in the subsample of eggs.

I was not able to use ultrasound images to estimate egg mass because I only saw a small portion of one ovary from each fish. Future studies could test if ovary size can be accurately estimated by collecting multiple jpeg images or by collecting images of multiple cross sections along the left lateral side of fish.

Although I was able to successfully scan endangered fish in a remote field environment, there were several drawbacks that might not make ultrasound applicable to all situations where similar measurements are needed. The initial investment for equipment was high because we needed a rugged machine that could withstand field conditions and because we needed multiple batteries and a way to charge them. For others, this investment will depend on specific project objectives. Because the machine

we used was not waterproof, we had to have the motherboard in the machine replaced due to water damage. Biologists and researchers who plan to use this machine should be prepared to protect it from any exposure to water to avoid costly repairs.

Ultrasonic imaging provides a powerful tool that fisheries biologists, managers, and researchers can use to learn more about the reproductive cycle of fishes, even in remote field locations. This method minimizes stress for fish, which is important when handling threatened or endangered species. Biologists and researchers will have to work with female fish of the target species to refine a brightness index to identify potentially ripe individuals. My method, with little alteration, could probably be used to ultrasonically scan cyprinid fishes above 180 mm TL. However, modifications of this method would likely be required when scanning smaller fish or when scanning fish species with different body shapes like ictalurids (Bryan et al. 2005; Guitreau et al. 2012).

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Figures

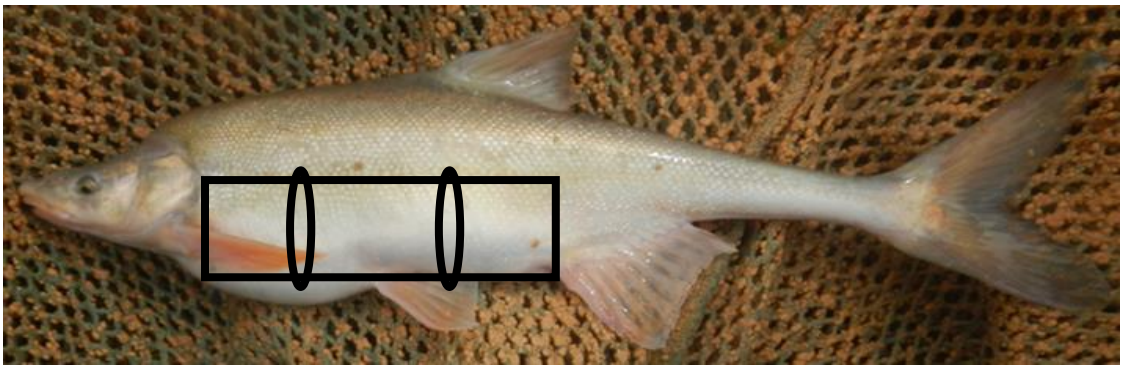

Figure 1.1—Location of ultrasound scans conducted on fish. The left lateral side of each fish was ultrasonically scanned twice. The inner area of the rectangle denotes the area of the first scan that collected a 10-15 s video clip. The area within the ellipses indicates the two locations captured with a jpeg image during the second scan.

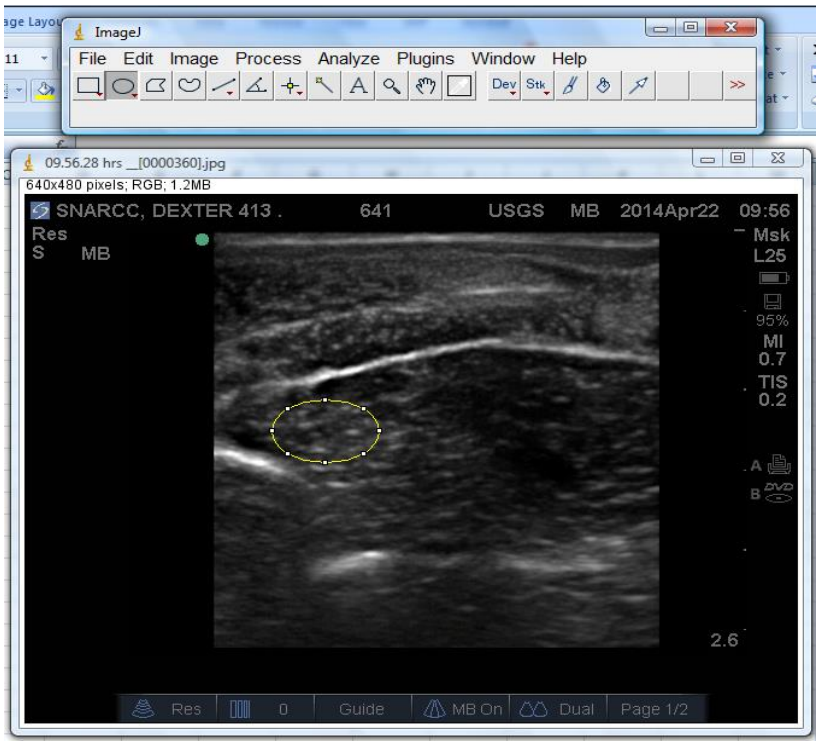

Figure 1.2—Screen capture of an ultrasound image showing an area (oval) immediately inside of the triangle created by the ovary lining at the anterior portion of the ovary. The area within the oval was selected and evaluated for brightness using ImageJ®.

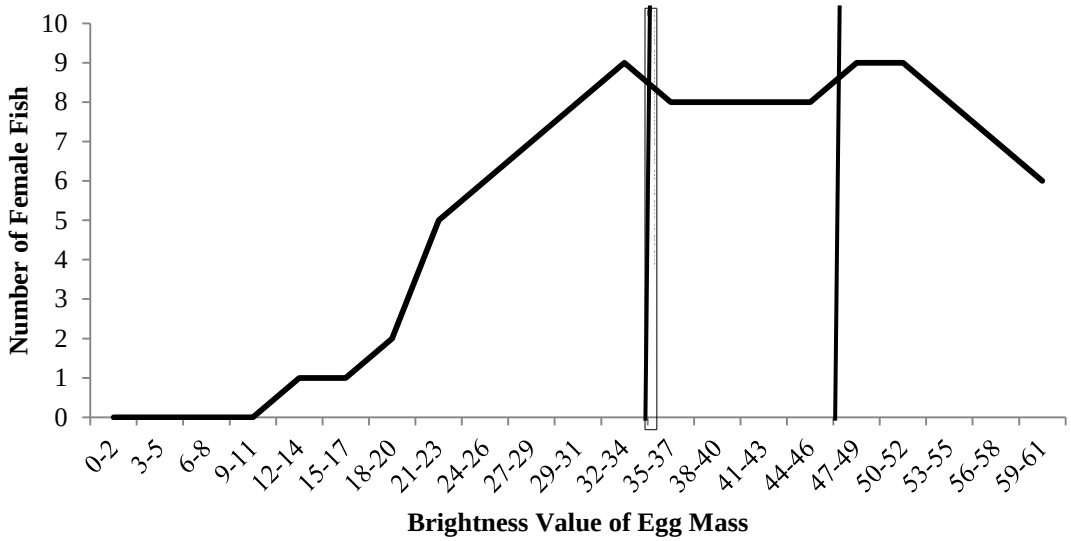

Figure 1.3—Brightness values for Common Carp varied between females due to differences in egg maturity. The vertical black lines denote the range of values (36-47) that encompassed ripe females.

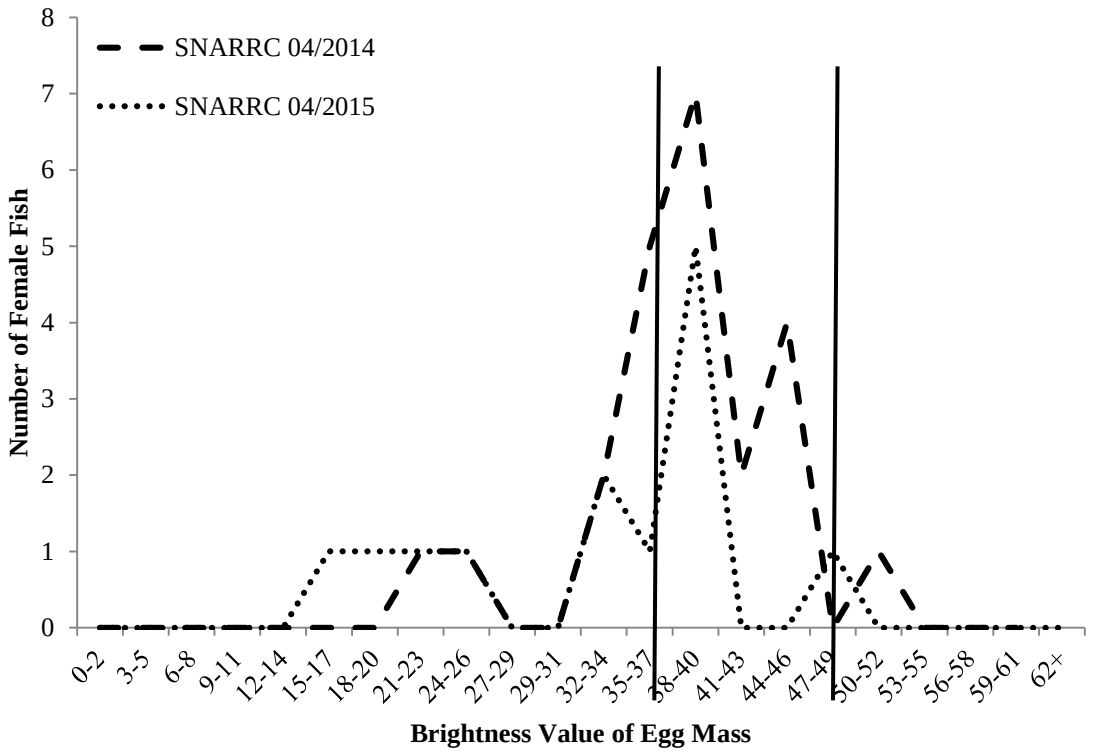

Figure 1.4—Brightness values of internal egg mass in female Humpback Chub held captive at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) and ultrasonically scanned April 2014 and 2015 varied. The vertical black lines denote the range of values (32-44) for ripe female Humpback Chub.

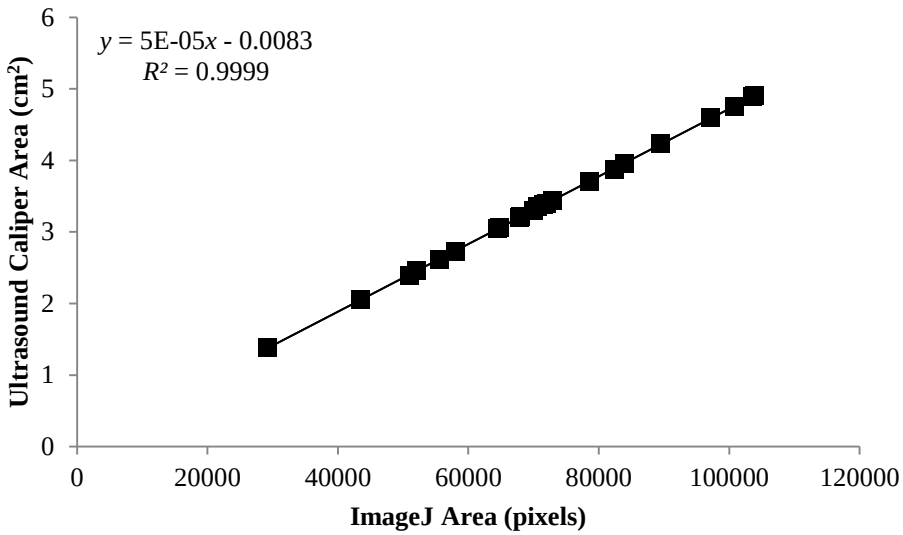

Figure 1.5—Relationship between area estimated in pixels with the use of ImageJ® and area estimated in cm^2 directly from the ultrasound machine for Common Carp. This relationship is specific for the settings described in this study which include a scanning depth of 2.6 cm.

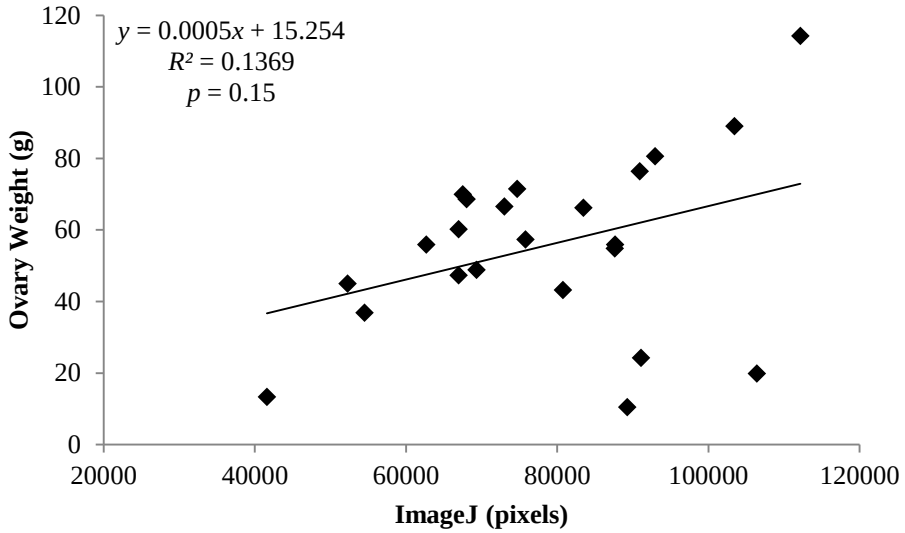

Figure 1.6—Relationship between area estimated with the use of ImageJ® and actual weight of the ovary for Common Carp.

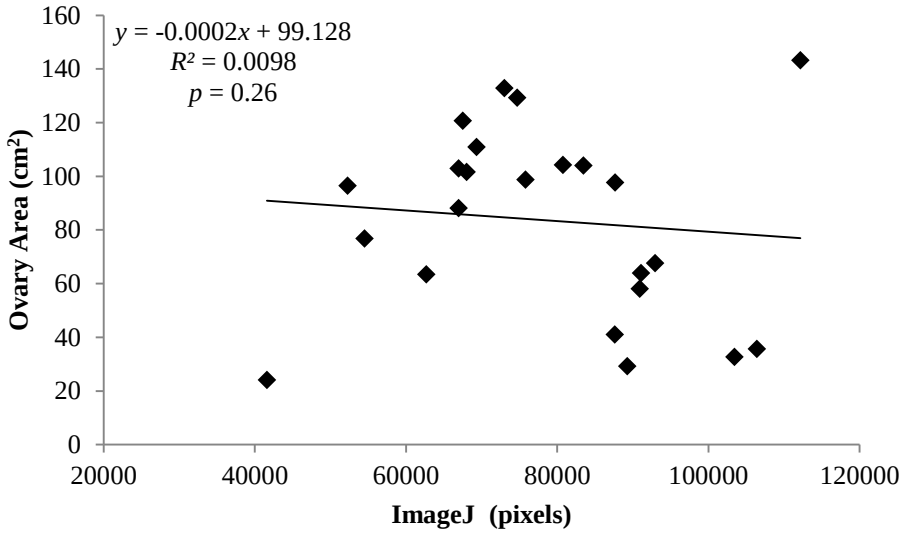

Figure 1.7—Relationship between area estimated with the use of ImageJ® and actual area of the ovary for Common Carp.

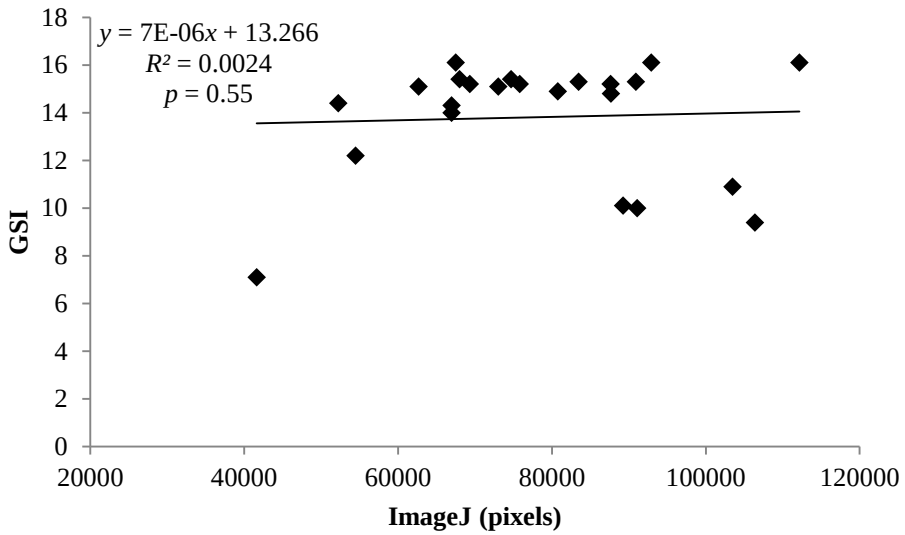

Figure 1.8—Relationship between area estimated with the use of ImageJ® and the gonadosomatic index (GSI) value for female Common Carp. GSI values were calculated as a proportion of the total body mass. $GSI = \frac{ovary\ weight}{total\ body\ weight} * 100$

Use of Ultrasonic Imaging to Evaluate Egg Maturation of Humpback Chub *Gila cypha* in Grand Canyon

Abstract

Humpback Chub *Gila cypha* are endangered cyprinids endemic to the Colorado River drainage and are adapted to live in fast currents of warm, turbid water. Although nine known aggregations of Humpback Chub currently exist in the main-stem Colorado River in the Grand Canyon, little is known about their reproduction. I hypothesized that recruitment of juvenile Humpback Chub in Grand Canyon is limited because hypolimnetic water releases from Glen Canyon Dam create water temperature conditions that are too cold for female Humpback Chub to develop mature eggs for spawning. My goal was to use ultrasonic imaging, a non-lethal method, to evaluate reproductive condition of female Humpback Chub in Grand Canyon to determine if water temperature limits egg development in female Humpback Chub. I documented egg development in female fish from the main-stem Colorado River, Little Colorado River, Havasu Creek, and Shinumo Creek. Egg development in Humpback Chub varies by location and time of year. Potentially ripe (stage 3) female fish were found at all sample locations and dates except at Shinumo Creek in 2013 and 2014. Potentially ripe females were also detected in every main-stem aggregation except for Pumpkin Springs and in two locations outside of established aggregations. My findings indicate that female Humpback Chub are able to produce eggs throughout the main-stem Colorado River and that internal egg development and egg production likely do not limit Humpback Chub recruitment in Grand Canyon. However, female fish may never experience the environmental triggers they need to spawn. If these fish do spawn, their eggs may not be able to hatch and

develop at the cold water temperatures currently present within much of the Colorado River in Grand Canyon.

Introduction

Humpback Chub are endangered fish endemic to the Colorado River Basin (U.S. Fish and Wildlife Service 2002). Historically, their range was thought to extend throughout the entire drainage; however, initial misidentification of many *Gila* species as Bonytail Chub *Gila elegans* and lack of fish collections may not support the hypothesis of a wide distribution (Miller 1946; Valdez and Clemmer 1982). Current understanding of historic distributions of Humpback Chub suggest they inhabited about 756 km of the Colorado River and its tributaries, starting at the Yampa River near its confluence with the Green River and extending downriver to the Grand Canyon (U.S. Fish and Wildlife Service 2002).

Currently, there are six wild, self-sustaining populations of Humpback Chub (U.S. Fish and Wildlife Service 2002). Five of these populations are located in the Upper Colorado River Basin, and the largest population is located in the Lower Colorado River Basin below Glen Canyon Dam in the Grand Canyon (U.S. Fish and Wildlife Service 2002).

Biologists have identified nine aggregations, or established sub-populations, of Humpback Chub in the main-stem Colorado River in Grand Canyon. Aggregations are defined as a group of fish that does not exchange individuals with another aggregation (Valdez and Ryel 1995). These aggregations have been named 30 Mile, Little Colorado River Inflow, Lava Chuar to Hance, Bright Angel Creek, Shinumo Creek, Stephen Aisle, Middle Granite Gorge, Havasu Creek, and Pumpkin Springs (Figure 2.1).

The largest of these aggregations is located at the confluence of the Little Colorado and Colorado Rivers where up to 95% of the entire population can be found (Valdez and Ryel 1995; Douglas and Marsh 1996). Individuals of this Little Colorado River Inflow aggregation are the only fish that biologists know consistently reproduce below Glen Canyon Dam (Coggins et al. 2006). They use the warm waters of the Little Colorado River to spawn and rear young (Gorman and Stone 1999). Adult fish typically spawn every spring from April through May in the Little Colorado River when water temperatures range from 16 to 22°C (Gorman and Stone 1999).

Humpback Chub can also be found in Havasu Creek and Shinumo Creek, which are tributary streams to the Colorado River. Both streams feature natural barriers near their confluences with the main-stem Colorado River. As part of restoration efforts to establish a second spawning population, the National Park Service began translocating juvenile Humpback Chub from the Little Colorado River into these streams in 2009 (Healy et al. 2013). Biologists found ripe male fish in Havasu Creek in May 2012 and both ripe male and female fish in May 2013 (Healy et al. 2013). In addition, untagged juvenile Humpback Chub were detected during the May 2013 sampling trip (Healy et al. 2013). No ripe individuals or untagged juvenile fish have been caught in Shinumo Creek (Healy et al. 2013). Spurgeon et al. (2015) found that due to high emigration rates, Shinumo Creek may only provide a suitable grow-out location for juvenile Humpback Chub before they disperse into the main-stem Colorado River.

Humpback Chub were listed as endangered under the Endangered Species Act in 1973 (U.S. Fish and Wildlife Service 2002). This species faces many threats including parasitism, hybridization with other members of the *Gila* genus, pollution, predation by

and competition with nonnative species, loss of habitat, and alteration of stream flow (Valdez and Clemmer 1982; U.S. Fish and Wildlife Service 2002).

Nineteen different parasites have been documented in Grand Canyon and its tributaries (Linder et al. 2012) and may also jeopardize the survival of Humpback Chub in this area. Three parasites—including Asian tapeworm *Bothriocephalus acheilognathi*, anchor worm *Lernaea cyprinacea*, and trematodes *Ornithodiplostomum* sp.—in particular affect Humpback Chub, especially resident fish in the Little Colorado River (Choudhury et al. 2004; Hoffnagle et al. 2006; Linder et al. 2012). Infected fish may exhibit poor body condition and can perish (Hoffnagle et al. 2006). The cold waters of the main-stem Colorado River are believed to inhibit the life cycle of these parasites and provide an important refuge for subadult and adult fish (Hoffnagle et al. 2006).

Another threat to the persistence of Humpback Chub is hybridization. Humpback Chub are able to hybridize with Bonytail and Roundtail Chub *Gila robusta* (U.S. Fish and Wildlife Service 2002). Hybridization can result in the loss of genetic diversity and further stress threatened populations of fish (U.S. Fish and Wildlife Service 2002).

Humpback Chub in Grand Canyon are less threatened by pollution than some populations located in the Upper Colorado River Basin; however, it is possible for pollutants to be washed down the Little Colorado River from Cameron, AZ (U.S. Fish and Wildlife Service 2002). Trucks carrying hazardous material across bridges could overturn and spill pollutants into the Little Colorado River approximately 65 km upstream of its confluence with the Colorado River (U.S. Fish and Wildlife Service 2002).

The presence of non-native fishes can also threaten populations of Humpback Chub. A myriad of non-native fishes have been introduced to the Colorado River, with the potential to negatively interact with native fishes (Gloss and Coggins 2005). Brown Trout *Salmo trutta*, Rainbow Trout *Oncorhynchus mykiss*, Channel Catfish *Ictalurus punctatus*, and Black Bullhead *Ameiurus melas* are the main species that threaten Humpback Chub in Grand Canyon (U.S. Fish and Wildlife Service 2002). In the Little Colorado River, Channel Catfish, Black Bullhead and Rainbow Trout are known to consume Humpback Chub (Marsh and Douglas 1997). In the main-stem Colorado River, Brown Trout, Rainbow Trout, and Channel Catfish prey upon Humpback Chub (Valdez and Ryel 1997, cited by U.S. Fish and Wildlife Service 2002; Yard et al. 2011). Common Carp *Cyprinus carpio* may eat chub eggs, and Fathead Minnow *Pimephales promelas*, Plains Killifish *Fundulus zebrinus*, and Red Shiner *Cyprinella lutrensis*, are known larvivores that consume early life stages of Humpback Chub. Rainbow Trout may also compete with Humpback Chub for limited food resources and rearing habitats (Valdez and Ryel 1997, cited by U.S. Fish and Wildlife Service 2002; Gloss and Coggins 2005).

The closure of Glen Canyon Dam in 1963 drastically altered the flow, turbidity, and temperature of the Colorado River through Grand Canyon. Prior to its closure, seasonal floods and variable flows were common, especially during monsoon season (Topping et al. 2003). Dam operations have removed natural high flood flows and increased base flows. Load following, or adjusting power output based on changing electricity demand, for hydroelectric generation has also caused extreme daily fluctuations in flow (Topping et al. 2003).

Water released from Glen Canyon Dam is now clear and cold. Sediment is still washed into the main-stem Colorado River through its tributaries; however, duration of turbidity is greatly reduced when compared to pre-dam conditions (Topping et al. 2000). Clear water conditions favor sight predators like Rainbow Trout and Brown Trout (Yard et al. 2011). Laboratory studies show that relatively low levels of turbidity reduce Rainbow Trout predation on juvenile Humpback Chub (Ward et al. 2016)

The average temperature of water released from Glen Canyon Dam is 9°C and fluctuations between 7°C and 12°C occur throughout the year with warmest temperatures typically recorded in late fall (Vernieu et al. 2005). Before the dam's closure, water temperatures averaged 14°C and fluctuations from 0°C to 27°C occurred with the warmest temperatures recorded in summer (Vernieu et al. 2005).

Cold water temperatures slow Humpback Chub growth, decrease larval survival (Clarkson and Childs 2000), and increase predation vulnerability of juvenile fish to rainbow trout (Ward and Morton-Starner 2015). Cold water temperatures also lengthen incubation period and reduce egg survival (Hamman 1982). Hamman (1982) found that 88% of Humpback Chub eggs will die in water temperatures of 12-13°C, which are similar to conditions in many parts of the main-stem Colorado River. He also found that eggs that do survive in 12-13°C water take over 200 hours longer to begin hatching and that 85% of the fry that hatch from those eggs perish (Hamman 1982).

Although spawning has been well documented in the Little Colorado River, little is known about reproduction of Humpback Chub in the main-stem Colorado River. After Glen Canyon Dam began its operations, reproduction in the main-stem has rarely been detected (Van Haverbeke et al. 2013). Since the dam's closure, the only possible

detection of reproduction in the main-stem has been near the 30 Mile aggregation where extensive spring systems create warm water refugia for fish (Valdez and Masslich 1999; Andersen et al. 2010). However, these fish could be upstream migrants from the Little Colorado River (Paukert et al. 2006). Based on the findings from these studies, water temperatures in the main-stem Colorado River below Glen Canyon Dam may be too cold to support egg and larval fish survival. Kaeding and Zimmerman (1983) speculate that females can normally develop eggs in the cold main-stem Colorado River, but mature fish must migrate to the Little Colorado River in order to successfully spawn. My study was designed to evaluate these hypotheses.

Fish biologists must understand reproductive development of a species in order to evaluate the dynamics of its populations (Bryan et al. 2007). Traditional methods used by biologists to determine sex and gonad maturity, like dissection, are often invasive (Blythe et al.1994) and impractical when working with endangered species (Bryan et al. 2007). The development and use of non-invasive and non-lethal methods like ultrasonic imaging are needed so biologists can collect reproductive information needed for management of endangered fishes.

Goal and Objectives

My goal was to use newly developed ultrasonic imaging techniques (Brizendine, Chapter 1, this thesis) to identify egg development in Humpback Chub in the Grand Canyon. My objectives were to:

1) Evaluate reproductive condition of Humpback Chub in the main-stem Colorado River, Little Colorado River, Shinumo Creek, and Havasu Creek.
2) Document locations of female Humpback Chub with eggs in the Grand Canyon.

Methods

Study Area

I sampled Humpback Chub in the main-stem Colorado River below Glen Canyon Dam between Lee's Ferry (river km 0.0) and Pearce Ferry (river km 452.2) (Figure 1). These locations include areas where aggregations have been identified and areas between these aggregations (Valdez and Ryel 1995). Additionally, I sampled the lower Little Colorado River (13.6-9.6 river km) where spawning activity has been documented (Gorman and Stone 1999). Finally, I sampled Havasu Creek below Beaver Falls (9.7-1.4 river km) and the lower reach of Shinumo Creek (6.1-0.4 river km).

Because the Grand Canyon is remote, access to field sites was difficult and limited. I sampled at various times of year, from April to October, to encompass probable spawning periods. The main-stem Colorado River was sampled July 20 - August 5 and September 4 - 22, 2013; July 18 - August 4 and September 4 - 24, 2014; and August 31 - September 18, 2015. I sampled the Little Colorado River April 20 – 24 and July 4 - 10, 2013; and April 16 - 20, May 15 - 18, and October 21 - 26, 2014. I sampled Shinumo Creek June 12 - 16, 2013; and June 11 - 18, 2014. Finally, I sampled Havasu Creek May 2 - 11, 2013; and May 5 - 14, 2014 (Table 2.1).

I used hoop nets baited with AquaMax® 600 sport fish pellets (Land O'Lakes, Inc., Purina Animal Nutrition LLC, Gray Summit, Missouri) and trammel nets to capture Humpback Chub in the main-stem Colorado River. I used unbaited hoop nets to capture fish in the Little Colorado River, Havasu Creek, and Shinumo Creek. All hoop nets and trammel net dimensions and set times were based on the standardized methods protocol for Grand Canyon fisheries research (Persons et al. 2013). Total length, fork length, sex,

sexual characteristics (indications of color or tuberculation), and a unique Passive Integrated Transponder (PIT) tag number were recorded for each fish per the Persons et al. protocol (2013). If a fish did not have a PIT tag, one was inserted. Fish were then scanned with ultrasound before release following the protocol of Brizendine (Chapter 1, this thesis). I linked unique PIT tag numbers to ultrasonic images to distinguish individual fish.

Analysis

I sexed all ultrasound video clips and images of fish and classified them as non-females or as females with eggs. The identified females were processed with ImageJ® following the protocol of Brizendine (Chapter 1, this thesis).

River range (km) for main-stem Colorado River aggregations was defined by Valdez and Ryel (1995). For this study, I extended some aggregation ranges downstream by several km to increase sample sizes per aggregation (Tables 2.2, 2.3).

I tested four different relationships. First, I tested the relationship between the proportion of potentially ripe (stage 3) females for different aggregations. I then evaluated the relationships between the proportion of potentially ripe females and month sampled and year sampled. Finally, I evaluated the relationship between the proportion of ripe females and accumulated degree days that individual female fish experienced.

All statistical analyses were performed by using the software package R 3.2.4 (R Core Team 2016) and the lme4, arm, and ggplot2 packages (Bates et al. 2015; Gelman and Su 2015; Wickham 2015). I used generalized linear regression for all analyses. I used

the proportion of potentially ripe females as the dependent variable, and depending on the analysis, aggregation, month, year, and degree days were used as independent variables. I used Akaike's Information Criterion (AIC) to compare models (Burnham and Anderson 2002).

For the degree days analysis, I first calculated degree days by using the averaging method (Herms 2004). I used the following formula:

$$Degree\ Days = \left(\frac{maximum\ daily\ temperature + minimum\ daily\ temperature}{2}\right) - \ base\ temperature\ (°C)$$

I used 14°C as the base temperature because that is the lowest temperature at which Humpback Chub have spawned (Kaeding et al. 1990).

I assigned a temperature gauge to each aggregation (Table 2.4) and downloaded data from the Grand Canyon Monitoring and Research Center's GCDAMP website (http://www.gcmrc.gov/discharge_qw_sediment/stations/GCDAMP). I used Microsoft Excel® to condense 15 min interval temperatures into daily maximum and minimum values. I calculated degree days for each calendar day. I added degree days accumulated from January 1 to the day prior to the beginning of a sampling trip. That value was associated with each main-stem aggregation from an individual sampling trip. I calculated degree days for each aggregation for every main-stem Colorado River sampling trip (Table 2.4).

Results

I identified four stages of egg development (Figure 2.2). Stage 0 fish had no eggs present and were classified as non-females. Stage 1 fish had a bright white ovarian lining in addition to a low density of eggs. Stage 2 fish had the ovarian lining and a higher

density of small eggs present. Stage 3 fish had the ovarian lining present and a large mass of bigger eggs. Each stage of egg development had a specific range of brightness values associated with it, as measured with ImageJ®. Stage 1 fish brightness ranged from 0-31, stage 2 fish ranged from 45-100, and stage 3 ranged from 32-44. The stage 3 fish were considered potentially ripe females based on laboratory evaluations (Brizendine, Chapter 1, this thesis).

The highest proportion of potentially ripe (stage 3) female Humpback Chub in the main-stem Colorado River was caught during the July/August 2013 sampling trip (Table 2.3). Potentially ripe females were also found outside of established aggregations near the confluence of the main-stem Colorado River and Deer Creek and near the confluence of the Colorado River and Kanab Creek. No potentially ripe fish were caught at the Pumpkin Springs aggregation.

The highest proportion of potentially ripe females was caught in the Bright Angel Creek aggregation (Table 2.5). All aggregations, except for Pumpkin Springs, had proportions of potentially ripe female fish that were significantly different than zero (Table 2.6). My model predicted that 50-60% of fish caught and ultrasonically scanned from each aggregation, except for Pumpkin Springs, should be potentially ripe female Humpback Chub (Figure 2.3).

A significantly higher proportion of potentially ripe females were caught during July/August than in September in the main-stem Colorado River (Tables 2.6, 2.7). The proportion of potentially ripe female Humpback Chub detected at the 30 Mile, Havasu Creek, LCR + Lava Chuar, and Stephen Isle aggregations was significantly different between July/August and September (Table 2.6). This model also predicted that roughly

50-60% of the total catch from these aggregations should be potentially ripe female fish (Figure 2.4).

The highest proportion of potentially ripe females was caught in 2015 (Table 2.8). The year sampled significantly affected the proportion of potentially ripe females detected in every aggregation except for Pumpkin Springs (Table 2.6). My model predicted that the highest proportions of potentially ripe females for each aggregation occurred in 2013 (Figure 2.5).

Fish located in the Pumpkin Springs aggregation experienced the most accumulated degree days (Table 2.4). The greatest number of accumulated degree days for each aggregation occurred during the September 2014 sampling trip (Table 2.4). I found an inverse relationship between degree days and proportion of ripe females (Tables 2.6, 2.8). My model predicted that the highest proportions of potentially ripe females were found in aggregations with the lowest number of degree days (Figure 2.6).

The highest proportion of stage 2 female Humpback Chub in the main-stem Colorado River was caught during the September 2015 sampling trip (Table 2.2). These moderately developed females were also found outside of established aggregations near the confluence of the main-stem Colorado River and Kanab Creek and in the main-stem Colorado River near Parashant.

Both stage 2 and stage 3 female Humpback Chub were found in all samples I collected from the Little Colorado River (Table 2.1). The highest proportion of stage 2 females was found in the Little Colorado River during the October 2014 sampling trip. Additionally, 15% of the fish scanned during this trip were stage 3 or potentially ripe

females. The highest proportion of potentially ripe females was found during the April 2014 sampling trip.

Both stage 2 and potentially ripe (stage 3) female Humpback Chub were found in the May 2013 and May 2014 samples from Havasu Creek (Table 2.1). No potentially ripe females were detected in Shinumo Creek during the June 2013 and June 2014 sampling trips, and no stage 2 females were detected during the June 2013 sampling trip (Table 2.1). However, six stage 2 females were found in the June 2014 sample. Both stage 2 and stage 3 or potentially ripe females were detected in the June 2013 and June 2014 main-stem samples near the confluence of the Colorado River and Shinumo Creek.

Discussion

Egg development in Humpback Chub varies by location and time of year. I found female fish with eggs at all sample locations during all sample dates. Moderately developed females (stage 2) were found at all sample locations and dates except in Shinumo Creek in 2014. Stage 2 females were also detected at all main-stem aggregations, and in two locations outside of these aggregations (Table 2.2). Potentially ripe (stage 3) female fish were found at all sample locations and dates except at Shinumo Creek in June 2013 and June 2014. Potentially ripe females were also detected in every main-stem aggregation except for Pumpkin Springs and in two locations outside of established aggregations (Table 2.3). It is likely that females from the Pumpkin Springs aggregation can ripen eggs due to warm water temperatures in that area. However, I scanned few adults from that aggregation because Humpback Chub over 180 mm TL are rarely caught in that area of the main-stem Colorado River.

An inverse relationship between accumulated degree days and the proportion of potentially ripe females is counterintuitive. For broodstock fish in aquaculture, a minimum number of degree days is needed for fish to mature gametes. For example, female Common Carp *Cyprinus carpio* need a minimum of 1,200 degree days before they can produce mature eggs (Bromage and Roberts 1995). The temperature data I used reflects only cold main-stem Colorado River water temperatures and does not include any warm springs or other warm-water inputs that may occur within the river channel. The most known warm springs were located in upstream locations near the 30 Mile aggregation where main-stem water temperatures are lowest. This problem combined with low sample sizes from some aggregations, especially Pumpkin Springs, likely explains this counterintuitive relationship.

During the Havasu 2013 and 2014 and Shinumo 2014 sampling trips, fish were scanned at depths other than the standard 2.6 cm (Table 2.1). During these trips, the ultrasound machine was hiked into and out of Grand Canyon and hiked around to hoop net sites. While the machine was being transported, the power button and scanning depth buttons were pressed. I did not double check the depth before I started scanning again. Even though I was scanning at different depths (2.2 cm and 3.1 cm), I did not realize my error until I downloaded the videos and images. Because National Park Service biologists stock subadult Humpback Chub in these tributaries, average fish size was smaller than other sample locations. In these smaller fish, it was difficult to discern egg development with a scan depth of 2.6 cm. The scan depth of 2.2 cm showed eggs more clearly. Therefore, I recommend not ultrasonically scanning Humpback Chub under 200 mm TL if biologists plan to use the method described by Brizendine (chapter 1, this thesis). If

biologists plan use this method to scan smaller fish, they should use a scan depth of 2.2 cm.

Management implications

My findings indicate that female Humpback Chub are able to produce eggs throughout the main-stem Colorado River and that internal egg development and egg production likely does not limit recruitment. However, I do not know if female fish ever experience the environmental triggers they need to spawn, or if their eggs are viable if they do spawn. Reproductive condition of fish can depend on multiple variables, including temperature, photoperiod, and stream flow (Helfman et al. 2009), and downstream effects of dams are not limited to modified thermal conditions. Unnatural flows can disrupt spawning, especially in fishes that respond to seasonal flow peaks (Helfman 2007). In the Little Colorado River, Humpback Chub spawn in April after late winter floods when water levels return to base flow and water temperature increases to 15°C (Gorman and Stone 1999). If females can successfully spawn in the main-stem Colorado River, their eggs and larval offspring will likely experience high mortality at current cold river temperatures which may lead to poor recruitment (Hamman 1982; Robinson and Childs 2001).

My findings of potentially ripe female fish in the main-stem Colorado River in June and July are similar to findings from previous studies. Suttkuss and Clemmer (1977) concluded that Humpback Chub in the main-stem Colorado River in Grand Canyon spawn during this time, and Hamman (1982) spawned fish captured from the main-stem in June in captivity when water temperatures were 21-22°C. I also found most of the

potentially ripe (stage 3) female Humpback Chub near areas with known warm springs or warm-water inputs from creeks. Valdez and Ryel (1995) noted that Humpback Chub gathered in areas with warm water refugia after Glen Canyon Dam was built. These small areas surrounding warm springs and other warm water inputs may play an important role in humpback chub reproduction and warrant further investigation. Biologists can now use advanced technologies to rapidly collect thermal images and identify small areas of warm water (Bonar and Petre 2015) which may help identify previously unknown areas that are important for female Humpback Chub egg maturation.

Some of the warmest water years on record since the construction of Glen Canyon Dam have occurred since 2003 (Figures 2.7 and 2.8) due to the combination of regional climate warming trends, drought conditions, and increasing water demands (Smerdon et al. 2007). These conditions may explain why I detected potentially ripe female Humpback Chub. In addition, recent samples of otolith microchemistry of fish indicate that juveniles are living in the main-stem Colorado River (Limburg et al. 2013). If this warming water trend continues and if female fish are able to spawn viable eggs, large numbers of Humpback Chub may begin reproducing and recruiting in the main-stem Colorado River.

Ultrasound is a valuable tool that can be used to monitor Humpback Chub reproductive status in Grand Canyon. In the main-stem Colorado River, additional samples from areas outside of established aggregations and from areas not adjacent to warm water inputs would further define where females are potentially ripe and spawning. Additionally, samples collected during years with cold water temperatures would inform biologists if female fish can develop eggs under varying river conditions or if egg

development in main-stem resident females is occurring because of drought conditions which lead to warmer water releases from Lake Powell. Furthermore, the detection of potentially ripe females in the Little Colorado River during the October 2014 sampling trips may indicate that some Humpback Chub spawn in the fall. More samples from the Little Colorado River over time could allow biologists to evaluate possible fall spawning and to give insight into the prevalence of skip spawning (Yackulic et al. 2014; Pearson et al. 2015).

My findings inform managers where and how many potentially ripe females occurred in the main-stem Colorado River. Managers and biologists may be able to target one area or aggregation in order to develop a second spawning population of Humpback Chub, which is needed for the recovery of this species (U.S. Fish and Wildlife Service 2002).

Future Research

Future research is needed to confirm and further explore the findings in this study. Conditions in the main-stem Colorado River below Glen Canyon Dam may cause female Humpback Chub to experience some form of reproductive dysfunction. Captive fish can display three types of reproductive dysfunction because their environment often lacks natural spawning cues (Zohar and Mylonas 2001). These types of reproductive dysfunction include failed vitellogenesis, failed final oocyte maturation, and failed spawning or egg release (Zohar and Mylonas 2001). The use of a spawning hormone like Ovaprim®, a synthetic hormone that has low mortality rates when used in cyprinid fishes and that does not cause a female fish to abort immature eggs (Hill et al. 2009), in order to

obtain eggs from wild female fish would allow researchers to confirm if eggs within the 32-44 brightness range were viable. Researchers could also use other more invasive methods to obtain eggs like a rigid catheter (Rottmann et al. 1991) or endoscopy (Bryan et al. 2007). Eggs could then be evaluated for viability by attempting to fertilize them or by histological analysis (Petochi et al. 2011). In addition, researchers could take blood samples to test the concentration of proteins like plasma vitellogenin and hormones like estradiol to determine egg maturity (Bangs and Nagler 2014). Finally, researchers could also monitor captive female Humpback Chub monthly with ultrasound over the course of 1-2 years to track changes in egg development.

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Tables

Table 2.1—Sampling dates, locations, and numbers of captured Humpback Chub with eggs detected using ultrasound. Female Humpback Chub with eggs were found in all sampling locations. Stage 2 females had moderate egg development, and stage 3 females were considered potentially ripe. Shinumo Creek (main-stem) refers to fish caught in the main-stem Colorado River above its confluence with Shinumo Creek. The percent of stage 2 and stage 3 females that were identified out of the total fish scanned is in parentheses.

Site	Sample Dates		Total Fish Scanned	Total Identified Females	Total Stage 2 Females	Total Stage 3 Females
	Month/Days	*Year*				
Colorado River	July 20–August 5	2013	67	46	7(*10%*)	29(*43%*)
Colorado River	July 18–August 4	2014	95	48	15(*16%*)	15(*16%*)
Colorado River	September 4-22	2013	73	42	10(*14%*)	13(*18%*)
Colorado River	September 4-24	2014	149	96	51(*34%*)	24(*16%*)
Colorado River	August 31-September 18	2015	93	63	35(*38%*)	12(*13%*)
Little Colorado River	April 20-24	2013	48	37	16(*33%*)	14(*29%*)
Little Colorado River	April 16-20	2014	39	23	13(*33%*)	18(*46%*)
Little Colorado River	May 15-18	2014	44	28	8(*18%*)	7(*16%*)
Little Colorado River	July 4-10	2013	57	37	7(*12%*)	2(*4%*)
Little Colorado River	October 21-26	2014	79	51	34(*43%*)	12(*15%*)
Havasu Creek[1]	May 2-11	2013	32	30	6(*19%*)	10(*31%*)
Havasu Creek[2]	May 5-14	2014	72	52	11(*15%*)	24(*33%*)

Shinumo Creek	June 12-16	2013	11	4	0(*0%*)	0(*0%*)
Shinumo Creek (main-stem)	June 12-16	2013	17	7	5(*29%*)	1(*6%*)
Shinumo Creek[3]	June 11-18	2014	32	12	6(*19%*)	0(*0%*)
Shinumo Creek[3] (main-stem)	June 11-18	2014	13	10	8(*62%*)	1(*8%*)

[1]Fish were ultrasonically scanned at different depths, including 2.6 cm, 1.9 cm, and 3.1 cm.
[2]Fish were ultrasonically scanned at different depths including 2.6 cm and 3.1 cm.
[3]Fish were ultrasonically scanned at different depths, including 2.6 cm and 2.2 cm.

Table 2.2—Stage 2 female Humpback Chub were identified using ultrasound and were found in established aggregations and outside of those aggregations in the main-stem Colorado River. Stage 2 female fish had moderate egg development. Dashes indicate the location was not sampled during that date. LCR is an abbreviation for Little Colorado River. Total S2F is the total number of stage 2 females detected at an aggregation during a sampling period. Total FS is the total number of fish scanned at an aggregation during a sampling period.

Location	**River Range (km)**	**July/August 2013**		**July/August 2014**		**September 2013**		**September 2014**		**September 2015**		**Total S2F**
		Total S2F	*Total FS*	*Total S2F*	*Total FS*	*Total S2F*	*Total FS*	*Total S2F*	*Total FS*	*Total S2F*	*Total FS*	
30 Mile	48-55	2	17	6	44	0	4	11	46	4	15	**23**
LCR + Lava Chuar	92-123	2	9	4	24	1	20	28	73	26	51	**61**
Bright Angel Creek	135-148	0	10	0	0	0	4	0	4	1	2	**1**
Shinumo Creek	174-175	0	2	0	3	0	9	1	2	0	1	**1**
Stephen Isle	185-193	0	0	0	11	0	2	3	4	2	6	**5**
Middle Granite Gorge	203-208	0	0	0	4	5	20	4	9	0	9	**9**
Havasu Creek	251-267	2	10	3	6	3	8	3	10	2	7	**13**
Pumpkin Springs	342-343	0	0	0	0	1	6	1	1	0	3	**2**
Deer Creek	218-220	0	11	-	-	-	-	-	-	-	-	**0**
Kanab Creek	227-232	1	8	-	-	-	-	-	-	-	-	**1**
Parashant	317-320	0	0	2	3	-	-	0	0	0	1	**2**
Total		**7**	67	**15**	95	**10**	73	**51**	149	**35**	93	**118**

Table 2.3—Stage 3 or potentially ripe female Humpback Chub were identified using ultrasound and found in several aggregations in the main-stem Colorado River. Dashes indicate the location was not sampled during that date. LCR is an abbreviation for Little Colorado River. Total S3F is the total number of stage 3 females detected at an aggregation during a sampling period. Total FS is the total number of fish scanned at an aggregation during a sampling period.

Location	**River Range (km)**	**July/August 2013**		**July/August 2014**		**September 2013**		**September 2014**		**September 2015**		**Total S3F**
		Total S3F	*Total FS*	*Total S3F*	*Total FS*	*Total S3F*	*Total FS*	*Total S3F*	*Total FS*	*Total S3F*	*Total FS*	
30 Mile	48-55	8	17	10	44	0	4	8	46	2	15	**28**
LCR + Lava Chuar	92-123	2	9	2	24	0	20	12	73	7	51	**23**
Bright Angel Creek	135-148	5	10	0	0	0	4	1	4	0	2	**6**
Shinumo Creek	174-175	0	2	0	3	3	9	0	2	0	1	**3**
Stephen Isle	185-193	0	0	2	11	0	2	0	4	1	6	**3**
Middle Granite Gorge	203-208	0	0	1	4	8	20	2	9	1	9	**12**
Havasu Creek	251-267	5	10	0	6	2	8	1	10	1	7	**9**
Pumpkin Springs	342-343	0	0	0	0	0	6	0	1	0	3	**0**
Deer Creek	218-220	4	11	-	-	-	-	-	-	-	-	**4**
Kanab Creek	227-232	6	8	-	-	-	-	-	-	-	-	**6**
Total		**30**	67	**15**	95	**13**	73	**24**	149	**12**	93	**94**

Table 2.4—A temperature gauge was assigned to each aggregation in order to calculate degree days. Degree days were calculated by adding values from January 1 to the day prior to the beginning of each sampling trip. LCR is an abbreviation for Little Colorado River. Female Common Carp *Cyprinus carpio* need a minimum of 1,200 degree days before they can produce mature eggs (Bromage and Roberts 1995).

Location	**River Range (km)**	**Temperature Gauge**	**Degree Days**				
			July/August 2013	*July/August 2014*	*September 2013*	*September 2014*	*September 2015*
30 Mile	48-55	09383050	0	0	0	2	0
LCR + Lava Chuar	92-123	09383100	0	4	0	31	5
Bright Angel Creek	135-148	09402500	0	38	2	111	39
Shinumo Creek	174-175	09403270	4	100	23	217	78
Stephen Isle	185-193	09403270	4	100	23	217	78
Middle Granite Gorge	203-208	09403270	4	100	23	217	78
Havasu Creek	251-267	09404120	47	161	101	312	150
Pumpkin Springs	342-343	09404220	219	349	353	586	363

Table 2.5—Stage 3 or potentially ripe females were found in all aggregations except Pumpkin Springs. The totals in this table include all fish caught during this study. LCR refers to the Little Colorado River aggregation. The data here were used to conduct the site-only effect generalized linear model analysis.

Location	Total Stage 2 Females	Total Non-Stage 2 Fish Scanned	Total Stage 3 Females	Total Non-Stage 3 Fish Scanned	Total Fish Scanned
30 Mile	23	103	28	98	126
Bright Angel Creek	1	19	6	14	20
Havasu Creek	13	28	9	32	41
LCR + Lava Chuar	61	116	23	154	177
Middle Granite Gorge	9	33	12	30	42
Pumpkin Springs	2	8	0	10	10
Shinumo Creek	1	16	3	14	17
Stephen Isle	5	18	3	20	23
Total	**115**	**84**	**372**	**341**	**456**

Table 2.6—Results of generalized linear models used to analyze factors related to the proportion of potentially ripe female Humpback Chub in main-stem Colorado River aggregations in Grand Canyon. Significance codes for p-values are as follows: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1. LCR refers to the Little Colorado River aggregation.

Model	R code	Coefficients	Estimate	Standard Error	Z-value	P-value	AIC
Site	CRaggmodel1 <- glm(cbind(ripefemale, nonripefemale)~-1+site, family="binomial", data=CRagg5)	30 Mile	-1.2528	0.2143	-5.846	5.03e-09 ***	42.53
		Bright Angel Creek	-0.8473	0.4880	-1.736	0.082485 .	
		Havasu Creek	-1.2685	0.3773	-3.362	0.000774 ***	
		LCR + Lava Chuar	-1.9015	0.2235	-8.506	< 2e-16 ***	
		Middle Granite Gorge	-0.9163	0.3416	-2.683	0.007305 **	
		Pumpkin Springs	-25.1184	54605.9245	0.000	0.999633	
		Shinumo Creek	-1.5404	0.6362	-2.421	0.015466 *	
		Stephen Isle	-1.8971	0.6191	-3.064	0.002183 **	
Site + Month	CRaggmodel2 <- glm(cbind(ripefemale, nonripefemale)~-1+site+month, family="binomial", data=CRagg6)	30 Mile	-0.9984	0.2462	-4.055	5.01e-05 ***	65.83
		Bright Angel Creek	-0.5960	0.5060	-1.178	0.23887	
		Havasu Creek	-0.9635	0.4063	-2.371	0.01773 *	
		LCR + Lava Chuar	-1.4860	0.3017	-4.926	8.38e-07 ***	
		Middle Granite Gorge	-0.4406	0.4176	-1.055	0.29135	
		Pumpkin Springs	-19.5875	4482.3273	-0.004	0.99651	
		Shinumo Creek	-1.1848	0.6616	-1.791	0.07331	
		Stephen Isle	-1.6461	0.6320	-2.605	0.00919 **	
		September	-0.5309	0.2689	-1.974	0.04836 *	
Site + Year	CRaggmodel3 <- glm(cbind(ripefemale, nonripefemale)~-1+site+year,	30 Mile	-0.6989	0.3112	-2.246	0.0247 *	80.78
		Bright Angel Creek	-0.6554	0.4973	-1.318	0.1875	
		Havasu Creek	-0.9048	0.4032	-2.244	0.0248 *	
		LCR + Lava Chuar	-1.3300	0.3106	-4.281	1.86e-05 ***	

	family="binomial", data=CRagg7)	Middle Granite Gorge	-0.5637	0.3690	-1.528	0.1265	
		Pumpkin Springs	-19.8382	4402.3196	-0.005	0.9964	
		Shinumo Creek	-1.3316	0.6446	-2.066	0.0388 *	
		Stephen Isle	-1.2670	0.6679	-1.897	0.0578 .	
		2014	-0.6643	0.2980	-2.229	0.0258 *	
		2015	-0.8216	0.3898	-2.108	0.0351 *	
Site + Degree Days	CRddmodel1 <- glm(cbind(ripefemale, nonripefemale)~-1+site+DD, family="binomial", data=CRagg4)	30 Mile	-1.248e+00	2.143e-01	-5.822	5.81e-09 ***	107.7
		Bright Angel Creek	-6.815e-01	4.965e-01	-1.373	0.1698	
		Havasu Creek	-2.968e-01	5.538e-01	-0.536	0.5919	
		LCR + Lava Chuar	-1.801e+00	2.281e-01	-7.898	2.84e-15 ***	
		Middle Granite Gorge	-3.911e-01	4.091e-01	-0.956	0.3391	
		Pumpkin Springs	-1.648e+01	2.649e+03	-0.006	0.9950	
		Shinumo Creek	-1.180e+00	6.575e-01	-1.794	0.0728 .	
		Stephen Isle	-1.190e+00	6.937e-01	-1.716	0.0862 .	
		Degree Days	-7.014e-03	3.235e-03	-2.168	0.0301 *	

Table 2.7—Stage 3 or potentially ripe females were found in all aggregations except Pumpkin Springs. The totals in this table include all fish caught in an aggregation during a specified month. July/Aug refers to fish caught during the July and August sampling trips in the main-stem Colorado River, and Sep refers to fish caught during the September sampling trips. LCR refers to the Little Colorado River aggregation. The data here were used to conduct the generalized linear model analysis with site and month effects.

Location	Month	Total Stage 2 Females	Total Non-Stage 2 Fish Scanned	Total Stage 3 Females	Total Non-Stage 3 Fish Scanned	Total Fish Scanned
30 Mile	July/Aug	8	53	18	43	61
Bright Angel Creek	July/Aug	0	10	5	5	10
Havasu Creek	July/Aug	5	11	5	11	16
LCR + Lava Chuar	July/Aug	6	27	4	29	33
Middle Granite Gorge	July/Aug	0	4	1	3	4
Pumpkin Springs	July/Aug	0	0	0	0	0
Shinumo Creek	July/Aug	0	5	0	5	5
Stephen Isle	July/Aug	0	11	2	9	11
30 Mile	Sep	15	50	10	55	65
Bright Angel Creek	Sep	1	9	1	9	10
Havasu Creek	Sep	8	17	4	21	25
LCR + Lava Chuar	Sep	55	89	19	125	144
Middle Granite Gorge	Sep	9	29	11	27	38
Pumpkin Springs	Sep	2	8	0	10	10
Shinumo Creek	Sep	1	11	3	9	12
Stephen Isle	Sep	5	7	1	11	12
Total	**NA**	**115**	**84**	**372**	**341**	**456**

Table 2.8—Stage 3 or potentially ripe females were found in all aggregations except Pumpkin Springs. The totals in this table include all fish caught in an aggregation during a specified year. LCR refers to the Little Colorado River aggregation. The data here were used to conduct the generalized linear model analysis with site and year effects.

Location	Year	Total Stage 2 Females	Total Non-Stage 2 Fish Scanned	Total Stage 3 Females	Total Non-Stage 3 Fish Scanned	Total Fish Scanned
30 Mile	2013	2	19	8	13	21
Bright Angel Creek	2013	0	14	5	9	14
Havasu Creek	2013	5	13	7	11	18
LCR + Lava Chuar	2013	3	26	2	27	29
Middle Granite Gorge	2013	5	15	8	12	20
Pumpkin Springs	2013	1	5	0	6	6
Shinumo Creek	2013	0	11	3	8	11
Stephen Isle	2013	0	2	0	2	2
30 Mile	2014	17	73	18	72	90
Bright Angel Creek	2014	0	4	1	3	4
Havasu Creek	2014	6	10	1	15	16
LCR + Lava Chuar	2014	32	65	14	83	97
Middle Granite Gorge	2014	4	9	3	10	13
Pumpkin Springs	2014	1	0	0	1	1
Shinumo Creek	2014	1	4	0	5	5
Stephen Isle	2014	3	12	2	13	15
30 Mile	2015	4	11	2	13	15
Bright Angel Creek	2015	1	1	0	2	2
Havasu Creek	2015	2	5	1	6	7
LCR + Lava Chuar	2015	26	25	7	44	51
Middle Granite Gorge	2015	0	9	1	8	9
Pumpkin Springs	2015	0	3	0	3	3
Shinumo Creek	2015	0	1	0	1	1
Stephen Isle	2015	2	4	1	5	6
Total	**NA**	**115**	**84**	**372**	**341**	**456**

Figures

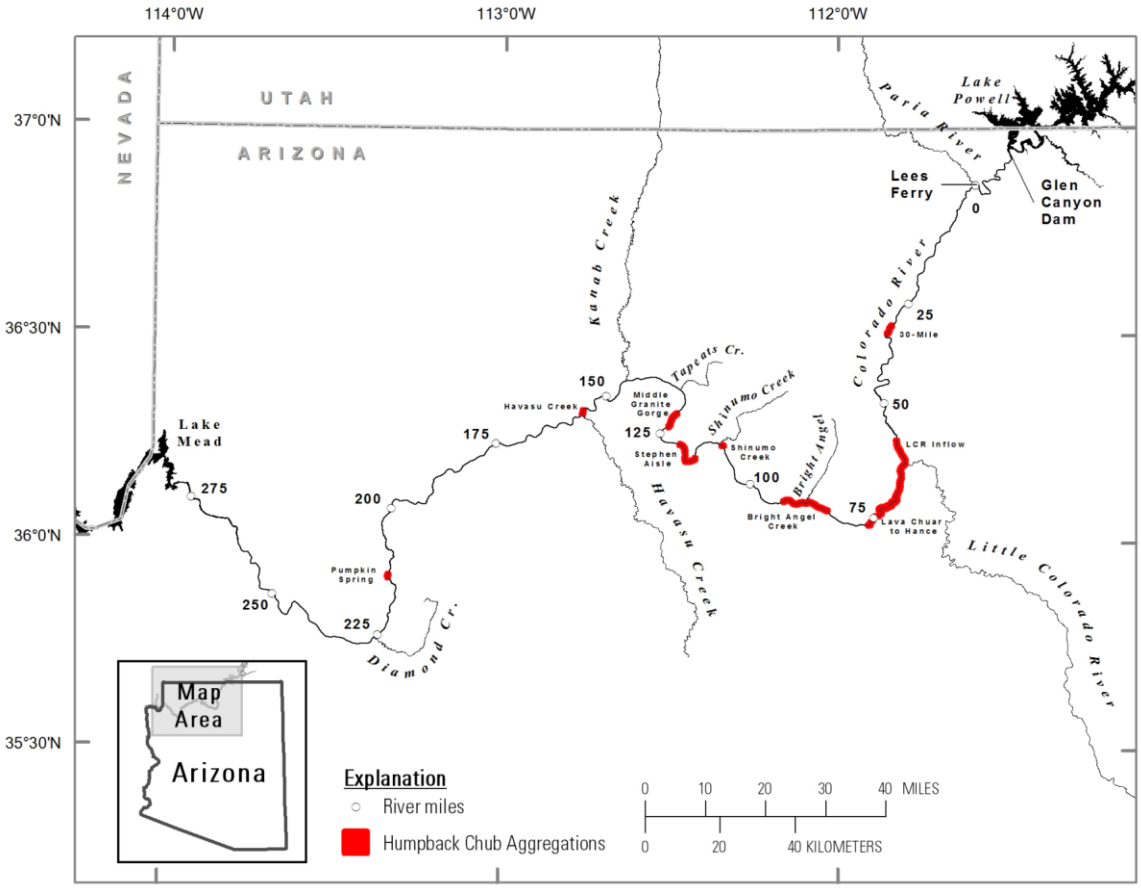

Figure 2.1—Map of areas sampled within the Grand Canyon, including the main-stem Colorado River, Little Colorado River, Shinumo Creek, and Havasu Creek. Map courtesy of Thomas Gushue, U.S. Geological Survey, Grand Canyon Monitoring and Research Center.

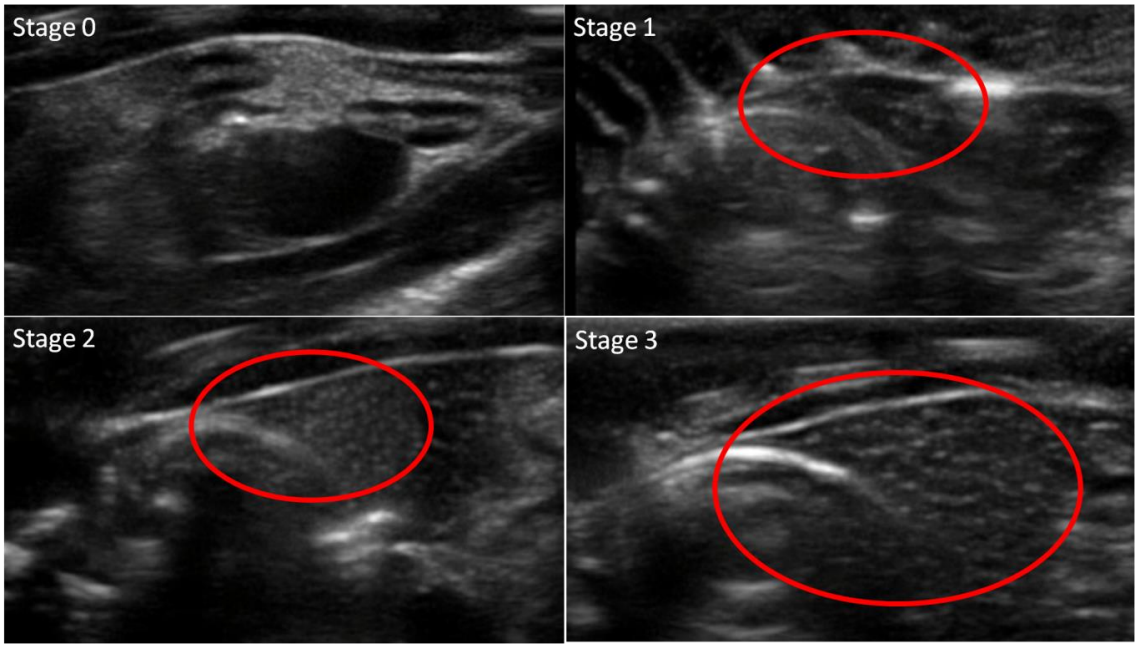

Figure 2.2—This illustration shows the four different stages of egg development in Humpback Chub identified in this study with ultrasound. The ellipses denote eggs. Stage 0 fish had no eggs and were considered non-females. Stage 1 fish had a bright white lining, which was likely outer ovarian lining, in addition to a low density of eggs. Stage 2 fish had the lining tissue and a higher density of small eggs present. Stage 3 fish had the lining present and a large mass of bigger eggs.

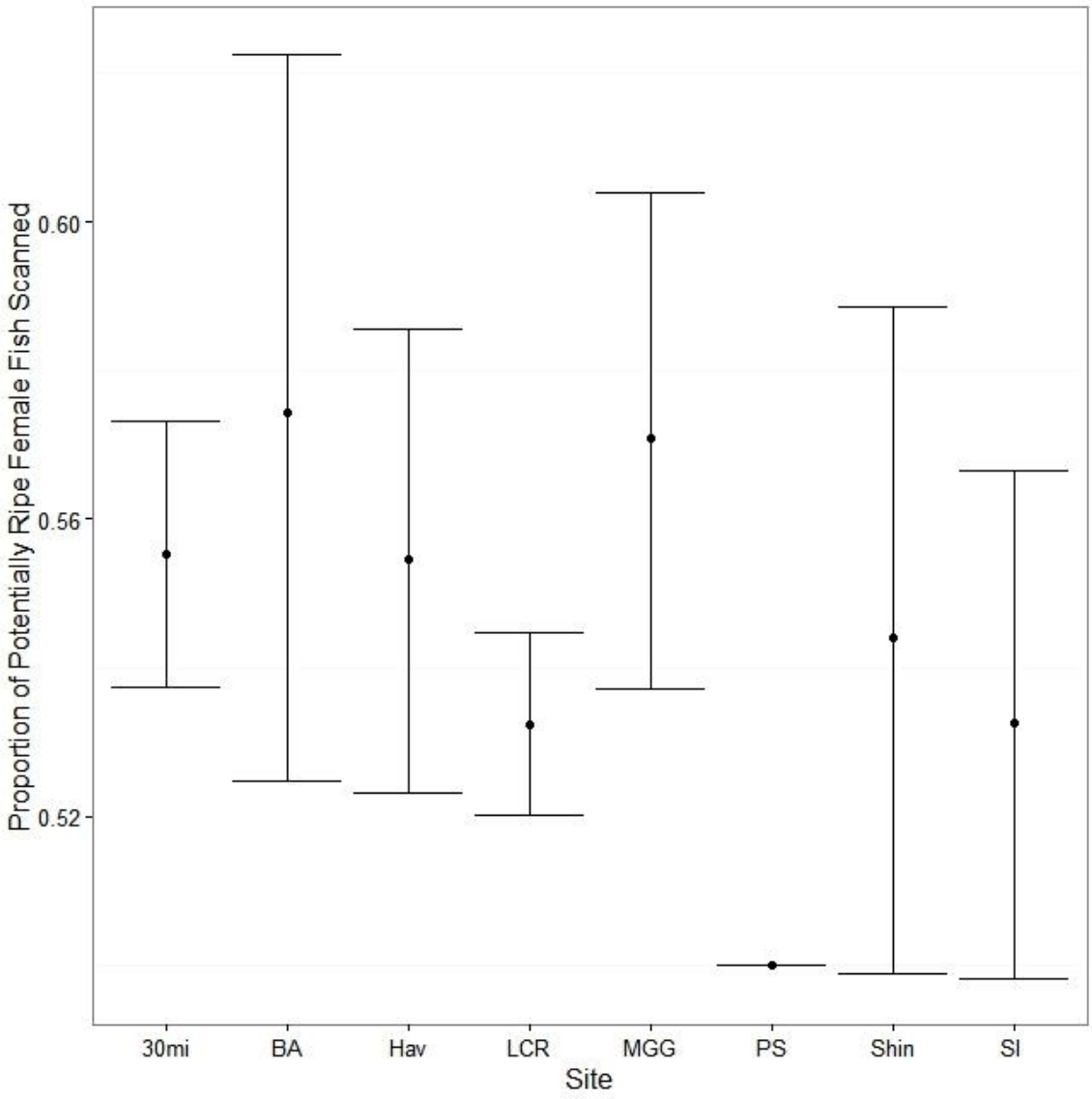

Figure 2.3—The proportion of potentially ripe female Humpback Chub was significantly different from zero for all main-stem Colorado River aggregations except for Pumpkin Springs. 30mi refers to the 30 Mile aggregation, BA to Bright Angel aggregation, Hav to Havasu Creek aggregation, LCR to Little Colorado River Inflow aggregation, MGG to Middle Granite Gorge aggregation, PS to Pumpkin Springs aggregation, Shin to Shinumo Creek aggregation, and SI to Stephen's Isle aggregation.

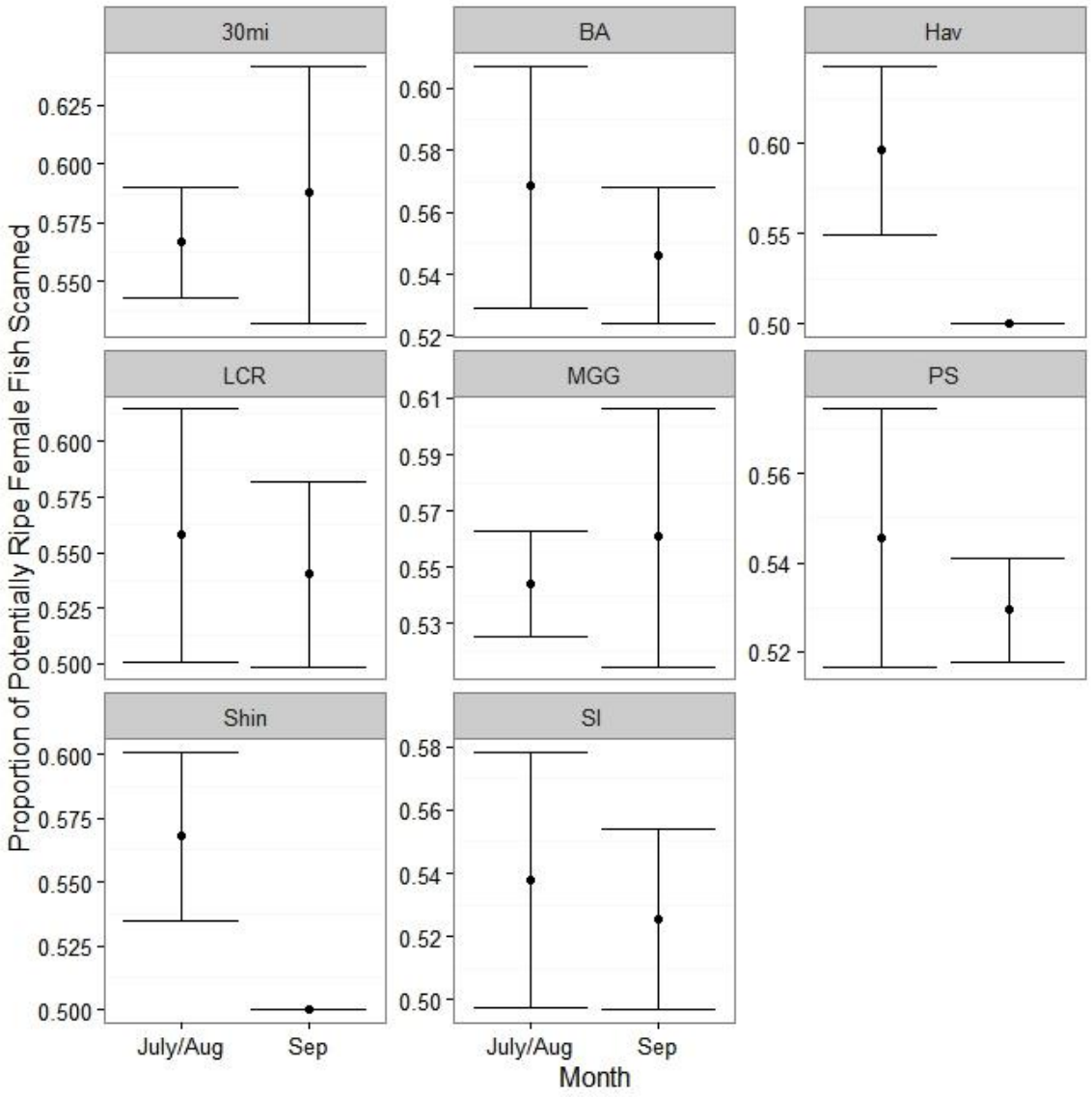

Figure 2.4—The proportion of potentially ripe female fish significantly varied between months sampled for each Humpback Chub aggregation in the main-stem Colorado River. 30mi refers to the 30 Mile aggregation, BA to Bright Angel aggregation, Hav to Havasu Creek aggregation, LCR to Little Colorado River Inflow aggregation, MGG to Middle Granite Gorge aggregation, PS to Pumpkin Springs aggregation, Shin to Shinumo Creek aggregation, and SI to Stephen's Isle aggregation.

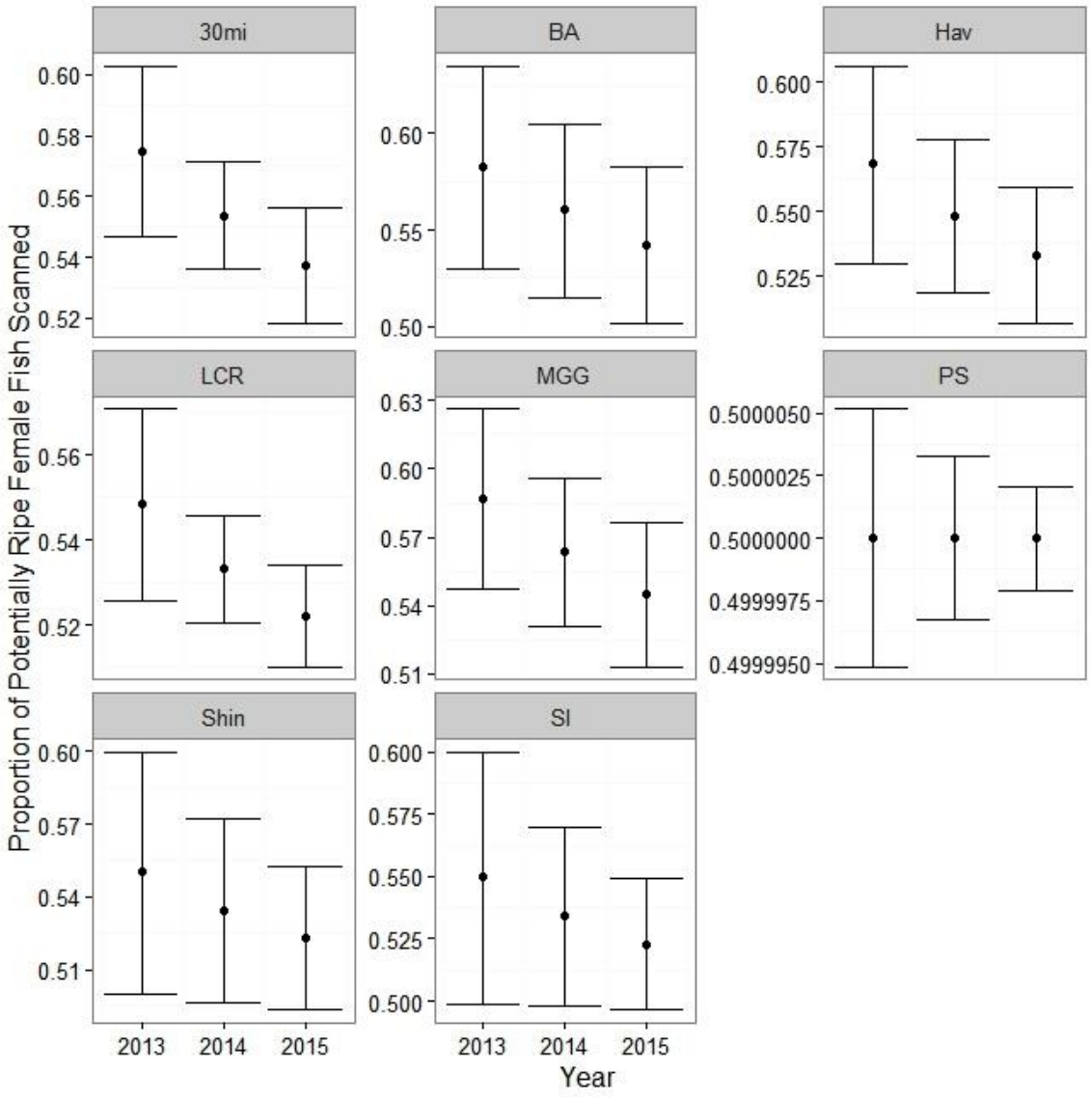

Figure 2.5—The proportion of potentially ripe female Humpback Chub varied between sampling years. The most potentially ripe female fish were captured in 2013 in each main-stem Colorado River aggregation except for Pumpkin Springs. 30mi refers to the 30 Mile aggregation, BA to Bright Angel aggregation, Hav to Havasu Creek aggregation, LCR to Little Colorado River Inflow aggregation, MGG to Middle Granite Gorge aggregation, PS to Pumpkin Springs aggregation, Shin to Shinumo Creek aggregation, and SI to Stephen's Isle aggregation.

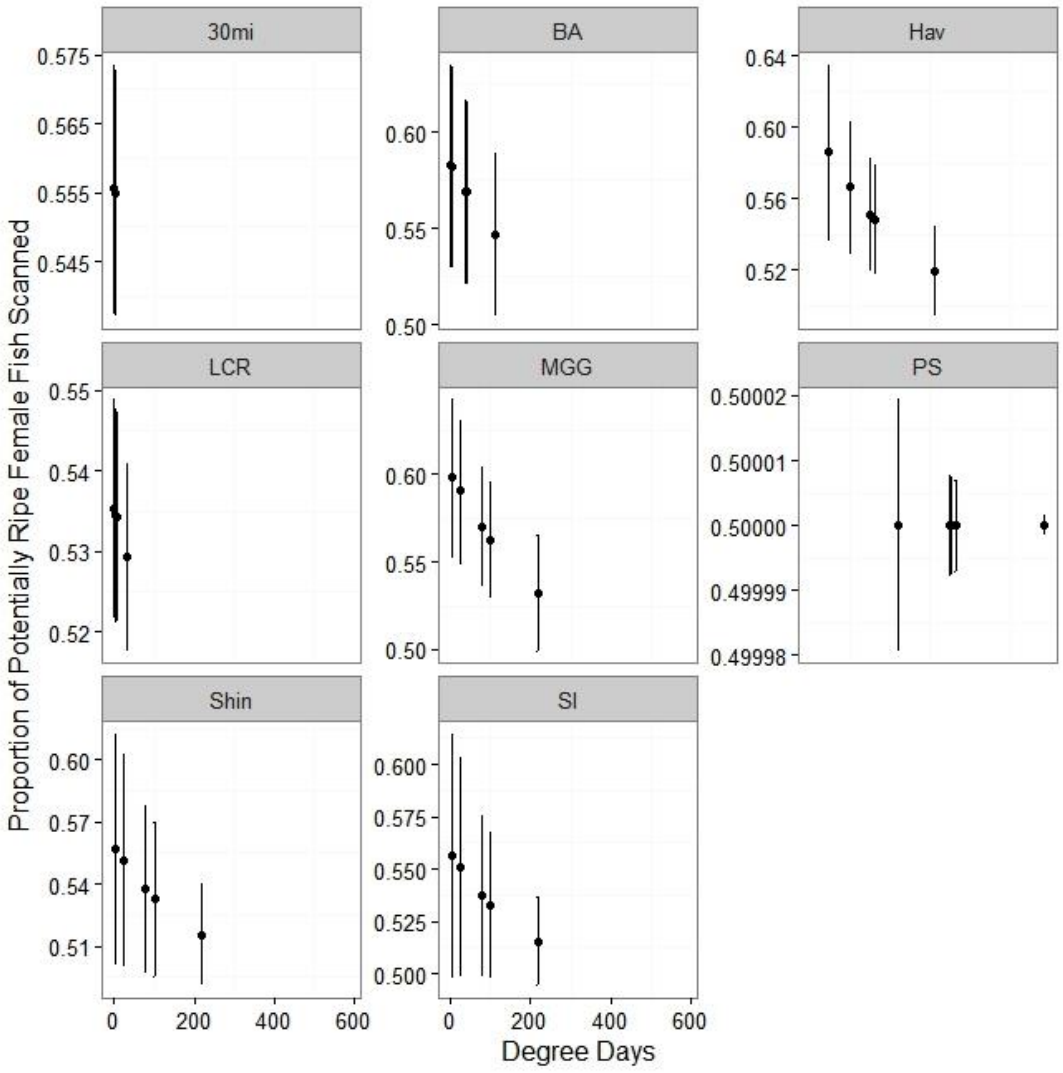

Figure 2.6—The main-stem Colorado River aggregations with the highest proportion of potentially ripe female Humpback Chub often experienced the least accumulated degree days. This inverse relationship is counterintuitive and is likely explained by a lack of potentially ripe female fish detected at Pumpkin Springs and by temperature data that does not reflect warm water inputs to the main-stem. 30mi refers to the 30 Mile aggregation, BA to Bright Angel aggregation, Hav to Havasu Creek aggregation, LCR to Little Colorado River Inflow aggregation, MGG to Middle Granite Gorge aggregation, PS to Pumpkin Springs aggregation, Shin to Shinumo Creek aggregation, and SI to Stephen's Isle aggregation.

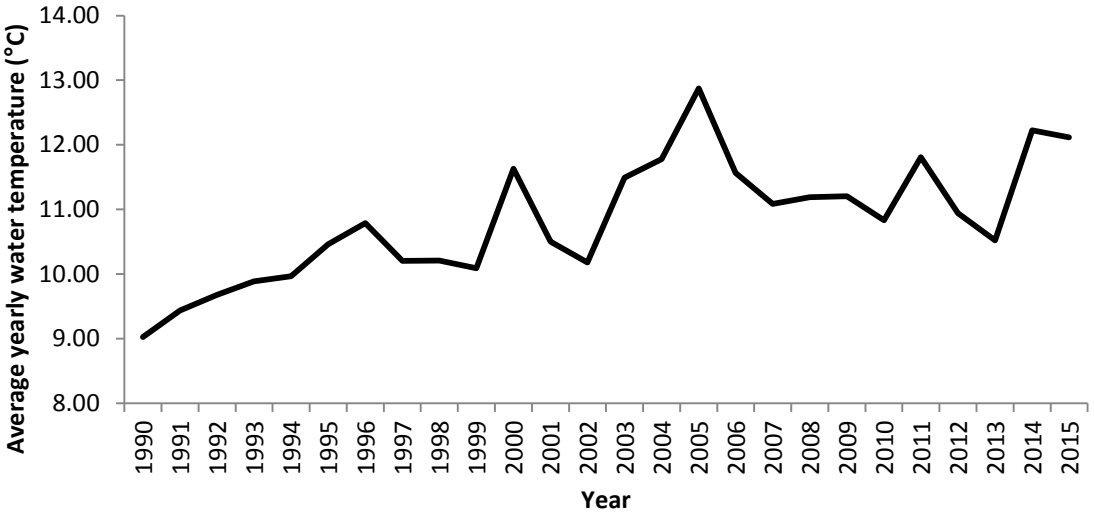

Figure 2.7—Average annual water temperature (°C) has increased over time since 1990 above the confluence of the main-stem Colorado River and the Little Colorado River. (Data source: U.S. Geological Survey, Grand Canyon Monitoring and Research Center, Grand Canyon Stations, Gauge 09383100: http://www.gcmrc.gov/discharge_qw_sediment/stations/GCDAMP). Average daily water temperatures were calculated for every day of the year and then averaged to calculate average annual water temperature.

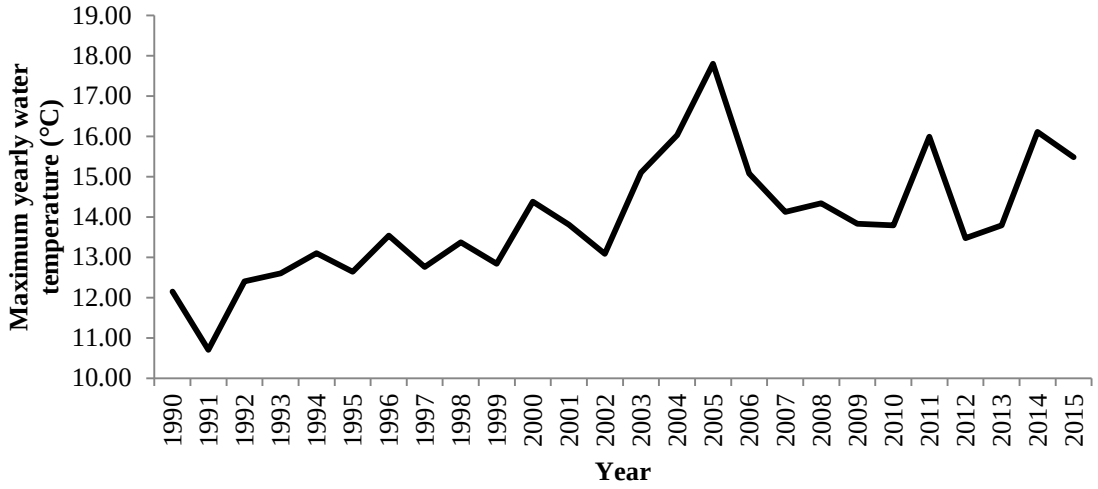

Figure 2.8—Maximum annual water temperature (°C) has increased over time since 1990 above the confluence of the main-stem Colorado River and the Little Colorado River. (Data source: U.S. Geological Survey, Grand Canyon Monitoring and Research Center, Grand Canyon Stations, Gauge 09383100: http://www.gcmrc.gov/discharge_qw_sediment/stations/GCDAMP). Average daily temperatures were calculated and then the highest average daily temperature was selected as the maximum annual water temperature.