

**VIRGIN RIVER RESOURCE MANAGEMENT AND RECOVERY PROGRAM
(VRRMRP)**

Project Title: Development and Optimization of Spawning and Intensive Culture Techniques for Woundfin

Project Number: V.09.02

Lead Agency: U.S. Fish and Wildlife Service

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Table Of Contents

RELATIONSHIP TO RECOVERY PROGRAM/CATEGORY:.....4

PROJECT BACKGROUND INFORMATION:4

GOALS, OBJECTIVES AND END PRODUCT(S):4

**OBJECTIVES/TASK AS PER APPROVED SCOPES OF WORK FROM
2008-20135**

**TASKS 1 AND 2. SUCCESSFULLY REAR WOUNDFIN IN INTENSIVE
CULTURE AND DESCRIBE THE REPRODUCTIVE CYCLE OF
WOUNDFIN.....6**

**TASK 3. EFFECT OF FEED PELLET MANUFACTURING ON
ACCEPTABILITY TO JUVENILE WOUNDFIN.20**

**TASK 4. THE ROLE OF CHASING BEHAVIOR AND SUBSTRATE IN
DETERMINATION OF SPAWNING READINESS AND SPAWNING29**

**TASKS 5, 7AND 12. USE OF THERMAL REGIME TO CONDENSE THE
SPAWNING SEASON FOR WOUNDFIN33**

**TASK 6. DETERMINE DIETARY PROTEIN AND ENERGY NEEDS OF
JUVENILE WOUNDFIN CULTURED AT THREE TEMPERATURES.38**

**TASK 8 AND 15. EFFECTS OF ULTRAVIOLET-B RADIATION ON
WOUNDFIN EMBRYOS AND LARVAE WITH APPLICATION TO
CONSERVATION PROPAGATION.....47**

**TASK 9. CANNIBALISM OF EMBRYOS AND LARVAE BY ADULT
WOUNDFIN IN INTENSIVE CULTURE: APPLICATION TO
CONSERVATION PROPAGATION.....59**

**TASK 10 AND 16. HORMONAL INDUCTION REGIME FOR
SPAWNING OF WOUNDFIN IN INTENSIVE CULTURE.64**

**TASK 11 AND 13. DEVELOP BROODSTOCK DIET FORMULATIONS
TO INCREASE FECUNDITY AND LARVAL SURVIVAL AND
PERFORMANCE.70**

**TASK 14. DETERMINE THE PROTEIN REQUIREMENT FOR
JUVENILE WOUNDFIN.....76**

TASK 18. WAHWEAP STATE FISH HATCHERY SITE VISIT82

TASK 19: WOUNDFIN INTENSIVE CULTURE DEMONSTRATION PROJECT...100

TASK 20. IMPROVE EGG QUALITY IN WOUNDFIN INTENSIVE CULTURE.....102

**TASK 21. CONTROL OF FUNGUS IN INTENSIVE CULTURE FOR
WOUNDFIN.....109**

OVERALL CONCLUSIONS AND RECOMMENDATIONS.....113

Relationship to Recovery Program/Category:

This project is an ongoing project at the request of the US Fish and Wildlife Service, Utah Field Office. This project addresses the following elements and objectives of the Recovery Action Plan:

IV. Protect and enhance native species communities

A. Re-establish native species communities

6. Restock reaches that have chemically eradicated nonnative species

B. Establish additional populations of native species within historically occupied habitat

Project Background Information:

The endangered woundfin *Plagopterus argentissimus* has been reared in captivity since the mid-late 1980s at Southwestern Native Aquatic Resources and Recovery Center (SNARRC). Initially, the captive rearing program was intended as a means to retain natural genetic diversity and maintain a refugial population in the event of catastrophic loss in the Virgin River. By the early 1990s, SNARRC had established a sustainable population ($N_e = 500$) through pond culture. As the wild woundfin population continued to decline and as reaches of the Virgin River have been reclaimed through nonnative fish eradication efforts, production numbers of woundfin have been requested for restocking into the Virgin River. The SNARRC has consistently provided approximately 5–10 thousand woundfin annually since the early 2000s. Pond culture of woundfin is also ongoing at (Wahweap State Fish Hatchery (WSFH), Utah, and Bubbling Ponds State Fish Hatchery (BPSFH), Arizona).

The Virgin River Resource Management and Recovery Program is in the process of determining how many fish will be needed for an effective restocking program, which could possibly require an order of magnitude or more increase in annual hatchery production with the target of 100,000 10-month olds spawning each spring in the river. The increased production may be achieved through intensive culture (e.g., tank rearing) versus extensive culture (e.g., ponds) or some combination of these techniques.

Goals, Objectives and End Product(s):

The original overall goal of this multi-year project conducted at the Bozeman Fish Technology Center (BFTC) is to establish intensive culture techniques for woundfin and future application of those techniques at production facilities (e.g., SNARRC) to help meet requirements of the restocking program. The current document describes the research activities performed at the Bozeman Fish Technology Center from 2008 through 2013 with conclusions on the utility and application of the research.

Objectives/Task as per Approved Scopes of Work from 2008-2013

Task #	Title
1	Successfully rear woundfin obtained from DNFHTC captive stock in test aquaria at the BFTC.
2	Describe the reproductive cycle of woundfin.
3	Determine optimal diets for larval and juvenile woundfin (2008-09).
4	Develop tool(s) to determine sexual dimorphism and assess spawning readiness.
5	Refine intensive conditions for successful woundfin spawning to maximize spawning success and condense the spawning season.
6	Determine dietary protein and energy needs of juvenile woundfin cultured at three temperatures.
7	Optimize thermal regime to maximize spawning potential.
8	Determine ultraviolet (UV) effects on embryo survival.
9	Determine predation rates on embryos and larvae by adult woundfin.
10	Develop hormonal injection regime for ovulation and spermiation of woundfin.
11	Initial funding to begin development of broodstock diet formulations to increase fecundity and larval survival and performance.
12	Cooperate and coordinate with DNFHTC, Wahweap, and Bubbling Ponds to ensure the necessary transfer of technology and information.
13	Develop broodstock diet formulations to increase fecundity and larval survival and performance. (See Task 11 note.)
14	Determine the protein requirement for juvenile woundfin. (This study was initiated in fall 2012 and completed in 2013.)
15	Determination of the minimum UV-B exposure resulting in mortality to woundfin embryos and larvae. (Study was completed in 2012.)
16	Optimization of hormonal induction for spawning of woundfin. (See Task 10 note.)
17	Prepare a comprehensive final report on 4 years of research.
18	Site visit and technology transfer to WSFH to improve conservation propagation of woundfin.
19	Woundfin intensive culture demonstration project.
	FY 2014 Projects
20	Improve egg quality in intensive woundfin culture.
21	Control of fungus in intensive culture for woundfin.

Tasks 1 and 2. Successfully Rear Woundfin in Intensive Culture and Describe the Reproductive Cycle of Woundfin

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Introduction

The woundfin is native to the lower Colorado River Basin, but the combined effects of habitat loss, flow alteration, and competition with invasive species has reduced the woundfin's distribution to a fraction of its historic range (USFWS 1994). Woundfin are now restricted to reaches of the mainstem Virgin River and were listed as endangered in 1970 (USFWS 1970); a designation that provides protection under the Endangered Species Act (ESA 1973). Recovery efforts for the federally endangered (ESA 1973) woundfin include captive rearing at three facilities: Southwestern Native Aquatic Resources and Recovery Center (SNARRC), New Mexico, Bubbling Ponds State Fish Hatchery (Bubbling Ponds), Arizona, and Wahweap State Fish Hatchery (Wahweap), Utah. Initially, the captive rearing program was intended as a means of retaining natural genetic diversity and as species refugia in the event of catastrophic loss. The principal goal of the Virgin River Resource Management and Recovery Program (Recovery Program) is to successfully rear woundfin at these facilities for release into its former range. The Recovery Program has a restocking program currently underway that calls for as many as 100,000 ten-month old woundfin to be produced annually (S. Meisner, personal communication).

Increase in production may be achieved through intensive culture (e.g. tank) verses extensive culture (e.g. ponds) or some combination of these. As of 2008, intensive culture of woundfin had been done only on a small scale. To successfully and consistently spawn woundfin, an understanding of the reproductive cycle was needed, hence, the objective of this study (which was conducted in 2008-2009) was to describe the reproductive cycle of woundfin.

Materials and Methods

Fish. Woundfin were imported from SNARRC to the Bozeman Fish Technology Center (BFTC; n=450) in October of 2008. Fifty six woundfin were held in a 70-gallon glass aquarium, and the remaining woundfin were maintained in 3-foot round tanks in the BFTC's quarantine facility (approximately 50 per tank). Fish were maintained on natural photoperiod similar to St. George, Utah and constant 22°C water temperature. Fish were fed Otohime and freeze dried Cyclo-peeze to excess. Overall survival was 95%.

Biological Sample Collection. In January 2009, monthly sampling of woundfin (8 months of age; n=4/month) was initiated. Fish were sampled on 22 January, 2 March, 7 April, 4 May, 5

June, 3 July, 26 August, 15 October, and 17 November 2009. At each sampling time, four fish were randomly captured from each tank and body weight (± 0.001 g) and length (fork length and total length, ± 1 mm) were measured. To determine the timing of sexual differentiation, age-0 woundfin that resulted from the BFTC spawning events in 2009 were sampled in October (n=2) and November (n=4) 2009.

Gonadal tissue was collected and stored in phosphate-buffered formalin or Davidson's for histological analysis. Gonadal tissue was embedded in paraffin, sectioned at 5 μ m, and stained by hematoxylin and eosin (Luna 1968). Slides were examined under a compound scope (Leica, 10x-1000x), and the germ cells were scored for stage of maturation.

Plasma Vitellogenin Assay Development. In order to induce Vtg synthesis, female and male woundfin were exposed to the birth control pill estrogen, ethinyl estradiol (500 ng/L) in water for 2 weeks. Plasma was collected and shipped to the University of Florida for plasma yolk purification. Measurement of Vtg will allow for another mechanism to determine first sexual maturity and endocrine disruption in the wild.

Results/Discussion

Growth. Woundfin body weight and length were monitored in the fish sampled each month for gonadal development (2008-year class). Given the small sample size and the fact that weight and length was not evaluated in the same fish over time, population level growth rates at the BFTC could not be evaluated.

Table 1. Body weight and length (fork and total length) of the 2008 year class woundfin maintained at constant 22°C at the Bozeman Fish Technology Center (8 to 18 month old fish). Fish were sampled monthly from January to November 2009 (data are means \pm sd; n=4).

Date	Body Weight (g)	Fork Length (mm)	Total Length (mm)
January 22	1.95 \pm 0.80	53.75 \pm 7.18	59.50 \pm 9.00
March 2	1.93 \pm 0.53	53.75 \pm 4.03	58.75 \pm 5.32
April 7	2.73 \pm 1.02	60.50 \pm 7.59	69.00 \pm 7.70
May 4	2.73 \pm 1.07	62.50 \pm 5.97	68.50 \pm 6.56
June 5	2.63 \pm 0.46	61.75 \pm 6.85	69.75 \pm 6.34
July 3	2.55 \pm 0.24	60.00 \pm 1.63	66.75 \pm 2.50
August 26	3.83 \pm 1.16	64.25 \pm 4.11	72.50 \pm 6.14
October 15	4.70 \pm 0.96	71.25 \pm 1.89	79.25 \pm 2.63
November 17	3.55 \pm 0.26	67.50 \pm 3.11	74.00 \pm 3.74

Sexual dimorphism was observed early in the monitoring effort and changed dramatically with females developing ripe ovarian follicles at first maturity. Females were the smallest individuals with little visceral fat and energy primarily being shuttled to egg development, while males were the largest individuals with significant amounts of visceral fat early in the season. When females developed ripe ovarian follicles, they were easily identified by distended abdomens. The mean body weight of the fish did decrease following the spawning season in July (Table 1).

The body weight and length of the 2009-year class produced at the BFTC was measured in October (6 months of age) and November (7 months of age) of 2009 (Table 2). On average, these fish increased in size by 0.5 g and 1 mm in total length in one month.

Table 2. Body weight and length (fork and total length) of woundfin from the 2009 year class sampled in 2009 (data are means \pm sd; n=2 in October and n=4 in November).

Date	Body Weight (g)	Fork Length (mm)	Total Length (mm)
October 15	0.65 \pm 0.21	39.50 \pm 2.12	44.00 \pm 2.83
November 17	0.70 \pm 0.42	41.00 \pm 5.66	45.00 \pm 6.36

Gonadal Histology. A total of 36 woundfin from the 2008-year class were sampled to describe gametogenesis over a 10-month period. A total of 6 woundfin from the 2009-year class were sampled to determine the timing of sexual differentiation. Stage of maturity was classified according to Table 3.

Of the 2008-year class, 15 of the woundfin were female and 13 were male. Eight of the gonadal samples did not contain enough gonial cells to determine sex or stage of maturity; these samples were primarily adipose tissue. The sex ratio of the 28 woundfin that were able to be sexed was 1.15:1 females to males. At the end of January, the majority of the females had perinuclear oocytes (Stage 1), though one female did have oocytes that had initiated endogenous growth (Stage 2). Spermatogonia (Stage 1) were present in the testes of males in January.

Table 3. Stages of gonadal maturity in woundfin.

	Developmental Stage	Description
Females		
Differentiation	1	Clusters of perinuclear oocytes
Endogenous Growth	2	Endogenous growth of oocyte, small oocytes with visible lipid droplets
Early vitellogenic	3	Enlarged oocytes with vitelline envelope surrounded by granulosa cells; small yolk globules
Mid-vitellogenic	4	Yolk globules present throughout cytoplasm; centrally located nucleus
Post-vitellogenic	5	Fully grown ovarian follicles with differentiated chorion and germinal vesicle displaced to animal pole
Oocyte maturation	6	Ovulated oocytes that have undergone germinal vesicle breakdown
Post-ovulatory	7	Numerous empty post-ovulatory follicles and the next generation of oocytes similar to Stage 2
Atretic	8	Atretic follicles or atretic bodies containing residual yolk and lipid
Males		
Differentiation	1	Clusters of primary spermatogonia
Mitotic	2	Proliferation of spermatogonia within testicular cysts
Onset of Meiosis	3	Spermatogonia (~50%) and spermatocytes
Meiotic	4	Majority of cysts contain spermatocytes and spermatids, less than 25% of cysts contain spermatogonia
Spermiation	5	Testicular cysts and ducts contain spermatozoa
Post-spermiation	6	Regressed testicular cysts with residual spermatozoa

By the beginning of March, woundfin ovaries contained oocytes that had initiated endogenous growth (Stage 2). One female was just initiating vitellogenesis (Stage 3; first maturity) as seen by the presence of yolk platelets. Proliferation of spermatogonia in testicular cysts had occurred in males (Stage 2).

In April, females were vitellogenic (Stages 3 and 4), and males had initiated meiosis (Stage 3; first maturity). The first spawning event occurred on 30 April 2009. Histological sections prepared from gonads collected in April revealed the presence of at least two clutches of developing ovarian follicles within the same female indicating batch spawning.

In May, females had late vitellogenic ovarian follicles (Stage 4), follicles undergoing oocyte maturation (Stage 5), and post-ovulatory follicles (Stage 7). One female had eggs that were oviposited during handling. Histological analysis of the ovary of this female revealed late vitellogenic ovarian follicles (Stage 4), follicles undergoing oocyte maturation (Stage 5), and ovulation (Stage 6). All males were mid-spermatogenic (Stage 4) or ripe with testicular cysts containing spermatozoa (Stage 5) in May, except one male which had testes containing spermatogonia and a high degree of adipose tissue (Stage 1).

In June, the one female sampled had ovaries containing perinuclear oocytes (Stage 2) and atretic bodies (Stage 8); no post-ovulatory follicles were seen though the gonad was very small. Two of the three males had testes containing spermatozoa (Stage 5), while one male contained spermatogonia (Stage 2).

Of the fish collected in July, one had such a regressed gonad, sex and stage of maturity could not be determined. The other fish were females with perinuclear oocytes (Stage 1). There was evidence of atretic bodies in one of these females.

Gonadal recrudescence continued in August with ovaries containing perinuclear oocytes (Stage 1) and testicular tissue containing spermatogonia (Stage 2). Biological samples were not collected in September.

In October, gonadal tissue in females contained both perinuclear oocytes and oocytes that had initiated endogenous growth (Stage 2). Testicular tissue in males still contained spermatogonia (Stage 2). Meiosis was initiated in males (Stage 3) in November, however, females still remained in Stage 2 (oocytes undergoing endogenous growth).

In the 2009-year class of woundfin, sexual differentiation had occurred by the first sampling time in October (5 months of age). Females contained perinuclear oocytes in October with endogenous growth of oocytes initiated by November. Testicular tissue in males contained spermatogonia that had proliferated in October, and one male had initiated meiosis in November. Woundfin spawned during the 2010 spawning season will be sampled in June, July, and August 2010 to determine the timing of sexual differentiation.

Determination of Germinal Vesicle Position. The germinal vesicle migrates to the animal pole prior to ovulation and oviposition in fishes and has been used as a means to assess spawning readiness in many species. To visually identify the position of the germinal vesicle in woundfin ovarian follicles relative to the animal pole, Pankhurst and Stockard solutions were used to clear the ovarian follicles as woundfin follicles are opaque. Stockard solution allowed for the clearing of the ovarian follicle as seen in Figure 1.

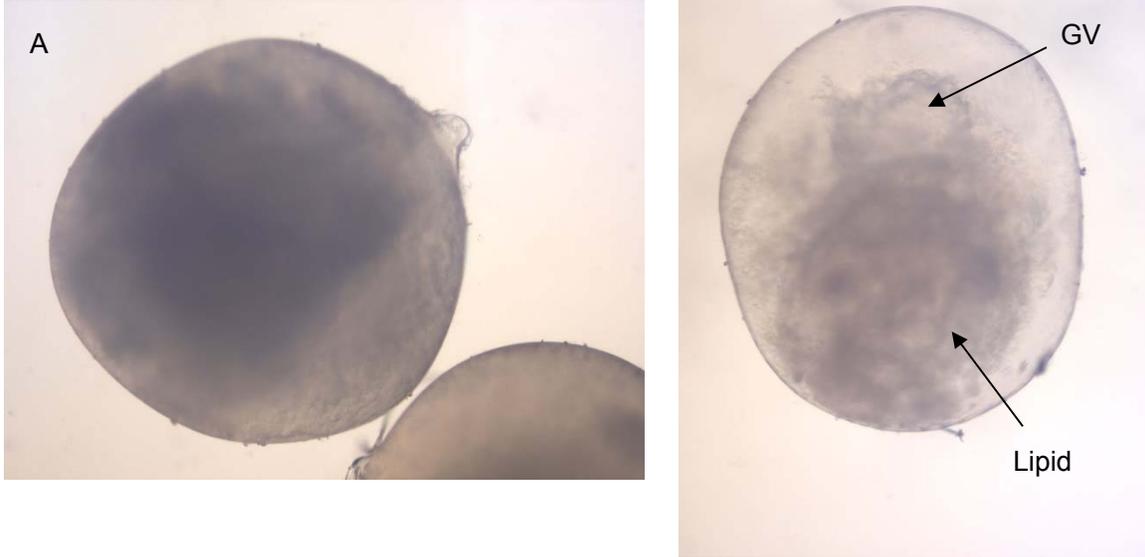


Figure 1. An uncleared (A) and cleared (B) woundfin ovarian follicle. In the cleared ovarian follicle, the germinal vesicle (GV) and the lipid may be seen inside the follicle.

Spawning of Age-0 Woundfin. Following histological confirmation of maturing females and males and chasing behavior in the age-0 2008-year class woundfin, substrate was placed in the bottom of each tank on 13 April 2009. Both marbles and rocks of various colors were placed in metal baskets and utilized as substrate (Figure 2). The first spawning event was documented on 30 April 2009. During the first week of the spawning season, each tray of substrate was removed from the tank and examined for embryos (Figure 3). Each embryo was then carefully removed from the substrate and placed into an incubation condo to hatch.

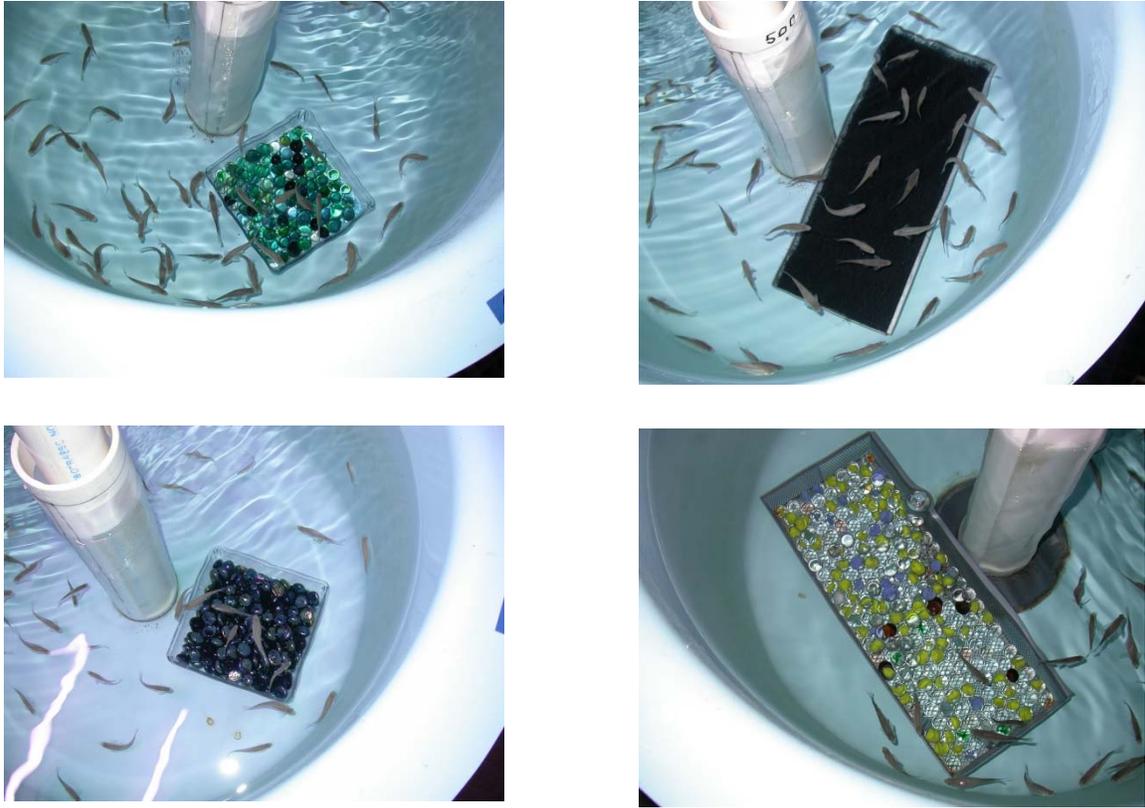


Figure 2. Various types and colors of substrate for natural woundfin tank spawning.

Following the first week of the spawning season, a less labor-intensive hatching protocol was developed. Seven days of feed was given to adults in a 5-day period. After the 5th day, food was removed from the tank and substrate was placed in the tank. The substrate remained in the tank for 3 days. On the fourth day, the substrate tray was removed and placed into a separate hatching tank. This strategy was used to reduce the amount of fungus that could grow in the spawning substrate due to uneaten feed and waste. The last spawning event was seen 12 June 2009. Spawning substrate was placed in the tanks until 19 June with no further spawning events, therefore substrate trays were no longer placed in the tanks.

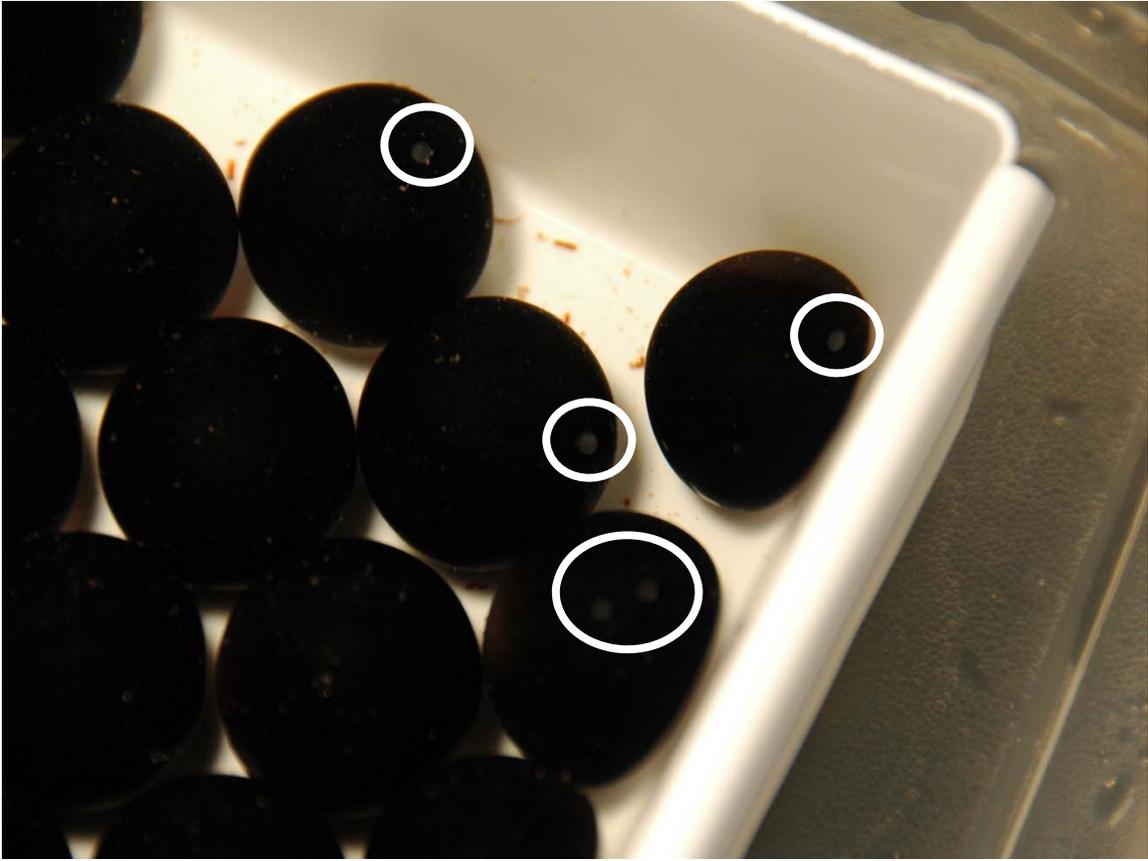


Figure 3. Adhesive woundfin embryos (in circles) on substrate following a natural tank spawning event.

Embryo Development and Hatch Success. Embryo development was observed during the spawning season. The two-cell stage (Figure 4A), blastodisc formation (Figure 4B), late blastula and the beginning of epiboly (Figure 4C), and formation of the eye and heart (Figure 4D) were captured. A total of 661 woundfin embryos were hatched at the BFTC. At 22°C, embryos hatched in 5 days. Larvae were exposed to feed one-day post-hatch. Larvae were fed 1/3 freeze dried Cyclo-peeze, 1/3 freeze dried rotifers, and 1/3 Otohime. Larvae were hand fed three times per day until active feeding was seen. At that time, belt feeders were used to introduce feed to tanks. Greater than 90% of the larvae initiated to feed. Developmental abnormalities (e.g. skeletal deformities, shortened opercles) were observed in 5% of the larvae.

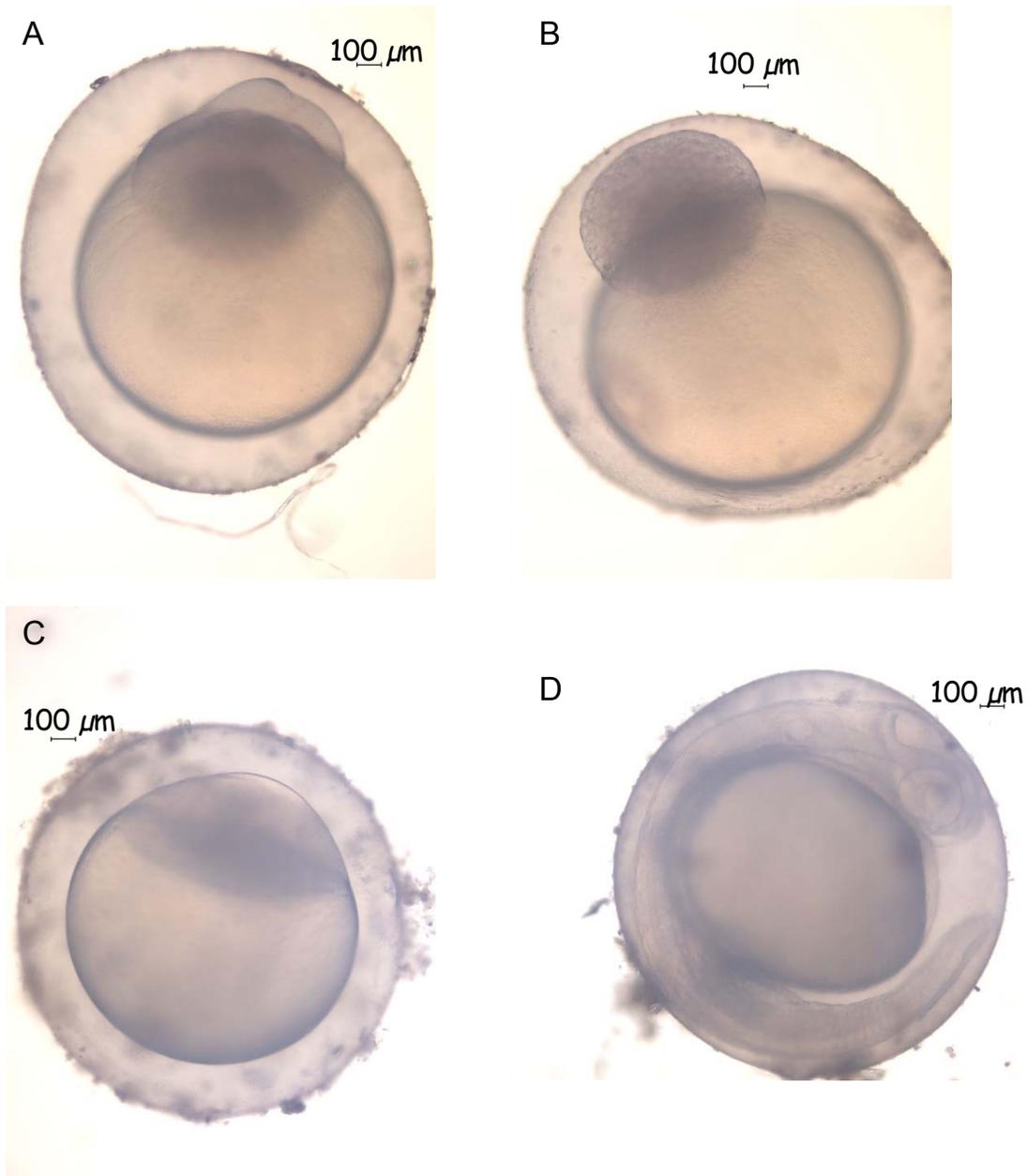


Figure 4. Woundfin embryo development: A 2-cell stage, B blastodisc formation, C late blastula and beginning of epiboly, D formation of the eye and heart.

Fecundity Estimates in Woundfin from SNARRC. Fecundity estimates for woundfin provided from SNARRC to the BFTC were conducted. Of the 25 fish sent to the BFTC, 5 females had eggs comparable in size to the woundfin eggs spawned at the BFTC in 2009. All other females had ovarian follicles that did not appear to be full grown or their gonads were devoid of eggs; fecundity was not estimated in these fish. In the 5 females that had the largest ovarian follicles, gonadosomatic index (GSI) ranged from 17 to 35% with 4 of the females having a GSI of 31-35%. Estimated fecundity ranged from 1,593 to 4,611 eggs. The largest female (body weight 9.642 g after fixation) did have the highest GSI (35.04%) and the highest estimated fecundity of 4,611 eggs. The high variability in both GSI and estimated fecundity appears to be due the presence of multiple clutches of ovarian follicles present in the gonad. Two to three distinct size classes of ovarian follicles were present in all females.

Plasma Vitellogenin Assay Development. Vtg was purified using anion exchange chromatography and the BIOCAD Perfusion System as described by Denslow et al. (1999). Plasma was diluted 1:10 in a running buffer (20mM bis-tris-propane, 50 mM NaCl, pH 9) and loaded onto a strong anion exchange resin (POROS 20 HQ). Non-binding proteins were eluted with several washes of running buffer. The Vtg was eluted using a linear gradient of NaCl (50-800 mM). The Vtg peak was identified by comparison to non-induced male profiles (Figure 5) pH adjusted to 7.0, and concentrated using a 50,000 MWCO Microcon. The Vtg integrity was maintained by adding protease inhibitor- Aprotinin (10 KIU/ml), bactericide- sodium azide (0.02%), and cryoprotectant- glycerol (1:2). The Vtg quality was verified by polyacrylamide electrophoresis (SDS-PAGE) and Western Analysis (Figure 6). It is apparent that Vtg is not present in males (Vtg peak indicated in Figure 6), induced by estrogen and is recognized by an anti-Vtg antibody developed in another fish species (Figure 7; Western Analysis). Based on the PAGE and Western data displayed in Figure 6, Vtg and lipovitellin both appear to be a dimers.

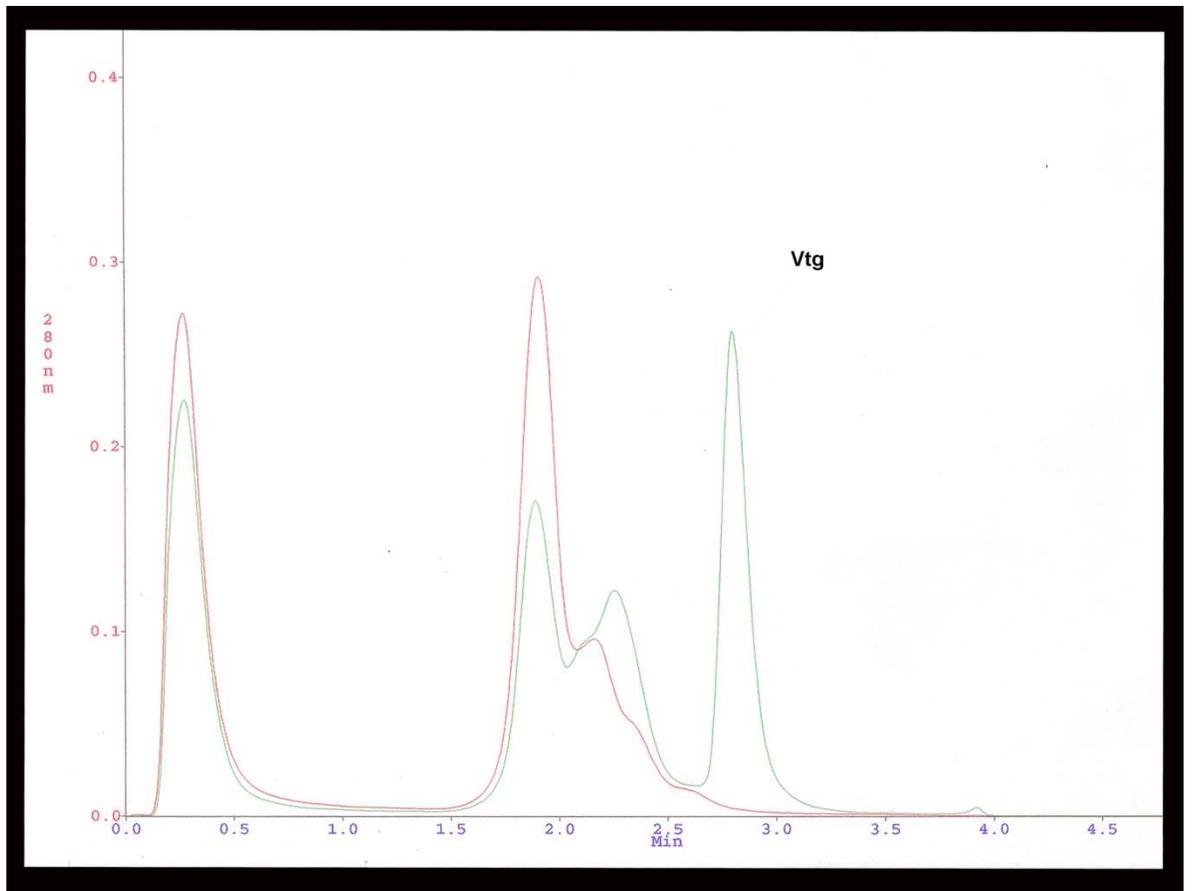


Figure 5. Anion exchange chromatograph displaying elution profile of a male and estrogenized male plasma. The Vtg peak is indicated. The male protein elution profile is red and the estrogenized male is green.

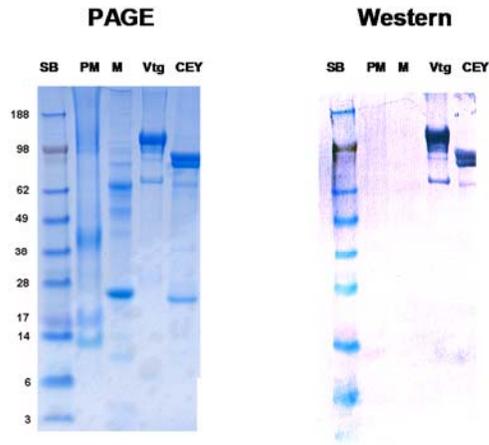


Figure 6. Polyacrylamide electrophoresis and Western blot of woundfin plasma (M), purified Vtg, and crude egg yolk (CEY). Molecular weight marker (See Blue 2 , SB) and Peppermint phosphoprotein marker (PM) are also indicated.

A standard curve (0, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 $\mu\text{g/ml}$) using purified woundfin Vtg was tested against a panel of 12 fish monoclonal antibodies (carp I, carp II, striped bass, white sucker, swordfish, gar, sturgeon, killifish, sheepshead minnow, catfish, quail) by direct ELISA. In brief, the standards were loaded onto an ELISA plate (NUNC-ImmunoSorp) and incubated overnight at 4°C in a humidified Tupperware container. The following day, the plate was washed four times with 100 mM tris, 150 mM NaCl, 0.5% tween-20, 0.02% azide, pH 7.6 (TBSTZ) and blocked with 1% BSA in TBSTZ for 2 hours at room temperature. Twelve different monoclonal antibodies (Mabs) were tested against the standard curves by adding 1.0 $\mu\text{g/ml}$ of each antibody diluted in blocking buffer. After 2 hours of incubation, the plates were washed and the secondary antibody, anti-mouse IgG (H&L)-biotin was added. After 2 hours, the final reagent, alkaline phosphatase-strep-avidin conjugate was added. The plates were rewashed and the developing solution, 4-nitrophenyl in 300 mM carbonate buffer, 20 mM MgCl_2 , pH 9.6 was added. Color intensity was measure using a SpectraMax 384 plate reader and data analyzed with the SoftMax Pro program (Molecular Devices).

Three different Mabs cross-reacted with woundfin Vtg. Carp, swordfish egg yolk, and white sucker anti-Vtg Mabs recognized woundfin Vtg. However, the Mab against white sucker Vtg showed the strongest affinity and gave the best standard curve by direct ELISA (Figure 3).

All samples were run in triplicate and coefficient of variation was <10%. The curves correlation coefficient was >0.95. Intra and inter-assay variation using positive controls is typically <5% and <10%, respectively.

The development of the Vtg ELISA allows for a biochemical marker for onset of maturation in woundfin. This ELISA will also allow for detection of endocrine disruption in wild woundfin exposed to estrogenic compounds.

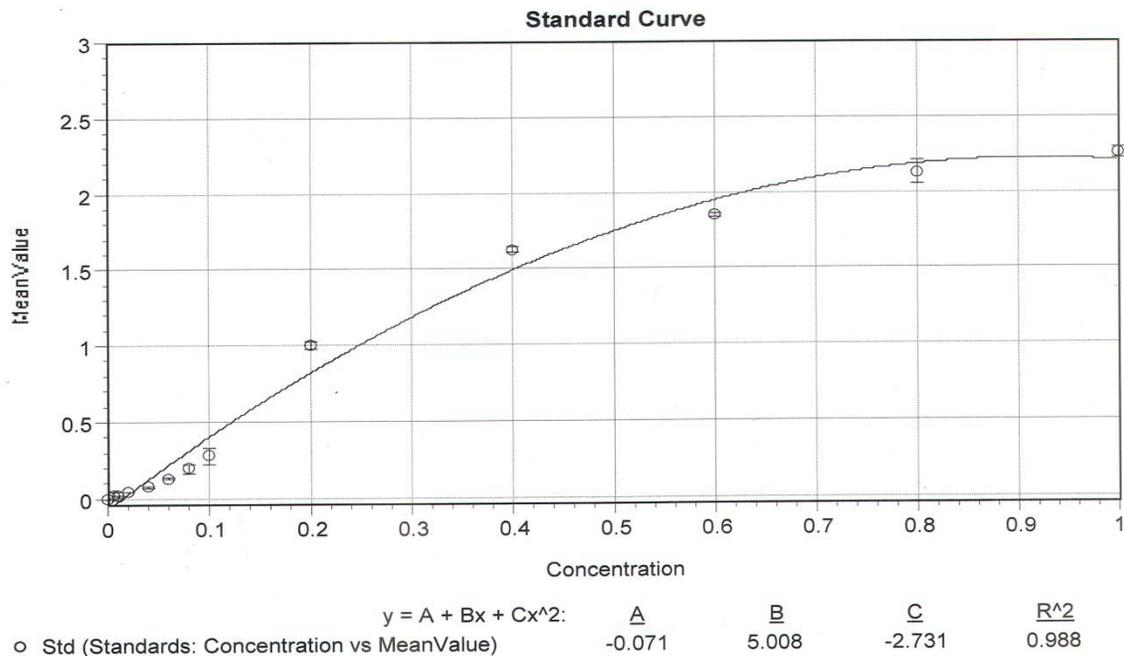


Figure 7. Woundfin Vtg standard curve using a monoclonal antibody developed against white sucker Vtg. X-axis is Vtg concentration ($\mu\text{g}/\text{ml}$) and Y-axis is absorbance at 405 nm.

The BFTC successfully reared and spawned age-0 woundfin (2008-year class) from SNARRC in 2009. The maturation cycle for woundfin has been described which provides critical information that will allow for successful and consistent spawning of woundfin in intensive culture. Woundfin had sexually differentiated by 5-months of age. Females reached first sexual maturity by 10-months of age, and males reached first sexual maturity by 11-months of age. It is unusual that males would reach first sexual maturity after females; this may be an artifact of low sample size. Histological sections prepared from gonads of females revealed the presence of at least two clutches of developing ovarian follicles within the same female indicating batch spawning. An ELISA for the detection of Vtg in woundfin has been successfully developed. Measurement of plasma Vtg allows for a biochemical marker to detect the onset of maturation in woundfin and the detection of endocrine disruption in wild woundfin exposed to estrogenic compounds. To simplify the intensive culture of a species that is a batch spawner, such as woundfin, entrainment of the maturation cycle may allow for the spawning season to be condensed to 2 or 3 mass spawning events.

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Task 3. Effect of feed pellet manufacturing on acceptability to juvenile woundfin

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Introduction

Woundfin (*Plagopterus argentissimus*) is a small scale-less fish historically found in the lower Colorado River basin and have an omnivorous feeding habit consuming insects, insect larvae, seeds and organic debris and detritus (Greger and Deacon 1988). Woundfin was listed as a federally endangered fish species in 1970 (USFWS 1994) and were brought into captivity at the Dexter National Fish Hatchery and Technology Center (DNFHTC) in 1979 as a refugial population. Fish culture efforts have primarily focused on spawning woundfin in ponds and collecting juvenile fish to augment wild populations. Stocking efforts began in 1993 from the DNFHTC and are ongoing.

Juvenile woundfin were held at BFTC following successful spawning during the '09 spawning season. Interest in evaluation of currently available commercial feeds on the growth and condition of juvenile woundfin has been expressed by SNARRC personnel. Rio-Grande Silvery Minnow (RGSM) flake feed has been utilized for production of woundfin juveniles in pond culture. In contrast, juveniles held at the BFTC are fed Otohime C-1 + Cyclop-eeze which is a 1 mm sinking marine fish diet. Due to the demersal nature of woundfin, feed production characteristics other than nutrient specifications also may affect feed consumption and thus fish growth and condition. In order to test the hypothesis that woundfin prefer a sinking feed to a floating or slow sink feed, four diets processed by different manufacturing techniques were used to produce flakes, slow sink pellets, floating pellets and fish growth and survival was compared to fish fed two commercially available hatchery feeds.

Materials and Methods

To evaluate the performance of woundfin juveniles fed commercially available feeds in intensive recirculating aquaculture systems, six diets were tested. The feeds were based on the open formula Rio Grande Silvery minnow formulation, developed at BFTC (Table 1), and Otohime+Cyclopeeze (BFTC standard culture diet):

- (1) Dexter-RGSM Rio-Grande Silvery Minnow (RGSM) flake from Silver Cup Fish Feeds (Nelson and Sons Inc, Murray, UT) (DNFHTC control),
- (2) BFTC-RGSM - RGSM formulation produced as a flake feed at BFTC,
- (3) MEM - RGSM formulation sinking pellet produced utilizing microextrusion marumerization technology (MEM),
- (4) PARA - RGSM formulation as a lower density sinking pellet produced by particle assisted rotational agglomeration (PARA),

- (5) BFTC Formula - BFTC formulation utilizing alternative ingredients manufactured by MEM.
(6) Otohime - Otohime+Cyclo-peeze (BFTC control)

Dexter-RGSM, BFTC-RGSM, MEM and PARA were formulated to the RGSM open formula in Table 1. All RGSM formulation based diets were mixed as a single batch and further processed using flake or pelleting equipment. The BFTC formula diet was formulated to meet be nutritionally equivalent to the RGSM formula but utilized different ingredient combinations to meet the nutritional specifications. BFTC RGSM was produced utilizing a double drum dryer (BuffaloVac, Buffalo, NY) flaking process. The MEM diet and BFTC formula were processed by microextrusion marumerization (MEM) to produce pellets between 500-850 µm. The PARA (Particle Assisted Rotational Agglomeration) diet produced low-density, slow sinking partilces between 500-850 µm. Moisture addition was variable across each pelleting process due to process differences required to form a pellet or flake. All diets were air dried to less than 8% moisture.

Table 1. Diet formulation for juvenile woundfin feeding trial to assess diet manufacturing procedure on woundfin performance.

Rio Grande Silvery Minnow		BFTC Formula	
Ingredient	% of diet dry	Ingredient	% of diet dry
Krill meal	18.97	Krill meal	10.00
Menhaden fish meal-Special Select	24.46	Wheat gluten meal	5.00
Squid meal	7.28	Corn gluten meal	15.00
EGG Solid	7.98	Soy protein concentrate	23.00
Spirulina	10.98	Spirulina	10.98
Wheat Starch, pregelatinized	4.99	Wheat Starch, pregelatinized	4.99
Wheat flour	14.26	Wheat flour	9.12
Menhaden fish oil	5.24	Menhaden fish oil	11.22
Stay-C 35	1.24	Stay-C 35	1.24
Vitamin premix ARS	4.50	Vitamin premix ARS	4.50
Charophyll® Pink - DSM	0.1	TM ARS 640	0.20
		Dicalcium Phosphate	2.50
		Charophyll® Pink - DSM	0.10
		Taurine	1.00
		DL-Methionine	0.25
		Lysine HCl	0.70
		Threonine	0.20

Table 2. Analyzed composition of diets fed to juvenile woundfin.

	Dexter RGSM	FTC RGSM	MEM	PARA	BFTC Formula	Otohime
Moisture	3.66	5.87	7.22	4.45	7.2	9.66
Crude Protein (% dry weight)	55.0	49.7	48.8	48.3	50.8	61.8
Crude Fat (% dry weight)	14.0	14.0	14.5	14.6	15.4	16.4

Culture system: Woundfin were cultured in a modified zebra fish tank system consisting of six 9.5 L tanks per row and three rows connected to a common bio-filter. Water quality was maintained at near lower limits of detection for ammonia, nitrite and nitrate. Dissolved oxygen was maintained near saturation and nitrogen gas saturation was maintained below 101% utilizing compressed oxygen. Water temperature was maintained at $22 \pm 1^\circ\text{C}$.

Experimental design: A randomized block design was employed with block being assigned as a row of tanks with each row containing one replicate per dietary treatment. Three replicate tanks of fish were assigned to each dietary treatment (one diet per tank per block). Twenty juvenile woundfin ($1.2 \text{ g} \pm 0.2$, mean \pm s.d) were stocked into each tank. The fish were conditioned to the culture system for 2 weeks prior to initiation of the 12-week feeding trial and fed Otohime C-1 + Cyclopeeze to slight excess twice daily. During the experiment fish were fed their respective diets to slight excess, twice daily for 12-weeks.

Fish count and total weight of fish for each tank was determined initially and every four weeks for the 12-week trial. Survival, weight gain, feed conversion ratio, morphological quality (i.e. evaluation of lesions, skeletal deformity or other deformities present) was quantified at the end of the study. All fish at the end of the trial were euthanized and stored at -20°C for proximate analyses.

Hematocrit and Proximate analysis: Whole blood was centrifuged in hematocrit tubes to determine packed cell volume. Whole fish were ground and homogenized by tank. Whole body moisture, lipid, protein and energy were determined by standard AOAC methodology.

Statistics: Differences among response variables were evaluated by ANOVA complete block design and deemed significant at $P < 0.05$ (SAS software program Proc GLM, Version 7, SAS Institute, Cary, NC). Where significant effects were detected, comparisons were performed using Tukey's means separation.

Results and Discussion

Diet protein ranged from 48.3 to 49.7% for FTC-RGSM, MEM and PARA with Dexter RGSM having higher protein at 55%. The BFTC formula contained 50.8% crude protein and Otohime contained 61.8% protein. Lipid content of all diets were less variable ranging from 14-14.6 for all RGSM formulations, 15.4% for the BFTC formulation and 16.4% for Otohime (Table 2).

At the end of the 12 week trial, final fish weight and specific growth rate were not different across treatments, ranging from 0.75 to 0.89 % day⁻¹ (Table 3). Survival also was not different across treatments (98-100%). Hematocrit was not affected by dietary treatment for juvenile woundfin (Table 4). Whole body protein and lipid also were unaffected by dietary treatment, but whole body moisture was affected with Dexter RGSM having higher moisture than FTC-RGSM, MEM, PARA and BFTC Formula treatments. The Otohime diet yielded intermediate whole body moisture.

Table 3. Growth and survival of juvenile woundfin fed diets manufactured by different processing techniques.

Diet	Initial fish mass ^a	Final fish mass ^a	SGR ^b	Survival
	g	g	%/day	%
Dexter RGSM	1.19	1.77	0.75	100
FTC RGSM	1.23	2.01	0.85	100
MEM	1.18	1.92	0.87	98
PARA	1.24	1.95	0.86	98
BFTC Formula	1.22	2.01	0.84	100
Otohime	1.27	2.12	0.89	100
<i>Pr>F</i>	0.5148	0.0924	0.7867	0.6187

^a Mean weight of individual woundfin in a tank

^b Specific Growth Rate

Table 4. Hematocrit and whole body compositional analysis of juvenile woundfin fed diets manufactured by different processing techniques.

Diet	Hematocrit	Moisture	Protein	Lipid
	%	%	%	%
Dexter RGSM	45.2	64.5 ^a	14.4	16.6
FTC RGSM	43.6	60.5 ^b	13.1	20.9
MEM	42.3	58.1 ^b	12.4	23.2
PARA	42.8	60.4 ^b	13.7	21.1
BFTC Formula	43.3	59.8 ^b	13.9	21.0
Otohime	42.5	61.3 ^{ab}	13.4	20.0
<i>Pr>F</i>	0.1100	0.0497	0.3797	0.1525

Utilization of three diet processing techniques to present feeds in differing forms does not appear to influence growth of juvenile woundfin cultured in tanks. The diets were presented in three unique forms. (1) A semi-floating flake feed represented by the Dexter RGSM and BFTC RGSM diets. These feeds were included as the control formulations currently utilized for feeding woundfin at Dexter National Fish Hatchery and support fish growth and composition

equivalent to any feeds tested in the current trial. (2) The MEM feeds were the densest feeds, sinking to the bottom of the tanks most rapidly and necessitating feeding from the bottom of the tank by the fish. (3) The PARA feed was intended to be a slow-sinking pellet but sinking rate was not notable different from the MEM diets. Therefore, the fish consumed most of the feed from the bottom of the tanks.

Compositional analysis of the diets demonstrated that protein contents of the diets differed but lipid was only slightly altered. The protein content of the diets ranged from 48 to 62% protein while lipid ranged from 14-16%. These limited changes in composition had no notable effects on growth or composition of the fish, but the uniqueness of the formulations indicate that woundfin can utilize nutrients from a variety of ingredient sources equally well or potentially that the diets contain an excess of nutrients. The later hypothesis may be supported by the high whole body lipid content of the woundfin across all dietary treatments and observational data on high levels of lipid accumulation of woundfin under culture at the Bozeman Fish Technology Center (Cal Fraser, personal communication). In conclusion, current diets appear to support adequate growth of juvenile woundfin but may not be optimal for fish condition based on observations of high fat accumulation in the body and liver.

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Task 3. Effect of feed lipid source on providing essential fatty acids for larval woundfin.

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Introduction

Nutritional needs of woundfin maintained in refugia are currently unknown. Thus, the desire to increase production of this species for stock enhancement and reestablishing the fish in its historical range necessitates determination of the nutritional requirements for woundfin to ensure optimal fish growth and health and reproductive fitness. Providing adequate amounts of essential fatty acids is a key limiting factor in survival of the larval stage for a variety of fish species. Most commercially available larval fish feeds contain high levels of omega-3 fatty acids, predominately eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) provided through inclusion of marine fish oils. However, freshwater ecosystem foodwebs contain limited EPA and DHA and are predominated by the omega-6 fatty acids linoleic acid (LA) and arachidonic acid (ARA). The fatty acid requirements for fish of the family Cyprinidae to which woundfin is a member are largely unknown with the exception of common carp, *Cyprinus carpio*, which have been estimated to require 1% omega-6 fatty acids and 1% omega-3 fatty acids (linolenic acid, LNA) in the diet. Thus, identification of the essential fatty acid requirements is one step in dietary optimization for woundfin.

Materials and Methods

Diets (Dietary evaluation of essential fatty acid requirements): A series of four practical-type diets (Table 1) were formulated to contain graded levels of n-6 to n-3 fatty acid ratios in a 12% lipid diet with 55% crude protein. Lipid sources included terrestrial commercially available sources that mimic the predominant fatty acid profile of freshwater ecosystems; specifically soybean oil to supply LA, flax oil to supply LNA and fish oil which supplies EPA and DHA fatty acids to mimic currently available larval fish feeds. A control diet of Otohime with Cyclo-peeze (6 parts Otohime: 1 part Cyclopeeze) served as a reference feed to assess relative performance based on historical data obtained for woundfin in indoor culture at the BFTC. Experimental diets were manufactured by Particle Assisted Rotational Agglomeration (PARA). The PARA method (U.S. Patent no. 5,851,574) was developed to increase the yield of particles less than 400 μm and using a low pressure method produce a particle that sinks slower (Barrows and Lellis 2006). The diets were air dried to less and 8% moisture and sifted to yield particle sizes of between 250-355 μm for first feeding larva.

Table 1. Diet formulations for larval woundfin with varied amounts of fish oil, soybean oil and flaxseed oil to alter dietary fatty acid profiles.

Ingredient	% of diet dry				
	Fish Oil	Soy Oil	Flax Oil	Soy-Flax	Otohime
Krill meal	10.0	10.0	10.0	10.0	
Wheat gluten meal	10.0	10.0	10.0	10.0	
Select menhaden fish meal	18.0	18.0	18.0	18.0	
Squid meal	16.0	16.0	16.0	16.0	
Soy protein concentrate	16.0	16.0	16.0	16.0	
Yeast protein, NuPro™	3.0	3.0	3.0	3.0	
Spirulina	3.0	3.0	3.0	3.0	
GemGel Wheat Starch	9.3	9.3	9.3	9.3	
Stay-C 35	0.3	0.3	0.3	0.3	
Vitamin premix ARS	1.0	1.0	1.0	1.0	
Choline chloride 50%	0.6	0.6	0.6	0.6	
Trace mineral premix - ARS 640	0.1	0.1	0.1	0.1	
Dicalcium Phosphate	2.5	2.5	2.5	2.5	
Taurine	1.0	1.0	1.0	1.0	
DL-Methionine	0.17	0.17	0.17	0.17	
Lysine HCl	0.93	0.93	0.93	0.93	
Threonine	0.10	0.10	0.10	0.10	
Menhaden fish oil	8				
Soy oil		8			
Flax			8		
Soy/Flax blend				8	
Analyzed Composition					
(% Dry weight)					
Crude protein	58.7	58.5	58.5	58.7	65.5
Lipid	11.6	11.5	11.5	11.1	12.6

Experimental design: Fish were assigned to diets using a randomized block design with fifty (50) newly hatched woundfin larvae stocked into each of 15 tanks (total of 15 tanks with 3 replicate tanks per treatment) at the beginning of the study. These fish were the F1 and F2 progeny of parents imported from DNFHTC and spawned on location at the BFTC. A randomized block design was utilized with replicate tanks of fish being blocked for all treatments over time. Individual spawning events were insufficient to stock all tanks on a single day; therefore, three spawning events were utilized to stock the experiment (Table 2).

Table 2. Randomized Block Design utilized to assess fatty acid needs of woundfin fry.

Block (time)	Dietary treatment ¹				
1	2	4	1	5	3
2	1	3	5	4	2
3	4	5	3	2	1

¹ Each cell represents an individual tank connected to a common biofiltration system.

Culture conditions: Woundfin fry were cultured in 15, 100-L tanks connected to a common boiler. Water quality was maintained at near limits of detection for ammonia, nitrite and nitrate. Water temperature was maintained at $22 \pm 1^\circ\text{C}$. The five diets, described above, were fed to three replicate lots of newly hatched woundfin larvae. Feeding began 5 days post hatch and feeding continued for 16 weeks. Fish were offered their respective diets utilizing belts feeders to apparent excess. All tanks were cleaned of uneaten feed and feces daily. Mortalities were removed and counted daily.

Water quality parameters were measured to ensure optimal DO, pH, total ammonia, nitrite and nitrate levels were maintained at optimal levels. Fish were counted and total weight for each tank recorded at the end of the 16-week trial. Survival, weight gain, morphological quality (i.e. evaluation of lesions, skeletal deformity or other deformities present) and fish total length variation were determined on all fish from each tank at the end of the study. A sample from each diet was stored for proximate analyses.

Chemical analyses: Whole fish were ground and homogenized by tank to determine fatty acid profiles. Diet moisture, lipid, protein and energy were determined by standard AOAC methodology (AOAC 1995).

Statistical analyses: Differences among response variables were evaluated by ANOVA complete block design and deemed significant at $P < 0.05$ (SAS software program Proc GLM, Version 7, SAS Institute, Cary, NC). Where significant effects are detected, comparisons were performed using Tukey's means separation.

Results and Discussion

Dietary lipid source had no apparent effect on fish mass or length at termination of the 12-week trial (Table 3). Fish fed the Otohime control diet grew faster than fish fed any of the experimental formulations. No significant dietary effects were noted on larval deformities. The deformities that were observed manifested as spinal deformities. Survival of larval woundfin was affected by fatty acid composition of the diets. Feeding diets having higher linolenic acid (Flax and Soy Flax blend) resulted in higher survival (~30%) than the fish oil diet (9%) and the soy oil diet (15%). Feeding the Otohime control diet resulted in the best survival (96%).

Table 3. Performance of juvenile woundfin fed diets with differing lipid sources and fatty acid profiles.

Diet	Fish mass (g)	Fish length (mm)	Percent deformity	Survival
Fish oil	0.107 ^b	26 ^b	29.2	9 ^c
Flax	0.097 ^b	25 ^b	19.3	32 ^b
Soy	0.081 ^b	23 ^b	20.8	15 ^c
Soy Flax	0.102 ^b	24 ^b	23.6	29 ^b
Otohime	0.374 ^a	38 ^a	7.6	96 ^a
<i>Pr>F</i>	0.0001	0.0001	0.7144	0.0001

Larval woundfin fed the Otohime control diet had the highest survival and fastest growth compared to fish fed any of the experimental diets. The formulation utilized to test the fatty acid needs, irrespective of lipid source, performed poorly relative to the Otohime control diet. The reduced performance of fish fed these diets indicates that potentially one or several ingredients sourced for the formulation may have been of lower quality than expected. The reduced quality may have impaired feed acceptability which could not be directly measured in the larval fish. The data observed for larval survival, although lower than the Otohime control fed larva, does indicate that a balanced omega-3 and omega-6 fatty acid profile (Soy-Flax and Flax diets) improved survival of larval woundfin compared to diets receiving fish oil or soy oil as the supplemental dietary fat source.

Task 4. The Role of Chasing Behavior and Substrate in Determination of Spawning Readiness and Spawning

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Introduction

The woundfin is native to the lower Colorado River Basin, but the combined effects of habitat loss, flow alteration, and competition with invasive species has reduced the woundfin's distribution to a fraction of its historic range (USFWS 1994). Woundfin are now restricted to reaches of the mainstem Virgin River and were listed as endangered in 1970 (USFWS 1970); a designation that provides protection under the Endangered Species Act (ESA 1973). Recovery efforts for the federally endangered (ESA 1973) woundfin include captive rearing at three facilities: Southwestern Native Aquatic Resources and Recovery Center (SNARRC), New Mexico, Bubbling Ponds State Fish Hatchery (Bubbling Ponds), Arizona, and Wahweap State Fish Hatchery (Wahweap), Utah. The principal goal of the Virgin River Resource Management and Recovery Program (Recovery Program) is to successfully rear woundfin at these facilities for release into its former range. The Recovery Program has a restocking program currently underway that calls for as many as 100,000 ten-month old woundfin to be produced annually (S. Meismer, personal communication).

In 2009, the Bozeman Fish Technology Center (BFTC) received woundfin from SNARRC, and their reproductive cycle was described. For conservation propagation, knowledge about spawning readiness, i.e. the proper time to spawn fish, is essential for successful and consistent production of larval fish. During the 2009 spawning season, chasing behavior was seen in both male and female woundfin at the BFTC but was not monitored extensively. No spawning was seen without the presence of substrate in 2009 indicating a potential means by which to control the timing of spawning. The goal of this research was to test two questions to determine whether chasing behavior was a potential non-invasive means to determine spawning readiness and the appropriate time to expose females to males to trigger a spawning event as well as whether the use of substrate could trigger a spawning event. The first question was do males chase females due to spawning readiness (visual cues and pheromones)? The second question was is substrate necessary to induce a spawning event or will oviposition occur regardless of the presence or absence of substrate?

Materials and Methods

Four gravid females were randomly selected from the 2008 year class and placed in separate tanks (n=1 per tank). For two of these tanks, four male fish that demonstrated chasing behavior in the master tank were selected and added to the tanks with gravid females (n=2 males per tank; Tanks 1 and 2). For the other two tanks, four males (chosen based on body conformation) that did not demonstrate any chasing behavior in the master tank were selected and added to the tanks with gravid females (n=2 males per tank; Tanks 3 and 4). The fish were observed for 8 weeks beginning April 5, 2010. Each week, spawning substrate (glass marbles

in a 6 X 4 inch plastic tray) was added to one tank with chasing males (Tank 2) and one tank with non-chasing males (Tank 3). The remaining two tanks did not receive substrate (Tanks 1 and 4). All fish were monitored for 30 minutes three times per day to observe chasing behavior and determine if a spawning event had taken place. A camera was set up every evening to try to photo-document a spawning event. The cameras did not have infrared capabilities and could therefore not capture a spawning event in the dark. Spawning substrate was left in the tanks for 72 hours with minimal feeding to decrease fungal growth in the substrate should a spawning event occur. After the 72-hour trial, substrate was removed and the fish were then put back on a normal feeding regime for 3 days. Following the 3 days, the trial was again initiated. Substrate was added to the same tanks throughout the trial (Tanks 2 and 3).

To further determine whether there is a correlation between chasing behavior of males and stage of spawning readiness in females and determine whether ultrasound could potentially be used to determine sex and stage of maturity, we placed 1) a non-gravid female (based on body conformation) in a tank with a male that was observed to be chasing in the master tank, and 2) a gravid female in a separate tank with a male that was observed to be chasing in the master tank. After 30 minutes of observation for each tank, we then euthanized the fish with MS-222 to scan the gonad using ultrasound (Sonosite Titan) and remove the gonadal tissue for histological analysis to determine sex and stage of maturity.

Results and Discussion

In the tank (Tank 1) which had a gravid female with two chasing males and no substrate, there was very minimal chasing behavior monitored during the first 5 weeks. After 5 weeks, we added three more chasing males to increase the sex ratio to 5:1 males to females. Once the ratio was increased, the female was observed to hide in the corner of the tank due to continual chasing by all males. By week 8, the female had not released any eggs and appeared to have undergone follicular atresia potentially due to chronic stress (confirmed 1 week later by abdominal pressure and the release of resorbing ovarian follicles). This suggests oviposition by females appears to require the presence of substrate and that male density may detrimentally affect female spawning success as seen with medaka (*Oryzias latipes*; Spence and Smith, 2005; Weir, 2013). The lack of chasing by the original two males placed in the tank with the female is not well understood. These males were chasing in the master tank prior to transfer to the experimental tank. The addition of three more males immediately resulting in chasing behavior by all males suggests an increase in male mating competition.

In the second tank (Tank 2) which had a gravid female with two chasing males and substrate, significant chasing behavior was observed throughout the study. During week 6 of the 8-week trial, a spawning event occurred, but soon after, the female had eaten all of her eggs that were oviposited. This egg/embryo predation was photo-documented (Figure 1).

In the third tank (Tank 3) which had a gravid female and two non-chasing males with substrate, very minimal to almost no chasing behavior was observed during the 8-week trial. Oviposition of eggs never occurred during the trial.

In the fourth tank (Tank 4) which had a gravid female with two non-chasing males and no substrate, very little chasing behavior for the 8-week trial was observed. The trials with non-chasing males suggest that chasing behavior may be a good indicator for male spawning readiness (Tank 3 and 4), however the non-chasing behavior in the tank with the original two males that chased in the master tank prior to the initiation of the trial (Tank 1) suggests that chasing behavior does not confer male spawning readiness in all cases. The chasing behavior by males in the tank with the gravid female (Tank 2) that occurred 6 weeks prior to the spawning suggests that chasing behavior does not confer immediate female spawning readiness (i.e., immediate oviposition).



Figure 1. Egg predation by female woundfin. Female is seen grazing the eggs throughout the tray. To play video, ensure that the file is saved to your hard drive. Right click on photo. Use the menu “video clip object” and choose “play”. If the video does not play, you will need to click on the “avi” file on your hard drive.

In the trial to further determine whether there is a correlation between chasing behavior of males and stage of spawning readiness in females and determine whether ultrasound could potentially be used to determine sex and stage of maturity, we observed very little chasing behavior in the tank with the non-gravid female. Dissection of the suspected non-gravid female confirmed that the female was in the early stage of gonadal maturity (pre-vitellogenic). Significant chasing behavior occurred in the tank with the gravid female, and dissection confirmed that the female was mature and the ovary contained post-vitellogenic follicles. We were capable of differentiating the immature female, mature female and males using ultrasound (Figure 2).



Figure 2. Ultrasound conducted on adult woundfin to determine sex and stage of maturity.

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Tasks 5, 7 and 12. Use of Thermal Regime to Condense the Spawning Season for Woundfin

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Introduction

The principal goal of the Virgin River Resource Management and Recovery Program (Recovery Program) is to successfully rear woundfin at conservation propagation facilities for release into its former range. The woundfin is native to the lower Colorado River Basin, but the combined effects of habitat loss, flow alteration, and competition with invasive species has reduced the woundfin's distribution to a fraction of its historic range (USFWS 1994). Woundfin are now restricted to reaches of the mainstem Virgin River and were listed as endangered in 1970 (USFWS 1970). The current goal of the Recovery Program's woundfin restocking program is to release 100,000 ten-month old woundfin annually (S. Meisner, personal communication).

Woundfin are clutch or batch spawners as seen through histological analysis conducted during the first year of spawning at the Bozeman Fish Technology Center (2009) which can increase the spawning season as females become ripe multiple times during optimal spawning conditions. The conservation propagation programs (Southwestern Native Aquatic Resources and Recovery Center (SNARRC), New Mexico; Bubbling Ponds State Fish Hatchery (Bubbling Ponds), Arizona; Wahweap State Fish Hatchery (Wahweap), Utah) are currently responsible for spawning multiple spring spawning species, which is challenging when faced with a species such as woundfin that trickle spawn over a several-month period (May-July). The goal of this project was to entrain the maturation cycle and condense the spawning season of woundfin allowing for spawning to occur during a 1- to 2-week interval rather than over several months. During the spring of 2010, experiments were run at the Bozeman Fish Technology Center (BFTC) to develop a thermal protocol. During the spring of 2011, experiments were run simultaneously at the BFTC, SNARRC, and Bubbling Ponds to apply the protocol developed in 2010 to the conservation propagation programs.

Materials and Methods

Temperature was used to entrain the maturation cycle using a controlled thermal unit at the BFTC (Figure 1). Woundfin adults (2008 year class) were maintained at 22°C through the fall of 2009. Photoperiod was equivalent to that in St. George, Utah. Fish were sexed by morphological characters, and a total of 120 females and 60 males were transferred into the thermal unit on February 19, 2010. A small subsample of fish was euthanized at the time of transfer of fish to the thermal unit to confirm sex determination by visual characters (i.e., extended abdomen, slender body). The temperature in the thermal unit was 22°C at the time fish were transferred, and the photoperiod mimicked that of Bozeman, Montana. Females were

randomly assigned to either a control group or one of three treatment groups (10 females per group; all groups run in triplicate). Males were randomly assigned to either a control group or one of the three treatment groups (60 males total; 15 males per group). All males assigned to a treatment group were kept separate in a single tank (4 tanks total) until the spawning trial. Temperature remained at 22°C in the control group throughout the 2010 spawning season. Temperature in the 3 treatment groups was slowly dropped to 16°C over a 1-week period after transfer to the thermal unit in February 2010. Temperature was then raised over a 1-week period from 16 to 22°C during the first week of May in Treatment 1, the second week of May in Treatment 2, and the third week of May in Treatment 3 (Figure 2). The temperature in the assigned tank of males was ramped simultaneously with the respective female tanks. Control females were spawned at the same time as Treatment 2 females, as this was the peak spawning time for females maintained at 22°C in 2009. When temperature reached 22°C in each treatment group, four to seven males were introduced into each control or treatment tank of females and substrate (tray of glass marbles) was placed into the tanks.



Figure 1. Controlled thermal unit at the Bozeman Fish Technology Center in which woundfin were exposed to temperatures ranging from 16 to 22°C.

Substrate trays placed in the Treatment 1 tanks remained Monday through Thursday during week 1 (10 May–13 May), and fish were not fed during this time. Embryos were observed each morning but not enumerated. It became apparent that embryo predation by the adults was occurring due to the disappearance of embryos on a daily basis, which prompted hourly observation of the tanks on Thursday (13 May). Direct predation on embryos by females was observed. As a result of the predation, substrate was placed in the tanks at 3 PM on each Monday, Tuesday, and Wednesday and removed at 9 AM on Tuesday, Wednesday, and Thursday, respectively, for the remainder of the study. Substrate trays with embryos were labeled and placed in a separate hatching tank. Fish were fed each day immediately following removal of the substrate tray, and feed was removed prior to addition of the substrate. All groups were spawned over a 2-week interval. The rate of embryo predation during week 1 for Treatment 1 is unknown. We assumed that the rate of embryo predation was equivalent for Treatment 1 week 2, Control weeks 1 and 2, Treatment 2 weeks 1 and 2, and Treatment 3 weeks 1 and 2.

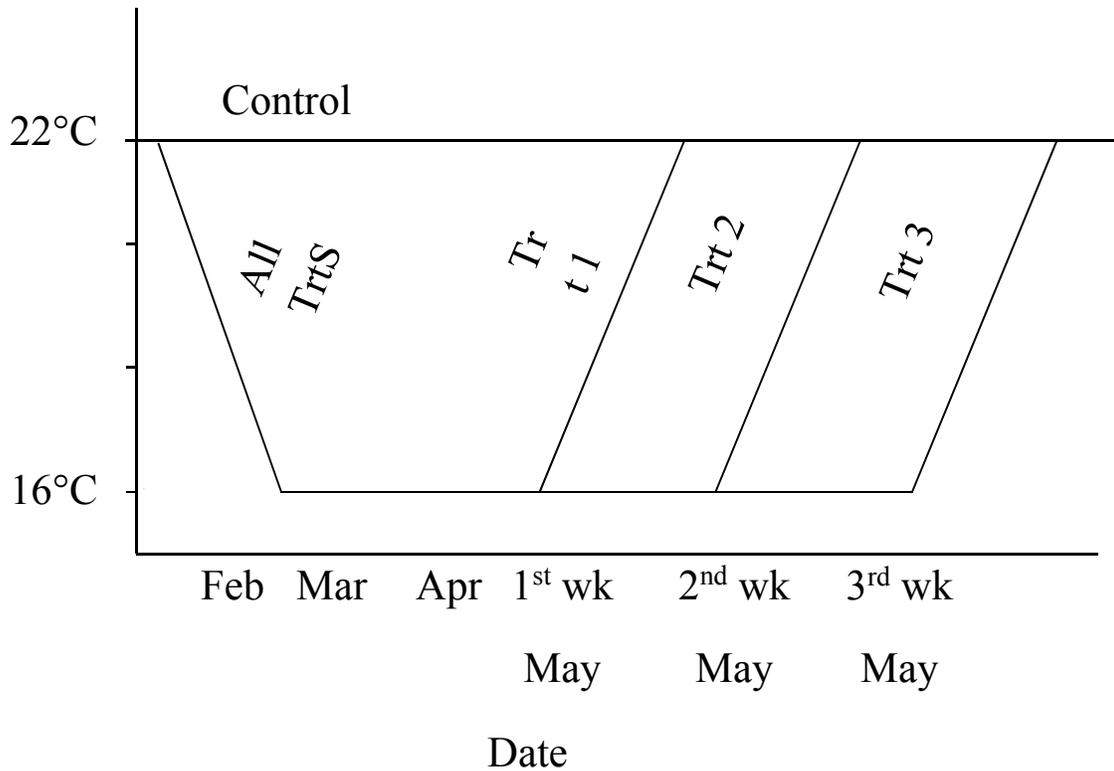


Figure 2. Temperature regimes for control and treatment groups of adult woundfin to entrain the maturation cycle. Control group was maintained at 22°C throughout the spawning season and was spawned the second week of May. Temperature in the 3 treatment groups was slowly dropped to 16°C over a one-week period after transfer to the thermal unit in February. Temperature was then raised over a one-week period from 16 to 22°C the first week of May in Treatment 1, the second week of May in Treatment 2, and the third week of May in Treatment 3. The temperature in the assigned tank of males was ramped simultaneously with the respective female tanks.

Due to daily feeding, fungal outbreaks did occur during week 2 for Treatment 1 and week 1 for Treatment 2 and the Control. As the blastopore was observed in the embryo, we assumed the fungus was due to feces accumulation within the tray and not poor egg quality or fertilization success. We developed a washing technique for each tray prior to placement in the hatching tank, and fungus decreased latter during the second week of May and throughout the remainder of the study. As a result of the fungus, we counted both fungal-infected embryos and hatched larvae. Fungal-infected embryos once counted were removed from the substrate tray.

In 2011, the thermal regime experiment was conducted simultaneously at the BFTC, SNARRC, and Bubbling Ponds. All facilities maintained the same water temperature profiles and photoperiod. In January, 30 woundfin (1:2 sex ratio using body conformation as an indicator of sex) were placed into each of two tanks (60 fish total). The water temperature was decreased (no more than 1°C per day) to 8-9°C depending on the facility. One tank was randomly assigned as the control tank and the other tank as the treatment tank. The control

tank had a naturally vernalized water temperature profile throughout the spring (8-22°C), while the treatment tank had a temperature profile to entrain the maturation cycle (8-16°C followed by a quick ramp from 16-22°C).

During the last full week of May, the water temperatures were ramped in the treatment tank from 16 to 22°C (1°C/day). The following Monday (May 30) after the water temperature reached 22°C, spawning substrate was placed in both the control and treatment tanks by 2 PM. On Tuesday (May 31) morning, the substrate tray was removed, rinsed of feces, and placed in a separate incubation/hatching tank (22°C). The control and treatment substrate trays were kept in separate incubation/hatching tanks. Fish were fed several pinches of feed in the morning after removal of the substrate tray. Tanks were cleaned prior to addition of the fresh spawning substrate by 2 PM each day. Fish were placed on a normal feeding regime over the weekend, and the spawning regime was repeated the next week.

Results and Discussion

The total number of fungal-infected embryos and hatched larvae collected in 2010, herein referred to as the “spawn” is shown in Table 1. The total spawn was very low in Treatment 1 due to the unregulated predation upon embryos during week 1, and as a result, no further comparisons with Treatment 1 were made. The spawns in Treatment 2 and 3 were much higher compared to the Control. These results suggest that the maturation cycle of woundfin can be entrained using temperature to increase the number of eggs spawned by females. The largest spawns occurred during the third week of May in both Treatments 2 and 3, suggesting that this may be the optimal time to spawn woundfin given the temperature treatment and photoperiod.

Table 1. The total number of fungal-infected embryos and hatched larvae referred to as “spawn” in woundfin exposed to four different treatment conditions at the Bozeman Fish Technology Center in 2010. Fish were allowed to spawn over two consecutive weeks (3 days each week). The asterisk denotes unregulated predation rate in Treatment 1.

Treatment Group	Spawn
Control (constant 22°C)	105
Treatment 1 (ramp 16-22°C first week May)	42*
Treatment 2 (ramp 16-22°C second week May)	1,180
Treatment 3 (ramp 16-22°C third week May)	1,769

If all 30 females per treatment group spawned in 2010, 1.4 eggs/female were spawned in the Control group, 39 eggs/female were spawned in Treatment 2, and 59 eggs/female were spawned in Treatment 3. Given the low number of eggs spawned per female, it would seem

that not all females within a treatment group spawned. We would like to explore if sex ratio in a tank is a key driver in natural spawning success in the future.

In 2011, fish did successfully spawn at the BFTC and SNARRC but not at Bubbling Ponds. At the BFTC, 2.7% more eggs were spawned in the treatment group compared to the control. At SNARRC, significantly more eggs were spawned in the treatment group compared to the control group. The SNARRC did apply the ramping temperature to their main production and reported that the maturation cycle was compressed. Three times more young-of-year woundfin were produced in 2011 (~15,000 young-of-year) compared to the average production of other years (~5,000 young-of-year).

As of 2012 spawning season, SNARRC has incorporated the thermal regime and removal of larval fish into a separate pond from the spawning pond (see Task 9). As a result, ~30,000 young-of-year woundfin were produced in each 2012, 2013, and 2014.

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Task 6. Determine dietary protein and energy needs of juvenile woundfin cultured at three temperatures

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Introduction

Limited information is available on the nutritional requirements of woundfin and therefore no commercial feeds are available specifically formulated for woundfin. Current feeds utilized for woundfin culture include a diet developed for the Rio Grande Silvery Minnow or trout feeds. These feeds contain protein levels in excess of 45% crude protein and 14% lipid and are notably higher than required nutrient levels for other genera in the family *Cyprinidae* for which nutrient requirements have been determined (Lochmann and Phillips 2002; Takeuchi et al 2002). Although feed costs are not a limiting factor in rearing fish for population restoration, the effects of metabolic waste on the rearing environment and excessive energy deposition in the fish may not be optimal for culture and final fish health.

It has been widely established that culture temperature is a major factor affecting fish growth due to their poikilothermic nature. Woundfin culture is conducted primarily in ponds where water temperature cannot be controlled. Several researchers have addressed the effects of temperature on the biology of woundfin. Deacon et al (1987) determined that the critical thermal maximum (defined as loss of equilibrium) was 39.5 °C for woundfin acclimated to 25 °C while Addley et al 2005 determined thermal limits (defined as weight loss) to be above 4.6 and below 32.4 °C for lower and upper limits, respectively. Addley et al 2005 further addressed the optimal temperatures for growth and feed consumption and found both temperatures to be around 23.4 °C with a notable decline in growth occurring when temperature increased to 29.5 °C. Feed consumption did not decline until temperatures exceeded 32.4 °C. This suggests the possibility that the metabolic rate of woundfin was up regulated at the higher culture temperatures but feed consumption and thus nutritional plane was not capable of maintaining adequate supplies for metabolic functions and growth.

Optimal diet formulations for woundfin as culture temperatures vary are currently unknown. It may be possible to refine dietary protein and energy levels in order to optimize woundfin growth, feed efficiency, while minimizing metabolic waste as culture temperature varies. Therefore, the current experiment was designed to address the effect of dietary protein and fat alterations on growth performance and nitrogenous waste output from juvenile (age-0) woundfin cultured at three temperatures (20, 24 and 28 °C) spanning the range of culture temperatures observed in spring thru fall in outdoor ponds.

Materials and Methods:

Experimental design. In order to test the level of dietary protein and lipid that supports good growth and minimizes excessive levels of fat deposited in various tissues of woundfin cultured at 3 water temperatures, a 2 by 2 by 3 factorial experimental design was utilized to test the effects of 2 dietary protein levels (45 and 35% crude protein), 2 dietary lipid levels (8 and 16% crude lipid), and 3 culture temperatures (20, 24 and 28 °C) on the growth and nutrient deposition rates in juvenile (Age-0) woundfin.

Diet formulation. Diets were formulated with the same ingredients that are included in the Rio Grande Silvery Minnow formula currently used for woundfin culture. Modifications in inclusion levels were made to yield 45 or 35% digestible protein and 8 or 16% crude lipid in the diets (Table 2). Amino acids were balanced on a digestible amino acid basis (Gaylord et al. 2010). All other nutrients were formulated to meet or exceed known requirements for fish (NRC 1993). The upper level of digestible dietary protein (45%) was chosen to meet the minimum crude protein specified for the Rio Grande Silvery Minnow formula and because no growth differences were observed a juvenile feeding trial (unpublished results) with diets containing up to 62% dietary protein. All diets were mixed utilizing a paddle mixer (Marion Mixers, Inc. Marion, IA), pelleted with a cold-pelleted pasta extruder (Italgi P35A, Italgi US LLC, Spring Valley, NY) through a 1 mm die, and force air dried to <8% moisture (OTW pulse bed drier, Buhler Inc., Plymouth, MN). All diets were stored in a cool dry place until fed.

Fish Culture. Age-0 woundfin were second generation offspring from wild fish obtained from Dexter National Fish Hatchery, Dexter, NM. Woundfin were stocked at 25 fish per tank into a temperature controlled flow through culture system consisting of 36, 100 L rectangular tanks. Temperature was maintained at 20, 24, and 28 °C for each of 12 culture tanks. Water quality parameters were measured to ensure adequate DO, pH, total ammonia, nitrite and nitrate levels were maintained. Diets were randomly assigned to three replicate tanks of fish at each culture temperature. The fish were conditioned to the culture system/temperatures for 2 weeks with temperatures adjusted 1 °C daily to attain treatment temperatures from an initial temperature of 20 °C prior to initiation of the feeding trial and fed a control diet at a rate of 3% body weight per day. Fish were fed 3% bw day⁻¹ their respective diets for 4-weeks then fed 5% bw day⁻¹ 8 weeks. At the beginning of the 8-week feeding trial mean fish weight was 1.28 g ± 0.21 (mean ± sd). Every 4 weeks fish in each tank were counted and weighed to assess growth. At the termination of the trial, all fish were counted and weighed to determine survival and growth rates. Remaining fish were euthanized, ground and assayed for proximate composition (dry matter, crude protein, crude lipid and gross energy).

Water quality and ammonia excretion rates. Water quality parameters (dissolved oxygen, pH, ammonia and nitrite-N) were measured weekly to ensure optimal culture conditions. Four hour ammonia-N excretion rates were determined at the completion of the experiment. In order to obtain a quantifiable ammonia rate all fish in each tank were placed in 36 individual, 1 L Erlenmeyer flasks. Each flask was filled with 800 mL of tank water and submerged in their respective tanks to keep consistent temperatures throughout the test. Air stones, connected to an air pump, were placed in each flask to maintain dissolved oxygen levels. Over a 4 hour period, ammonia was measured initially, at 2 hours and at 4 hours. Total ammonia-N was

measured by the salicylate method (method 8155) utilizing a Hach DR850 colorimeter (Hach Company, Loveland, CO, USA).

Proximate analysis. Proximate analysis of diets and whole body composites of fish were analyzed according to the following procedures. Dry matter was determined according to standard methods (AOAC, 1995). Crude protein (N x 6.25) was determined by the Dumas method (AOAC, 1995) on a Leco Truspec CN determinator (LECO Corporation, St. Joseph, MI, USA). Crude lipid was determined by ether extraction utilizing an Ankom XT10 fat extractor (Ankom ANKOM Technology, Macedon, NY). Gross energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, IL, USA).

Weight gain and feed conversion ratio (FCR) were calculated according to the following formulas:

$$\text{Weight gain} = \frac{(\text{final weight (g)} - \text{initial weight (g)}) \times 100}{\text{initial weight (g)}}$$

$$\text{FCR} = \frac{\text{g dry feed fed}}{\text{g wet weight gain}}$$

Statistical analyses. Differences among response variables were evaluated by factorial ANOVA and deemed significant at $P < 0.05$ (SAS software program Proc GLM, Version 8, SAS Institute, Cary, NC). When significant effects of dietary treatment were detected comparisons were performed using Tukey's means separation.

Table 1. Formulation and proximate composition of diets fed to juvenile woundfin.

Ingredient	Diet			
	45/8	45/16	35/8	35/16
Corn Protein Concentrate	8.00	8.00	6.22	6.22
Squid Meal	7.00	7.00	5.44	5.44
Earthrise Spirulina	11.00	11.00	8.56	8.56
Krill Meal	17.00	17.00	13.22	13.22
Menhaden Fish Meal, Special Select™	5.00	5.00	3.89	3.89
Wheat Gluten Meal	5.00	5.00	3.89	3.89
Chicken 42, American Dehydrated Foods	14.40	14.40	11.20	11.20
Wheat Starch Pre-gelatinized	10.00	10.00	10.00	10.00
Wheat Starch (Non-Gelatinized)	12.97	4.97	25.86	17.86
Menhaden Fish Oil	0.26	8.26	1.87	9.87
Lecithin – Alcolec S	1.00	1.00	1.00	1.00
Ascorbic Acid Polyphosphate, Stay-C 35	0.50	0.50	0.50	0.50
Vitamin Premix ARS 702	3.00	3.00	3.00	3.00
Trace Mineral Premix, ARS 640	0.10	0.10	0.10	0.10
Dicalcium Phosphate	1.20	1.20	2.10	2.10
Choline Cl 50%	1.00	1.00	1.00	1.00
DL-Methionine	0.17	0.17	0.14	0.14
Lysine HCl	1.82	1.82	1.43	1.43
Taurine	0.50	0.50	0.50	0.50
Astaxanthin	0.08	0.08	0.08	0.08
Estimated digestible protein	45	45	35	35
Analyzed composition (as-fed)				
Moisture	3.31	2.07	3.07	2.88
Crude Protein	49.74	50.73	43.18	37.03
Crude Fat	9.54	16.9	9.3	15.6
Gross Energy	5005	5450	4893	5174

Results and Discussion

Weight gain of woundfin reared on two dietary protein concentrations and two dietary lipid concentrations at three temperatures are shown in Figure 1. Dietary protein affected growth rate with 45% protein diets supporting greater growth than 35% protein diets when fed to woundfin juveniles. Lipid concentration of the diets had no effect on fish growth rates. Woundfin grew to a greater final fish mass during the experiment when reared at 28 °C compared to either 24 or 20 °C at which temperatures the fish had equivalent growth rates. No interactions were observed between diet protein, lipid or culture temperature on growth rates of woundfin.

Compositional analyses of woundfin at completion of the feeding trial are reported in Table 2 on a wet weight basis. Dry matter content of woundfin was influenced by culture temperature but was not by diet composition, either protein or fat. Fish fat content increased from 20 to 25% as culture temperature increased from 20 to 28 °C. Fish protein content decreased from 13 to 12.3% as culture temperature increased. Whole body energy mimicked the increase in whole body fat and increased with increasing culture temperature. Whole body energy was higher for fish consuming 45% protein diets compared to 35% protein diets. Culture temperature influenced all response variables for body composition of woundfin. Increasing temperature increased the dry weight of woundfin by increasing the deposition of fat from 20.8% to 25% which yielded concomitant increase whole body energy levels. Increasing temperature decreased protein concentrations in whole body composition of woundfin from 13.3 to 12.3% as culture temperature rose from 20 to 28 °C.

Woundfin appear to be unique in the family Cyprinidae in their need for higher dietary protein concentrations to support maximal weight gain. Reduced dietary protein reduced the rate of ammonia excretion but also elevated feed conversion ratios and reduced growth rates. Although feeding the lower protein diets would allow for reduced nitrogen loading into the culture environment, reduced growth by juvenile woundfin would be counterproductive in attaining target stocking size. Dietary lipid had no effect on growth rates, feed conversion or ammonia excretion rates. Current formulations fed to woundfin often contain dietary fat contents around 15% (such as the Rio Grande silvery minnow formulation or Otohime discussed previously). It appears from the current experiment that this level of dietary fat may not be needed to obtain optimal fish growth rates or fish condition. The unresponsiveness of woundfin to dietary fat concentrations, either positive or negative, was unexpected. It is unclear from the research to date whether the lack of any effect of dietary fat levels is due to the general plasticity of metabolism of woundfin or an inability of woundfin to adequately digest and assimilate the higher levels of dietary fat.

Increasing culture temperature from 20 to 28 °C increased growth rates, had no effect on feed conversion ratios, but increased ammonia excretion rates. No interactive effects of dietary protein, dietary lipid or culture temperature were observed across any response variable. The lack of interactive effects between dietary protein, fat and culture temperature was also unexpected. One may have expected to observe an increased demand for dietary nutrients as culture temperature increased as metabolic rate increased to support the elevated growth rates at higher culture temperature. One explanation for the lack of interaction may be that woundfin met the increased demands for nutrients associated with an elevated growth rate

largely by consuming more feed. Measuring feed consumption was not performed in the current trial due to the technical difficulty in obtaining accurate estimates of feed consumption of 2 g fish. Therefore, the fish were fed a fixed percentage of their body weight per day which appeared to be in slight excess, and it was indiscernible how much in excess each treatment group was fed.

Based on the results, it appears that juvenile woundfin feeds should be formulated to contain a minimum of 45% digestible protein (based on trout digestibility data approximately 50% crude protein utilizing the ingredients chosen for this trial) to optimize fish growth rates and feed efficiency. Dietary fat levels can be reduced to 8% compared to some of the current formulations utilized for woundfin culture. Although culture temperatures largely cannot be regulated in pond production systems where a majority of woundfin culture occurs, currently, a culture temperature of 28 °C supports good growth and feed efficiency of juvenile woundfin.

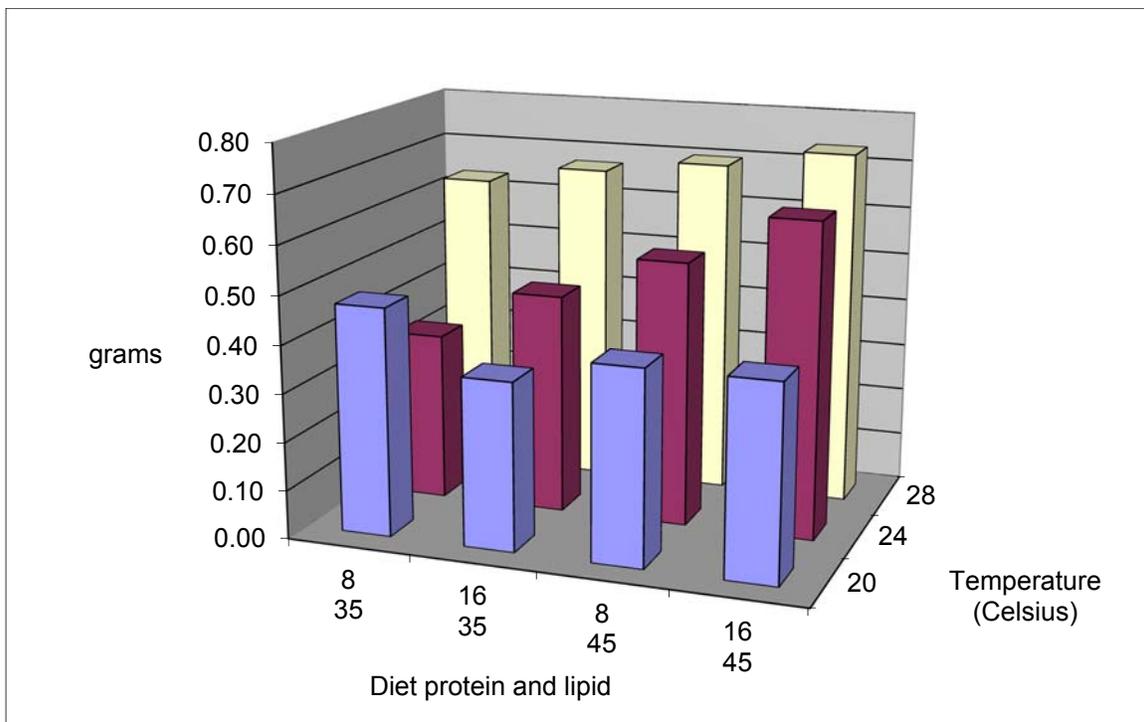


Figure 1: Woundfin weight gain (g) per fish consuming diets with variable dietary protein and lipid concentrations reared at three temperatures.

Table 2. Growth, feed conversion and ammonia excretion by juvenile woundfin fed diets containing differing protein and lipid when reared at three temperatures.

Treatments													Pooled SEM	Pr>F ¹				
Temperature	20	20	20	20	24	24	24	24	28	28	28	28						
Protein	35	35	45	45	35	35	45	45	35	35	45	45						
Lipid	8	16	8	16	8	16	8	16	8	16	8	16						
														Temp	Protein	Fat		
Initial weight	1.51	1.70	1.42	1.54	1.77	1.63	1.40	1.53	1.74	1.69	1.60	1.47	0.116	² P=0.3689				
Final weight (g)	1.99	2.05	1.94	1.95	2.12	2.09	1.95	2.18	2.37	2.36	2.29	2.20	0.075	0.0001	0.0909	0.5513		
Weight gain (g)	0.47	0.35	0.52	0.40	0.35	0.46	0.55	0.65	0.63	0.67	0.70	0.74	0.076	0.0003	0.0251	0.8602		
Percent Increase	31.34	22.07	36.62	25.84	20.81	30.91	39.71	43.79	36.40	40.08	44.31	52.23	6.865	0.0222	0.0172	0.8112		
Specific Growth Rate (g/fish/day)	0.49	0.35	0.56	0.41	0.33	0.47	0.59	0.64	0.56	0.60	0.65	0.74	0.091	0.0227	0.0168	0.9163		
Feed Conversion Ratio	1.94	3.31	1.56	2.12	3.41	2.21	1.57	1.39	1.58	1.48	1.29	1.16	0.608	0.1152	0.0313	0.9119		
Condition Factor	0.81	0.77	0.81	0.80	0.76	0.80	0.77	0.80	0.82	0.82	0.82	0.81	0.018	0.0269	0.7495	0.8313		
Ammonia Excretion Rate (mg/kg BW/h)	4.84	4.44	5.74	5.83	5.67	5.55	8.61	7.83	6.79	7.55	9.07	9.23	0.685	0.0001	0.0001	0.8505		

¹ Probability associated with the F statistic for factorial ANOVA. No interactions occurred between main effects; therefore, if P≤0.05 for the main effect then differences noted below P-values for treatments.

² P-value for full model effects to test for differences in fish weight at initiation of the experiment.

Table 3. Whole body proximate composition of juvenile woundfin fed diets containing differing protein and lipid when reared at three temperatures.

Treatment													Pooled SEM	Pr>F ¹		
Temperature	20	20	20	20	24	24	24	24	28	28	28	28		Temperature	Protein	Fat
Protein	35	35	45	45	35	35	45	45	35	35	45	45				
Lipid	8	16	8	16	8	16	8	16	8	16	8	16				
Dry Matter (%)	38.3	39.7	39.0	38.7	40.8	39.6	39.9	42.3	42.6	44.2	44.0	44.0	0.837	0.0001	0.3832	0.2053
Crude Fat (%) ²	20.7	21.2	20.9	20.2	21.5	21.3	22.4	23.4	23.2	24.8	26.7	25.3	0.932	0.0001	0.0664	0.8000
Crude Protein (%) ²	13.4	13.0	13.5	13.4	13.2	12.6	12.9	12.6	12.5	12.5	12.0	12.1	0.317	0.0005	0.6467	0.3112
Gross Energy (cal/g) ²	2821	2809	2814	2827	2863	2941	3011	3206	3118	3233	3246	3247	78.76	0.0001	0.0493	0.1671
														20<24<28	35<45	

¹ Probability associated with the F statistic for factorial ANOVA. No interactions occurred between main effects; therefore, if P≤0.05 for the main effect then differences noted below P-values for treatments.

² Expressed on a wet weight basis.

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Task 8 and 15.

Effects of Ultraviolet-B Radiation on Woundfin Embryos and Larvae with Application to Conservation Propagation

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Abstract

Endangered woundfin *Plagopterus argentissimus* embryos and larvae were exposed to artificial ultraviolet-B (UV-B) radiation to directly examine the effects on mortality. The experiment was part of a project assisting the Virgin River Resource Management and Recovery Program's efforts to increase hatchery production of this endangered fish. The UV-B radiation used in this experiment was administered in treatments of 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25% of the ambient irradiance levels documented in outdoor tanks and living streams at Bubbling Ponds State Fish Hatchery, in Arizona. Embryos and larvae were exposed for 14.5 h followed by 9.5 h of darkness, in correspondence with the daylight hours at Bubbling Ponds. No embryos survived UV-B treatments; mortality among control (UV-B-free) treatments varied (5–100%) among females, indicating that there may be important parental effects that influence embryo mortality. Larval mortality was also 100% for individuals exposed to any of the three UV-B treatments. In contrast to embryo trials, larval mortality in UV-B-free treatments approached 20% for 2-d-old larvae. These experiments provide evidence that woundfin embryos and larvae are sensitive to even low levels of UV-B when exposed for 14.5 h. Susceptibility of larvae to UV-B also appears to be a function of age at exposure, with older larvae exhibiting significantly lower levels of mortality during the initial days of exposure. Experiments with UV-B mitigation strategies indicated that shade cloth, Aquashade®, and elevated dissolved organic carbon can aid in the attenuation of UV-B, and these strategies may assist hatchery managers in implementing UV-B mitigation measures during periods when woundfin are most susceptible.

Keywords: woundfin; embryo; larvae; UV-B; UV-B mitigation strategies; ultraviolet radiation; *Plagopterus argentissimus*

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Introduction

The steady and significant depletion of stratospheric ozone has produced a measurable increase in the levels

of ultraviolet-B (UV-B; 280–320 nm) radiation entering the earth's freshwater habitats (Cracknell and Varotsos 2009). Measurements of elevated UV-B have been accompanied by studies documenting the deleterious

effects of UV-B on susceptible life stages of aquatic organisms (Häder et al. 1995; Bancroft et al. 2007; Dong et al. 2007; Romansic et al. 2009; Calfee et al. 2010). Studies conducted with fish embryos and young larvae show increased mortality and impaired development as a result of exposure to ambient and elevated UV-B (Hunter et al. 1979; Kouwenberg et al. 1999; Häkkinen et al. 2002; Weigand et al. 2004; Dong et al. 2007), and combined, these factors may limit recruitment to adult populations (Béland et al. 1999). For example, recent evidence shows that Atlantic cod *Gadus morhua* embryos are particularly susceptible to UV-B radiation during gastrulation (Kouwenberg et al. 1999).

The principal effect of UV-B on fish and other aquatic organisms is the formation of pyrimidine dimers that manifest as lesions and alter the structure of DNA (Douki 2010). Specifically, pyrimidine dimers inhibit replication and transcription of the DNA (Buma et al. 2003) and can lead to deformities and/or increased mortality (Bancroft et al. 2007). Because of the harmful and potentially lethal effects of UV-B, many organisms have physiological strategies that assist in repairing or preventing UV-B induced damage (Häkkinen et al. 2002; Wiegand et al. 2004; Bancroft et al. 2007) or have altered activity patterns (e.g., seeking UV-B-free refugia or modifying periods of activity) to minimize exposure to elevated UV-B (Ylönen et al. 2004; Garcia et al. 2009). Despite the documented effects of UV-B, studies to date have been limited to a relatively small number of aquatic vertebrates.

The effects of UV-B on woundfin *Plagopterus argentissimus* are unknown. The woundfin is native to the lower Colorado River basin, but the combined effects of habitat loss, flow alteration, and competition with invasive species have reduced the woundfin's distribution to a fraction of its historic range (USFWS 1994). Woundfin are now restricted to reaches of the mainstem Virgin River (USFWS 1994) and were listed as endangered in 1970 (USFWS 1970), a designation that provides protection under the Endangered Species Act (ESA 1973). Recovery efforts for the federally endangered (ESA 1973) woundfin include captive rearing at three facilities: Southwestern Native Aquatic Resources and Recovery Center, New Mexico; Bubbling Ponds State Fish Hatchery (Bubbling Ponds), Arizona; and Wahweap State Fish Hatchery, Utah. The principal goal of the Virgin River Resource Management and Recovery Program (Recovery Program) is to successfully rear woundfin at these facilities for repatriation to its former range. The Recovery Program has a restocking program currently underway that calls for as many as 100,000 10-mo-old woundfin to be produced annually (S. Meisner, Virgin River Resource Management and Recovery Program, personal communication).

The culture practices and conditions for rearing woundfin at the production facilities increase the likelihood that embryos and larvae are exposed to UV-B. The production of woundfin at the aforementioned facilities is done through the use of outdoor ponds and streams. These habitats lack the physical complexity of natural habitats and have low concentrations of dissolved organic carbon (DOC); organic debris and DOC aid in blocking or attenuating UV-B (Williamson et al. 1996;

Bukaveckas and Robbins-Forbes 2000). At Bubbling Ponds, few viable woundfin larvae have been found in outdoor ponds and streams following the spawning season. Given the reported effects of UV-B on developing embryos and larvae, we hypothesized that UV-B may contribute to poor survival at the conservation propagation facilities. To test this hypothesis, we examined how varying levels of UV-B affect captive-reared woundfin embryo mortality immediately following fertilization and larval mortality at different ages posthatch. Ambient UV-B radiation at Bubbling Ponds during the woundfin spawning season was documented in 2011, and we used these ambient levels as a guide for our study. Accordingly, three UV-B irradiance levels were included to better understand what UV-B levels are lethal to woundfin. Additionally, we also explored the use of three UV-B mitigation techniques that could be used to reduce incoming UV-B levels on captive reared woundfin.

Materials and Methods

Ultraviolet-B exposure chamber

We established the UV-B treatments using a four-chamber exposure system. This system was composed of an aluminum water bath (35.5 cm × 26.2 cm × 121.9 cm) separated into four discrete chambers using sheets of 2-mm fiberglass-reinforced plastic that were secured to the water bath with a silicone sealant. The system was located in a quarantine room at Bozeman Fish Technology Center, and therefore, the system had a dedicated water system. Water flowed through the system at a rate of 7.5 L/min and the temperature averaged 21.1°C (range: 18.5 to 24.5°C) for all trials. At the initiation of the experiments, source water pH was 8.7, NO₃-N concentration was 0.01 mg/L, NH₃-N concentration was below detection levels, and the DOC was 6.9 mg/L. Temperature loggers (iButton, Maxim, Sunnyvale, CA) were also used to document water temperatures within each of the four exposure chambers.

In 2011, we designed treatments to simulate ambient UV-B levels (approximately 0.060 mW/cm²) measured at Bubbling Ponds facility during spawning. We took measurements of UV-B at a depth of 60 cm, where embryos have been detected in previous years. Treatment levels in this experiment were 0.060, 0.030, 0.015, and 0.000 mW/cm², designed to simulate 100, 50, 25, and 0%, respectively, of the ambient UV-B levels documented at Bubbling Ponds. We used a UV-B-free treatment (0% of ambient UV-B) as a control in all trials. We made UV-B measurements using a 2100 PMA (personal measuring assistant) meter and a 2102 UV-B detector (Solar Light, Philadelphia, PA).

The three different UV-B irradiance levels were achieved by suspending one UV-B-313-EL light bulb (Q-Lab, Cleveland, OH) and one Verilux Instant Sun Full Spectrum model F40T12SUN light bulb (Verilux Inc., Stamford, CT) in a common ballast established at different distances from test embryos or larvae held in incubation cups. The UV-B-313-EL bulbs produce a small level of detrimental UV-C (< 280 nm) radiation that is not present in natural systems because stratospheric ozone

absorbs UV-C before it reaches the earth's surface (Lepre et al. 1998). Pyrex glass dishes have been shown to be an effective filter of UV-C radiation (Lepre et al. 1998), and we used Pyrex dishes in follow-up trials (completed in 2012) to confirm that mortality observed in 2011 was not UV-C related.

We constructed incubation cups using a 5-cm-diameter polyvinyl chloride coupler with a nylon screen (500 μm) inserted 3.8 cm from the bottom of the coupler. The control chamber consisted of two Verilux full-spectrum light bulbs suspended at a distance equal to the 50% UV-B treatment. We randomized the order of treatments between trials by reestablishing unique irradiance levels in each chamber. We set the photoperiod at 14.5 h light and 9.5 h dark, which was maintained with an automatic timer. This photoperiod corresponded to the average number of daylight hours during spawning season at the Bubbling Ponds rearing facility. We documented variation in irradiance levels for the entire 14.5-h irradiance period once for each of the four UV-B treatments.

For both embryo and larval trials, we checked woundfin daily for mortality until there were no embryos or larvae remaining in the treatment chambers or until the trial was over. We removed incubation cups containing embryos from the system (embryos were maintained in water during transport and assessment) and checked for mortalities under a dissecting microscope. We conducted observations of mortality for larvae during larval trials by shining a light-emitting diode light with a red filter over the tops of the cups and documenting the number dead. We recorded the number of mortalities for each incubation cup, and removed nonviable embryos and larvae to eliminate mortality caused by fungus spreading from nonviable woundfin to viable woundfin resulting in non-UV-B related mortality.

Spawning

Woundfin embryos and larvae in both 2011 and 2012 were progeny from the 2008 and 2009 year class (origin: Southwestern Native Aquatic Resources and Recovery Center) maintained in indoor tanks at the Bozeman Fish Technology Center. Before the start of each embryo trial, we hormonally injected 30 fish (20 females and 10 males) with 20 $\mu\text{g/g}$ carp pituitary extract (Argent Chemical Laboratories, Redmond, WA). Twenty-four hours postinjection we strip-spawned the fish. The eggs were immediately fertilized, and fertilization success was assessed (presence of first cleavage) within 30 min.

Embryo trials

We conducted embryo trials using multiple females and approximately 20 embryos from each female per UV-B treatment. To avoid density-related mortality, we split embryos from each female between two incubation cups (approximately 10 embryos per cup; see *Supplemental Material, Figures S1, S2, S3, S4, S5 and S6*) for each treatment. For all trials, we attempted to include embryos from three females. When successful (trials 3, 4, and 5), this resulted in a total of 240 embryos per trial: 20 embryos per

female \times three females \times three UV-B treatments and one control treatment. We summarized mortality results by female by pooling the mortality information across both cups. For trials 1, 2, and 6, we were only able to successfully spawn two females; inclusion of only two females in these trials resulted in the use of 160 embryos (20 embryos per females \times two females \times two UV-B treatments and two control treatments).

For embryo trials 1 and 2 in 2011 and trial 6, conducted in 2012, we ran the experiment for 5 d. In trials 3, 4, and 5 in 2011, we carried out the experiments for 9, 11, and 10 d, respectively. We chose to use a more protracted observation period in later trials to more carefully assess mortality of control embryos. Because we saw no mortality in control embryos past 5 d, all data are reported up to 5 d. The UV-C was not blocked in trials 1–5 but was for trial 6.

Trial 6, which was conducted in 2012, included Pyrex dishes filtering out UV-C radiation. A total of 160 embryos were collected from two different females, and 10 embryos were placed into each of the 16 incubation cups. This resulted in fewer incubation cups per exposure chamber than in the 2011 trials but we were constrained by a limited number of embryos available in 2012.

Larval trials

We placed embryos not used for embryo trials in 10-cm incubation jars and allowed them to hatch. After hatching, we put larvae into 61-cm-diameter fiberglass tanks with 20 cm of water. The tanks contained larvae from multiple females and therefore contained a mixture of progeny. In trials 1 and 2, we exposed larvae to UV-B 2 d after hatching. For trials 3, 4, and 5, exposed larvae were 5–6 d, 27–30 d, and 41–44 d posthatch, respectively. For the larval trials, we lowered water levels to 5.4 cm inside the exposure chambers to prevent larvae from swimming out of the incubation cups. Because of the reduction in water levels, we were unable to produce a treatment that was 25% of the ambient UV-B levels documented at Bubbling Ponds. Treatment levels for larval trials were limited to 100, 50, and 0% of the ambient intensity. With the exception of trial 1, we used three incubation cups per exposure chamber for larval trials; this resulted in a total of nine cups and 45 larvae per trial (five larvae per cup \times three cups per treatment \times two UV-B treatments and one control treatment). In trial 1, a limited number of larvae available restricted the number of incubation cups to one per treatment (five larvae per cup \times one cup per treatment \times two UV-B treatments and one control treatment).

In the final two larval trials (trials 4 and 5), we produced a second 0% treatment using multiple sheets of acetate (nine sheets) to shield the larvae from UV-B emitted from the combination of UV-B-313-EL and full-spectrum light bulbs. This 0% UV-B control contrasted with the other 0% UV-B irradiance produced using two full-spectrum bulbs. Because of this additional treatment, these final two 2011 larval trials required an additional three incubation cups and an expanded total of 60 larvae (five larvae per cup \times three cups per treatment \times two UV-B treatments and two control treatments). These

trials also allowed us to compare mortality among the two different 0% UV-B treatments and assess whether bulb type (full-spectrum vs. UV-B bulbs) contributed to the results in our 0% UV-B treatments. We used Pyrex dishes to shield UV-C only in the 2012 embryo trial (trial 6). We did not use Pyrex dishes in any of the larval trials.

Ultraviolet-B mitigation strategies

We examined the ability of three different UV-B mitigation strategies: 90% black knitted shade cloth, Aquashade® aquatic dye, and DOC (introduced as sucrose [C₁₂H₂₂O₁₁]) to attenuate UV-B. We briefly describe the experiments for each strategy below. All experiments were conducted outside in 3.5-m³ tanks (external dimensions 1.2 m × 2.4 m × 1.2 m) that were filled to a depth of 60 cm to mimic water depths present at Bubbling Ponds rearing facility and using source waters described above.

Aquashade. Prior to introduction of Aquashade, we took UV-B measurements in untreated source waters at the surface and at a depth of 60 cm in order to obtain a ratio between the two measurements. We then introduced Aquashade in aliquots that would increase the concentration of the dye in the tank by 1 mg/L. After its introduction, we mixed Aquashade for 5 min and allowed it to dilute evenly for approximately 1 h. After 1 h, we took UV-B measurements at a depth of 60 cm and at the surface. We used the surface reading to document any possible changes in ambient UV-B that occurred over the course of 1 h. By using the surface UV-B measurement and the ratio of UV-B absorbed in 60 cm of untreated water, we were able to calculate UV-B levels at a 60-cm depth in untreated water. This step was important because we conducted the experiments outside and UV-B levels were subject to natural fluctuations. After we recorded readings for each Aquashade concentration, we raised the concentration 1 mg/L, and repeated the steps until we reached a final concentration of 9 mg/L. We repeated this experiment in triplicate for concentrations ranging from 1 to 4 mg/L and only once for the remaining concentrations up to 9 mg/L.

Dissolved organic carbon. Natural levels of DOC in aquatic environments lead to the attenuation of UV-B in freshwater (Morris et al. 1995; Williamson et al. 1996). We introduced sucrose to manipulate DOC in source waters. Our approach for measuring and estimating UV-B attenuation and increasing DOC concentrations followed the methods described for treatments of Aquashade. Although sucrose alone does not reflect the natural complexity of DOC in the environment, it provided an opportunity for us to examine how the intentional manipulation of DOC may influence UV-B levels in outdoor rearing facilities. We manipulated DOC levels increasing concentrations in DOC between background levels in source water to background + 6.5 mg/L DOC as sucrose. We repeated the entire experiment twice.

Shade cloth. We characterized the reduction in UV-B provided by 90% black knitted shade cloth (EnviroCept Greenhouse & Supply, Benton City, WA). We floated shade cloth at the water surface and held it in place during measurements. As described previously, we

completed measurements at a depth of 60 cm and at the surface both before and after the treatments were imposed to assist in documenting natural changes in UV-B in addition to quantifying the treatment effect.

Data analysis

We used fixed effect one-way analysis of variance models to characterize differences in the mortality of embryos or larvae among UV-B treatments for each trial and across trials to examine differences in mortality for individual exposure days. In the embryo trials, we used females as the unit of replication. In the larval trials, mixed progeny prevented us from tracking larvae by individual, and we therefore used the incubation cups as the unit of replication. For some trials, limited sample sizes (larval trial 1 and embryo trials 1, 2, and 6) or lack of within group variation (larval trials 3–5) prevented us from using inferential statistics. In these instances, we used descriptive statistics to summarize mortality by treatment. We arcsine square-root transformed all percentage data prior to analysis (French and Lindley 2000). We used correlation and regression analysis to examine the relationship between larval age and mortality during each 24-h exposure period. Finally, we used a nonlinear exponential decay function to model the attenuation of UV-B with different concentrations of Aquashade and DOC. We analyzed embryo and larval mortality data using Microsoft Excel 2007 software (Microsoft Corporation, Redmond, WA).

Results

Environmental conditions

Water temperatures averaged 21.1°C (range: 18.5 to 24.5°C) across treatments, and the mean measured water temperature differed by less than 0.2°C among the four exposure chambers (21.2, 21.0, 21.1, and 21.0°C). More than 90% of the 535 total readings from each chamber were ≤ 22°C. Time series plots of experimental chamber temperatures showed no indication of a UV-B treatment effect; however, these plots did indicate that there was variation in source water temperature over the course of the 10 trials. Although this variation was measurable, it would have been experienced by all organisms in a trial regardless of the UV-B treatment.

The 100% UV-B treatment had a mean (± 1 SD) irradiance of 0.0563 ± 0.0006 mW/cm² (range 0.0541–0.0583 mW/cm²), which was slightly lower than our target treatment of 0.0600 mW/cm². The 50 and 25% treatments had mean irradiance levels of 0.0297 ± 0.0006 mW/cm² and 0.0164 ± 0.0003 mW/cm², respectively. Although some variation in irradiance levels were documented over the 14.5-h dosing period, the coefficients of variation for the mean irradiance levels were low and similar across treatments (Figure 1); coefficients of variation were 1.64 (25%), 2.02 (50%), and 1.01 (100% UV-B), respectively.

Embryo results

Across all trials, embryo exposure to any level of UV-B resulted in complete mortality (Table 1; Tables S1, S2, S3, S4, S5, and S6, *Supplemental Material*). Mortality of

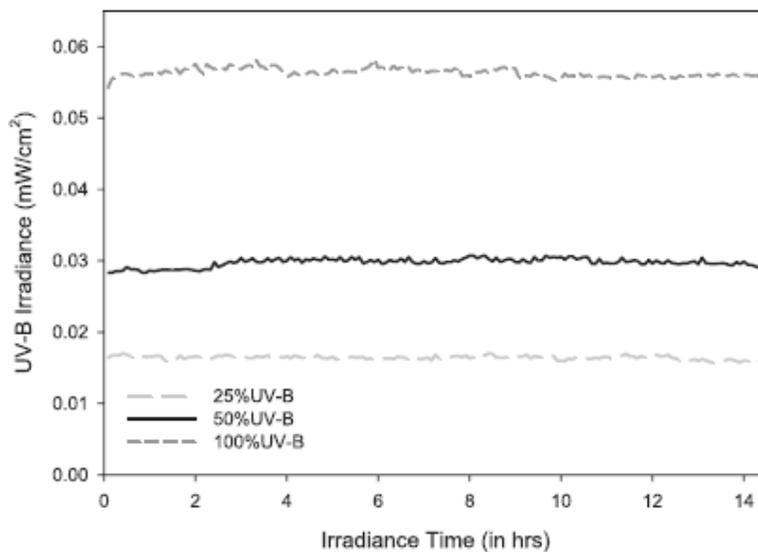


Figure 1. Irradiance levels summarized over the 14.5-h irradiance period for each of the ultraviolet-B (UV-B) radiation treatment levels to which woundfin *Plagopterus argentissimus* were exposed. The treatment levels were 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. The control is 0% UV-B. We exposed woundfin larvae and embryos to UV-B radiation in a controlled experimental exposure chamber at the Bozeman Fish Technology Center in Bozeman, Montana, in June and July of 2011 and 2012.

control treatments (0% UV-B) varied markedly by female and ranged from 5.0% to 100.0% for individual females across all trials; mean embryo mortality was 64.6% in control treatments. Across all 2011 trials and treatments, mortality was highest ($P < 0.001$, $F = 35.576$) on day 2 of UV-B exposure ($76.5 \pm 6.1\%$; Figure 2a). In contrast, mean mortality for day 1 and 3 of UV-B exposure was $11.5 \pm 6.6\%$ and $3.0 \pm 1.3\%$, respectively. Although trials 1 and 2 were carried out for only 5 d, trials 3, 4, and 5 were run for 9, 11, and 10 d, respectively. Results from these latter trials indicate that no additional mortality occurred after 5 d in the control group. Trial 6, conducted in 2012, had the same exposure doses (UV-B treatments

× exposure time) as the 2011 trials but included a Pyrex UV-C filter; UV-B related mortality patterns observed in these trials were nearly identical to trends in mortality documented in 2011 (Figure 2b).

Larval results

Exposure to any level of UV-B resulted in 100% mortality of larval woundfin (Table 2; Tables S7, S8, S9, S10, and S11, *Supplemental Material*). Across all trials, larval mortality differed by day ($P = 0.021$, $F = 3.691$), and mean mortality tended to be highest on days 2 and 3 ($22.7 \pm 9.9\%$ and $21.0 \pm 5.7\%$, respectively). Despite these patterns of mortality, larval mortality was docu-

Table 1. Mean percentage of mortality of woundfin *Plagopterus argentissimus* embryos following 5 d of exposure to four levels of the ambient ultraviolet-B (UV-B) radiation for 14.5 h daily. We exposed woundfin to UV-B radiation treatments of 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 6 at the same dose and exposure time as the previous five trials; however a Pyrex dish was used to eliminate ultraviolet-C (UV-C). We conducted all trials at the Bozeman Fish Technology Center in Bozeman, Montana, from May to July of 2011 and June 2012.

Treatment (% UV-B)	Trial					
	1	2	3	4	5	6 ^a
0%	88.8	95.2	53.5	46.7	57.1	56.1
25%	100.0	100.0	100.0	100.0	100.0	100.0
50%	100.0	100.0	100.0	100.0	100.0	100.0
100%	100.0	100.0	100.0	100.0	100.0	100.0
ANOVA results	— ^b	— ^b	$P < 0.001$, $F = 9.96$	$P < 0.001$, $F = 14.06$	$P < 0.001$, $F = 134.71$	— ^b

^a In trial 6, which was conducted in 2012, we utilized Pyrex dishes to block UV-C for all treatments to determine if UV-C influenced the results from the previous five trials conducted in 2011.

^b Summarized using embryos from only two females. Because of the limited number of replicates, we did not use inferential statistics.

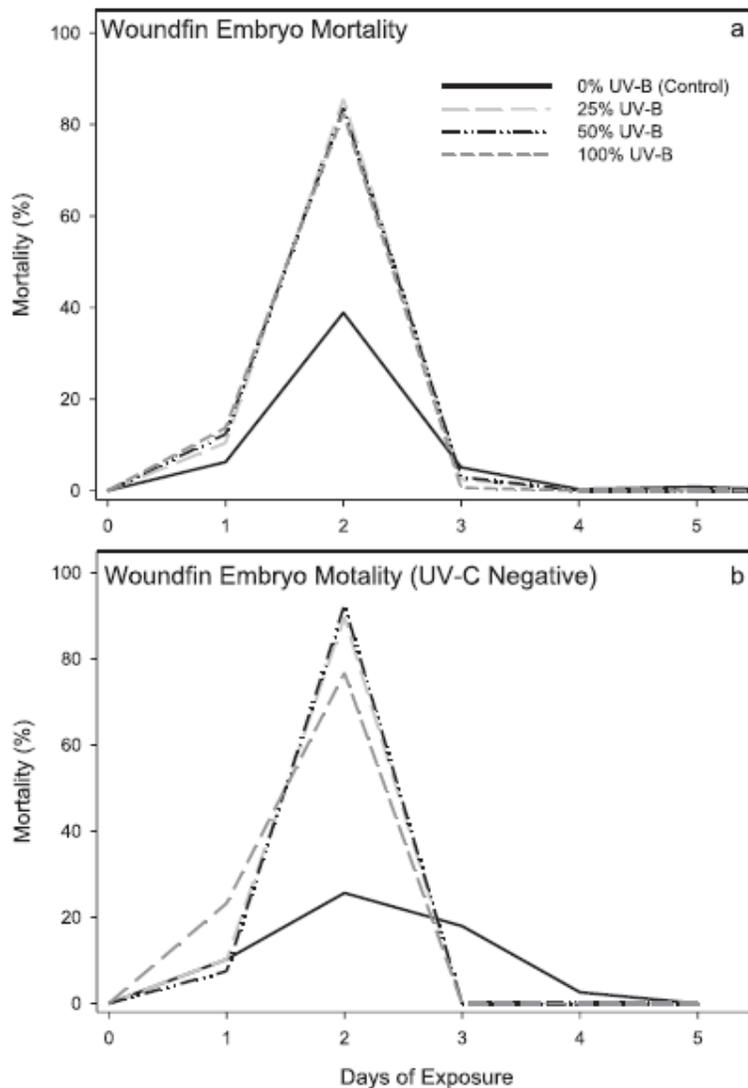


Figure 2. Woundfin *Plagopterus argentissimus* embryo mortality summarized by 14.5-h daily ultraviolet-B (UV-B) treatment over a 5-d exposure period. We exposed woundfin to UV-B radiation treatments of 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25% of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. The control is 0% UV-B. Panel (a) presents results from experimental exposures that did not include an ultraviolet-C (UV-C) shield. Panel (b) presents results from embryos that were covered by Pyrex dishes used to block UV-C across all UV-B treatments and the control. Embryo mortality is expressed as a percentage. We conducted trials in a controlled experimental UV-B exposure chamber at the Bozeman Fish Technology Center in Bozeman, Montana, during June and July of 2011 and 2012.

mented on all 5 d of UV-B exposure (Figure 3; Figure S1, *Supplemental Material*). For the 50% UV-B treatments, no mortality was documented within the first 24 h of exposure and only 1.7% of total mortality was documented on day 2; peak mortality was seen on day 4. In contrast, mortality was greatest ($P < 0.001$, $F = 23.33$) on days 2 ($49.3 \pm 12.0\%$) and 3 ($49.3 \pm 10.9\%$) of 100% UV-B treatments, and by day 5, we documented complete mortality of all individuals exposed to 100% UV-B

treatments. In trials 4 and 5 which contained two 0% UV-B controls, none of the larvae experienced mortality in either method of obtaining 0% UV-B.

Susceptibility of larvae to UV-B appears to be a function of larval age during the first few days of exposure (Figure S2, *Supplemental Material*). Larvae used in these trials varied in age from 2 days in trials 1 and 2 to 41–44 days in trial 5. For the 100% UV-B treatments, larval age explained $\geq 78\%$ of the variation in the

Table 2. Mean larval woundfin *Plagopterus argentissimus* percentage of mortality following exposure to 14.5-h doses at two levels of ultraviolet-B (UV-B) and two different UV-B controls (full-spectrum light or acetate-blocked UV-B). We exposed woundfin to UV-B radiation treatments of 0.060 and 0.030 mW/cm² to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. All trials also included a 0% UV-B control. We carried out all experiments over a 5-d exposure period. Larvae were 2 d old at the start of trials 1 and 2, 5–6 d old for trial 3, 27–30 d old for trial 4, and 41–44 d old for trial 5. We conducted all trials at the Bozeman Fish Technology Center in Bozeman, Montana, in June and July of 2011.

Treatment	Trial				
	1	2	3	4	5
0% UVB (fullspectrum lights)	20.0	13.3	0.0	0.0	0.0
0% UV-B (acetate-blocked UV-B) ^a	NA ^b	NA	NA	0.0	0.0
50% UV-B	100.0	100.0	100.0	100.0	100.0
100% UV-B	100.0	100.0	100.0	100.0	100.0
ANOVA results	— ^c	$P < 0.001, F = 66.65$		— ^d	— ^d

^a A second 0% UV-B treatment was produced by using multiple sheets of acetate (nine sheets), which effectively blocked 100% of the UV-B radiation emitted from a UV-B-313-EL light bulb and full-spectrum bulb pairing used in all > 0% UV-B treatments.

^b NA, not applicable.

^c Limited number of larvae available for this trial (only five larvae per treatment chamber). Because of the limited number of replicates, we did not use inferential statistics.

^d We did not generate *F* statistics because there was no within-group variation (i.e., there was no variation in mortality among experimental units within a given UV-B treatment).

mortality seen in day 2 and day 3. For example, on day 2 of the experiment, documented mortality averaged 66.7% for 2-d-old larvae to 6.7% for 43.5-d-old larvae in the 100% UV-B treatments ($R = -0.887, P = 0.045$).

Ultraviolet-B mitigation strategies

All three UV-B mitigation strategies reduced incoming levels of UV-B. A single treatment of 90% black knitted shade cloth eliminated $92.9 \pm 0.4\%$ of the pretreatment

levels UV-B levels at a depth of 60 cm. In addition, UV-B levels were inversely related with concentration of both Aquashade aquatic dye ($P < 0.001; R^2 = 0.967$) and DOC ($P = 0.002; R^2 = 0.480$; Figure 4). At a depth of 60 cm, an Aquashade concentration of 9.0 mg/L attenuated approximately 95% of pretreatment UV-B. In contrast, DOC concentrations attenuated approximately 20 to 25% of UV-B at concentrations ≥ 4 mg/L (Figure 4).

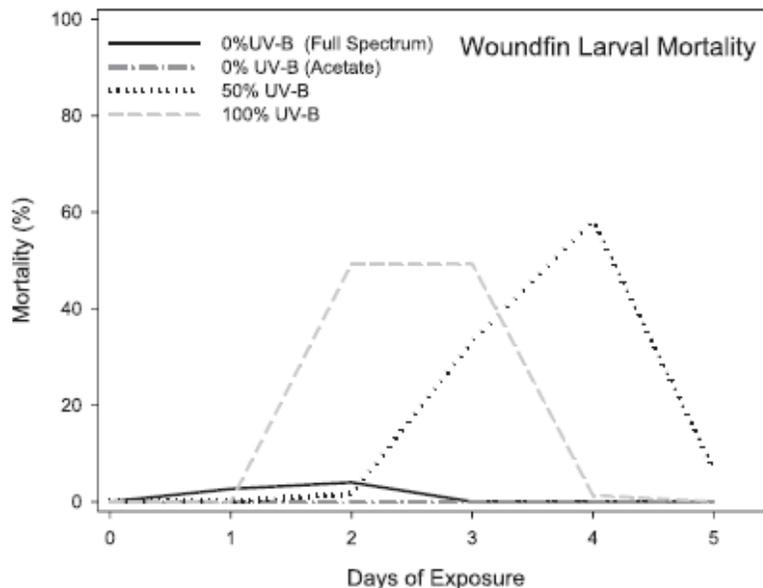


Figure 3. Woundfin *Plagopterus argentissimus* larval mortality summarized by 14.5-h daily ultraviolet-B (UV-B) treatment over a 5-d exposure period. We exposed woundfin to UV-B radiation treatments of 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. The control is 0% UV-B. Larval mortality is expressed as a percentage. We conducted trials during June and July of 2011 at the Bozeman Fish Technology Center in Bozeman, Montana.

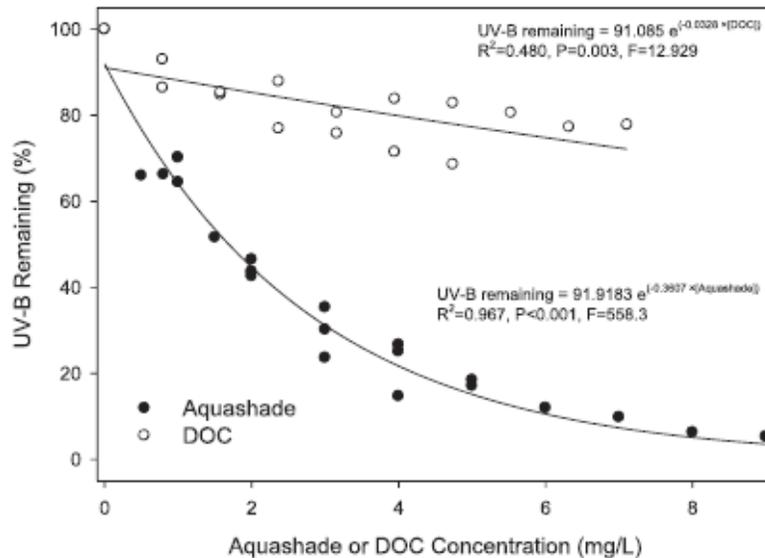


Figure 4. We explored mitigation techniques as methods to reduce incoming ultraviolet-B (UV-B) radiation that woundfin *Plagopterus argentissimus* were exposed to during captive rearing at conservation propagation facilities. The UV-B irradiance is expressed as a function of Aquashade[®] and dissolved organic carbon (DOC) concentrations, which were manipulated using sucrose (C₁₂H₂₂O₁₁). We tested mitigation techniques at the Bozeman Fish Technology Center in Bozeman, Montana, in July 2011. We took all UV-B measurements in outdoor tanks at a depth of 60 cm.

Discussion

There is evidence that UV-B has detrimental effects on early life stages of fishes (Hunter et al. 1979; Kouwenberg et al. 1999; Häkkinen et al. 2002; Weigand et al. 2004; Dong et al. 2007). Specifically, UV-B increased mortality in northern anchovy *Engraulis mordax* (Hunter et al. 1979; Béland et al. 1999; Kouwenberg et al. 1999), Pacific mackerel *Scomber japonicus* (Hunter et al. 1979), bluegill sunfish *Lepomis macrochirus* (Gutiérrez-Rodríguez and Williamson 1999), and zebrafish *Danio rerio* (Dong et al. 2007) and elicited avoidance behavior in whitefish *Coregonus albula* and *Coregonus lavaretus* larvae (Ylönen et al. 2004). We show here that embryos and larvae of the endangered woundfin are sensitive to UV-B at a 14.5-h exposure time, and that all woundfin embryos and larvae exposed to UV-B during our trials experienced mortality. The UV-B-free treatments produced using full-spectrum lights or by shielding woundfin with acetate film resulted in significantly lower levels of mortality. Although woundfin in the 2011 trials were also exposed to UV-C, the trial in which we excluded UV-C with Pyrex dishes showed that the mortality patterns observed were nearly identical to 2011 results and confirmed that mortality was induced by UV-B rather than UV-C. Our work also demonstrates that larvae differ in their sensitivity to UV-B; susceptibility of larvae to UV-B appears to be a function of age at exposure. Here, we show that the youngest larvae (2 d posthatch) experienced an order of magnitude greater mortality than 43.5-d-old larvae after just 2 d of UV-B exposure. This finding is consistent with work with other fish species

(Kouwenberg et al. 1999; Wiegand et al. 2004). For example, goldfish *Carassius auratus* embryo sensitivity to UV-B increased with increased cumulative exposure and embryos that were 25-h postfertilization were the most vulnerable to even a short (2- to 4-h) exposure to elevated UV-B. For zebrafish, the hatching success, incidence of malformations, and mortality varied significantly among embryonic stage classes. Interestingly, a greater incidence of malformations and higher mortality occurred when embryos were exposed more than 3 h postfertilization.

Recent evidence shows that zebrafish embryos raised in outdoor ponds were more tolerant to UV-B than laboratory-raised embryos (Dong et al. 2007). One explanation for the documented differences in zebrafish survival is individual variability in the expression of screening pigments. Screening pigments such as melanin can reduce the effects of UV-B radiation on fish and amphibians (Häkkinen et al. 2002; Garcia et al. 2009). However, Dong et al. (2007) found no measurable differences in zebrafish embryo pigmentation that would explain differences in mortality. Although larval woundfin begin developing visible dorsal melanophores within a few days of hatching (Snyder et al. 2011), both embryos and larvae were vulnerable to UV-B. Larval woundfin raised in outdoor ponds at the aforementioned facilities experience phytoplankton blooms (i.e., Southwestern Native Aquatic Resources and Recovery Center) and therefore may have less pigmentation than larval woundfin raised indoors, a phenomenon observed in razorback sucker *Xyrauchen texanus* (R. Muth, U.S. Fish and Wildlife Service, unpublished data). Alternatively, in

outdoor ponds or streams that are clear (i.e., Bubbling Ponds) and have low DOC levels, woundfin larvae may have more pigmentation than conspecifics raised indoors. Because pigmentation may offer photoprotection, future work should attempt to quantify the pigmentation levels for woundfin used in experiments.

Our results show that the survivable level of UV-B radiation for developing woundfin embryos is < 0.015 mW/cm² or $< 25\%$ of the ambient levels present at Bubbling Ponds at ≥ 14.5 -h exposures. We realize that the environmentally relevant exposure period would be much lower than a 14.5-h exposure, and we have initiated preliminary research to examine the response of woundfin to exposure times and doses that more closely mimic natural, daily changes in UV-B. For example, recent work at the Bozeman Fish Technology Center demonstrates that exposing woundfin to the ambient levels of UV-B radiation found at Bubbling Ponds during the spawning season for ≤ 5 h seems to induce very low UV-B related mortality (M.A.H. Webb, N. Pinkham, and A.M. Ray, unpublished). As a result of these preliminary findings, we believe that the UV-B related mortality threshold for ambient levels of UV-B present at Bubbling Ponds is between 5 and 14.5 h of exposure. Further work is needed to document the actual survivable level of UV-B in order to decrease the negative effects of UV-B radiation on production. In addition, current levels of UV-B at other conservation propagation facilities should be documented to better understand UV-B factors limiting production elsewhere. Furthermore, quantifying levels of UV-B in the Virgin River system at spawning and rearing locations would be beneficial for understanding if UV-B radiation is limiting natural recruitment of woundfin.

Given our results, future work should examine whether reductions in UV-B radiation lead to decreases in woundfin mortality and increases in production in the captive rearing facilities described above. Ultraviolet-B radiation has also been shown to interact synergistically with other stressors (including pH and contaminants; Bancroft et al. 2008). Our data suggest that woundfin embryos and larvae are sensitive to UV-B and could therefore experience higher mortality rates at low UV-B doses if other stressors are present. Reduction in UV-B radiation could be accomplished in a number of ways, including the creation of UV-B-free refugia (i.e., areas of decreased UV-B; Ylönen et al. 2004) in individual rearing ponds.

In summary, our work shows that woundfin embryos and larvae are vulnerable to UV-B levels > 0.015 mW/cm² at temperatures ranging from 18.5 to 24.5°C and that the survivable threshold for exposure time is less than 14.5 h for this dose. This work will help raise awareness for propagation facilities about the effects of UV-B on early developmental stages of the federally listed woundfin but also provide insight on UV-B mitigation strategies that could be deployed to minimize exposure in outdoor rearing facilities.

Supplemental Material

Please note: The *Journal of Fish and Wildlife Management* is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

Reference S1. [USFWS] U.S. Fish and Wildlife Service. 1994. Virgin River fishes recovery plan. U.S. Fish and Wildlife Service, Salt Lake City, Utah.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S1> (2.6 MB PDF).

Figure S1. Woundfin *Plagopterus argentissimus* larval mortality summarized by 14.5-h daily ultraviolet-B (UV-B) treatment over a 5-d exposure period. We exposed woundfin to UV-B radiation treatments of 0.060, 0.030, and 0.015 mW/cm² to simulate 100, 50, and 25% of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. The control is 0% UV-B. Larval mortality is expressed as a percentage. We conducted trials during June and July of 2011 at the Bozeman Fish Technology Center in Bozeman, Montana.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S2> (23 KB DOCX).

Figure S2. Woundfin *Plagopterus argentissimus* embryo mortality at different ages posthatch when exposed to 14.5 h daily ultraviolet-B (UV-B) treatments imitating 100% of the ambient UV-B (0.060 mW/cm²) radiation present at a depth of 60 cm in ponds at Bubbling Ponds State Fish Hatchery, Arizona. All trials were conducted for 5 d. Larvae were 2, 5–6, 27–30, and 41–44 d old when exposed to UV-B. We exposed all larvae to UV-B in an experimental exposure chamber at the Bozeman Fish Technology Center, Bozeman, Montana in June and July of 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S3> (56 KB PDF).

Table S1. Trial 1 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 1 from two females. Ultraviolet-B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 1 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on May 26, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S4> (13 KB DOCX).

Table S2. Trial 2 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 2 from two females. Ultraviolet-B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 2 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 7, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S5> (13 KB DOCX).

Table S3. Trial 3 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 3 from three females. Ultraviolet -B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 3 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 11, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S6> (13 KB DOCX).

Table S4. Trial 4 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 4 from three females. Ultraviolet -B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 4 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 15, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S7> (13 KB DOCX).

Table S5. Trial 5 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 5 from three females. Ultraviolet -B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We conducted trial 5 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 21, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S8> (13 KB DOCX).

Table S6. Trial 6 summary of woundfin *Plagopterus argentissimus* embryo mortality. Mortality data are summarized by female and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We collected embryos for trial 6 from two females. Ultraviolet -B radiation treatments were 0.060, 0.030, and 0.015 mW/cm², and we used these treatments to simulate 100, 50, and 25%, respectively, of the ambient

irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. All treatments contained a Pyrex ultraviolet-C filter to determine if ultraviolet-C influenced the results of trials 1–5. We conducted trial 6 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 15, 2012.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S9> (13 KB DOCX).

Table S7. Trial 1 summary of woundfin *Plagopterus argentissimus* larval mortality. Mortality data are summarized by incubation cup and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We selected larvae from a tank containing mixed progeny from multiple females. Ultraviolet -B radiation treatments were 0.060 and 0.030 mW/cm², and these treatments were used to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. Larvae were 2 d old at the start of trial 1. Each exposure chamber for trial 1 contained only one incubation cup with five larvae because of the limited number of larvae available for this trial. We conducted trial 1 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 3, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S10> (12 KB DOCX).

Table S8. Trial 2 summary of woundfin *Plagopterus argentissimus* larval mortality. Mortality data are summarized by incubation cup and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We selected larvae from a tank containing mixed progeny from multiple females. Ultraviolet-B radiation treatments were 0.060 and 0.030 mW/cm², and these treatments were used to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. Larvae were 2 d old at the start of trial 2. We conducted trial 2 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on June 30, 2011. 3B2\ERS\erj\non-issue 3d\2014-5-14.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S11> (12 KB DOCX).

Table S9. Trial 3 summary of woundfin *Plagopterus argentissimus* larval mortality. Mortality data are summarized by incubation cup and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed UV-B treatments using a 14.5-h exposure period each day. We selected larvae from a tank containing mixed progeny from multiple females. Ultraviolet-B radiation treatments were 0.060 and 0.030 mW/cm², and these treatments were used to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. Larvae were 5–6 d old at the start of trial 3. We

conducted trial 3 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on July 5, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S12> (12 KB DOCX).

Table S10. Trial 4 summary of woundfin *Plagopterus argentissimus* larval mortality. Mortality data are summarized by incubation cup and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed treatments using a 14.5-h exposure time at two levels of UV-B and two different UV-B controls (full-spectrum light [FSL] or acetate-blocked UV-B [acetate]). Ultraviolet-B radiation treatments were 0.060 and 0.030 mW/cm², and these treatments were used to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We selected larvae from a tank containing mixed progeny from multiple females. Larvae were 27–30 d old at the start of trial 4. We conducted trial 4 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on July 11, 2011.

Found at DOI: <http://dx.doi.org/10.3996/042013-JFWM-030.S13> (12 KB DOCX).

Table S11. Trial 5 summary of woundfin *Plagopterus argentissimus* larval mortality. Mortality data are summarized by incubation cup and ultraviolet-B (UV-B) exposure treatment over a 5-d observation period. We imposed treatments using a 14.5-h exposure time at two levels of UV-B and two different UV-B controls (full spectrum light [FSL] or acetate-blocked UV-B [acetate]). Ultraviolet-B radiation treatments were 0.060 and 0.030 mW/cm², and these treatments were used to simulate 100 and 50%, respectively, of the ambient irradiance levels documented in outdoor ponds at Bubbling Ponds State Fish Hatchery. We included a 0% UV-B treatment as a control. We selected larvae from a tank containing mixed progeny from multiple females. Larvae were 41–44 d old at the start of trial 5. We conducted trial 5 at the Bozeman Fish Technology Center in Bozeman, Montana, beginning on July 25, 2011.

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Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Task 9.

Cannibalism of Embryos and Larvae by Adult Woundfin in Intensive Culture: Application to Conservation Propagation

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Abstract

Woundfin *Plagopterus argentissimus* are a small, endangered cyprinid native to the Colorado River basin. Woundfin occur only in the Virgin River in Utah, Arizona, and Nevada, and habitat degradation and competition with invasive species threaten their survival. Three facilities raise woundfin in captivity for use in conservation propagation projects. A suspected limiting factor to pond culture production of woundfin is cannibalistic predation on embryos and larvae. We experimentally measured rates of predation on embryos and larvae by adult woundfin at the Bozeman Fish Technology Center in Montana. Predation was a significant source of mortality on both embryos ($W = 210, P < 0.001$) and larvae ($W = 45, P = 0.004$). These rates of predation could translate into the loss of thousands of fish over the course of a spawning season at the conservation propagation facilities. We recommend removing embryos from spawning ponds and rearing them in separate tanks or ponds to reduce predation loss.

Keywords: *Plagopterus argentissimus*; predation; woundfin

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Introduction

The woundfin *Plagopterus argentissimus* is a small (generally under 75 mm) omnivorous cyprinid in the Plagopterini tribe (Miller and Hubbs 1960; Figure 1). Native to the lower Colorado River basin, the species probably once ranged from the confluence of the Salt and Verde rivers to the mouth of the Gila River in Arizona, and up the Colorado River into the Virgin River and some of its tributaries in Utah (Miller and Hubbs 1960; Hickman 1987). The species faces serious threats from habitat modification and competition with a nonnative species (red shiner *Cyprinella lutrensis*; Deacon 1988; U.S. Fish and Wildlife Service [USFWS] 1995). When the species listed as endangered in 1970 (USFWS 1970), woundfin had already realized an 88% range reduction and persisted only in 141 km of the mainstem Virgin River in Utah, Nevada, and Arizona (USFWS 2008). Since its listing as endangered, woundfin have disappeared

from at least an additional 56 km of critical habitat in the lower river, and their abundance has declined to precariously low levels elsewhere (USFWS 2008).

The main elements of the woundfin recovery plan are to establish additional populations within the historic woundfin range, maintain two broodstock refugia, and identify sites and protocols for reintroductions (USFWS 1995). Woundfin culture at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) in New Mexico started in 1987 (USFWS 1995). The stock serves as a genetically diverse refugium population and augments conservation propagation efforts for wild populations (Chen et al. 2011). The facility now operates four culture ponds with 500–1,000 adult fish each, and over the past 5 y, it has produced an average of 10,000 age-0 woundfin annually for conservation stocking efforts in the Virgin River (fish stocked at 4 mo; M. Ulibarri, USFWS, personal communication). Woundfin from SNARRC have served to establish additional refugia populations at Wahweap





Figure 1. Adult woundfin *Plagopterus argentissimus* reared in captivity at the Bozeman Fish Technology Center, Montana, in 2011.

State Fish Hatchery (Wahweap), Utah and at Bubbling Ponds Native Fish Conservation Facility (Bubbling Ponds), Arizona. In 2010, Wahweap produced 1,055 age-0 fish for stocking (Z. Olsen, Utah Division of Wildlife Resources, personal communication), and Bubbling Ponds produces approximately 75 age-0 woundfin annually (M. O'Neill, Arizona Department of Game and Fish, personal communication). Augmentation efforts have successfully maintained woundfin in the Virgin River (Chen et al. 2011), but the estimated annual number of hatchery-produced woundfin potentially needed for future stocking to facilitate full re-establishment of wild, self-sustaining populations is 100,000–200,000 individuals, or approximately a 10- to 20-fold increase over current production levels (S. Meisner, Virgin River Resource Management and Recovery Program, personal communication). To help meet this potential increase in production, the Bozeman Fish Technology Center (BFTC), Montana, is conducting a multiyear project focused on developing intensive culture techniques for use at the conservation propagation facilities (Webb et al. 2011).

Predation on embryos and larvae by adult woundfin may be an important factor limiting pond culture production. Woundfin are batch spawners (Webb et al. 2009) and are known to prey upon their own offspring (Webb et al. 2011). We experimentally determined the rate of predation on embryos and larvae by adult woundfin under laboratory conditions.

Methods

We randomly assigned 10 adult (age-2) woundfin (90 ± 4 mm; mean \pm SD) to each of six 0.6-m diameter by 0.3-m-deep experimental tanks at the BFTC for the predation trials. Fish maintenance was at temperatures between 19 and 20°C, with flow-through water and natural photoperiod. Fish acclimated for 2 d before experiments began. We used belt feeders to provide feed (Otohime, C2, Japan) in excess both before and during each trial.

We obtained embryos for experiments by injecting hormones and strip-spawning 2- and 3-y-old fish. We injected males and females with 20 μ g/g carp pituitary extract (Argent Laboratories) and then strip-spawned them 24 h postinjection. We reared larvae in 0.6-m-diameter tanks and fed them a mix of Otohime and freeze-dried cyclop-eeze (Argent Laboratories). After all predation trials were complete, we used hand-stripping or ultrasound (SonoSite TITAN) to determine the sex of each adult in the predation tanks (Table 1).

We conducted eight embryo predation trials between May 26 and June 21, 2011. Each trial consisted of three randomly selected tanks out of the six tanks (10 adults per tank). In each of these three tanks, we placed a 15- by 23-cm plastic tray containing a layer of glass marbles to imitate spawning substrate. We used noneyed embryos (1–2 d postfertilization) for three trials, and eyed embryos (≥ 3 d postfertilization) for five trials. During a trial on May 27, the adult fish spawned on the marble substrate, resulting in more than 25 embryos at the end of the experiment. This tank initially held noneyed embryos, and it was not possible to distinguish between the original 25 and the newly fertilized embryos, so we removed this trial from analysis. For subsequent trials, we used either eyed embryos or noneyed embryos that

Table 1. Experimental tanks used for all embryo and larval predation trials with woundfin *Plagopterus argentissimus* at the Bozeman Fish Technology Center, Montana, in 2011. Ten adult woundfin (age 2 y) were randomly assigned to each tank for each trial.

Tank number	Number of males	Number of females
1	8	2
2	9	1
3	10	0
4	8	2
5	9	1
6	4	6

Table 2. Results from the woundfin *Plagopterus argentissimus* embryo predation trials conducted at the Bozeman Fish Technology Center, Montana, from May 26 to June 21, 2011. Columns show the date of each trial, the randomly selected experimental tanks, initial number (#) of embryos placed in each tank, number of embryos remaining in each tank after 4 h, number of embryos predated in each tank, and whether or not eyed-embryos were used (Y = yes, N = no).

Date	Tank	Initial embryos (n)	Remaining embryos (n)	Mortalities (n)	Eyed (Y/N)
May 26	1	25	18	7	N
May 26	2	25	21	4	N
May 26	3	25	24	1	N
May 27	2	25	17	8	N
May 27	4	25	24	1	N
May 31	1	17	15	2	Y
May 31	2	17	17	0	Y
May 31	4	17	17	0	Y
June 2	2	25	20	5	Y
June 2	4	25	23	2	Y
June 2	5	25	21	4	Y
June 8	1	25	14	11	N
June 8	4	25	25	0	N
June 8	6	25	17	8	N
June 14	4	25	19	6	Y
June 14	5	25	23	2	Y
June 14	1	25	22	3	Y
June 16	5	25	24	1	Y
June 16	1	25	24	1	Y
June 16	6	25	24	1	Y
June 21	1	25	16	9	Y
June 21	5	25	21	4	Y
June 21	6	25	22	3	Y

were at least undergoing blastulation to distinguish from newly spawned eggs and fertilized embryos. We randomly placed 25 embryos throughout each tray, except for the trial on May 31 when we used 17 eyed embryos per tray. We left the tanks undisturbed for 4 h before collecting the trays. We then enumerated the remaining embryos and determined their stage of development under a microscope.

We used the same experimental setup and design as the embryo trials to conduct five larval predation trials from June 10 to June 30, 2011. In each trial, we released 15 larvae into three randomly selected tanks of adults. The mesh covering the center of each tank standpipe was 500 μ m and precluded larval escapement. Following each trial, we captured the remaining larvae by hand with pipets and hand-nets. At least two people inspected each trial tank to ensure that no individuals, including dead larvae, remained.

We used Wilcoxon rank-sum tests to test for significance of predation on embryos and larvae, as well as to compare predation on noneyed versus eyed embryos. We used a bootstrap with replacement analysis to create 95% confidence intervals for medians. We also used a Kruskal-Wallis test to determine whether there was a relationship between adult sex ratio and predation rate in the experimental tanks. We performed all tests using the statistical program R version 2.11.1 (R Development

Core Team 2008) with the exactRankTests package (Hothorn and Hornik 2010).

Results

We detected significant predation effects on both embryos ($W = 210, P < 0.001$) and larvae ($W = 45, P = 0.004$). There was a median of three (95% CI, 1–4) embryos eaten per tank ($n = 8$ trials; Table 2). We found no significant difference in predation rate on noneyed compared with eyed embryos ($W = 32, P = 0.07$). There was a median of one (95% CI, 0.5–2) larva eaten per tank ($n = 5$ trials; Table 3). In both embryo and larval trials, no relationship existed between adult sex ratio and predation rate ($P = 0.829$ and 0.276 , respectively).

Discussion

In our experiments, predation on embryos and larvae was substantial. Adults consumed a median of three embryos over 4 h in a tank containing 10 adults, which translates into an average of 0.075 embryos eaten per adult per hour. Using the same calculation, the estimated rate of larval predation was 0.025 larvae eaten per adult per hour.

It is important to note that conditions in the experimental tanks at the BFTC were different from those at the propagation facilities. Most notably, the experimental tanks had very low water turbidity and

Table 3. Results from the woundfin *Plagopterus argentissimus* larval predation trials conducted at the Bozeman Fish Technology Center, Montana, from June 10 to June 30, 2011. Columns show the date of each trial, the randomly selected experimental tanks, initial number (#) of larvae placed in each tank, number of larvae remaining in each tank after 4 h, and number of larvae predated in each tank.

Date	Tank	Initial larvae (n)	Remaining larvae (n)	Predated (n)
June 10	1	15	13	2
June 10	2	15	12	3
June 10	3	15	11	4
June 24	3	15	15	0
June 24	4	15	15	0
June 24	5	15	13	2
June 29	1	15	14	1
June 29	3	15	14	1
June 29	5	15	14	1
June 30	1	15	15	0
June 30	4	15	13	2
June 30	6	15	14	1

artificial feed; live food is available in pond culture at the propagation facilities. There is approximately 43 cm of secchi disk visibility at Wahweap (Z. Olsen, Utah Division of Wildlife Resources, personal communication), and there is 46–90 cm of secchi disc visibility at SNARRC (M. Ulibarri, USFWS, personal communication). The water in our experimental tanks had >150 cm of secchi disc visibility. Other studies have found that increased turbidity can significantly decrease rates of predation on larvae by visual predators (e.g., Ohata et al. 2011). If woundfin are locating embryos and larvae visually, then an increase in water turbidity may significantly decrease predation rates. Therefore, the predation rate at the BFTC may represent a maximum or elevated predation rate due to clearer water in a tank environment compared with the propagation facilities.

There are 3,500 adult woundfin in ponds at SNARRC. Assuming a 1:1 sex ratio, 50% ovulatory success in adult females, 100 eggs per spawning event (Deacon and Minckley 1973), and a single spawning event per female (though female woundfin are batch spawners), a very conservative estimate of the number of progeny from 3,500 adults is 87,500. However, the average annual production at this facility is only 10,000 4-mo-old woundfin for stocking into the river. Even if actual predation rates in the pond culture environment is a small fraction of the maximal rate observed in this study, cannibalistic predation of embryos and larvae could be a substantial contributor to production shortfalls. We recommend that production facilities remove woundfin embryos from the ponds and hatch them in separate ponds. Woundfin in conservation propagation programs currently spawn on gravel-filled baskets placed in ponds. Baskets could be removed daily or several times a week and placed in separate ponds for incubation and rearing. If spawning substrate

cannot be removed at least several times a week, personnel could seine the spawning ponds weekly throughout the season to remove larvae. Such seining activity does not appear to cause high rates of mortality or deformity (M. Ulibarri, personal communication).

Supplemental Material

Please note: The *Journal of Fish and Wildlife Management* is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

Reference S1. [USFWS] U.S. Fish and Wildlife Service. 1995. Virgin River Fishes Recovery Plan. Salt Lake City, Utah.

Found at DOI: <http://dx.doi.org/10.3996/042012-JFWM-029.S1>; also available at <http://vrhcrp.mesquitenv.gov> (4.3 MB PDF).

Reference S2. [USFWS] U.S. Fish and Wildlife Service. 2008. Virgin River fishes, woundfin *Plagopterus argentissimus* and Virgin River chub *Gila seminuda*, 5-year review: summary and evaluation. U.S. Fish and Wildlife Service, Utah Field Office, West Valley City, Utah.

Found at DOI: <http://dx.doi.org/10.3996/042012-JFWM-029.S2> (558 KB PDF).

Reference S3. Webb MAH, Kappenman K and Fraser C. 2009. Development of Intensive Culture Techniques for Woundfin. Annual Report (2008-2009). U.S. Fish and Wildlife Service, Bozeman, Montana.

Found at DOI: <http://dx.doi.org/10.3996/042012-JFWM-029.S3> (4.4 MB PDF).

Reference S4. Webb MAH, Gaylord G, Nistler A, and Fraser C. 2011. Development and optimization of spawning and intensive culture techniques for woundfin, Annual report (2010 - 2011). U.S. Fish and Wildlife Service, Bozeman, Montana.

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Tasks 10 and 16. Hormonal Induction Regime for Spawning of Woundfin in Intensive Culture.

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Introduction

The principal goal of the Virgin River Resource Management and Recovery Program (Recovery Program) is to successfully rear woundfin at conservation propagation facilities for release into its former range. The woundfin is native to the lower Colorado River Basin, but the combined effects of habitat loss, flow alteration, and competition with invasive species has reduced the woundfin's distribution to a fraction of its historic range (USFWS 1994). Woundfin are now restricted to reaches of the mainstem Virgin River and were listed as endangered in 1970 (USFWS 1970). The current goal of the Recovery Program's woundfin restocking program is to release 100,000 ten-month old woundfin annually (S. Meismer, personal communication).

Pond culture of woundfin has resulted in the release of ~5,000 to 30,000 10-month old fish annually. Intensive culture may allow for an increase in progeny production, and within intensive culture, the use of hormonal induction may be considered a viable option for spawning and development of a genetics management plan for woundfin. There are several hormones that are available to induce ovulation and spermiation in fishes in captivity. They include Common Carp Pituitary Extract (CPE), human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH), and ovaprim (an analogue of salmon GnRH plus a dopamine blocker). Pituitary extracts and hCG are used extensively and bypass the hypothalamus-pituitary hormonal axis to act directly on the ovary. Problems with purity, specificity, and potency occur with pituitary extracts. However Chorulon®, a particular brand of hCG, is of known and consistent potency. Recently, synthetic hypothalamic releasing hormones have been manufactured and are widely used to induce ovulation and spermiation in fishes. These analogs referred to as GnRH-a or luteinizing hormone-releasing hormone (LHRH) last longer in the fish's system and are often more potent compared to the non-synthetic hypothalamic releasing hormones. These hormones act at the level of the pituitary stimulating the fish's own pituitary to produce and release gonadotropic hormones. Ovaprim is a synthetic hormone preparation of salmon GnRH plus a dopamine blocker called domperidone. The dopamine blocker in combination with the GnRH has been found to be necessary in cyprinids to induce ovulation.

The objective of this study was to develop a hormonal induction protocol for woundfin. An initial hormonal induction trial with woundfin was conducted at the Bozeman Fish Technology Center (BFTC) during the 2011 spawning season. Optimization of the hormonal induction protocol was conducted during the 2012 spawning season.

Materials and Methods

In 2011, three hormones (CPE, LHRH, and hCG) were screened at each of two doses to determine their efficacy in induction of ovulation and spermiation. The dosages are standard doses used for each chemical in fish culture. Ovaprim was not tested in 2011 due to a limited number of fish at the BFTC. Treatments included:

Treatment 1: LHRH 0.01 µg/g (10 µg/kg) with strip spawn,

Treatment 2: LHRH 0.01 µg/g (10 µg/kg) with natural spawn,

Treatment 3: LHRH 0.05 µg/g (50 µg/kg) with strip spawn,

Treatment 4: LHRH 0.05 µg/g (50 µg/kg) with natural spawn,

Treatment 5: hCG 1 IU/g on two consecutive days with strip spawn,

Treatment 6: hCG 1 IU/g on two consecutive days with natural spawn,

Treatment 7: hCG 2 IU/g with strip spawn,

Treatment 8: hCG 2 IU/g with natural spawn,

Treatment 9: CPE 5 ug/g with strip spawn,

Treatment 9: CPE 5 ug/g with natural spawn,

Treatment 11: CPE 20 ug/g with strip spawn,

Treatment 12: CPE 20 ug/g with natural spawn.

Each treatment group was replicated to allow natural spawning over substrate or strip spawned. The 2009 year class of woundfin was used for these trials. Woundfin females and males were maintained at temperatures of 9-22°C with natural vernalization of temperatures from 9 to 16°C (January to May) and a temperature ramping period from 16 to 22°C the third week of May. Fish were exposed to the natural photoperiod of St. George, Utah. Fish (n=60 females and n=60 males) were randomly assigned to the 12 treatment groups (n=5 females and 5 males per

treatment). Fish were allowed to acclimate to their tanks overnight. Fish were anesthetized, weighed, and all injections were administered intramuscularly.

Fish were hormonally injected at 6 AM with their respective treatments in the hormonal trial. Spawning activity was observed hourly without disturbing the fish in their tanks in the treatments provided spawning substrate (glass marbles in a 6X4 inch plastic tray). At 8-hours, 10-hours, 12-hours, and 24-hours post-injection, fish in all treatments assigned to strip spawning were manually spawned (i.e. gentle pressure was applied to the abdomen in the direction of the urogenital opening). Sperm was collected in a non-heparinized hematocrit tube. Hematocrit tubes were maintained at 22°C until sperm motility could be assessed. If gametes were collected, eggs were counted, and sperm motility was assessed (number of seconds sperm was motile following activation with water). If no ovulation or spermiation occurred, fish were placed back in their respective tank. Fish assigned to natural spawning had their substrate trays removed at 24-hours post-injection. The substrate trays were gently rinsed with fresh water and the number of eggs were counted.

In 2012, the efficacy of ovaprim was assessed and compared to CPE, the development of a potential immersion protocol was conducted as a less invasive means to induce spawning in woundfin using CPE, and the efficacy of CPE injections was further examined. Woundfin females and males were maintained at temperatures of 9-22°C with natural vernalization of temperatures from 9 to 16°C and a ramping period from 16 to 22°C the third week of May. To assess the efficacy of ovaprim, fish were randomly assigned to treatment groups (control=no hormone, CPE 20 µg/g, and ovaprim 0.5 ml/kg in one injection; n=10 females and 10 males per treatment), and each treatment was run in duplicate. A saline injection as a positive control was not included due to lack of fish; however, the control (no hormone) group was handled identically to the hormonally injected fish minus the injection. Woundfin were anesthetized with MS-222 and injected intramuscularly (hormone treatments only). Fish were visually monitored continuously without disrupting normal behavior following the injections for 3 hours.

To develop an immersion protocol as a less invasive means to induce spawning in woundfin, CPE was used as domperidone is not soluble in water, and we found that ovaprim containing domperidone was lethal to woundfin. The CPE immersion doses were 0 (control), 50, 100, and 200 mg/L. Eight to 20 woundfin per treatment (each treatment in duplicate) were exposed to CPE for 1 hour with aeration. Fish were strip spawned 24 hours after exposure to CPE. The number of females that ovulated and the number of eggs within treatments was compared.

The efficacy of CPE injections was further examined in four trials. In each trial, fish were randomly assigned to the treatment groups (control=no hormone and CPE 20 µg/g; n=10 females and 10 males per treatment), and each treatment was run in duplicate. Woundfin were anesthetized with MS-222 and injected intramuscularly (hormone treatment only). Fish were strip spawned 24 hours after injection.

Data was square root or arcsin transformed, and Student's t-tests were performed to determine if differences existed between the total number of eggs collected, fertilization success, and hatch success between the CPE and control treatments.

Results and Discussion

In 2011, hormonal injection and strip spawning or natural spawning resulted in no mortality of woundfin. Sperm remained viable in non-heparinized vacutainers for up to 79 minutes. In the natural spawning treatments following hormonal induction, the CPE (20 ug/g) was the only treatment that resulted in the collection of embryos (total of 287). It is possible that predation on embryos in the natural spawning treatments significantly reduced our ability to assess the efficacy of the hormonal treatments. In the strip spawned treatments, CPE (20 ug/g) resulted in the highest ovulatory success (83%) and mean number of eggs released per female (500 eggs/female). The hCG had the lowest efficacy with 1 IU/g resulting in only 7% ovulatory success and 2 IU/g resulting in 22% ovulatory success. The LHRH resulted in 33% ovulatory success with 0.01 µg/g and 8% ovulatory success with 0.05 µg/g. The 5 ug/g dose of CPE resulted in 61% ovulatory success. Sperm motility ranged from 0 to 56 seconds and did not appear to differ among hormone treatments. However, sperm volume did appear to differ among hormone treatments and was examined in 2012.

Ovaprim was not used due to limited number of fish available in 2011. In 2012, the efficacy of ovaprim was assessed and compared to CPE. Ovaprim resulted in mortality (selective for females) within 15 minutes to 2 hours following injection. In replicate 1, 30% of the fish died, while in replicate 2, 20% of the fish died. The skin in all remaining fish in the ovaprim treatments became highly vascularized (red), and the study was terminated. No mortality in the control or CPE treatments occurred.

The immersion trial resulted in no substantial increase in the number of females that ovulated in response to the CPE treatments (Table 1). The mean number of eggs per female was similar among the control, 50 mg/L, and 100 mg/L CPE treatments; there was an increase in the mean number of eggs per female in the 200 mg/L CPE treatment compared to the other treatments (Table 1). The variability within a treatment was high due to the lack of a tool to assess spawning readiness and standardize the response of females to a treatment by spawning them at the ideal stage of oocyte maturation. The small size of fish and reproductive strategy (i.e. batch spawners) of woundfin results in challenges associated with collecting ovarian follicles which are needed to determine spawning readiness. Further work must be conducted to substantiate the increase in mean number of eggs per female as a result of immersion in 200 mg/L of CPE.

Table 1. The percent ovulation and mean number of eggs per female in the immersion trial with Common Carp Pituitary Extract (CPE) conducted at the Bozeman Fish Technology Center in 2012 with woundfin. Control=no hormone.

Treatment	Ovulation (%)	# Eggs/Female (mean \pm sem (range))
Control	38	87 \pm 41 (0-423)
CPE 50 mg/L	50	59 \pm 30 (0-256)
CPE 100 mg/L	25	32 \pm 27 (0-323)
CPE 200 mg/L	67	167 \pm 97 (0-328)

The trials examining the efficacy of CPE injections resulted in a significant increase in the number of ovulated females in response to CPE (87%) compared to the control (59%; no hormone) (Table 2). The number of eggs per female was significantly greater ($P=0.00$) in the CPE treatment compared to the control (Table 2). Though the fertilization success did not statistically differ between the treatments ($P=0.66$), the hatch success was significantly greater in the CPE embryos compared to the control embryos ($P=0.04$).

Table 2. The percent ovulation and mean number of eggs per female in the injection trials with Common Carp Pituitary Extract (CPE) conducted at the Bozeman Fish Technology Center in 2012 with woundfin. Control=no hormone.

Treatment	Ovulation (%)	# Eggs/Female (mean \pm sem (range))
Control	59	180 \pm 41 (0-834)
CPE (20 μ g/g)	87	390 \pm 43 (0-969)

The spermiation success for males increased in response to CPE (88%) compared to the control (41%; no hormone) (Table 3). The volume of sperm per male was greater (65 μ l) in the CPE treatment compared to the control (9 μ l; Table 3), and the motility of sperm was two times greater in the CPE treatment (32 seconds) compared to the control (16 seconds, Table 3).

Table 3. The percent spermiation, volume of sperm per male, and motility (time to 90% immotile) in the injection trials with Common Carp Pituitary Extract (CPE) conducted at the Bozeman Fish Technology Center in 2012 with woundfin. Control=no hormone.

Treatment	Spermiation (%)	Sperm Volume (μl) (mean \pm sem (range))	Motility (seconds) (mean \pm sem (range))
Control	41	9 \pm 2 (0-50)	16 \pm 2 (0-38)
CPE (20 μ g/g)	88	65 \pm 9 (0-240)	32 \pm 2 (0-45)

The use of hormonal induction can synchronize spawning in woundfin and increase the number of eggs that are released from a female in a spawning event resulting in compression of the spawning season. As well, if a genetics management plan were developed, the use of hormonal induction would allow for control of mating. The variability within a hormonal treatment was high in the woundfin studies due to the lack of a tool to assess spawning readiness and

standardize the response of females to a treatment by spawning at the optimal stage of oocyte maturation. The small fish size and reproductive strategy (i.e. batch spawners) of woundfin results in challenges associated with collecting ovarian follicles which are needed to determine spawning readiness. Regardless, the use of CPE did result in a significant increase in the number of females and males that ovulated and spermiated, respectively, the number of eggs per female, the volume of sperm collected per male, and sperm motility. These results suggest that CPE is an effective hormonal induction agent in woundfin. When or if the development of a tool to determine spawning readiness is plausible for woundfin, the application of CPE in conjunction with the proper time to hormonally inject will be highly beneficial to increase the number of progeny in a single spawning event.

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Tasks 11 and 13. Develop broodstock diet formulations to increase fecundity and larval survival and performance.

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Introduction

Fish broodstock nutrition is a relatively understudied field due to the facilities, expense and time required to do so. No information is currently available for woundfin on the effects that nutrition will have on broodfish health, fecundity, hatchability and larval survival. Female woundfin broodfish maintained at BFTC have high fat depots in the peritoneal cavity. The effects of these fat depots on ovarian maturation and egg development are unknown, but the fat deposition may be detrimental. Therefore the hypothesis to be tested is that dietary nutrient profile and energy density will influence deposition of fat stores in the peritoneal cavity and impact egg production, hatchability and larval survival.

Material and Methods

A 2 by 2 factorial experimental design was utilized to test the effects of two dietary lipid levels (8 and 16%) and two fatty acid compositions (flaxseed/soy oil blend and fish oil) on woundfin fecundity, larval survival and larval performance. The design was based on results from our previous studies on juvenile and larval woundfin, and research on a variety of fish species that demonstrated substantial shifts in nutrient partitioning and allocation during reproduction. Our goals were 1) to assess the appropriate nutrient density of the diet to optimize fitness while limiting excessive energy accumulation that may impede gonadal development, and 2) assess the effects of altering the dietary fatty acid profile on egg development and embryo survival. Otohime C-2, which is used at BFTC for broodstock maintenance, was the control diet. Treatment diets were formulated with the same ingredients that comprise the RGSM diet currently used for woundfin culture at SNARRC, with modifications in inclusion levels to yield 45% digestible protein and 8 or 16% crude lipid. A 50:50 mixture of soybean oil: flaxseed oil was used to balance the omega-6 and omega-3 fatty acid profile in supplemental oil in substitution for menhaden fish oil (Tables 1 and 2).

Table 1. Diet formulations for woundfin broodstock diets containing either 8 or 16% lipid from a combination of flaxseed oil and soy oil or menhaden fish oil.

Ingredient	8 FlxSO ¹	16 FlxSO	8 FO	16 FO
Krill meal	19.00	19.00	19.00	19.00
Fish meal - Peruvian Anchovy	23.50	23.50	23.50	23.50
Squid meal	7.00	7.00	7.00	7.00
Chicken meal- ADF	8.00	8.00	8.00	8.00
Spirulina	11.00	11.00	11.00	11.00
Wheat Starch	18.18	10.18	18.18	10.19
Wheat Starch – pregelatinized	5.00	5.00	5.00	5.00
Flaxseed oil	0.82	4.82	0.00	0.00
Soy oil	0.82	4.82	0.00	0.00
Menhaden fish oil	0.00	0.00	1.64	9.63
Stay-C 35	1.25	1.25	1.25	1.25
Vitamin premix ARS 702	1.50	1.50	1.50	1.50
Trace mineral premix - ARS 640	0.10	0.10	0.10	0.10
Monocalcium Phosphate	1.10	1.10	1.10	1.10
Choline Cl 50%	1.00	1.00	1.00	1.00
DL-Methionine	0.12	0.12	0.12	0.12
Lysine HCl	0.97	0.97	0.97	0.97
Threonine	0.06	0.06	0.06	0.06
Taurine	0.50	0.50	0.50	0.50
Astaxanthin	0.08	0.08	0.08	0.08

¹ FlxSO – a combination of flaxseed oil and soy oil in a 1:1 ratio.

Table 2. Compositional analysis of experimental diets.

Lipid		8	16	8	16	Otohime
Lipid type		FlxSO	FlxSO	FO	FO	marine
Moisture	%	6.5	6.8	6.2	4.6	5.4
Fat	% dry weight	9.7	17.7	10.2	17.9	17.6
Protein	% dry weight	51.1	50.9	50.8	50.4	59.6
Gross energy	cal/g dry feed	5098	5523	5069	5466	5439

Age-1 woundfin were visually sorted as to gender, and females and males at a 1:1 ratio were randomly assigned to diets, with 24 individuals in each of 15, 100-L tanks connected to a common biofilter water system. Feeding was initiated at 5% body weight per day on September 7, 2011, and was adjusted every 4 weeks to compensate for growth and thereby ensure adequate nutrition from the respective diets for early egg development. A water temperature regime was implemented to prepare fish for spawning according to protocols developed by Molly Webb (personal communication). Woundfin were subjected to a thermal and photoperiod regime to mimic St. George photoperiod and thermal profile of Dexter NFH through the winter with the exception of the final week in which temperatures were ramped more rapidly to condense spawning.

Upon completion of the photo-thermal regime to induce spawning, all individuals in each tank were anesthetized in a bath of 50 ppm MS-222 (tricaine methanesulfonate) until loss of equilibrium, and intramuscularly injected with 20 µg carp pituitary extract/g fish. Seven tanks of fish were injected on day 1 and eight tanks of fish were injected on day 3 in order to allow strip spawning on the subsequent day within a 2-3 hour time period post-injection. Approximately twenty-four hours after injection of common carp pituitary extract, gametes were hand stripped from fish in eight tanks on June 5, 2012, and from fish in the remaining seven tanks on June 7, 2012. Viable milt from all males in a tank were pooled into a single micro-centrifuge tube and used to fertilize eggs from an individual female. Fertilized eggs were placed in individual hatching containers by female and incubated at 22 °C until hatched. Larvae were quantified and then transferred into a zebra fish system to assess larval survival through first feeding; survival rates were recorded after 7 days. A second spawn was attempted by providing marble substrate to the remaining fish in a tank. After spawning ended, broodstock females and males used in the study were euthanized and separated by tank number and gender for proximate-composition analysis.

Results and Discussion

No significant differences in spawning efficiency were noted among the five experimental diets, and no significant differences in growth, practical fecundity, embryo development, or level of fat deposition were noted between dietary lipid levels and between fatty acid compositions. Final fish mass (pre-spawn) ranged from 5.53 to 6.09 g ($P = 0.2449$). There was no dietary effect on mean number of eggs per tank, which ranged from 523 to 1,600 ($P = 0.3708$). Woundfin showed no dietary effects in number of eggs spawned per female, which ranged from 141 to 236 ($P = 0.4984$). Hatch-rate percentages were very poor for all dietary treatments, ranging from 3% to 31%. Larval survival through first feeding was not affected by dietary treatment and ranged from 22% to 70% ($P = 0.6426$). Percentage of male woundfin with good milt was not influenced by dietary treatment and ranged from 78% to 100%, ($P = 0.06$, Table 3).

Table 3. Effects of dietary lipid levels and source on final fish mass and woundfin egg production, hatchability, survival of fry and male ripeness.

Diet								
Lipid		8	16	8	16	Otohime		
Lipid type		FlxSO	FlxSO	FO	FO	marine	Pr>F	Pooled SEM
Final fish mass (pre-spawn)	g	5.82	6.09	5.53	5.9	5.6	0.2449	0.18
Mean # of eggs per tank		834	819	1524	523	1600	0.3708	435
Total females		8	7	8	7	10		
Females releasing eggs	%	71	51	82	53	64	0.3059	10.9
Eggs spawned/female		160	197	214	141	236	0.4984	41
Hatch rate	%	3	31	8	8	5	0.4916	11.78
Fry on feed		25	73	51	30	50	0.8452	32.4
Fry on feed	%	28	45	22	52	70	0.6426	23.94
Total survival from spawn		2	25	5	7	4	0.5437	10.17
Number males		10	10	11	10	8		
Males with good milt	%	79	100	78	87	96	0.0622	5.5

Proximate composition of post-spawned females and males fed diets with differing lipid levels and sources showed no significant differences among treatments. For females (Table 4), whole body fat percentages ranged from 18% to 23.2% ($P = 0.2281$), protein percentages ranged from 13.7% to 14.3% ($P = 0.9215$), and energy levels ranged from 2,664.2 to 3,144 cal/g ($P = 0.2286$). For males (Table 5), whole-body fat percentages ranged from 17.5% to 21% ($P = 0.6482$), protein percentages ranged from 14.7% to 15.2% ($P = 0.7031$), and energy levels ranged from 2,622 to 3,009 cal/g ($P = 0.5405$).

We concluded that the RGSM diet formula currently used at SNARRC for woundfin culture is satisfactory in providing nutrients for reproduction, fecundity, and embryo and early larval development and survival. However, a reduction in lipid level would be possible as we found that woundfin brood fish appeared to utilize the higher lipid or lower lipid diets equally well.

Table 4. Proximate composition of post-spawning female woundfin fed diets with differing lipid levels and sources.

Diet		8	16	8	16	Otohime		
Lipid level		FlxSO	FlxSO	FO	FO	marine	Pr>F	Pooled SEM
lipid type		Female	Female	Female	Female	Female		
Moisture	%	58.9	59.5	60.1	57.2	62	0.2857	1.47
Fat	% wet weight	20.3	19.3	20.1	23.2	18	0.2281	1.47
Protein	% wet weight	14.3	14	13.7	13.9	14.1	0.9215	0.46
Energy	cal/g	2779	2862	2838	3144	2664	0.2286	137

Table 5. Proximate composition of post-spawning male woundfin fed diets with differing lipid levels and sources.

Diet		8	16	8	16	Otohime		
Lipid level		FlxSO	FlxSO	FO	FO	marine	Pr>F	Pooled SEM
Lipid type		Male	Male	Male	Male	Male		
Moisture	%	61.1	55.9	59.8	58.8	58.4	0.3092	1.65
Fat	% wet weight	17.5	21	18.8	20	20.2	0.6482	1.7
Protein	% wet weight	14.8	14.7	15.2	14.9	14.1	0.7031	0.55
Energy	Cal/g	2622	2805	2841	2853	3009	0.5405	153

Additional Notes.—Fungal growth and a high level of immature and/or post-ripe eggs collected by hand stripping appeared to greatly affect our data for egg production, hatchability, and survival of fry. After hand spawning for Task 13 was performed, a follow-up trial was performed to see if woundfin would spawn naturally on marbles in trays following hand spawning. This was performed in conjunction with preliminary tests on the use of 1.5% sodium sulfite for de-adhesion of eggs from the marbles and to prevent eggs from sticking together in incubation wells. A formalin bath of Paracide-F at 2,000 ppm also was used to help control fungal growth on eggs. The use of sodium sulfite was very successful, and we are confident that this can be used as a technique for de-adhesion of woundfin eggs. Results were variable using Paracide-F to decrease fungal growth.

Task 14. Determine the protein requirement for juvenile woundfin

T. Gibson Gaylord and Aaron Nistler

USFWS Bozeman Fish Technology Center, Bozeman, MT 59715

Introduction

To date a woundfin specific feed does not exist, but could be beneficial for this species. SNARRC and WSFH, where propagation of woundfin for reintroduction into the Virgin River occur, have shown specific interest in having a diet specific for woundfin during the juvenile stage where the use of supplemental commercial feeds are necessary. Results from previous trials at the Bozeman Fish Technology Center indicate that the physical attributes of feeds such as flakes or pellets does not greatly affect the growth of juvenile woundfin (<1 yr). Another study was performed to address dietary protein and lipid levels across multiple culture temperatures. In that trial, juvenile woundfin grew fastest at all culture temperatures when dietary protein was 45% vs. 35%, but dietary lipid had no effect on the growth rate or composition of gain. This indicates that the metabolism of woundfin is shifted toward amino acid catabolism for an energy yielding substrate instead of lipid. Based on this finding determining the dietary protein requirement of juvenile woundfin would be warranted when dietary lipid is maintained at 8%.

Materials and Methods

Experimental design. In order to determine the protein requirement of juvenile woundfin, a completely randomized experimental design was employed in which graded levels of dietary protein were fed to juvenile woundfin.

Diet formulation. Diets were formulated to be equivalent in nutrient composition and meet or exceed known nutritional needs of other finfish except for dietary protein. Eight diets were formulated to supply protein from 30 to 54% in 4% increments (Table 1). Diets contained 8% lipid and wheat starch content increased as protein content decreased. A commercially available control diet, Otohime S2, also was fed and contained 58% protein and 14% lipid.

Feeding and fish culture. Duplicate tanks of juvenile woundfin were assigned to diets using a completely randomized design. Fifty juvenile woundfin (mean length 31mm mean weight 0.28g per fish) were stocked into each of 15, 100 L tanks connected to a common biofilter. Water temperature was maintained at 26 °C. Feeding rate was 10% body weight per day. Water quality parameters were measured to ensure optimal DO, pH, total ammonia, nitrite and nitrate levels are maintained.

Survival, weight gain, morphological quality (i.e. evaluation of lesions, skeletal deformity or other deformities present) and fish total length variation were determined on all fish from each

tank at the end of the study. Five fish per tank were euthanized and ground as a group to determine composition of fish to include moisture, protein, lipid and whole body energy.

Chemical analyses. Ingredient, diet and fish moisture, lipid, protein and energy were determined by standard AOAC methodology.

Statistical analyses. Dietary protein requirement was determined by regression analysis for each response variable with protein requirements defined as the level of dietary protein eliciting 95% of maximal or minimal response and deemed significant at $P < 0.05$ (JMP, Version 11, SAS Institute, Cary, NC).

Results and Discussion

Final mean fish mass per treatment group ranged from 1.07 to 1.27 g per fish (Table 1). Using a fourth order polynomial regression analysis protein requirement based on final fish mass was estimated at 50% crude protein when estimated as 95% of maximal response (Figure 1). Using fish total length and a fourth order polynomial regression model, the protein requirement of juvenile woundfin was 52% when estimated as 95% of maximal response (Figure 2). Fish length ranged from 49.3 to 53.8 mm. Whole body crude protein ranged from 13.6 to 14.4% with a quadratic regression model providing the best fit (Figure 3). The quadratic model for whole body protein estimated a protein requirement of 41.7% (95% of maximum response) that maximized whole fish protein concentration. Whole body fat, moisture and energy regression analysis resulted in p-values > 0.05 with r^2 ranging from 0.77 to 0.84.

Currently, extensive pond culture is utilized to provide nutrition to woundfin fry with supplemental feeding starting at the juvenile stage. Estimates of protein requirement for juvenile fish of 50 to 52% of the diet maximize fish mass and length, respectively, but a lower dietary protein concentration of approximately 42% maximized protein deposition and minimized fat accumulation. Conservative estimates of 45% dietary crude protein should provide adequate nutrition to juvenile woundfin while minimizing the potential for excessive fat accumulation from either a low protein nutritional plane or an excessive protein nutritional plane.

Table 1. Experimental diet formulations with a range of dietary crude protein from 33 to 54%.

Ingredient	30	34	38	42	46	50	54
	% Dietary crude protein						
Corn protein concentrate	2.78	3.15	3.52	3.89	4.26	4.63	5.0
Squid meal	6.67	7.56	8.44	9.33	10.22	11.11	12.0
Spirulina	2.22	2.52	2.81	3.11	3.41	3.7	4.0
Fish meal, Peruvian anchovy	11.11	12.59	14.07	15.56	17.04	18.52	20.0
Krill meal	2.78	3.15	3.52	3.89	4.26	4.63	5.0
Wheat gluten meal	2.78	3.15	3.52	3.89	4.26	4.63	5.0
Chicken meal	11.11	12.59	14.07	15.56	17.04	18.52	20.0
Wheat Starch, pregelatinized	43.01	39.33	35.69	31.97	28.38	24.67	20.82
Fish oil, menhaden	3.76	3.26	2.76	2.26	1.76	1.26	0.76
Lecithin	1	1	1	1	1	1	1
Ascorbic acid polyphosphate, Stay-C 35	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix ARS 702	3	3	3	3	3	3	3
Trace mineral premix, ARS 640	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Monocalcium Phosphate	3.7	3.2	2.65	2.2	1.6	1.1	0.6
Choline Cl 50%	1	1	1	1	1	1	1
DL-Methionine	0.58	0.48	0.39	0.29	0.2	0.11	0.01
Lysine HCl	2.57	2.26	1.95	1.64	1.33	1.02	0.71
Threonine	0.84	0.67	0.5	0.32	0.15	0	0
Tryptophan	0	0	0	0	0	0	0
Taurine	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Analyzed composition							
Moisture (%)	8.8	9	9.9	9.2	10.5	9.4	10.7
Crude protein (% dwb ¹)	34.8	37.5	41.3	45.1	48.7	52.6	56.6
Crude fat (% dwb)	5.4	5.4	5.9	6.3	6.6	7	7.2
Gross energy (cal/g dwb)	4840	4847	4909	4941	5036	4978	5126

¹ Dry weight basis (dwb)

Table 2. Fish performance metrics for woundfin fed differing concentrations of dietary protein

Diet	30	34	38	42	46	50	54	Pr>F	Regression Model	R ²	Requirement Estimate ^a
Initial fish mass	0.28	0.30	0.30	0.28	0.28	0.31	0.27	0.5247			
Week 4 fish mass	0.47	0.51	0.49	0.47	0.50	0.53	0.46	0.9533			
Week 8 fish mass	0.68	0.71	0.69	0.69	0.73	0.80	0.68	0.3778			
Week 12 fish mass	0.84	0.91	0.87	0.88	0.98	1.01	0.91	0.1117			
Week 16 fish mass	1.07	1.14	1.13	1.16	1.22	1.27	1.19	0.0099	quartic	0.995	50.2
Fish final length ^b (mm)	50.5	50.4	50.5	51.2	52.1	53.1	52.0	0.0040	quartic	0.998	52.1
Whole body proximate											
Moisture	59.3	60.8	63.4	62.3	61.1	62.3	60.8	0.1616	quadratic	0.777	37.9
Fat	22.0	20.5	18.3	19.4	20.7	20.0	21.0	0.3426	quartic	0.811	39.1
Protein	14.0	14.2	14.4	14.4	14.4	13.9	13.6	0.0034	quadratic	0.942	41.7
Energy	2852	2810	2651	2689	2746	2751	2733	0.2806	quartic	0.848	41.4

^a Requirement estimates were defined as 95% of maximal or minimal response, depending on response variable, of the regression model

^b Initial fish length on a 50 fish subsample was 31.1 mm ± 5.3 (mean±stdev) with a maximum of 42 mm and minimum of 22 mm.

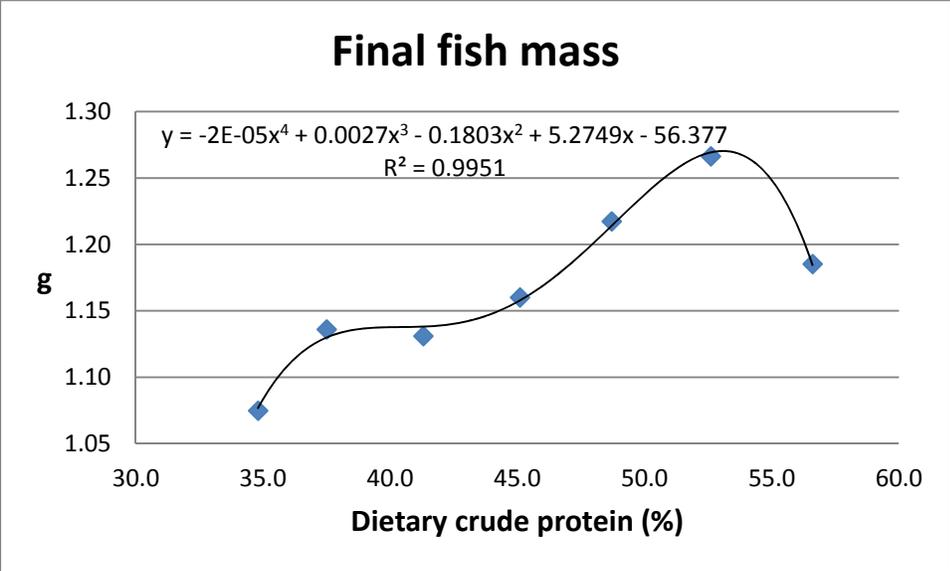


Figure 1. Final woundfin mass when fed diets with increasing crude protein.

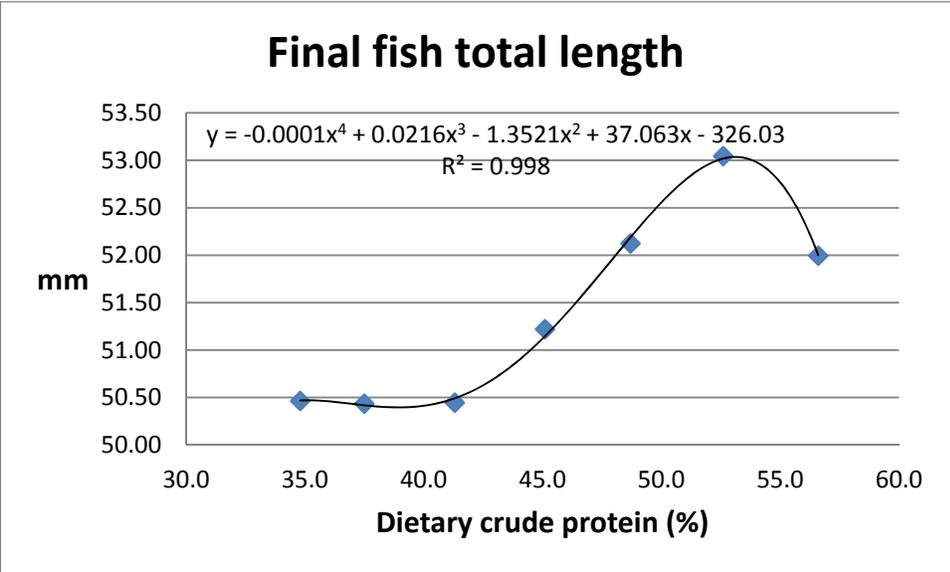


Figure 2. Final woundfin total length when fed diets with increasing crude protein.

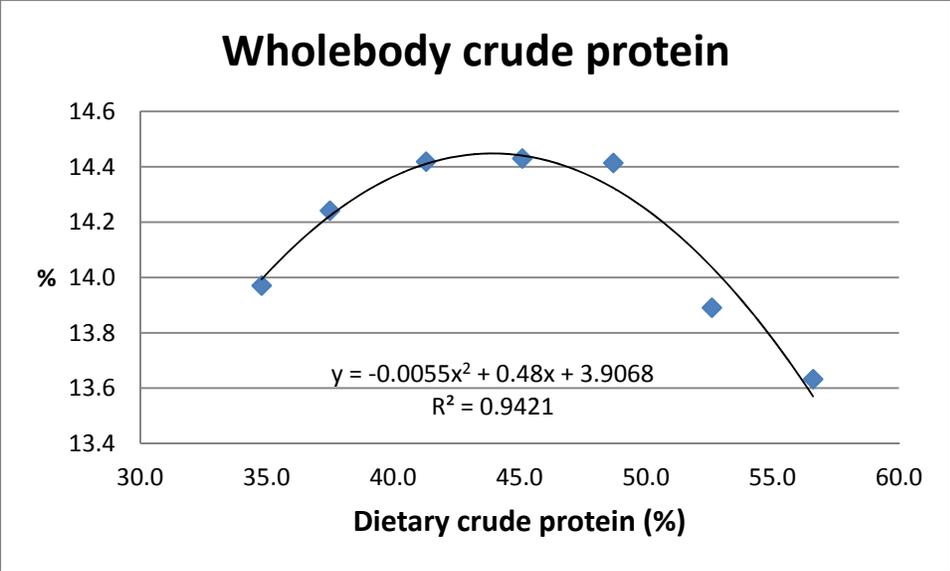
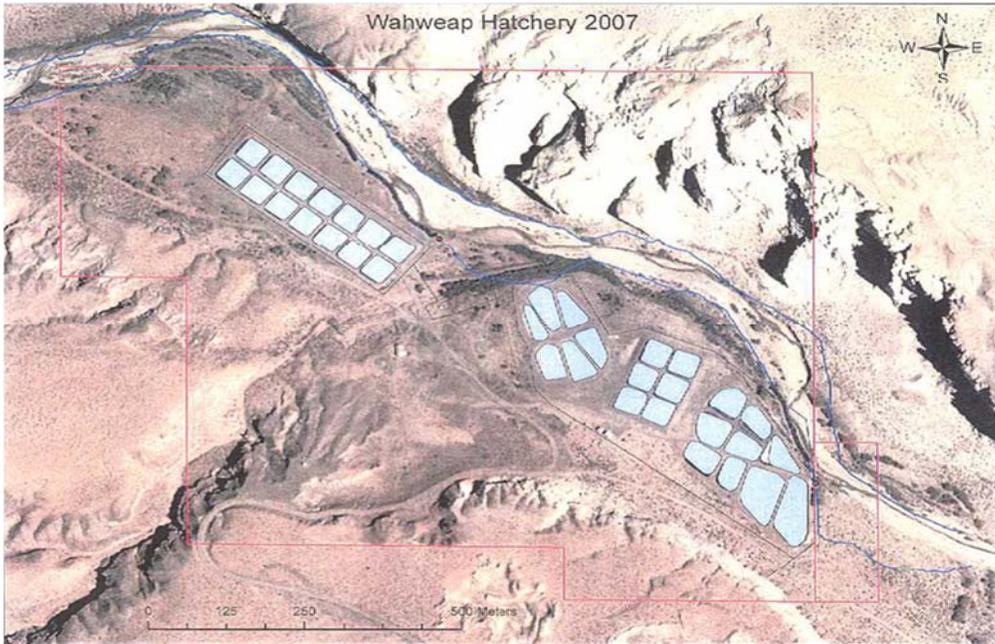


Figure 3. Crude protein concentration of woundfin fed diets with increasing crude protein.

Task 18. Wahweap State Fish Hatchery Site Visit

Molly Webb and T. Gibson Gaylord

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Preface

The USFWS Southwestern Native Aquatic Resource and Recovery Center (SNARRC) in Dexter, NM and the Utah Division of Wildlife Resources, Wahweap State Fish Hatchery (WSFH) are two conservation propagation facilities tasked with spawning, rearing, and releasing the endangered woundfin into their native habitat in the Virgin River. Currently, the majority of woundfin progeny from the conservation propagation program are produced at SNARRC. The WSFH maintains woundfin broodstock but has not yet played a significant role in the stocking of progeny into the river. To reach the stocking target of 100,000 one-year olds in the river each spring in the river, the conservation propagation program has decided to increase stocking rates of woundfin. One way to accomplish the increase in current production is to increase the number of progeny from WSFH.

The Virgin River Program (VRP) funded a site visit to WSFH to evaluate the management practices, capacity, and infrastructure which can be modified to increase woundfin production at this facility. The evaluation team included: Zane Olsen (Wahweap State Fish Hatchery, Hatchery Superintendent), Barry Nielsen (Wahweap State Fish Hatchery, Assistant Project Leader), Henry Maddux (Virgin River Program, Program Director), Steve Meismer, (Virgin River Program, Local Coordinator), Krissy Wilson (Utah Department of Natural Resources, Native Aquatic Species Program Coordinator), Terry Howick (Utah Department of Natural Resources, Fish Culture Supervisor), William Knight (Southwestern Native Aquatic Resources and Recovery Center, Fish Biologist), Molly Webb (USFWS Bozeman Fish Technology Center, Research Fish Biologist), and Gibson Gaylord (USFWS Bozeman Fish Technology Center, Physiologist). Manuel Ulibarri (Southwestern Native Aquatic Resources and Recovery Center, Center Director) and Chris Wilson (Fisheries Experiment Station, Utah Department of Natural Resources, Director/Fish Pathologist) were able to join by phone.

Over a 2-day period, the evaluation team examined the current infrastructure, capacity, management practices, and current protocols used to manage ponds and spawn and rear woundfin. Several other species are reared at WSFH, but this report focuses solely on woundfin. This report summarizes the findings of the evaluation team and defines needs and a decision flow chart aimed at increasing the number of progeny released from the woundfin conservation propagation program.

Virgin River Program Goals/Needs from Wahweap State Fish Hatchery: The Virgin River Program's (VRP) priority is to release reproductively capable woundfin into the Virgin River in March so that reproduction may occur in May-June. Woundfin are reproductive at 65-70 mm. Fish stocked in March are technically age-0 because they are stocked at 10 months of age but will be referred to as age-1. Approximately 70-100% of WSFH age-1 woundfin stocked in March are reproductive. The VRP would like to stock up to 100,000 age-1 woundfin.

Broodstock are maintained at the WSFH and SNARRC. Broodstock at WSFH are supplemented with age-1.5 (n=600) woundfin from SNARRC every year. Based on the current facility, the maximum fish that can be overwintered is 3,000-3,500 YOY. The maximum number of adult broodstock that can be maintained overwinter in the woundfin shop tank is 4,000. If the adults can be overwintered in the pond, this number could increase to 5,000-6,000 broodstock that could potentially be placed into ponds for overwinter only.

Old Well: The water in the "old well" on-site supplies 1,100 gallons/minute of water at 64°F/17.8°C (Figure 1). The water is artesian flow and is not affected by environmental conditions year-round. The water from the well is pumped up to the water tank (12,690 gallon capacity) on the hill (Figure 2). The water tank on the hill is filled once a day. The water for the on-site house is also supplied by the old well. Pumping water from the old well costs approximately \$1,500/month, which is the highest electrical cost on station.



Figure 1. The old well at the Wahweap State Fish Hatchery.

Last year the old well was rehabilitated due to gradual flow decrease over a 4-month period of time. The well was reconstructed in the spring of 2012 with new well lining, bowls, drive shaft, and pump column. The new bowls installed were engineered incorrectly causing “upthrust” on the pump and will rapidly increase premature wear out as upthrust during pumping causes the bowls to bounce. The bowls will need to be replaced for a second time. This mistake is the result of 2 separate contractors working on the well. An estimate to fix the bowls or add a restrictor plate has been requested. A solution to this problem must be found. The old well has no back-up power. If expansion of the facility occurs, a bigger storage tank is required.



Figure 2. The water tank that is supplied water from the “old well” at Wahweap State Fish Hatchery.

Needs:

- Replace bowls or add a restrictor plate to the old well.
- Alarm system for the old well to detect electrical failure(s).

New Well: The “new well” on site supplies 600 gallons/minute of water at the same temperature as the old well (Figure 3). The new well is 800 feet deep. If both wells are running simultaneously, it does affect the artesian flow. There is back-up power to the new well, but if the power goes out on-site, manual restart of the new well is required. It is unknown whether the generator for the new well is big enough to run the old well should the power fail. There is a SeQual Oxygen Generator in the generator room of the new well which is used to oxygenate

water for the raceway. This oxygen generator provides up to 84 cfm of 85% pure oxygen with all 6 units running and can be adjusted for smaller fish load in the raceway.



Figure 3. New well at Wahweap State Fish Hatchery.

Needs:

- Alarm system for the new well to detect electrical failure(s).

Raceway: The raceway was built in 2010 (Figure 4). The raceway is used for harvest, sorting, PIT tagging, and treatment of fishes. The raceway requires 24 hours to fill and recirculates 100 to 300 gallons/min. There are three pumps in the raceway. The oxygen line runs through the low head (85% pure oxygen). In addition to the low head, there are 3 air curtains that are placed throughout the raceway that help boost the dissolved oxygen and mix water during treatments. The raceway maintains dissolved oxygen level of > 6.0 mg/L. The raceway is used to treat fish for Asian tapeworm with praziquantel (24-hour static treatment @ 2.5 mg/L). It is thought that because this is a static treatment as worms are purged, uptake of worms can occur at the same time; this issue needs to be resolved for all species on station.



Figure 4. The raceway at Wahweap State Fish Hatchery.

Needs:

- French drain for the raceway effluent.
- Increased flow to improve purge rate of shed Asian tape worm.

Woundfin Trailer: This is the fourth year of overwintering woundfin in the trailer (Figure 5), and 2013-2014 will be the last year for use of the trailer. The woundfin trailer cannot have water flowing at the same time as the raceway. A Variable Frequency Drive (VFD) along with some replumbing would allow water to be controlled so that the raceway and woundfin trailer could be run simultaneously. The trailer requires 20-25 gallons/min of water that is degassed before entering the tanks. The water temperature of inflowing water is 19°C, but the temperature drops at night to 15°C. The young-of-year (YOY) fish from SNARRC are put directly into the trailer tanks and maintained from November to March. All YOY from WSFH are placed into tanks in the woundfin trailer in November. If recruitment is low, YOY are maintained with the adults. Fish have grown 11-14 mm during this time period in the trailer. In the past, 1000 fish/tank (4 tanks total) were maintained in the trailer. Zane would like to drop the numbers to 800 fish/tank to increase growth. Water quality is monitored daily in the trailer while woundfin are present. The water discharges into the willows. Black mold is present in the trailer, and iron deposits in the tanks preclude fish from being able to see food. The pumps need to be replaced. There is no security for water flow in the trailer.

Needs:

- The trailer and tanks need to be replaced.
- A VFD/plumbing is needed if the trailer remains to be able to use the raceway simultaneous to having fish maintained in the trailer.
- Alarm system to detect flow failure.
- A French drain should be installed for water discharge to contain potential pathogens and their spread across the facility.



Figure 5. Tanks in the woundfin trailer.

Ponds: There are 35 ponds at WSFH (Figure 6). Ponds 1-4 and 16-21 are sportfish ponds for wiper and channel catfish, 2 ponds out of Ponds 5-9 are for the VRP, the Least Chub Program rents one pond from the Upper Colorado River Program (UCRP), and the UCRP uses Ponds 10-15 and 21-35. Only Ponds 10-15 and 21-35 function properly. The UCRP ponds are newer and lined. The pond liners in Ponds 3-9 do not function, and the drains in Ponds 1-9 do not function. Replacing liners and drains are not worth the cost, therefore Ponds 1-9 need to be replaced. A flow event in Wahweap Creek causes Ponds 6-9 to fill via the drains, resulting in an uncertified water source into the facility. Ponds 1-9 need to be reconstructed.

All ponds are static ponds. Make-up water is added only as needed. The ponds do ice over in the winter. In the summer, there is a 4°C temperature fluctuation in a 24-hour period. Water quality is monitored in every pond every day. Fish in ponds are not fed when dissolved oxygen is < 6 ppm. No backup aeration is available in the ponds. Ponds are cleaned with a fire hose. Currently, there are too many fish (primarily bonytail) on station to dry and disinfect any of the ponds. When harvesting, traversing the stairs in the ponds is challenging. A driveway/harvesting equipment is needed to eliminate having to traverse the stairs. Stairs are an accident waiting to happen.

Horseshoe crabs are present in Ponds 4 and 16-21. Eradication has not been successful. If ponds are reconstructed, horseshoe crabs may be able to be eradicated.

Currently pond stocking density has to be maintained relatively low. This is primarily due to the lack of aeration equipment to maintain D.O. levels above critical minimums. No electrical power is available at each pond except for the UCRP ponds. Providing aeration could decrease the risk of losses due to D.O. crashes and increase the carrying capacity of the ponds.



Figure 6. Ponds at Wahweap State Fish Hatchery. Ponds 1-4 and 16-21 are sportfish ponds, 2 ponds out of 5-9 are for the Virgin River Program, the Least Chub Program rents one pond from

the Colorado River Program, and the Colorado River Program uses 3 ponds out of 5-9, 10-15, and 22-35.

Needs:

- Ponds 1-9 should be replaced.
- Ponds 16-21 need to be replaced.
- Driveways and harvest equipment are needed to eliminate use of stairs while harvesting.
- Horseshoe crab eradication.
- Electrical power could be supplied to ponds with supplemental aeration.

Isolation Building: The isolation building was built 3 years ago from a sea container (Figure 7). Oxygen is difficult to maintain in the tanks in the isolation building, and bottled oxygen is currently utilized to maintain DO levels. To maintain appropriate dissolved oxygen concentrations in the water, the water exchanges must be so fast that the fish must swim constantly. Woundfin treatments for Asian tapeworm occur in the isolation building.



Figure 7. Isolation building at Wahweap State Fish Hatchery.

Needs:

- SeQual Oxygen Generator with two compressors.
- Low head oxygenation system (LHO).

Shop: Woundfin adults from WSFH are overwintered inside the shop (Figure 8). The water is flow through and supplemental oxygen is applied. Hatching of catfish eggs in the spring is also performed in the shop. Fish culture in the shop area is far from ideal but has had to suffice due to indoor space limitations. Last year, approximately 2,300 adult woundfin were maintained indoors from November to March. The current tank cannot support more than 4,000 adult woundfin overwinter.

Need:

- \$800 pump box to aerate woundfin pond overwinter.



Figure 8. Woundfin tank in shop at Wahweap State Fish Hatchery.

Woundfin Transfers: The pond is cleaned before woundfin broodstock are moved outside in the spring. There are currently 1,908 adult and 270 young of year woundfin in the pond. In the spring of 2014, Zane will keep one pond open with aeration over the winter. The hypothesis has been that overwintering woundfin in ponds has not worked due to ice-over killing the fish. He will maintain the 1,908 adult and 270 young of year woundfin on station outside. Bird netting

will be placed over the pond. The 600 adults that will be sent from SNARRC will be maintained inside in the shop. A pump box is needed for aerating the woundfin pond overwinter.

Water Quality: Water chemistry analysis was conducted in 2002 (see results in Figure 9). The selenium concentration in the old well was 0.4 µg/l higher than the recommended guidelines for aquatic life. It is recommended that another water chemistry panel be conducted.

**UTAH STATE DEPARTMENT OF HEALTH
DIVISION OF LABORATORY SERVICES
Environmental Chemistry Analysis Report**

WAHWEAP WELL
UTAH DIVISION OF WILDLIFE
PO BOX 1446
PAGE

AZ 86040

928-645-2392

Lab Number: 200207137 Sample Type: 04 Cost Code: 900B
Description: OLD WELL - WAHWEAP STATE FISH HATCHERY
Collector: QUENTIN BRADWISCH

Site ID:	Source No: 00	Organic Review:	
Sample Date: 09/04/02	Time: 04:35	Inorganic Review:	10/10/02
		Radiochemistry Review:	
		Microbiology Review:	

TEST RESULTS:

Cyanide	<0.01 mg/l	Fluoride	0.356 mg/l
Turbidity	0.44 NTU	TDS @ 180C	1042 mg/l
T-Arsenic	<5.0 ug/l	T-Barium	0.017 mg/l
T-Beryllium	<1 ug/l	T-Cadmium	<1 ug/l
T-Chromium	9.1 ug/l	T-Copper	<12.0 ug/l
T-Lead	<3.0 ug/l	T-Mercury	<0.2 ug/l
T-Nickel	<10.0 ug/l	T-Selenium	2.4 ug/l
T-Antimony	<3.0 ug/l	T-Thallium	<1.0 ug/l
T-Sodium	148.0 mg/l	Sulfate IC	333.0 mg/l

QUALIFYING COMMENTS (*) on test results: NO COMMENTS

END OF REPORT

Figure 9. Water quality report for Wahweap State Fish Hatchery from 2002.

Needs:

- Conduct water quality assessment including trace elements.

Phytoplankton Bloom: The phytoplankton bloom is managed in the sportfish wiper ponds and UCRP ponds (Figure 10). Blooms are difficult to manage in the catfish and woundfin ponds due to the amount of fresh water that has to be added daily due to loss from leaky ponds. Without the phytoplankton blooms, the filamentous algal cannot be controlled and zooplankton is difficult to manage.

Ponds are fertilized with an inorganic granular fertilizer that is purchased from the IFA store in St. George, Utah. Fertilizer used is 11-52-0 (phosphorus, pot ash based) and 46-0-0 (nitrogen, urea based). Alga bloom is measured with a secchi disk (Table 1) and a colorimeter (Module: Orion AQ4000). Fertilizer is added on a percentage of alga bloom 2 times a week if needed. Algae blooms vary from pond to pond throughout the year and require different amounts of fertilizer. Pond fertilization targets are 600 ug/l for N and 30 ug/l for P. Ponds clear up very quickly for woundfin production and do not regain their bloom with subsequent fertilization.

Table 1. Secchi disk reading chart for pond fertilization.

Secchi Disk Reading	Condition of Bloom	Pond Recommendation
Greater Than 24-Inches	Inadequate	Fertilize Pond
18 to 24-Inches	Healthy	Continue Monitoring
12 to 18-Inches	Dense	Increase Monitoring and Top Off Ponds
6 to 12-Inches	Excessive	Flush with Water, Aerate, and Find Cause
Less than 6-Inches	Critical	Maximum Flushing and Increase Aeration



Figure 10. Woundfin pond with good phytoplankton bloom.

Needs:

- Determine why phytoplankton blooms cannot be maintained in woundfin ponds.

Predator Control: WSFH has a federal bird permit to reduce the number of predatory birds. They also have trapping permits for fur bearers. Some bird netting is used on site (Memphis nets 2 inch by 2 inch square with cable grid). The bird netting is used on ponds 10-15 which are UCR ponds. Herons will still perch around sides and on cable. Netting is expensive, but SNARRC has seen significantly higher returns over-winter when netting is used. The woundfin broodstock pond will be netted overwinter. It is difficult to maintain netting in spring and summer when fish are actively feeding, as nets make it difficult to get close to the feeding area to observe fish behavior.

Existing Facility Priorities: Currently, production at WSFH is 63% Colorado River fishes, 28% sportfish, 6% Virgin River woundfin, and 3% Least Chub. The WSFH is a Virgin River chub refuge. There is another chub population at SNARRC that is also a refuge population

which is supplemented with wild fish (n=100). Discussions about the WSFH chub population being moved offsite have occurred with no resolution.

The Review Team prioritized the facility needs based on sport fishes, woundfin, and other native fishes. The following are the prioritized lists with the smallest number representing the highest priority.

Sport Fish

1-Rebuild sport fish Ponds 1-4

1-Residence

1-Treatment facility

2-Rebuild sport fish Ponds 16-20

3-New hatchery building (part of the recirculating system listed for woundfin; need biosecurity)

Woundfin

1-New hatchery building with recirculating system (estimated 1,500 cubic feet of rearing space; 11,221 gallons of water; engineer will estimate costs and O&M; this space would allow 55-4 ft circular tanks or a mix of tanks; ballpark estimate is \$300,000-500,000; heating water would have to be with propane, electricity, or solar.

1-Residence

Other Natives

1-Treatment facility

Staffing Needs: Currently at WSFH, there are 3 full-time permanent employees and 1 half-time employee. If a hatchery building is built onsite, another residence and 1 more full-time permanent employee will be needed.

Fish Health: A Health Condition Profile (HCP) developed for trout has been incorporated at WSFH. External and internal organs are examined through necropsy. The HCP is a critical tool to evaluate nutrition and growth and provide a benchmark for overall fish health. The HCP

allows for maximization of fat content to overwinter but not allow for pathological fat accumulation. The HCP is conducted just prior to stocking.

In 2010, Ich was detected in woundfin at WSFH. Ich has been an issue in the past when water temperatures shift. This will need to be monitored closely with woundfin being maintained outside in 2013-2014 as uncontrolled temperature fluctuations will occur.

The routine inspection in 2011 resulted in detection of Asian tapeworm in Virgin River chub. This is a legally restricted pathogen. Eradication of Asian tapeworm was not 100% successful with praziquantel treatment of 0.75 mg/L. Subsequent treatments at 2.5 mg/L were successful in eradicating Asian tapeworm in the chub, however, the fish are being reinfected at a low rate potentially during the 24-hour flush after treatment. A 100% flush of worm segments in the raceway is not possible. Lake Powell could also be a wild source of reinfection. There is now a critical mass of infection and transfer of Asian tapeworm from pond to pond is occurring. To completely eradicate Asian tapeworm from the facility, there are logistic challenges. The entire facility would need to be vacated at single time to eradicate Asian tapeworm. All ponds need to be cleaned and dried or allowed to freeze. If ponds remain wet, the copepods, which are the intermediate host, do not die. There are enough trucks in Utah to hold all fish at WSFH, but fish would need to be held for 2-3 weeks which is prohibitive, and conducting this extreme possibility in the summer poses a problem because maintaining dissolved oxygen in warm waters is challenging. No detection of Asian tapeworm in woundfin, razorback, wipers, or catfish has occurred.

The routine inspection of WSFH in 2012 resulted in no detection of pathogens.

If more fish are reared on station, there will be a need for larger disinfection and quarantine capabilities.

There is currently a biosecurity issue at WSFH in that river water can back flush into Ponds 6-9. This must be remediated.

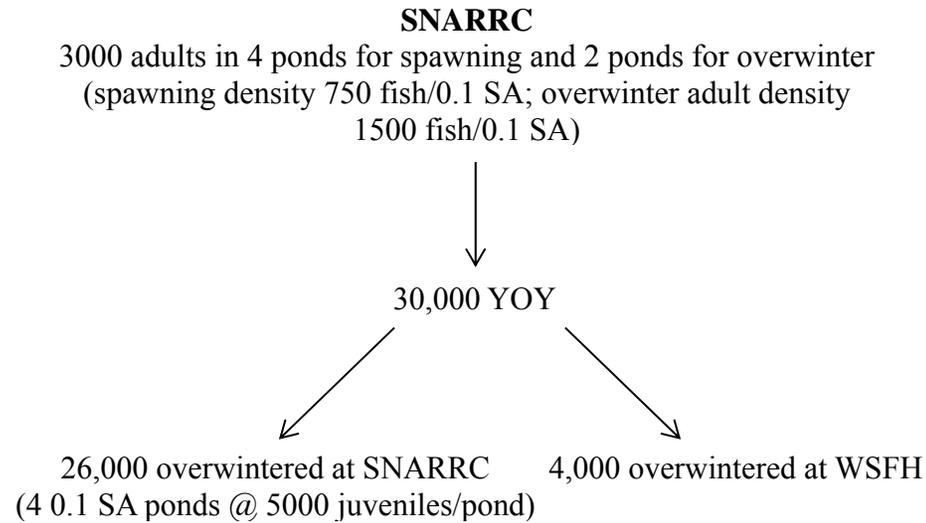
Needs:

- If more fish are reared on station, there will be a need for larger disinfection and quarantine capabilities.

Potential Short- and Long-Term Solutions to Meet VRP Goals: The following diagram depicts the potential short-term and long-term solutions for the VRP to meet the goal of 100,000 YOY woundfin stocked into the Virgin River in March.

**To Achieve Current 30,000 YOY in March
Through Extensive Culture**

Caveats: WSFH will try to overwinter adult woundfin outside in 2014



**Short-Term Solution to Achieve 50,000 YOY
in March Through Extensive Culture**

SNARRC will require 2 extra ponds
for spawning and an increase in
broodstock (n=2,000 additional adults)
in addition to current extensive culture plan

WSFH becomes a grow out facility
(no spawning) and requires 2 extra
ponds with power in addition to
current extensive culture plan

20,000 overwintered at SNARRC
(4 0.1 SA ponds @ 5000 juveniles/pond)

30,000 maintained at WSFH
for two years*

**Long-Term Solution to Achieve Additional 50,000
YOY in March Through Intensive Culture**

Caveat: Intensive culture must be proven to be successful
(broodstock number for 50,000 YOY through intensive
culture have not yet been determined)

Hatchery Building at WSFH

FES as Potential Grow Out Facility

*water temperatures in ponds at WSFH will not allow for growth to 65-70 mm

**To Achieve Current 100,000 YOY in March
Through Extensive Culture**

Caveats: WSFH needs to be able to overwinter woundfin outside

SNARRC and WSFH

9,000 adults in 12 ponds for spawning and 6 ponds for overwinter at SNARRC
(spawning density 750 fish/0.1 SA; overwinter adult density
1500 fish/0.1 SA)



100,000 YOY



100,000 YOY maintained at WSFH in
10 grow out ponds at 10,000 fish/pond
for two years*

Single Facility (10 ponds total)

9,000 adults in 10 ponds for spawning
(spawning density 900 fish/0.4 SA)



100,000 YOY



100,000 YOY maintained in
8 grow out ponds at 12,500 fish/ 0.4 SA
for two years*
Adults overwintered in 2 ponds at
4,500 fish/X SA

*water temperatures in ponds at WSFH will not allow for growth to 65,700 g

Prioritized Needs List for Wahweap State Fish Hatchery

The following is a prioritized list of needs that were generated following the Wahweap State Fish Hatchery site visit. The needs were prioritized by Zane Olsen into high, medium, and low priority needs. Needs are not prioritized within the categories.

High Priority

- Replace bowls or add a restrictor plate to the old well. **(Need Further Investigation)**
- The trailer and tanks need to be replaced in the woundfin trailer. **(2013 is the last year for the trailer.)**
- Ponds 1-9 should be replaced.
- Ponds 16-21 need to be replaced.
- Driveways and harvest equipment are needed to eliminate use of stairs while harvesting.
- Horseshoe crab eradication.
- SeQual Oxygen Generator with two compressors needed for Isolation Building.
- Low head oxygenation system (LHO) needed for Isolation Building.
- Determine why phytoplankton blooms cannot be maintained in woundfin ponds.
- If a hatchery building is built onsite, another residence and 1 more full-time permanent employee will be needed.
- If more fish are reared on station, there will be a need for larger disinfection and quarantine capabilities.
- Additional WF ponds. **(High priority for additional WF)**

Medium Priority

- Alarm system for the old well to detect electrical failure(s).
- Alarm system for the new well to detect electrical failure(s).
- Alarm system to detect flow failure in woundfin trailer.
- Conduct water quality assessment including trace elements.
- Hatchery building construction. **(If WF can be reared with “Intensive Aquaculture”, then this is a priority.)**

Low Priority

- A VFC/plumbing is needed if the woundfin trailer remains to be able to use the raceway simultaneous to having fish maintained in the trailer.
- A French drain from the woundfin trailer should be installed for water discharge to contain potential pathogens and their spread across the facility.

Task 19. Woundfin Intensive Culture Demonstration Project

William C. Fraser, Aaron Nistler, Mariah Talbott, Gibson Gaylord and Molly Webb

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Introduction

Woundfin (*Plagopterus argentissimus*) are a small scaleless fish historically found in the lower Colorado River basin that have an omnivorous feeding habit consuming insects, insect larvae, seeds and organic debris and detritus. Woundfin were listed as a federally endangered fish species in 1970 (USFWS 1994) and were brought into captivity in 1979 to create a refugial population. Fish culture efforts have primarily focused on spawning woundfin in ponds and collecting juvenile fish to augment wild populations. Stocking efforts began in 1993 from the Southwestern Native Aquatic Resources and Recovery Center (formerly Dexter National Fish Hatchery and Technology Center) and are ongoing.

The purpose of this project was to combine all woundfin culture techniques that had been developed at the Bozeman Fish Technology Center (BFTC) since 2008 to determine if woundfin could be produced intensively. Our goal was to produce progeny in a reuse aquaculture system to determine whether intensive culture may be incorporated in the conservation propagation program as a means to meet the recovery program's goal of 100,000 10-month olds annually.

Materials and Methods

The 2008-2009 and 2010-2011 adult woundfin were used during the 2013 demonstration project. The 2008 and 2009 adults were combined into one 8'x2'x2' tank in 96 gallons of water. The 2010 and 2011 adults were combined into an identical tank. The tanks received six gpm flows throughout the experiment. Temperature was controlled by combining cold (8°C) and warm spring water (22°C; Figure 1) and using a chiller or heater to maintain the temperature profile described in Tasks 5, 7, and 12.

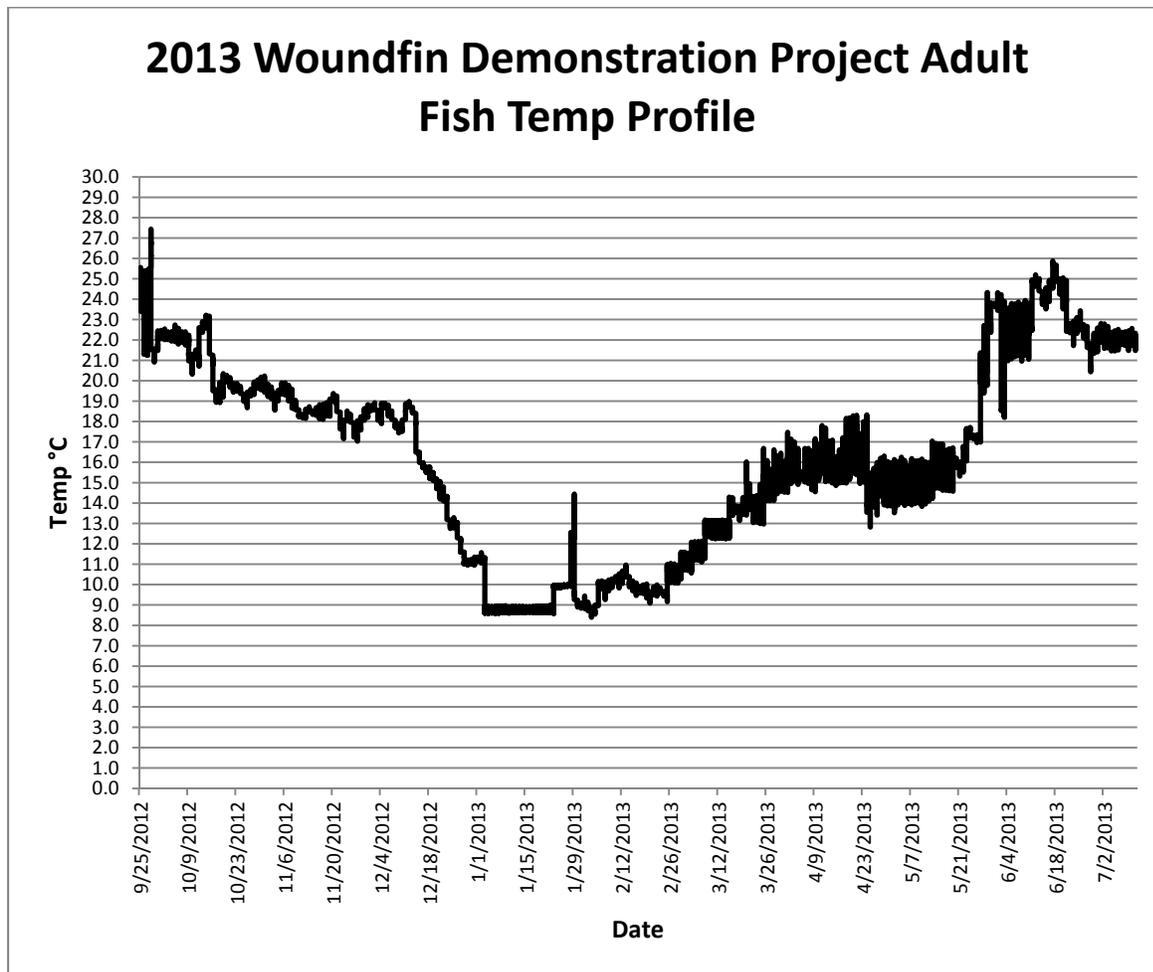


Figure 1. Water temperature profile to which woundfin adults were exposed during the demonstration project conducted in 2013 at the Bozeman Fish Technology Center.

Woundfin adults (n=307 2008 and 2009 and n=284) were placed in the spawning tanks three months prior to the projected spawning season to allow fish to acclimate to the new environment. When the temperature reached 16°C, the fish were held at this temperature for one month. After the one-month holding period, the fish were quickly ramped (1°C per day) to a target temperature of 22°C. During the ramping period, fish were observed daily for chasing behavior. At the first signs of chasing, 21 9"x6"x2" plastic fork trays half filled with 9/16" multicolored marbles were added to the tanks alternating the 2008-2009 and 2010-2011 adult tanks. Once spawning started, spawning trays were checked every morning for embryos. If no embryos were seen, spawning trays and marbles were cleaned and dried. Fish were not fed when the spawning trays were in the tank. When embryos were found, a screen was placed over trays immediately in the morning (7 AM) to reduce predation. To remove embryos from the spawning substrate, the trays were soaked in 1.5% sodium sulfite for 15 minutes. The trays were gently rinsed 3 times in system water over a collection tub, and the embryos were counted (alive and dead). The embryos were incubated in either McDonald jars with a flow through formalin treatment or

condos (PVC pipe with small mesh bottom) with a static formalin treatment. For the flow through formalin treatment, embryos were treated with 2000 ppm formalin (Paracide F; (flow rate x treatment time x final concentration x correction factor)/chemical concentration) in a flow-through bath for 15 minutes to reduce fungus. Embryos in the condos were treated with 1500 to 2000 ppm formalin (Paracide F; (volume of water to be treated x final concentration x correction factor)/chemical concentration) in a static bath for 15 minutes to reduce fungus. The static formalin treatment was conducted twice a day for two days or as needed until embryos hatched. All dead embryos were removed from the McDonald jars or condos daily. Formalin treatments ceased upon hatching.

Results/Discussion

Based on the number of adults at the end of the experiment, woundfin in the intensive culture system at the Bozeman Fish Technology Center produced 36.37 eggs/adult with 6.70 larvae/adult initiating to feed (Table 1). On average, the person hours to produce the larvae and care for the culture systems amounted to 8.26 person hours per day for 53 days.

The project experienced significant fungal outbreaks for the duration of the experiment. Any dead embryos were encased in fungus very quickly trapping live embryos in the fungal mass. Embryos often died during epiboly; we determined this mortality was not due to handling. Mortality and fungus complicated embryo care and significantly affected successful hatch of those embryos undergoing normal embryogenesis. Fungal outbreaks must be controlled prior to a complete assessment of intensive culture systems for woundfin production.

Table 1. Number of adult woundfin spawned and the number of embryos and larvae produced in the demonstration project at the Bozeman Fish Technology Center in 2013.

Age Class	2008 and 2009	2010 and 2011	Total
Adult at beginning	307	284	591
Adults at end	262	261	523
Embryos	5,863	13,156	19,019
Larval on feed for both groups			3,450

References

USFWS. 1994. Virgin River Fishes Recovery Plan. US Fish and Wildlife Service, Salt Lake City, Utah. 45pp.

Task 20. Improve Egg Quality in Intensive Culture of Woundfin

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Introduction

Egg quality is determined by many factors including but not limited to genetic and nutritional make-up of broodstock, stress load of broodstock, size and/or age of broodstock, contaminant load of eggs, and overripening of eggs (Lubzens et al. 2010). There are several causes of arrested embryogenesis in fishes which include factors that affect egg quality listed above as well as exposure to contaminants and ultraviolet-B radiation, handling, temperature, fertilization success, and salinity. Reproductive success, specifically fertilization success, can be affected by density and sex ratio (i.e. male-male competition; e.g. Spence and Smith 2005; Castranova et al. 2011; Weir 2013; Billman et al. 2014). The optimal density and sex ratio for spawning of woundfin in conservation propagation has never been determined.

In previous years of intensive culture at the Bozeman Fish Technology Center (BFTC), significant fungal outbreaks during embryo incubation have occurred. Dead embryos were encased in fungus very quickly trapping live embryos in the fungal mass. It was determined that a high number of embryos died during epiboly due to a cause(s) other than handling. The cause of mortality has not been determined but egg quality and fertilization success are of concern.

To better understand the potential causes of mortality during embryogenesis and assess egg quality, we described embryogenesis to identify the key stages of development and their timing and tested the following null hypotheses: 1) sex ratio of adults has no effect on egg quality as assessed by fertilization success, 2) at optimum sex ratio, tank density has no effect on egg quality as assessed by fertilization success, and 3) current intensive culture handling protocols (deadhesion and incubation) have no effect on embryogenesis and hatch success.

Methods and Materials

All 2010-2011 woundfin at the BFTC were exposed to water temperatures of 9-22°C with natural vernalization of temperatures from 9 to 16°C during January to May and a temperature ramping period from 16 to 22°C the third week of May. Fish were exposed to the natural photoperiod of St. George, Utah. This year class of fish was used for all trials. To describe embryogenesis, 3 females and 4 males were hormonally injected with common carp pituitary extract (20 µg/g) on June 22, 2014. Fish were anesthetized, weighed, and all injections were administered intramuscularly. Following fertilization, embryos were transferred in hatchery water to an incubator in the laboratory (22°C). Photos were taken hourly using a Leica DM2000 compound scope (10-100x) for 27 hours.

The sex ratio trials were conducted June 1-June 6 (Trial 1) and June 8-June 13, 2014 (Trial 2). Fish (n=55 females and n=125 males) were randomly assigned to the 3 treatment groups (n=12 fish/tank total) except for sex. Treatments were run in quintuplicate. The 1:1 sex ratio treatment

had 6 females and 6 males, the 1:3 sex ratio treatment had 3 females and 9 males, and the 1:5 sex ratio treatment had 2 females and 10 males. Fish were allowed to acclimate to their tanks for three days before the addition of spawning substrate. Each trial lasted 5 nights and 6 days with spawning substrate placed in the tank at 3 PM every afternoon, and the substrate removed at 7 AM every morning.

The density trial was conducted using the 1:3 sex ratio on June 15-June 20, 2014. Fish (n=77 females and 154 males) were randomly assigned to the 3 treatment groups, and treatments were run in quadruplicate. The density treatments were 0.05 fish/L (4 fish/tank), 0.21 fish/L (16 fish/tank), and 0.49 fish/L (38 fish/tank). Fish were allowed to acclimate to their tanks for three days before the addition of spawning substrate. The trial lasted 5 nights and 6 days with spawning substrate placed in the tank at 3 PM every afternoon, and the substrate removed at 7 AM every morning. One trial was conducted.

Following the initial density trial, the fish were placed in holding tanks, and randomly chosen for a single tank trial of 0.95 fish/L (76 fish/tank). Not enough males were available to replicate the treatment at the 1:3 sex ratio. Fish were allowed to acclimate to their tank for three days before the addition of spawning substrate on June 22-June 27, 2014. The trial lasted 5 nights and 6 days with spawning substrate placed in the tank at 3 PM every afternoon, and the substrate removed at 7 AM every morning.

Each morning (7 AM) following the addition of spawning substrate, covers were placed over substrate to end spawning. Substrate trays were removed and treated with sodium sulphite solution to deaden eggs/embryos. All eggs/embryos were gently decanted from individual spawning trays and individually counted. Fertilization success (expressed as a percent) was calculated at the 4-cell stage (3 hours at 22°C) from three individual counts of 30-40 embryos each and averaged for the tank. Only fertilization success was assessed in the trials as embryos were needed for the fungal trials. At the end of each trial, sex was confirmed by expressing gametes from the urogenital pore.

One way analysis of variance was used to compare total number of eggs and total number of fertilized eggs among sex ratio treatments and density treatments. The 0.95 fish/L density could not be compared to the other three densities as only one replicate was possible due to limited number of males.

Results and Discussion

The optimal stages of embryogenesis to assess developing woundfin embryos were: 8-cell stage (3 hours at 22°C; fertilization success), high stage of cleavage (5 hours at 22°C), 90% epiboly (10 hours at 22°C), and eyed embryos (24 hours at 22°C) (Kimmel et al. 1995; Figure 1).

Data from the sex ratio Trials 1 and 2 were combined. All sex ratios (1:1, 1:3, and 1:5) tested resulted in fertilized embryos. There was no significant difference among the total number of eggs spawned per treatment with the highest number of eggs found in the 1:5 sex ratio (n=1,413)

followed by the 1:3 (n=1,309) and the 1:1 sex ratios (n=1,197) (P=0.8951; Figure 2). Fertilization success was highest in the 1:1 sex ratio treatment followed by the 1:5 and then the 1:3 sex ratio treatments but was not significantly different among treatments (1:1 = 91%; 1:3 = 82%; 1:5 = 86%; P=0.5376).

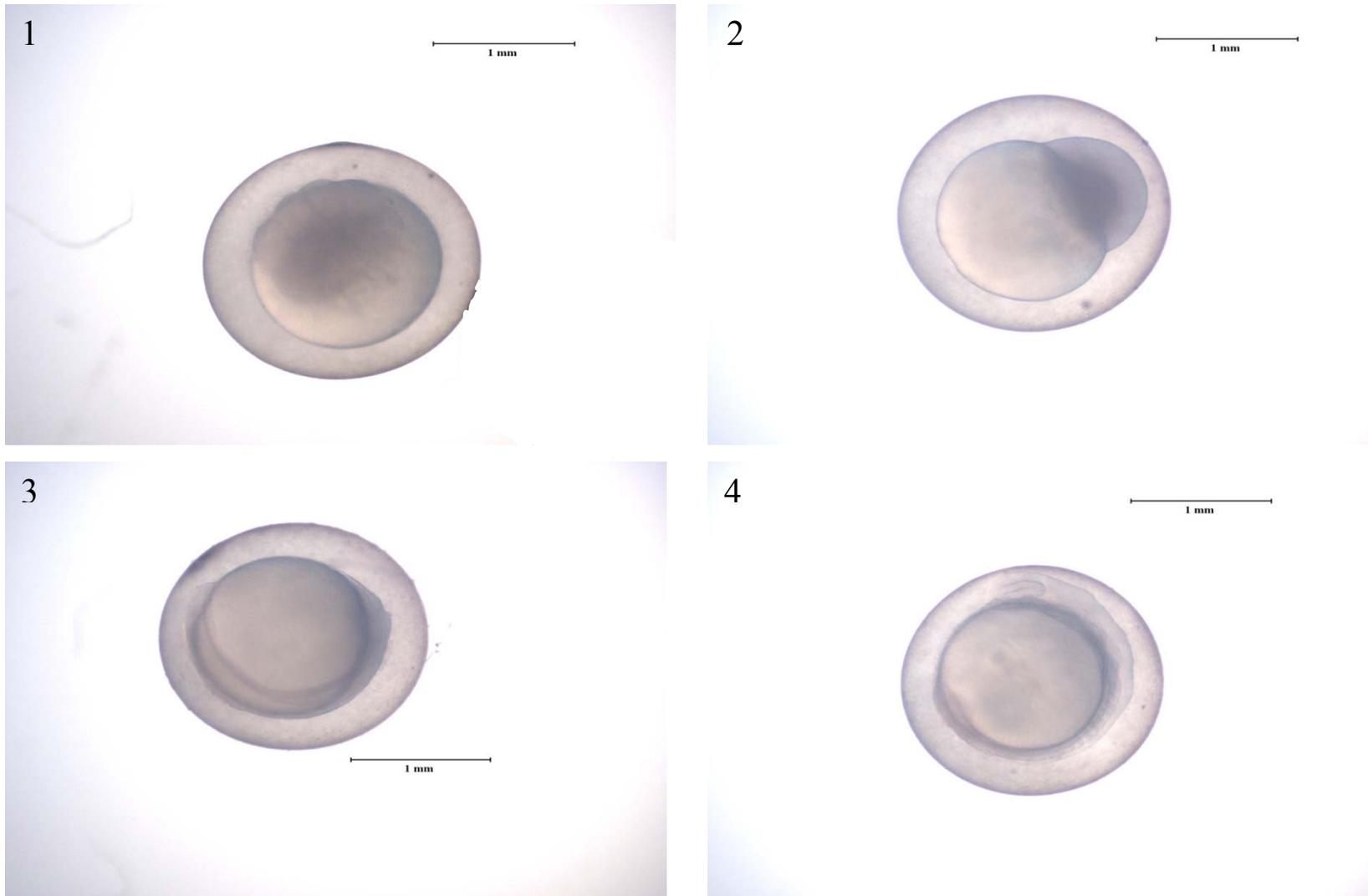


Figure 1. Optimal stages of embryogenesis to assess developing woundfin embryos due to ease of visual identification. 1) 8-cell stage (3 hours at 22°C; fertilization success), 2) high stage of cleavage (5 hours at 22°C), 3) 90% epiboly (10 hours at 22°C), and 3) eyed embryos (24 hours at 22°C).

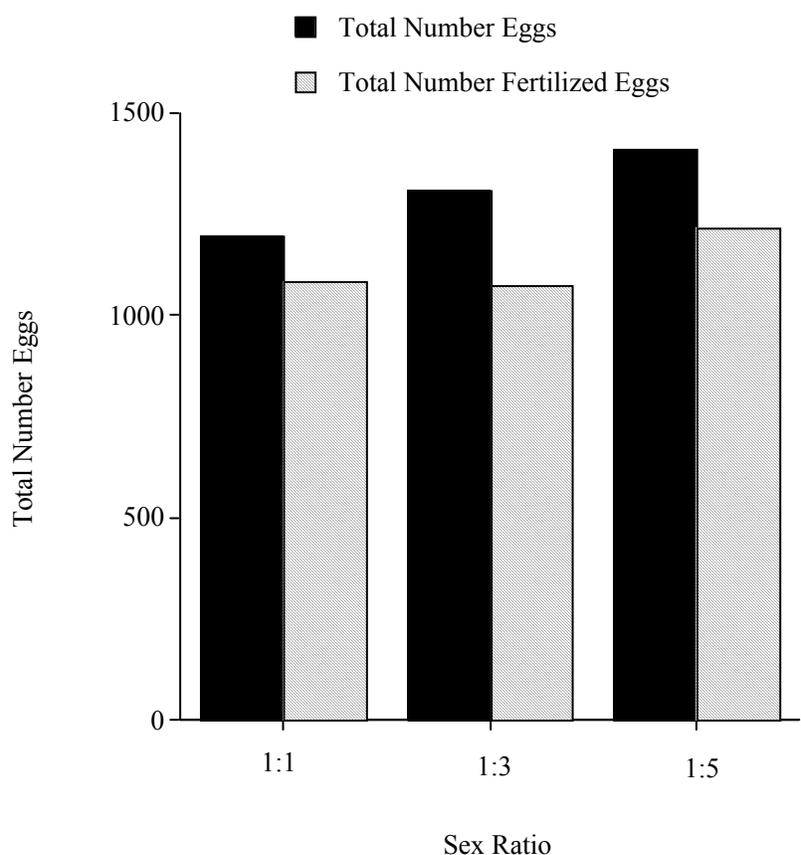


Figure 2. Total number of eggs and fertilized eggs from woundfin spawned at different sex ratios (1:1=6 females and 6 males, 1:3=3 females and 9 males, 1:5=2 females and 10 males).

At a sex ratio of 1:3, tank density (0.05 fish/L = 4 fish/tank, 0.21 fish/L = 16 fish/tank, 0.47 fish/L = 38 fish/tank) did effect spawning ($P=0.0003$) with the 0.05 fish/L treatment resulting in zero eggs/embryos. The fertilization success was highest in the 0.21 fish/L treatment (86%) as compared to the 0.47 fish/L treatment (75%), but the fertilization success of these two treatments did not differ significantly ($P=0.1184$). We did assess one tank of woundfin at 0.95 fish/L which resulted in 1,624 eggs and 80% fertilization. We did not see a negative effect of the highest density we could achieve on spawning and egg quality as assessed by fertilization success. Statistics were not possible to compare the four density treatments as only one tank at 0.95 fish/L was possible due to limited fish number.

These results indicate that woundfin in intensive culture may be spawned at a sex ratio between 1:1 and 1:5 and densities of 0.21 fish/L to 0.47 fish/L, possibly as high as 0.95 fish/L, with no detrimental effects on spawning and egg quality as assessed by fertilization success. Hatch success was not assessed as embryos from these trials were used to address task 21.

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Task 21. Control of Fungus in Intensive Culture for Woundfin

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Introduction

One limiting factor on increasing embryo survival in intensive culture of woundfin has been our ability to control fungal overgrowth of fertilized eggs and embryos prior to hatching. Treatment of fungus in fish hatcheries has been widely described across multiple fish species with varying degrees of success depending on the chemical utilized and fish species. Treatment options have included formalin, hydrogen peroxide, copper sulfate, malachite green, sodium chloride, sodium and potassium dichromate among others. Malachite green was at one time the fungicide of choice for treating fish and eggs. It has largely been replaced by other treatment options that due to its toxic effects (Sudova et al 2007). Bozwell et al (2009) demonstrated 400 and 800 ppm hydrogen peroxide were efficacious in controlling fungus in a single static bath treatment to improve hatch rates to approximately 70% with golden shiner eggs. Marking et al (1994) demonstrated the efficacy of both hydrogen peroxide and formalin as flow through treatments for controlling saprolegnia, both of which are now considered standard procedures for fungus control on trout eggs. Copper treatment also has been shown to be effective in controlling fungus on fish eggs and improving hatchability (Straus et al 2009; Mitchel et al 2010). Dichromate compounds of sodium and potassium also have been reported to reduce fungus infections in eggs using a static bath. To date, the most widely used treatments in a hatchery setting appear to be formalin and hydrogen peroxide.

Materials and Methods

In order to test the efficacy of various treatments for fungus control, woundfin were spawned 30 May to 19 Jun 2014. Two substrate trays filled with marbles were placed in each tank in the morning and left in the tanks for 24 h. The following morning egg trays were covered with a metal screen until removal to decrease predation on eggs by adults. A 1.5% sodium sulfite solution was utilized to remove eggs from marble substrate. Egg trays were placed in solution for 10min to eliminate adhesiveness of eggs to the marbles. Marble trays were agitated and rinsed until all eggs fell off marbles. Eggs were gathered, counted, and cleaned three times with flow through water. Eggs were prescreened for fertilization success (according to methods above, Task 20) and only viable eggs utilized in the trial. Once all eggs were gathered, they were counted into lots of 50 eggs and placed into condos (plastic PVC coupler with nylon screen). Each condo was placed in a zebra fish tank (9 liters - 2.38 gallons) receiving a constant flow of UV treated spring water at 22 °C. Each tank was assigned one of 7 treatments to control fungus growth. Each condo was an experimental unit and up to four condos were present in each treatment tank. Replication was performed over time as eggs were available to stock condos.

Flow through water was utilized for all treatment groups except sodium dichromate and potassium dichromate treatments. Treatments included a negative control (no chemical treatments), formalin 2000 ppm for 15 min daily, hydrogen peroxide 1000 ppm for 15 min daily, sodium dichromate at 125 ppm continuous bath, potassium dichromate at 125 ppm continuous bath, and copper continuous treatment utilizing copper scrub pads in the bottom of the tank. Thirteen replicate groups of embryos were utilized in the control treatment, Copper pad, and malachite green treatment. Eleven replicate groups of embryos were utilized for formalin, hydrogen peroxide, sodium dichromate and potassium dichromate treatments. A relative fungus growth score was developed and percent eyed embryos and hatched fry were quantified. Fungus score was simply defined as visual observation of formation of fungus overgrowth on eggs defined as yes or no.

Statistics

Analysis of variance (JMP 11, SAS Institute Cary, NC) was utilized to test treatment effects on percent eyed eggs and percent hatch. Fungus score was analyzed by contingency analysis of categorical data.

Results and Discussion

The efficacy of formalin, hydrogen peroxide, copper, sodium dichromate, potassium dichromate and malachite green in preventing mortality of embryos putatively caused by fungal overgrowth was tested. The research demonstrated that treatment of eggs with formalin, sodium dichromate or potassium dichromate improved the percent eyed egg and percent hatch above control treatment levels over the course of the experiment (Table 1). The untreated controls had 1.2 and 1.1% of the embryos developing to the eyed egg stage and hatching, respectively. Equivalent results were observed for development of embryos treated with hydrogen peroxide (12.1% eyed eggs and 9.5% hatch rate), malachite green (5.6% eyed eggs and 5.2% hatch rate), and copper pads (7.8% eyed eggs and 5.4% hatch rate).

Table 1. Embryos surviving to the eyed egg stage or hatching and relative observation of fungus for woundfin embryos treated with various antifungal compounds.

Treatment	Eyed eggs (%)		Hatched fry (%)		Fungus score ⁶ (%)
	mean	± stdev	mean	± stdev	
Control	1.2	3.3	1.1	3.3	100
Formalin ¹	21.1*	17.6	17.3*	18.6	60*
Hydrogen peroxide ²	15.5	10.5	9.5	11.8	100
Sodium dichromate ³	23.5*	19.4	20.7*	19.6	66.7*
Potassium dichromate ³	22.9*	19.8	20.9*	19.7	71.4*
Malachite green ⁴	5.6	8.7	5.2	8.8	100

Copper pads ⁵	7.8	9.7	5.4	9.7	100
Root MSE	13.6		14.0		
Pr>F or ChiSq	0.0001		0.0015		0.0228

¹ Parasite-S, Western Chemical, Ferndale, WA, USA

² 35% PEROX-AID®, Western Chemical, Ferndale, WA, USA

³ Fisher Scientific, Pittsburgh, PA, USA

⁴ Rid-Ich Plus, 4.26% Formalin and 0.038% chloride salt of malachite green, Kordon LLC, Hayward, CA USA

⁵ Copper scrub pads, Chore Boy, Prestige Brands Holdings Inc., Tarrytown, NY USA

⁶ Fungus score is a subjective rating of presence of fungus overgrowth on embryos as yes (observed large fungus easily visible) or no (limited fungus visible with the naked eye)

* Indicates difference from untreated controls at P<0.05 with analysis of variance and contrast statement.

Fungus score was rated as yes or no for severe fungal overgrowth was evaluated by contingency analysis. Fungus was observed in 60% or greater of all treatment group replicates with controls, hydrogen peroxide, malachite green and copper treatments having severe fungus observed in all replicates. Formalin, sodium dichromate and potassium dichromate treatments reduced incidence of severe fungus overgrowth to 60, 66.7 and 71.4% occurrence.

Although formalin, sodium dichromate and potassium dichromate were able to improve the number of woundfin embryos developing to the eyed egg stage as well as the hatch rate compared to untreated embryos, the incidence of fungal overgrowth was still severe in over 60% of the replicates. It is unclear if fungus overgrowth was the main cause of low survival observed in the trial but treating embryos to reduce fungus did improve survival. Average survival rates of less than 20% were observed in the formalin, sodium dichromate and potassium dichromate treatments compared to 1% for the control embryos. Peak hatch rates of for the best replicates were approximately 50% compared to only 12% in the control treatment. The prevalence of fungus may be in part to a number of factors. One may be due to the inability to effectively remove dead eggs or embryos from our culture condos in a manpower efficient manner. This effort is more easily performed with trout eggs in which dead eggs either float out of the incubators as with MacDonald jar or by picking eggs in Heath trays. The small egg size of woundfin makes pick a tedious labor intensive endeavor. Other species with small eggs (e.g. Walleye and hybrid striped bass) are routinely hatched in MacDonald jars where dead eggs and embryos flow out to prevent contamination of other embryos. Hatching with MacDonald jars has not proven effective in our hatchery with woundfin due to the limited number of eggs. Our experience with hatching woundfin in jars has been that it is difficult, even in 1 L jars, to maintain even flow through the eggs without excessive eggs disturbance. Refining our ability to remove dead eggs early should prove beneficial in decreasing fungal overgrowth and improving embryo hatch rates when performed in tandem with some of the classical antifungal treatments such as formalin baths.

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Overall Conclusions and Recommendations

A series of research trials have been performed at the Bozeman Fish Technology Center since 2008 when woundfin were first brought on station. The trials can be generally grouped into three main classes of research with an overall goal of improving the ability to intensively produce woundfin for stock enhancement. The first research area was to address reproductive success of woundfin and optimize methodology for spawning success. The second research area was to develop protocols for embryo and larval culture and rearing fingerlings to maturity. The third research area was to assess the efficacy of current diets utilized in larval, juvenile and adult stages and establish general recommendations for nutrient targets for feeds. This section will give an overall synopsis of the results and recommendations for potential implementation in the culture of woundfin.

Reproduction

The BFTC successfully reared and spawned age-0 woundfin (2008-year class) from SNARRC in 2009. The maturation cycle for woundfin has been described which provides critical information that will allow for successful and consistent spawning of woundfin in intensive culture. Woundfin sexually differentiated by 5-months of age and reached first sexual maturity by 10-months of age at 22°C. Histological sections prepared from gonads of females revealed the presence of at least two clutches of developing ovarian follicles within the same female indicating batch spawning. An ELISA for the detection of Vtg in woundfin has been successfully developed. Measurement of plasma Vtg allows for a biochemical marker to detect the onset of maturation in woundfin and the detection of endocrine disruption in wild woundfin exposed to estrogenic compounds.

Oviposition (i.e. release of ovulated eggs) by woundfin females appears to require the presence of substrate. Chasing behavior by ripe males does occur but does not confer immediate female spawning readiness (i.e. immediate oviposition). Ultrasound can be used to successfully differentiate non-reproductive females, mature females and males. Testicular size does not confer stage of maturity, and therefore, ultrasound cannot be used to differentiate non-reproductive males from reproductive males.

The maturation cycle of woundfin can be entrained using temperature to increase the number of eggs spawned by females while compressing the spawning season. The temperature profile to entrain the maturation cycle begins by following the natural vernalization of water temperature from 8-16°C from winter to spring; when water temperature reaches 16°C, temperature is maintained at that temperature until mid-May at which time temperature is quickly ramped from 16-22°C during a one-week period. At SNARRC, significantly more eggs were spawned when woundfin were exposed to this water temperature treatment compared to a control group exposed to the natural water temperature. Three times more young-of-year woundfin were produced in

2011 (~15,000 young-of-year) compared to the average production of other years (~5,000 young-of-year).

Based on observations of predation of embryos at the BFTC, a suspected limiting factor to pond culture production of woundfin was cannibalistic predation on embryos and larvae. We experimentally measured rates of predation on embryos and larvae by adults and found that predation was a significant source of mortality on both embryos ($W = 210$, $P < 0.001$) and larvae ($W = 45$, $P = 0.004$). In tank trials, a median number of three embryos consumed over 4 hours in a tank containing 10 adults translated into an average of 0.075 embryos eaten per adult per hour. Using the same calculation, the estimated rate of larval predation was 0.025 larvae eaten per adult per hour. These predation rates may represent a maximum or elevated predation rate due to clear water in a tank environment compared to the propagation facilities. Even if actual predation rates in the pond culture environment is a small fraction of the maximal rate observed in this study, cannibalistic predation of embryos and larvae could be a substantial contributor to production shortfalls, and it was recommended that spawning substrate be removed daily and/or newly hatched larvae be removed as soon as possible following hatch. As of the 2012 spawning season, SNARRC incorporated the thermal regime and removal of larval fish into a separate pond from the spawning pond to avoid predation on embryos and larvae. As a result, ~30,000 young-of-year woundfin were produced in each 2012, 2013, and 2014.

The UV-B related mortality threshold for ambient levels of UV-B present at the woundfin conservation propagation facilities is between 5 and 14.5 hours of exposure. We determined that the survivable level of UV-B radiation for developing woundfin embryos is $< 0.015 \text{ mW/cm}^2$ or $< 25\%$ of the ambient levels present at Bubbling Ponds at ≥ 14.5 -hr exposures, while the effects of ambient UV-B radiation detected at SNARRC for 5 hours resulted in very low UV-B related mortality. This suggests that short-term, high intensity UV-B damage is far less detrimental than prolonged exposure. Susceptibility of larvae to UV-B appears to be a function of age at exposure with older larvae exhibiting significantly lower levels of mortality during the initial days of exposure, and short-term, environmentally relevant doses of UV-B do not appear to cause mortality in larval woundfin.

A hormonal injection regime was developed for woundfin. A Common Carp Pituitary Extract (CPE) injection of 20 ug/g administered intramuscularly resulted in the highest ovulatory success (83%) and mean number of eggs released per female (500 eggs/female). The trials examining the efficacy of CPE injections resulted in a significant increase in the number of ovulated females in response to CPE (87%) compared to the control (59%; no hormone), and the number of eggs per female was significantly greater in the CPE treatment (mean \pm sem = 390 ± 43) compared to the control (mean \pm sem = 180 ± 41). Though the fertilization success did not statistically differ between the treatments ($P=0.66$), the hatch success was significantly greater in the CPE embryos compared to the control embryos ($P=0.04$). The spermiation success for males increased in response to CPE (88%) compared to the control (41%; no hormone). The volume of sperm per male was greater (65 μl) in the CPE treatment compared to the control (9 μl), and the

motility of sperm was two times greater in the CPE treatment (32 seconds) compared to the control (16 seconds). The variability within a hormonal treatment was high in the woundfin studies due to the lack of a tool to assess spawning readiness and standardize the response of females to a treatment by spawning at the optimal stage of oocyte maturation. When or if the development of a tool to determine spawning readiness is plausible for woundfin, the application of CPE in conjunction with the proper time to hormonally inject will be highly beneficial to increase the number of progeny in a single spawning event.

The optimal stages of embryogenesis to assess developing woundfin embryos were: 8-cell stage (3 hours at 22°C; fertilization success), high stage of cleavage (5 hours at 22°C), 90% epiboly (10 hours at 22°C), and eyed embryos (24 hours at 22°C). Woundfin in intensive culture may be spawned at a sex ratio between 1:1 and 1:5 and densities of 0.21 fish/L to 0.47 fish/L, possibly as high as 0.95 fish/L, with no detrimental effects on spawning and egg quality as assessed by fertilization success.

Embryo, larval and juvenile culture

Generally, woundfin were found to be quite adaptable to intensive culture from the larval stages through first feeding. One limitation we have consistently encountered is variable hatching success. One key component we found to improve hatching success was our ability to remove the eggs from tanks housing the adult fish. This simple effort prevents predation by adult fish during embryo development as it may take up to seven days for the eggs to hatch. Another limitation we have encountered was consistent fungal contamination during egg incubation. Although a number of anti-fungal treatments were attempted, limited but significant improvements were observed with formalin treatments.

Upon hatching, fry readily accept dry feed when the yolk sac reserves are depleted. Once weaned onto formulated dry feeds survival rates were high, generally above 90% to the juvenile fish stages. The optimal diet utilized during transition from the yolk sac stage to first feeding was Otihime-B1. Initial trials utilized a mixture of freeze-dried cyclopeeze and rotifers in combination with Otihime due to concerns about feed acceptability, but later trials demonstrated that first feeding woundfin grew with a 96% survival rate when fed 6 parts Otihime + 1 part cyclopeeze. Later trials were able to omit the cyclopeeze.

Once the fish were transitioned to feed, normal fish culture practices could be utilized to maintain growing, healthy fish. In general, feeding was performed throughout the day to slight excess utilizing belt feeders. Temperature is widely known to influence growth rates. In one of our trials, woundfin grew best at 28°C compared to 20 or 24°C, but we generally maintained fish at 24°C due to near maximal growth rates and our ability to utilize available spring water to maintain this culture temperature. Neither tank construction material nor shape appeared to influence our ability to grow woundfin from larval stages to adult fish. Although self-cleaning circular tanks are easier to maintain, we were able to culture fish in circular fiberglass tanks, long aluminum troughs, and glass aquaria with apparently equal success.

Maintaining and growing woundfin from the first feeding stages to reproductively successful adults was done successfully in intensive recirculating aquaculture systems. Although manpower efforts are increased when growing fish in recirculating systems, advantages include the ability to maintain control over many environmental factors that will influence fish growth and health. There is also a saving in total space needed to produce fish. Although efforts to produce large numbers of woundfin in limited indoor facilities were not completely successful, it appears that our main limitations are in improving hatchability.

Nutrition

A number of trials were performed to assess the nutritional needs of woundfin across life stages, from first feeding fry, through juvenile grow out, to reproductively active adults. For first feeding larval woundfin Otohime, a commercially available marine larval feed, provided the best total performance. Although diets produced at the Bozeman Fish Technology Center supported notably lower growth and survival compared to the commercial feed, it did appear that a blend of lipid sources to provide a more balanced Omega-3 to Omega-6 fatty acid profile improved woundfin survival.

Juvenile woundfin appear to be adaptable in intensive culture to a variety of feed types from pelleted feeds to flake feeds. Compared to some cyprinids, juvenile woundfin larger than 1 g have a relatively high protein requirement. It was found that approximately 50% dietary protein supported maximal growth of woundfin cultured at 26 °C, while lower dietary protein in the range of 38 to 42% appeared to optimize body composition. The lower protein levels may be beneficial in reducing excessive fat deposition we observed with long term feeding of Otohime to juvenile and adult woundfin. Culture temperature is well known to affect growth rates of fish. Our research indicates that at culture temperatures as high as 28 °C, dietary protein concentrations as high as 50% are beneficial as well as dietary fat levels of 16% to supply nutrients and energy. At lower culture temperatures (around 20 °C), less nutrient and energy supplies are needed, therefore a low protein (35%), low fat (8%) feed can be effectively provided to woundfin.

Adult woundfin appear to be accepting of a variety of feeds and nutrient target to support egg development and spawning. Otohime was used commonly in our efforts to culture woundfin and maintain healthy reproductively active adults. In our one nutrition trial with adult woundfin, formulations comparable the commercially available Rio Grande silvery minnow feeds with alternative dietary fat sources and levels were inconclusive as the additional benefit of fatty acid alterations or total fat levels on alterations in spawning success or hatchability, with all results being equivalent to fish consuming Otohime as a control. Therefore it appears that reduced dietary fat levels may be utilized in broodstock diets.

In conclusion woundfin appear adaptable to intensive, indoor culture in recirculating aquaculture systems. Nutrient requirements for protein are in around 45% digestible protein for juvenile fish larger than 1 g when cultured at 24-28 C but can be reduced as water temperatures are lowered indoors or during fall and winter in pond culture. Woundfin appear to tolerate a wide range of dietary fat levels from 8 to 16% with minimal effects on growth and efficiency. A diet providing 45% digestible protein and 8% dietary fat from highly palatable ingredients appears to be

adequate for culturing juvenile and adult woundfin, while fry will require a higher protein feed similar to Otohime-B1 which contains ~60% protein and 16% fat.