

Project Title: Development of Intensive Culture Techniques for Woundfin

Project Number: V.09.02

Contact Person(s): Molly Webb, Kevin Kappenman, and Cal Fraser,
Bozeman Fish Technology Center, U.S. Fish and Wildlife Service, 4050 Bridger
Canyon Road, Bozeman, MT 59715

Collaborator: Kevin Kroll, University of Florida, Department of Physiological
Sciences and Center of Environmental and Human Toxicology, Gainesville, P.O.
Box 110885 Gainesville, FL 32611

Project Title: Development of Intensive Culture Techniques for Woundfin



Project Summary:

The endangered woundfin minnow has been reared in captivity since the mid- to late 1980s at Dexter National Fish Hatchery and Technology Center (Dexter NFHTC). Initially, the captive rearing program was intended as a means of retaining natural genetic diversity and as a species refugia in the event of catastrophic loss. By the early, 1990's Dexter NFHTC had established a sustainable population ($N_e = 500$) through pond culture. As the wild woundfin population continued to decline and as reaches of river have been reclaimed through nonnative eradication efforts, increased production numbers of woundfin have been requested for re-stocking into the Virgin River. Dexter NFHTC has consistently provided approximately five to ten thousand woundfin annually over the past 5 years. The Virgin River Resource Management and Recovery Program is in the process of determining how many fish may be needed for an effective restocking program which could require an order of magnitude or more woundfin through production.

Increase in production may be achieved through intensive culture (e.g. tank) verses extensive culture (e.g. ponds) or some combination of these. To date, intensive culture of woundfin has been done on a small scale. To employ a successful intensive culture program, basic culture and rearing information should be investigated and rearing and spawning techniques developed and refined. These include 2 categories of information: 1) rearing conditions (densities, temperature, water quality requirements, photoperiod, diet, and stress effects on physiology) for egg, embryo, larval, and juvenile life-stages; and 2) spawning conditions (staging, spawning readiness, spawning techniques, embryo incubation and hatch success).

Project Status/Anticipated/Expected Date of Completion:

The overall goal of this multi-year project is to establish intensive culture techniques that allow for the production of 100,000 to 200,000 woundfin annually. The specific tasks in Year 1 of this multi-year project were:

Task 1. Successfully rear woundfin obtained from Dexter NFHTC captive stock in test aquaria at the Bozeman Fish Technology Center (Bozeman FTC).

Task 2. Describe the reproductive cycle of woundfin and spawn any reproductively mature fish.

Task 3. Determine effects of specially formulated diets on larval and juvenile woundfin.

To date, we have successfully completed tasks 1 and 2. The determination of the effects of specially formulated diets on juvenile woundfin (Task 3) is currently

underway. The determination of the effects of specially formulated diets on larval woundfin will occur following the 2010 spawning season.

Accomplishments/Recommendations/Results:

Task 1. Successfully rear woundfin obtained from Dexter NFHTC captive stock in test aquaria at the Bozeman FTC. Woundfin were imported from Dexter NFHTC to Bozeman FTC (n=450). Fifty six woundfin were held in a 70-gallon glass aquarium, and the remaining woundfin were maintained in 3-foot round tanks in the Bozeman FTC'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%. Woundfin were observed daily for behavior, health, and mortality. Conditions appeared to be optimal as the age-0 woundfin became sexually mature and were successfully spawned (see Task 2).

Task 2. Describe the reproductive cycle of woundfin and spawn any reproductively mature fish. To successfully and consistently spawn woundfin, an understanding of the reproductive cycle is needed. In January 2009, monthly sampling of woundfin (n=4) from the aquarium 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 determined. To determine the timing of sexual differentiation, age-0 woundfin from the Bozeman FTC spawning events in 2009 were sampled in October (n=2) and November (n=4) 2009. Blood was collected from the caudal vasculature using a heparinized hematocrit tube. Blood was centrifuged and plasma collected and stored for analysis of vitellogenin (Vtg), the egg yolk precursor protein. 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. Gonad and liver tissue were also collected, flash frozen, and are currently stored at the Bozeman FTC for any future gene expression analyses of interest.

Growth. Woundfin body weight and length was 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 Bozeman FTC cannot be evaluated. It is interesting to note that sexual dimorphism was observed early in the monitoring effort and changed dramatically with females developing ripe ovarian follicles. 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

Table 1. Body weight and length (fork and total length) of woundfin from the 2008 year class sampled in 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

Abdomens. The mean body weight of the fish did appear to decrease following the spawning season in July (Table 1). The body weight and length of the 2009 year class produced at the Bozeman FTC was measured in October and November of 2009 (Table 2).

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

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.

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 1) 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).

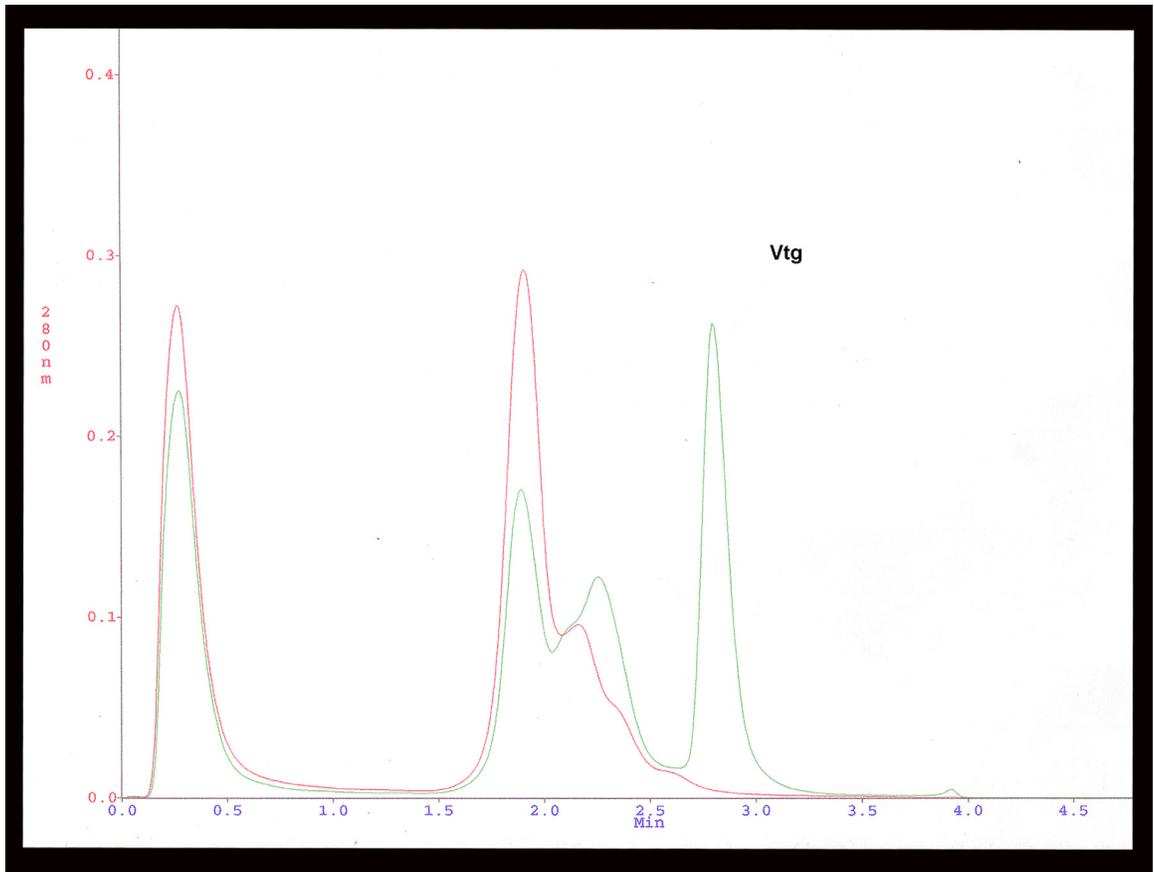


Figure 1. Anion exchange chromatograph displaying elution profile of a male and estrogenized male plasma. The Vtg peak is indicated. The male protein elution profile is red and the estrogenized male is green.

The Vtg quality was verified by polyacrylamide electrophoresis (SDS-PAGE) and Western Analysis (Figure 2). It is apparent that Vtg is not present in males (Vtg peak indicated in Figure 2), induced by estrogen and is recognized by an anti-Vtg antibody developed in another fish species (Figure 3; Western Analysis). Based on the PAGE and Western data displayed in Figure 2, Vtg and lipovitellin both appear to be dimers.

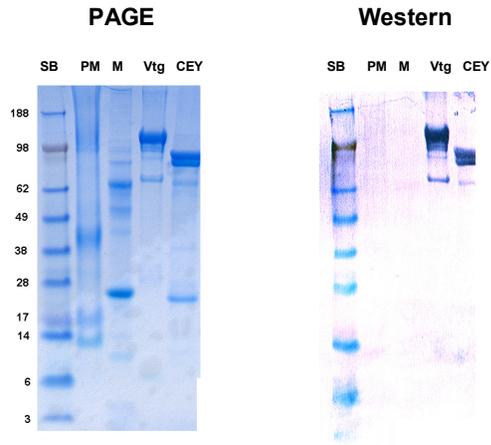


Figure 2. Polyacrylamide electrophoresis and Western blot of woundfin plasma (M), purified Vtg, and crude egg yolk (CEY). Molecular weight marker (See Blue 2 , SB) and Peppermint phosphoprotein marker (PM) are also indicated.

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 MgCl₂, 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.

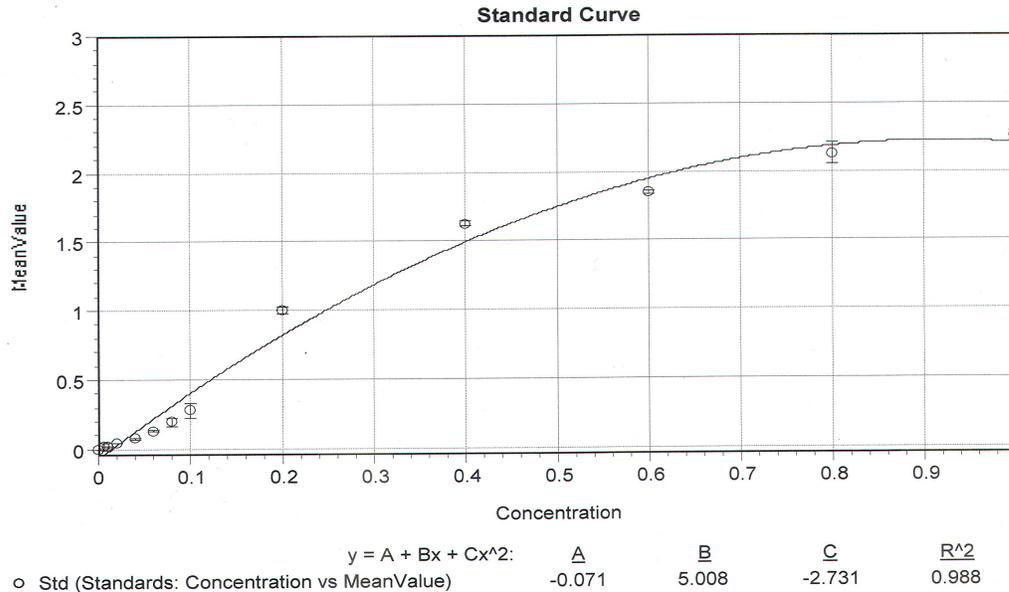


Figure 3. Woundfin Vtg standard curve using a monoclonal antibody developed against white sucker Vtg. X-axis is Vtg concentration (µg/ml) and Y-axis is absorbance at 405 nm.

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.

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.

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

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. By the beginning of March, woundfin ovaries contained oocytes that had initiated

endogenous growth (Stage 2). One female was just initiating vitellogenesis (Stage 3) 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). 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 blood collection. 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 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. 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 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 4.

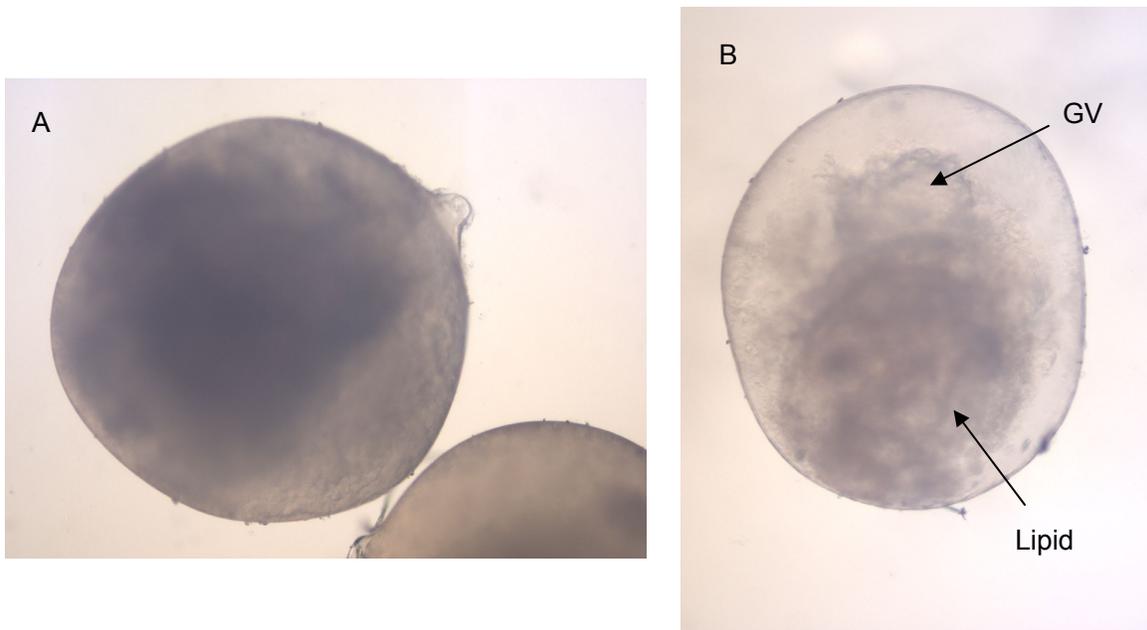


Figure 4. An uncleared (A) and cleared (B) woundfin ovarian follicle. In the cleared ovarian follicle, the germinal vesicle (GV) and the lipid may be seen inside the follicle.

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 5). 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 6). Each embryo was then carefully removed from the substrate and placed into an incubation condo to hatch.

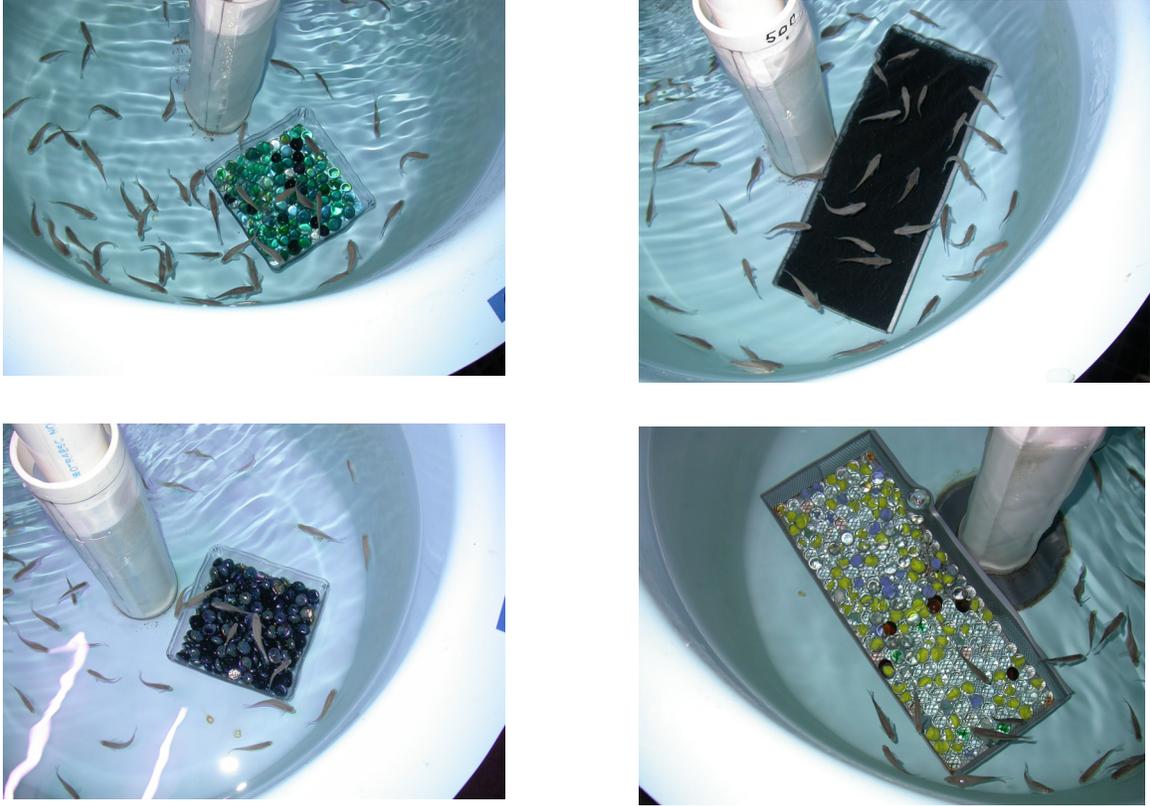


Figure 5. Various types and colors of substrate for natural woundfin tank spawning.

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.

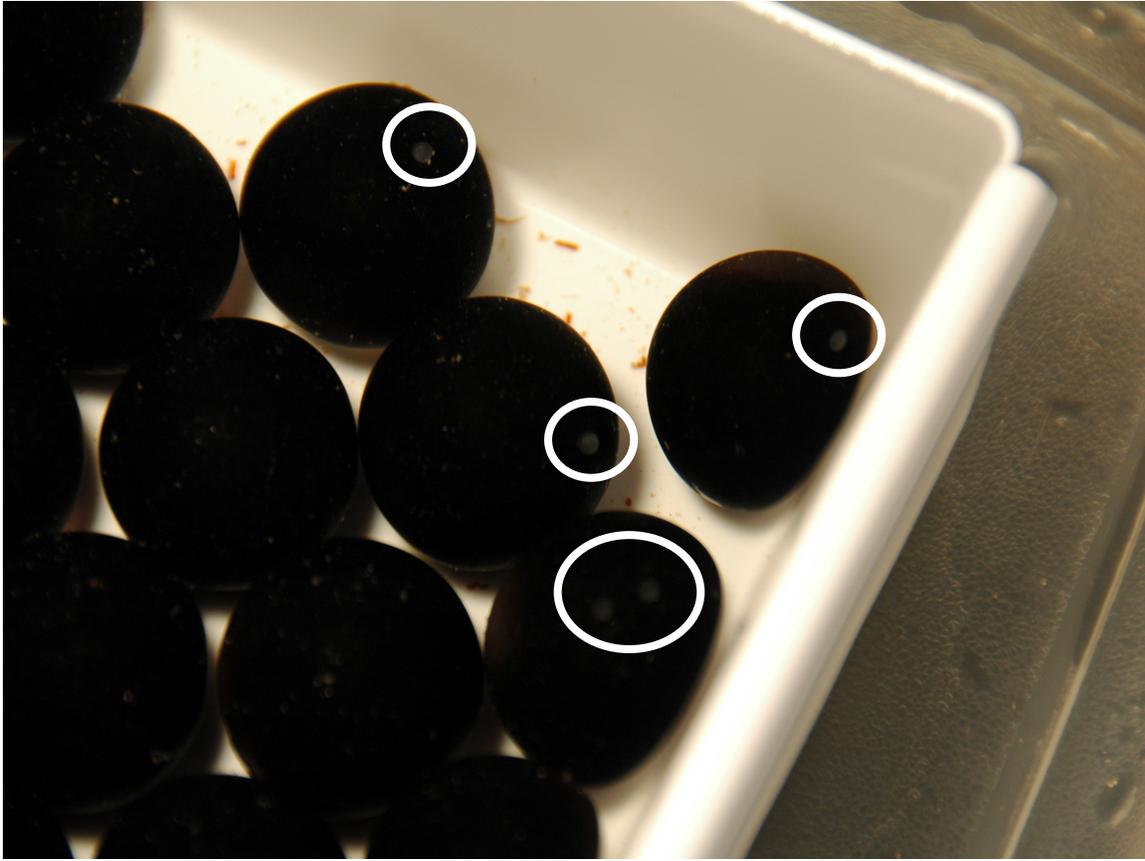


Figure 6. Adhesive woundfin embryos (in circles) on substrate following a natural tank spawning event.

Embryo Development and Hatch Success. Embryo development was observed during the spawning season. The two-cell stage (Figure 7A), blastodisc formation (Figure 7B), late blastula and the beginning of epiboly (Figure 7C), and formation of the eye and heart (Figure 7D) were captured. A total of 661 woundfin embryos were hatched at the Bozeman FTC. 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.

Fecundity Estimates in Woundfin from Dexter NFHTC. Fecundity estimates for woundfin provided from Dexter NFHTC to the Bozeman FTC were conducted. Of the 25 fish sent to the Bozeman FTC, 5 females had eggs comparable in size to the woundfin eggs spawned at the Bozeman FTC 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. A small subsample of the 2008 and 2009 year class females at the Bozeman FTC will be euthanized in the spring of 2010 for further elucidation of fecundity in woundfin.

Major Accomplishments and Recommendations: The Bozeman FTC successfully reared and spawned age-0 woundfin from Dexter NFHTC 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. 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. Histological sections prepared from gonads collected from woundfin before and during the spawning season revealed the presence of at least two clutches of developing ovarian follicles within the same female indicating batch spawning. 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. The Bozeman FTC will conduct research to refine the intensive conditions for successful spawning of woundfin in 2010.

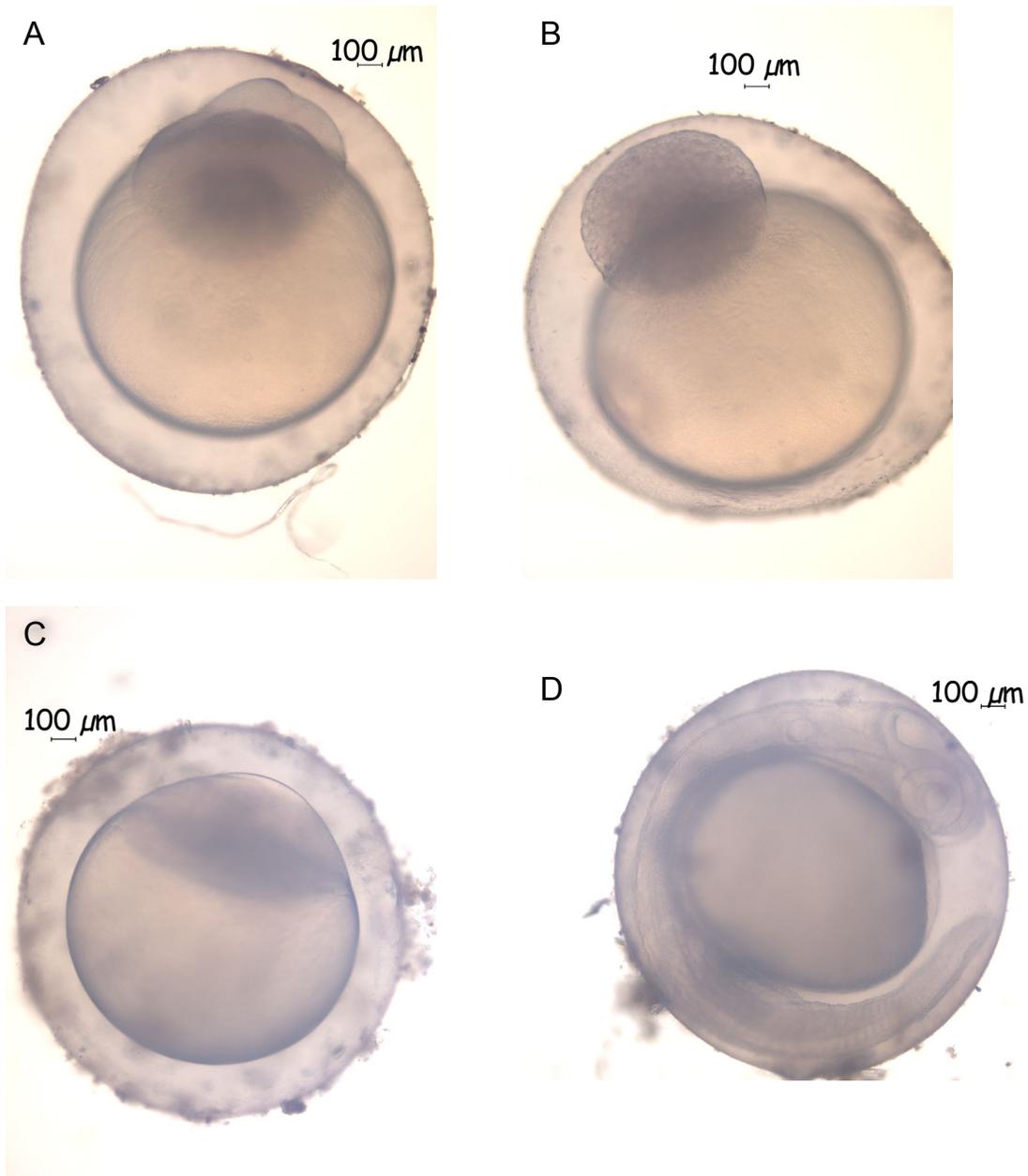


Figure 7. Woundfin embryo development: A 2-cell stage, B blastodisc formation, C late blastula and beginning of epiboly, D formation of the eye and heart.

Task 3. Determine effects of specially formulated diets on larvae and juvenile woundfin. The determination of the effects of specially formulated diets on juvenile woundfin (Task 3) is currently underway. The determination of the effects of specially formulated diets on larval woundfin will occur during the 2010 spawning season.

References

Denslow, N.D., Chow M.C., Kroll K.J., and Green L. 1999. Vitellogenin as a biomarker for estrogen or estrogen mimics. *Ecotox* 8, 385-398.

Luna, L. G. 1968. *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill Book Company, New York.

Budget:

Funds Provided: \$26,800

Funds Expended: \$26,800

Remaining Balance: \$0