

## Artificial Spawning and Larval Rearing of Klamath River Green Sturgeon

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**Abstract.**—Two female and five male prespawning green sturgeon *Acipenser medirostris* that were caught and held in cages in the Klamath River, California, were induced to spawn by injections of gonadotropin releasing hormone analog (GnRH $\alpha$ ) and domperidone. All broodfish produced mature gametes for artificial fertilization and were sampled for age, body size, sperm motility, hatchery fecundity, and oocyte diameter. The females were estimated to be 25 and 32 years old, respectively; they weighed 38 and 48 kg and produced 52,000 and 82,000 ova. The mean diameters of fully grown oocytes in the two females were 4.52 and 4.24 mm. The males ranged from 18 to 30 years in age and from 23 to 55 kg in weight. Their sperm exhibited 100% motility in river water for up to 5 min. Ovulated eggs were fertilized with milt pooled from all five males, and the eggs were transported to university facilities in oxygenated bags and incubated in MacDonald jars. Fertilization rates were 26% and 41% for the two females' eggs. In all, 23,000 (28%) normal embryos hatched from the female with the higher fertilization rate; the eggs from the other female were discarded at 4 d owing to the low number of viable embryos (<5%). Five-d post hatch larvae were reared in circular flow-through tanks and fed a commercial semimoist diet, either alone or in combination with live *Tubifex* worms. The survival to metamorphosis (age 35 d, length 66.4 mm, and weight 1.78 g) was significantly higher for fish in the treatment with the combined commercial and live diet (74.2% versus 85.5%,  $P < 0.05$ ), but there was no difference in the length and weight of juveniles.

The green sturgeon *Acipenser medirostris* is an anadromous species inhabiting Asian and American shorelines of the northern Pacific Ocean. Despite its wide geographic distribution and the occasionally large landings in the commercial fisheries of the West Coast (Houston 1988), the green

sturgeon is considered a rare or vulnerable species in the United States and Canada (Birstein 1993; Moyle et al. 1994; Campbell 1997) and an endangered species in Russia (Artyukhin and Andronov 1990). Unfortunately, there is a scarcity of literature on green sturgeon life history and population biology (Houston 1988; Moyle et al. 1994). The only known spawning populations in North America are in the Klamath, Rogue, and Sacramento rivers, California, all of which have flow regimes that are affected by water projects (Moyle et al. 1994). There is no information on the reproductive characteristics, spawning migrations, spawning and nursery habitats, and early life stages of the green sturgeon. Of the eight members of North American *Acipenseriformes*, the green sturgeon is the only species that has never reproduced in captivity. The Asian stock appears to be less abundant and is known to spawn in only one river. Artyukhin and Andronov (1990) described the spawning run of the green sturgeon in the Tummin River and succeeded in artificially spawning two females.

The green sturgeon supports a minor commercial fishery on the Pacific coast, with the majority of fish being caught in the lower Columbia River. During the period 1995–1998, annual commercial catches of green sturgeon ranged from 400 to 1,600 fish (Washington Department of Fish and Wildlife and Oregon Department of Fish and Wildlife 1999), while the fishery of the sympatric white sturgeon *Acipenser transmontanus* harvests about 46,000 annually from the same portion of the river (DeVore et al. 1995). However, this minor fishery is important to Native Americans. The Klamath River population of green sturgeon has been central to the life of the Yurok tribe for more than a thousand years, and its fishery remains an integral

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part of the tribe's subsistence, culture, and economy. Their gill-net fishery has had an annual catch of about 250 fish for the past 15 years (Hillemeier, unpublished data). In addition to using the meat of the sturgeon for food, the Yurok make traditional "sturgeon bread" from the eggs and process other parts to make a glue for manufacturing regalia and tools such as sinew back bows, arrow shafts, and wood plugs for canoes. According to Yurok tradition, everything used to make regalia is sacred.

A research project was initiated to investigate the reproductive and environmental physiology of green sturgeon, with one objective being to develop artificial spawning and culture techniques. This paper provides the details on our artificial spawning of wild green sturgeon and presents data on hormone-induced ovulation and spermiation, egg size, fertilization and hatching rates, and larval survival and growth to metamorphosis.

### Methods

Wild broodfish for this study were obtained from the Yurok tribe's gill-net fishery on the lower Klamath River and spawned on site; the fertilized eggs were transported for incubation and larval rearing to the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis. Migratory broodfish were caught from May 8 to May 14, 1999 in the Weitchpec area between river kilometers 56 and 66, which is downstream from the confluence of the Klamath and Trinity rivers. The fishers used anchored gill nets that were 10–15 m long and 7–9 m deep, with a stretch-bar mesh of 17–19 cm. Nets were typically set along the shoreline in back eddies that were 3–6 m deep. The five males and two females that were caught were placed into two wood frame cages measuring  $1.2 \times 2.1 \times 1.2$  m that were covered with a plastic-coated, 16-gauge wire mesh (2.5 cm square) and a hinged plywood cover 1.3 cm thick. Cages were submerged in the river and anchored with 14-kg cement weights and river anchors. The maximum holding time in a cage was 10 d.

On May 18, 1999, all seven fish were removed from the cages, weighed ( $\pm 0.1$  kg), measured ( $\pm 0.5$  cm), and tagged for identification during spawning procedures. At this time, 50 fully grown ovarian follicles were collected by catheter (Conte et al. 1988) and placed in a Ringer solution (Detlaff et al. 1993) containing 0.03 g/L of penicillin and 0.05 g/L of streptomycin sulfate. Thirty follicles were boiled, chilled on ice for 20 min, and

stored in a 10% solution of phosphate-buffered formalin for measuring the polarization index (PI, a ratio of the distance of the germinal vesicle from the animal pole to the oocyte animal-vegetal diameter), and 20 were fixed directly in formalin for measuring oocyte diameter. Ten boiled eggs were bisected on site before spawning to estimate the stage of germinal vesicle migration under a dissecting microscope. The remainder were measured ( $\pm 0.01$  mm) in the laboratory using a dissecting scope with camera lucida and a digital image-analyzing tablet (Nikon Microplan II) with micro-computer interface. The condition factor of the broodstock was calculated as  $100 \times [\text{body weight (g)}/\text{fork length}^3 \text{ (cm)}]$ .

The spawning protocol followed Conte et al. (1988) and Webb et al. (1999), with the following modifications: The males received a single injection of 10  $\mu\text{g}/\text{kg}$  body weight of mammalian gonadotropin releasing hormone analog (GnRHa) [D-Ala<sup>6</sup>, Des-Gly<sup>10</sup>]-LH-RH Ethylamide (Peninsula Laboratories), and the females received a priming injection of 2  $\mu\text{g}/\text{kg}$  GnRHa plus 1 mg/kg of domperidone (a dopamine antagonist; Research Diagnostics, Inc.) followed in 12 h with a resolving injection of 18  $\mu\text{g}/\text{kg}$  GnRHa plus 3 mg/kg domperidone. All injections were intramuscular and given underwater to minimize handling stress. River temperature during the injection period ranged from 12.9°C to 13.6°C. Spawning mats, made of weighted cotton rope and placed in the female cage, were checked for released eggs beginning 20 h after the second injection. Ovulated eggs were removed surgically from each fish, which was placed into a hooded stretcher (Conte et al. 1988). Because of the human health risk associated with the release and potential capture of fish containing residues of injected drugs, broodfish were euthanatized during surgery by an overdose of anesthetic (500 mg/L tricaine methanesulfonate), which was pumped across the gills with a submersible pump with a vinyl tube 5 cm in diameter and a 100-L ice chest as a sump. Age was estimated from the cross sections of the dried base portions of pectoral fin rays, as described in Van Eenennaam et al. (1996b).

Milt was collected from each male with a 60-mL plastic syringe and a 4-cm-long vinyl catheter inserted into the urogenital pore (Conte et al. 1988). Sperm was evaluated for percent initial motility and the time to less than 50% motility under a compound microscope, using 5  $\mu\text{L}$  of semen diluted immediately with 200  $\mu\text{L}$  of river water. Eggs were fertilized with 20 mL of pooled milt (4 mL

from each male) diluted 1:200 with river water. After 3 min of fertilization, eggs were gently mixed in a water suspension of Fuller's Earth (100–200 mesh size; Sigma Chemical Company) for 80 min to prevent egg adhesion. The number of ova collected from each female was estimated volumetrically (egg count in five 5-mL subsamples). All the above procedures were conducted under field conditions.

Approximately 50% of the eggs from each female were placed into each of two oxygenated polyethylene bags (38 × 30 × 58 cm, with a square bottom) containing 8 L of river water. The bags were placed into 100-L ice chests (43 × 94 × 41 cm) with one 3.3-kg ice block attached to the inside cover to maintain a cool environment during transport to CABA (6 h transit time). Water temperature in the bags was 13–14°C before and 12–12.5°C after transportation. In addition to silt-treated eggs, approximately 50 mL of ova from each female were fertilized without silt treatments and transported in glass crystallizing dishes 190 mm in diameter for incubation in the adhered state (Van Eenennaam et al. 1996a). Upon arrival at CABA, the embryos (Stage 6–7, third and fourth cleavage) were acclimated to the hatchery water temperature (15°C) and incubated in MacDonald jars or in dishes submerged in cylindrical larval rearing tanks 1.2 m in diameter. Eggs were examined every 2 h in transit, and random samples collected at Stage 5–6 (second and third cleavage) to determine fertilization rates. Stages of development followed the classification of Dettlaff et al. (1993), and a temperature-dependent time to sampling was estimated by exponential equations for white sturgeon (Wang et al. 1985). The embryos were also sampled from hatching jars at stage 22–23 (late neurulation) to determine post-gastrulation survival. The larvae were counted after hatching and randomly sampled on day 5 post-hatch ( $N = 25$ ) for length ( $\pm 0.01$  mm) and weight ( $\pm 0.1$  mg).

Two diet regimes were tested to compare survival and growth to 35 d post hatch. Two thousand 5-d-old larvae were stocked into each of six round flow-through tanks 1.2 m in diameter (355 L) with internal spray bars that created a gentle circular current (Conte et al. 1988, fig. 102). Tanks were housed in a building with skylight windows and exposed to natural photoperiod. Larval behavior was observed during the day and at night with a flashlight. Each tank had three automatic feeders (the Fish Sitter) placed around its outside edge an equal distance apart. Three tanks (treatment

“SC”) were fed ad libitum a Silver Cup semimoist fry feed (Nelson & Sons, Inc.) that was distributed every hour over a 24-h period. Daily feed rates per tank ranged from 15 g at the beginning to 150 g at the end of the experimental period (30 d). Feeding was started with small amounts on day 8 posthatch to ensure availability of food at the onset of exogenous feeding, which was observed on days 10–15. Three other tanks (treatment “SC + TB”) received the same amount of Silver Cup diet fed ad libitum, supplemented with 5 g of chopped live *Tubifex* worms fed by hand each morning and afternoon. Daily mortalities were recorded and subsamples of 15–25 larvae per tank were collected at age 14, 28, and 35 d (larvae sampled at 14 d were examined for the presence of a melanin plug in the spiral intestine and for food in the gut). Juveniles were counted at 35 d and transferred to outdoor tanks for further rearing. The effect of dietary treatment on larval length and weight was examined by one-way analysis of variance (ANOVA) with tanks as samples and individual observations as subsamples. Larval survival data at 35 d were arcsine-transformed and compared by a Student's *t*-test. The accepted significance level was  $P < 0.05$ .

## Results

Characteristics of the spawned broodfish are given in Table 1. The two females weighed 38 and 48 kg and were 190 and 207 cm long (total length) and 25 and 32 years old, respectively. The males were generally similar to the females in size and age. Both females and males had streamlined bodies and similar condition factors (females,  $0.71 \pm 0.08$ ; males,  $0.68 \pm 0.05$  [mean  $\pm$  SD]). The oocytes sampled before spawning were in an advanced stage of germinal vesicle migration, with PI values of 0.03 and 0.04. Fully grown ovarian follicles were large (mean diameters 4.52 and 4.24 mm, respectively) and had an ovoid shape and a light-brown to olive-green coloration.

Forty to 60 mL of semen was collected from each male at 38 h postinjection and stored in 250-mL Erlenmeyer flasks on wet ice. Sperm from all males exhibited 100% motility up to 5 min after activation with river water and 50% motility at approximately 7 min (Table 1). Small numbers of weakly adhesive eggs were observed on the spawning mats at 26 h (2 eggs) and 28 h (30 eggs) after the second injection. Both females were gently palpated underwater and found to have soft abdomens indicating ovulation. The ovulated eggs were removed at 28.5 h (female 1) and 29.5 h

TABLE 1.—Characteristics of green sturgeon spawned on the Klamath River.

Characteristic	Female 1	Female 2	Males (mean $\pm$ SD and range; $N = 5$ )
Age (years)	25	32	23 $\pm$ 5 (18–30)
Body weight (kg)	38	48	40 $\pm$ 14 (23–55)
Fork length (cm)	180	185	177 $\pm$ 18 (154–199)
Total length (cm)	190	207	192 $\pm$ 20 (166–212)
Condition factor <sup>a</sup>	0.65	0.76	0.68 $\pm$ 0.05 (0.63–0.76)
Oocyte polarization index <sup>b</sup>	0.03	0.04	
Oocyte diameter (mm)	4.52	4.24	
Ova collected ( $10^3$ )	52.1	81.6	
Spermiation volume (mL)			52 $\pm$ 8 (40–60)
Sperm motility (s)			396 $\pm$ 29 (360–430)

<sup>a</sup>  $100 \times$  body weight (g)/fork length<sup>3</sup>.

<sup>b</sup> The ratio of the distance of the germinal vesicle from the animal pole to the oocyte animal-vegetal diameter.

(female 2) after the second injection; 52,100 eggs were collected from female 1 (4,250 mL, 12.27 eggs/mL) and 81,600 eggs from female 2 (5,800 mL, 14.07 eggs/mL). Both fish underwent complete ovulation and released eggs from the vent during handling. The fertilized eggs from both females exhibited poor adhesion and did not develop a thick jelly coat, as in white sturgeon (Cherr and Clark 1985). The eggs that were incubated in crystallizing dishes were easily separated from the glass substrate with a slight shaking motion.

The fertilization rate was 25.5% and 41.2% in the eggs of females 1 and 2, respectively, and survival to neurulation was 4.9% and 31.5%. Because of the low number of viable embryos and fungal growth, the eggs of female 1 were discarded 4 d after fertilization. Approximately 23,000 larvae (28%) hatched from the eggs of female 2 at 168–216 h after fertilization. Larvae did not exhibit a swim-up hatching behavior but remained on the bottom of the hatching jar. Newly emerged larvae (Stage 36) were 14–15 mm long, had a large and ovoid-shaped endodermal “yolk sac,” and had scarce pigmentation compared with white sturgeon at a similar stage.

At age 5 d (Stage 44), larvae were 21.8 mm in length and weighed 65 mg (Table 2). They devel-

oped dark pigmentation and exhibited nocturnal behavior patterns that persisted until metamorphosis (limited mobility and clumping during the day and vigorous swimming at night). Exogenous feeding was initiated at age 10–15 d. Mortality was low at ages 5–15 d (2–15 individuals/tank daily), peaked at ages 16–25 d in the SC + TB tanks (3–46 daily) and ages 16–29 d in the SC tanks (12–90 daily), and ceased during the last 6–10 d of rearing. At age 14 d, the dietary effect was highly significant (ANOVA;  $P < 0.005$ ), with a greater mean length (28.7 versus 27.4 mm) and weight (129 versus 105 mg) in the SC + TB diet (Table 2). At this age, 73% of the larvae fed the SC + TB diet treatment had food in their gut, compared with 40% of the larvae fed the SC diet treatment; approximately 2% of the former still retained a melanin plug, compared with 16% of the latter. The fish in the SC + TB treatment were observed to have eaten both food sources. Lengths and weights at ages 28 and 35 d were not greater for fish fed the combination of artificial and live food, but survival at 35 d was significantly ( $P < 0.05$ ) higher:  $74.2 \pm 2.1\%$  with the SC diet versus  $85.5 \pm 2.8\%$  with the SC + TB diet. Juveniles reached a mean length of 66.4 mm and a mean body weight of 1.78 g at age 35 d.

TABLE 2.—Length and weight of green sturgeon reared on Silver Cup semimoist fry feed (SC) or Silver Cup supplemented with live *Tubifex* worms (SC + TB). Data are means  $\pm$  SD for pooled populations of three tanks. Data for a given age followed by different letters are statistically different (ANOVA;  $P < 0.05$ ). Length and weight at the start of the experiment (5 d posthatch;  $N = 25$ ) were  $21.8 \pm 0.4$  mm and  $65 \pm 3$  mg.

Age (d)	$N$	Total length (mm)		Wet weight (mg)	
		SC	SC + TB	SC	SC + TB
14	45	27.4 $\pm$ 0.9 z	28.7 $\pm$ 1.7 y	105 $\pm$ 17 x	129 $\pm$ 40 w
28	75	50.4 $\pm$ 6.2	51.8 $\pm$ 6.5	1,024 $\pm$ 312	1,072 $\pm$ 337
35	75	66.5 $\pm$ 8.8	66.3 $\pm$ 7.7	1,794 $\pm$ 611	1,767 $\pm$ 497

### Discussion

Broodfish used for spawning were generally similar in age and size to white sturgeon (Kohlhorst et al. 1980; Chapman et al. 1996). All seven fish that were captured and held in the cages for this project were candidates for spawning. The males all had mature testes and the two females had large oocytes with a low PI. It appears that all the broodfish captured in this region of the river are mature and ready to spawn. Necropsy samples collected during May 1999 by the Yurok fishers from an additional 12 females and 18 males support this observation (Van Eenennaam, unpublished data). The condition factor of the green sturgeon was low compared with that of other species, which supports our field observations on slim body shapes in both sexes of mature broodstock. In comparison, mature female and male Atlantic sturgeon in the Hudson River had condition factors that were higher and significantly ( $P < 0.05$ ) different from each other (0.94 for females versus 0.83 for males; Van Eenennaam et al. 1996b). The low condition factor of and modest number of ovulated eggs collected from both females in our study suggests a low fecundity for green sturgeon. However, the number of eggs collected does not account for losses during ovulation in the cage and handling procedures prior to egg collection. Artyukhin and Andronov (1990) reported the fecundity of two females spawned on the Tumnin River as 60,000 and 160,000 eggs, respectively, and commented that the large size of the oocytes and the body conformation of green sturgeon contribute to their low fecundity compared with other anadromous species. The mean diameters of fully grown oocytes in the two females were 4.52 and 4.24 mm (Table 1), and the ongoing sampling of migratory females in the tribal fishery confirms a large egg size in this population (average diameter,  $4.34 \pm 0.15$  mm;  $N = 14$ ; Van Eenennaam, unpublished data). To our knowledge, these are some of the largest recorded oocytes in acipenserids. In comparison, the beluga *Huso huso* from the Caspian Sea has ova ranging in diameter from 3.6 to 4.3 mm (Pirogovskii et al. 1989). Female green sturgeon invest a greater amount of their reproductive resources in maternal yolk for nourishment of the embryo, which results in larger larvae. This reproductive strategy is in great contrast with that of the anadromous Atlantic sturgeon *Acipenser oxyrinchus* and European sturgeon *A. sturio*, which have much higher fecundity and smaller eggs and

larvae (Holčík et al. 1989; Van Eenennaam et al. 1996b).

Spawning induction techniques established for white sturgeon (Conte et al. 1988; Webb et al. 1999) were successfully used for green sturgeon in this study. The peripheral position of the germinal vesicle in the oocyte indicates spawning readiness in a sturgeon female, and an oocyte PI less than 0.07 is considered a threshold for oocyte maturation and ovulation (Dettlaff et al. 1993). Both the low PI values in both females used in this study and the 100% response to hormonal treatment in all broodfish indicate that they were captured in the vicinity of their natural spawning grounds; the long duration of sperm motility may indicate that spawning usually occurs in strong river currents (Dettlaff et al. 1993). The poor adhesion of fertilized eggs that was observed in both females, which is not seen in white or Atlantic sturgeon eggs, appears to be a distinct characteristic of green sturgeon. Preliminary histological observations of the egg chorion revealed that the outer layer, which forms the adhesive coat after fertilization, was approximately half the thickness seen in white sturgeon. Knowledge of species' differences in egg adhesiveness and substrate for egg attachment (Vorobyeva and Markov 1999) is important for characterization and protection of sturgeon spawning habitat; these differences need to be further investigated.

The rates of fertilization observed in this study were generally similar to the variable rates (20–95%) found when spawning wild-caught white sturgeon (Doroshov et al. 1983). The low egg fertility in female 1 was probably caused by faster than expected ovulation and the delayed removal of ovulated eggs, which may undergo activation prior to fertilization (Dettlaff et al. 1993). Species-specific and temperature-dependent ovulation periods must be determined for green sturgeon to optimize egg fertility.

Despite the larger egg size, the rate of embryo development and hatching time in green sturgeon at a temperature of 15°C appears to be similar to that of white sturgeon (Wang et al. 1985). Newly emerged larvae of green sturgeon are large, possess a significant amount of yolk, and unlike white sturgeon larvae, do not swim up and out of hatching jars. Similar behavior was reported for the green sturgeon in Asia (Artyukhin and Andronov 1990).

Throughout their rearing in tanks, green sturgeon larvae and juveniles exhibited nocturnal behavior characterized by low activity during the day

and vigorous swimming during the night, and feeding was observed both during the day and at night. Artyukhin and Andronov (1990) also noted, "limited mobility" in larvae and juveniles, apparently based on observations during the daytime.

Five-day-old green sturgeon larvae had almost twice the weight (65 versus 34 mg) of white sturgeon larvae at Stage 44 (Wang et al. 1987). This greater reserve of maternal yolk and larger larvae could provide an advantage in larval feeding and survival. Compared with other acipenserids, green sturgeon larvae appear to be more robust and easier to rear. The ones in our study exhibited fast growth on artificial feed, reaching a length of 66 mm and a weight of 1.8 g during 3 weeks of exogenous feeding. Supplementation of an artificial diet with chopped *Tubifex* worms significantly increased survival to metamorphosis but did not affect the length and weight of juveniles (Table 2). The early onset of exogenous feeding and the significantly larger size of 14-d-old larvae in the SC + TB treatment indicate that the major advantage of supplemental feeding with a natural diet is in reducing mortality during transition to exogenous feeding. Higher daily mortality in the SC treatment during transition to exogenous feeding supports this conclusion. Observations on the discharge of the melanin plug from the spiral intestine also confirm that this discharge is not the best diagnostic criterion to use to start feeding in larval sturgeon (Gisbert and Williot 1997).

In conclusion, the first artificial spawning and hatchery rearing of green sturgeon from the Klamath River suggests a unique reproductive strategy for this species as well as a similarity in the reproductive biology of the North American and Asian stocks. Studies of reproductive biology in this species may be critical for stock management because its reproductive potential is greatly affected by the availability of spawning habitat (Detlaff et al. 1993) and fishing mortality (Boreman 1997), and the effects of these factors on the sturgeon fishery may not be apparent for years. Several important species (eulachon *Thaleichthys pacificus*, lamprey *Lampetra* sp., steelhead *Oncorhynchus mykiss*, coho salmon *O. kisutch*, and chinook salmon *O. tshawytscha*) have declined dramatically in the Klamath River over the past few decades, causing concern about the health of the Klamath basin ecosystem. Although the decline is attributable to several factors, the primary cause is the loss or degradation of freshwater habitat. Studies are currently under way to determine the river flow required to provide sufficient habitat for robust

tribal fisheries and recovery of the coho salmon, which is listed as a threatened species under the Endangered Species Act. Unfortunately, the needs of the green sturgeon are not included in these studies owing to the scarcity of scientific information on this species' spawning and nursery habitats. Investigations are needed to determine the status of the green sturgeon in the Klamath River and other Pacific Northwest streams as well as to identify the optimal habitat requirements for the spawning and early life stages of this valuable fish.

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