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[Article]

Induced Spawning, Artificial Fertilization, and Egg Incubation Techniques for Green Sturgeon

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Abstract.—Establishment of hatchery breeding techniques for the threatened green sturgeon *Acipenser medirostris* is important for research and conservation hatcheries. Injections of either gonadotropin-releasing hormone analog (GnRHa) or GnRHa plus domperidone were used to induce ovulation in 13 female Klamath River green sturgeon and to induce spermiation in 19 males. Ovulated eggs were either rinsed in water or not rinsed before fertilization, and the eggs were fertilized with different milt dilutions and for different lengths of time. After fertilization, eggs either were allowed to adhere to the bottom of glass dishes or were silted for 1 h and then incubated in McDonald or upwelling jars. All broodfish ovulated or spermiated in all hormonal treatments, and the best treatment was GnRHa injected alone in a single dose of 10 μg/kg for males or in a 1-μg/kg priming dose and a 19-μg/kg resolving dose for females. Females were held at 12–13°C, and ovulation was observed 14 ± 3 h (mean ± SD) after the second injection. Domperidone was not required for successful ovulation and appeared to reduce the adhesion of ovulated eggs. From 49,000 to 115,000 eggs were collected from each female, and from 30 to 300 mL of milt were collected from each male. Sperm cell concentrations in milt ranged from 2.9×10^8 to 5.4×10^9 sperm/mL, and the sperm exhibited 90–100% motility for up to 5 min. In all experiments, egg rinsing improved fertilization success by 5–12%. Embryo survival to neurulation in the McDonald jars was lower (5–32%) than that in the upwelling incubators (60–82%). Green sturgeon eggs were sensitive to the high-impact rolling action at the bottom of the McDonald jars, probably due to the large egg diameter (mean ± SD = 4.33 ± 0.14 mm) and thinner chorion (42 ± 4 μm) relative to eggs of white sturgeon *A. transmontanus* (diameter = 3.79 ± 0.03 mm; chorion thickness = 115 ± 6 μm).

The anadromous green sturgeon *Acipenser medirostris* inhabits the North American shorelines of the Pacific Ocean from the Bering Sea to Mexico (Moyle 2002) and is considered rare or vulnerable in the United States and Canada (Birstein 1993; Campbell 1997; Musick et al. 2000). The average annual harvest has decreased from 6,494 fish in 1985–1989 to 1,072 fish in 2000–2003; the Klamath River and Columbia River tribal fisheries account for 65% of the total catch (Adams et al. 2007). In 2001, a petition was made to list the green sturgeon under the Endangered Species Act; after the Biological Review Team's updated status report (BRT 2005), the National Marine Fisheries Service listed the southern distinct population segment (DPS) as threatened (NMFS 2006). The southern DPS encompasses the region south of the Eel River, California, and includes the coastline, San Francisco Bay, and the Sacramento River (Israel et al. 2004).

Green sturgeon are highly migratory, able to enter seawater at a young age (Allen and Cech 2007) and to migrate long distances along the Pacific coast (Moser and Lindley 2007). The migratory behavior of green sturgeon is similar in many respects to that of the European sturgeon *A. sturio* and Atlantic sturgeon *A. oxyrinchus* (Gessner et al. 2007). In contrast to Atlantic sturgeon, green sturgeon have very large eggs and low fecundity (Van Eenennaam et al. 2006). Their newly emerged larvae are not pelagic and are highly sensitive to light (Deng et al. 2002; Kynard et al. 2005). The only known breeding grounds of green sturgeon are the Klamath and Sacramento rivers in California (Moyle 2002; Van Eenennaam et al. 2006; Brown 2007) and the Rogue River in Oregon (Moyle 2002; Erickson and Webb 2007; Webb and Erickson 2007). There is also some evidence for spawning in the Umpqua River, Oregon, and the Trinity and Eel rivers, California (Adams et al. 2007). It is believed that green sturgeon spawn in fast-flowing rivers; however, there is no published information on spawning and larval nursery habitat.

Natural reproduction of green sturgeon is compromised by altered river flows, reduced spawning habitat, and climate change (Bartholow 2005; Van Eenennaam et al. 2005). The development of techniques for artificial breeding and culture is critical for conservation and future rehabilitation of this unique fish. Some life history traits of green sturgeon, such as large eggs, robust and easily reared larvae, and fast-growing

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juveniles (Deng et al. 2002; Van Eenennaam et al. 2006), are attractive for conservation hatcheries and potentially for aquaculture.

The first artificial reproduction of green sturgeon from the Klamath River was reported by Van Eenennaam et al. (2001). However, this early work encountered problems with suboptimal egg quality, low fertilization rates, and high embryo mortality in jar incubators. After this first attempt, the techniques for spawning induction, egg fertilization, and embryo incubation were modified. This paper summarizes results of the past 7 years of spawning induction, artificial fertilization, and egg incubation, providing a basis for recommended hatchery techniques for green sturgeon.

TABLE 1.—Mean (±SD) size and reproductive characteristics of wild-caught Klamath River, California, green sturgeon (13 females; 19 males) used for artificial spawning experiments during 1999–2005. Only four males were examined for sperm concentration.

Characteristic	Females	Males
Live weight (kg)	46 ± 8	32 ± 9
Fork length (cm)	180 ± 11	165 ± 14
Age (years)	29 ± 5	20 ± 4
Oocyte polarization index	0.04 ± 0.01	
Oocyte diameter (mm)	4.35 ± 0.15	
Number of ova collected ($\times 10^3$)	82 ± 23	
Volume of sperm collected (mL)		165 ± 115
Initial sperm motility (%)		92.8 ± 4.6
Time to 50% sperm motility (s)		323 ± 74
Sperm concentration ([cells/mL] $\times 10^9$)		2.2 ± 2.2

Methods

Broodfish collection, holding, and transport.—Wild adult green sturgeon were obtained from the Yurok Tribe gill-net fishery on the lower Klamath River, California, during the spring spawning migration (Van Eenennaam et al. 2006). Mature fish were caught during April and May for seven consecutive years (1999–2005), and usually two females and two males were provided annually by the tribal fishery program for artificial spawning. The fish were captured between river kilometers 32 and 72 using anchored gill nets (length = 10–15 m; depth = 7–9 m; stretched bar mesh = 17–19 cm) set along the shoreline in back eddies (3–6 m deep). Fish were placed into two wood-frame cages (1.2 × 2.1 × 1.2 m) that were covered with plastic-coated wire mesh (16-gauge wire; 2.5-cm square mesh) and a hinged plywood cover (thickness = 1.3 cm). Cages were submerged in the river and anchored with multiple 14-kg cement weights and river anchors. The maximum holding time in a cage was 10 d in the early trials but only 1–4 d during the final 4 years. Some fish (4 females; 7 males) were spawned in the cages in the river, and fertilized eggs were transported for incubation to the Center for Aquatic Biology and Aquaculture (CABA) facilities at the University of California, Davis (Van Eenennaam et al. 2001). Most of the fish (9 females; 12 males) were transported to CABA and were held in flow-through, fiberglass tanks (diameter = 3.7 m) during the spawning induction period. The flow rate of surface irrigation water was 80–120 L/min, and tank temperature was 12–15°C. Fish were transported in a marine-grade aluminum live-haul tank (volume = 1,500 L; dimensions = 1.9 × 1.1 × 0.7 m) with double walls and foam insulation (thickness = 5 cm). The tank was filled with river water (12–14°C), and dissolved oxygen was maintained at 100% air saturation by oxygenation. Transportation time was approximately 6 h.

Assessment of maturity.—All fish were weighed (nearest 0.1 kg), measured for fork length (nearest 0.5 cm), and tagged. Approximately 40 fully grown ovarian follicles were biopsied from each female, and a core sample of testis was biopsied from each male (Conte et al. 1988); biopsy samples were stored in 10% phosphate-buffered formalin. Sex had to be verified by biopsy because mature female and male green sturgeon have similar body conformations (Van Eenennaam et al. 2006) and a previous attempt to externally determine sex of mature green sturgeon was only 79% accurate (Benson et al. 2007). To determine female maturity, 15 eggs were bisected to measure egg polarization index (PI; relative distance of the germinal vesicle from the animal pole) and another 15 eggs were used to measure egg diameter (Van Eenennaam et al. 2006). Measurements (nearest 0.005 mm) were made by means of a dissecting scope equipped with camera lucida and a digital image analyzing tablet (Nikon Microplan II).

Induced spawning.—The general spawning procedures followed those described by Conte et al. (1988), Webb et al. (1999), and Van Eenennaam et al. (2001). All males received a single injection of mammalian gonadotropin-releasing hormone analog (GnRHa; [D-Ala^6, Des-Gly^{10}]–luteinizing hormone releasing hormone ethylamide; Peninsula Laboratories) at a concentration of 10 μg GnRHa/kg of fish weight. The females received a priming injection of GnRHa and domperidone (a dopamine antagonist; Research Diagnostics, Inc.); each female received a second injection 12 h after the priming injection (Table 2). The domperidone was used to potentiate the effect of GnRHa, but the treatment dose was gradually reduced over the study years and the drug was not applied during the last 2 years (Table 2). All injections were intramuscular and given underwater by use of a 1-mL syringe equipped with a 23-gauge, 3.8-cm-long needle.

TABLE 2.—First and second doses of gonadotropin-releasing hormone analog (GnRHa; alone or in combination with domperidone [domp]) used to induce spawning of female green sturgeon; observed fertilization rates (FRs); and estimated egg adhesiveness (W = weak; S = strong). Individual fish ($n = 13$) are coded by year of capture (Y; 1999 [99] to 2005 [05]); asterisks indicate fish that were spawned in cages in the Klamath River, California.

Fish (Y–number)	Injection 1		Injection 2			
	GnRHa (μg/kg)	Domp (mg/kg)	GnRHa (μg/kg)	Domp (mg/kg)	FR (%)	Adhesiveness
99–1*	2.0	1.0	18.0	3.0	20–26	W
99–2*	2.0	1.0	18.0	3.0	38–42	W
00–3*	2.0	1.0	18.0	3.0	34–47	W
00–4	2.0	1.0	18.0	3.0	38–42	W
01–5*	2.0	1.0	18.0	3.0	50–77	W
02–6	1.0	1.0	19.0	3.0	58–62	W
02–7	0.6	-	19.0	1.0	78–82	W
03–8	0.6	-	19.0	1.0	31–44	W
03–9	0.6	-	19.0	1.0	60–75	W
04–10	1.0	-	19.0	-	75–77	S
04–11	1.0	-	19.0	-	65–78	S
05–12	1.0	-	19.0	-	39–95	S
05–13	1.0	-	19.0	-	49–84	S

For in-river spawning in cages, egg mats made of weighted cotton rope were placed in the female cage and were periodically checked for adhered eggs. To detect ovulation and oviposition in tanks, the tank bottom was scraped hourly with a fine-mesh dip net (20 × 15 cm) beginning at 7–10 h after the second injection. Ovulated eggs were removed surgically from anesthetized fish that were placed in a hooded stretcher (Conte et al. 1988; Webb et al. 1999; Van Eenennaam et al. 2001). The broodfish were euthanized by an overdose of tricaine methanesulfonate (500 mg/L of water) that was pumped across the gills by means of a submersible pump with a 5-cm-diameter vinyl tube and a 100-L ice chest as a sump. Sacrifice of broodfish was necessary because of the human health risk associated with the release and potential capture of fish containing residues of injected drugs. Holding the broodfish was not feasible, as the wild-caught green sturgeon refused to feed in captivity and the cesarean incision healing would certainly have been impaired in nonfeeding fish; this impairment would have led to suffering and mortality. Age was estimated from cross sections of the dried base portions of pectoral fin rays (Van Eenennaam et al. 2006).

Milt was collected with a plastic syringe (60 mL) and a vinyl catheter (length = 4 cm; diameter = 4 mm) inserted into the urogenital duct (Conte et al. 1988; Van Eenennaam et al. 2001). Sperm motility was subjectively evaluated (Rurangwa et al. 2004) under a compound microscope by examination of a 5-μL sample of semen diluted in 500 μL of river or hatchery water. Percent initial motility and time to 50% motility were recorded as described by Park and Chapman (2005). Sperm concentration in milt was calculated as the mean of triplicate counts made with a hemacytometer (Hausser Scientific); milt was diluted 1:1,000 in a 4% buffered formalin solution (i.e., 1 μL in 1 mL). Sperm : egg ratio was then determined as [(sperm/mL) × (milt volume)]/[(eggs/mL) × (egg volume)].

The number of ovulated eggs collected from each female was estimated volumetrically based on the average egg count in five 5-mL subsamples and the total volume of eggs collected. The eggs either were rinsed with freshwater or were not rinsed before artificial fertilization by the semidry or dry method (Dettlaff et al. 1993). Eggs were incubated in McDonald jars, upwelling incubators (designed for trout; Eagar, Inc.), or glass crystallizing dishes submerged in 1.2-m-diameter larval rearing tanks. McDonald jars and upwelling incubators were both 16 cm in diameter and 46 cm tall and had a 9-L capacity. Crystallizing dishes were 19 cm in diameter and 10 cm deep and had a 2.8-L capacity. Eggs that were incubated in McDonald jars and upwelling incubators were silted after fertilization by gently mixing them with a water suspension of fuller's earth (particle size = 100–200 mesh; Sigma Chemical Co.) for 60 min to prevent adhesion to each other. Eggs in the crystallizing dishes were allowed to adhere to the glass bottom in a single layer.

Fertilization trials (2004).—Trials were conducted in crystallizing dishes and examined two full-sibling crosses and a factorial design for each cross. Two factors (2 levels/factor) were tested: egg rinsing (unrinsed and rinsed) and milt dilution (1:50 and 1:200). Replications were three random subsamples of the gametes fertilized in three dishes. Milt was divided into quantities of 2.5 and 10.0 mL and was kept in beakers on wet ice. Freezing damage was unlikely since the milt column depths in the beakers were 7 and

14 mm, and the sperm always had high motility. The first 400 mL of ovulated eggs collected from each female were placed into a 4-L glass beaker and were gently mixed; 20 mL of eggs (528 eggs for cross 1; 390 eggs for cross 2) were then placed into each dish. Eggs in dishes were rinsed (each rinse ≈ 10 s) twice by adding 1 L of hatchery water, gently swirling the eggs, and then pouring off the water. Milt mixed with 500 mL of hatchery water was then gently poured into the dishes containing unrinsed or rinsed eggs. After 4 min, the milt water was poured off and the eggs were rinsed twice with 1 L of hatchery water. During these final rinses, egg adhesiveness was subjectively evaluated as strong or weak. Eggs that were strongly adhesive would remain stuck to the dish bottom when the dish was tilted for pouring out the rinse water, whereas weakly adhesive eggs would not. A final 1 L of water was added, and the dish was carefully submerged to the bottom of a 1.2-m-diameter, flow-through tank with a temperature of 14–15°C and dissolved oxygen at greater than 85% saturation. All eggs in each dish were fixed in 10% formalin at early cleavage (development stages 5–6: Dettlaff et al. 1993) and were examined for fertilization rate.

Fertilization trials (2005).—Similar to 2004, a factorial design with two full-sibling crosses was applied using fertilization in dishes to test the effects of egg rinsing and fertilization time (2.5 and 5.0 min; milt dilution = 1:200).

To simulate production hatchery conditions, the remaining eggs from the same females were fertilized by sperm from the same males in 7-L stainless-steel bowls. The eggs collected from each female were gently mixed with a feather in one bowl and equally divided among six bowls. For cross 1, each bowl received 400 mL of eggs (12,560 eggs); for cross 2, which used a less-fecund female, 250 mL (8,275 eggs) were added to each bowl. The eggs either were unrinsed or were rinsed twice with 2 L of water, and the semidry or dry fertilization method was used (Dettlaff et al. 1993). There were two replications for cross 1 and three replications for cross 2. For semidry fertilization, 10 mL of milt diluted in 2 L of water (1:200) were slowly poured over the eggs and gently mixed with the eggs, and the mixture was allowed to stay undisturbed for 1 min. Gentle mixing was repeated three more times at 1-min intervals for a total time of 4 min. For dry fertilization, 10 mL of milt were poured onto the eggs and gently mixed with the eggs; 2 L of water were then slowly added, and gentle mixing was conducted at 1-min intervals for 4 min total. After fertilization, the eggs were rinsed with 2 L of water and treated with fuller's earth (100 g in 3 L of water) by gentle, slow, continuous stirring with a feather for the first 15 min and in 3–5-min intervals thereafter (total time = 60 min). Three changes of water were made at approximately 15, 30, and 45 min to maintain the temperature between 15.7°C and 16.2°C. Eggs from each bowl were placed into an individual upwelling incubator, and a random egg sample (~700 eggs) was collected at early cleavage (stages 5–6) to determine proportions of normally fertilized and polyspermic eggs, which were identified by the presence of supernumerary cleavage furrows (Dettlaff et al. 1993).

Egg incubation trial (2002).—The eggs from two females and milt from three males were used in the 2002 egg incubation trial. One liter of ovulated eggs from each female was placed into a stainless-steel bowl (two bowls for female 1; three bowls for female 2), rinsed twice in hatchery water, and fertilized (4 min) with pooled milt from two males (cross 1) or the pooled milt of three males (cross 2); milt was diluted 1:100 with water. After the silt treatment, the eggs were combined into one bowl for each cross and gently mixed, and 800 mL of eggs (~11,000 eggs) were placed into McDonald jars (flow rate = 1 or 6 L/min) or upwelling incubators (flow rate = 15 L/min). Two replications for cross 1 and three replications for cross 2 were used. Approximately 700 eggs were randomly sampled from each incubator at stages 5–6 and stages 22–23 (closure of neural tube) for postgastrulation survival; the latter stages tend to give a good estimation of hatching rates (Dettlaff et al. 1993). The eggs were collected by gentle suction using clear, rigid aquarium tubing (length = 40 cm; diameter = 1 cm) with a bulb on one end. Eggs were suctioned, beginning from the bottom and continuing through the entire column of eggs, and this procedure was repeated six times in different areas of the incubator. Incubation temperature ranged from 14.5°C to 15.5°C.

Egg coat thickness.—The eggs of green sturgeon appeared to have a thinner egg coat relative to the eggs of white sturgeon *A. transmontanus* (Van Eenennaam et al. 2006), and therefore this difference should be considered when handling green sturgeon eggs in the hatchery. To ascertain the difference between the two species, we measured the total egg coat thickness in eggs from 10 Klamath River green sturgeon (Van Eenennaam et al. 2006) and 10 farmed white sturgeon (Sterling Caviar, Elverta, California). All females were preovulatory, and mean egg PI was less than 0.09. The eggs were fixed in 10% buffered formalin and later dehydrated and infiltrated with paraffin in a vacuum tissue processor. A single egg (n = 10 eggs/fish), oriented by the animal–vegetal axis, was (1) embedded in a paraffin block, (2) sectioned stepwise at a thickness of 5 μm until the egg nucleus was reached, (3) cut approximately in half, and (4) stained by the

TABLE 3.—Percent fertilization (mean ± SD) in crystallizing dishes ($n = 3$) for green sturgeon eggs from two full-sibling crosses (see Methods) used in experiments during 2004–2005. Rinsed versus unrinsed eggs were compared during both years; two levels of sperm dilution were examined in 2004 only, and two fertilization periods (2.5 and 5.0 min) were examined in 2005 only.

	Rinsed		Unrinsed		Rinsed		Unrinsed	
Cross	1:50 dilution	1:200 dilution	1:50 dilution	1:200 dilution	2.5 min	5.0 min	2.5 min	5.0 min
1	67.4 ± 2.4	67.4 ± 1.4	60.8 ± 0.2	60.8 ± 1.4	93.6 ± 0.5	94.8 ± 2.8	88.7 ± 1.1	88.6 ± 2.6
2	76.6 ± 1.3	77.8 ± 3.0	71.8 ± 1.9	71.0 ± 4.7	53.9 ± 2.0	65.0 ± 1.4	49.1 ± 2.9	60.1 ± 3.0

periodic acid Schiff method (Sheehan and Hrapchak 1980). Images from the histological slides were gathered with a digital camera (Nikon Digital Sight DS-U1) and Olympus BH-2 microscope (objective = 40×). Measurements of chorion thickness (calibrated by a stage micrometer) were performed using Adobe Photoshop software. Twenty repeated measurements yielded an accuracy of ±0.3 μm.

Statistical analysis.—The effects of factors and interaction in the fertilization trials with crystallizing dishes were tested by a two-factor analysis of variance (ANOVA) with equal replication. For the egg incubation trial, we used one-factor ANOVA with survival at two stages nested within incubator type as a random effect factor. Data on egg fertilization in the bowls were analyzed by one-factor ANOVA. If the effect of treatment was significant, pairwise comparisons by Tukey's honestly significant difference test were performed. In all trials, data were analyzed separately for each cross. Percent fertilized eggs data were normalized by arcsine–square root transformation (Zar 1984) and tested for homogeneity of variance by a Bartlett's test. Difference in the egg coat thickness between green sturgeon and white sturgeon was tested for significance by the ANOVA with subsamples. The accepted significance level for all statistical tests was 0.05. Data in tables, figures, and the Results are presented as untransformed means (±SD). The statistical software package JMP version 4 (SAS Institute, Cary, North Carolina) was used for all analyses.

Results

Induced Spawning

General characteristics of broodfish and their gametes are shown in Table 1. The females were larger and older than the males, and all had fully matured eggs (large diameter and low PI). All males spermiated, and 30–300 mL of semen were collected at 26–32 h after a single GnRHa injection at 10 μg/kg. Sperm had an average initial motility of 93%, and the time to 50% motility was greater than 5 min. The sperm cell concentration in milt of four males used in the fertilization trials averaged 2.2×10^9 sperm/mL (range = 2.9×10^8 to 5.4×10^9 sperm/mL).

Ovulation was induced in all females, regardless of the hormonal treatment (Table 2). The reduction in dose and elimination of domperidone injections did not affect ovulatory response. The four females treated with only GnRHa in 2004 and 2005 produced good-quality eggs with much greater adhesiveness than that of the eggs from females treated with domperidone (Table 2). The mean latent time between the second injection and ovulation was 14.2 ± 2.5 h at holding temperatures of 12.3–13.4°C. Once the first eggs were observed in the holding tank, 1.2–1.8 h was allowed to elapse before egg collection began. On average, 82,000 eggs/female were collected (range = 49,000–115,000 eggs/female) .

Fertilization Trials (2004)

Rinsing eggs before fertilization improved fertilization rates in both crosses (Tables 3, 4). The effects of milt dilution and the interaction were not significant (Table 4). The sperm : egg ratio for cross 1 was 25.6×10^6 at the 1:200 milt dilution and 102.4×10^6 at the 1:50 dilution; the sperm : egg ratio for cross 2 was 9.3×10^6 at 1:200 dilution and 37.2×10^6 at 1:50 dilution (Table 5).

Fertilization Trials (2005)

Similar to the previous experiment, rinsing eggs improved fertilization rates in both crosses (Tables 3, 4). The effect of fertilization time was not significant in cross 1, but a 5-min exposure to sperm significantly improved fertilization rate in cross 2 (Tables 3, 4). Interaction effects were not significant in either of the crosses (Table 4). Sperm : egg ratios were 12.3×10^6 for cross 1 and 2.2×10^6 for cross 2 (Table 5).

The ANOVA did not reveal a significant treatment effect on fertilization rate in bowls, although the fertilization rate of rinsed eggs from cross 1 tended to be higher than that of unrinsed eggs (Table 6). Treatments with dry fertilization had significantly higher proportions of polyspermic eggs in both cross 1 ($P < 0.05$) and cross 2 ($P < 0.001$; Table 6).

TABLE 4.—Analysis of variance examining the effect of egg rinsing, sperm dilution level, and fertilization period (see Table 3) on percent fertilization of green sturgeon eggs in crystallizing dishes (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant). Eggs were from two full-sibling crosses.

		Cross 1		Cross 2	
Source of variation	df	Mean square	*F*	Mean square	*F*
Egg rinsing	1	47.52	52.8***	43.25	11.0*
Sperm dilution	1	<0.01	<0.1 NS	0.07	<0.01 NS
Egg rinsing × dilution	1	<0.01	<0.1 NS	1.22	0.3 NS
Error	8	0.90		3.94	
Egg rinsing	1	103.47	22.1**	24.26	12.3**
Fertilization period	1	2.32	0.5 NS	123.37	62.3***
Egg rinsing × period	1	2.21	0.5 NS	0.01	<0.1 NS
Error	8	4.69		1.98	

Interestingly, cross 2 had higher fertilization rates than cross 1 in the bowls, whereas cross 1 tended to have the higher fertilization rates in crystallizing dishes (Tables 3, 6); this could have occurred as a result of different loading rates (400 mL of eggs/bowl in cross 1; 250 mL of eggs/bowl in cross 2) or changes in egg fertility during the period between egg collection and fertilization. Sperm : egg ratios were 1.2×10^6 for cross 1 and 0.7×10^6 for cross 2 (Table 5).

Egg Incubation Trial

The ANOVA revealed a significant effect of incubator type ($P < 0.001$) for both crosses (Figure 1). The percentage of developing eggs at cleavage was similar in all incubator types within each cross, but neurulation survival was significantly lower in McDonald jars than in upwelling incubators. Cross 1 had less than 1% neurulation survival in McDonald jars with a flow rate of 6 L/min, 32.2% neurulation survival in McDonald jars with a flow rate of 1 L/min, and 60% neurulation survival in upwelling incubators. The neurulation survival rate for cross 2 was 4.7% in McDonald jars (6 L/min) and 80.3% in upwelling incubators. Percentages of normally developing embryos at cleavage and neurulation were not significantly different between crosses in the upwelling incubators.

TABLE 5.—Sperm concentrations (cells/mL) in milt and fertilization mixtures (FMs) and the sperm : egg ratios observed in green sturgeon fertilization trials, 2004–2005. Eggs were from two full-sibling crosses, and fertilization was performed in crystallizing dishes or stainless-steel bowls. Two levels of sperm dilution (1:50 and 1:200) were examined in 2004.

	Sperm concentration		
Trial and cross	Milt	FM ($\times 10^6$)	Sperm : egg ratio ($\times 10^6$)
2004 (dishes)			
Cross 1 (1:50)	5.4×10^9	108.2	102.4
Cross 1 (1:200)	5.4×10^9	27.1	25.6
Cross 2 (1:50)	1.5×10^9	29.1	37.2
Cross 2 (1:200)	1.5×10^9	7.3	9.3
2005 (dishes)			
Cross 1	1.5×10^9	7.7	12.3
Cross 2	2.9×10^8	1.4	2.2
2005 (bowls)			
Cross 1	1.5×10^9	7.7	1.2
Cross 2	2.9×10^9	2.9	0.7

Chorion Thickness

Egg coat thickness was significantly lower in green sturgeon (42.2 ± 4.3 μm) than in white sturgeon (115.1 ± 6.3 μm; $P < 0.001$). The average chorion thickness in individual green sturgeon ranged from 38 to 48 μm and that in white sturgeon ranged from 108 to 127 μm. Both species had an egg coat composed of three layers (L1–L3); the L3 was distinctly thinner in green sturgeon than in white sturgeon (Figure 2).

Discussion

Spawning Induction

Mean body size and age of broodfish used for spawning were similar to those of the natural breeding

TABLE 6.—Mean (±SD) percent fertilization and polyspermy of green sturgeon eggs fertilized in stainless-steel bowls during 2005. Eggs were rinsed or unrinsed prior to dry or semidry fertilization. Eggs were from two full-sibling crosses ($n = 2$ bowls for cross 1; $n = 3$ bowls for cross 2); one-way analysis of variance was performed separately for each cross. Differing letters indicate significantly different means (Tukey's honestly significant difference test: $P < 0.05$).

Cross and treatment	Fertilization success (%)	Polyspermy (%)
Cross 1		
Rinsed; dry	51.2 ± 15.4	4.04 ± 1.28 y
Rinsed; semidry	44.6 ± 6.1	0.27 ± 0.38 z
Unrinsed; semidry	39.2 ± 0.7	0.37 ± 0.17 z
Cross 2		
Unrinsed; dry	83.9 ± 7.6	1.05 ± 0.14 y
Unrinsed; semidry	72.9 ± 5.9	0.32 ± 0.18 z

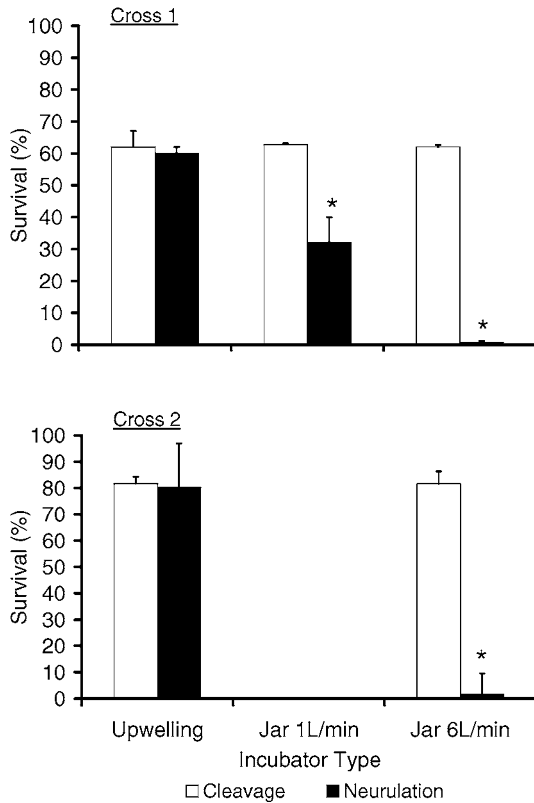

FIGURE 1.—Percent survival (mean ± SD) at cleavage (development stage 5) and neurulation (stages 22–23) for green sturgeon embryos incubated in McDonald jars (cross 1: flow rate = 1 or 6 L/min; cross 2: flow rate = 6 L/min only) or upwelling incubators. Nested analysis of variance (stage nested within incubator type) showed a significant effect (*P* < 0.001) of incubator type for both crosses; asterisks indicate a significant decrease in neurulation survival for embryos in McDonald jars relative to those in upwelling incubators.

population (Van Eenennaam et al. 2006). The peripheral position of the egg germinal vesicle (mean PI = 0.04; Table 1) indicated maturation competence (Dettlaff et al. 1993), in agreement with the 100% ovulation rate in all hormonal treatments. Males produced large quantities of milt (up to 300 mL), and the sperm of all males had high motility that lasted over 5 min. Motile sperm traveled quickly in a linear trajectory; no side-to-side motion was observed until the time when only 10% of the sperm remained motile. The sperm concentration in milt of four males ranged from 2.9×10^8 to 5.4×10^9 sperm/mL and was within the range reported for Eurasian and North American sturgeons (Dettlaff et al. 1993; Cosson et al. 2000; Park and Chapman 2005).

Domperidone was used in this study to enhance pituitary response to GnRHa injections, based on the reported dopaminergic inhibition of gonadotropin release in sturgeon (Pavlick and Moberg 1997) and application of dopamine antagonists for induced spawning of other fishes (Peter et al. 1988; Dufour et al. 2005). With a limited annual supply of broodfish (two pairs), there was a concern that capture and handling stress would affect GnRH-induced ovulation and therefore the annual production of green sturgeon juveniles needed for research projects. While the domperidone treatment could have assured ovulation in the early phase of this study, it was suspected to negatively affect egg quality. The withdrawal of domperidone treatment during the last 2 years had no effect on ovulation rate and appeared to improve egg quality. The recommended total GnRHa doses of 20 μg/kg for female green sturgeon and 10 μg/kg for males are the dosages currently used in the aquaculture industry to induce spawning of domestic white sturgeon (Van Eenennaam et al. 2004). The most apparent effect of domperidone was the reduced adhesiveness of green sturgeon eggs. A distinct feature

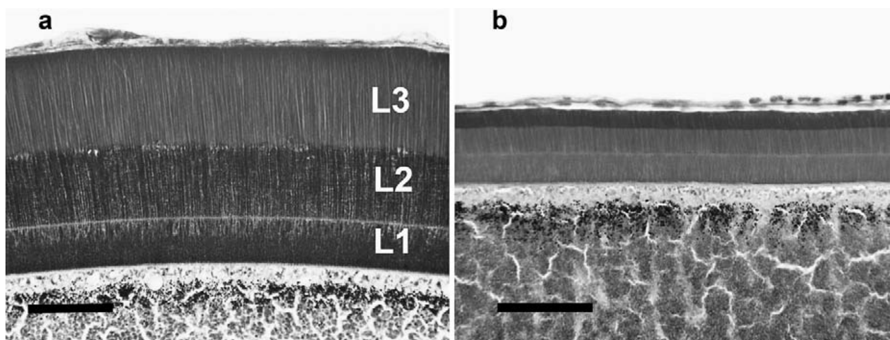

FIGURE 2.—Transverse histological sections of preovulatory ovarian follicles, showing three layers (L1–L3) of the egg coat in **(a)** white sturgeon and **(b)** green sturgeon examined under the same level of magnification (scale bar = 50 μm).

of sturgeon eggs is the adhesive jelly coat released from the ducts of L3 (Figure 2) upon exposure of an egg to freshwater (Markov 1978; Cherr and Clark 1984b). In white sturgeon, the release of the jelly is dependent on a trypsinlike enzyme and divalent cations and occurs in both fertilized and unfertilized eggs (Cherr and Clark 1985). Markov (1978) reported release of jelly in fertilized and unfertilized eggs but noted that fertilized eggs formed a large, uninterrupted substrate attachment area, whereas unfertilized eggs had a "punctuated" (interrupted) attachment area due to incomplete release of the adhesive material from the L3 ducts. We observed a similar weak attachment in both unfertilized and fertilized eggs obtained from all females injected with domperidone. Our previous interpretation that this weak egg adhesiveness was a unique characteristic of green sturgeon (Van Eenennaam et al. 2001; Deng et al. 2002) was apparently erroneous, as the last four females did not receive domperidone injections and all produced highly adhesive fertilized eggs. The effect of domperidone, however, was not experimentally tested in this study, and therefore other factors (holding time in cages, optimal timing of hormonal injection, and egg collection) may have produced a similar effect. Vorobyeva and Markov (1999) reported that sturgeon species spawning in fast-flowing rivers tended to have strongly adhesive eggs. The Klamath River has a fast flow, but flow varies considerably (100–1,700 m^3/s) depending on the rainfall and water releases from dams (Wallace and Collins 1997; Benson et al. 2007).

Our attempts to hand strip the ovulated eggs from green sturgeon were ineffective and yielded a very small quantity (sometimes released as a single row of eggs) due to the small urogenital opening. The cesarean surgery method appears to be most practical, while the internal incision of the oviduct (Mims et al. 2004) might be possible, but because of the small urogenital opening, care must be taken to ensure that the incision is made in the urogenital duct (Wrobel 2003). Ultimately, a noninvasive, natural tank spawning method must be developed, especially for sturgeon conservation hatcheries, based on early studies of Caspian Sea sturgeons (N. A. Derzhavin, unpublished, cited by Dettlaff et al. [1993]).

Fertilization Trials

A brief rinse of eggs in freshwater increased fertilization rates by 5–12% in all trials that used dishes. An effect of milt dilution (1:50 or 1:200) was not detected, and the increase in fertilization time from 2.5 to 5.0 min improved fertilization in one cross but had no significant effect in another cross with overall higher fertilization rates (Table 3). Fertilization trials in bowls had limited replication and an effect of treatment on fertilization rate was not detected, but these trials confirmed previous reports that the percentage of polyspermic eggs increases when dry fertilization is used for sturgeon (Ginsburg 1972). Dettlaff et al. (1993) concluded that dry fertilization is less suitable for sturgeon, and the best and most consistent results are obtained using the semidry method (i.e., milt diluted with water).

The effect of egg quality on fertilization rate was not evaluated in this study. Quality of sturgeon eggs obtained by hormonal induction of ovulation is affected by many factors, including variable water quality (river versus irrigation water), initial stage of maturity, condition of fish (capture and holding stress), timing of injection and egg collection, and even the time between egg collection and fertilization, which can decrease or increase fertilization rate depending on the continuing egg cytoplasm maturation (Dettlaff et al. 1993). Spawning induction procedures do not replicate natural spawning, which in sturgeon involves a complex mechanism of egg maturation, ovulation, and egg transport.

The rinsing of eggs affected fertilization rates in all dish experiments (Table 3). Sturgeon eggs do retain fertilization capacity after exposure to freshwater (Ginsburg 1972; Cherr and Clark 1985; Dettlaff et al. 1993), and the brief exposure of green sturgeon eggs to freshwater consistently increased fertilization success in many other hatchery trials conducted during the past 7 years. Rinsing could enhance fertilization by removing viscous coelomic fluid that inhibits sturgeon sperm motility (Cherr and Clark 1984a; Dettlaff et al. 1993; Lahnsteiner et al. 2004; Park and Chapman 2005). Dettlaff et al. (1993) observed a strong negative effect of coelomic fluid on fertilization in Russian sturgeon *A. gueldenstaedtii*, which produce abundant and viscous fluid at ovulation. We observed a minimal amount of coelomic fluid in all ovulated green sturgeon, but the fluid was more viscous than that of white sturgeon (J.P.V., unpublished data). It was also possible that egg exposure to freshwater before fertilization enhanced gamete interaction by stimulating the eggs to release a sperm attractant or sperm inducer, such as the 66-kilodalton glycoprotein that induces the acrosome reaction in white sturgeon (Cherr and Clark 1984a). With the exception of white sturgeon (Cherr and Clark 1985), biochemical mechanisms of gamete interaction in sturgeons remain largely unknown, in contrast to other animals (Vacquier 1998; Eisenbach 1999; Coward et al. 2002; Riffell et al. 2002, 2004).

The sperm : egg ratios in this study (0.7×10^6 to 102.4×10^6) were far above those used in laboratory experiments with white sturgeon (10^1–10^6: Cherr and

Clark 1985; 10^3: Conte et al. 1988), Siberian sturgeon *A. baerii*, and sterlet *A. ruthenus* (19–20 × 10^4: Tsvetkova et al. 1996). In the wild, sturgeon tend to spawn in parts of the river with strong currents that quickly disperse the eggs and sperm. Sturgeon possess several properties that increase the effectiveness of natural spawning, such as eggs with numerous micropyles, large ejaculation volume, and the ability of eggs and spermatozoa to remain in water for a considerable time without losing fertilizing capacity (Cherr and Clark 1985; Dettlaff et al. 1993; Vorobyeva and Markov 1999). The only estimate of sperm : egg ratio under natural spawning conditions was reported for lake sturgeon *A. fulvescens* by Bruch and Binkowski (2002), who observed that intermittent oviposition lasted 8–12 h and that sperm discharged by multiple males resulted in a ratio of approximately 500 × 10^6.

Egg Incubation

The standard McDonald jars used in sturgeon hatcheries are not suited for incubation of green sturgeon eggs. The sensitivity of green sturgeon embryos to the high-impact rolling action at the bottom of the McDonald jars is probably due to the large egg size (diameter = 4.33 ± 0.14 mm; Van Eenennaam et al. 2006) and thinner chorion (42.2 μm) relative to those of white sturgeon eggs (diameter = 3.79 ± 0.03 mm: Lutes et al. 1987; chorion thickness = 115.1 μm). High mortalities in McDonald jars occurred at gastrulation, a highly sensitive period in embryonic development of sturgeon (Bolker 2004). The large mass (40 mg: Deng et al. 2002) and high density of green sturgeon eggs appeared to increase the effect of the eggs' collision with the McDonald jar's spherical bottom. The upwelling incubators, which were designed for use with trout eggs, did not have such an effect. However, a high flow rate (15 L/min) was needed to keep green sturgeon eggs suspended, apparently due to the high density of the eggs (47% dry mass: Van Eenennaam et al. 2005) relative to that of trout eggs (34–36% dry mass: Barrett et al. 2001) undergoing significant hydration at maturation (Milla et al. 2006). The embryos of other sturgeon species incubated in McDonald jars tend to swim up at hatch and flow out of the jars into larval tanks. However, green sturgeon larvae do not swim up after hatch (Deng et al. 2002) and must be poured or siphoned out of the upwelling incubators into the rearing tanks.

Egg Coat Thickness

The total thickness of the green sturgeon egg coat was approximately one-third that of the white sturgeon egg coat, which probably contributed to the sensitivity of green sturgeon eggs to handling. The sturgeon egg coat undergoes significant modification after fertilization, including hardening of the two inner layers (Zotin 1953) and hydration of L3 (Cherr and Clark 1985). Our preliminary observations on the postfertilization egg coat similarly revealed a difference in total thickness between green sturgeon (60–80 μm) and white sturgeon (180–200 μm; J.P.V., unpublished data). Dettlaff et al. (1993) reported increases in total postfertilization egg coat thickness from 70 to 180–200 μm in stellate sturgeon *A. stellatus* and from 110–120 to 360–400 μm in Russian sturgeon, largely due to the swelling of L3. There are obvious differences among species, but their adaptive significance is not understood. One could hypothesize that larger eggs with a smaller surface : volume ratio require more oxygen and thus benefit from a thinner egg coat, which allows faster diffusion of oxygen; however, this was questioned for salmonid eggs (Einum et al. 2002). It is also possible that thickness and adhesiveness of the egg coat are related to egg substrate and flow velocity. The thickness ratio of L3 : (L1 + L2) in sturgeon species with highly adhesive eggs and that spawn in strong currents was lower than the ratio in species that spawn in slow currents (Vorobyeva and Markov 1999); this finding appears to be in agreement with the observed thin L3 in green sturgeon eggs (Figure 2).

Conclusion

To induce spawning of female green sturgeon, we recommend use of a 1-μg/kg GnRHa priming injection and a second, 19-μg/kg injection 12 h later. To induce spawning of males, a single GnRHa injection of 10 μg/kg is recommended. Ovulated eggs should be collected no later than 1.5 h postovulation. The eggs should be briefly rinsed in freshwater and fertilized with milt (diluted 1:200) for at least 4 min or until the eggs start to adhere to the sides of the fertilization container. Fertilized eggs should be silted for 1 h and incubated in upwelling incubators. Optimally, all these procedures should be performed within the temperature range of 12–16°C. Green sturgeon eggs must be handled very gently during egg collection, rinsing, fertilization, silting, and incubation. In contrast to the highly sensitive eggs, the rearing of green sturgeon larvae and juveniles presents few difficulties relative to the rearing of other sturgeon species (Van Eenennaam et al. 2001; Deng et al. 2002).

The successful artificial reproduction of green sturgeon has supported many recent research projects with various life stages of this species (Kynard et al. 2005; Van Eenennaam et al. 2005; Allen et al. 2006; Allen and Cech 2007; Kaufman et al. 2007; Werner et al. 2007). While some aspects of artificial reproduction

should be further investigated (egg collection in vivo) or optimized (in vitro fertilization), the techniques recommended here for green sturgeon can be used in conservation hatcheries and aquaculture.

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