

Induced Meiotic Gynogenesis of Paddlefish *Polyodon spathula*

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Abstract

Viable, diploid gynogenetic (gynogenotes) paddlefish *Polyodon spathula* were produced by activating eggs with ultraviolet-irradiated shovelnose sturgeon *Scaphirhynchus platyrhynchus* spermatozoa and heat shocking. Without irradiation treatment, sturgeon spermatozoa appeared to activate the eggs (up to gastrulation), but did not result in any viable hybrids. Experiment 1 determined that heat-shock treatment of 35 C for a 2-min duration within the interval of 2–22 min post-activation resulted in highest yield of gynogenotes (12–19%) from eggs incubated at 18 C. Experiment 2 applied the heat shock treatment at 35 C from 14.0 to 28.0 min in 2-min intervals after activation at 18 C for a larger scale of gynogenetic production. Both experiments showed that the best yields of gynogenotes were obtained when the heat shock treatment occurred at 16, 18, and 20 min after activation. When these times were expressed in terms of τ_0 units (duration of one mitotic cycle of synchronous cell division related to water temperatures), optimal activations were 0.26, 0.29, and 0.32 τ_0 (τ_0 @ 18 C = 63.5 min). Experiment 3 tested the utility of τ_0 at two different pre-shock incubation water temperatures of 18 C and 16 C, and determined that there was no significant interaction in percentage of viable gynogenotes between two different incubation temperatures and the mitotic intervals (0.21, 0.26, 0.31, 0.36, 0.41 τ_0) tested. Best survival of gynogenotes occurred when eggs held at either pre-shock incubation water temperatures were shocked at 0.26 τ_0 . All gynogenotes examined were histologically confirmed to have ovarian tissue and were determined to have similar oocyte development to that of normal female (control) paddlefish.

Paddlefish *Polyodon spathula* is a primitive chondrosteian found primarily in large rivers of the Mississippi River drainage and a few rivers that run directly into the Gulf of Mexico (Carlson and Bonislawsky 1981). Female fish are valued for their roe as a commercial caviar and are currently available only from the wild populations. Overexploitation (Carlson and Bonislawsky 1981) and contamination of the role by organochlorine pollutants (Gundersen and

Pearson 1992) have required that many state agencies in the United States close down this valuable fishery. Demand for commercial farming is apparent but the main problem of raising these fish for caviar is their long maturation period of 8–14 yr (Russell 1986). Further, about half of the cultured stock will be males with no economic value for caviar. Production of all-female paddlefish would be most economical for the aquacultural industry.

Induced meiotic gynogenesis is a chromosome manipulation technique with potential for all-female inheritance, which could have direct application for the caviar industry. The technique is accomplished by activating egg development with irradiated spermatozoa and then restoring diploidy to the developing zygote. Spermatozoa from heterologous, but related species should be used to prevent production of viable hybrid offspring or to provide morphologically or biochemically recognizable markers (Nagy et al. 1979; Chourrout et al. 1986; Kaastrup and Horlyck 1987; Linhart et al. 1986, 1989, 1995a). Radiation treatments such as gamma rays from ^{60}Co or ^{137}Cs sources (Chourrout et al. 1980; Ijiri 1980; Refstie et al. 1982; Pipota and Linhart 1986), X-rays (Romashov et al. 1963; Stanley and Sneed 1974), and UV light (Chourrout 1982; Shelton 1989; Christensen and Tiersch 1994) have been used to inactivate the paternal genome of the spermatozoa without affecting their physical integrity to initiate egg development for gynogenesis.

After activation of the egg, diploidy must be restored by suppressing the second meiotic division or second polar body extrusion in the egg (Thorgaard 1983). Retention of the second polar body is accomplished by thermal-shock (heat or cold) or hydrostatic pressure-shock treatments. The type of shock, intensity, time of application and duration varies among fish species and these manipulation parameters must be optimized for successful production of diploid gynogenotes (gynogenotes). Romashov et al. (1963) was first to report spontaneous gynogenetic sterlet *Acipenser ruthenus* when X-ray was used to irradiate the spermatozoa. Vasetskii (1967) reported that heat shock treatment of 34 C produced triploid sterlet *Acipenser ruthenus* larvae. Kowtal (1987) attempted to induce gynogenesis in white sturgeon *Acipenser transmontanus* but was not successful in producing viable diploids. There have been no previous studies on the induction of gynogenesis for paddlefish.

This paper reports on three experiments

that provide information on successful induction of meiotic gynogenesis with the paddlefish and on the histological verification of ovarian development in gynogenotes: Experiment 1 objective was to determine a heat-shock water temperature and shock duration for direct induction of paddlefish gynogenesis; Experiment 2 objective was to verify the mitotic intervals for heat shocking to increase the scale of gynogenote production; and Experiment 3 objective was to obtain preliminary data for assessing the utility of τ_0 as a means of adjusting absolute shock time at different pre-shock incubation temperatures (Shelton et al. 1997).

Materials and Methods

All experiments and analyses were conducted at the Aquaculture Research Center (ARC), Kentucky State University, Frankfort, Kentucky, USA, from 1993 to 1996. Brood paddlefish from 5 to 22 kg were captured during spring migration below Uniontown Dam on the Ohio River and Cumberland Lake, Kentucky, USA. Shovelnose sturgeon *Scaphirhynchus platyrhynchus* from 1 to 3.5 kg also were captured during the spring migration below Smithland and Uniontown Dams on the Ohio River. Broodfish were transported to and held in 400-m² holding ponds at ARC. Two female paddlefish and two male paddlefish were selected and held separately in 3,000-L circular metal tanks with water flow rate of 12 L/min, 9.0-mg O₂/L, and controlled water temperature of 17–19 C. Ten female and 20 male sturgeon were separated by sex and kept in two circular metal tanks (3,000 L) with water flow rate of 12 L/min, 9.0-mg O₂/L and controlled water temperature of 17–19 C. Broodfish were injected intraperitoneally with LHRH analogue of des-Gly10(D-Ala6) LHRH ethylamide (Graham et al. 1986). Female paddlefish and sturgeon received a total dosage of 100 µg/kg body weight (BW) administered in a priming injection (10 µg/kg BW) and a resolving injection (90 µg/kg BW) 12 h apart.

Male paddlefish and sturgeon received a single dose of 50 $\mu\text{g}/\text{kg}$ BW when the females were given the priming injection.

UV-Irradiated Spermatozoa

Shovelnose sturgeon spermatozoa were irradiated with UV-light to deactivate the genome without affecting motility as described by Mims and Shelton (in press). In brief, milt was collected in 3-cc syringes and immediately placed on wet ice. Individual milt samples were examined under a light microscope at $200\times$ in dark field and only those samples determined to have 90–100% spermatozoal motility were combined (Mims 1991; Linhart et al. 1995b). Because milt of *Acipenseriformes* is naturally dilute compared to teleost, a portion of the pooled sample was placed in polystyrene disposable cuvette and was measured for percent transmittance with a Milton Roy Digital Spectronic 401 spectrophotometer (Rochester, New York, USA). Since percent transmittance correlates with density of spermatozoal cells, the transmittance was used as an independent variable on which to base the dosage of UV. Ultraviolet dosage (Joules/m^2) for the pooled sample (40% of the lethal dosage) was calculated using the following linear regression equation: $\text{Dosage} (\text{Joules}/\text{m}^2) = 3590.88 - 575.00X$ (X = percent transmittance). The pooled sample was then subdivided into 2-mL aliquotes in 5-cm glass petri dishes, agitated on a shaker table and irradiated under a UV germicidal lamp (254 nm) at the calculated dose. Irradiated milt was placed on wet ice in a dark insulated container until eggs were ready to be activated. Motility was confirmed microscopically before and after UV treatment.

Meiotic Gynogenesis

Experiment 1. Various combinations of heat shock water temperatures and shock durations were tested in 1993 (trial 1) and 1994 (trial 2) to determine a suitable heat shock and duration for paddlefish gynogenesis (Table 1). Each trial was evaluated with

TABLE 1. Experimental heat shock water temperatures (C), durations (min) and time intervals and ranges after eggs activation at 18 C tested for developing gynogenetic procedures for paddlefish.

	Shock temperature (C)	Shock duration (min)	Time intervals after eggs activation (min)	Time range after eggs activation (min)
1993	30	1	1	1–22
(trial 1)	34	1	1	1–22
1994	28	2	2	2–22
(trial 2)	35	2	2	2–22
	40	2	2	2–22
1994	28	4	2	2–22
(trial 3)	35	4	2	2–22
	40	4	2	2–22

eggs from one paddlefish female without replication. The fertilized and activated batches of eggs (196–356) were placed into 15-cm diameter PVC incubation units and allowed to attach to the nylon screen bottoms. The units were submerged into a flow-through water bath and kept at 18 ± 0.3 C in each experimental trial prior to the start of the temperature shock, and after the shock treatment in order to ensure standard embryonic development. The τ_0 unit values (the duration of one mitotic cycle during synchronous cell division; Dettlaff and Dettlaff 1961) for paddlefish were used as described by Shelton et al. (1997). The τ_0 at 18 C was 63.5 min. In the first trial, water temperatures of 30 C and 34 C for heat shocking were tested based on an early effort to induce ploidy alteration in *Acipenser gueldenstaedti* (Vasetskii 1967). Heat shocks were administered for 1-min duration at 1-min intervals beginning 1 min after gamete activation and continuing for 22 min (Table 1). In the second trial, heat shocks of 28, 35 and 40 C were applied for 2- and 4-min durations in 2-min intervals starting at 2 min after gamete activation and continuing for 22 min (Table 1). Control groups included C_1 = female paddlefish (PF) \times male PF (no shock), C_2 = PF \times PF (shock), C_3 = PF \times shovelnose sturgeon

(SS, no shock), and $C_4 = PF \times$ irradiated SS (no shock). Control-1 (C_1) was to test the viability of eggs, C_2 tested the effect of shock temperature on survival, C_3 provided a test for hybridization potential, and C_4 was to examine potential for spontaneous gynogenesis. Hatched larvae (swimming) were counted and relative percentages of viable gynogenotes, i.e., gynogens, were determined using (number of hatched gynogenotes/number of total activated eggs) divided by the (number of hatched C_1 /number of total C_1 fertilized eggs) $\times 100$.

Experiment 2. Three gynogenesis trials were conducted, each using gametes from one female paddlefish and three to five male sturgeon. This large-scale production experiment was undertaken as an extension of the trials in experiment 1 and was possible because of the large number of eggs produced per female (50,000 to 350,000). This experiment was also necessary for verification of gynogenetic optimization since experiment 1 was based on low numbers of eggs per treatment. Activated eggs (6,000 to 10,000 per treatment replicate) were maintained at 18 ± 0.3 C. The eggs, held in screen-bottom wood-frame boxes, were submerged into a 300-L water bath and each batch was heat shocked at 35 ± 0.2 C for a 2-min duration based upon experimental results (τ_0 @ 18 C was 63.5 min). Time of temperature shock ranged from 14 to 28 min in 2-min intervals or in mitotic intervals of 0.23 to $0.44\tau_0$ (τ_s/τ_0 ; where τ_s = time of shock in min) in $0.03\tau_0$ increments, respectively. Three females served as the replication over time. Each trial had a control of $PF \times PF$ (no shock) to verify the quality of the eggs. Other controls, as tested in experiment 1, were not examined. The treated eggs were incubated in 10-L McDonald jars receiving non-recirculating, dechlorinated tap water held at 18 C. At hatch, larvae swam out of the hatching jars into hapas (net enclosures) suspended in 100-L conical-shape fiberglass tanks. Larvae were counted and percentage of viable gynogenotes calculated as in experiment 1.

Percentage of hatch were analyzed by general linear model (GLM) for a completely randomized block design. The relationship between percentage of viable gynogenotes and shock at mitotic intervals (τ_0) was examined by regression (SAS Institute Inc. 1988).

Experiment 3. Two gynogenesis trials were conducted to obtain preliminary data for assessing the utility of τ_0 as a method of adjusting absolute shock time at different preshock incubation temperatures. Eggs from one female were collected and divided into two lots (60,000/lot). Irradiated milt pooled from three to five shovelnose sturgeon was added to each lot of eggs and then activated with water at temperatures of either 16 or 18 C (± 0.3 C). At an incubation temperature of 16 C, mitotic intervals (τ_0) equals 74.5 min (Shelton et al. 1997) and at 18 C, τ_0 equals 63.5 min. The eggs were then volumetrically subdivided into five experimental shock groups based on tau (τ_s/τ_0): 0.21, 0.26, 0.31, 0.36, 0.41 τ_0 and a control group for each of the two water temperature treatments, and placed into 20-cm glass bowls. Eggs were coated with Fuller's earth suspension to eliminate adhesiveness. After 10 min, eggs were carefully poured into screen-bottom wooden frame boxes, maintained at pre-shock incubation temperature of 16 or 18 C until submerged for 2 min in a 300-L water bath held at 35 ± 0.2 C at each of the respective τ_0 treatments. After heat shock, eggs were returned to either 16 or 18 C water bath for incubation of the activated eggs until they were transferred to 10-L McDonald jars at their respective (pre-shock) incubation water temperatures. At hatch, larvae swam up and over into hapas suspended in 100-L conical shape fiberglass tanks. Larvae were counted and percentage of viable gynogenotes calculated as above. The relationship between percentage of viable gynogenotes and mitotic intervals (τ_s/τ_0) were examined by regression, and the differences between preshock incubation temperatures were exam-

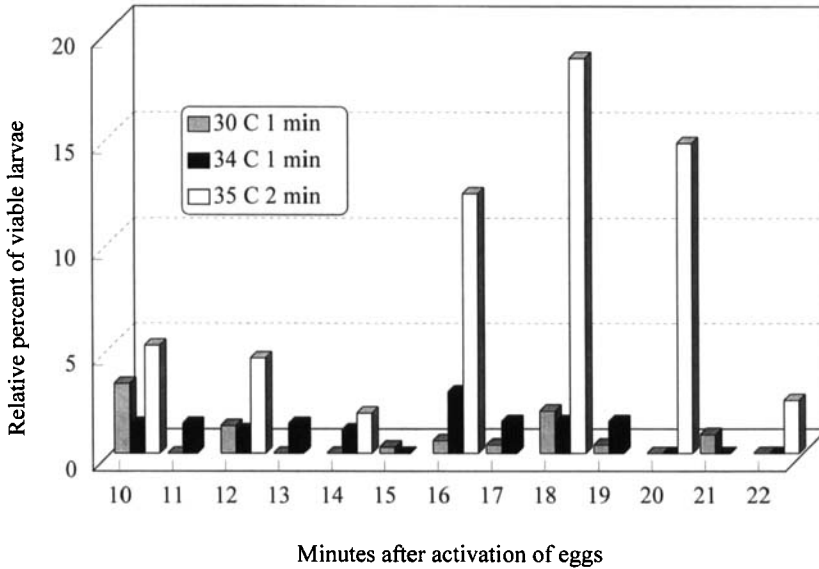


FIGURE 1. Heat shock water temperatures and shock durations on paddlefish eggs activated with irradiated shovelnose sturgeon spermatozoa that produced viable diploid gynogenotes relative to Paddlefish \times Paddlefish control. Control (Paddlefish \times Paddlefish - C_1) samples were 56% for 30 C for 1-min duration and 61% for 28 C for 2-min and 35 C for 2-min. The PF \times PF (shock) controls had relative hatch percentages of 44% at a heat shock of 34 C for 1 min (trial 1) and 30% and 16% at 28 C for both 2- and 4-min duration (trial 2 and 3, respectively). Pre-shock incubation water temperature was 18 ± 0.3 C.

ined by homogeneity regression (SAS Institute Inc. 1988).

Histology of Gonadal Tissue

Gynogenetic paddlefish and conventionally-propagated paddlefish (control), hatched in 1994 and 1995, were cultured at a stocking rate of 3 fish/m² in five 400-m² earthen ponds as described by Mims et al. (1991) and were later fed a 1.5-mm extruded diet (50% protein) formulated for paddlefish. After 70 wk in ponds, fish were sacrificed and gonads removed. We have found that sex of paddlefish can be determined through histological examination of gonadal tissues at 70 wk of age (unpublished data). Therefore, gonads of 45 gynogenotes and 50 control fish from each experimental year were fixed by a LX 120 Fisher Tissue Processor, embedded in paraffin on a Leica Histoembedder, sectioned serially at 10 μ m and stained with hematoxylin and eosin. Female sex was indicated when the gonadal section showed lamellar structure and pri-

mary oocytes under a low-power light microscope (40 \times).

Results

Meiotic Gynogenesis

Experiment 1. Of the shock temperatures and shock durations tested (Table 1), gynogenotes were produced at 30 C for 1 min, 34 C for 1 min, and 35 C for 2 min (Fig. 1). Other shock temperatures and durations (Table 1) did not produce viable gynogenotes. Best survival of viable gynogenotes was achieved when eggs were shocked at 35 C for a 2-min duration (trial 2) which was significantly higher ($P = 0.004$) than survival of diploid gynogenotes from eggs shocked at 30 C or 34 C for 1-min durations (trial 1). Highest survivals from the 35 C, 2-min treatment occurred when eggs were shocked at 16, 18, or 20 min (i.e., at 0.26, 0.29 or 0.32 τ_0 , respectively) after activation of the eggs with irradiated milt (Fig. 1). The PF \times PF control (C_1) percentages of actual hatch were 56% for trial

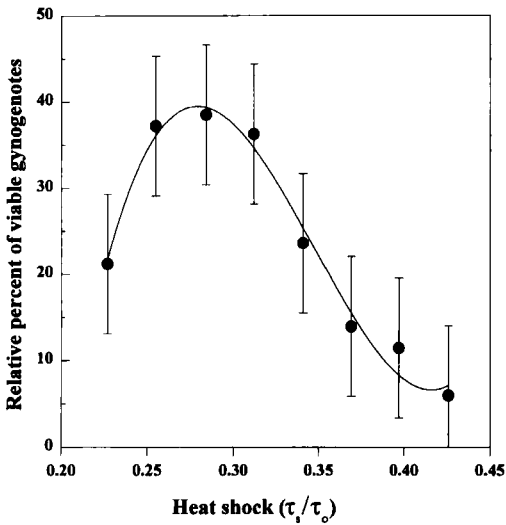


FIGURE 2. The response of heat shock initiation (τ_s/τ_o) on the percentage of viable, diploid gynogenotes when pre-shock incubation water temperature was 18 ± 0.3 C (where $\tau_o = 63.5$ min) in experiment 2. Control (Paddlefish \times Paddlefish - C_1) samples had a hatching mean of $55 \pm 18\%$ and were not exposed to heat shock treatment.

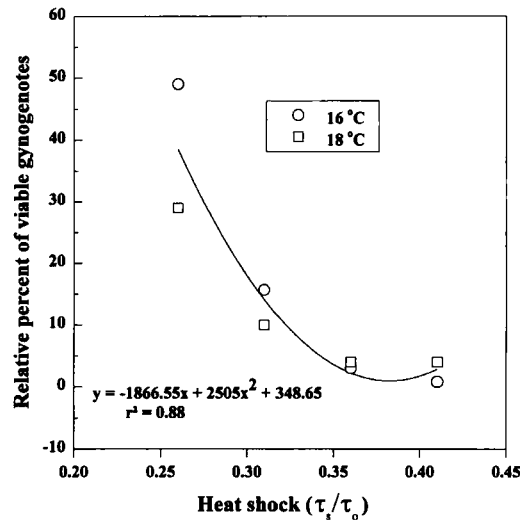


FIGURE 3. The response of heat shock initiation on the percentage of viable, diploid gynogenotes when activated eggs were incubated at two pre-shock incubation water temperatures of 16 C (where $\tau_o = 74.5$ min) and 18 C (where $\tau_o = 63.5$ min) in experiment 3.

1 and 61% for trial 2. No hatch was observed for the PF \times SS (C_3) or PF \times irradiated SS (C_4) controls, suggesting a low or non-viability of diploid hybrids and a low number of spontaneous gynogenotes. The PF \times PF (shock) controls (C_2) had relative hatch percentages of 44% at a heat shock of 34 C for 1 min (trial 1) and 30% and 16% at 28 C for both 2- and 4-min duration (trial 2). No larvae were observed in the other PF \times PF (shock) controls.

Experiment 2. The percentage of viable gynogenotes responded cubically to the tau index (τ_s/τ_o) when shock treatments were applied (Fig. 2). The highest percentages of gynogenotes were obtained from eggs shocked at 16, 18, and 20 min (i.e., at 0.26 , 0.29 , $0.32\tau_o$, respectively), whereas the lowest percentages of gynogenotes were obtained from eggs shocked at 24, 26 and 28 min (i.e., at 0.38 , 0.41 and $0.44\tau_o$, respectively).

Experiment 3. There was no significant interaction between pre-shock incubation temperature and the shock time (when ref-

erenced to mitotic intervals; τ_s/τ_o) when the shock treatment was applied on percentages of viable gynogenotes. There was a significant effect of the absolute time when the shock treatment was applied. The effect of pre-shock incubation temperature and the shock time (when referenced to mitotic intervals; τ_s/τ_o) were not significant because similar percentages of gynogenotes were obtained at both incubation water temperatures (Fig. 3). The relationship between percentages of viable gynogenotes and the shock time (τ_s/τ_o) can be represented by a quadratic equation within the range of time examined. Homogeneity of regression indicated that the data obtained at both incubation temperatures can be fitted together to a single regression line. The highest yield of diploid gynogenotes occurred at $0.26\tau_o$, indicating the shock time was 19 min or 16 min after activation when the pre-shock incubation temperature was 16 C or 18 C, respectively.

Histology of Gonadal Tissue

The 70-wk-old gynogenetic paddlefish ($N = 45$) were histologically determined to

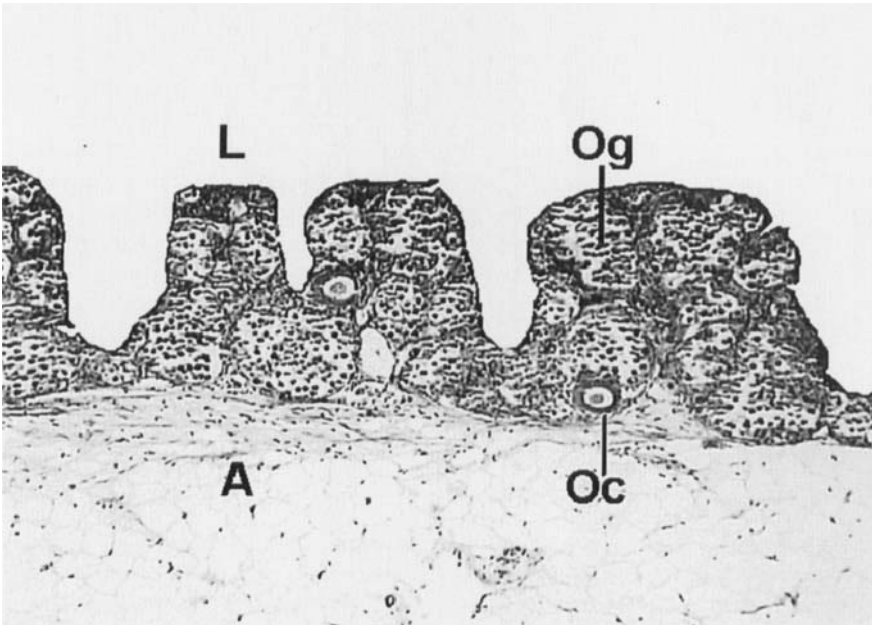


FIGURE 4. Longitudinal section of ovarian tissue from viable, diploid gynogenotes. Bar = 100 μ m. Development of lamellae (L) with oogonia (Og) and primary oocytes (Oc) are similar to that of ovarian tissue found in traditionally-produced female paddlefish. A identifies adipose tissue.

have ovarian tissue dominated with primary oocytes (Fig. 4). The control groups of fish had 24 females and 26 males from 1994 hatch and 27 females and 23 males from 1995 hatch. No anatomical or cytological differences were observed between ovarian tissues of gynogenotes and normal female (control) fish.

Discussion

The application of UV-irradiated shovelnose sturgeon spermatozoa and heat shock was effective in producing viable, paddlefish gynogenotes. Without irradiation treatment, sturgeon spermatozoa appeared to activate the eggs (development up to gastrulation), but no viable hybrids hatched in this control (C_2) cross. Reciprocal hybridization (sturgeon female \times paddlefish male) was not tested, but could result in viable progeny as reciprocal crosses are not always equally viable (Linhart et al. 1995a). In experiment 1, heat shock at 35 C for a 2-min duration gave the highest yields (12–19%) of gynogenotes when eggs were

shocked at 16–20 min after fertilization (i.e., at 0.26 to 0.32 τ_0 , respectively). When experimental protocol was tested on larger quantities of eggs (6,000 to 10,000), best results were also obtained at 16–20 min after fertilization with increased yields (36–39%) of gynogenotes produced. Although triploids should have been expected in PF \times PF (shock) control (C_2), we did not test for these larvae. Production of triploid hybrids also is possible, even when diploid hybrids are not viable (Marian and Krasznai 1978; Shelton 1989), triploid hybrids might have been expected in the PF \times SS (C_3) group, but no viable larvae hatched. The probability of producing triploid hybrids would have been increased, had we heat shocked activated eggs from this group. Triploidy would result in sterility and thus, have an adverse effect in a population of gynogenotes; however, UV-deactivation of the sturgeon genome and the evidence for inviable hybrids would suggest that this is a minimal problem.

Direct induction of gynogenesis requires

that several variables such as intensity of shock, shock duration and the shock time must be empirically determined (Shelton et al. 1997). Optimum induction of chromosomal manipulation requires development of a specific treatment for each fish species, but the temperature-dependent measure of the mitotic interval (τ_0) can improve standardization of chromosomal manipulation for all-female production and facilitate preliminary trials in closely related species (Shelton et al. 1997). Time of shock is species-specific but is also subject to variation caused by environmental factors. Pretreatment incubation temperature will change the rate of development and thus, the optimum absolute time for shocking. Reporting shock application in terms of τ_0 provides a dimensionless unit for this treatment variable (Shelton et al. 1997). In this study, highest percentages (49 ± 9.4 at 16 C and 29 ± 9.4 at 18 C) of viable gynogenotes were produced when eggs were shocked at $0.26\tau_0$, whether incubation water temperature was 16 or 18 C. In absolute time, optimal shock initiation for sperm-activated paddlefish eggs at 18 C would be about 16 min at 18 C, but about 19 min at 16 C. These results verify the utility of τ_0 in improving treatment standardization and will aid in refining treatment protocol.

Maternal inheritance and production of monosex culture are the major rationales for the interest in gynogenesis (Thorgaard 1983). The application of induced gynogenesis for production of all-female progenies is possible only in the case of female homogamy. Up to now there were no data on the mechanisms of sex determination in chondrosteans. The present study reveals that gynogenetic progenies of paddlefish are all-female, and this indicates the occurrence of female homogamy (and correspondingly male heterogamy) in this species. The results from this study suggest that induced diploid gynogenesis could be feasible for commercial quantities of gynogenotes because mature female paddlefish grow large

(10–70 kg) and have large numbers of eggs (50,000 to 350,000 eggs per fish; or 2 to 8 kg of roe) and the optimized protocol for mass production could provide producers with relatively high percentages (12–49%) of gynogenotes. No differences were found between the ovarian tissues from treated and control fish which should indicate that similar development of oocytes will occur, and mature gynogenetic fish would be suitable for caviar production. Growth performance testing will be examined in later studies, since inbreeding through gynogenesis might be expected to have some deleterious effects.

Further optimization of direct induction could increase the percentage of gynogenetic paddlefish. Improvement in irradiation of heterologous spermatozoa and further testing of the mitotic intervals (τ_0) would benefit the refinement of the gynogenesis protocol. Sex reversal of gynogenetic paddlefish could also provide broodstock to be used in a breeding program to produce all-female progeny. Mims et al. (1995) reported that paddlefish implanted with two 17- α methyltestosterone (MT) capsules (each capsule with 5 mg of MT) was an effective method for producing phenotypic males with maternal genomes similar to that accomplished with grass carp, *Ctenopharyngodon idella* (Shelton 1986). Male broodstock take 6 to 9 yr to become sexually mature (Russell 1986), consequently such a monosex paddlefish breeding program will be a long-term commitment. Therefore, direct induction of gynogenesis, as defined in this study, will provide all-female paddlefish that can be cultured for the caviar industry before an all-female breeding program can be established with mature sex-reversed gynogenetic fish.

Acknowledgments

This work was supported by USDA 1890 Capacity Building Grant Nos. 92-38814-7484 and 96-38814-2852 and USDA Foreign Agricultural Service Research and Scientific Exchange Division International Co-

operation and Development Contract No. 58-319R-4-042. Special thanks to Jim Dan-
cy for the capture of paddlefish broodstock
and Craig Preston for capture of sturgeon
broodstock. Thanks also to Rick Onders,
Julia Clark, Kenny Thompson, David Mahl
and Leigh Ann Vitatoc who provided assis-
tance to this project.

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