

**National Fish and Wildlife Foundation  
Final Programmatic Report**

**Project Name and Number:** Distribution & Movement of Shortnose Sturgeon III (2006-0087-001)

**Recipient Organization/Agency:** Maryland Department of Natural Resources

**Recipient Organization Web Address:** www.dnr.state.md.us

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**1) Summary of Accomplishments**

Anesthetic and laparoscopic procedures were adapted for use in shortnose sturgeon to identify the sex, reproductive status and general health of fish. Laparoscopy allows for high quality imagery of the gonad and other internal organs, requires smaller incisions than what are used to implant acoustic transmitters and enables collection of tissue biopsies to positive identify the sex of immature fish. Fish are released within minutes following completion of procedures with little risk of post-operative complications. In addition, hematologic, plasma chemistry and sex hormone reference intervals were generated from captive shortnose sturgeon, which may be useful to identify and monitor disease and reproductive status in fish. Annual and/or seasonal hormone, hematologic and plasma chemistry values were obtained from Delaware Cooper River populations. General and reproductive health assessments can be incorporated into existing research and monitoring programs providing additional useful information about the status of fish populations.

**2) Project Activities & Results**

**Fig. 1: Logic framework table with indicators.**

Activities	Short-Term Outputs	Long-Term Outcomes	Indicator	Baseline Value	Predicted Value of Project Output	Actual Value of Project Output
Determine movement patterns and fish aggregation sites of shortnose sturgeon by use of sonic telemetry	Tag and track shortnose sturgeon by use of stationary and mobile receivers	Capacity to possibly identify foraging, spawning or over-wintering habitat sites in Chesapeake Bay	# of sturgeon captured and implanted with tracking tags	0	5	0
Determine if the Chesapeake Bay population is genetically distinct from that of the Delaware River	Determine genetic origin of sturgeon through tissue	Evidence on distinct populations or common grouping as a single population segment	# of tissue samples collected from sturgeon	35	45	62*
Determine to what extent shortnose sturgeon use the C&D canal	Monitor fish movement through the canal via stationary receivers	Determine if fish commonly move between the Delaware river and the Chesapeake Bay	# of hits on stationary acoustic equipment in C&D Canal	2	unknown	0

\*Actual value includes samples collected from the Delaware River only. Baseline value includes samples collected prior to 2002 by the US Fish and Wildlife Service.

## Activities

### *Project Indicators*

The scope of activities proposed in the logic framework applies to fish captured within the Chesapeake Bay. These activities were not implemented because MD DNR has had no access to shortnose sturgeon in the Chesapeake Bay during the project period. All but two shortnose sturgeon examined and tagged in the Chesapeake Bay since 1996 were obtained as commercial by-catch through a reward program (Skjeveland et al., 2000). However, the NMFS suspended access to incidentally-captured fish until a Habitat Conservation Plan (HCP) for endangered species in the Chesapeake Bay is drafted and approved, and an incidental take permit (ITP) for shortnose sturgeon is granted. The HCP for the Chesapeake Bay has not been completed to date, and therefore, an ITP allowing MD DNR to collect data from these fish has not been granted. As a result, no samples have been collected for genetic comparison to Delaware River fish, and no fish were tagged for acoustic telemetry.

Alternate indicators of the progress of this project may include completion of reference intervals for hormone, hematologic and plasma chemistry values, and the number of population segments assessed in this project. All three types of reference intervals were generated using hatchery-reared fish. Determination of spring hormone levels in hatchery-reared fish was not done because established research protocols prevented phlebotomy during spawning season. Fish from the Delaware and Cooper Rivers were assessed using laparoscopy and blood sample analysis. In addition to these population segments, we anticipated collecting data from the Altamaha, Ogeechee and the Savannah Rivers. However, Scientific Research Permits for work on these populations were not granted with sufficient time to allow samples to be collected and analyzed.

### *Anesthesia*

Fish were anesthetized with tricaine methanesulfonate (MS-222, Argent Laboratories) until stage 4 (surgical phase) of anesthesia was reached (Summerfelt and Smith, 1990). Fish were placed in a trough (induction tank) containing a 250 mg/L solution of MS-222 buffered with 500 mg/L of sodium bicarbonate. Fish were removed from the induction tank when equilibrium was lost, respiratory rate decreased, fin and tail movement ceased and no response to tactile stimulation was observed. Movement, respiratory rate, equilibrium and response to stimuli were monitored throughout the procedures to determine the depth of anesthesia. Once anesthetized, the animal was moved to a re-circulating anesthesia machine (Bakal and Stoskopf, 1994) containing an 85 mg/L solution of MS-222 buffered with 170 mg/L of sodium bicarbonate. While on the anesthesia machine heart rate was monitored by visualization of body wall excursions immediately cranial to the pectoral girdle, ventilation rate was monitored by visualization of opercular movements, signs of hypoxia were monitored by examination of gills and fins for development of pallor and fish were observed for other indicators of distress (e.g. erythemia). Observation of fin or body movement while on the anesthesia machine indicated that the fish was not properly anesthetized. In those cases, the outflow tube from the anesthetic machine was removed from the opercular cavity of the fish, the gills were drenched with water containing induction dose of anesthetic until movements ceased and the outflow tube replaced.

Upon completion of the sampling procedures the fish was returned to fresh water and assisted with ventilation by slowly moving the fish back and forth in the water while supporting it by the tail and under the body. Once the animal was conscious, regained equilibrium and attempted to swim on its own it was released.

### *Blood collection*

A blood sample was collected from the caudal vein of each fish while under anesthesia. This was achieved by inserting a needle attached to a syringe, perpendicular to the ventral midline at a point immediately caudal to the anal fin. Once the blood was collected direct pressure was applied to the veinipuncture site to ensure clotting and prevent subsequent blood loss. Needle and syringe size as well as blood volume collected was dependent on fish size (Fig. 2).

**Fig. 2: A guide to needle and sample sizes for the safe collection of shortnose sturgeon blood.**

Fish weight (Kg)	Sample Size* (ml)	Needle Size (Gauge x Length)	Syringe Size (ml)
0.5 – 1.0	3	22 x 3/4"	3
1.0 – 1.5	5	22 x 3/4"	6
1.5 – 2.0	7.5	22 x 3/4"	10
>2.0	10	20 x 1"	12

Immediately following blood collection, two blood smears were prepared on glass slides, two hematocrit tubes were filled, and 600 µl of blood was placed in a Microtainer tube (Becton, Dickinson and Company) containing lithium heparin. Remaining blood was placed into 1 or more 3-ml Vacutainer tubes (Becton, Dickinson and Company) containing lithium heparin. Microtainer and Vacutainer tubes were centrifuged for 5 min. Microtainer tube, blood smears and hematocrit tubes were submitted to Antech Diagnostics (Lake Success, NY) for analysis. Total and differential white cell counts were determined by enumerating cells visible on blood smears that were dried and stained with Wright's-Giemsa. The packed-cell volume of erythrocytes was measured directly from centrifuged micro-hematocrit tubes. Albumin, aspartate aminotransferase, urea nitrogen, calcium, chloride, creatinine phosphokinase, globulin, glucose, phosphate, potassium, sodium, total protein, and lactate dehydrogenase were measured using an Olympus AU5400 analyzer. Plasma from Vacutainers was transferred to cryovials, stored at -80°C and submitted to the Cooperative Oxford Laboratory (COL) for hormone and vitellogenin analysis.

### *Laparoscopy*

Once anesthetized, each fish was positioned in lateral recumbency on the anesthesia machine. A 5-mm incision was made in the ventral body wall slightly off midline approximately 1/3 of the way from the cloaca to the pectoral girdle. A 5 mm Endotip Cannula (Karl Storz Veterinary Endoscopy, Inc.) was then inserted through the incision in the skin. The body cavity was insufflated with ambient air by attaching the pressure port of a pipette pump (Bellco Glass, Model 1225-80003) to the insufflation port of the Cannula with air-line tubing. Pressurized ambient air from the pump was added until the body cavity was visibly distended without resulting in a taut ventral body wall. A 5 mm rigid laparoscope (Karl Storz Veterinary Endoscopy, Inc., Model Hopkins II) was then inserted through the cannula to allow visualization of the internal anatomy. To enhance visualization a digital video camera (JWIN Electronics Corporation, Model JV-AC820) was mounted to the eyepiece of the laparoscope and the image was projected to an external monitor (Inducomp Corporation, Model 12" RAINdrop™) mounted to the anesthesia machine.

In some fish the swim bladder was deflated to allow visualization of the internal anatomy. To accomplish this, a Veress Pneumoperitoneum needle (Karl Storz Veterinary Endoscopy, Inc.) was inserted through the ventral body wall, on the lateral aspect of the body between ventral scutes, approximately 12-15 cm cranial to the insertion of the cannula. Once inside the coelom, the needle is inserted into the swim bladder using the video image as a guide and suction is applied to the needle vacuum port from the pipette pump until the swim bladder collapsed within the coelomic cavity.

A general visual assessment of peritoneum, swim bladder, spleen, liver, intestine and gonad was made for each animal. In addition, gonad was assessed by palpation with the blunt tip of the Veress needle, to determine sex and reproductive status of the animal. In those instances where the sex of the animal could not be determined by visual assessment or palpation a biopsy of the gonad was collected using a 5 mm biopsy forceps (Karl Storz Veterinary Endoscopy, Inc.) for histological examination. An incision was made 24-30 cm cranial from the insertion of the first cannula and a second cannula was inserted through the incision. The biopsy forceps was inserted through the cannula and guided to the gonad using the video image, and a biopsy was collected from the gonad and placed in 10% Neutral Buffered Formalin.

Following completion of procedures the laparoscope, cannulas and Veress needle were removed from the body and each incision was closed with a single suture in a cruciate pattern using PDS II, size 0, suture material (Ethicon, Inc.). Betadine ointment was applied to each suture, incision and skin immediately surrounding the incision.

### *Determination of hematologic and plasma chemistry reference intervals*

Shortnose sturgeon used for reference intervals were sexually immature progeny of Savannah River brood stock housed at the Bears Bluff National Fish Hatchery, Wadmalaw Island, South Carolina. First-generation progeny (1988 and 1989 year classes) ranged in Total Length (TL) from 803 to 1043 and from 3.8 to 7.5 Kg in weight. Second-generation progeny (2002 year class) ranged from 444 to 610 mm in TL and from 0.4 to 1.5 Kg in weight. Fish were housed in outdoor, covered, fiberglass tanks with flow-through well water. Well-water was treated with a low-head oxygenator to increase dissolved oxygen and lower carbon dioxide concentrations prior to outflow into holding tanks. Fish were fed daily with a commercial pelleted diet (38% protein, 10% fat; Melick Aquafeeds Inc., Catawissa, PA). Blood was collected for this study in January, 2008. Salinity was measured at 1.8, and did not vary during January prior to this study. Water temperature was 20° C and dissolved oxygen was at or above 6.2 mg/L in all holding tanks.

Individual fish were anesthetized in MS-222 (see *anesthesia*), transferred to an anesthesia machine, and 5-ml of blood was collected (see *blood collection*). Samples from a total of 61 fish (26 male, 35 female) were processed for

submission to Antech Diagnostics. Following completion of sampling, fish were euthanized and necropsied to determine sex.

Hematology and plasma chemistry reference intervals were determined according to methods established by the National Committee for Clinical Laboratory Standards (NCCLS, 2<sup>nd</sup> Edition, 2000). Data from all fish were pooled within each hematologic and plasma chemistry category and all values that were greater than 1/3 of the total range of that category were considered outliers and were discarded. Remaining values were ranked and remaining values that were outside the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentile were discarded. The range of the remaining values was used as the sex-independent reference interval. Data was then pooled, by sex, for each category, and a separate reference interval determined for male and female fish.

Mean values for each reference interval were tested to determine gender-based differences. For each category, normality of distribution was determined by the Wilks–Shapiro test and homogeneity of variances was determined by Bartlett's test (Davis, 2007). Data failing these tests were log transformed or square root transformed and transformed data were retested. For transformed and non-transformed data determined to be normally distributed and to have homogeneous variances, a t-test was used to compare results for gender. Data not normally distributed or with heterogeneous variances after transformation were analyzed with the Kruskal–Wallis and Median one-way nonparametric procedures (Davis, 2007). Differences were considered significant at  $P < 0.05$ .

### *Skin and biopsy healing*

To determine the healing rates of skin incisions and biopsied gonads, anesthetic and laparoscopy procedures were performed on captive Atlantic sturgeon (*Acipenser oxyrhincus*) maintained at the University of Maryland Center of Environmental Science, Horn Point Laboratory, Cambridge, Maryland. Although there are differences in the thickness and structure of the integument between Atlantic and shortnose sturgeon, the captive population of Atlantic sturgeon contained individuals of similar size to adult shortnose sturgeon, was readily accessible to MD DNR staff, and could be observed regularly for months.

The Atlantic sturgeon maintained at Horn Point Lab consisted of fish that were captured incidentally by commercial fishermen in the Chesapeake Bay and transferred to MD DNR as part of a Reward Program. Fish were maintained in indoor, round, 2.7 m diameter fiberglass tanks containing flow-through river water from the Choptank River, Maryland. Fish were fed frozen natural foods and transitioned to a commercial pelleted diet.

Anesthetic and Laparoscopy procedures were performed on nineteen sub-adult Atlantic sturgeon. Either one or two cannulas and a Veress needle were installed in each fish. A single biopsy was collected from the gonad from 2 sub-adult male fish, 2 sub-adult female fish, and 2 juvenile fish of undetermined sex. A single suture was used to close each incision, using a cruciate pattern for each cannula incision and a simple pattern for each Veress incision. Betadine ointment was applied to each suture, incision and skin immediately surrounding each incision. Fish were re-examined periodically for a year. Gonads from the 6 fish that were biopsied were re-examined laparoscopically 30 days after biopsies were collected and condition of biopsy locations were observed.

### *Comparison of anesthetic protocols*

The efficacy of Metomidate Hydrochloride (Aquacalm<sup>®</sup>, Syndel Laboratories Ltd., Qualicum Beach, BC, Canada) as an anesthetic suitable for use in laparoscopy was compared to MS-222 in sub-adult and juvenile Shortnose sturgeon maintained at Bears Bluff National Fish Hatchery. Fish used in this study consisted of two groups of sexually immature progeny of Savannah River brood stock. One group of 24 fish was first-generation progeny (1988 and 1989 year classes) ranging from 803 to 1043 mm in total length (TL) and from 3.8 to 7.5 Kg in weight. A second group of 81 fish was second-generation progeny (2002 year class) ranging from 444 to 610 mm in TL and from 0.4 to 1.5 Kg in weight.

Three anesthetic treatments were compared: 1) induction with 250 mg/L MS-222 and maintenance with 85 mg/L MS-222, 2) induction with 15 mg/L metomidate and maintenance with 8 mg/L Metomidate; and induction with 15 mg/L Metomidate and maintenance with 85 mg/L MS-222. Eight sub-adult fish and 27 juvenile fish were assigned to each treatment. Fish selected for treatment 1 were induced with MS-222 as described above and time of induction was recorded. Fish selected for treatments 2 or 3 were induced by immersion of the head in a solution of 15 mg/L Metomidate and circulating that solution over the gills with a submersible pump and vinyl tube, and time of induction was recorded. Once induced, fish were weighed and measured and transferred to one of three anesthesia machines, identical in design, containing a 60-L solution of well water and a maintenance dosage of either MS-222 or Metomidate.

Once on the anesthesia machines, laparoscopy was simulated, blood samples were collected and observed morbidity was recorded. Ventilation rates of fish were measured for 1 min after the first min and again after the ninth min on the anesthesia machines. Ventilation rate was the number of times the operculum opened and closed,

either fully or partially, during a 1-min period. A 5-ml blood sample was collected within the first 2 min on the anesthesia machine. After the 5<sup>th</sup> minute on the anesthesia machine, an Endotip cannula was installed and the coelom was insufflated. After the 10<sup>th</sup> min on the anesthesia machine, the cannula was removed and the incision closed. The fish was recovered in fresh water by assisted ventilation and time to recover from anesthesia was recorded. Following all anesthetic trials each fish was euthanized for necropsy and sex determination.

#### *Assessments of Delaware River fish*

Shortnose sturgeon were collected over 1 or 2 days in the spring and fall, 2006-2008. Adult shortnose sturgeon were collected using anchored bottom-set gill nets deployed at two locations (Duck Island Range and Biles Island Channel) in the upper tidal Delaware River, in the vicinity of Bordentown, New Jersey. Two nets were set parallel to the current at navigation channel depth at each location. The nets were 100 m long by 1.8 m deep and consisted of either 12.7 or 15.2 cm stretched monofilament mesh. Captured fish were transported in a holding tank with oxygenated river water to an on-shore location where procedures could be performed. Water samples were collected from fish capture locations, frozen and submitted to Cooperative Oxford Laboratory.

Each sturgeon was measured for fork (FL) and total length (TL), weighed, and tagged with a numbered T-bar tag (Floy Tag and Manufacturing, Inc.) and a Passive Integrated Transponder (PIT) tag. The PIT tags were Destron 14 mm tags, activated at a radio frequency of 125 kHz. Laparoscopy was performed on each fish and blood samples collected and processed for submission to Antech Diagnostics and the Cooperative Oxford Laboratory. Fish were recovered from anesthesia, transported to the capture site and released after a brief observation period. All sampling and handling of shortnose sturgeon was performed in accordance with Permit to Take Endangered Species No. 1486-2 issued by the National Marine Fisheries Service (NMFS).

#### *Assessments of Cooper River fish*

Twelve adult Shortnose sturgeon were collected annually, over 3 days in February, in 2007 and over 4 days in 2008. Fish were collected using anchored, experimental multi-filament gill nets with six 50-foot panels of alternating mesh sizes (5, 7, and 10"). Nets were deployed below Pinopolis Lock and Dam, Cooper River, South Carolina. Captured fish were transported to an on-shore location in a holding tank with oxygenated river water. Water samples were collected, frozen and submitted to Cooperative Oxford Laboratory for analysis.

Each sturgeon was measured (FL and TL), weighed, and PIT tagged. Laparoscopy was performed on each fish and blood samples collected and processed for submission to Antech Diagnostics and the Cooperative Oxford Laboratory. Once recovered, fish were released at the capture site. All sampling and handling of shortnose sturgeon was performed in accordance with Permit to Take Endangered Species No. 1505 issued by NMFS.

#### *Plasma hormone assay*

Testosterone (T) and 17 $\beta$ -estradiol (E2) were measured from plasma samples using competitive enzyme immunoassays specific to each steroid (Cayman Chemical). Plasma samples were extracted using diethyl-ether to separate T and E2 from any binding proteins. Five ml of diethyl-ether was added to 0.5 ml plasma and vortexed. The steroid-containing supernatant was removed and transferred to a clean glass test tube. A second extraction was performed on the plasma sample by adding an additional 5 ml of diethyl-ether, the sample was vortexed, the supernatant was transferred to the test tube containing the first supernatant and the sample was stored at -80° C until analysis could be performed. Samples were placed in a 30°C water bath and the Diethyl-ether was evaporated leaving the steroid residue in the test tube. This residue was reconstituted in 0.5 ml buffer provided by Cayman Chemical for use in immunoassays.

Extraction efficiency was determined by spiking replicate ultra-pure water samples with a known concentration of either T or E2, and extracting those samples with diethyl-ether. An average proportion of recovery was determined for T and E2 and these values were applied to derived T and E2 data to correct sample steroid values for efficiency of extraction.

Samples were assayed for T and E2 according to directions provided by Cayman Chemicals. For each steroid, a serial dilution of the steroid standard was prepared and each transferred to a microtiter plate in duplicate. Samples were diluted with ultrapure water, 1:20 for E2 and 1:1000 for T, and diluted and non-diluted samples were transferred to microtiter plates in triplicate. AChE-tracer and antiserum specific for each assay were added to the samples and steroid standards and the microtiter plates were incubated on an orbital shaker, 2 hours for T and 1 hour for E2. Plates were washed 5 times with a wash buffer (provided by Cayman Chemicals) following incubation and microtiter plates were developed with Ellman's reagent until the B<sub>0</sub> wells were >0.3 Absorbance Units (blank subtracted). Plates were read on a spectrophotometer at a wavelength of 410 nm. A standard curve of each steroid was used to calculate steroid concentrations in the unknown samples.

The reported sensitivity of T was 32 pg/ml at a %B/B<sub>0</sub> of 50%, and 6 pg/ml at a %B/B<sub>0</sub> of 80%. The sensitivity of E2 was 129 pg/ml at a %B/B<sub>0</sub> of 50%, and 19 pg/ml at a %B/B<sub>0</sub> of 80%. The cross reactivities of the antibodies used in this assay with other similar steroids is reportedly ≤27.4% for T and ≤14% for E2.

#### *Total estrogens assay*

Total estrogens (ES) consisting of Estrone (E1), 17β-estradiol (E2) and Estriol (E3) were measured from water samples using a competitive enzyme immunoassay (Japan EnviroChemicals, Ltd). Water samples were filtered with glass-fiber filters (1 μm pore diameter) and ES were concentrated 1000-fold using following solid phase extraction (SPE) procedures provided by Japan EnviroChemicals, Ltd. Pretreated samples were filtered through C18 cartridges, cartridges were washed with distilled water and then hexane and analyte was eluted from the C18 cartridges into glass test tubes with dichloromethane. The dichloromethane was evaporated in a 40° C water bath in a nitrogen-enriched atmosphere. Once evaporated, 1 ml of 100% methanol was added to each tube and vortexed until the ES residue went into solution. The methanol-residue solution was filtered through an aminopropyl cartridge and the filtrate was collected in a glass test tube. The aminopropyl cartridge was then rinsed with 5 ml of 100% methanol, combining the filtrate with the previously obtained filtrate, the methanol was evaporated and the residue was reconstituted in 10% methanol.

Extraction efficiency was determined by spiking replicate ultra-pure water samples with a known concentration of E2, filtering the water through glass-fiber filters and concentrating E2 into 10% methanol using C18 and aminopropyl SPE. An average proportion of recovery was determined for E2 and these values were applied to derived ES data to correct sample steroid values for efficiency of extraction.

Water samples were assayed for ES according to directions provided by Japan EnviroChemicals Ltd. E2 standards and samples were transferred to a microtiter plate in duplicate and triplicate respectively. Enzyme-antigen conjugate solution was added to standards and samples and the microtiter plate was incubated for 1 hour on an orbital shaker. Plates were washed 3 times, developed with color solution for 30 min, and the stop solution was added to terminate the reaction. Plates were read on a spectrophotometer at a wavelength of 450 nm. A standard curve was used to calculate total concentration of ES in the unknown samples.

The reported sensitivity for the ES assay is 300 ng/L at a %B/B<sub>0</sub> of 50%, and 90 ng/L at a %B/B<sub>0</sub> of 80%. The cross reactivities of the antibodies used in this assay with other similar steroids is reportedly ≤0.03%.

#### *Vitellogenin*

Measurement of vitellogenin (VTG) concentration was attempted by electrophoresis and differential staining of the protein (Van Veld et al., 2005). Purified VTG from multiple sources was loaded into pre-electrophoresed, discontinuous, 8% polyacrylamide SDS-Page gels. Gels were run from 45 to 60 min to separate VTG from other plasma protein bands. Once run, the gels were fixed overnight in 50% methanol and 10% acetic acid in ultrapure water and rinsed 3 times. Gels were stained with Pro-Q Diamond® stain (Invitrogen) for 90-180 min and destained with Pro-Q Diamond destaining solution for 120-240 min. Gels were imaged with a UV transillumination system at variable wavelengths. Separation and identification of known VTG bands was not achieved. Therefore plasma samples were not assayed by this technique.

## **Results**

#### *Anesthesia*

Time to induce surgical level anesthesia in fish ranged from 1 min 30 sec to 9 min 35 sec. Most fish exhibited a brief excitement phase, lasting no longer than 10 sec, approximately 30 sec to 2 min after being immersed in the induction solution. Following the excitement phase, fish lost equilibrium causing them to roll into lateral or dorsal recumbency in the induction tank. After equilibrium was lost ventilation rate and fin and body movements decreased. Once fin and body movements ceased, fish did not respond to tactile stimuli along the tail and fins, and ventilation rate was typically around 2 cycles/min. At this point, fish were considered to be in stage-4 level of anesthesia. Excitement phase was not observed in all fish. These fish began losing equilibrium soon after immersion in the induction tank and generally reached stage-4 level anesthesia sooner than fish that experienced excitement phase, but this was not always the case.

Time to induce stage-4 level anesthesia increased with increasing weight of fish and decreased with increasing water temperature (Fig. 3). Mean time to induce anesthesia in fish of similar TL and weight was 6 min 20 sec at 10° C, and 3 min 56 sec at 22° C. Recovery time from anesthesia increased with increasing weight of fish and decreased as water temperature increased (Fig. 4). Greater variability was evident in recovery times than was seen in induction

times for each water temperature. This was likely the result of variation in total time under anesthesia for each temperature, and variability in the fish's physiological response to the anesthetic.

During the maintenance phase of anesthesia (85 mg/L MS-222), ventilation rate gradually increased, and in a few fish resulted in hyperventilation. In some fish, the operculum did not fully open and close during a ventilation cycle. In these fish, either the operculum opened partially or only the opercular margin moved during ventilation. Heart rate, when evident as body wall excursions, remained steady and did not appear to change dramatically during procedures. In some fish, mild to moderate erythema developed ventrally on the head and body while on the anesthesia machine. Pallor of the gills or fins, indicating hypoxia, was not evident in any fish. Rarely, a single or few localized mild contractions (muscle spasm) in the ventral body wall were observed when incising fish or when instruments were placed in the incision. In those cases, the muscle spasm was brief, lasting no longer than a couple of seconds.

### *Laparoscopy*

Laparoscopic examination of gonads was useful in identifying the sex and reproductive status of shortnose sturgeon. Gonads were assessed in fish ranging in size from 542 to 1191 mm TL. Gonads were identified as paired structures located laterally within the coelom, along either side of the intestine. Video imagery of gonads was sufficient to differentiate testis and ovary based on morphology, color, and texture, in most fish (Figs. 5 and 6). Moderate to well-developed testis was white in color, had a smooth texture, had a lobular organization and occasionally had little to no amounts of brown-colored melanin and yellow-colored adipose tissue on the outer surface. Less-developed testis had variable amounts of adipose tissue and little to no observable melanin. Size of testis tended to increase with increasing fish size, and amount of gonadal adipose tissue tended to decrease with increasing fish size, however this was not true in all fish.

Mature ovary was enlarged, had numerous large oocytes (white or tan in color) or eggs (brown to black in color) that were easily visible and had variable amounts of melanin on the surface. Moderately-developed ovary had a granular to nodular appearance and was light-pink to tan in color. Oocytes were visible in moderately developed ovaries and varied in number and size. Ovary appeared granular when oocytes were small, and nodular as oocytes increased in size. Less-developed ovary tended to have a smooth to lightly granular texture, was pink in color and occasionally had small amounts of adipose tissue on the surface. Lamella-like folds were evident on the surface of less-developed ovary. These folds were numerous, located in a transverse orientation to the long-axis of the gonad, and could be visualized or revealed by palpating the surface of the gonad with the blunt tip of the Veress needle.

Undeveloped gonad had a thin ribbon-like appearance, was yellow in color and had a smooth texture (Fig. 5). The germinal tissue of undeveloped gonad could not be visualized or directly palpated because it was completely enveloped in adipose tissue. A single, 5 mm biopsy was sufficient to obtain germinal tissue in all cases. Although undeveloped gonad was more likely found in smaller fish, TL of fish was not a good indicator of developmental state of gonad in fish from 675-800 mm TL.

Histological examination of undeveloped gonad revealed a thick outer layer of adipose tissue and a narrow, inner band of germinal tissue (Fig. 7). Testis, collected from visibly undifferentiated gonad, consisted of a cyst-like arrangement of spermatogonia with no to few clusters of spermatids within developing seminiferous tubules. Ovary, collected from a visibly undifferentiated gonad, contained numerous clusters of oogonia and stage I-IV oocytes of variable numbers and sizes within a connective tissue stroma. The germinal layer of testis and ovary from visibly undifferentiated gonad was surrounded by a thin tunica albuginea.

Additional internal organs were examined visually, including the intestine, peritoneum, liver, spleen, pancreas, and stomach. Inter-coelomic parasites or lesions were not observed in any of these fish.

### *Skin and biopsy healing*

Incision containing sutures showed rapid initial, superficial healing, followed by gradual, complete healing over a period between 6 months and 1 year (Fig. 8). Sutures implanted in skin of captive Atlantic sturgeon remained intact for approximately 1 to month following closure of incisions. During this time a re-growth of epidermis was evident over the incision and needle holes. At 1 month post-closure (PC), skin and subjacent tissue layers were contracted and the sutures were generally loose in the skin. At 3 months PC, a distinct erythematous region was usually evident immediately surrounding the incision and suture. At this time less suture material was visible externally in all fish; in some fish knots were still evident while in other fish only the end of a suture strand was visible. At 6 months PC, suture material was not evident externally in most fish and the region of erythema was greatly reduced or absent. Skin at the site of the incision appeared normal in some fish by this time, or had a thin line with variable amounts of pigment and redness. At 9 months PC, a faintly-pigmented line was evident on the

skin in some fish, while other fish had normal appearing skin at the site of the incision. By 1 year PC, the skin at the site of incisions appeared normal in the majority of the fish.

In two fish, a mass composed of fibrosis had formed over the incision and part of the suture material, within the first month PC and increased in size over the next 30-45 days. The masses were raised, had a rough to lumpy texture, reddened and were irregular in shape. The mass on one fish reached approximately 4 cm in diameter and depth before it sloughed and the mass on the other fish was approximately 1 cm in diameter and depth before it sloughed. Once the masses had sloughed, skin re-growth over the incision sites occurred within 1 week. The fibrotic mass appeared to be a superficial reaction only. No additional signs of inflammation were evident in these fish. Healing of incisions, once the masses sloughed, progressed in the same manner as in the other fish except that suture material was still evident in the skin at 6 months PC and a visible line indicating the location of the incision was still evident after 1 year. In both cases, sutures were tied too tightly at closure causing uneven closure of the incision and the skin to bunch or wrinkle immediately surrounding the incision.

Removal of suture material was not done in this study, as this would not be possible in wild fish. Inflammatory response in the skin at the incision site may have been exacerbated by friction of the suture material against the fiberglass tank bottom. Abrasions along the ventral scutes and body wall were evident on some captive fish. However, when working with captive fish, sutures may be removed after 2-4 weeks potentially increasing the rate of healing in the skin.

Re-examination of gonads from fish that were biopsied revealed that tissue healing and re-growth occurs rapidly. By 30 days following biopsy collections, no evidence of biopsy collection, inflammation or lesions were evident on testes and ovaries. Biopsy site was identified on undeveloped gonad in both fish, as an indentation of the visible adipose tissue. No inflammatory response or lesions were evident. Because the biopsy sites were covered in adipose tissue, germinal layer of gonads could not be observed.

#### *Comparison of anesthetic protocols*

Body movements observed in fish during induction with MS-222 were useful in determining the depth of anesthesia and consisted of coordinated fin and body movements for propulsion (i.e. swimming activity), ventilatory movements of the operculum, and localized fin and body movements in response to stimuli. Swimming activity and ventilatory movements of the operculum typically increased briefly during an excitement phase within the first minute or two of MS-222 induction, and then gradually declined until only a slow ventilation rate was observed. After swimming activity ceased, localized response to tactile stimuli in the fins and caudal aspect of the body decreased until no reaction to stimuli was evident. At this point in MS-222 induction, fish were considered to be at stage-4 anesthesia. In addition to these body movements, repeated and regular excursions of the ventral body wall between the pectoral girdle were evident in most fish as a result of heart activity.

In contrast to MS-222 induction, body movements were less useful in determining depth of anesthesia during metomidate induction. Swimming activity ceased almost immediately upon exposure to metomidate and no excitement phase was observed. There was a slight decline in ventilatory movements of the operculum during metomidate induction. Localized fin and body movements in response to tactile stimuli were also evident during metomidate induction, and the degree of response gradually decreased until no reaction to stimuli was evident. Following this point, increased time spent in the metomidate induction trough resulted in erythemia in some fish and in general did not affect ventilation rates. Although fish lost equilibrium and peripheral reflexes during metomidate induction, near-normal ventilation rates indicated that fish did not fully reach stage-4 anesthesia (Summerfelt and Smith, 1990). Body wall excursions indicating heart activity were also evident during metomidate induction, but were not useful in determining depth of anesthesia. Finally, body flexions were observed in some fish during metomidate induction. A body flexion was a brief, rapid, powerful contraction of one side of a fish's body, often causing the fish to flip. Flexions typically occurred as a single event and there was no apparent trend in the occurrence of flexions by body size or time of induction. Occurrence of flexions may indicate that metomidate induced fish failed to reach stage-4 anesthesia.

MS-222 had a more pronounced affect on depressing ventilation rate than metomidate. Immediately prior to induction, some fish were hyperventilating (>60 cycles/min) following capture, while the majority of fish appeared to be ventilating normally (45-60 cycles/min). Mean ventilation rate of fish decreased to 2 cycles/min during induction with MS-222 (Fig. 9). During induction with metomidate, fish that were hyperventilating prior to induction showed a slight decline in ventilation rate. Ventilation rates did not change when fish that were ventilating normally were induced with metomidate. Following induction with MS-222, mean ventilation rate increased to 60 cycles/min while fish were maintained for 9 min on the lower MS-222 dosage (85 mg/L). Following induction with metomidate, mean ventilation rate decreased slightly while fish were maintained for 9 min on the lower metomidate

dosage (8 mg/L), and mean ventilation rate increased slightly while fish were maintained for 9 min on maintenance dose of MS-222.

Mean time of induction was significantly longer with MS-222 than with metomidate (Fig. 9). However, induction times were probably biased because general fin and body movements ceased almost immediately upon exposure to metomidate. Shorter induction times are preferred to reduce stresses associated with induction and the mean induction time of 4.1 for MS-222 is well within suggested guidelines for fish anesthetics (Marking and Meyer, 1985).

Fish recovered almost 3 times faster from MS-222 anesthesia than from metomidate anesthesia (Fig. 9). Mean recovery time of fish anesthetized with the combined treatment of metomidate induction and MS-222 maintenance was significantly lower than metomidate-only anesthesia and significantly higher than the MS-222-only anesthesia.

Morbidity, which is the development of physiological or anatomical lesions, occurred more frequently and often in greater severity in fish in the metomidate-only treatment and combination treatment than in the MS-222 treatment (Fig. 9). Some fish in all treatments developed erythema ventrally along the body or head after induction was initiated or after fish were transferred to the anesthesia machine. However, erythema occurred more frequently and the area and intensity of redness on the skin was typically greater in metomidate induced fish. Shallow ventilation, which was the failure of the operculum to fully open or close during a ventilation cycle, occurred most frequently in fish anesthetized entirely with metomidate, and least frequently in fish anesthetized entirely with MS-222. In some fish anesthetized entirely with metomidate, opercular movement was only detected at the caudal margin of the operculum, although the ventilation rate was at near-normal levels. Movements of the body wall immediately adjacent to the incision were observed frequently in metomidate induced fish when the cannula was inserting into the skin. This reflex response to cannula installation, which was rare in MS-222 induced fish, may indicate that metomidate has reduced analgesic properties compared to MS-222. Less fin movement, fewer body flexions, fewer episodes of muscle spasms, and fewer occurrences of shallow ventilation were observed in fish anesthetized with the combined treatment compared to the metomidate-only treatment.

Signs of morbidity observed in metomidate induced fish that were not observed in MS-222 induced fish included muscle spasms and cardiac arrhythmias. Muscle spasms consisted of episodes of repeating, mild contractions of muscle in the left lateral body wall near the incision, each episode lasting from a few seconds to more than a minute. Muscle spasms typically occurred after fish were placed on the anesthesia machine and were more commonly observed in fish anesthetized entirely with metomidate. Cardiac arrhythmias were evident as brief episodes of decreased or erratic excursions of the ventral body wall. Each episode lasted no longer than 20 sec and no more than 3 episodes were observed in any fish and in some fish, arrhythmic episodes were accompanied by brief periods with no apparent heart activity. Heart rates could not be determined in every fish. Body-wall excursions were not observed in few fish.

In general, metomidate was not as effective in anesthetizing fish, resulted in increased types and severity of morbidity, and required longer recovery times than MS-222. Few benefits were evident from the use of a combined anesthetic treatment designed to reduce induction times (metomidate induction) while providing analgesia (MS-222 maintenance), while cost and complexity increased. While apparent induction times were lower with metomidate, those fish were probably not at surgical level of anesthesia. Body movements of fish during surgical procedures increase the risk of trauma to organs or major blood vessels. Recovery times of fish from metomidate are longer than from MS-222, requiring increased resuscitation and handling of fish. Finally, morbidity was greater in fish during metomidate anesthesia, indicating increase adverse physiological reaction to metomidate compared to MS-222.

#### *Determination of hormone, hematologic, and plasma chemistry reference intervals*

Sex ratio of fish used in this study was 1:1.4 females to males for F1 progeny and 1:1.1 females to males for F2 progeny. No gross signs of disease were evident in fish when necropsied. Reference intervals, mean, median and standard deviation are presented for each hematologic and plasma chemistry category (Fig. 12). Mean values for male and female fish are presented in Figs. 13 and 14.

White blood cell types identified in this study included heterophils, lymphocytes, monocytes, and eosinophils. Cells identified as heterophils had multi-lobed nuclei and faint pink cytoplasm with few granules. Neutrophils were differentiated from heterophils by having more nuclear lobes and less cytoplasmic granulation. In addition, the nuclei of neutrophils were more likely to be segmented than in heterophils. Neutrophils and heterophils were approximately the same size and were round to ovoid. Knowles et al. (2006) did not describe heterophils, which are usually described from avian species, as a blood-cell type. Heterophils and neutrophils are closely related morphologically. Variation in neutrophil appearance and lack of piscine hematologic experience by clinicians at Antech Diagnostics most likely accounted for the inclusion of heterophils as a cell-type in their reports.

Lymphocytes were variable in size and contained large, dark-blue, round nuclei with little visible cytoplasm that often contained pseudopodia. Monocytes were large cells with abundant, dark-blue cytoplasm that was usually vacuolated. Eosinophils were similar in size to neutrophils, the cytoplasm contained numerous eosinophilic granules, and the nucleus was multi-lobed.

Reference intervals for total white blood cells are given in Fig. 12. Values obtained in this study for total white blood cells and each cell-type is approximately an order of magnitude lower than reference intervals reported by Knowles et al. However, different quantitative techniques were used in these studies. Knowles et al. counted white blood cells directly using Natt-Herrick's solution as a diluent stain, while Antech Diagnostics used a modified count following an eosinophilic stain. Differences in these techniques account for the different results obtained. However, the purpose of this study was to provide reference intervals using a large, widely-available diagnostic laboratory where samples from any population can be submitted to for analyses. The reference intervals reported here are useful for interpreting results obtained from Antech Diagnostics (or other laboratory using similar techniques). Differential cell counts require specialized training and equipment and are not practical when assessing fish in remote locations. Collection and submission of blood samples to a diagnostic laboratory such as Antech Diagnostics require less training, equipment and time. Also, results obtained from multiple populations can be directly compared when using a common diagnostic laboratory.

Plasma chemistry reference intervals are given in Fig. 12, and are generally in close agreement to intervals reported by Knowles et al. Two intervals that differed markedly between this study and Knowles et al. however, were aspartate aminotransferase (AST) and glucose. AST interval was 2-3 times higher in this study and could have been induced in-vitro during sample processing. AST release from red blood cells can be triggered with little hemolysis, which can occur during sample processing. A wider glucose interval was obtained in this study than that of Knowles et al. Glucose measurement can be affected by a number of factors including the dietary state of the animal, if the animals are excited before sample collection, and as handling time of blood increases. Although the interval obtained in this study was wider, mean and median values were similar in both studies. Because the animals used in both studies were on standard diets, the relatively high standard deviation reported here for glucose suggests greater variation in handling and processing of samples in this study, rather than increased variation in dietary state of the fish.

Testosterone (T) concentrations increased in the plasma of male fish, from 153 ng/ml in July, to 729 ng/ml in February, while female fish showed no increase over this period (Fig. 15). Estradiol (E2) concentrations increased in the plasma of female fish, from 8.2 ng/ml in July, to 34 ng/ml in February (Fig. 15). A slight increase in E2 occurred in male fish, although the increase was not significant. T and E2 concentrations and E2:T ratios were not measured in the spring because spawning protocols prevented the collection of blood samples.

#### *Assessments of Delaware River fish*

Fish were collected from a total of two sites in the Delaware River, in the vicinity of Bordentown, New Jersey (Fig. 17), over a period of 1 or 2 days in November, 2006, May and November 2007, and June, 2008 (Fig. 18). Mean weight of fish captured in the fall, 2006, was larger than any other sampling period, even though fish examined in the spring, 2008 were longer in TL and fish examined in the spring, 2007 did not differ significantly in TL. Fish collected in each sampling period generally appeared healthy externally, although minor lesions were noted infrequently from this population, including abrasions along the ventral scutes and missing barbels. Moderate erosion of the pectoral fin and small, reddened, multi-focal ulcers were observed around the eyes of a single fish.

Sex was determined by visual assessment of the gonad, through the laparoscope, in 56 of the 62 fish examined. A complete or nearly complete layer of adipose tissue covered the germinal tissue layer of the gonad in the remaining 6 fish, and therefore sex could not be determined by visual assessment or by palpation of the gonad. Histological examination of gonadal biopsies from these 6 fish indicated that four were immature males and the remaining 2 fish were immature females.

A greater proportion of the fish examined in the fall of each year were male (Fig. 18). The sex ratio (male:female) of all fish collected in the fall of both years was 8.5:1. The sex ratio of fish examined in the spring decreased from 1.2:1 in 2007 to 0.6:1 in 2008 (Fig. 18).

Reproductive state varied in the fish examined during each sampling period. Of the 3 female fish captured in the fall, 2 were gravid with large black eggs, while the third was a less-developed female with a pink, granular ovary. Males examined in the fall were typically mature, although some fish had smaller gonads with an incomplete layer of adipose tissue on the surface, and a few were immature with a complete layer of adipose tissue. The majority of females examined in the spring had well developed ovaries with numerous large, white oocytes, which gave the ovaries a nodular appearance, while few of the females were immature with pink, granular ovaries. Testis

from males examined in the spring was typically mature (enlarged and smooth white surface), although a few males were immature with a complete or nearly-complete layer of yellow adipose tissue on the surface.

Proficiency in performing laparoscopy on Delaware River fish increased from 2006-2008, which led to reduced handling time, time under anesthesia and decreased total time in captivity for fish. Average total time to assess fish, which was the time required to induce anesthesia, perform laparoscopy, and recover fish, decreased from 27 min in fall, 2006, to 19 min in spring, 2008. No fish died during procedures or while under observation, and all fish released appeared healthy and swam away unaided.

Seasonal differences in blood cell counts were noted within the Delaware River population as well as between Delaware River fish and reference intervals (Fig. 19). Total white blood cell counts were significantly lower in the spring and fall from the reference intervals. In addition there were a greater proportion of lymphocytes in both seasons. Decreased WBC production may reflect differences in environmental conditions between hatchery and Delaware River. It is also possible that decreased WBC counts in Delaware River fish indicates decreased immune response. Seasonal differences were also detected in most plasma chemistry values in the Delaware population. However, fewer differences from the reference intervals were detected in the fall than in the spring. In general, a number of factors, such as differences in diet and localized water chemistry, may affect hematology and plasma chemistry leading to tributary and seasonal differences. A greater understanding of how these values change in different conditions, in sturgeon, is necessary. However, there is an increasing awareness of hematologic and plasma chemistry response to disease in fish, and a comparison of values obtained in this study will be useful in the event of potential epizootics or other sub-lethal health conditions.

Concentration of T increased in male fish, from 147 ng/ml in the spring, to 377 ng/ml in the fall (Fig. 23). In female fish, T concentrations decreased from 21 ng/ml to 3 ng/ml over this period. E2 concentration in male fish did not change from spring to fall. E2 increased in female fish from 30 ng/ml to 62 ng/ml from spring to fall, but did not change in male fish between these seasons. T concentrations from Delaware River fish are similar to concentrations obtained from BBNFH fish. However, E2 concentrations from Delaware River fish were approximately 3.5 times greater in the spring and were more than 2 times greater in the fall, than BBNFH fish. Hormone concentrations were measured from Delaware River fish in May/June, while the closest reference concentration from BBNFH was obtained in July. The increased E2 measured in Delaware River fish in the spring may be the result of sampling fish closer to the spawning season. E2 concentrations should peak in the spring during spawning season and decline to a summer-time low. Peak E2 concentrations during spawning season are unknown. However, the higher E2 concentration measured from Delaware River fish in the fall cannot be explained by differences in the timing of sample collections. Differences in seasonal hormone concentrations may be expected between these populations as they reside in different geographical areas. However, E2 appears to be disproportionately higher than T in Delaware River fish than in BBNFH fish. E2 may be more variable than T in sturgeon, and the higher E2 concentrations may be explained by differences in natural environmental conditions. However, the increased E2 concentrations may have been stimulated by exposure to environmental estrogens or estrogen-mimicking contaminants in the Delaware River. If true, than exposure to these contaminants may depress seasonal T in male fish, and data do indicate that T was slightly lower in Delaware River males than in BBNFH males in both the spring and fall.

#### *Assessments of Cooper River fish*

A total of 12 fish were captured over a period of three days in February, 2007, and an additional 12 fish were captured over a period of four days in February, 2008. Fish collected in 2008 were greater in mean TL but smaller in mean weight than those collected in 2007 (Fig. 21). In 2007, no external or internal lesions were observed on any of the 12 fish examined. In 2008, one of the 12 fish examined had a laceration-like wound on the rostrum, and a second fish had a damaged operculum. Otherwise all fish appeared healthy and no internal lesions were observed.

Sex and reproductive state was determined by visual assessment of the gonad, through a laparoscope, in all fish. Gonads were well-developed or mature in all fish with little or no adipose tissue evident. Biopsies were not collected from any fish in 2007 and 2008. Sex ratio of fish assessed in the Cooper River changed from 2:1 (male:female) in 2007 to 0.5:1 in 2008. In 2007, 7 of the 8 female fish were gravid, while the eighth female had moderately developed ovaries with large white oocytes. All 4 male fish assessed in 2007 had large, well-developed testes. In 2008, all 4 female fish examined were gravid, and all 8 male fish had well-developed testes.

Differences in blood cell counts and plasma chemistry values were detected between Cooper River fish and BBNFH reference intervals (Fig. 22). WBCs were significantly reduced in numbers although the proportion of heterophils and lymphocytes did not differ. The majority of plasma chemistry values were significantly greater than reference values. Although reference and Cooper River fish reside in the same general latitude and were sampled

during the same season, differences in diet, water chemistry and other factors probably contributed to the observed differences.

Concentrations of T in males and females from the Cooper River were slightly higher than fish from BBNFH (Fig. 23). E2 concentrations were approximately 2 times greater in females from the Cooper River and were about the same in males in comparison to BBNFH fish. Although the Cooper River and BBNFH are geographically close to each other and samples were collected at the same time of year, differences in diet and environmental conditions, presence of estrogens or estrogenic mimicking contaminants in the Cooper River, or other factors, may explain the higher E2 concentrations in females from the Cooper River.

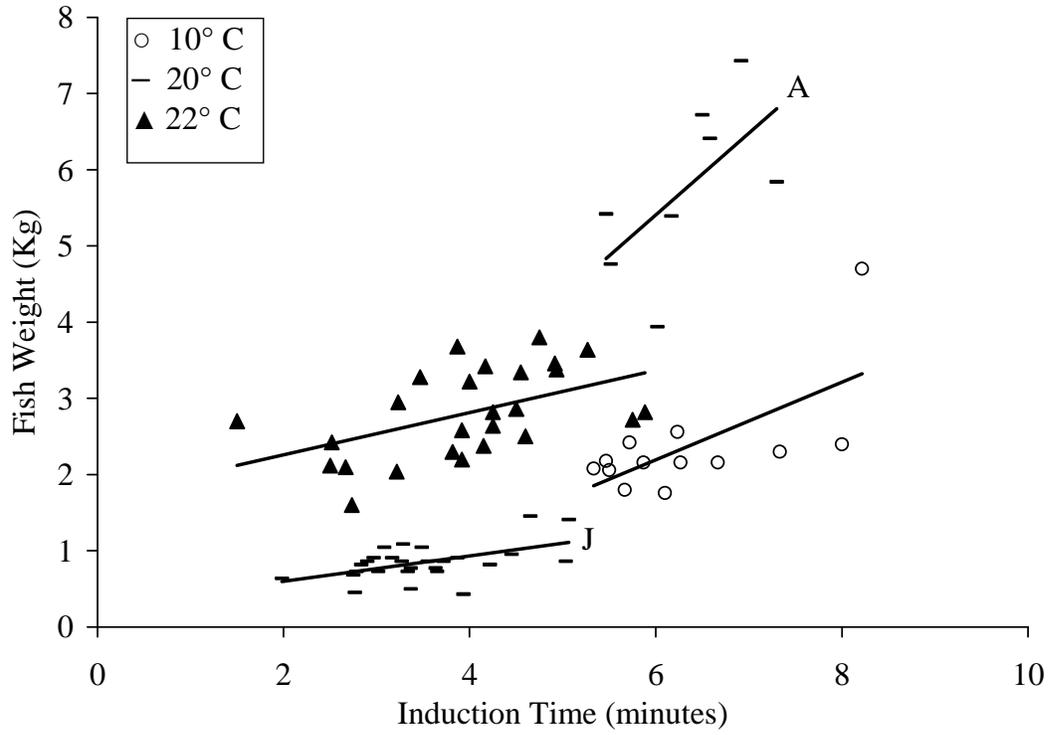
#### *Water assay for total estrogens*

Concentration of total estrogens did not exceed the minimum detection threshold of 80-300 ng/L in water samples collected from Bears Bluff National Fish Hatchery, the Delaware River, and the Cooper River. It is likely that water-borne concentrations diminish rapidly following release of estrogens as effluent. In addition, Extraction efficiencies using a solid phase extraction process were very low (<10%), which may have hindered detection of estrogens. Contamination of sediments may be a more important source and exposure route to benthic fish such as sturgeon. Future analysis of sediments at fish aggregation sites may provide more useful information on contaminant exposures.

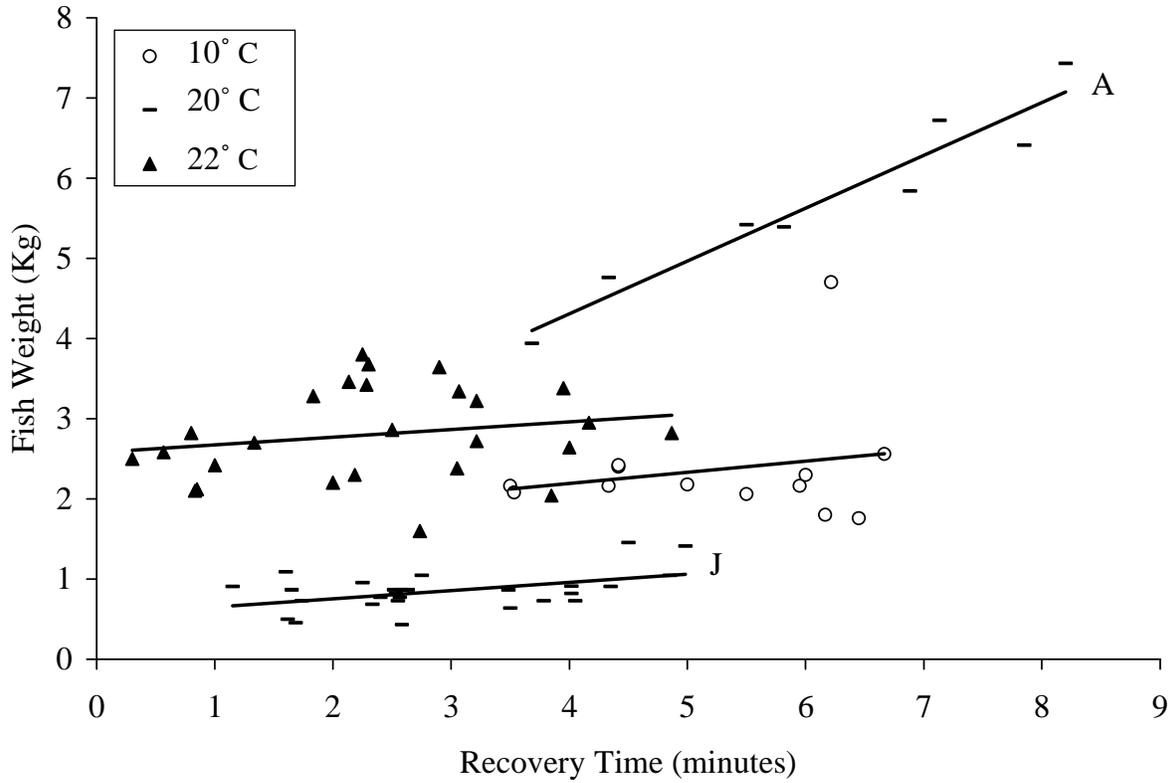
#### *Vitellogenin*

Vitellogenin expression in male fish may indicate exposure to environmental estrogens or estrogenic-mimicking compounds (Folmar et al., 1996). Application of electrophoresis and protein staining techniques to separate and quantify VTG were not successful. Distinct VTG bands, using positive controls, were not visible following a wide range of staining protocols and when transilluminated using a range of ultraviolet active wavelengths. Therefore samples collected from fish were not analyzed for VTG. Preserved plasma samples collected from fish from Delaware River, Cooper River and BBNFH may be analyzed for VTG in the future.

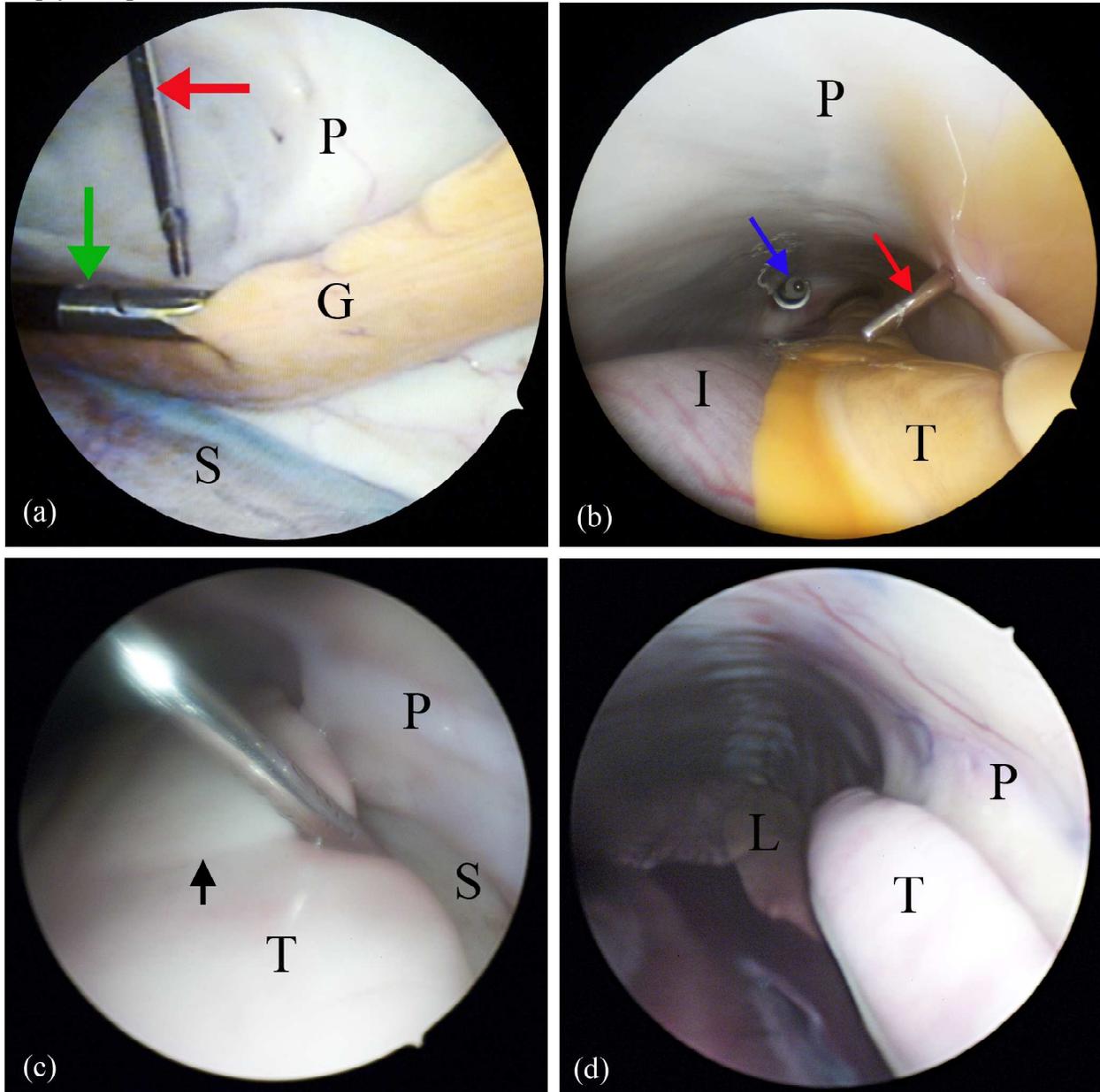
**Fig. 3: Times required to induce surgical level of anesthesia in Shortnose sturgeon. Anesthetic solution consisted of 250 mg/L MS-222 buffered with 500 mg/L sodium bicarbonate. Separate trend lines are shown for adult (A) and juveniles (J) induced at 20° C.**



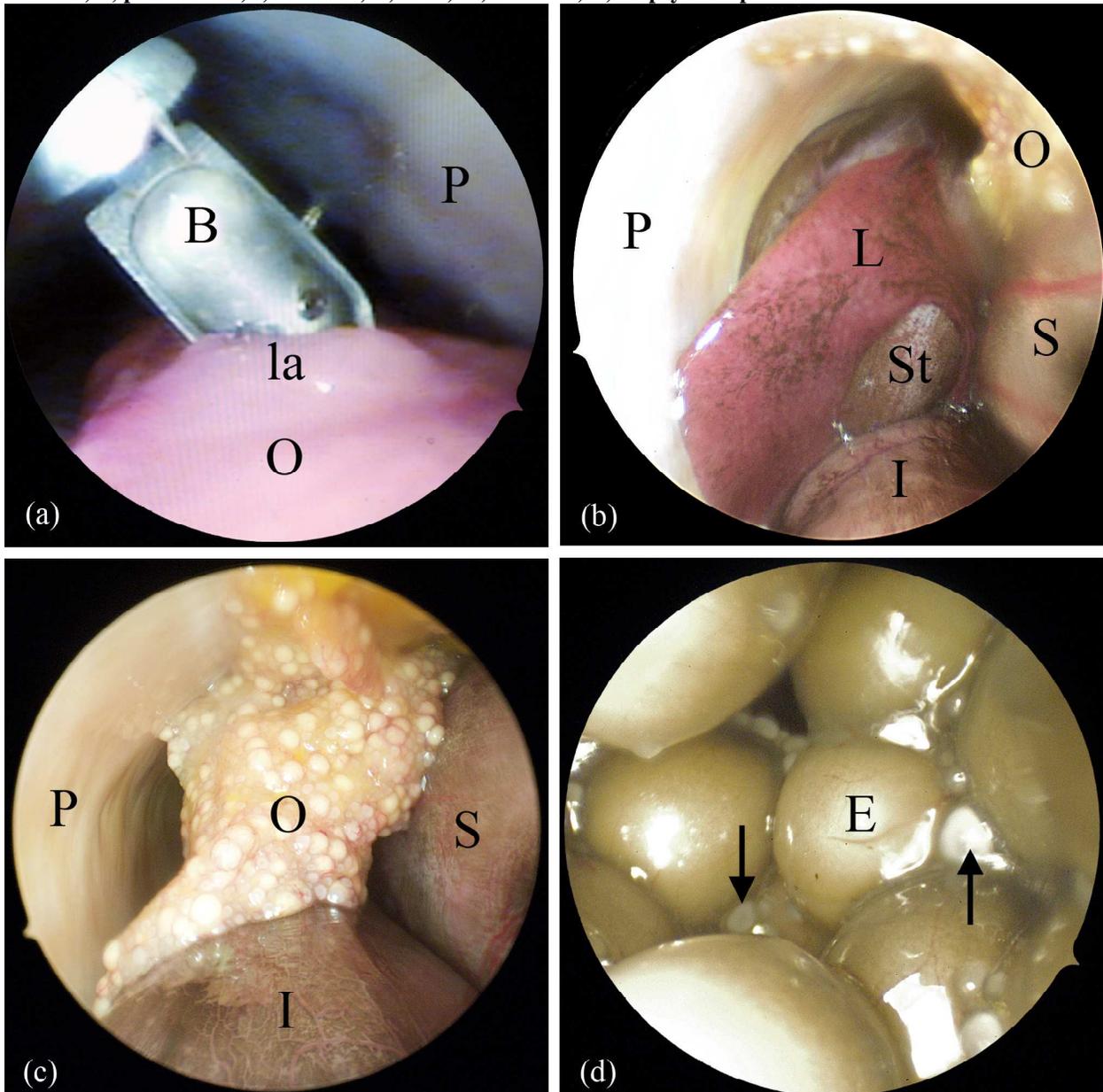
**Fig. 4:** Times required for fish to recover from surgical level of anesthesia in Shortnose sturgeon. Anesthetic solution consisted of 250 mg/L MS-222 buffered with 500 mg/L sodium bicarbonate. Separate trend lines are shown for adult (A) and juveniles (J) anesthetized at 20° C.



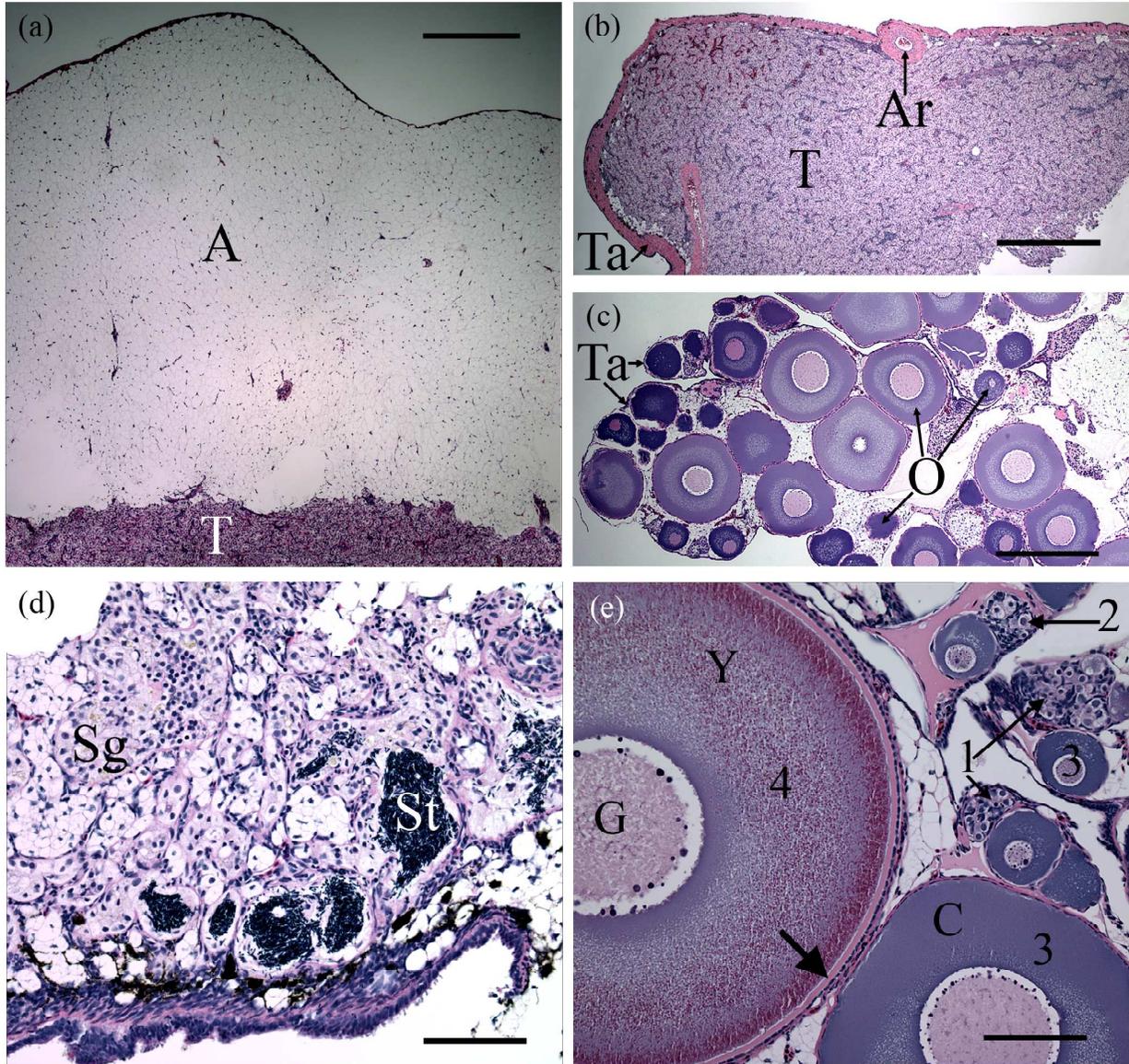
**Fig. 5. Laparoscopic images of gonads in shortnose sturgeon: (a) undeveloped gonad grasped with biopsy forceps (green arrow), (b) less-developed testis with incomplete layer of adipose tissue, (c) mature testis before swim bladder was deflated, and (d) mature testis after swim bladder was deflated. G, gonad; T, testis; S swim bladder; P, peritoneum; I intestine; L, liver; red arrow, Veress needle; blue arrow, laparoscope; green arrow, biopsy forceps; black arrow, testicular lobe**



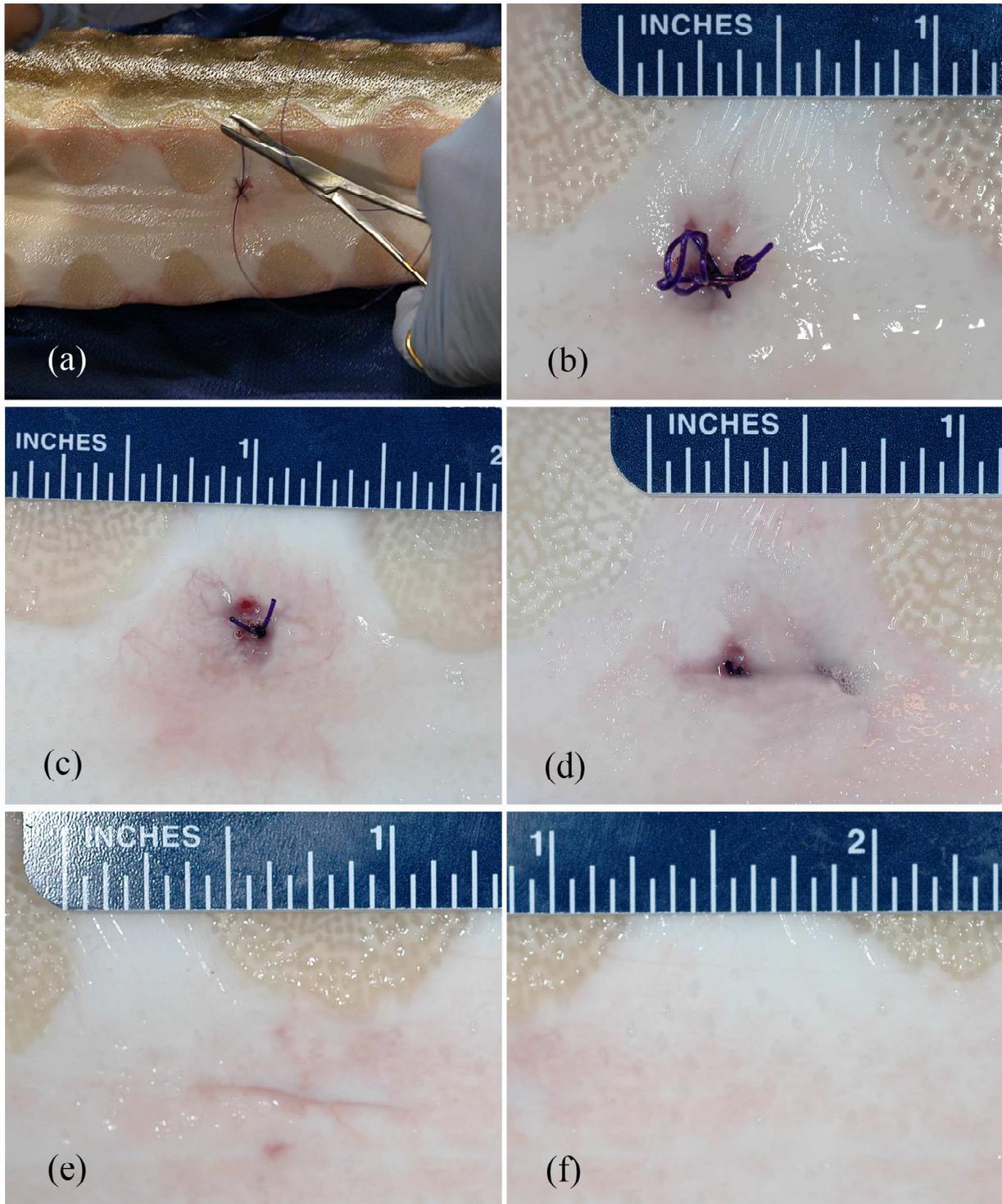
**Fig. 6. Laparoscopic images of gonads in shortnose sturgeon: (a) immature ovary with lamella-like fold (la) revealed, (b) moderately-developed ovary with swim bladder deflated, (c) moderately-developed ovary before swim bladder was deflated, and (d) mature ovary with oocytes (black arrows) and eggs (E). O, ovary; S, swim bladder; P, peritoneum; I, intestine; L, liver; St, stomach; B, biopsy forceps**



**Fig. 7:** Histological images of gonadal biopsies collected from shortnose sturgeon: (a) undeveloped gonad with prominent layer of adipose tissue (A) covering testis (scale bar = 750  $\mu$ m), (b) biopsy from testis (scale bar = 750  $\mu$ m), (c) biopsy from ovary (scale bar = 750  $\mu$ m), (d) testis with spermatogonia (Sp) in cyst-like arrangement and spermatids (St) within developing seminiferous tubules (scale bar = 150  $\mu$ m), and (e) ovary with stage 1-4 oocytes. Ar, Artery; Ta, tunica albuginea; G, germinal vesicle; Y, yolk vacuoles; C, cytoplasm



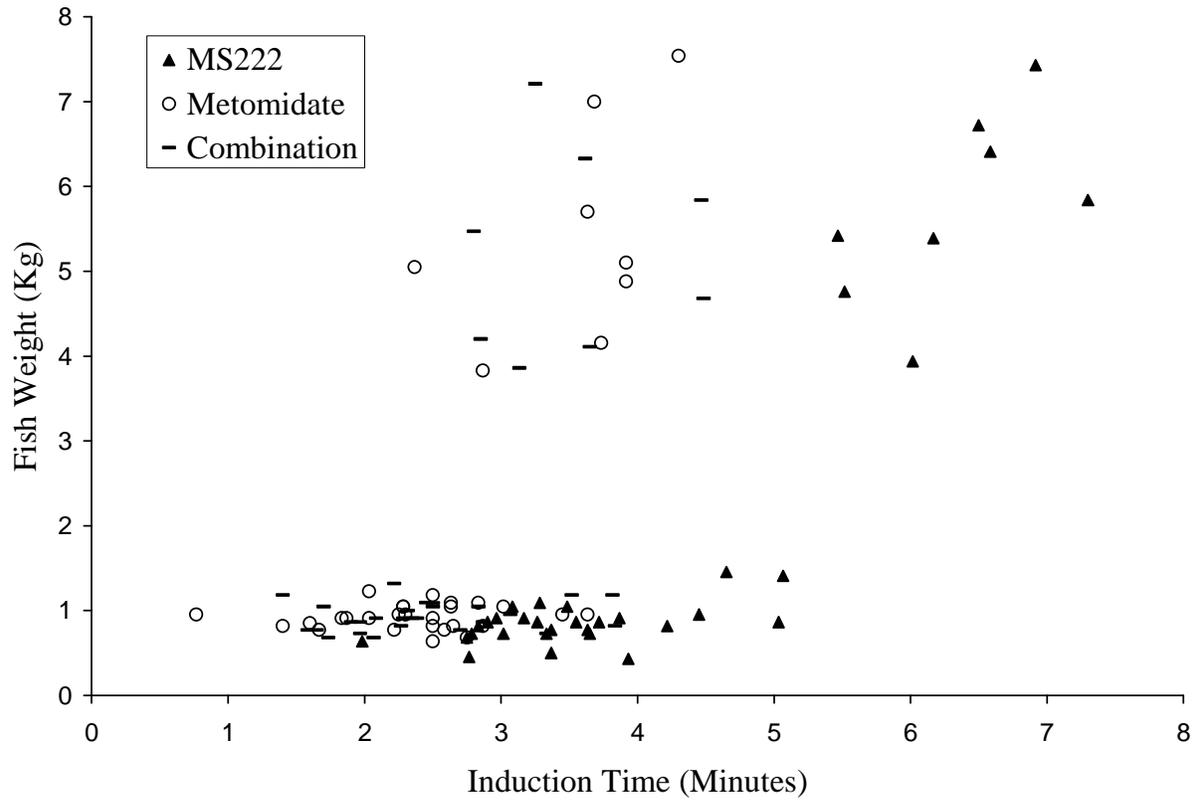
**Fig. 8:** Skin condition on the ventral body wall of Atlantic sturgeon after closing a full-thickness incision with PDS II suture material in a cruciate pattern: (a) tying knots, and (b) at 1 month, (c) at 3 months, (d) at 6 months, (e) at 9 months, and (f) at 12 months following closure



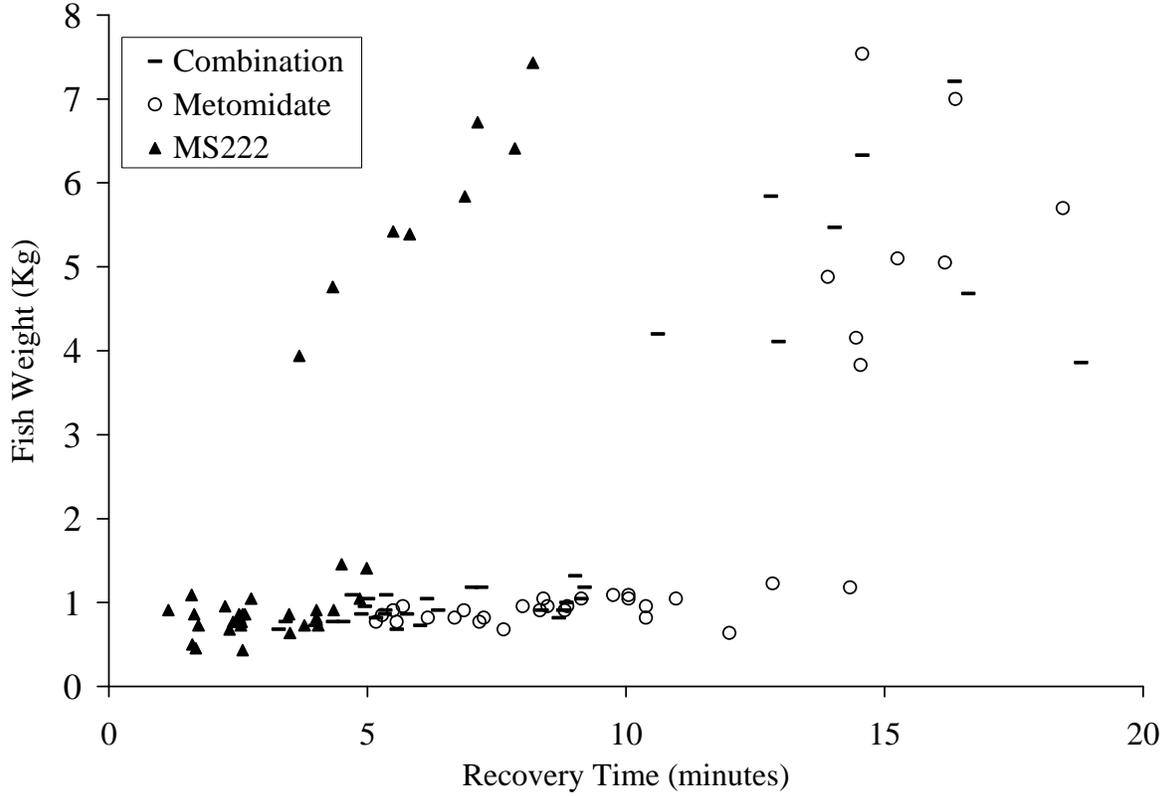
**Fig. 9: Comparison of anesthetic treatments in Shortnose sturgeon. Qualitative observations were recorded following induction and during a 10-min maintenance phase. Lesions or reflex responses were either not observed (-) or varied in frequency and/or intensity within each treatment from low (+) to high (+++++). Mean times or rates with a different letter indicate a significant difference ( $\alpha=0.05$ ). Water temperature was 20°C and flow rate of water over the gills containing the maintenance dose was 2.4-3 L/min in all treatments.**

Dosages	Anesthetic Treatment		
	MS-222	Metomidate	Metomidate Induction/ MS-222 Maintenance
Induction Dose	250 mg/L	15 mg/L	15 mg/L (metomidate)
Maintenance Dose	85 mg/L	8 mg/L	85 mg/L (MS-222)
Qualitative Observations			
Body flexion	-	++	+
Fin movement	-	+++	++
Reflex to instrument insertion	+	+++++	+++++
Muscle spasm	-	++	+
Cardiac arrhythmia	-	+	+
Erythema	+	++	++
Shallow ventilation	+	+++	++
Quantitative Observations			
Induction time (mean min $\pm$ SD)	4.1 $\pm$ 1.4a	2.6 $\pm$ 0.8b	2.7 $\pm$ 0.8b
Ventilation rates (mean $\pm$ SD)			
1 min post induction	2.0 $\pm$ 4.2a	53.1 $\pm$ 9.5b	54.7 $\pm$ 11.9b
9 min post induction	59.8 $\pm$ 11.8a	44.3 $\pm$ 10.0b	59.9 $\pm$ 7.5a
Recovery time (mean min $\pm$ SD)	3.7 $\pm$ 1.8a	10.1 $\pm$ 4.1b	8.1 $\pm$ 3.7c

**Fig. 10: Time required to induce surgical level anesthesia in fish at 20°C using MS-222 (250 mg/L), metomidate (15 mg/L), and a combination protocol involving induction with 15 mg/L Metomidate (Maintenance dose to follow at 85 mg/l MS-222).**



**Fig. 11: Time required for fish to recover from surgical level of anesthesia at 20°C using MS-222 (Induction dose = 250 mg/L, Maintenance dose = 85 mg/L), Metomidate 222 (Induction dose = 15 mg/L, Maintenance dose = 8 mg/L), and a combination of the two anesthetic drugs 222 (Induction dose = 15 mg/L Metomidate, Maintenance dose = 85 mg/l MS-222). Fish were maintained under anesthesia for a total of 10 min and laparoscopy was simulated by installing a cannula and insufflating each fish.**



**Fig. 12: Blood cell and plasma chemistry reference intervals for cultured juvenile shortnose sturgeon (n=61).**

Analyte	Reference Interval	Median	Mean	Standard Deviation
Hematocrit (%)	23-51	34	34	6
Total WBCs (cells/ $\mu$ l)	7,500-10,750	9,500	9,123	929
Heterophils (% of WBC)	47-84	70	67	12
Lymphocytes (% of WBC)	13-50	28	30	11
Monocytes (% of WBC)	1-9	2.0	3.2	2.2
Eosinophils (% of WBC)	0-0.5	0	0.5	0.3
Neutrophils (cells/ $\mu$ l)	3,845-8,225	6,300	6,096	1,240
Lymphocytes (cells/ $\mu$ l)	1,140-4,600	2,660	2,725	1,081
Monocytes (cells/ $\mu$ l)	90-875	200	296	200
Eosinophils (cells/ $\mu$ l)	0-45	0	4.6	26.7
<hr/>				
Total protein (g/dl)	2.8-6	3.7	4.0	0.9
Albumin (g/dl)	0.7-1.85	1.1	1.2	0.3
Glucose (mg/dl)	22-104	55	58	20
Urea nitrogen (mg/dl)	1-3	2	2	0.5
AST <sup>1</sup> (U/L)	212-802	330	353	141
Calcium (mg/dl)	7.9-16.6	8.7	9.3	2.1
Phosphorus (mg/dl)	6.8-14.2	9	9.4	1.8
Sodium (mEq/L)	132-146	138	138	4
Potassium (mEq/L)	2.5-3.6	3.3	3.2	0.3
Chloride (mEq/L)	110-125	118	117	0.7
Globulin (g/dl)	2.1-4.4	2.6	2.8	0.7
CPK <sup>2</sup> (U/L)	1,723-8,632	4,788	4,834	2114
Uric acid (mg/dl)	0-0.4	0	0.05	0.09

<sup>1</sup> aspartate aminotransferase<sup>2</sup> creatinine phosphokinase

**Fig. 13: Comparison of mean relative blood cell counts for male (n=26) and female (n=35) cultured juvenile shortnose sturgeon. P-value determined from T-test or Kruskal-Wallis non-parametric test.**

Analyte	Sex	Mean	Standard Deviation	P-value (t-test)	Pr>X <sup>2</sup> (Kruskal-Wallis)
Hematocrit (%)	M	35	6	-	0.5197
	F	34	7		
Total WBCs (cells/μl)	M	9,200	900	-	0.1548
	F	9,000	900		
Heterophils (% of WBC)	M	66	12	-	0.6986
	F	67	12		
Lymphocytes (% of WBC)	M	31	11	0.5397	-
	F	29	11		
Monocytes (% of WBC)	M	3.1	1.9	-	0.6443
	F	3.4	2.3		
Eosinophils (% of WBC)	M	0.04	0.2	-	0.7728
	F	0.06	0.34		
Neutrophils (cells/μl)	M	6,134	1,271	0.8358	-
	F	6,067	1,234		
Lymphocytes (cells/μl)	M	2,870	1,100	0.3704	-
	F	2,617	1,071		
Monocytes (cells/μl)	M	281	171	-	0.9125
	F	307	220		
Eosinophils (cells/μl)	M	3.5	17.7	-	0.6237
	F	5.4	32.1		

**Fig. 14: Comparison of mean blood cell chemistry values for male (n=26) and female (n=35) cultured juvenile shortnose sturgeon. P-value determined from t-test or Kruskal-Wallis non-parametric test.**

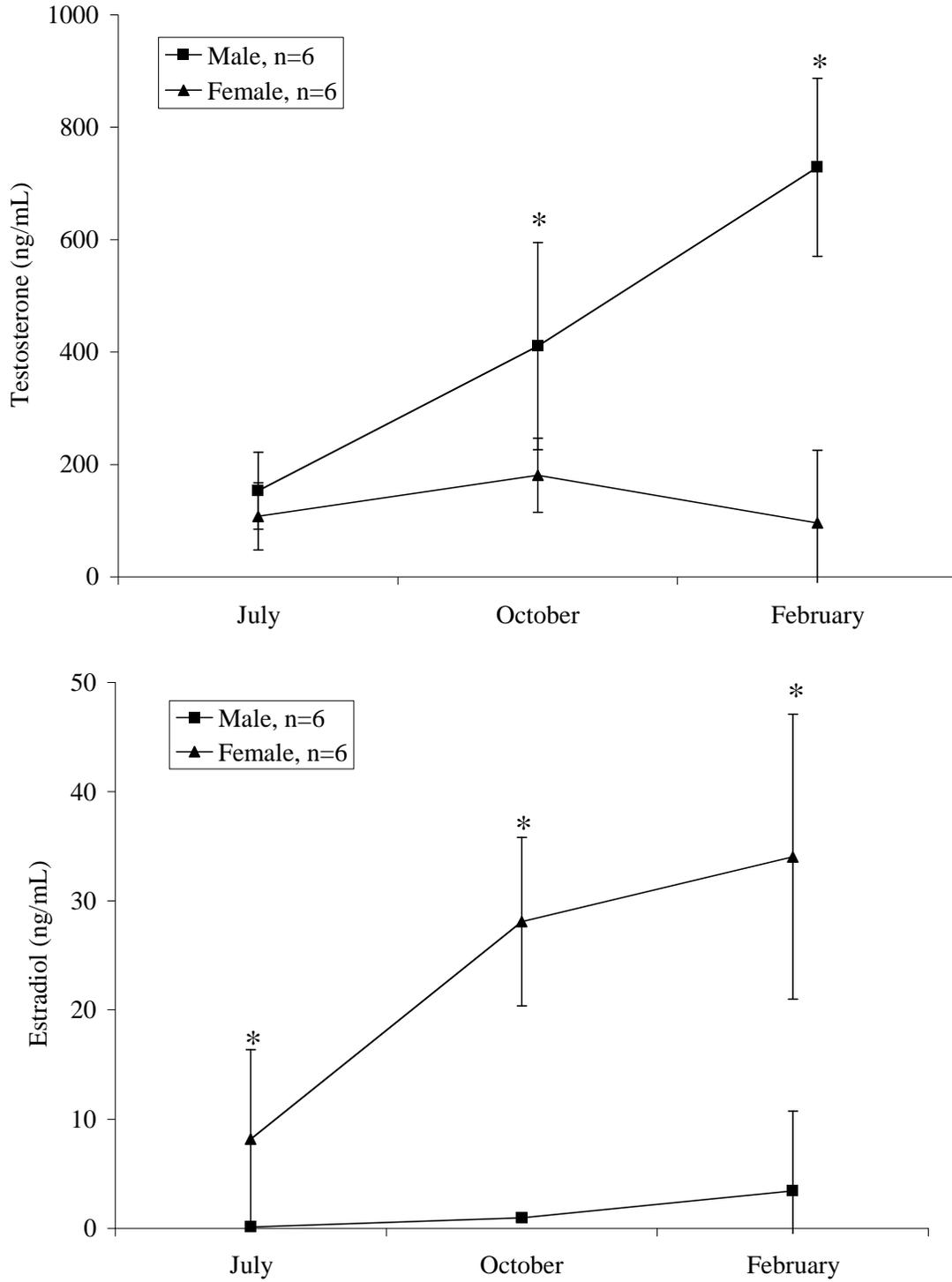
Analyte	Sex	Mean	Standard Deviation	P-value (t-test)	P-value (Kruskal-Wallis)
Total protein (g/dl)	M	4.0	0.9	0.6465	-
	F	3.9	0.9		
Albumin (g/dl)	M	1.2	0.3	0.4168	-
	F	1.1	0.4		
Glucose (mg/dl)	M	57	22	0.7618	-
	F	58	18		
Urea nitrogen (mg/dl)	M	2.0	0.5	-	0.5578
	F	1.9	0.5		
AST <sup>1</sup> (U/L)	M	321	73	-	0.2274
	F	376	172		
Calcium (mg/dl)	M	8.7	0.4	-	0.0614*
	F	9.9	2.6		
Phosphorus (mg/dl)	M	9.1	1.6	0.2072	-
	F	9.7	1.9		
Sodium (mEq/L)	M	138	2.8	0.9437	-
	F	138	4.1		
Potassium (mEq/L)	M	3.2	0.3	-	0.8023
	F	3.2	0.3		
Chloride (mEq/L)	M	117	3.8	0.9311	-
	F	117	4.1		
Globulin (g/dl)	M	2.8	0.6	0.7382	-
	F	2.8	0.4		
CPK <sup>2</sup> (U/L)	M	4,739	1,862	-	0.9125
	F	4,904	2,307		
Uric acid (mg/dl)	M	0.05	0.10	-	0.6312
	F	0.04	0.07		

<sup>1</sup> aspartate aminotransferase

<sup>2</sup> creatinine phosphokinase

\*Potential difference between male and female fish

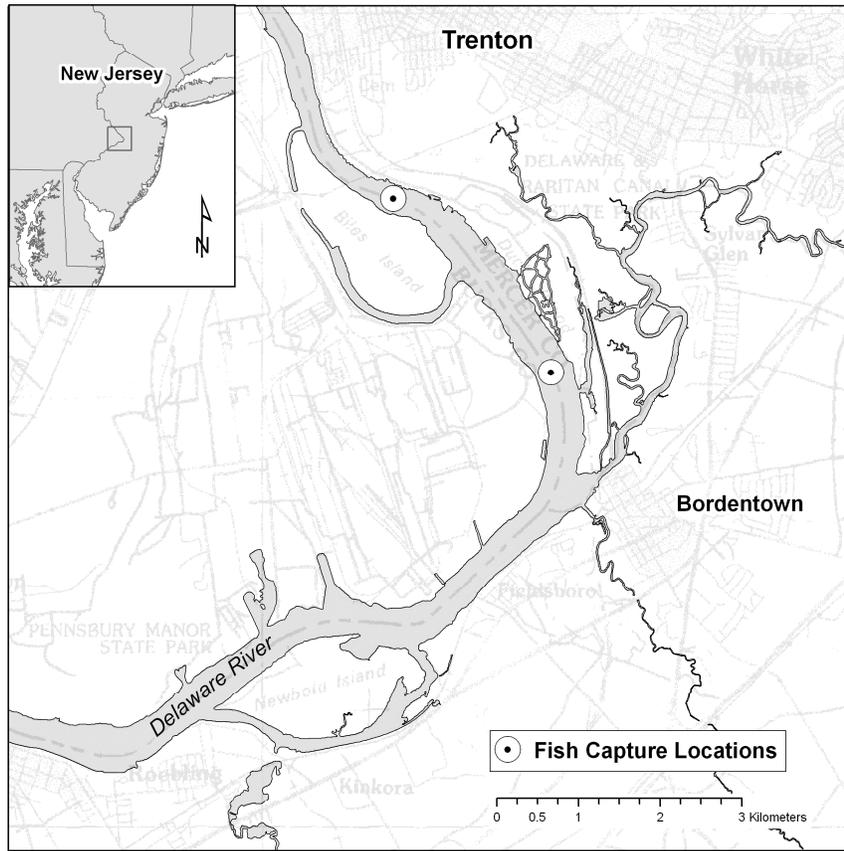
**Fig. 15: Mean plasma concentration of testosterone and estradiol in captive adult Shortnose sturgeon. Error bars indicate 95% confidence intervals. Means with \* indicate significant difference ( $\alpha=0.05$ ).**



**Fig. 16: Mean and 95% confidence intervals of estradiol:testosterone ratios in captive adult male and female Shortnose sturgeon.**

	n		July	October	February
Male	6	Mean	0.0007	0.0027	0.0051
		95% CI	± 0.0005	± 0.0016	± 0.0119
Female	6	Mean	0.0667	0.2040	0.6103
		95% CI	± 0.0552	± 0.1817	± 0.3673

**Fig. 17: Fish capture locations in the Delaware River, 2006-2008.**



**Fig. 18: Morphometrics, sex ratios, and times required to induce surgical level anesthesia, recover from anesthesia and the total time required to assess fish (elapsed time from induction to recovery) captured in the Delaware River, 2006-2008.**

Fish capture periods	Water temp. (°C)	Mean TL ± SD (mm)	Mean weight ± SD (Kg)	Sex ratio (M:F)	Mean induction time ± SD (min:sec)	Mean recovery time ± SD (min:sec)
Fall, 2006	12	775 ± 104	3.40 ± 1.52	5:1	7:04 ± 2:04	6:26 ± 3:16
Spring, 2007	19	767 ± 54	2.14 ± 0.38	1.2:1	4:18 ± 1:03	2:45 ± 1:39
Fall, 2007	10	756 ± 78	2.36 ± 0.74	12:1	6:20 ± 0:57	5:14 ± 1:06
Spring, 2008	22	797 ± 75	2.73 ± 0.72	0.6:1	3:58 ± 0:04	2:23 ± 0:05

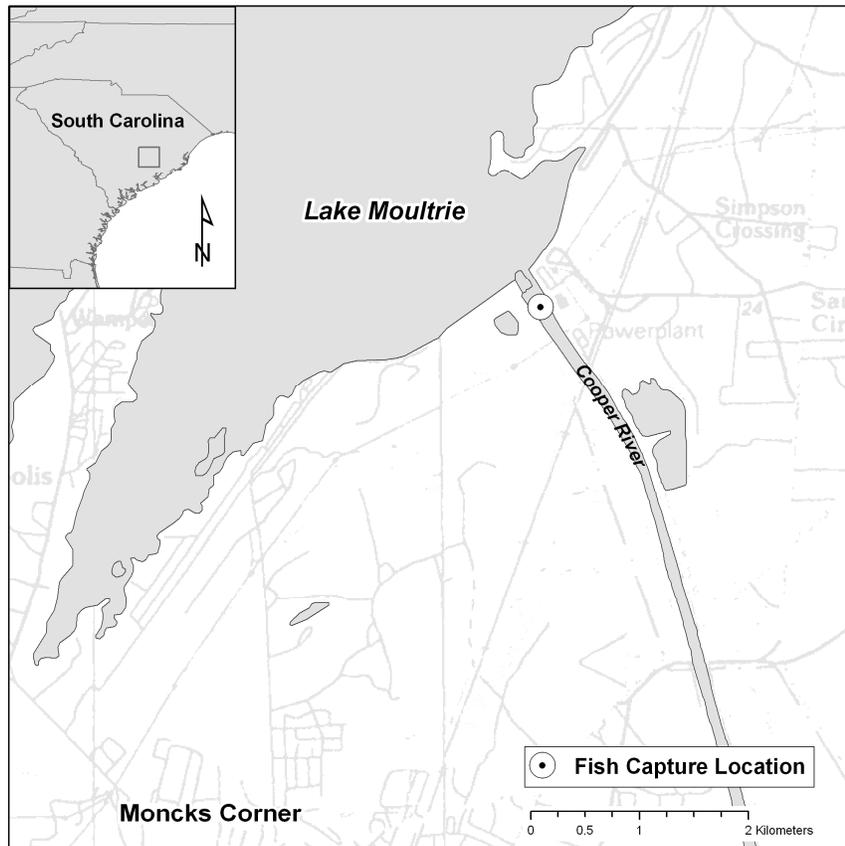
**Fig. 19: Mean relative blood cell counts and plasma chemistry values for shortnose sturgeon collected in the Delaware River, 2006-2008. Enumeration based on dried and stained blood smears (Antech Diagnostics). Means with \* indicate a significant difference between seasons. Means with ▲ were significantly higher than mean values from reference fish (Bears Bluff National Fish Hatchery) and means with ▼ were significantly lower than reference means based on t-test or Kruskal-Wallis non-parametric tests.**

Analyte	Hatchery Fish		Delaware River			
	Winter n = 61		Spring n = 37		Fall n = 26	
	Mean	SD	Mean	SD	Mean	SD
Hematocrit (%)	34	6	31	7	32	5
Total WBCs (cells/μl)	9,123	929	6,000 * ▼	4,000	3,900 * ▼	1,600
Heterophils (% of WBC)	67	12	52	20	56	9
Lymphocytes (% of WBC)	30	11	45 ▲	21	41 ▲	10
Monocytes (% of WBC)	3.2	2.2	1.45	0.82	1	1
Eosinophils (% of WBC)	0.5	0.3	1.36	1.12	2	2
Neutrophils (cells/μl)	6,096	1,240	3,854 * ▼	3,975	2,182 * ▼	1,067
Lymphocytes (cells/μl)	2,725	1,081	2,020	450	1,553 ▼	530
Monocytes (cells/μl)	296	200	79 ▼	44	69 ▼	44
Eosinophils (cells/μl)	4.6	26.7	109 ▲	134	101 ▲	121
Total protein (g/dl)	4.0	0.9	3.1 * ▼	0.52	4.8	0.96
Albumin (g/dl)	1.2	0.3	0.73 * ▼	0.16	1.3 *	0.35
Glucose (mg/dl)	58	20	79 * ▲	24	56 *	17
Urea nitrogen (mg/dl)	2.0	0.5	3.1 * ▲	1.4	2.0 *	1.3
AST <sup>1</sup> (U/L)	353	141	495 * ▲	114	365 *	104
Calcium (mg/dl)	9.3	2.1	8.5 ▼	0.49	8.8	1.2
Phosphorus (mg/dl)	9.4	1.8	12 * ▲	1.2	10.5 *	3.1
Sodium (mEq/L)	138	4	139 *	5	147 * ▲	5
Potassium (mEq/L)	3.2	0.3	2.9 ▼	0.24	2.8 ▼	0.3
Chloride (mEq/L)	117	0.7	119 *	5	125 * ▲	5
Globulin (g/dl)	2.8	0.7	2.3 * ▼	0.39	3.5 * ▲	0.8
CPK <sup>2</sup> (U/L)	4,834	2,114	2,368 * ▼	1,836	1,243 * ▼	994
Uric acid (mg/dl)	0.05	0.09	1.1 * ▲	0.58	0.2 *	0.1

<sup>1</sup> aspartate aminotransferase

<sup>2</sup> creatinine phosphokinase

**Fig. 20: Fish capture location in the Cooper River, 2007-2008.**



**Fig. 21: Morphometrics and sex ratios of fish captured in the Cooper River, 2007-2008.**

Fish capture periods	Water temp. (°C)	Mean TL ± SD (mm)	Mean weight ± SD (Kg)	Sex ratio (M:F)
Winter, 2007	9	983 ± 82	9.15 ± 3.72	2:1
Winter, 2008	12	1034 ± 130	7.21 ± 3.11	0.5:1

**Fig. 22: Mean relative blood cell counts and plasma chemistry values for shortnose sturgeon collected in the Cooper River, 2007-2008. Means with ▲ were significantly higher than mean values from reference fish (Bears Bluff National Fish Hatchery) and means with ▼ were significantly lower than reference means based on t-test or Kruskal-Wallis non-parametric tests.**

Analyte	Hatchery Fish		Cooper River	
	Mean	SD	Mean	SD
Hematocrit (%)	34	6	35	8
Total WBCs (cells/μl)	9,123	929	4,700 ▼	2,300
Heterophils (% of WBC)	67	12	59	20
Lymphocytes (% of WBC)	30	11	29	13
Monocytes (% of WBC)	3.2	2.2	6 ▲	10
Eosinophils (% of WBC)	0.5	0.3	2 ▲	5
Neutrophils (cells/μl)	6,096	1,240	3,055 ▼	2,180
Lymphocytes (cells/μl)	2,725	1,081	1,317 ▼	758
Monocytes (cells/μl)	296	200	242	340
Eosinophils (cells/μl)	4.6	26.7	29 ▲	67
<hr/>				
Total protein (g/dl)	4.0	0.9	5.3 ▲	1.0
Albumin (g/dl)	1.2	0.3	2.0 ▲	0.4
Glucose (mg/dl)	58	20	78 ▲	32
Urea nitrogen (mg/dl)	2.0	0.5	2.0	1.1
AST <sup>1</sup> (U/L)	353	141	353	159
Calcium (mg/dl)	9.3	2.1	14.8 ▲	5.2
Phosphorus (mg/dl)	9.4	1.8	13.3 ▲	3.0
Sodium (mEq/L)	138	4	153 ▲	7
Potassium (mEq/L)	3.2	0.3	2.2 ▼	0.7
Chloride (mEq/L)	117	0.7	127 ▲	6
Globulin (g/dl)	2.8	0.7	3.3 ▲	0.8
CPK <sup>2</sup> (U/L)	4,834	2,114	2,384 ▼	2,255
Uric acid (mg/dl)	0.05	0.09	1.7 ▲	1.2

<sup>1</sup> aspartate aminotransferase

<sup>2</sup> creatinine phosphokinase

**Fig. 23: Shortnose sturgeon plasma concentrations and ratio of testosterone (T) and estradiol (E2) from Bears Bluff National Fish Hatchery (collected in 2004), Delaware River (collected 2006-2008), and Cooper River Collected, 2007-2008).**

Location	Month	Sex	n	Testosterone (ng/ml)		Estradiol (ng/ml)		E2:T	
				Mean	± 95% CI	Mean	± 95% CI	Mean	± 95% CI
Reference	July	M	6	153	69	0.11	0.09	0.0007	0.0005
		F	6	108	60	8.2	8.2	0.0667	0.0552
	October	M	6	411	185	1.0	0.36	0.0027	0.0016
		F	6	181	66	28	7.7	0.2040	0.1817
	February	M	6	729	158	3.5	7.3	0.0051	0.0112
		F	6	95.8	129	34	13	0.6103	0.3673
Delaware River	May/June	M	15	147	56	1.6	1.1	0.0109	0.0060
		F	21	162	69	30	11	0.1852	0.0436
	November	M	23	377	143	1.3	0.5	0.0034	0.0012
		F	3	168	89	62	30	0.3690	0.5249
Cooper River	February	M	12	810	437	4.1	2.3	0.0051	0.0005
		F	12	113	94	67	29	0.5929	0.1804

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## **2) Lessons Learned**

The laparoscopic procedures developed for this project provide a safe and effective way of obtaining sex and reproductive information from fish that was previously more difficult to obtain (e.g. outside of spawning season) or not possible (e.g. juvenile fish) with live fish. Previous techniques used to determine sex in sturgeon were either not sufficiently sensitive when used on juveniles or reproductively inactive fish (e.g. boroscopy), or were more invasive than laparoscopy thus increasing risk of infections/post operative complications (e.g. open coelomic surgery). In addition, histological examination of gonad biopsies, which can be safely collected during laparoscopy, is required to positively identify sex in fish with undifferentiated gonads. The equipment design employed in this study allows use of laparoscopy in remote locations or on a small vessel.

Determination of normal hormone, hematologic and plasma chemistry values will be useful in identifying future sub-lethal health conditions and reproductive state of fish. However more work is needed to determine how these values vary from hatchery to wild fish, by season, by latitude, and other factors.

## **3) Dissemination**

The techniques developed in this project for the assessment of shortnose sturgeon, have been documented for distribution and use by other state and federal agencies, Universities, or organizations engage in sturgeon research or monitoring activities. Standard operating procedures and a video training guide (DVD) have been prepared providing a comprehensive overview of anesthetic, laparoscopic and blood sample collection and processing techniques. The SOP and DVD have been provided to the sturgeon technical committee, Atlantic States Marine Fisheries Commission, for incorporation of these techniques into a revised guide to accepted research practices for sturgeon. A manuscript is in preparation for submission to the Journal of Applied Ichthyology detailing application of anesthetic and laparoscopic procedures to both shortnose and Atlantic sturgeon. A continuing education course was conducted at the American Fisheries Society Annual Meeting, in Ottawa, Canada, 2008. Attendees were given an 8-hour course on anesthesia and laparoscopy procedures. Finally, the training video has been provided to NMFS for duplication and distribution.