

APPENDIX

METHODS

Specimen Collection

Male SMB (n = 79) were collected during 2014 and 2015 by boat electrofishing from several rivers in the Mid-Atlantic region and by electrofishing and hook-&-line in a single river in Vermont. Male LMB (n = 44) were collected in the spring by boat electrofishing from several sites within the Potomac River system (2014) and from a lake in Georgia (2015). An attempt was made to collect a minimum of ten male fish from each sample location, the minimum used in analysis was seven per site. To increase the likelihood of TO detection, collection of fish occurred in the early spring prior to spawning season and only fish of sufficient size to be reproductively mature (total length ≥ 250 mm) were included. Specific collection locations of wild-caught fish are not reported because it was not the intent of this study to compare TO results of field-collected fish populations with those of reference populations, as in traditional site characterization. Rather, the design was meant to compare effectiveness of tissue collection and examination method (biopsy vs. transverse) at TO quantification. An additional batch of male LMB was sourced from a hatchery (n = 24; Smartfish Farms, Auburn KY) exclusively for use in the first recovery and survival study.

Tissue Collection

In accordance with University of Maryland Institutional Animal Care and Use Committee (IACUC; Project Reference No: R-13-16) requirements all fish were anesthetized prior to tissue collection. Fish were anesthetized either, in a water bath with buffered tricaine methane sulfonate (100 mg/L MS-222; Finquel[®], Argent Laboratories,

Redmond, WA) or with a portable electro-immobilization system (EIS) (Hudson et al., 2011; Matsche, 2013). Following anesthesia, fish were measured for total length (mm) and weight (g) (Table 1) before testis tissue was collected both by biopsy (see *Biopsy Tissue Collection* below) and by conventional excision and transverse sectioning. For SMB, biopsies were taken from excised testes either immediately after sacrifice or from archived tissue several months after fixation (RNAlater or 10% neutral buffered formalin) and transverse sectioning (details in Table 1). In contrast, for most LMB, tissue biopsies were taken *in situ* prior to testis removal, fixation (10% neutral buffered formalin), and transverse sectioning. Those LMB that were intended for post-biopsy survival studies (hatchery batch as well as a subset of eight field-collected fish from the Potomac system) were allowed to recover (~1 week) in clean water immediately after completion of the laparoscopic procedure before being returned to holding tanks or raceways for approximately one month. Therefore, for these fish, sacrifice and excision of testes for transverse sectioning occurred approximately one month after testis tissue collection by biopsy. In all instances, fish were anaesthetized and euthanized by decapitation prior to testis excision. On sacrifice, fish were dissected for positive identification of sex and subsequent removal of the testes. Entire testes were excised, weighed (for calculation of gonadosomatic index, GSI; only LMB), and placed in pre-labeled bottles of fixative for a minimum of 48 hours, right lobe was archived, left lobe (previously biopsied) was transverse-sectioned and positioned in tissue cassettes for histological processing. Fish condition ($k = [\text{body weight}/\text{length}^3] \times 100$) and gonadosomatic index ($\text{GSI} = [\text{gonad weight}/\text{body weight}] \times 100$) were calculated to compare fish responses across sites.

Biopsy and Transverse Tissue Collection

Samples obtained by biopsy were collected using several different procedures depending on the objective of the collection (survival/reproduction or increasing sample size), specimen condition on arrival, and time constraints in the field. Biopsy collection was performed *in situ* on LMB by laparoscopic surgery on live specimens or post-mortem by removal of the ventral body wall and direct tissue collection.

Laparoscopic Tissue Collection

Tissue collection by laparoscopy followed the methods of Matsche (2013) with several modifications. Briefly, the laparoscope set-up employed a 4.8 mm x 190 mm sheath housing the 2.7 mm 30° rigid endoscope, portable LED light source and 1.7 mm flexible oval biopsy forceps (Medit, Inc.; www.meditinc.com) (Figure 1). The imaging system consisted of an ImagePro USB endoscope camera (Medit, Inc.) and laptop computer (Figure 2). The instrument was introduced via the urogenital pore into the urinary bladder (Figure 3A) and the bladder wall was perforated by biopsy forceps (Figure 3B). Once inside the body cavity five biopsies were collected approximately equidistant along the length of the left testis lobe (Figure 3C). Direct tissue collection involved removal of the ventral body wall allowing collection of the five biopsies using the forceps independent of the biopsy sheath and scope (Figure 3B).

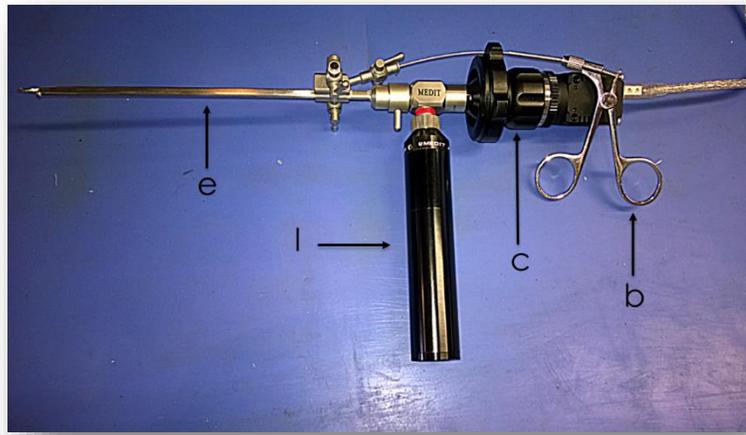


Figure 1. A laparoscope equipped with a 2.7 mm 30° rigid telescope (w/in sheath). (e) 4.8 mm examination sheath; (l) LED light source; (c) digital camera; (b) 1.7 mm flexible biopsy forceps.

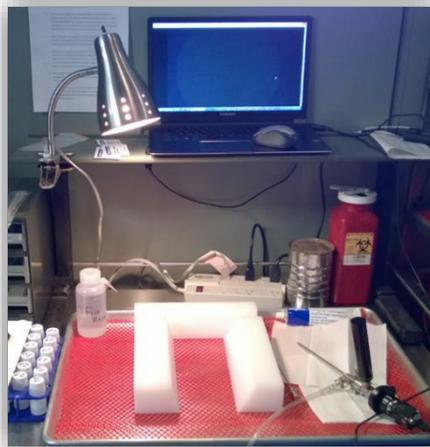


Figure 2. Benchtop laparoscopy setup for use with chemically anesthetized largemouth bass *Micropterus salmoides*, computer used for viewing and blocks for holding fish.



Figure 3. Laparoscopic testis tissue collection from largemouth bass *Micropterus salmoides*: **A)** examination sheath inserted into the urogenital pore; **B)** testis sampling via biopsy forceps (body-wall removed and

surgical clamp employed for demonstration only); C) image capture of video from laparoscope showing protruding biopsy forceps approaching testis.

Two methods of anesthesia, chemical and physical, were employed for laparoscopic surgery. Initially, chemical anesthetic, MS-222 was used for out-of-water surgery, with the fish placed on its back between two angled blocks (high-density polyethylene plastic) to hold it in position. For field sampling, a physical electric anesthetic, portable EIS was used for water bath surgery, placing the fish on its back into a sling, angled to allow water flow over the gills while the vent protruded above the water line (Matsche, 2013; Hudson et al., 2011). The EIS consisted of a cooler with two metal plates equipped to deliver a continuous direct current with a low voltage gradient of around 0.30 V cm^{-1} (Matsche, 2013; Hudson et al., 2011) depending on water conductivity. In addition to the previously mentioned attributes of electro immobilization, this method minimized the number of females sacrificed since they could be quickly identified and immediately released. When performing laparoscopy for detection of TO, it is important to make sure that the fish are reproductively mature and collected prior to spawning which generally coincides with the optimal testis size, reducing the chance of complications.

Detailed Laparoscopic Procedure

Once the fish was anesthetized and in position, the urogenital opening was disinfected with a 70% alcohol wipe then slightly dilated using a blunt obturator lightly coated with anti-bacterial surgical lubricant (Surgilube® Nycomed US Inc). The blunt obturator was placed back within the examination sheath, sealing the end, and was inserted into the urogenital opening at a 90° angle to the fish and advanced into the urinary bladder (Benz and Jacobs, 1986; Matsche, 2013). The examination sheath was

then angled 60° towards the anterior of the fish so that the tip of the sheath was directed to the head of the fish, then adjusted 90° to point towards the body wall at 30°, prior to perforation of the urinary bladder to protect from damaging organs (Figures 4 & 5). The blunt obturator was then removed from the examination sheath to open the channel and kept steady with the sheath in the vent and carefully transitioning the laparoscope into the space from the blunt obturator then inserted the biopsy forceps into a channel on the sheath. Once situated, the urinary bladder was perforated using the biopsy forceps, removing a small piece of the bladder to gain direct access into the body cavity (coelom). To assist with laparoscopic viewing, the coelom was slightly inflated using a low-pressure air supply, connected to a stopcock on the examination sheath with vinyl tubing (Matsche, 2013). The examination sheath was advanced into the coelom caudally along the body wall on the underside of the fish, the gonads were examined, sex confirmed, and five biopsies were collected along the length of the left testis, at an approximately equidistant transect and placed biopsies in 10% neutral buffered formalin. The examination sheath remained in the body cavity during collection of all biopsies, only removing the flexible forceps between biopsies to store the collected tissue. Occasionally, it was followed by placement of passive integrated transponder (PIT) tag into the coelom to track individuals for survival and recovery of the urinary bladder; this was

accomplished by removing the laparoscope from the sheath and gently letting the PIT tag slide into the coelom before removing the examination sheath from the vent.



Figure 4. Proper orientation of the fish and the instrument to perforate the urinary bladder without causing injury, the direction is evident by the light shining through the body wall at the tip of the instrument.



Figure 5. Field laparoscopy setup, photo depicts largemouth bass collected in Georgia, Spring 2015.

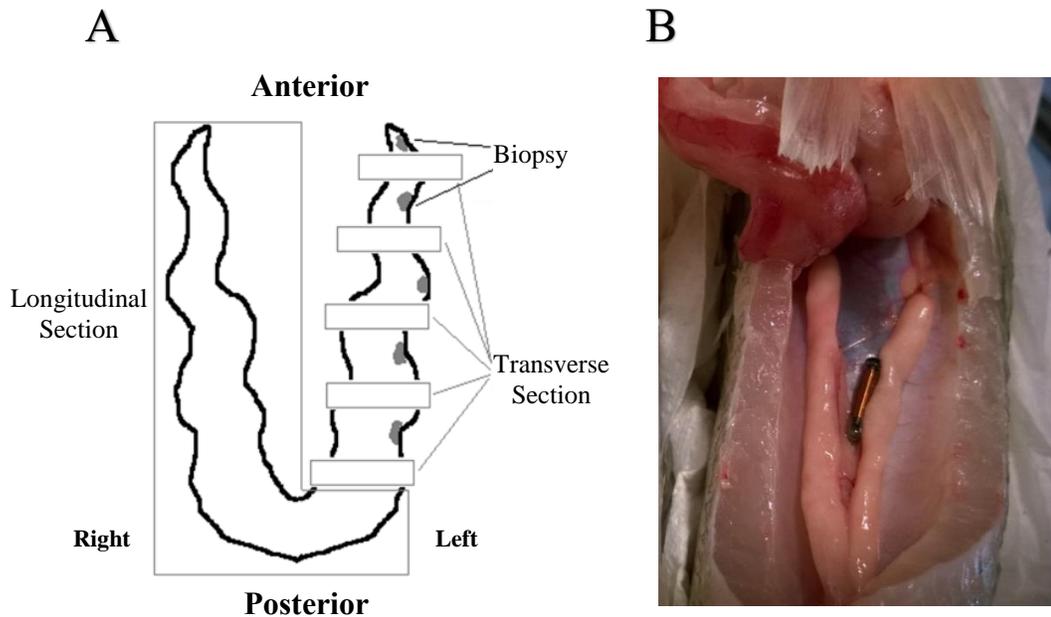


Figure 6. Experimental design for tissue collection by biopsy, transverse, and longitudinal sectioning, A) tissue collection strategy depicted in diagram, B) actual view of largemouth bass *Micropterus salmoides* body cavity after removal of the body wall, with testes connected to swim bladder, left testis (seen on the right ventral view).

In specific circumstances following laparoscopy, fish were anesthetized using MS-222 and decapitated, then necropsied and the entire testes were excised for histological examination. Sex was determined by examination of morphological features of the gonads and confirmed by microscopic examination of biopsy sections. Each left lobe of the testes (laparoscopic biopsied) was transverse sectioned (3-5 mm thick) between each biopsy, fixed in 10% neutral buffered formalin and processed for routine histology (Figure 6; Presnell et al., 1997).

Histological Tissue Processing

After adequate fixation, tissues were processed for routine histological evaluation. Briefly, preserved tissue was dehydrated in alcohol, embedded in paraffin wax, sectioned at 6 μm , and stained with hematoxylin and eosin (Luna, 1992; Presnell et al., 1997). For each specimen, all transverse segments were embedded in one or more paraffin blocks

and a single section of each was mounted for examination by light microscopy. Biopsies were substantially smaller than transverse sections, so all five biopsies from a given fish were blocked together and six step sections (three sections spaced at 30 μm then at 120 μm to represent tissue from end to end) were mounted on two glass slides (3 sections/slide) for examination by light microscopy. Briefly, both detection methods were performed on each individual for comparison, this resulted in a total of 5 transverse sections (each equal to 1 unit) and 5 biopsies (1 unit together) step-sectioned 6 times (30 small pieces of tissue). LMB from a lake in Georgia, were subjected to an additional method of sectioning, the right testis lobe was sectioned longitudinally down the length, each section was embedded in one paraffin block and a single section was mounted for examination by light microscopy.

Testicular Oocyte Quantification and Comparison

Histological sections of multiple transverse segments and of multiple biopsies were examined for assessment of TO occurrence, as determined by the observation of one or more discernible oocytes within preserved testicular tissue from individual specimens. Tissue sections of adequate quality from each specimen were scanned for the presence of oocytes under low and moderate magnification (4 \times and 10 \times objectives, respectively) with confirmation of presumptive oocytes made under high magnification (40 \times objective). Results were used to estimate site TO prevalence (calculated as the proportion of individuals from each collection location in which at least one oocyte was encountered) determined independently by each detection method.

In the instance of transverse sections, severity was determined by enumerating all observable TO and averaging between the number of sections (generally five) as well as

by scoring each section using the ranking system described by Blazer et al. (2007) for use with SMB. The central regions (concentration of TO) of transverse sections were examined under moderate magnification (10× objective) and TO observed within a single field of view were ranked as follows: focal distribution (score 1), occurrence of a single oocyte; diffuse distribution (score 2), more than one spatially distinct oocytes; cluster distribution (score 3), more than one but fewer than five closely associated oocytes; and zonal distribution (score 4), multiple oocyte clusters (Figure 8, see Blazer et al. 2007 for detailed description). This resulted in a single mean count value based on enumeration per section, and a mean rank value based on the score per section for each individual.

Biopsy intersex severity was formulated by taking the first five sections of adequate quality were examined and TO enumerated was similarly divided by five, to generate a single mean count value based on TO enumeration per section. It is important to recognize that each of these sections included all five spatially distinct biopsies, so the units of observation (five biopsies vs. one transverse section) are qualitatively and quantitatively different (Figure 7). Due to these issues and other differences associated with tissue collection, such as blocking and sectioning methods, no ranking system for severity was applied to biopsied tissues, only enumeration.

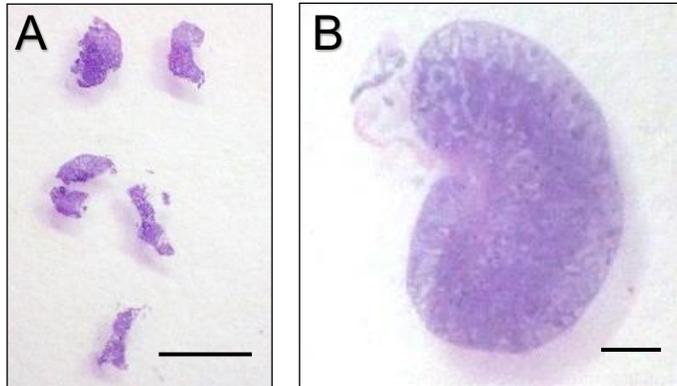


Figure 7. A cluster of five biopsies (A) collected from distinct regions along the length of a largemouth bass (*Micropterus salmoides*) testis was determined to be approximately equivalent in area to a single transverse section (B) as the unit of observation for testicular oocytes (TO) presence and abundance (5- μ m sections glass-mounted and stained with hematoxylin and eosin; bar = 2 mm). Five step-sections (30 – 120 μ m spacing) of biopsy clusters was equated to five equidistant transverse sections (i.e., five units of observation of each method). Assessment of TO prevalence and severity was calculated by averaging TO counts from five individual units of observation (transverse section or 5-biopsy cluster) for each specimen to produce a mean value for each tissue collection method.

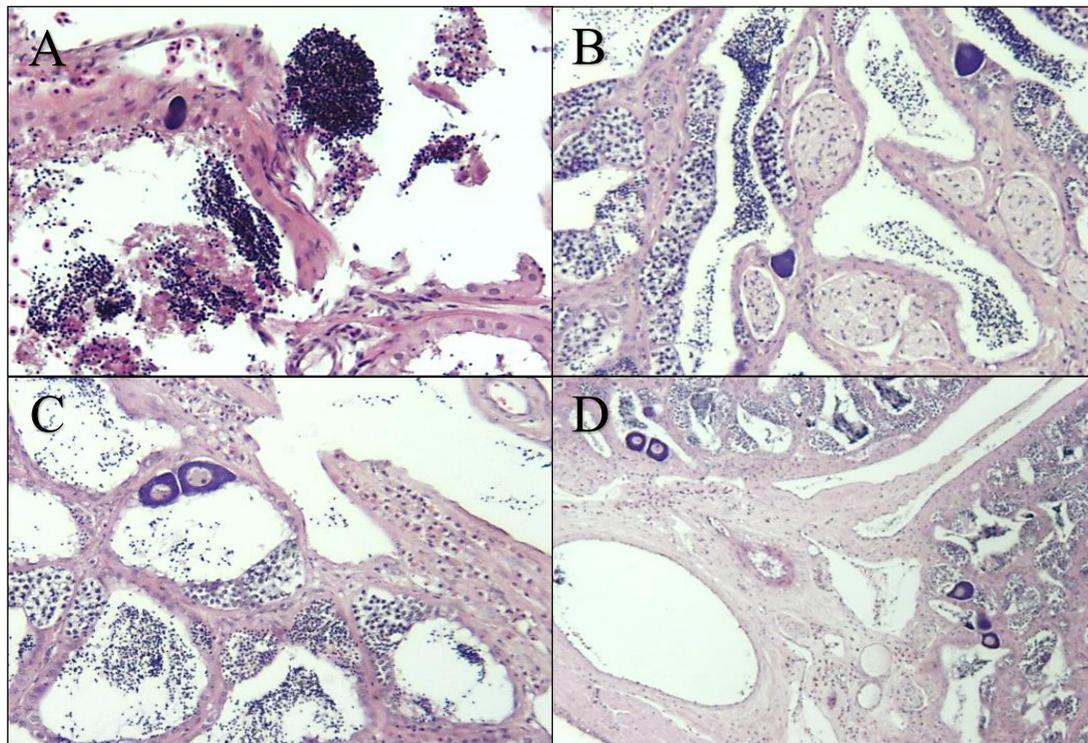


Figure 8. Examples of testicular oocytes in step-sections of biopsies from largemouth bass *Micropterus salmoides*. To depict different scenarios for transverse section ranking, each section represents a different rank based partially on enumeration of testicular oocytes and spatial distribution, a) Rank 1, b) Rank 2, c) Rank 3, and d) Rank 4.

Intersex severity for longitudinal sections was determined by enumeration only, similarly to biopsies, disregarding the rank severity index developed for transverse sections due to the constraints of the nature of the tissue. Alternatively to the mean oocyte count for biopsies, longitudinal section oocytes per testis lobe were only enumerated and summed to report individual severity. To reiterate, longitudinal sections were assessed on one occasion in LMB only.

Survival and Healing Study – Largemouth Bass

In the first survival study the viability of laparoscopy was assessed on live hatchery-reared LMB to monitor healing of the perforated urinary bladder and survival over a 28-day period. Fish were anesthetized, males were identified via laparoscopy and approximately five biopsies were obtained from each individual fish (n=24) equidistantly along the length of the left lobe of the testis. A subset (n=7) of fish were sacrificed immediately as controls to observe and to hone the technique. At one week post-laparoscopy intervention, a subset (n=5) were sacrificed to investigate testis condition after multiple biopsy and repair of the urinary bladder integrity. The remainder (n=12) were intended for sacrifice at 28 days to further investigate testis condition, repair of the perforated urinary bladder and establish likelihood of long-term survival. Fish used in this study (285-360 mm total length; 350-811 g total wet weight) were obtained in early March 2014, tagged with a PIT tag, and maintained in a controlled lab tanks containing aerated de-chlorinated city water in a flow-through system with a biofilter (Figure 9).



Figure 9. *Micropterus salmoides* largemouth bass held in three circulating fiberglass tanks (2000 L each) plus reservoir and biofilter. Fish density at 7 days into the first survival and healing study fish density was 353 L/Fish, after 7 days the density reduced to 500 L/Fish.

In the second survivorship study laparoscopy was performed on wild-caught LMB from the Potomac and Anacostia rivers with the goal of assessing potential effects to the spawning capability of the male fish. Field-collected LMB were laparoscopically viewed to definitively establish sex. Females (360-480 mm total length; 397-1531 g total wet weight) were identified by endoscopic viewing and received no additional treatment, thereby leaving the urinary bladder intact. In males (280-400 mm total length; 284-1021 g total wet weight) five biopsies were collected from the left testis lobe. Fish were segregated by sex and held for a short recovery period (~1 week) before pairing in flow-through spawning raceways to monitor for survival although females did not lay eggs after the allotted 28 days.

STATISTICAL ANALYSIS

Quantitative data (e.g., morphometric characteristics; TO counts), if normally distributed, were analyzed by one-way analysis of variance (ANOVA) followed by Holm-Šídák pairwise multiple-comparison test for significance. Where data failed

assumptions of normality or homogeneity of variance, estimations used REML (restricted maximum likelihood), Kruskal-Wallis one-way ANOVA on ranks was performed, and significant differences were discerned by Dunn's pairwise multiple-comparison test for site differences. A linear regression (r^2) was used to test for the relation of TO metrics between transverse and biopsy methods at $p = 0.05$. All analyses were performed using SigmaStat version 12.0 (Systat Software, Inc., San Jose, CA, USA) with statistical significance reported.

RESULTS

Comparison of Histological Methods: Longitudinal, Transverse, and Biopsy Sections

The Georgia site was the only population sampled using all three methods for TO detection employed in this study and resulted in superior detection using longitudinal sections, somewhat lower in transverse sections and much lower in biopsies (Figure 11). The longitudinal sections most frequently detected intersex (75%) compared to 50% and 17% in transverse sections and biopsies, respectively (Table 1). The longitudinal sections did not detect all intersex males, as determined by comparing all individuals detected using all three methods and increased prevalence to 83% indicating that the longitudinal sections missed two individuals (Figure 10). The inclusion of longitudinal sections derived a much higher mean severity of 7 compared to 0.4 and 0.1 for transverse sections and biopsies, respectively (Table 1; Figure 11).

Table 1. Method comparison in all *Micropterus salmoides* largemouth bass populations sampled with intersex. Data are presented as means and (standard deviation). *Longitudinal mean severity is derived from each individual severity; not each individual mean severity.

	Longitudinal Section*	Transverse Section	Biopsy
Mean Severity	7	0.4	0.1
STD	12.95	0.82	0.32
Prevalence (%)	75%	50%	17%

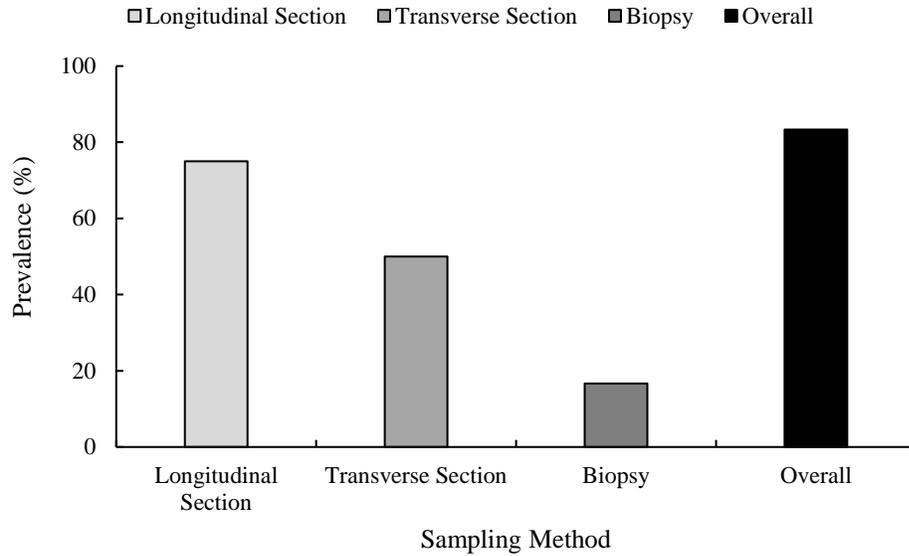


Figure 10. Percent occurrence of intersex in male largemouth bass *Micropterus salmoides* using longitudinal, transverse, and biopsy sections for oocyte quantification from the Georgia discrete population (n=24).

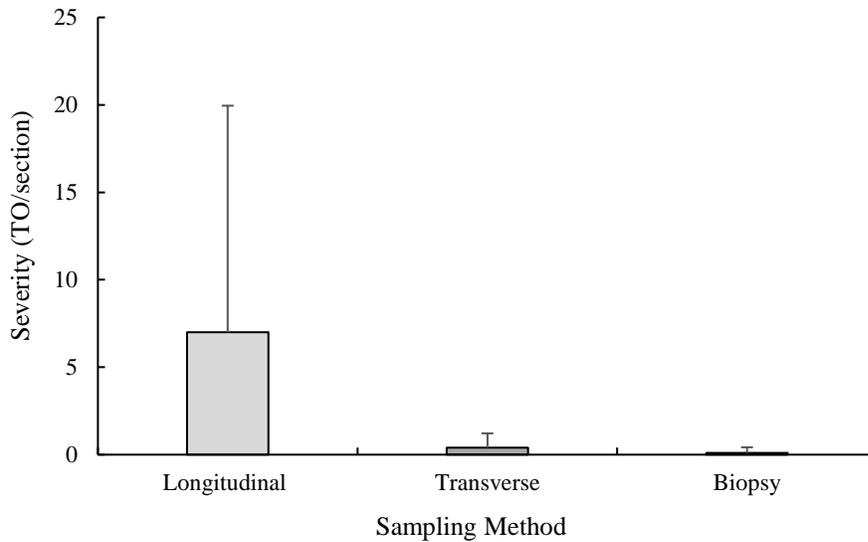


Figure 11. Degree of intersex severity in male largemouth bass *Micropterus salmoides* using longitudinal, transverse, and biopsy sections for oocyte quantification from the Georgia discrete population (n=24).

Survival and Healing (Largemouth Bass Only)

Post-operative recovery and healing were assessed at intervals up to 28 days in a lab-controlled setting using hatchery-reared male LMB (Table 2). Post-surgery healing

was high based on integrity of the urinary bladder (90%) for the 9 out of 10 checked (Table 2).

Table 2. Experimental design and physiological parameters for a twenty-eight day survival and healing experiment post-laparoscopy in *Micropertus salmoides* largemouth bass. Healing is described by repair of the urinary bladder, and hemorrhaging is described by evidence of bleeding, these diagnostic characteristics were measured at each time-point. All organisms survived to their prescribed time point except one in the final group. Mean procedure duration is in minutes and the range in parentheses.

Time Point	Sample Size	Urinary Bladder Recovered	Hemorrhage	Mean Procedure Duration (minutes)
Control	7	-	2	11 (8-14)
7 days	5	N/A	1	15 (10-23)
28 days	12*	9~	4	10 (4-19)
Total	24	9	7	12 (4-23)

*1 fish lost equilibrium, sacrificed at 14 days

~Didn't check first two fish sacrificed

N/A – Did not check at 1 week for UB recovery

A spawning study was conducted using wild-caught LMB and placing them in raceways but spawning did not occur within the time allotted. It did, however, contribute to the healing and survival aspect by increasing the sample size of LMB survival post-laparoscopy. Assessing the data in this fashion results for LMB (n=20), was a high urinary bladder recovery (90%), a medium incidence of hemorrhage (47%), and high survival (90%) (Table 3).

Table 3. Survival and recovery data from healing study and wild caught reproduction study for males only and survived to at least 28 days.

Study	Sample Size	Urinary Bladder Recovered	# Hemorrhage	Survival
Survival	12	82*	64	92
Reproduction	8	100	25	88
Total	20	90%	47%	90%

*Didn't check first two fish sacrificed