

## SUPPLEMENTARY DATA

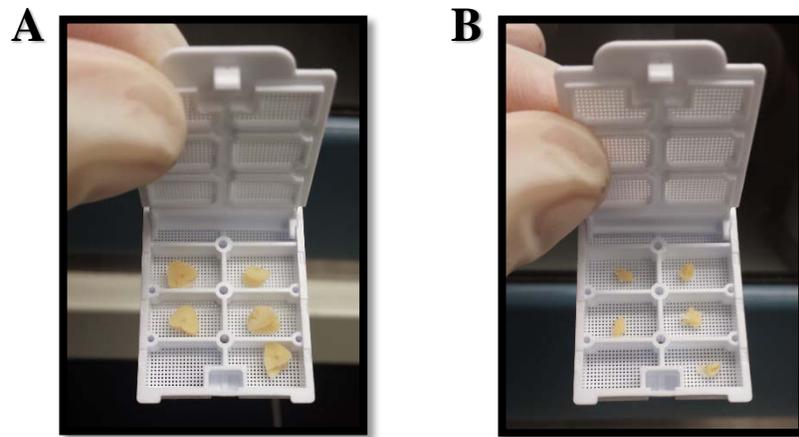


Figure 1. Fixed testis in cassette prior to embedding for histology, examples shown: left is of transverse sections and right is of biopsies.

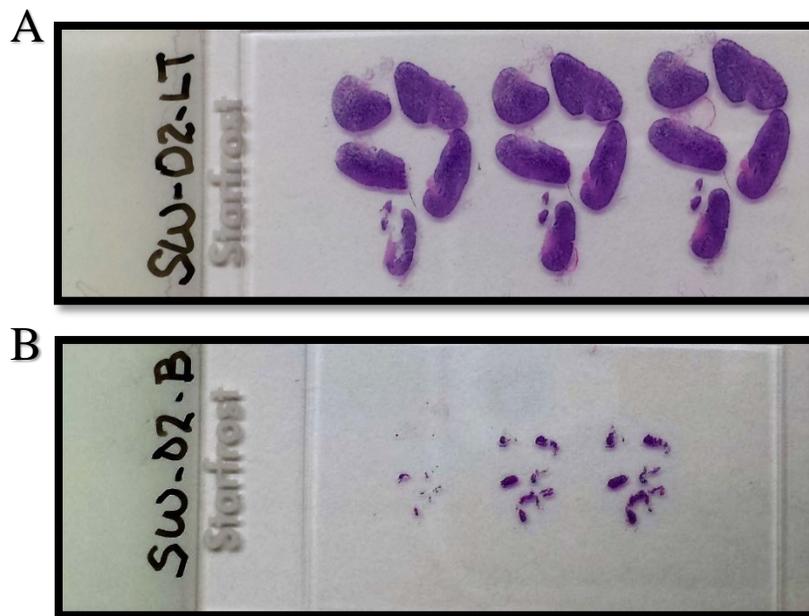


Figure 2. Example of the difference between stained biopsy sections and transverse sections, high variability using biopsies.

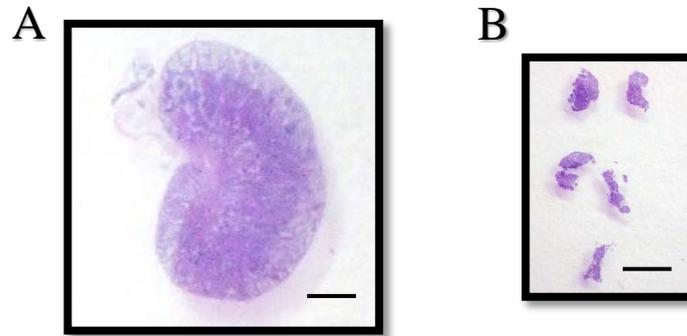


Figure 3. Five distinct transverse sections (A) were collected from unique regions along the entire testis, while five biopsies from unique regions were clustered (B) and subsequently step-sectioned. The resulting units of observation for comparison of TO counts were individual transverse sections vs. clusters of five biopsies. The TO counts from five units of observation were averaged to produce a mean value for each tissue collection method (bar = 2 mm; hematoxylin and eosin stain).

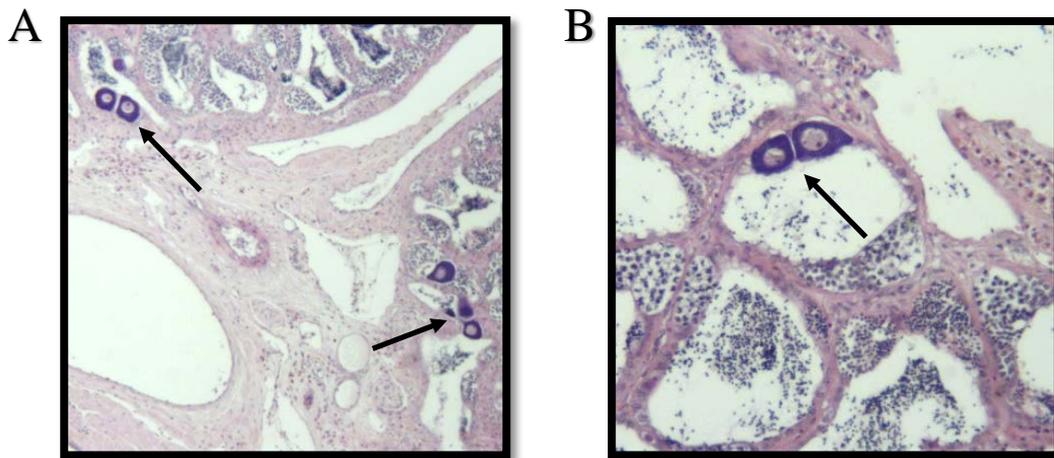


Figure 4. Microscopic view of undeveloped previtellogenic testicular oocytes (arrow) surrounded by testicular tissue and sperm in *Micropterus salmoides* largemouth bass, (a) transverse section, (b) biopsy taken adjacent to the transverse section.

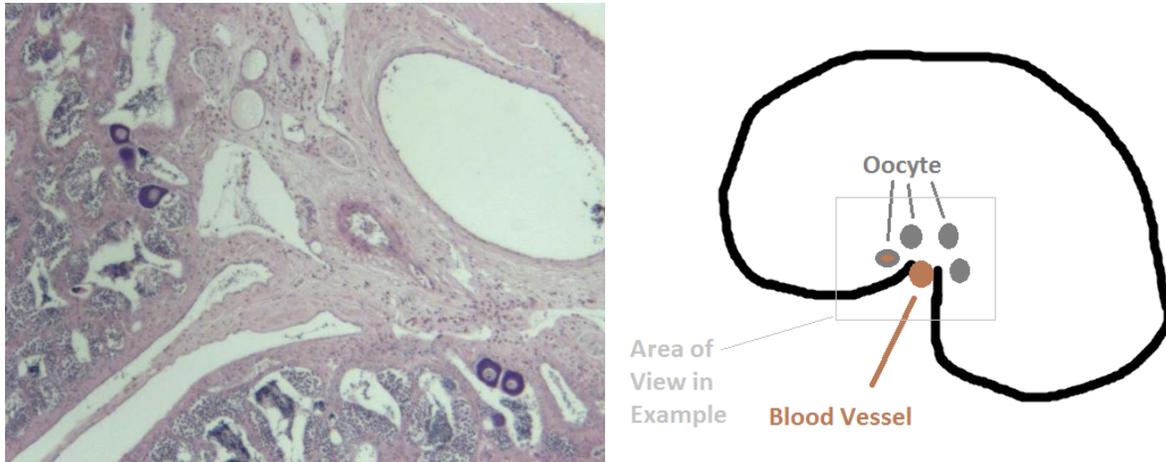


Figure 5. Tissue orientation becomes an issue with the biopsy tool if targeted approach fails to collect tissue from this location.

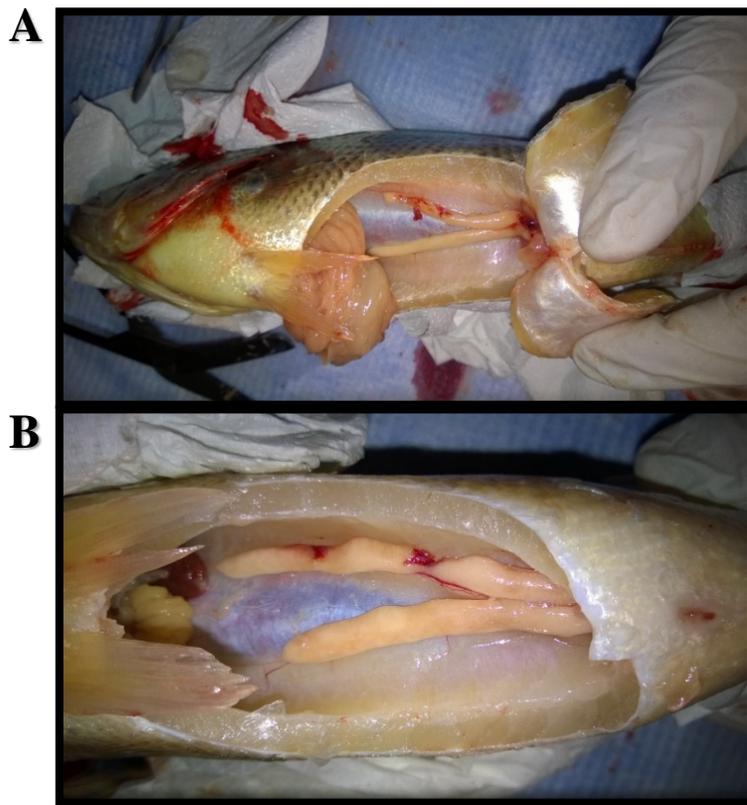


Figure 6. Variety of immediate effects after laparoscopic biopsies.



Figure 7. *Micropterus salmoides* largemouth bass anesthetized prior to laparoscopy.



Figure 8. Surgical setup in the lab and demonstration in the field on *Micropterus salmoides* largemouth bass.

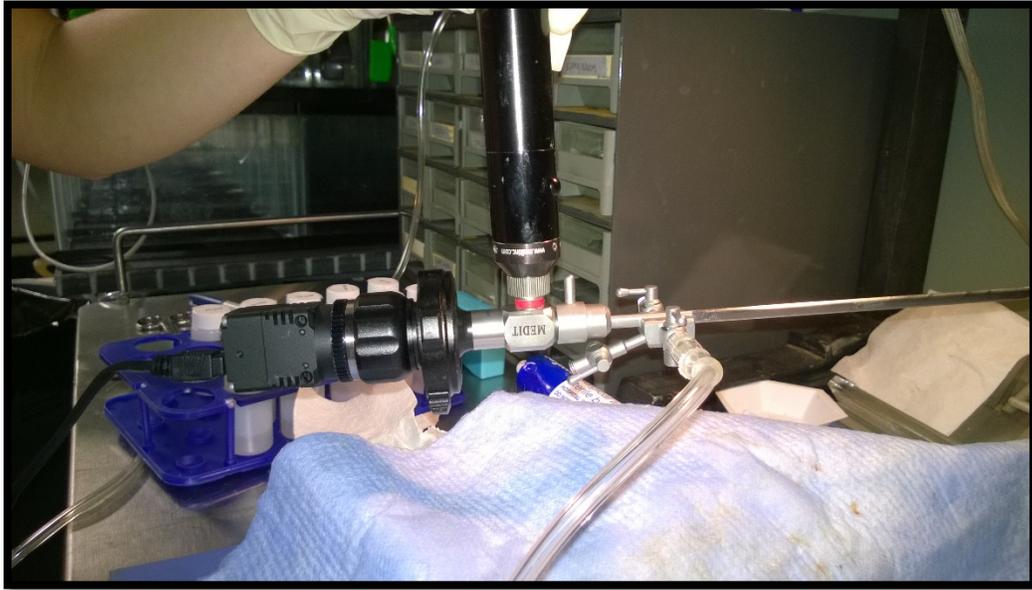


Figure 9. Proper orientation for laparoscopy, with 30 degree viewing downwards for optimal gonad inspection.

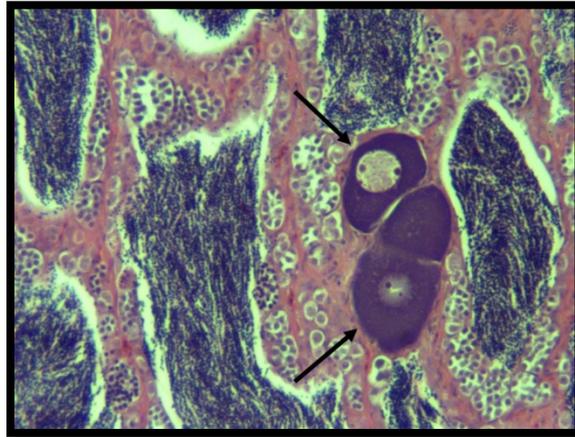


Figure 10. Testicular oocytes clustered surrounded by mature sperm and testicular tissue from hatchery reared *Micropterus salmoides* largemouth bass.

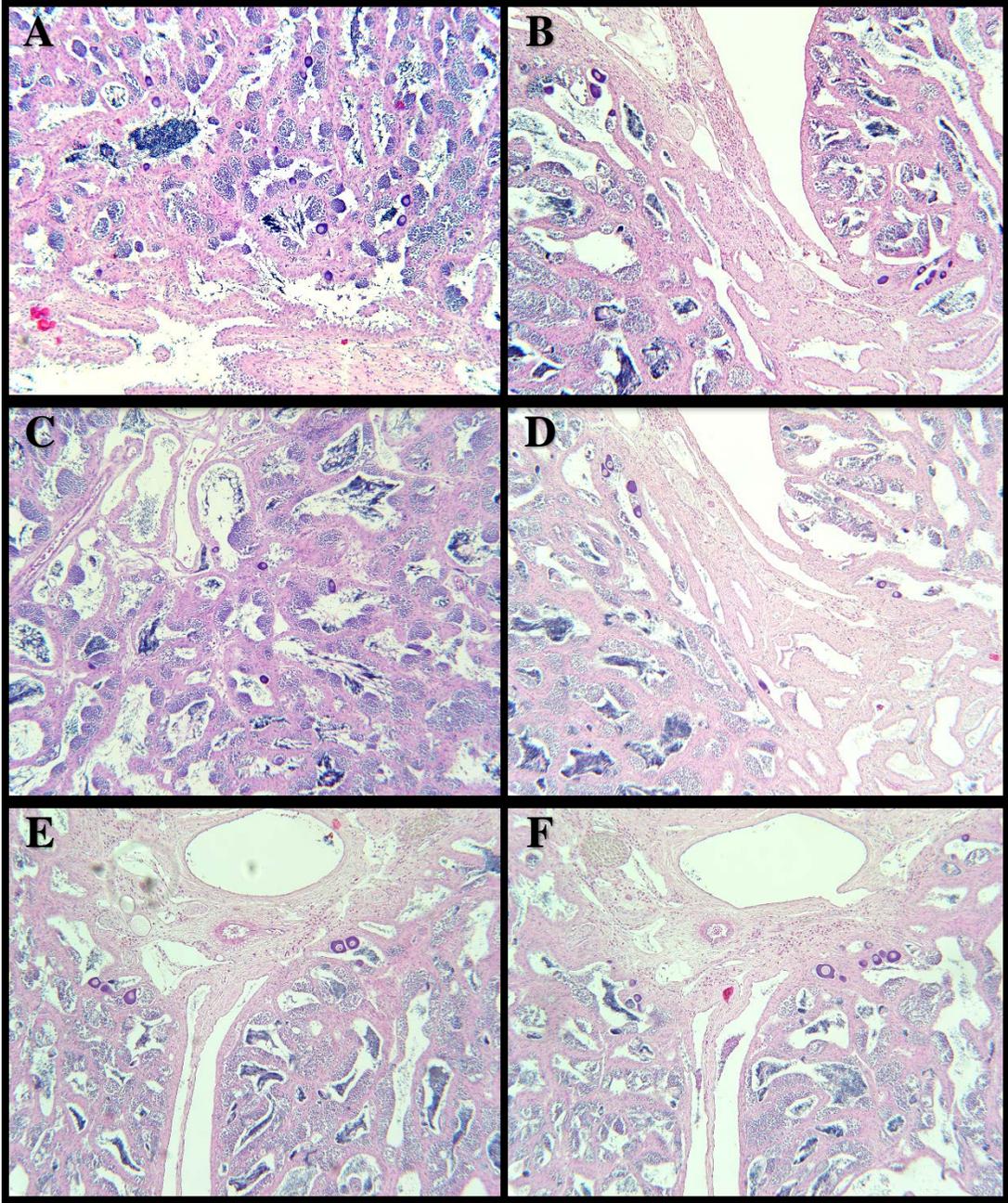


Figure 11. Series of microscopic images of testicular oocytes to show the variety between sizes, shapes, and color.

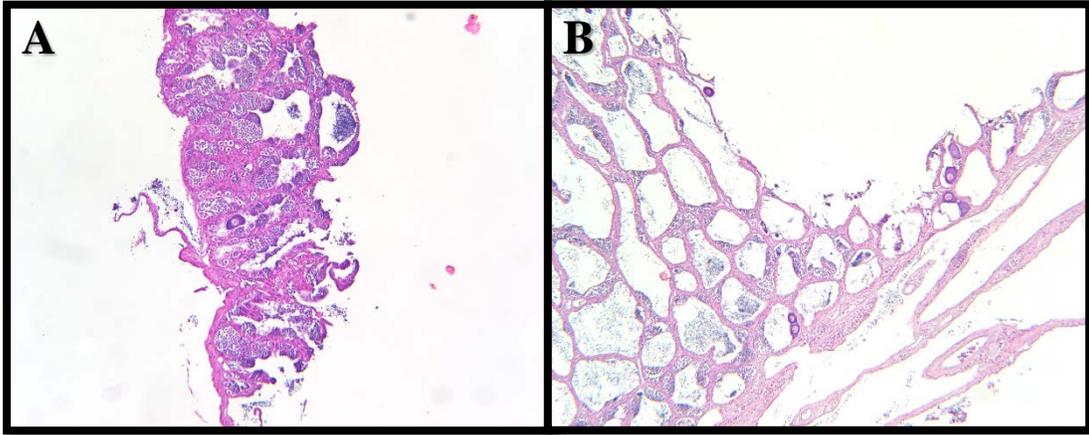


Figure 12. Microscopic images of testicular oocytes by biopsy from *Micropterus salmoides* largemouth bass.

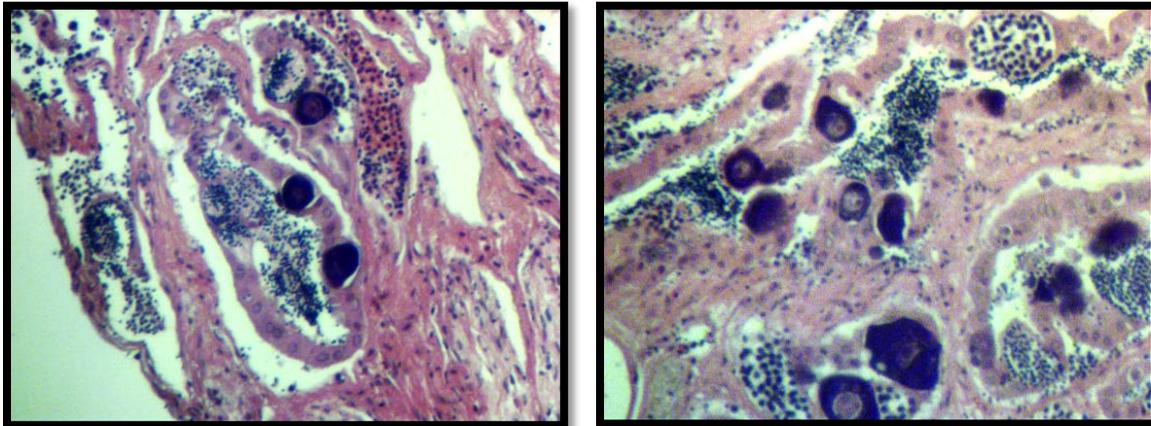


Figure 13. Two microscopic images of testicular oocytes to show the variety between sizes, shapes, and color from *Micropterus salmoides* largemouth bass.



Figure 14. Parasites filling *Micropterus salmoides* largemouth bass, impeding laparoscopic viewing.

Table 1. Prevalence and severity metrics summarized across all sites for method comparison in *Micropterus dolomieu* smallmouth bass and *Micropterus salmoides* largemouth bass.

Site	n	Testicular Oocyte				
		Prevalence		Severity		
		Biopsy	Transverse	Biopsy (#TO/section)	Transverse (#TO/section)	Transverse Rank
SMB	79	66%	76%	3.5 ± 4.87 (0-46)	3.4 ± 3.73 (0-23)	1.0 ± 0.81 (0-3.7)
LMB	44	18%	45%	0.1 ± 0.06 (0-1.4)	0.5 ± 0.23 (0-4)	0.4 ± 0.17 (0-2.2)
Mean		42%	61%	1.7 ± 2.19	2.0 ± 2.05	0.7 ± 0.42

\*Mean ± SD (Range)

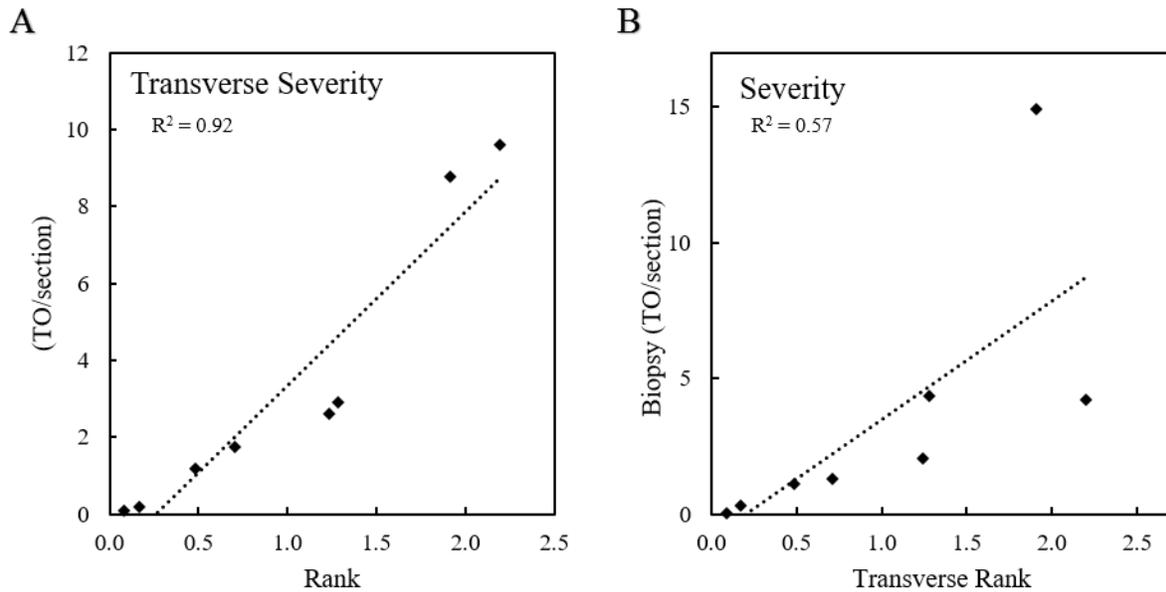


Figure 15. Relationship between severity measures in *Micropterus dolomieu* smallmouth bass, A) transverse severity (number TO per section) versus transverse rank, B) biopsy severity (number TO per section) versus transverse rank. Linear regression.

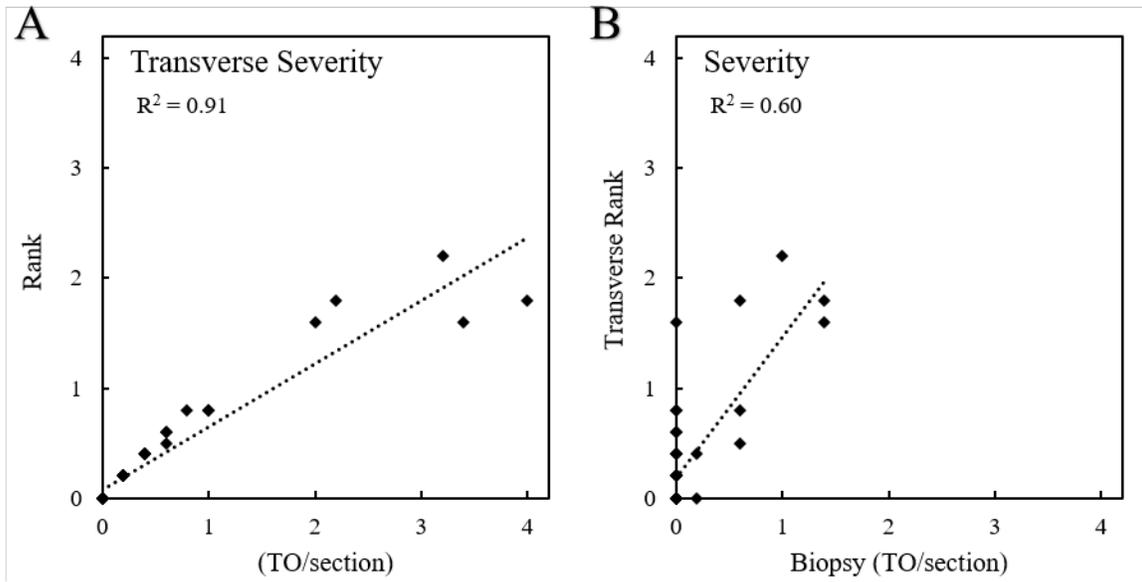


Figure 16. Relationship between severity measures in *Micropterus salmoides* largemouth bass, A) transverse severity (number TO per section) versus transverse rank, B) biopsy severity (number TO per section) versus transverse rank. Linear regression.

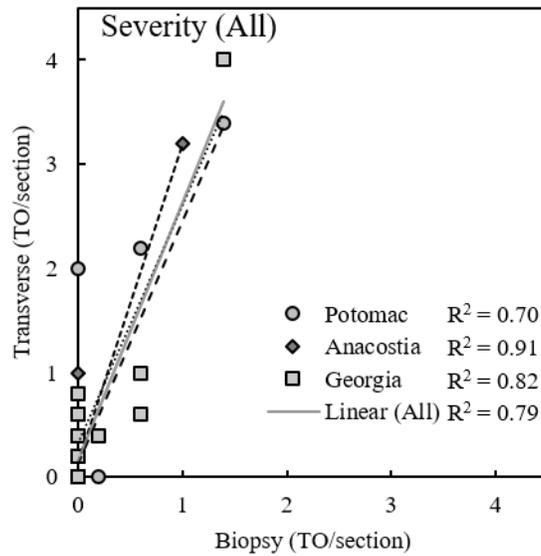


Figure 17. Relationship between the biopsy method and transverse sections for A) severity (TO count per individual) from the same individual *Micropterus salmoides* largemouth bass compared within sites and with TO pooled across sites (linear All). Linear regression.

**Wilcoxon / Kruskal-Wallis Tests (Rank Sums)**

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
A	14	229.000	560.000	16.3571	-4.332
D	7	285.000	280.000	40.7143	0.079
F	11	564.500	440.000	51.3182	1.793
H	8	558.000	320.000	69.7500	3.941
B	7	173.500	280.000	24.7857	-1.867
C	12	395.000	480.000	32.9167	-1.178
E	11	542.000	440.000	49.2727	1.468
G	9	413.000	360.000	45.8889	0.827

**1-way Test, ChiSquare Approximation**

ChiSquare	DF	Prob>ChiSq
39.1881	7	<.0001*

**Nonparametric Comparisons For All Pairs Using Dunn Method For Joint Ranking**

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
H	A	53.2946	9.96208	5.34975	<.0001*
B	H	-44.8304	11.63320	-3.85366	0.0033*
F	A	34.8799	9.05644	3.85139	0.0033*
E	A	32.8344	9.05644	3.62553	0.0081*
C	H	-36.7292	10.25952	-3.58001	0.0096*
G	A	29.4405	9.60343	3.06562	0.0608
H	D	28.9018	11.63320	2.48442	0.3633
B	F	-26.4156	10.86772	-2.43065	0.4220
D	A	24.2500	10.40505	2.33060	0.5537
E	B	24.3701	10.86772	2.24243	0.6981
G	H	-23.7431	10.92209	-2.17386	0.8320
G	B	20.9762	11.32759	1.85178	1.0000
H	F	18.3239	10.44439	1.75442	1.0000
C	A	16.4821	8.84259	1.86395	1.0000
E	C	16.2689	9.38263	1.73394	1.0000
G	C	12.8750	9.91164	1.29898	1.0000
F	D	10.4870	10.86772	0.96497	1.0000
E	D	8.4416	10.86772	0.77676	1.0000
B	A	8.3214	10.40505	0.79975	1.0000
C	B	8.0179	10.69017	0.75002	1.0000
G	D	5.0476	11.32759	0.44560	1.0000
E	F	-1.9545	9.58443	-0.20393	1.0000
G	E	-3.2828	10.10288	-0.32494	1.0000
G	F	-5.3283	10.10288	-0.52740	1.0000
C	D	-7.6845	10.69017	-0.71884	1.0000
B	D	-15.7857	12.01472	-1.31386	1.0000
C	F	-18.3144	9.38263	-1.95195	1.0000
E	H	-20.3693	10.44439	-1.95026	1.0000

Figure 18. Example of statistical analysis used to compare TO enumeration across sites.

Table 2. Series of multivariate and pairwise correlations between all three severity measures for *Micropterus dolomieu* smallmouth bass.

**Multivariate Correlations**

	<b>Transverse Mean Count</b>	<b>Biopsy Mean Count</b>	<b>Transverse Rank</b>
Transverse Mean Count	1.0000	0.6596	0.8898
Biopsy Mean Count	0.6596	1.0000	0.5518
Transverse Rank	0.8898	0.5518	1.0000

**Covariance Matrix**

	<b>Transverse Mean Count</b>	<b>Biopsy Mean Count</b>	<b>Transverse Rank</b>
Transverse Mean Count	27.31381	24.94359	5.13936
Biopsy Mean Count	24.94359	52.35177	4.41233
Transverse Rank	5.13936	4.41233	1.22126

**Pairwise Correlations**

<b>Variable</b>	<b>by Variable</b>	<b>Correlation</b>	<b>Count</b>	<b>Lower 95%</b>	<b>Upper 95%</b>	<b>Signif Prob</b>
Biopsy Mean Count	Transverse Mean Count	0.6596	79	0.5134	0.7686	<.0001*
Transverse Rank	Transverse Mean Count	0.8898	79	0.8325	0.9283	<.0001*
Transverse Rank	Biopsy Mean Count	0.5518	79	0.3767	0.6889	<.0001*

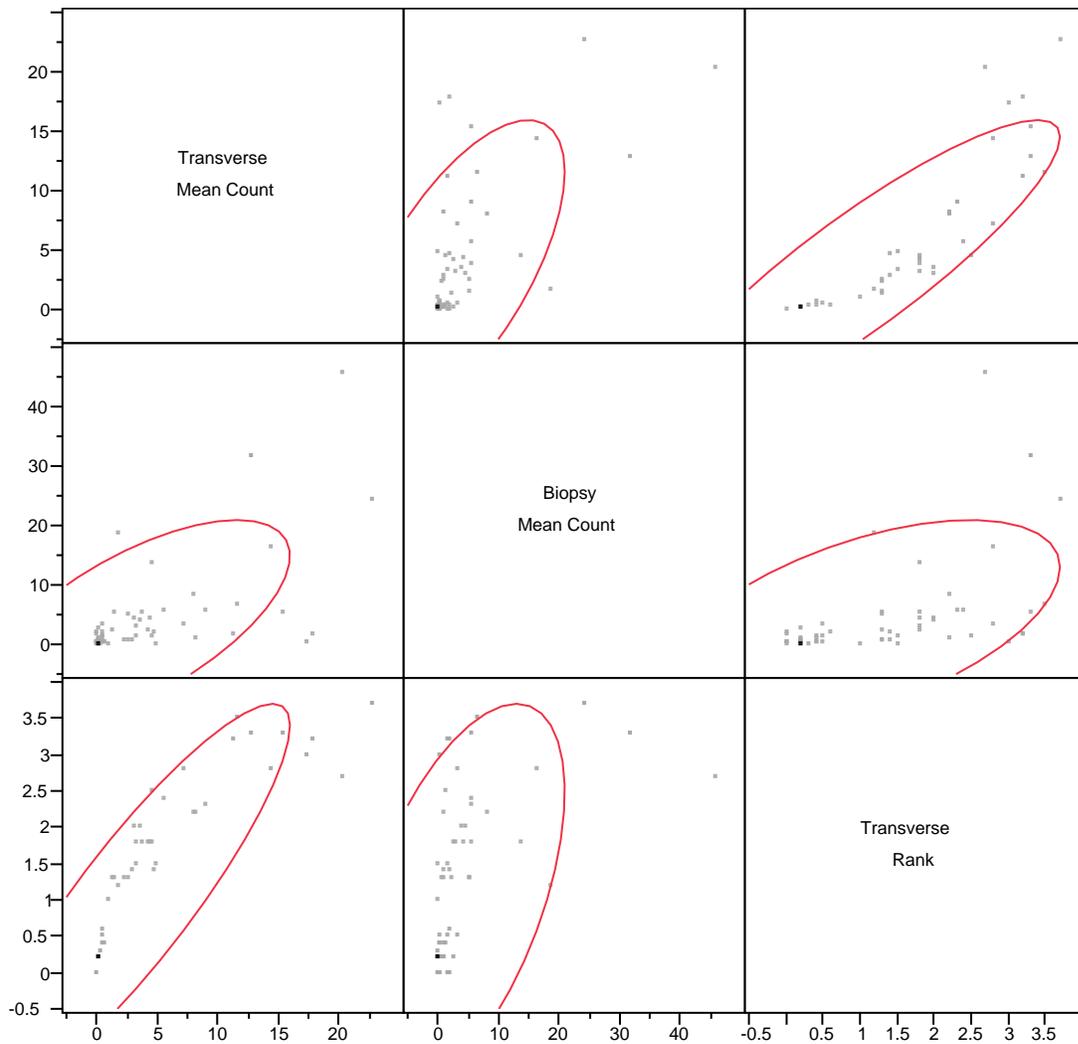


Figure 19. Scatterplot matrix of all three severity measures from *Micropterus dolomieu* smallmouth bass. Table 3. Three-way method detection of intersex comparison from *Micropterus salmoides* largemouth bass collected in Georgia. Data are presented as (+) detected and (-) not detected intersex.

Table 4. Severity metrics summary across all *Micropterus dolomieu* smallmouth bass sites for biopsy sampling method.

Individual	Prevalence		
	Transverse	Longitudinal	Biopsy
1	+	+	-
2	+	-	+
3	+	+	-
4	+	+	+
5	+	-	-
6	+	+	-
7	-	+	-

8	-	+	-
9	-	+	-
10	-	-	-
11	-	+	-
12	+	+	-
13	+	+	-
14	-	-	-
15	-	+	-
16	-	+	-
17	-	+	-
18	-	+	-
19	-	-	-
20	+	+	+
21	+	+	-
22	+	+	+
23	+	+	-
24	-	-	-

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*Methodological Approach for Reconciling Transverse Sections to Biopsies*

To reconcile the type of information collected between methods for consistent interpretation of intersex in male black bass, transverse severity ranks were correlated to biopsy oocyte counts. Since the biopsy forceps generally retrieve the same size of tissue, the biopsy oocyte counts are easily comparable without the ambiguity of an index. Due to the nature of biopsied tissue several factors must be considered before reliable semi-quantitative results can be produced. Generally, five transverse sections are analyzed to evaluate for intersex and the area of a single transverse section is on average 6x the area of a biopsy. Therefore, in order to view the same total area, each biopsy (total of 5) must be viewed at different depths (step-sections) to make a comparison based on total observable surface area. Thus, the unit that acts as an equivalent to a transverse section is five biopsies placed in a cassette, embedded in paraffin and then all five pieces of tissue are step-sectioned simultaneously with the number of TO used to relate to severity. Using the five, 6 µm thick step-sections of all five biopsies (unit for severity comparison) employed in this study, is still substantially less observable area as transverse sections (Figure 1 & 2). Since the observable area of the five biopsies is much smaller than the

transverse sections, this results in an estimation of at least 20 and up to 60 times the tissue that is observable in a transverse section. Considering that we only used 5 step-sections for TO quantification, we were extremely conservative in how they are ~~ranked~~ compared to transverse sections. For this purpose the group of five biopsies per organism were step-sectioned at least six times because the tissue is not uniform, thus it is not uncommon that the first step-section does not adequately represent all five biopsies. For this study, each step-section of the five biopsies functioned as the unit equivalent to a transverse section used for TO detection and severity. Severity of TO occurrence using biopsies was determined by relating the number of oocytes, since tissue orientation in all five biopsies is not the same and many may not include the area of concern where TO are concentrated. Depending on the quality of the step-sections, the first five good quality sections were used for prevalence and severity comparison. The oocyte count associated to each step-section (unit – 5 biopsies) was used to calculate the mean, similarly to the ranks in the transverse section method (Figure 3). The development of a predictive model will provide pathologists with a robust tool for the grading of the intersex condition in black bass species using non-lethal laparoscopic biopsy collection. This approach may be applicable in other fish species depending on the resilience of the urinary bladder to perforation. Since it is difficult to discern orientation, enumeration of TO found in each unit of biopsies was used instead of spatial attributes.