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Development of Methods to Determine the Reproductive Status of Pallid Sturgeon in the Missouri River

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Contents

Executive Summary 1
Study Area 2
General Methods 2
Results and Discussion 7
Conclusions 8
Chapter 1 Sex identification of shovelnose sturgeon using ultrasonic and endoscopic imagery, importance of reproductive stage, and application to the pallid sturgeon 9
Abstract 9
Introduction 9
Methods and Materials 10
Study Area 10
Sampling 10
Ultrasonic Imaging 11
Endoscopic Imaging 11
Sex Validation 12
Data Analysis 13
Results 13
Shovelnose Sturgeon 15
Pallid Sturgeon 18
Discussion 18
Ultrasound Effectiveness 22
Endoscope Effectiveness 23
Chapter 2 Gonadal development and seasonal plasma sex steroid and vitellogenin profiles in Missouri River sturgeons (*Scaphirhynchus* spp.) 25
Abstract 25
Introduction 25
Methods and Materials 26
Study Area and Fish Collection 26
Blood Collection and Field Processing 26
Measurements and Tissue Sampling 26
Histological Analysis 27
Sex Steroid Extraction and Analysis 27
17-β Estradiol 28
11-Ketotestosterone 28
Quality Assurance Procedures and Assay Performance Characteristics 28
Vitellogenin Analysis 28
Statistical Analysis 29
Discriminant Analysis 29
Results 30
Fecundity 30
Gonadosomatic Index and Gonadal Fat 30
Plasma Sex Steroid Concentrations 37
Plasma Vitellogenin Concentrations 41
Discriminant Analysis 41
Discussion 49
Females 49

Males 50
Sex Steroid and Vitellogenin Profiles 50
Chapter 3 Visual guide for identifying shovelnose sturgeon reproductive stages 53
Abstract 53
Introduction 53
Methods and Materials 54
Study Area and Sampling 54
Blood Collection and Field Processing 54
Ultrasonic Imaging 54
Endoscopic Imaging 55
Measurements and Tissue Sampling 56
Histological Analysis 56
Sex Steroid Extraction and Analysis 57
17-β Estradiol 57
11-Ketotestosterone 58
Quality Assurance Procedures and Assay Performance Characteristics 58
Vitellogenin Analysis 58
Analysis 59
Results 59
General Observations by Method 59
Ultrasound 59
Endoscope 59
GSI, Histology, Sex Steroids, and Vitellogenin 60
Detailed observations 60
Reproductive Stage I Female Shovelnose Sturgeon (Fig. 25) 60
Reproductive Stage II Female Shovelnose Sturgeon (Fig. 26) 60
Reproductive Stage III Female Shovelnose Sturgeon (Fig. 27) 75
Reproductive Stage IV Female Shovelnose Sturgeon (Fig. 28) 75
Reproductive Stage V Female Shovelnose Sturgeon (Fig. 29) 75
Reproductive Stage VI Female Shovelnose Sturgeon (Fig. 30) 75
Reproductive Stage I Male Shovelnose Sturgeon (Fig. 31) 76
Reproductive Stage II Male Shovelnose Sturgeon (Fig. 32) 76
Reproductive Stage III Male Shovelnose Sturgeon (Fig. 33) 76
Reproductive Stage IV Male Shovelnose Sturgeon (Fig. 34) 76
Reproductive Stage V Male Shovelnose Sturgeon (Fig. 35) 77
Reproductive Stage VI Male Shovelnose Sturgeon (Fig. 36) 77
Conclusion 77
Acknowledgements 77
References Cited 77

Figures

1. Map of the Missouri River Basin. Sturgeon Collection sites noted on map 3
2. Collection of sturgeon by USFWS using drifting trammel nets 4
3. Collection of blood from adult shovelnose sturgeon 5
4. Preparation of blood for transport back to CERC for detailed analysis 5
5. Collection of internal images of a pallid sturgeon using a portable ultrasound unit 6

6. Collection of internal images of a shovelnose sturgeon using flexible endoscope through the gonoduct wall 6
7. Body cavity of gravid female shovelnose sturgeon 7
8. Transverse images of shovelnose sturgeon in spawning condition taken using ultrasound....15
9. Monthly average reproductive stage of shovelnose sturgeon 19
10. Percent correct sex identification of shovelnose sturgeon at each reproductive stage for four imaging methods 20
11. Monthly percent correct sex identification of shovelnose sturgeon for four imaging methods21
12. Total length of female and male shovelnose sturgeon with age and reproductive stage 33
13. Fecundity with length and total fecundity with age of female shovelnose sturgeon..............34
14. Mean total fecundity, mean relative fecundity, and mean follicle length of shovelnose sturgeon by age class....35
15. Mean vitellogenin, 17-β estradiol, and 11-ketotestosterone levels in female and male shovelnose sturgeon with reproductive stage....38
16. Mean 11-ketotestosterone levels in female and male shovelnose and pallid sturgeon by month and reproductive stage 39
17. Mean 17-β estradiol levels in female and male shovelnose and pallid sturgeon by month and reproductive stage 40
18. Relationship of 11-ketotestosterone and 17-β estradiol levels for male and female shovelnose sturgeon 41
19. Mean vitellogenin levels in female and male shovelnose and pallid sturgeon by month and reproductive stage 42
20. Mean (+ SE) plasma sex steroids (11-ketotestosterone and 17-β estradiol) and vitellogenin in shovelnose sturgeon males and females captured in the lower Missouri River............45
21. Collection of internal images of a shovelnose sturgeon along the transverse plane using ultrasound....55
22. Collection of internal images of a shovelnose sturgeon along the frontal plane using ultrasound....55
23. Frontal plane ultrasonic images of shovelnose sturgeon at different reproductive stages based on Moos (1978)....61
24. Endoscopic images of shovelnose sturgeon at reproductive Stage III based on Moos (1978)62
25. Four views of a reproductive Stage I female shovelnose sturgeon and blood plasma and GSI characteristics 63
26. Four views of a reproductive Stage II female shovelnose sturgeon and blood plasma and GSI characteristics 64
27. Four views of a reproductive Stage III female shovelnose sturgeon and blood plasma, PI, and GSI characteristics 65
28. Four views of a reproductive Stage IV female shovelnose sturgeon and blood plasma, PI, and GSI characteristics 66
29. Four views of a reproductive Stage V female shovelnose sturgeon and blood plasma, PI, and GSI characteristics 67
30. Four views of a reproductive Stage VI female shovelnose sturgeon and blood plasma characteristics 68
31. Four views of a reproductive Stage I male shovelnose sturgeon and blood plasma and GSI characteristics 69
32. Four views of a reproductive Stage II male shovelnose sturgeon and blood plasma and GSI characteristics 70

33. Four views of a reproductive Stage III male shovelnose sturgeon and blood plasma and GSI characteristics 71
34. Four views of a reproductive Stage IV male shovelnose sturgeon and blood plasma and GSI characteristics 72
35. Four views of a reproductive Stage V male shovelnose sturgeon and blood plasma and GSI characteristics 73
36. Four views of a reproductive Stage VI male shovelnose sturgeon and blood plasma and GSI characteristics 74

Tables

1. Mean weight, total length, and age by month and sex of the shovelnose sturgeon collected from the Lower Missouri River used in statistical analyses 14
2. Success rate for identifying sex of shovelnose sturgeon collected in the Lower Missouri River using four methods of examination 16
3. Frequency of occurrence for the 14 possible outcome combinations of success at identifying sex 16
4. Logistic analysis of covariance of frequency of correct shovelnose sturgeon sex identification for the four methods used 18
5. Mean total lengths and weights ± SD, with range in parenthesis for male and female shovelnose sturgeon captured in the Missouri River between May 2001 and June 2002 31
6. Monthly capture of male shovelnose by reproductive stage 32
7. Monthly capture of female shovelnose by reproductive stage 32
8. Mean gonadosomatic index (GSI), plasma sex steroids, vitellogenin, and percent fat of female and male shovelnose sturgeon at various reproductive stages 36
9. One-way analysis of variance conducted to determine whether there were differences between the $\log_{10}$-transformed sex steroids and vitellogenin concentrations among the 11 groups of sex and reproductive stage 37
10. Mean gonadosomatic index, sex steroids, and vitellogenin of female and male shovelnose sturgeon at various reproductive stages 44
11. One-way analysis of variance conducted to determine whether there were differences between the $\log_{10}$-transformed sex steroids and vitellogenin concentrations among the 4 groups of sex and maturity 44
12. Results from stepwise discriminant function analysis predicting sex only, sex and maturity or reproductive stage of shovelnose sturgeon captured in the Missouri River, which was conducted using males and females combined, male only, and female only 46
13. Classification summary for determination of sex from the quadratic discriminant function analysis for shovelnose sturgeon using log10-transformed plasma vitellogenin and 11-ketotestosterone or only vitellogenin as predictors 46
14. Classification summary for determination of sex and maturity from the quadratic discriminant function analysis for shovelnose sturgeon using the significant $\log_{10}$-transformed plasma 11-ketotestosterone, 17-β estradiol, and vitellogenin as predictors 47
15. Classification summary for determination of reproductive stage from the quadratic discriminant function analysis for shovelnose sturgeon using the significant $\log_{10}$-transformed plasma indicators from the stepwise selection procedure as predictors 48

Executive Summary

The collection of sex-specific population data is fundamental to managing fish irrespective of whether the goal is commercial harvest, recreation, or recovery. Information needed by fisheries biologists includes sex ratios, the percentage of a population that is reproductively active, and seasonal reproductive patterns. Obtaining sex and reproductive information on fish can be challenging because there is often no obvious sexual dimorphism, especially outside of the breeding season. Collecting such data has traditionally necessitated sacrificing the fish for gross visual examination of the gonads, a procedure that can be time-consuming, inaccurate depending on reproductive stage or size of fish, and prohibited if the species is rare and/or endangered. Alternative non-lethal methods have been used for identifying sex and reproductive stage in fish but have not been extensively applied in sturgeon monitoring and research.

Pallid sturgeon (*Scaphirhynchus albus*) and shovelnose sturgeon (*S. platorynchus*), endemic to the Missouri and Mississippi rivers, are two of the smaller Acipenserids (Bailey & Cross, 1954; Bemis et al., 1997). While much information exists on the life history and biology of other sturgeons (e.g., *Acipenser transmontanus, A. baeri*), less is known about the pallid and shovelnose sturgeons (Keenlyne, 1997; Mayden & Kuhajda, 1997). The pallid sturgeon is very rare and is listed as endangered (Dryer & Sandvol, 1993; Mayden & Kuhajda, 1997). The shovelnose sturgeon is more common but little is known about the quality and health of the Missouri River population(s) that today are subject to unusually heavy fishing pressures due to the collapse of the European caviar industry (Canon, 2002). It is closely related and common enough to be used as a biological surrogate for the pallid sturgeon (Hesse & Carreiro, 1997) for the study of some biological characteristics. As with many sturgeon species worldwide, habitat alteration and destruction is presumably a major factor in the decline of both species (Birstein, 1993).

In recent years, state and federal initiatives have been implemented to recover and protect the Missouri River sturgeons. The U.S. Fish and Wildlife Service (USFWS) recovery plan for the pallid sturgeon lists rehabilitation of habitat in the Upper Missouri River as necessary for reproduction and recruitment (Dryer & Sandvol, 1993). The Biological Opinion issued by USFWS with regard to the operation of the Missouri River Main Stem Reservoir System, operation and maintenance of the Missouri River Bank Stabilization and Navigation Project, and operation of the Kansas River Reservoir System requires the U.S. Army Corps of Engineers (USACOE) to modify the flows out of main stem reservoirs to provide spawning cues and conditions for pallid sturgeon reproduction (USFWS, 2000; 2003). Critical to these efforts are data that allow assessment of the reproductive capacity and likely recruitment to the reproductive population. Therefore, the goal of this study was development and assessment of non-lethal methodologies in identifying sex and reproductive stage in the two Missouri River *Scaphirhynchus* species. Because validation of these methods required sacrificing a large number of animals, the majority of our data are from the shovelnose sturgeon with data from the pallid opportunistically collected.

Ultrasonography, endosocopy, blood sex steroids, and vitellogenin (VTG — an egg protein precursor) levels are non-lethal methods that may be useful in identifying sex and reproductive stage of sturgeons. Ultrasonography and endoscopy are routinely used in reproductive assessments of mammalian wildlife but only limited instances of its use are reported for fish and very few for sturgeons (ultrasound: Reimers et al., 1987; Bonar et al., 1989; Mattson, 1991; Shields et al., 1993; Blythe et al., 1994; Karlsen & Holm, 1994; Sande & Poppe, 1995; Martin-Robichaud & Rommens, 2001; Bryan et al., 2005; Endoscope: Ortenburger et al., 1996). There has been one published attempt at using endoscopy to identify

sex in sturgeon in which shortnose sturgeon *A. brevirostrum* and one cultured pallid sturgeon were examined (Kynard & Kieffer, 2002) and two, though limited in temporal extent and number, attempts at using ultrasound to identify sex of sturgeon (stellate sturgeon *A. stellatus*, Moghim et al., 2002; shovelnose sturgeon, Colombo et al., 2004). These methods alone are never 100% effective for identifying sex and reproductive stage. This is especially true for early reproductive stage fishes. Gametogenesis and reproduction are under the control of a number of different steroids such that these relative sex steroid levels will correlate with sex and stage of gamete development. Plasma sex steroid measurements in hatchery fish, and less frequently in wild fish, have been used to determine sex and reproductive stage (Amiri et al., 1996; Webb et al., 2002). However, baseline information for both sexes at different reproductive stages and juvenile life stages collected during different seasons is necessary and, currently, shovelnose and pallid sturgeon gametogenesis and reproductive endocrinology have not been documented.

Sex steroid measurements or imagery alone are unlikely to differentiate between sex and reproductive stage. Rather, a suite of methods may provide the most accurate identification of sex and stage of gonadal development. This report presents results from our efforts in development of methods to use endoscopy and ultrasonic imagery to visually observe the gonads of adult shovelnose and pallid sturgeon at different reproductive stages. Concomitantly, we developed reproductive stage-specific profiles for sex steroids and VTG important in gametogenesis and gonadal development and assessed the predictive ability of these measurements to differentiate males from females at different reproductive stages. The temporal plasma profiles and gonad histology in tandem with the imagery measurements enabled us to validate the results from the images and assess their accuracy in determining sex and reproductive stage. As much as possible, the same information collected for shovelnose was collected from field-caught pallid sturgeon.

The results presented in this report confirm that a set of non-lethal methods can be used to assess reproductive status of male and female shovelnose sturgeon with the potential for using them on pallid sturgeon. The first chapter of this report provides a detailed comparison of the effectiveness of various imaging procedures for identifying sturgeon sex with non- to minimally-invasive procedures. The second chapter provides reproductive stage-specific profiles of monthly 17-β estradiol (E2), 11-ketotestosterone (KT), and VTG together with basic gonadosomatic indices (GSI) and fecundity information and an assessment of their effectiveness as predictors of sex and reproductive stage. The third chapter provides a set of gonadal images taken from ultrasonic, endoscopic, histological, and internal examinations for each reproductive stage of the shovelnose sturgeon based on Moos (1978) along with average GSI, E2, VTG, and KT of male and female shovelnose sturgeon. This last chapter is meant to serve as a reference atlas for those working with the *Scaphirhynchus* sturgeons.

Study Area

Fieldwork was conducted in the Upper and Lower Missouri River (Fig. 1). In the Upper Missouri River section, fish were collected in Montana and North Dakota from below Fort Peck Dam, from the Lower Yellowstone River, and from near the confluence of these two rivers. In the Lower Missouri River, fish were collected in Missouri between the confluences with the Chariton and Osage rivers (Fig. 1).

General Methods

Our study objectives were to develop and assess methods for determining the reproductive status of shovelnose and pallid sturgeons through the use and cross-validation of multiple indicators that define sex and reproductive stage. Our approach involved year-round,

monthly sampling of shovelnose sturgeon, and collection of pallid sturgeon as opportunities presented. This sampling strategy was used to develop monthly reproductive stage profiles for shovelnose sturgeon. The temporal profiles include data on sex steroids and VTG plasma levels, anatomical and histological descriptions of the gonads, GSI, and laboratory and field ultrasonic and endoscopic imagery. These data were used to compare the effectiveness of ultrasonic and endoscopic technology and plasma indicators for identifying the sex of adult shovelnose sturgeon at different reproductive stages. Additionally, to a limited extent we were able to validate the methods we developed for shovelnose sturgeon for application to pallid sturgeon using fish collected for broodstock from the Missouri and Yellowstone rivers by the USFWS for their pallid sturgeon propagation and recovery program.

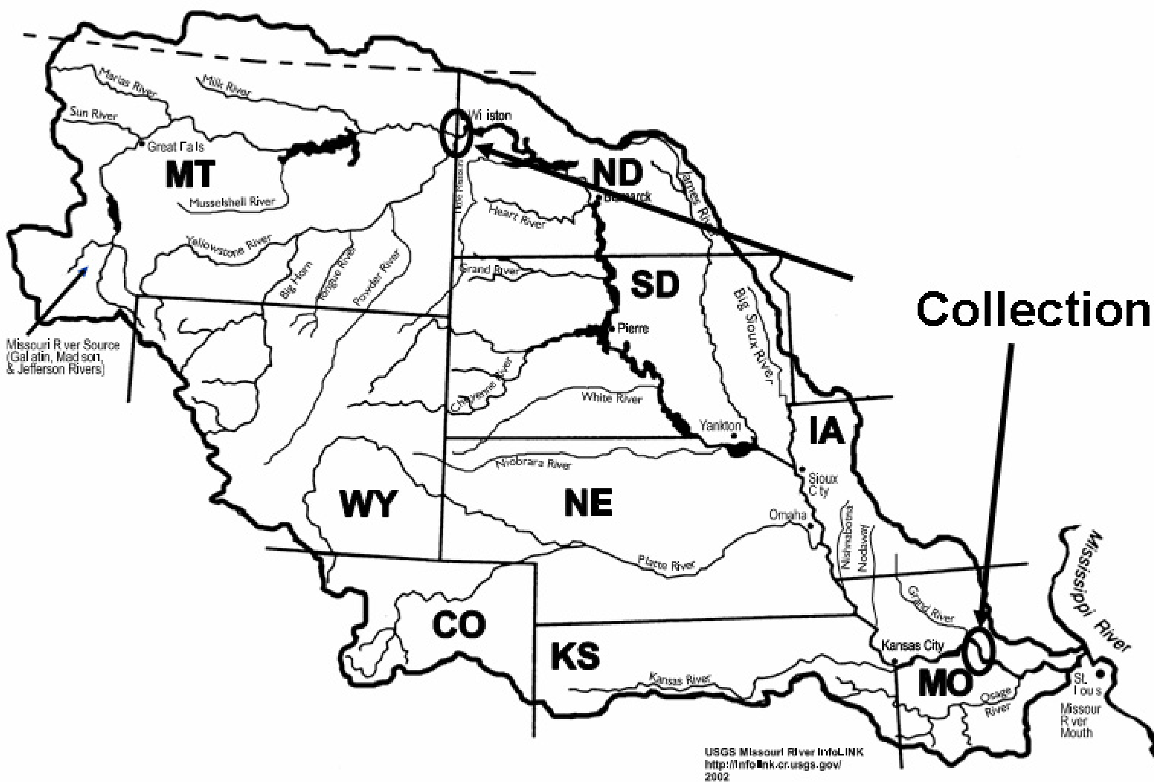


Figure 1. Map of the Missouri River basin. Sturgeon collection sites noted on map.

The majority of the data collected for this study were from shovelnose sturgeon with data from pallid sturgeon opportunistically collected. Monthly from May 2001 to June 2002, attempts were made to collect up to 30 shovelnose sturgeon (15 males and 15 females) ≥ 550 mm total length (i.e., adult fish) from the Lower Missouri River. Fish were collected using multiple gears (i.e., overnight hoopnet, trotline, stationary gillnet sets, and drifting trammel nets—Fig. 2) depending on season or behavior of the fish (e.g., hoopnets and gillnets in spring when sturgeon are migrating and trammel nets and trotlines during non-migratory periods). Pallid sturgeon samples, along with data from a limited number of shovelnose sturgeon, were collected in April 2002 from the Upper Missouri River. Though limited, these data were used to assess the application of the techniques developed on shovelnose sturgeon for use on pallid sturgeon.

Immediately after capture, shovelnose sturgeon were uniquely tagged with a Floy® tag for future reference and placed in 378.5-L tanks constantly supplied with fresh river water until the fish could be examined on shore. Within 4 h after capture 6 – 8 ml of blood were collected (Fig. 3) and processed to collect plasma (Schmitt et al., 1999; Fig. 4). To test the effect of handling and holding on sex steroid measurements, fish collected in October were bled a second time after 48 h of holding in tanks at the Columbia Environmental Research Center (CERC). River bank (i.e., field) examination was accomplished using a portable ultrasound unit (Fig. 5) and, in the case of fish collected in the Upper Missouri River, an endoscope through the gonoduct (Fig. 6) . Shovelnose sturgeon from the Lower Missouri River collections were transported to CERC where they were held at river temperatures in fiberglass flow-through circular tanks for 24-48 hrs until sex was re-examined. In the laboratory, the sex of the shovelnose sturgeon was determined using ultrasonic and endoscopic imagery. For most fish, individual ultrasonic and endoscopic analysis was videotaped to provide a documented series for later analyses.

Figure 2. Collection of sturgeon by USFWS using drifting trammel nets.

After imagery and external examination, the shovelnose sturgeon were euthanized, total length measured (tip of rostrum to where uppermost ray joins caudal filament; does not include filament), and weighed to the nearest 50 grams using a hanging scale. The body cavity was slit open and a voucher photo was taken of the gonad prior to removal. Gonads were weighed to the nearest 0.05 grams for calculating GSI (GSI = [gonad and associated fat weight/body weight]*100). Pieces of gonadal tissue were collected and preserved in 10% neutral buffered formalin (NBF) until processing for histology and determination of fecundity and polarization index (PI). The PI is determined by measuring the long diameter of the oval follicle from animal to vegetal pole and dividing this into the distance between the germinal vesicle and the animal pole (Dettlaf et. al., 1993). The first ray of the left pectoral fin was removed at the joint for age

analysis (described in Whiteman et. al., 2004). In the Upper Missouri River, pallid sturgeon were bled, examined with an ultrasound unit and endoscope then either released to the wild or utilized for broodstock.

Detailed methods for the procedures and techniques used in this study are found in each chapter. Some repetition of methods among chapters exists so that the individual chapters are complete and can stand alone.

Figure 3. Collection of blood from adult shovelnose sturgeon.

Figure 4. Preparation of blood for transport back to CERC for analysis.

Figure 5. Collection of internal images of a pallid sturgeon using a portable ultrasound unit.

Figure 6. Collection of internal images of a shovelnose sturgeon using a flexible endoscope through the gonoduct wall.

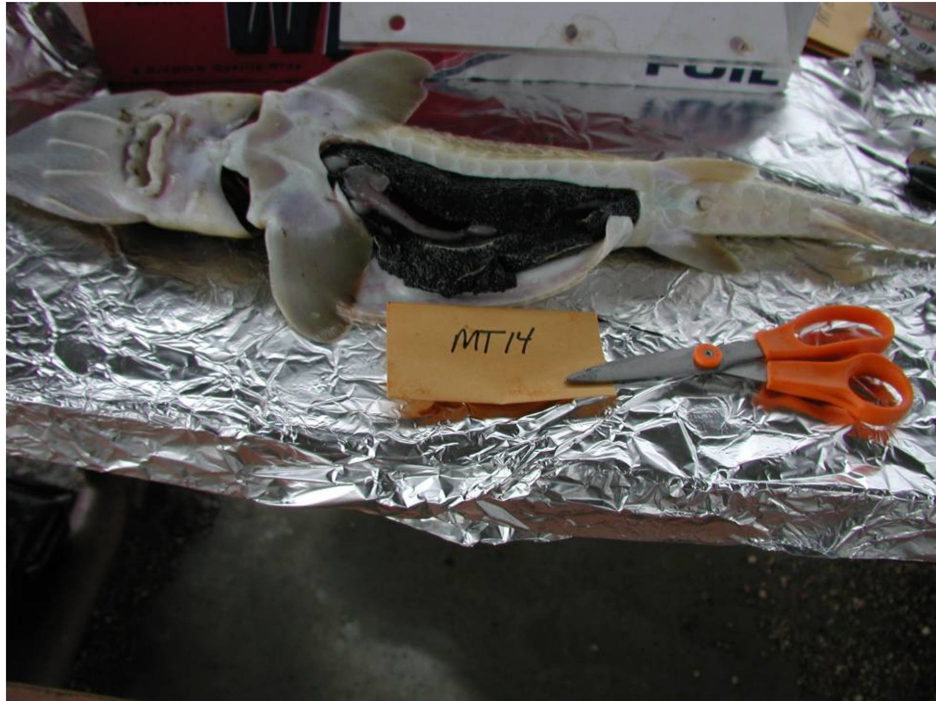


Figure 7. Body cavity of gravid female shovelnose sturgeon.

Results and Discussion

As described in Chapter 1 and by Wildhaber et al. (2005), ultrasonic and endoscopic methods were effective to some degree at sex identification of shovelnose sturgeon. The least invasive method (i.e., ultrasound scan) was least effective while the most invasive (i.e., endoscope through an abdominal incision) was the most effective for identifying shovelnose sturgeon sex. In most cases, success rate for identifying males was greater than females, with greater success at identifying both sexes in more advanced reproductive stages. Concomitantly, for most months average reproductive stage was higher for males than females. April and May were the months with the greatest average reproductive stage (i.e, closest to spawning), and were the months when the ultrasound scan was most effective. Methods were also applied to sturgeon in the Upper Missouri River to validate their use on pallid sturgeon. The ultrasound scan was effective for identifying pallid sturgeon sex; however, endoscopic examination through the gonoduct was only effective for identifying pallid sturgeon sex when the gonoduct was not opaque. This research provides data on the annual profile of reproductive stage of Missouri River shovelnose sturgeon, an evaluation of ultrasonic and endoscopic technologies for sex identification of fish, and guidance as to when, during reproductive cycles, these methods should be the most effective. Results reported here can also be found in Wildhaber et al. (2005).

In Chapter 2, seasonal and reproductive stage-specific plasma sex steroid and VTG profiles have been developed for shovelnose sturgeon from the Missouri River. Sex steroid levels fluctuated as expected during gametogenesis and were characteristically different between males and females. Pallid sex steroid levels were comparable to those of the shovelnose. Sturgeons, as with many other fish, are difficult to identify as to sex and reproductive stage because there are no obvious external sex- or reproductive stage-specific characteristics. The VTG protein was the best single predictor of shovelnose sturgeon sex with a success rate > 99%. KT, E2, and VTG were all selected for the model predicting sex (with fish

grouped into one of two maturity categories) with an 87 % success rate. Prediction of male shovelnose sturgeon reproductive stage had the lowest success rate of 32% with females having a 68% success rate. The baseline hormonal profiles developed here should be useful as indicators to assess whether the physical and environmental conditions necessary for successful reproduction exist.

In Chapter 3, a comprehensive reference set of images, plasma sex steroid, and VTG data for assessing shovelnose sturgeon sex and reproductive stage is presented. This reference set includes ultrasonic, endoscopic, histologic, and internal images of male and female gonads of shovelnose sturgeon at each reproductive stage along with complementary data on average E2, KT, VTG, and GSI. Results reported here can also be found in Wildhaber et al. (2006).

Conclusions

Histological analysis coupled with blood chemistry provided the most accurate assessment of sex and reproductive stage. Nevertheless, ultrasonography and endoscopy are useful screening tools, especially for identifying later reproductive stages. Ultrasonography and endoscopy are seemingly the least invasive techniques, particularly when the endoscope is inserted into the gonoduct. Tissue collection for histology may require killing the fish or invasive procedures (e.g., abdominal surgery, catheterization through the gonoduct). Although both methodological approaches require specialized equipment and training, ultrasonography and endoscopy, in contrast to histology and blood chemistry, provide immediate information about sex and reproductive stage. Ultimately, the research scientist, field biologist, or hatchery manager will want to choose the most appropriate method or combination of methods for their specific needs.

Efforts to protect and restore endangered pallid sturgeon populations in the Missouri River have intensified in recent years. The U.S. Fish and Wildlife Service, the Army Corps of Engineers, and state resource agencies have created habitat, and initiated monitoring, propagation and stocking programs with the goal of pallid sturgeon recovery to a self-sustaining population. Critical to evaluating the success or failure of these efforts within an adaptive management framework is an understanding of the basic biology and life cycle of the Missouri River pallid sturgeon. Given the difficulties associated with monitoring the response of populations of rare sturgeon to specific management actions, it is necessary to develop techniques to more directly measure individual reproductive response to environmental changes. These techniques should allow for the direct assessment of the reproductive stage of pallid sturgeon in the field with minimal impact to the fish. Ideally these tools should be minimally invasive, yet sufficiently sensitive to allow investigators to track the progress of individual fish through the reproductive cycle and to determine whether spawning has occurred. The rarity and endangered status of the pallid sturgeon necessitates that a closely-related, surrogate species, such as the shovelnose sturgeon, be used to develop these assessment tools. These results will assist the USACOE, in cooperation with the USFWS, in implementation of the Missouri River Biological Opinion and also assist them as they work with all of their partners to recover the pallid sturgeon. The information will also be useful for artificial propagation of both species.

CHAPTER 1

Sex Identification of Shovelnose Sturgeon Using Ultrasonic and Endoscopic Imagery, Importance of Reproductive Stage, and Application to the Pallid Sturgeon

Abstract

Monthly sampling of shovelnose sturgeon, a biological surrogate for the endangered pallid sturgeon, was conducted to develop a multi-seasonal profile of reproductive stage. Data collected included histological characteristics of gonads from wild-caught fish and laboratory and field ultrasonic and endoscopic images. These data were used to compare effectiveness of ultrasonic and endoscopic techniques for identifying sex of adult shovelnose sturgeon at different reproductive stages. The least invasive method (i.e., ultrasound scan) was least effective while the most invasive (i.e., endoscope through an abdominal incision) was the most effective for identifying shovelnose sturgeon sex. In most cases, success rate for identifying males was greater than females, with greater success at identifying both sexes in more advanced reproductive stages. Concomitantly, for most months average reproductive stage was higher for males than females. April and May were the months with the highest reproductive stage, and were the months when the ultrasound scan was most effective. Methods were also applied in the Upper Missouri River to validate their use on pallid sturgeon. The ultrasound scan was effective for identifying pallid sturgeon sex; however, endoscopic examination through the gonoduct was only effective for identifying pallid sturgeon sex when the gonoduct was not opaque. This research provides data on the annual profile of reproductive stage of Missouri River shovelnose sturgeon, an evaluation of ultrasonic and endoscopic technologies for sex identification of fish, and guidance as to when, during reproductive cycles, these methods should be the most effective. Results reported here can also be found in Wildhaber et al. (2005).

Introduction

Ultrasonic and endoscopic methods have been previously used in sex identification of fishes (e.g., Reimers et al., 1987; Ortenburger et al., 1996). Ultrasonic technology has been used successfully to identify sex of various non-sturgeon species such as Neosho madtom *Noturus placidus* (Bryan et al., 2005), Atlantic salmon *Salmo salar* (Mattson, 1991; Sande & Poppe, 1995), Atlantic halibut *Hippoglossus hippoglossus* (Shields et al., 1993; Martin-Robichaud & Rommens, 2001), striped bass *Morone saxatilis* (Blythe et al., 1994), cod *Gadus morhua* (Karlsen & Holm, 1994), Pacific herring *Clupea harengus pallasi* (Bonar et al., 1989), winter *Pleuronectes americanus* and yellowtail flounders *P. ferruginea* and haddock *Melanogrammus aeglefinus* (Martin-Robichaud & Rommens, 2001), and rainbow trout *Oncorhynchus mykiss* (Reimers et al., 1987). Successful use of endoscopic technology to identify sex of non-sturgeon species was reported in only one species, Arctic charr, *Salvelinus alpinus* (Ortenburger et al., 1996). There has been one documented attempt at using endoscopy on shortnose sturgeon *A.*

brevirostrum and one cultured pallid sturgeon (Kynard & Kieffer, 2002), however no published works on the use of endoscopy on shovelnose sturgeon. Though limited in temporal extent and number, there are two published assessments of the effectiveness of ultrasound for identifying sex of sturgeon (stellate sturgeon *A. stellatus*, Moghim et al., 2002; shovelnose sturgeon, Colombo et al., 2004).

The overall goal of this aspect of our research was to evaluate the application of ultrasonic and endoscopic methods to visually observe the gonads of adult shovelnose and pallid sturgeons for sex identification. The specific objective was to compare the effectiveness of using endoscopic and ultrasonic imagery to accurately identify sex of shovelnose and pallid sturgeons at different reproductive stages throughout the year in the laboratory and the field. The tools being assessed in this study could ultimately be used to identify breeding sturgeons, especially pallids, and in monitoring the effects of river management strategies on reproductive activity.

Methods and Materials

The first aspect of this research involved year-round, monthly sampling of shovelnose sturgeon, the procedural surrogate for pallid sturgeon, to develop reproductive stage profiles. The temporal profile data collected on these fish included histological descriptions of the gonads and laboratory- and field-collected ultrasonic and endoscopic imagery. These data were used to compare the effectiveness of ultrasonic and endoscopic technology for identifying the sex of adult shovelnose sturgeon at different reproductive stages. The second aspect of this research involved validating the methods we developed for shovelnose sturgeon for application to pallid sturgeon. These data were obtained from fish collected for broodstock from the Missouri and Yellowstone rivers by the USFWS for their pallid sturgeon propagation and recovery program.

Study Area

Fieldwork was conducted in the Upper and Lower Missouri River (Fig. 1). In the Upper Missouri River section, fish were collected in Montana and North Dakota from below Fort Peck Dam, from the Lower Yellowstone River, and from near the confluence of these two rivers. In the Lower Missouri River, fish were collected in Missouri between the confluences with the Chariton and Osage rivers (Fig. 1). The geographic extent from which the shovelnose sturgeon collections were taken was limited to a short reach of the Missouri River to control for potential population differences in reproductive status that may be present at any given time as a result of the latitudinal temperature gradient found in the Missouri River (Galat et al., 2001).

Sampling

The great majority of the data collected for this study were from shovelnose sturgeon with data from pallid sturgeon opportunistically collected. Monthly from May 2001 to June 2002, attempts were made to collect up to 30 shovelnose sturgeon (15 males and 15 females) $\geq$ 550 mm total length (i.e., adult fish) from the Lower Missouri River. Fish were collected using multiple gears (i.e., overnight hoopnet, trot line, stationary gillnet sets, and drifting trammel nets) depending on season or behavior of the fish. Pallid sturgeon samples, along with data from a limited number of shovelnose sturgeon, were collected in April 2002 from the Upper Missouri River. These fish were collected for broodstock by the USFWS in their pallid sturgeon propagation and recovery programs. Though limited, data from pallid sturgeon were used to assess the application of the techniques developed on shovelnose sturgeon for use on pallid sturgeon.

Immediately after capture, shovelnose sturgeon were uniquely tagged with a Floy® tag for future reference. Tagged sturgeon were held in 378.5-L tanks constantly supplied with fresh river water until the fish could be examined on shore using a portable ultrasound unit and, in the case of fish collected in the Upper Missouri River, with the endoscope through the gonoduct. The shovelnose sturgeon collected in the Lower Missouri River were transported to the Columbia Environmental Research Center (CERC) where they were held at river temperatures in fiberglass flow-through circular tanks for 24-48 hrs until the sex was re-examined. In the laboratory, the sex of these fish was determined using ultrasonic and endoscopic imagery that was later verified through histological examination. Histological samples were taken on site from the shovelnose sturgeon collected in the Upper Missouri River. All shovelnose sturgeon collected were humanely euthanized prior to collection of histological samples. After examination with ultrasound and endoscope units, pallid sturgeon were released to the wild or utilized for broodstock by the USFWS in their pallid sturgeon propagation and recovery programs.

Ultrasonic Imaging

Ultrasonic imagery was collected in the laboratory using a clinical laboratory ultrasound unit (Shimadzu SDU-400 Plus with a 7.5 Mhz linear probe). In October 2001 the use of a portable ultrasound unit in the field began (Sonosite 180 Plus with a 5-10 MHz linear probe—Fig. 5). Application of ultrasound followed basic methods (Zwiebel & Sohaey, 1998) with slight modifications. Prior to scanning the body cavity of the fish, the scanning surface of the ultrasound transducer was first covered with ultrasound gel followed by a plastic sheath over the entire ultrasound transducer. During scanning, focus depth, output power, and frame rate were all kept constant. The other ultrasound settings varied slightly from what our preliminary sturgeon examinations identified as optimal ultrasound settings: frame averaging on, gain of 16, and whole body.

In order to acquire an interpretable ultrasonic image of sturgeon internal organs, ultrasonic images had to be collected from the abdominal surface of the fish. Ultrasonic signals cannot penetrate the calcified surface of the scutes found on sturgeon back and sides and thus, limited images of internal organs can be generated by ultrasound through these surfaces. To gain the highest resolution of internal organs possible with ultrasound, the fish were held ventral side up just below the surface of the water. The fish were docile enough in this position that no anesthesia was necessary. Whether in the field or in the laboratory, a full body scan along the abdominal surface was performed on each fish from vent to operculum with the ultrasound probe oriented parallel to the transverse plane (Fig. 5). Images were recorded for later analyses.

Endoscopic Imaging

The endoscopes initially used were rigid borescopes, 18 mm in length and 2 mm in diameter with a standard light source (Zibra Corporation). The first borescope had a 10-mm focal length with a view directly from the end of the scope. The second borescope had a 4-mm focal length and a view that was at a 70° angle to the end of the probe. Experience with the previous two scopes prompted having a custom built flexible borescope produced by Zibra Corporation to meet our specifications (i.e., Milliwand flexible probe, model number P20.250.70 FMW2 with a 1-mm focal length and a view that was at a 70° angle to the end of the probe—Fig. 6) with which use began in October of 2001. For all the endoscopes used, the endoscope was covered by a glass tube that was sealed on one end in order to acquire a clearly focused image.

Using the endoscope, attempts were made to view the gonads of each fish in two ways: through the gonoduct wall (shovelnose and pallid sturgeon) and through a small ventral incision in the body cavity wall (shovelnose sturgeon only). During examination, the fish was oriented ventral side up with the tail of the fish being raised out of the water while the head and gills were kept submerged. The result was a fish that was at a 45° angle to the water surface. This angle increased the ease of insertion of the endoscope.

The endoscope was inserted into the urogenital pore immediately posterior to the vent. The urogenital pore opens into a short sinus that branches into paired gonoducts. The paired gonoducts are located dorso-laterally between the body cavity wall and mesonephros and run from the urogenital sinus to the approximate mid-point of the body cavity (Hoar, 1969; Moos, 1978). The gonoducts terminate in a collapsible funnel-like structure, or ostia, that acts as a one-way valve to release mature oocytes from the coelomic cavity while preventing external materials from entering. The gonoducts in male sturgeon are vestigal and do not appear to have a function. To view the gonads through the gonoduct wall, the probe was inserted into the urogential pore and then directed into the right or left gonoduct.

Viewing of the gonads through an abdominal incision was only performed in the laboratory. In order to view the gonads through the body cavity wall, first a small anterior to posterior incision was made in the ventral wall of the body cavity towards the posterior end of the fish just off the midline of the body using a scalpel. The incision was approximately 1-2 cm long and was just deep enough to penetrate the body cavity wall. The endoscope was inserted through the incision and run simultaneously posterior to anterior and ventral to dorsal along the full length of the body cavity.

Sex Validation

Histological analysis was conducted on the gonads of the sturgeon to accurately determine sex and reproductive stage. Samples were preserved and processed according to methods outlined by Blazer (2002). Briefly, after an initial fixation of 48 h in 10% neutral buffered formalin, samples were transferred through a series of Hepes Buffer rinses to 70% ethanol (EtOH). The samples were stored in the 70% EtOH until additional processing occurred. Routine processing of tissue involved trimming into small pieces, dehydration through a series of alcohols followed by immersion in an organic solvent, then infiltration with paraffin. Paraffin blocks containing the tissues were cut into 5-μm slices. Sections were taken at three different depths to ensure the microscopic evaluations were representative of the entire tissue. These sections were placed on glass slides, allowed to dry, and then deparaffinized. The slides were stained with hematoxylin and eosin (Luna, 1968).

Light microscopy was performed to evaluate each fish as to sex and reproductive stage according to Moos (1978) without date of capture considered. Gonads of male fish were classified as Stage I- Immature: lots of fat, few spermatogonia; Stage II-Developing: germ cells consist mostly of spermatogonia in nests or singly; Stage III-Spermatogenic: lobules filled with spermatocytes, spermatids or developing spermatozoa; Stage IV- Pre-spawning: most lobules filled with spermatozoa; Stage V- Spawning: all lobules are filled with or releasing spermatozoa; Stage VI-Post-spawn (Spent): collapsed lobules empty or containing some residual spermatozoa. Gonads of female fish were classified as Stage I-Immature: large fat body, few small oogonia; Stage II-Developing: pre-vitellogenic oocytes abundant, less fat than Stage I and lamellar structure of gonad obvious (i.e., ovigerous folds); Stage III-Vitellogenesis: yolk deposition, oocytes greatly increase in size and are enveloped by follicular cells, and the germinal vesicle is near center of follicle; Stage IV-Pre-spawning: follicles enlarge, begin to turn black, and the germinal vesicle begins to shift towards the animal pole; Stage V-Spawning:

germinal vesicle continues to migrate closer to animal pole where it will ultimately breakdown as meiosis resumes, follicles are black; Stage VI-Post-spawn (Spent): no to little fat, empty follicles, few atretic follicles, and early oocytes.

Data Analysis

The statistical analyses conducted were designed to compare and contrast the frequency of correct identification of shovelnose sturgeon sex by field ultrasound (FU), laboratory ultrasound (LU), endoscope through gonoduct (GE), and endoscope through abdominal incision (IE). Categorical model analysis was done to directly compare the success rate for each of the four methods. This was done by looking at the frequency of occurrence of the 14 possible outcomes of correct versus incorrect sex identification for each fish using the four different methods. Two-way (i.e., month and sex) logistic analysis of covariance with reproductive stage as a covariate was done for each of the four techniques individually to determine which factors best described the observed results. Two-way (i.e., month and sex) logistic analysis of variance of reproductive stage was also conducted to assess the influence of month and sex on the reproductive stage. Statistical significance was tested at the $p<0.05$ level with $p<0.10$ considered marginally significant.

Results

For Wildhaber et al. (2005), 13 months of data on 405 individual fish were collected (data from 10 fish were not included due to a total length < 550 mm and one additional due to total length not being recorded). The data included 369 shovelnose sturgeon caught in the Lower Missouri River and 9 shovelnose and 16 pallid sturgeon from the Upper Missouri River. Upon internal examination of the shovelnose sturgeon from the Lower Missouri River, 25 (7%) were found to have intersex gonads (i.e., characteristics of both male and female gonads) and one was too young for sex assessment**.** Statistical analysis of the various methods used to identify the sex of individual shovelnose sturgeon was thus focused on the resulting 343 adults from the Lower Missouri River. At time of publication (Wildhaber et al., 2005), histological reproductive stage data was only available for 334 of the 343 shovelnose sturgeon from the Lower Missouri River and 8 of the 9 shovelnose sturgeon collected in the Upper Missouri River. Of the shovelnose sturgeon, 1 (0.3 %) female and 17 (5 %) males were identified as Stage VI by histological criteria. Stages II and VI can be distinguished from each other through histological examination but not through gross visual examination (Moos, 1978); the ultrasound and endoscope units provide gross visual examination of gonadal tissue. Therefore, the 18 Stage VI fish were grouped with Stage II fish for analysis of ultrasonic and endoscopic results. Delayed acquisition of a portable ultrasound unit meant only 224 of adult shovelnose sturgeon used in the analyses were examined with all four methods.

After release of Wildhaber et al. (2005), and upon reexamination by a second individual, there were 47 fish that had reproductive stage and/or sex changes. The corrected data included: 4 female fish that did not have a stage were assigned one, 39 female reproductive stages were changed (I to II = 12, II to I = 2, II to III = 10, III to II = 3, III to IV = 5, IV to III = 5, IV to V = 2), 2 fish that were intersex were changed to female stage III, 1 fish that was a female Stage III was changed to an intersex fish, 1 fish that was a female Stage VI was changed to a male Stage II. As expected, since the overwhelming majority of the changes were only one reproductive stage, included increases and decreases in stage and represented less than 15% of the data used in Wildhaber et al. (2005), a reanalysis of Wildhaber et al. (2005) produced essentially identical results and are presented in this report to provide the most up to date data.

Table 1 contains length, weight, and age of the shovelnose sturgeon collected in this study based on the updated data.

During scanning with ultrasound a search was done for gonadal structures throughout the body cavity. On the monitor, individual oocytes or follicles of both species appeared as a pair of light colored parabolas (Fig. 8). For females in the later reproductive stages, these paired parabolas were visible throughout the body cavity. The resulting image presented a body cavity with fairly uniform light/dark contrast throughout. The testes in the later reproductive stages were large and well-defined paired structures that were generally oval in shape that most often occurred along the entire dorsal side of the body cavity (Fig. 8). The resulting male image presented a body cavity with a pair of fairly well-defined oval structures with fairly uniform light coloration throughout.

Table 1. Mean weight, total length, and age by month and sex of the shovelnose sturgeon collected from the Lower Missouri River used in statistical analyses in Chapter 1. Standard deviation and N for each mean, respectively, are given in parentheses.

	Weight (g)		Total Length (mm)		Age (yr)	
Month	Female	Male	Female	Male	Female	Male
January	1025.00	900.00	738.79	710.71	18.71	18.86
	(216.4, 14)	(185.2, 14)	(46.1, 14)	(44.0, 14)	(3.8, 14)	(2.8, 14)
February	757.69	746.15	676.15	685.54	17.15	17.23
	(186.9, 13)	(163.9, 13)	(49.1, 13)	(48.5, 13)	(3.6, 13)	(2.8, 13)
March	825	891.67	682.5	702.44	18.2	17.72
	(226.4, 10)	(320.5, 18)	(41.7, 10)	(67.4, 18)	(2.9, 10)	(3.9, 18)
April	1114.71	945	740.59	713.9	18.71	18.3
	(243.5, 17)	(149.9, 10)	(43.2, 17)	(37.6, 10)	(4.4, 17)	(2.4, 10)
May	998.81	1024.07	699.48	710.85	18.78	17.93
	(364.9, 21)	(368.0, 27)	(47.6, 23)	(66.4, 27)	(3.0, 23)	(2.9, 27)
June	815.38	744.44	684.62	676.83	16.15	16.72
	(203.2, 13)	(169.0, 18)	(50.1, 13)	(37.7, 18)	(2.8, 13)	(3.39, 18)
July	792.5	709.09	682.25	664.73	18.75	16.27
	(211.9, 4)	(159.4, 11)	(72.9, 4)	(40.8, 11)	(2.9, 4)	(2.3, 11)
August	825.00	711.11	688.20	663.67	18.22	16.95
	(332.7, 10)	(163.5, 9)	(65.0, 10)	(57.7, 9)	(3.6, 9)	(3.5, 9)
September	784.38	745.45	667.74	666.39	16.95	17.23
	(292.0, 16)	(150.8, 11)	(63.5, 19)	(32.1, 13)	(3.4, 19)	(3.3, 13)
October	786.54	830.59	686.54	679.29	16.62	16.81
	(295.7, 13)	(262.1, 17)	(55.7, 13)	(55.7, 17)	(3.3, 13)	(2.7, 16)
November	675	700	652.08	661.38	15.58	16.19
	(290.9, 11)	(189.3, 16)	(73.9, 12)	(49.4, 16)	(2.9, 12)	(3.5, 16)
December	754.17	764.44	673.17	686.72	17.33	17.11
	(205.6, 12)	(155.4, 18)	(60.2, 12)	(41.8, 18)	(2.1, 12)	(3.2, 18)

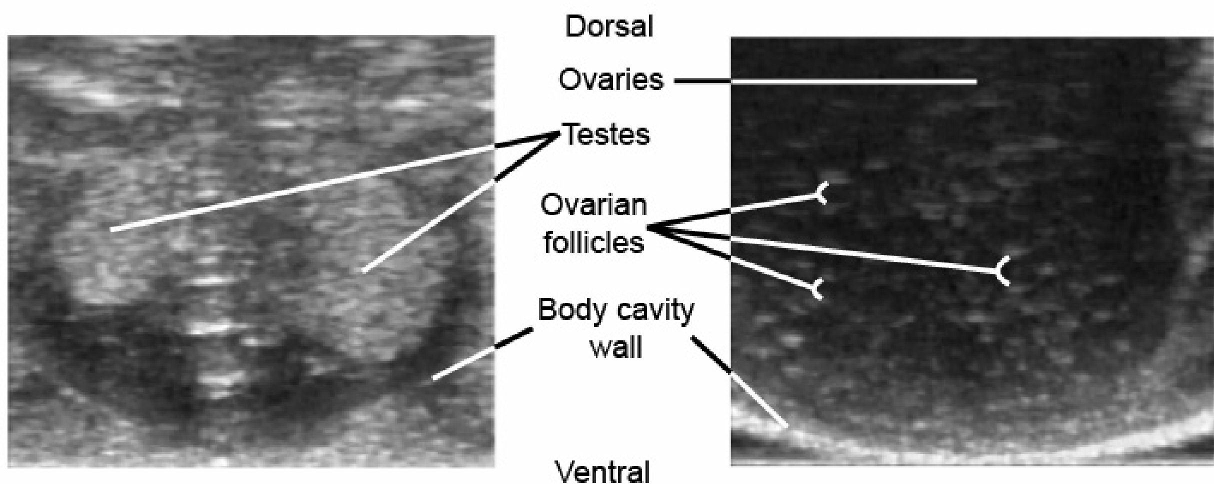


Figure 8. Transverse images of shovelnose sturgeon in spawning condition taken using ultrasound. The left image is of a male collected in October 2001 and the right is of a female collected in July 2001; both were identified as reproductive Stage IV (i.e., pre-spawning).

During viewing with an endoscope a search was done for gonadal structures along the length of the body cavity of each fish indirectly through both gonoducts and directly through the abdominal incision. Oocytes or follicles of the female sturgeon were obvious when present. The oocytes and follicles ranged from small yellow particles (oocytes at a very early reproductive stage), to medium-sized light gray circles (maturing follicles), to dark black circles in which one follicle alone filled the entire viewing area of the endoscope (mature follicle). For females in the later reproductive stages, follicles dominated the image seen through the endoscope along the entire length of the body cavity. The ovaries of females in the earliest reproductive stages and in the post-spawning reproductive stage contained small white oocytes or black particles (melanin pigment) leftover after spawning, respectively; these reproductive stages also contained lamella-like folds. Later reproductive stage male testes appeared as large yellow surfaces. The testicular surface was finely textured and lacked the granular texture that the presence of oocytes and follicles in females created. The testes also lacked the lamella-like folds seen in ovaries. Early reproductive stage testes typically had a large amount of fat attached. Fat was distinguishable from testicular tissue by having a smoother surface and fewer blood vessels than testes.

Shovelnose Sturgeon

Sex identification of Lower Missouri River shovelnose sturgeon was possible by all four methods evaluated (FU, LU, GE, and IE); the more invasive the method, the more effective it was (Table 2). Categorical analyses further supported these results through direct comparison among the four methods for those fish on which all four methods were applied (Table 3). Analysis of the 14 possible outcomes of correct versus incorrect sex identification showed that, for the 225 fish on which all four methods were used, the sex of just over half was correctly identified by all four methods.

Table 2. Success rate for identifying sex of shovelnose sturgeon collected in the Lower Missouri River using four methods of examination.

	Field Ultrasound (FU)	Laboratory Ultrasound (LU)	Gonoduct Endoscope (GE)	Incision Endoscope (IE)
% correctly sex identified (correct/total)	68	70	85	92
	(154/225)	(240/344)	(292/344)	(302/327)
% correctly identified males (correct/total)	76	75	93	93
	(91/119)	(139/184)	(172/184)	(161/174)
% correctly identified females (correct/total)	59	63	75	92
	(63/106)	(101/160)	(120/160)	(141/153)

Table 3. Frequency of occurrence of the 14 possible outcome combinations of success for identifying sex. This is based on the 225 (of the 339 used in this study) Lower Missouri River shovelnose sturgeon on which all four methods of internal examination (i.e., field ultrasound, laboratory ultrasound, endoscope through gonoduct, and endoscope through abdominal incision) were used. Also given is the parameter estimate, Chi-square value, and associated p-value of the frequency of occurrence (with percent of the 225 observations in parentheses) of each combination from the categorical analysis of variance. 'Correct' represents correct sex identification.

Combination Number	Field Ultrasound (FU)	Laboratory Ultrasound (LU)	Gonoduct Endoscope (GE)	Incision Endoscope (IE)	Parameter Estimate	Chi-square	P value	Frequency of Occurrence (%)
1	Correct	Correct	Correct	Correct	3.11	276.23	<0.0001	115 (51.1)
2	Correct	Correct	Correct	Incorrect	-0.24	0.24	0.6	4 (1.8)
3	Correct	Correct	Incorrect	Correct	-0.93	1.91	0.17	2 (0.9)
4	Correct	Correct	Incorrect	Incorrect	-1.63	2.99	0.08	1 (0.4)
5	Correct	Incorrect	Correct	Correct	1.71	49.84	<0.0001	28 (12.4)
6	Correct	Incorrect	Incorrect	Correct	-0.93	1.91	0.17	2 (0.9)
7	Correct	Incorrect	Incorrect	Incorrect	-0.93	1.91	0.17	2 (0.9)
8	Incorrect	Correct	Correct	Correct	1.59	40.86	<0.0001	25 (11.1)
9	Incorrect	Correct	Incorrect	Correct	-0.93	1.91	0.17	2 (0.9)
10	Incorrect	Correct	Incorrect	Incorrect	-1.63	2.99	0.08	1 (0.4)
11	Incorrect	Incorrect	Correct	Correct	1.42	29.31	<0.0001	21 (9.3)
12	Incorrect	Incorrect	Correct	Incorrect	-0.93	1.91	0.17	2 (0.9)
13	Incorrect	Incorrect	Incorrect	Correct	1.26	21.18	<0.0001	18 (8.0)
14	Incorrect	Incorrect	Incorrect	Incorrect	0			2 (0.9)
Total								225

Endoscopic methods were the most consistent for identifying shovelnose sturgeon sex (Table 3). Analyses showed that IE was the most accurate method for identifying sex, with none of the occurrences where IE provided incorrect sex identifications significant (i.e., frequencies were not significantly greater than 0). Endoscope through the gonoduct was also effective for identifying sex but was slightly less accurate than IE, with significant frequencies of incorrect sex identifications only when sex identifications by FU and LU were also incorrect (combination 13). Further evidence for the more accurate nature of the endoscope over ultrasound examination comes from the fact that there were significant occurrences where GE and IE gave correct sex identifications while FU and LU gave incorrect sex identifications (combination 11); this was one of the only other three situations that occurred at a significant frequency. Furthermore, occurrences where IE and/or GE gave incorrect sex identifications while FU and LU gave correct sex identifications did not occur at significant frequencies. Although four out of the five occurrences that had frequencies significantly greater than zero included FU, LU, or both as incorrect, ultrasound was still an effective method for sex identification of shovelnose sturgeon. The effectiveness of ultrasonic methods was supported by the fact that the two occurrences with significant frequencies where sex was correctly identified by either FU (combination 5) or LU (combination 8) included correct identification of sex by both GE and IE and occurred in similar frequencies. Furthermore, a significant frequency was observed for the combination in which GE was incorrect when the frequency of occurrence of FU and LU were both incorrect (combination 13).

Reproductive stage was the factor that overwhelmingly determined the success of all four methods of shovelnose sturgeon sex identification even after accounting for month and sex factors (Table 4). Logistic analysis of variance showed that month accounted for most of the variation in reproductive stage (df = 11, $X_2 = 52.88$, $P < 0.0001$) with sex also accounting for a marginally significant amount of the variance (df = 1, $X_2 = 3.67$, $P < 0.056$; Fig. 9). The greatest average reproductive stages for both sexes were observed in April and May (Fig. 9). Only in May, June, and August was the average reproductive stage slightly higher for females than males; for 8 of the 9 other months average reproductive stage for males was greater than for females. As reproductive stage increased so did our ability to correctly identify the sex of a shovelnose sturgeon using all four methods (Fig. 10). Simultaneously, male shovelnose sturgeon were more often correctly identified than females with the lesser invasive methods at earlier reproductive stages (I and II). Only the IE had a greater than 80% success rate for reproductive Stages II through V. For all methods, except IE, overall success rates for identifying males were higher than for females (Table 2). Month was not even marginally significant for any of the methods after accounting for sex and reproductive stage (Table 4 and Figs. 9 and 11).

The nine shovelnose sturgeon collected from the Upper Missouri River were all identified as females upon histological examination. In the field, the sex of the same six shovelnose sturgeon was correctly identified with FU, GE, and IE. The sex of a seventh fish was correctly identified with GE. Of the six shovelnose sturgeon for which sex was correctly identified by all methods, there was reproductive stage data for five. Four of these five fish were at reproductive Stage IV and one was found to be Stage VI; this latter fish was found to contain a large amount of fat. The three shovelnose sturgeon for which sex was incorrectly identified by at least one method were at reproductive Stages I through III.

Table 4. Logistic analysis of covariance of frequency of correct shovelnose sturgeon sex identification for the four methods used.

	Method used to determine shovelnose sturgeon sex Chi-square (P value)			
	Field Ultrasound (FU)	Laboratory Ultrasound (LU)	Gonoduct Endoscope (GE)	Incision Endoscope (IE)
Month	13.17	12.32	7.39	9.4
	(0.106)	(0.340)	(0.766)	(0.585)
Sex	3.78	6.47	16.76	0.66
	(0.052)	(0.011)	(<0.0001)	(0.415)
Reproductive stage	32.56	56.04	26.28	15.89
	(<0.0001)	(<0.0001)	(<0.0001)	(<0.0001)
R-square	0.27	0.29	0.23	0.14
Percent concordance	83.2	83.9	86.8	85.8
N	225	339	339	327

Pallid Sturgeon

Of the 16 pallid sturgeon collected in Upper Missouri River, 11 were verified to be males and three as females. The sex of two pallid sturgeon had not been verified at the time of publication of Wildhaber et al. (2005; Steve Krentz, USFWS, personal communications). Subsequently, one of these fish was identified as a female and was incorrectly identified as a male by both methods. The sex of 12 of the 14 fish of known sex was correctly identified using ultrasound. Using the endoscope through the gonoduct, six of these 14 pallid sturgeon had opaque gonoducts and thus, their gonads could not be clearly seen. Of the eight pallid sturgeon for which we could see through the gonoduct with an endoscope the sex of all were correctly identified as six males and two females.

Discussion

This study presents the first direct comparison of the effectiveness of ultrasonic and endoscopic technology for identifying the sex of fish. More importantly, this research is the first documented attempt to directly compare the effectiveness of endoscope and ultrasound units. All four methods tested in this study were effective to some degree for sex identification of shovelnose sturgeon. As might be suspected, success associated with application of a method was dependent on their invasiveness. The least invasive method (i.e., ultrasound scan) was the least effective while the most invasive (i.e., endoscope through an abdominal incision) was the most effective. Except for the IE, all methods were more effective for identifying males than females with greater effectiveness for identifying sex in later reproductive stages. At the earlier reproductive stages, testes were more easily identifiable by all methods due to their size and definition compared to ovaries. As reproductive stage advanced the size and definition of the testes increased as did the number, size, and extent of oocytes or follicles within the body cavity and thus, our ability to identify both sexes increased for all methods. Concomitantly, for most months average

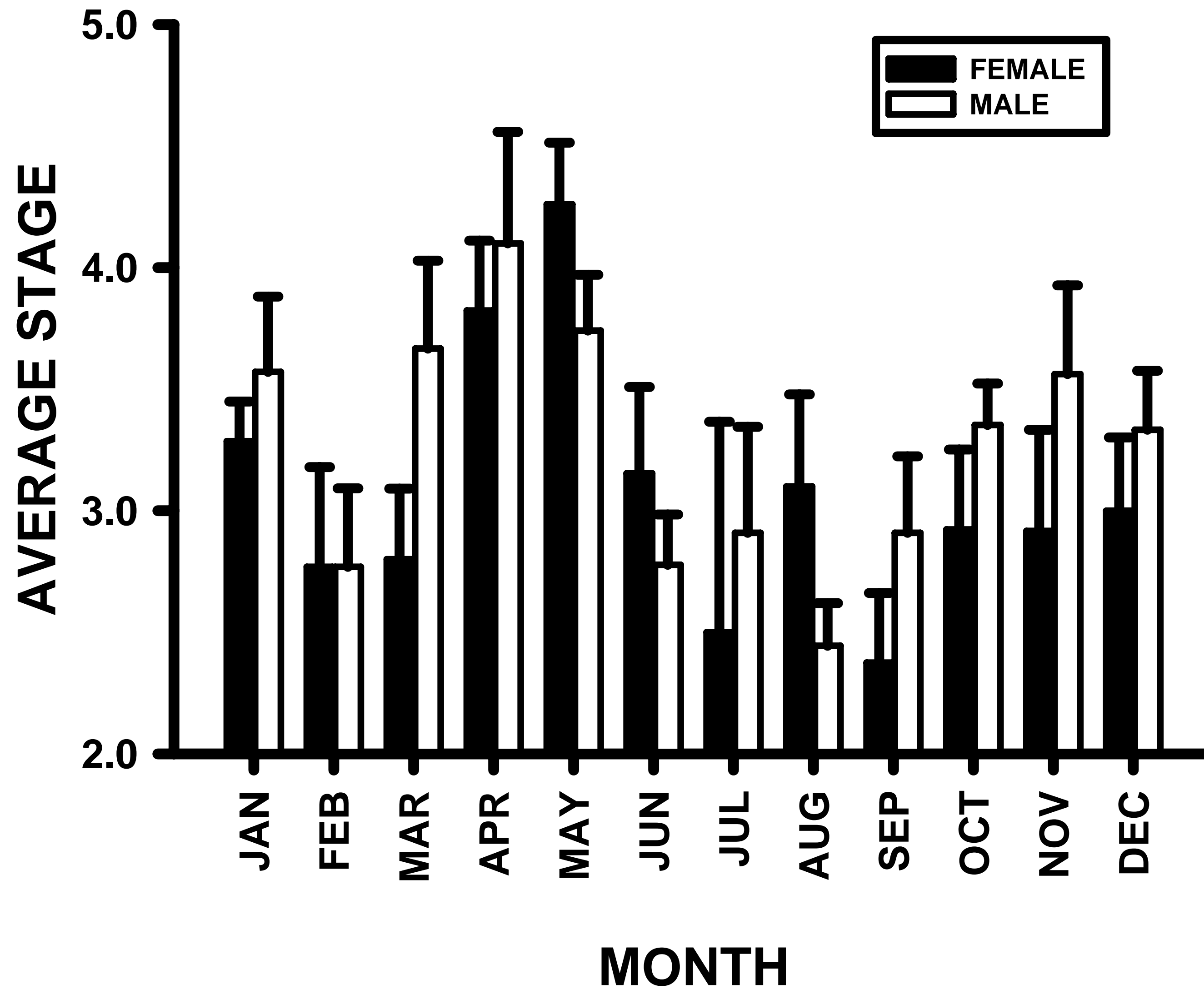


Figure 9. Monthly average and standard error of the reproductive stage of shovelnose sturgeon. These fish were collected between May 2001 and June 2002 in the Lower Missouri River. The number of observations used to calculate the average for each month and sex combination was the same as that for the mean weights given in Table I except May and November averages each had 2 and 1 more females, respectively.

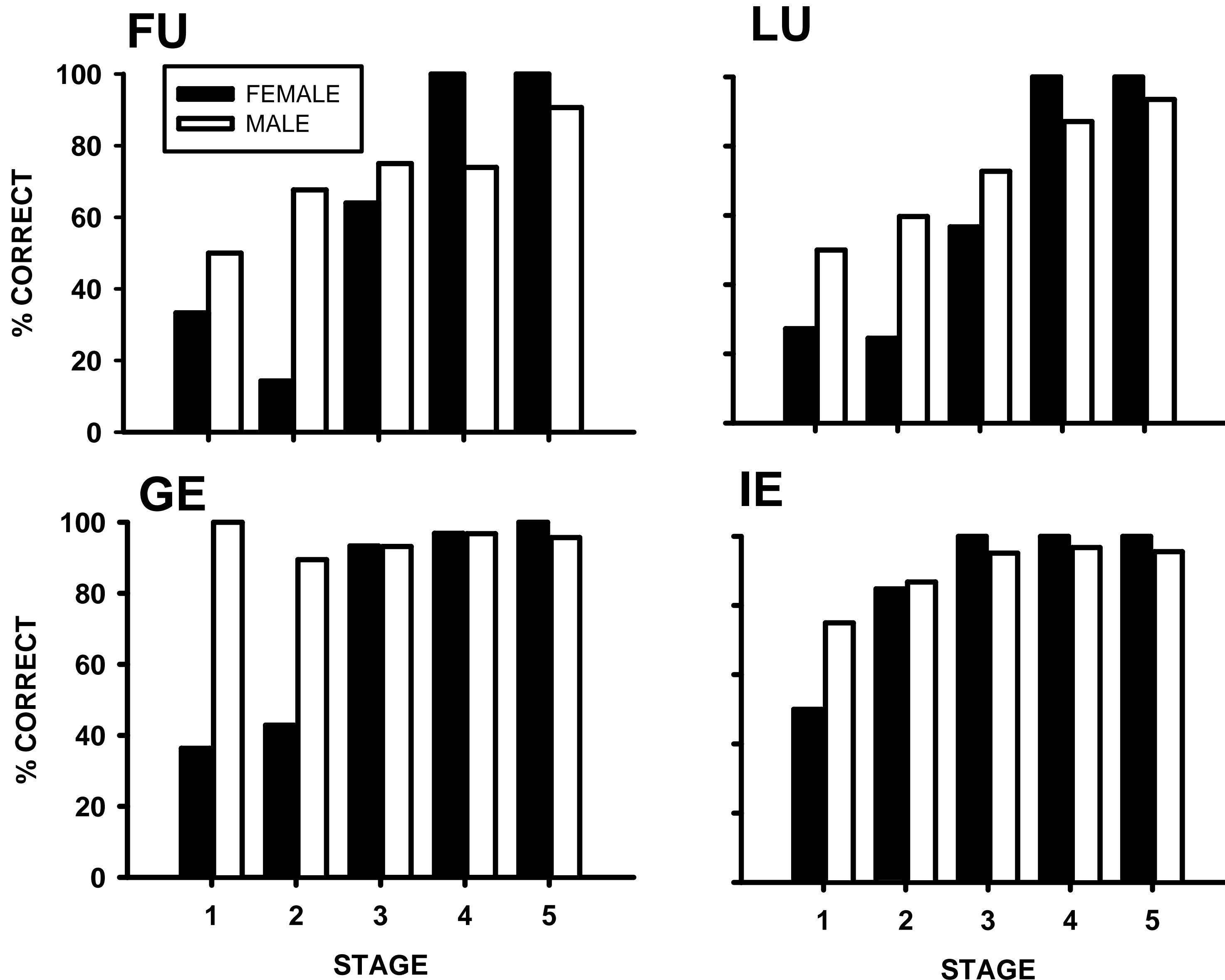


Figure 10. Percent correct sex identification of shovelnose sturgeon at each reproductive stage for four imaging methods. The methods used were: FU) field ultrasound, LU) clinical laboratory ultrasound, GE) endoscope through the gonoduct, and IE) endoscope through an abdominal incision. These fish were collected between May 2001 and June 2002 in the Lower Missouri River.

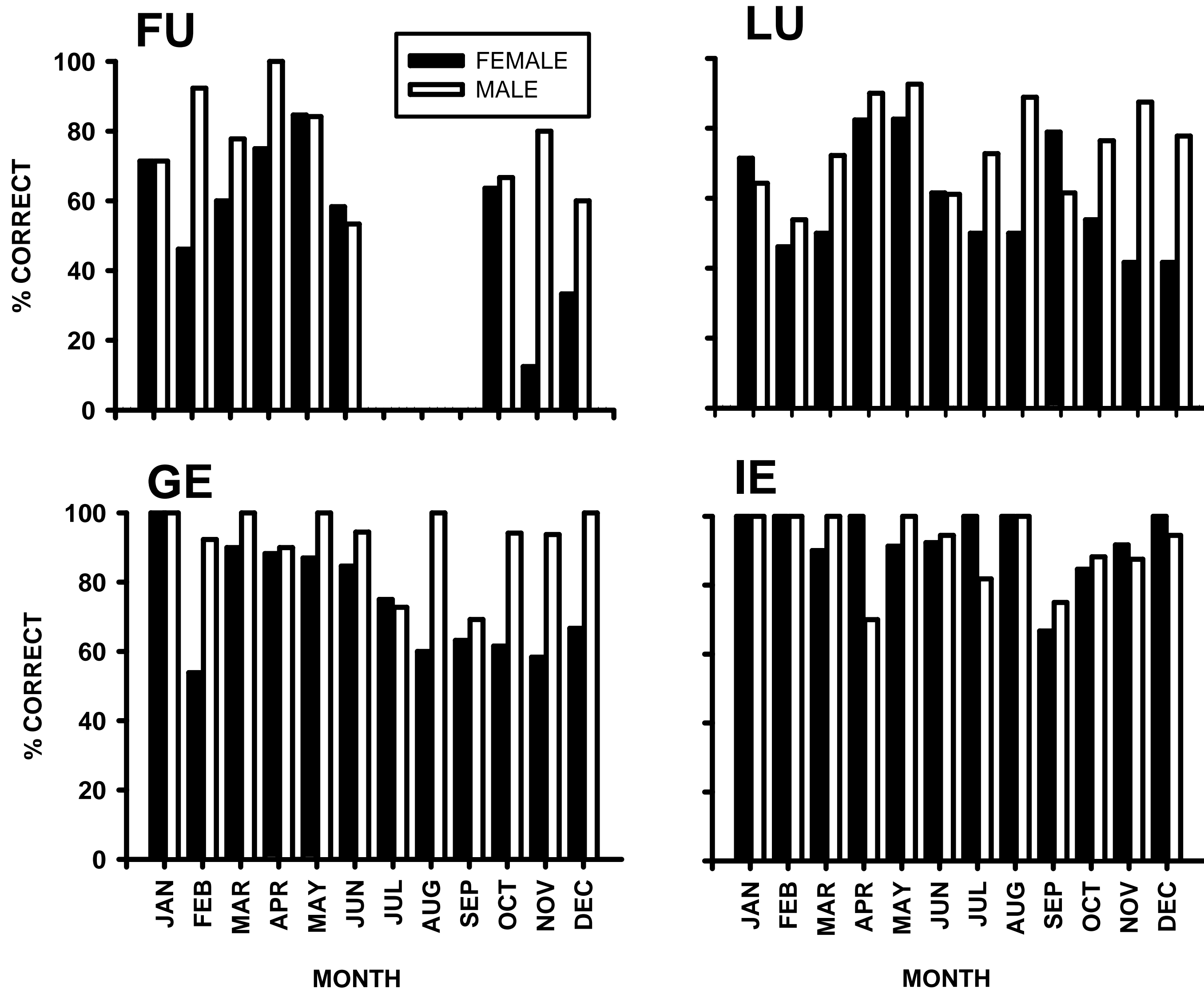


Figure 11. Monthly percent correct sex identification of shovelnose sturgeon for four imaging methods. The methods used were: FU) field ultrasound, LU) clinical laboratory ultrasound (corrected from Wildhaber et. al. (2005) where the % CORRECT axis was labelled from 0 to 100% when in actuality it ranged from 40 to 100%), GE) endoscope through the gonoduct, and IE) endoscope through an abdominal incision. These fish were collected between May 2001 and June 2002 in the Lower Missouri River.

reproductive stage was higher for males than females. April and May were the months with the highest reproductive stage, the same months when use of ultrasound was most effective. Ultrasonic and endoscopic methods gave similar results on shovelnose sturgeon collected in the Upper Missouri River as was observed for the shovelnose sturgeon collected in the Lower Missouri River. For pallid sturgeon collected in Upper Missouri River ultrasound was effective for identifying sex; however, the endoscope through the gonoduct was only effective for identifying sex when the gonoduct was not opaque.

Ultrasound Effectiveness

Overall, ultrasound allowed viewing of gonads from both sexes of shovelnose and pallid sturgeon. The primary limitation of the ultrasound units used in this study was the limited ability to use them to distinguish among males and Stage I, II, or VI female sturgeon. Effectiveness of using ultrasound for sex identification of shovelnose sturgeon compares favorably with previous work done on fishes. Previous studies that demonstrated a higher success rate for sex identification of fish using ultrasound than observed in this study were performed on larger fish species suggesting increased ease with which gonads can be identified with increased fish size (i.e., stripped bass and stellate sturgeon, Blythe et al., 1994; Moghim et al., 2002). Similar to this study, research on broodstock held in a laboratory setting have documented an increased success rate at identification of fish sex using ultrasound just prior to and during the spawning period with the lowest success rate during the period just following reproduction (Blythe et al., 1994; Karlsen & Holm, 1994). In addition, previous studies have found that the success rate for identification of males using ultrasound was either similar to or greater than for females (Blythe et al., 1994; Karlsen & Holm, 1994; Moghim et al., 2002; Colombo et al., 2004) except perhaps during spawning (Karlsen & Holm, 1994). These last findings are likely the combined effect of the fact that males tended to exhibit a higher reproductive stage throughout most of the year and the tendency of observers to identify females without any oocytes or follicles as males. Ultimately, in this study, if the gonads were not easily distinguishable from the other tissues within the body cavity using ultrasound and there were no signs to indicate the presence of female gonad tissue (i.e., egg-like structures or ovigerous folds) in the ultrasound image the fish was classified as a male.

Though the overall ability to identify sex using portable and clinical laboratory ultrasound units was similar, the image quality of the portable ultrasound unit was not quite as good as the clinical laboratory unit. The clinical laboratory ultrasound unit provided a higher image resolution, making it easier to distinguish oocytes, follicles, and gonad than with the portable unit. It is important to note that clinical ultrasound units are also much more expensive than portable units.

There were various limitations associated with using ultrasound on sturgeons due to the body make-up of these and many other fish species. First, the various tissues and organs within the body cavity (i.e., air bladder, fat, intestines, etc.) often obscured the view of the gonads using ultrasound. During this study, the air bladder of the shovelnose sturgeon often expanded to the point that the gonads were confined to a very small area on the ventral side of the body cavity. Second, identifying females using ultrasound is difficult when they are spent, pre-vitellogenic, or immature (i.e., Stages VI, II, or I, respectively) because the gonads are not substantial enough to identify. Third, the ability to use ultrasound to distinguish between fat and gonad was limited. In the shovelnose sturgeon, fat reserves were located directly near and often interwoven with the gonads which hindered sex determination. The effectiveness of ultrasound is limited by the ability to use it to distinguish between these types of tissues due to its process of measuring differences in density and texture to produce images of those tissues.

One possible way to eliminate some of these errors would be by using an additional view perpendicular to the transverse plane of the body cavity so that a better view of the lamellae-like folds of the ovaries (Moghim et al., 2002; and see Chapter 3) is obtained.

Even with the limitations described above, there are a number of factors that make the portable ultrasound unit a very useful, non-invasive tool for internal examination of fish while in the laboratory and the field. The current and improving capabilities of portable ultrasound units make it possible to use them in the field, even on a boat. The unit used in this study had a multiple range transducer. This was important because the optimal transducer for a given fish is dependent on its size and shape. The depth to which a transducer can image fish tissue is primarily dependent upon the frequency of the transducer; the higher the frequency the shallower the depth to which ultrasound signal can penetrate the tissue. The minimal range of transducer frequency needed for an ultrasound unit when used on a fish was found to be 5-7.5 MHz (personal observations). The unit used in this study contained on-screen measurement capabilities and an alpha numeric keyboard for image identification, and was capable of storing images in its own memory for later download to a computer. Finally, the portable ultrasound unit used in this study was light-weight (i.e., less than 4.5 kg) and could be powered by battery or alternating current.

Endoscope Effectiveness

The endoscope was effective in visualizing the gonads of shovelnose sturgeon through an incision in the abdomen and through the gonoduct but was of limited utility for visualizing pallid sturgeon gonads through the gonoduct. In shovelnose sturgeon not in spawning condition, the endoscope through an abdominal incision produced the best view and ultimately the most confident assessment of the sex of the fish. Through the abdominal incision, Stage IV and V males and females, one Stage VI female, and most Stage III fish were successfully distinguished. Overall, the effectiveness of endoscopy for sex identification of shovelnose sturgeon observed in this study was similar to that seen by Ortenburger et al. (1996) on Arctic charr and much more effective than had been found in previous work on shortnose sturgeon (Kynard & Kieffer, 2002).

Like the ultrasound units, the endoscope can be a valuable tool for field and laboratory determination of the sex of sturgeons. At least for shovelnose sturgeon, use of the endoscope was more effective for sex identification than using the portable ultrasound unit in the field. With a video camera connected, the endoscope could be effectively used in the field even in a boat on a river. Unlike the ultrasound units, the endoscope allows one to directly assess the color of the oocytes and follicles and thus, potentially gain a better estimate of their reproductive stage of development (Bryan, et al., 2006). To gain the most effective images from the endoscope, it was found that viewing through the end of the endoscope at 70° from horizontal, allowed one to see slightly forward while still having the perpendicular view. The perpendicular view was important because the endoscope view was from the gonoduct which lies on the side of the gonad. Furthermore, with a focal length of ~1 mm, one can see through the gonoduct while still being able to have the gonads in the field of focus.

There were various limitations associated with using endoscopic techniques on sturgeons due to the body make-up of the species. The ability to use the endoscope to distinguish between fat and gonad was limited. More importantly, as previously mentioned, in the larger pallid sturgeon the opaque coloration and thickness of the gonoduct obscured the view into the body cavity. This was not a problem with shovelnose sturgeon where, in most cases, the gonoduct was a clear thin membrane through which gonads could be seen. Occasionally the gonoduct of the shovelnose sturgeon was darkly pigmented with occasional

blood vessels that slightly obstructed the view of the gonads. Because all of the pallid sturgeons examined using an endoscope were very old adults it was not possible to say whether the difference in opaqueness of gonoducts observed between pallid and shovelnose sturgeons was due to species differences and/or age differences. As with the ultrasound units, if gonads were not easily distinguishable from the other tissues within the body cavity and there was no indication of the presence of female gonad tissue (i.e., oocytes, follicles, or ovigerous folds), the fish was classified as a male. This likely accounted for some of the increased ability to successfully identify male sturgeon compared to female sturgeon when the endoscope was used to view the gonads through the gonoduct. This is due to the fact that fish that could not be definitively identified as either of the two sexes, but were biologically male, were always identified as males while such fish that were biologically females were also identified as males.

The results of this study provide further support for the use of ultrasonic and endoscopic technologies as effective non-invasive and minimally-invasive tools that can be used to identify sexually mature fishes in the field. Such information is essential for assessment of the effects of river management strategies on the reproductive activity of riverine fishes. The level of success observed in this study at identifying sex in sturgeons, and the success at using ultrasound on even very small species (e.g., Bryan et al., 2005) suggests that these methods can be applied to most species of fish. Directly, this study should assist the USFWS as they work with all of their partners on the recovery of the pallid sturgeon and other threatened or endangered fish species. The results of this research provide useful information and tools that can be used for artificial propagation efforts. It provides an evaluation of the effectiveness of ultrasonic and endoscopic technologies for sex identification of fish as well as guidance as to when, during reproductive cycles, these methods should be the most effective. These results suggest these methods would be useful to monitor age and sex-specific fisheries impacts on, and progress in, recovery efforts of fish populations. Continued research is being done to determine whether additional measurements made during endoscopy and ultrasonography may allow assessment of not only reproductive stage but also gonadal size and female fecundity (Bryan et al. 2006).

CHAPTER 2

Gonadal Development and Seasonal Plasma Sex Steroid and Vitellogenin Profiles in Missouri River Sturgeons (*Scaphirhynchus* spp.)

Abstract

Sex and reproductive stage data provide important information for evaluating and managing fish populations. In this study, seasonal and reproductive stage-specific plasma sex steroid and vitellogenin (VTG, an egg protein precursor) profiles have been developed for shovelnose sturgeon from the Missouri River. Due to its rarity, limited pallid sturgeon data is also presented. Sex steroid and VTG levels fluctuated as expected during gametogenesis and were characteristically different between males and females. Pallid sex steroid levels were comparable to those of the shovelnose. Sex and reproductive stage are difficult to identify in sturgeons, as well as many other fish, because there are no obvious external sex- and reproductive stage-specific characteristics. The VTG protein was the best single predictor of shovelnose sturgeon sex with a success rate > 99%. 11-ketotestosterone, VTG, and 17-β estradiol were all selected for the model predicting sex (with fish grouped into one of two maturity categories) with a 87 % success rate. Prediction of male shovelnose sturgeon reproductive stage had the lowest success rate of 32% with females having a 68% success rate. The baseline hormonal profiles developed here should be useful as indicators to assess whether the physical and environmental conditions necessary for successful reproduction exist.

Introduction

Knowledge of a fish's reproductive cycle is a key to determining what physical and environmental conditions are necessary for reproduction and whether management strategies for creating or maintaining these conditions have been successful. Identification of sex and reproductive stage using methods that can be easily implemented during population monitoring and research studies can contribute valuable biological information (Young et al., 1988). While direct gross or microscope examination of gonads is the most definitive means to determine fish sex and reproductive stage, such examination is stressful and may necessitate killing the fish. Circulating blood levels of sex steroids and proteins (e.g., vitellogenin-VTG, an egg protein precursor) reflect sex and reproductive stage and their measurement can be an alternative to gonad inspection (Amiri et al., 1996; Cornish, 1998; Pavlidis et al., 2000). Techniques for measuring blood sex steroid levels in fish are well established (McMaster et al., 1992); however, cross validation of sex steroid levels with reproductive stage has not been reported for shovelnose sturgeon (*Scaphirhynchus platorynchus*).

Moos (1978) provides a thorough macro- and microscopic analysis of the reproductive stages of the shovelnose sturgeon. The present study advances this work by relating reproductive stage-specific plasma sex steroid and VTG profiles over the course of a year to gonad development as determined by macro and microscopic examination. Our objectives were to 1) describe seasonal changes in plasma levels of sex steroids and VTG at all reproductive stages and 2) begin to develop non-lethal, minimally invasive methods to classify sex and reproductive stage using blood parameters. The shovelnose sturgeon was used as a surrogate

for the rare pallid sturgeon for developing the monthly profiles, however limited sex steroid and VTG data from pallid sturgeon has been included.

Methods and Materials

Study Area and Fish Collection

Shovelnose sturgeon were collected from the Missouri River in the reach between the confluence of the Missouri River with the Chariton and Osage rivers (Fig. 1). We attempted to collect a total of 30 individuals (15 of each sex) monthly beginning in May 2001 and ending in June 2002. Adult fish were targeted by attempting to collect only fish $\geq$ 550 mm total length, however, 9 fish used in this chapter consisted of adults ranging in size from 350 to 550 mm. Fish were collected using multiple gears (i.e., overnight hoopnet, set line with multiple baited hooks, stationary gillnet sets, and drifting trammel nets) depending on season or behavior of the fish. Blood from a limited number of pallid sturgeon was also collected and used in some analyses. These fish were collected in April 2002 in the Upper Missouri River for broodstock by the US Fish and Wildlife Service (USFWS) as part of their pallid sturgeon propagation and recovery program.

Immediately after capture, each shovelnose sturgeon was uniquely tagged with a Floy® tag for individual identification. Tagged fish were held in aerated 378.5-L tanks constantly supplied with fresh river water. Live fish were subsequently transported to the Columbia Environmental Research Center (CERC) where they were held at river temperatures in circular fiberglass flow-through tanks for 24-48 h at which time they were euthanized.

Blood Collection and Field Processing

While in the field, within 4 h after capture 6 – 8 ml of blood were collected (Fig 3) and processed to collect plasma (Schmitt et al., 1999; Fig. 4). To test the effect of handling and holding on sex steroid measurements, fish collected in October were bled a second time after 48 h of holding in tanks at CERC. Blood samples were kept on wet ice until centrifugation at 3500 rpms for 10 minutes (Fig. 4). Plasma was transferred to 1.8-ml cryovials and snap frozen in a slurry of ethanol and dry-ice, then transferred to CERC to be stored in a -80°C freezer until steroid extraction and analysis.

Measurements and Tissue Sampling

After shovelnose sturgeon were humanely euthanized, total length was measured (tip of rostrum to where uppermost ray joins caudal filament; does not include filament), and fish were weighed to the nearest 50 grams using a hanging scale. The body cavity was slit open and a voucher photo was taken of the gonad prior to removal. Gonads with associated fat were weighed to the nearest 0.05 grams for calculating the gonadosomatic index (GSI = [gonad and associated fat weight/body weight] * 100). Fat was trimmed off the gonad and the gonads reweighed to obtain an estimate of percent fat composition of the gonad. Five 1-cm^3 pieces of gonadal tissue were collected along the anterior to posterior axis. The gonad tissue was preserved in 10% neutral buffered formalin (NBF) until processing for histology. Additionally, three 5-gram pieces of ovaries containing gray to black follicles were preserved in NBF and later the number of follicles in each sample piece was determined. Average number of follicles per gram was multiplied by the total grams of ovary minus fat to estimate the total number of follicles, or fecundity of a female. Fecundity and follicle size (length of oblong follicle) are also reported relative to age of fish. The first ray of the left pectoral fin was removed at the joint for age analysis (described in Whiteman et. al., 2004).

Histological Analysis

Histological analysis was conducted on the gonads of the sturgeon to accurately determine sex and reproductive stage. Samples were preserved and processed according to methods outlined by Blazer (2002). Briefly, after an initial fixation of 48 h in 10% NBF, samples were transferred through a series of Hepes Buffer rinses to 70% ethanol (EtOH). The samples were stored in the 70% EtOH until analysis was performed. Routine processing of tissue involved trimming into small pieces, dehydration through a series of alcohols followed by immersion in an organic solvent, then infiltration with paraffin. Paraffin blocks containing the tissues were cut into 5-μm slices. Sections were taken at three different depths to ensure the microscopic evaluations were representative of the entire tissue. These sections were placed on glass slides, allowed to dry, and then deparaffinized. The slides were stained with hematoxylin and eosin (Luna, 1968).

Light microscopy was performed to evaluate each fish as to sex and reproductive stage according to Moos (1978) with date of capture considered only in the case of the females. Gonads of male fish were classified as Stage I-Immature: lots of fat, few spermatogonia; Stage II-Developing: germ cells consist mostly of spermatogonia in nests or singly; Stage III-Spermatogenic: lobules filled with spermatocytes, spermatids or developing spermatozoa; Stage IV-Pre-spawning: most lobules filled with spermatozoa. Stage V-Spawning: all lobules are filled with or releasing spermatozoa; Stage VI-Post-spawn (Spent): collapsed lobules empty or containing some residual spermatozoa. Gonads of female fish were classified as Stage I-Immature: large fat body, few small oogonia; Stage II-Developing: pre-vitellogenic oocytes abundant, less fat than Stage I and lamellar structure of gonad obvious (i.e., ovigerous folds); Stage III-Vitellogenesis: yolk deposition and oocytes greatly increase in size and are enveloped by follicular cells, and the germinal vesicle is near the center of follicle; Stage IV-Pre-spawning: follicles enlarge, begin to turn black, and the germinal vesicle begins to shift towards the animal pole; Stage V-Spawning: germinal vesicle continues to migrate closer to animal pole where it will ultimately breakdown as meiosis resumes, follicles are black. Because vitellogenesis ends the fall/winter prior to the next year's spawning, and because the ovaries physically change little over the winter months, fish with follicles beginning to turn black before January 1 are assigned to Stage IV and after January 1 are assigned to Stage V; Stage VI-Post-spawn (Spent): no to little fat, empty follicles, few atretic follicles, and early oocytes.

Sex Steroid Extraction and Analysis

Plasma samples were extracted using diethyl-ether to separate the sex steroids from the binding proteins. The steroid-containing ether phase was snap frozen and the resulting supernatant was decanted and placed in a 30°C water bath and evaporated under nitrogen. The steroid residues were then reconstituted in either Cayman Chemical's competitive enzyme immunoassay (EIA) Buffer for 17-β estradiol (E2) analysis or PBSG (phosphate buffered saline pH 7.0 with 1 % gelatin) for 11-ketotestosterone (KT). Extracted E2 samples were stored at 4° C and KT samples were stored at -20° C until assayed.

Extraction efficiency was determined by spiking the plasma with a known concentration of tritiated steroid (10,000 DPMs). The spiked sample then underwent the extraction procedure as outlined above, with a sub-sample of the ethylether supernatant taken for radioactive analyses. An additional scintillation vial containing a 10,000 DPM spike was also analyzed. The resulting radioactive count was calculated for recovery rate by comparing the recovered radioactive values to that of the known 10,000 DPM spike. A laboratory average was determined by averaging the recovery rates of several extractions to obtain an average of

95.1% for E2 and 80.0% for KT. These values were applied to the derived steroid data to correct sample steroid values for efficiency of extraction.

17-β Estradiol

The E2 concentrations were determined by EIA using a kit from Cayman Chemical (Ann Arbor, Michigan). Steroid concentration was determined through the competition between the variable amount of free steroid (plasma sample or standard) and a constant amount of steroid tracer (steroid linked with acetylcholinesterase (AChE)) available for binding to the steroid-specific rabbit antiserum. The steroid antiserum complex (free or tracer) binds to a mouse anti-rabbit antibody attached to a 96-well microtiter plate. Following plate washing to remove unbound antigen, Ellman's Reagent was used to activate the AChE of the steroid tracer complex, producing a yellow color the absorbance of which was measured spectrophotometrically. The absorbance value is inversely proportional to the amount of free steroid in the plasma sample. The E2 concentration in a sample was determined from a standard curve of known concentrations of E2 plotted against corresponding absorbance values. Each sample was measured in duplicate and corrected for extraction efficiency.

11-ketotestosterone

The KT was assayed using the radioimmunoassay method (McMaster et al., 1992). Steroid concentration was determined through competition of the standard or sample KT and a constant volume of radiolabeled steroid for a fixed titer of antibody. Extracted samples were incubated with the antibody (anti-white sturgeon KT developed in rabbit, a gift from Dr. Tim Gross, USGS Florida Integrated Science Center) and radiolabeled steroid (Amersham Biosciences) overnight at 4°C. The following day a chilled solution of dextran-coated activated charcoal in PBSG (0.4% dextran, 0.625% charcoal) was added and allowed to incubate before centrifugation at 0°C for 20 minutes at 2800 rpm. A 500-µL portion of the resulting supernatant was added to a scintillation vial containing 5 mL of scintillation cocktail (Ecolume). Radioactivity was determined by scintillation spectroscopy with the resulting values indicating the amount of antibody bound to labeled steroid, which is inversely proportional to the amount of free steroid in the sample. A standard curve of a serial dilution of KT standard (Sigma-Aldrich, St. Louis, MO) was used to allow calculation of steroid concentrations in the unknown samples.

Quality Assurance Procedures and Assay Performance Characteristics

Samples in all assays were run in duplicate and included measurement of blanks which were subtracted from all absorbance values. The reported kit sensitivity for E2 was 89 pg/mL at a %B/Bo of 50%, and 9 pg/mL at a % B/Bo of 80%. The KT sensitivity was 19 pg/mL and 3 pg/mL at 50% and 80% binding, respectively. Cross-reactivities of the antibodies used in these assays with other similar steroids are reportedly ≤ 17% for the E2 antiserum (Cayman Chemical, Ann Arbor, MI) and < 10% for the KT antiserum (Sepulveda et al., 2002). The E2 intra-assay variation was 1.2% and inter-assay variation was 6.6%. The KT intra-assay variation was 18% and inter-assay variation was 10%. Levels of KT were validated by verifying that serial dilutions of sample were parallel to a standard curve. A slope of 0.8 was obtained from the regression of steroid measured and concentration of steroid added to a plasma sample.

Vitellogenin Analysis

The VTG analysis was conducted using a sandwich Enzyme-Linked-Immunosorbant Assay as described by Folmar et al., (1996). Microtiter 96-well plates were coated with a mouse anti-shovelnose sturgeon VTG protein (custom made by Abraxis, LLC, Pennsylvania) and

incubated overnight. After washing the plate in a wash buffer (tris-buffered saline/Tween 20) and blocking non-specific binding by incubating with bovine serum albumin, 2 dilutions of each plasma sample were added in duplicate to the plate and incubated for 1 h at room temperature. A standard curve was created with serial dilutions of purified shovelnose sturgeon VTG (see Kroll, 1990) and processed in the same manner. The plate was then washed with the washing buffer before the addition of the rabbit anti-shovelnose sturgeon antibody. After 1 h of incubation at room temperature, the plate was washed as before. A secondary antibody (goat anti-rabbit horseradish peroxidase) was added and incubated for an additional hour prior to washing. A 20-minute incubation in TMB solution (Tetramethylbenzidine free base chromogen in a hydrogen peroxide-citrate buffer) allowed color development. A stop solution (2N HCl) was added prior to reading the plate at 450 nm on a spectrophotometer. As with the E2 EIA described above, the VTG concentrations were determined by quantifying the absorbance values in relation to the known values of the standard curve.

Statistical Analysis

Plasma sex steroids, VTG, fecundity and fat were log_{10}-transformed and GSI was squareroot-transformed to meet normality and homogeneity of variance assumptions. Plasma sex steroids and VTG were compared among the eleven classes of sex and reproductive stage using one-way analysis of variance (ANOVA). Mean comparisons were conducted using the Bonferroni procedure (alpha = 0.05). Time-related differences in sex steroid concentrations were tested using a repeated measures ANOVA. Regression analysis was used to obtain estimates for the equations of the relationships between fecundity and body size and fecundity and age. Means and standard deviations are reported throughout the text unless otherwise specified.

Discriminant Analysis

For discriminant analysis, we followed the methods used by Webb et al. (2002) for white sturgeon (*Acipenser transmontanus*) to predict shovelnose sturgeon sex and reproductive stage using two plasma sex steroids, KT and E2, and one plasma protein, VTG. As did Webb et al. (2002), we grouped reproductive stages into two groups of maturity by sex with Immature consisting of Stages I, II, and VI and Maturing consisting of Stages III – V (we did not catch any Stage VI females, consequently Immature females only contained Stage I and II). Even though Webb et al. (2002) used fish length in their analysis, we did not due to our selection of shovelnose sturgeon $\geq$ 550 mm. In addition, Webb et al. (2002) did not use date of capture to influence reproductive stage, consequently our analysis did not include date of capture in the determination of reproductive stage.

Plasma sex steroids and VTG were compared among the four groups of sex and maturity using one-way analysis of variance (ANOVA). Mean comparisons were conducted using the Bonferroni procedure. Discriminant function analysis (DFA) was used to develop a set of discriminating functions to predict sex or sex and maturity. To attain multivariate normality, the logarithms (log_{10}) of the variables VTG, KT, and E2 were considered for analysis. Stepwise DFA was conducted using log_{10}-transformed VTG, KT, and E2 concentrations to choose the best predictor(s) of sex or sex and reproductive stage. The significance level to enter and remain in the model was alpha = 0.05. A quadratic DFA was then conducted with the variables chosen in the stepwise procedure to determine the number of observations and percent classified into the two groups of sex or four groups of sex and reproductive maturity. The error rate associated with predicting sex or sex and maturity using the chosen discriminant functions was accomplished through cross-validation (see Khattree & Naik 2000). We used Bartlett's

modification of the likelihood ratio test (Morrison, 1976; Anderson, 1984) to test the homogeneity of the within-group covariance matrices (alpha = 0.05). Because five out of the six tests were significant ($P < 0.001$), we used quadratic DFA and the within-group covariance matrices to calculate the distances. The DFA was also conducted on the female and male fish alone. All analyses were conducted using Statistical Analysis Systems for Windows, release 9.10 (SAS Institute, Cary, North Carolina).

Results

Three-hundred eighty shovelnose sturgeon from the Lower Missouri River were caught. Upon internal examination, 24 (6%) were found to have intersex gonads (i.e., characteristics of both male and female gonads), another two were juveniles (i.e., too young for sex assessment) and 5 fish had no gonads preserved; thus, these fish were not included in analyses. Although 9 fish did not meet the ≥ 550 mm target size and 1 had no length reported, they were nevertheless included in the analyses because sex and reproductive stage could be identified. Additionally, of 16 pallid sturgeon collected from the Upper Missouri River, blood was collected from 8 males and 4 females. Therefore, sturgeon analysis for this chapter was based on 349 shovelnose sturgeon and 12 pallid sturgeon collected during a 13-month period.

A total of 190 male and 159 female shovelnose sturgeon averaging 681 mm and 866 mm, respectively, were sampled between May 2001 and June 2002 from the Missouri River (Table 5). Fish at Stages II-V were well represented among the 349 fish we captured (Tables 6 and 7). At least 49% of the all the females we caught had spawned previously based on black pigmented remains of resorbed oocytes. We rarely observed spent males and did not capture any spent females. Stage IV and V fish comprised the greatest percentage of those collected in March through May and April through May, for males and females, respectively. A single female with Stage V follicles was observed as late as August. Some Stage II of both sexes were caught every month of the year. Probably because of our minimum size target for fish, both male and female Stage I and II fish were rare. Total length seems to exponentially increase with age and then levels-off in males and females (Fig. 12); however, because of the very limited number of fish less than 10 years in age resulting from our minimum size target, this relationship is tentative. Stage IV and V fish were not seen in the youngest fish caught due to our target size requirement.

Fecundity

Fecundity measurements (total number of advanced yolked oocytes matured per female) were made on 67 Stage IV and V females ranging in size from 605 to 825 mm TL. Estimated average number of follicles in a female was 21,091 ± 7,085. Total fecundity measurements (total number of vitellogenic oocytes at any time in the ovary) were made on 74 Stage III, IV and V females ranging in age from 10 to 31 years old (Fig. 13). The number of follicles per female increased linearly with length of fish (Fig. 13). Length was a better predictor of fecundity than age (Fig. 13). Total fecundity tended to increase in older fish up to 28 years old, while relative fecundity (mean of 20/g fish; number of follicles per gram of fish) remained the same up to age 24 years (Figs. 13 and14). Although total fecundity of fish >28 years and relative fecundity of fish >24 years old decreased, the data may be unreliable given the small sample sizes. Follicle length remained the same for all ages of fish.

Gonadosomatic Index and Gonadal Fat

Testes of males at Stages III, IV, and V averaged 3% of body weight while those at earlier reproductive stages or after spawning were ≤ 2 percent (Table 8). Ovaries of females at

Stage V were the largest at a mean of 19% of body weight, while Stages I and II were the lowest averaging 2% (Table 8). Percent gonadal fat decreased as reproductive stage increased (Table 8).

Table 5. Mean total lengths and weights ± SD, with range in parenthesis for male and female shovelnose sturgeon captured in the Missouri River between May 2001 and June 2002. The differences in this table versus Table 1 in Chapter 1 are due to the slight difference in number of fish used in this study and the separation of May and June 2001 and 2002.

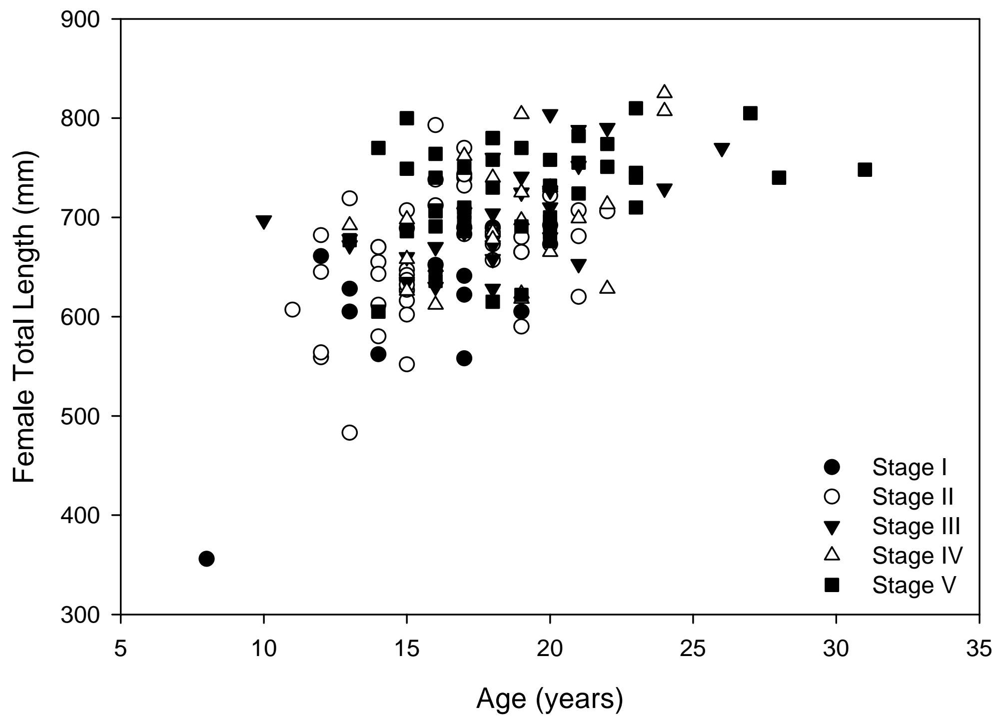
Date Captured	Sex	Weight (g) ± SD (Min.-Max.)	Length (mm) ± SD (Min.-Max.)	Sex	Weight (g) ± SD (Min.-Max.)	Length (mm) ± SD (Min.-Max.)
May-01	M	1425.0 ± 337.0	692.8 ± 58.2	F	1240.0 ± 284.6	675.2 ± 63.8
		(900-1900)	(596-795)		(900 -1800)	(598-810)
Jun-01	M	550.0 ± 244.9	590.4 ± 90.5	F	600.0	621.0
		(200-800)	(452-665)		(600)	(621)
Jul-01	M	662.5 ± 221.7	646.3 ± 74.9	F	792.5 ± 211.9	682.3 ± 72.9
		(150-900)	(443-712)		(620-1100)	(620-782)
Aug-01	M	660.0 ± 223.4	646.1 ± 78.8	F	825.0 ± 332.7	681.2 ± 65.0
		(200-1050)	(488-776)		(400-1600)	(602-807)
Sep-01	M	680.8 ± 211.7	643.2 ± 64.0	F	743.6 ± 330.1	650.2 ± 99.7
		(250-950)	(504-713)		(83-1400)	(356-804)
Oct-01	M	830.6 ± 262.1	679.3 ± 55.7	F	786.5 ± 295.7	686.5 ± 55.7
		(550-1450)	(609-820)		(425-1475)	(622-825)
Nov-01	M	679.4 ± 202.0	654.7 ± 55.2	F	639.6 ± 303.3	639.1 ± 84.9
		(350-975)	(548-734)		(250-1200)	(483-753)
Dec-01	M	764.4 ± 155.4	686.7 ± 41.8	F	754.2 ± 205.6	673.2 ± 60.2
		(550-1100)	(625-761)		(400-1175)	(580-804)
Jan-02	M	900.0 ± 185.0	710.7 ± 44.0	F	1025.0 ± 216.4	738.8 ± 46.1
		(600-1400)	(631-808)		(550-1300)	(630-793)
Feb-02	M	746.2 ± 163.9	685.5 ± 48.5	F	757.7 ± 186.9	676.2 ± 49.1
		(500-1000)	(621-775)		(450-1100)	(605-745)
Mar-02	M	891.7 ± 320.5	702.4 ± 67.4	F	825 ± 226.4	686.0 ± 41.6
		(550-1900)	(619-890)		(500-1300)	(590-740)
Apr-02	M	945.0 ± 149.9	721.2 ± 44.0	F	1114.7 ± 243.5	732.9 ± 45.3
		(700-1150)	(596-795)		(750-1700)	(665-805)
May-02	M	855.3 ± 223.5	686.7 ± 54.2	F	881.8 ± 223.9	697.2 ± 51.2
		(500-1300)	(596-795)		(500-1300)	(616-810)
Jun-02	M	798.4 ± 184.3	681.9 ± 39.2	F	833.3 ± 201.2	689.9 ± 48.4
		(400-1025)	(610-742)		(500-1100)	(628-758)
Total	M	809.5 ± 269.8	677.7 ± 61.5	F	866.1 ± 297.5	686.0 ± 67.2
		(150-1900)	(443-890)		(83-1800)	(356-825)

Table 6. Monthly capture of male shovelnose by reproductive stage.

	Month																									
	J		F		M		A		M		J		J		A		S		O		N		D		All months	
Stage	Num	%	num	%	num	%	num	%	Num	%	Num	%	num	%	num	%	num	%	num	%	num	%	num	%	num	%
I	0		0		0		0		0		2	10	1	8	0		2	15	0		1	6	0		6	3
II	2	14	5	38	5	28	1	10	3	11	6	29	6	50	4	40	1	8	2	12	5	29	3	17	43	23
III	1	7	2	15	0		0		8	30	11	52	2	17	4	40	6	46	7	41	0		4	22	45	24
IV	6	43	1	8	0		0		3	11	1	5	0		0		2	15	8	47	5	29	7	39	33	17
V	3	21	2	15	10	56	7	70	11	41	1		3	25	0		1	8	0		6	35	2	11	46	24
VI	2	14	3	23	3	17	2	20	2	7	0		0		2	20	1	8	0		0		2	11	17	9
Total	14		13		18		10		27		21		12		10		13		17		17		18		190	100

Table 7. Monthly capture of female shovelnose by reproductive stage.

	Month																									
	J		F		M		A		M		J		J		A		S		O		N		D		All months	
Stage	Num	%	num	%	num	%	num	%	num	%	Num	%	num	%	num	%	num	%	num	%	num	%	num	%	num	%
I	0		2	15	0		0		0		1	8	1	25	0		5	29	2	15	1	8	0		12	8
II	1	7	6	46	5	50	3	18	4	17	3	23	2	50	5	50	6	35	3	23	7	54	5	42	50	31
III	8	57	1	8	2	20	4	24	2	9	5	38	0		0		2	12	2	15	1	8	3	25	30	19
IV	0		0		0		0		0		0		0		4	40	4	24	6	46	4	31	4	33	22	14
V	5	36	4	31	3	30	10	59	17	74	4	31	1	25	1	10	0		0		0		0		45	28
VI	0		0		0		0		0		0		0		0		0		0		0		0		0	0
Total	14		13		10		17		23		13		4		10		17		13		13		12		159	100



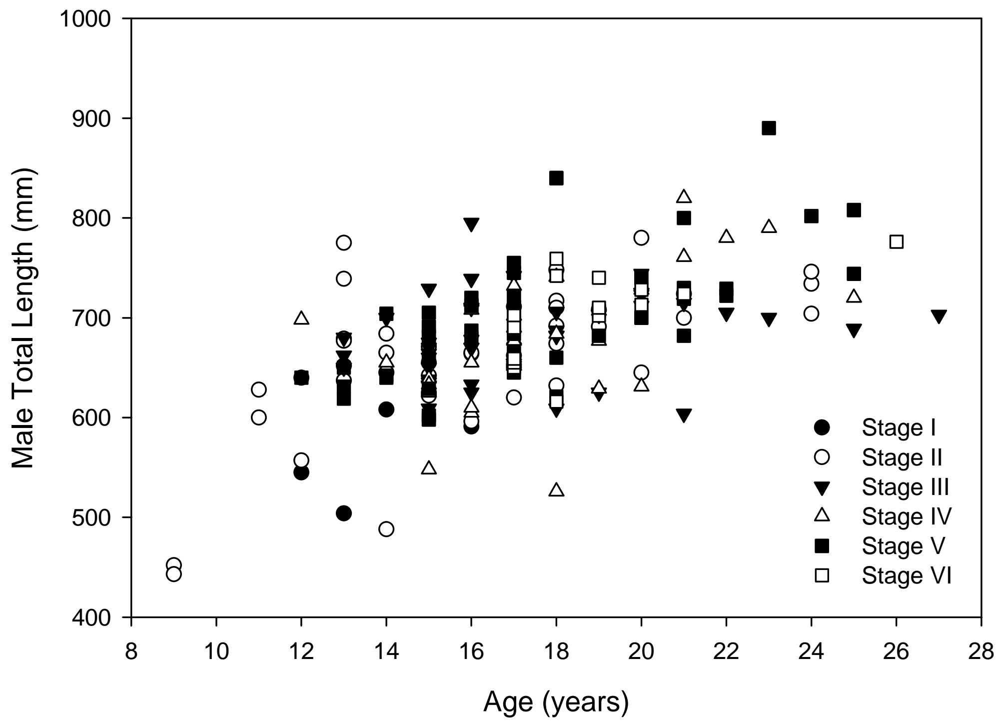
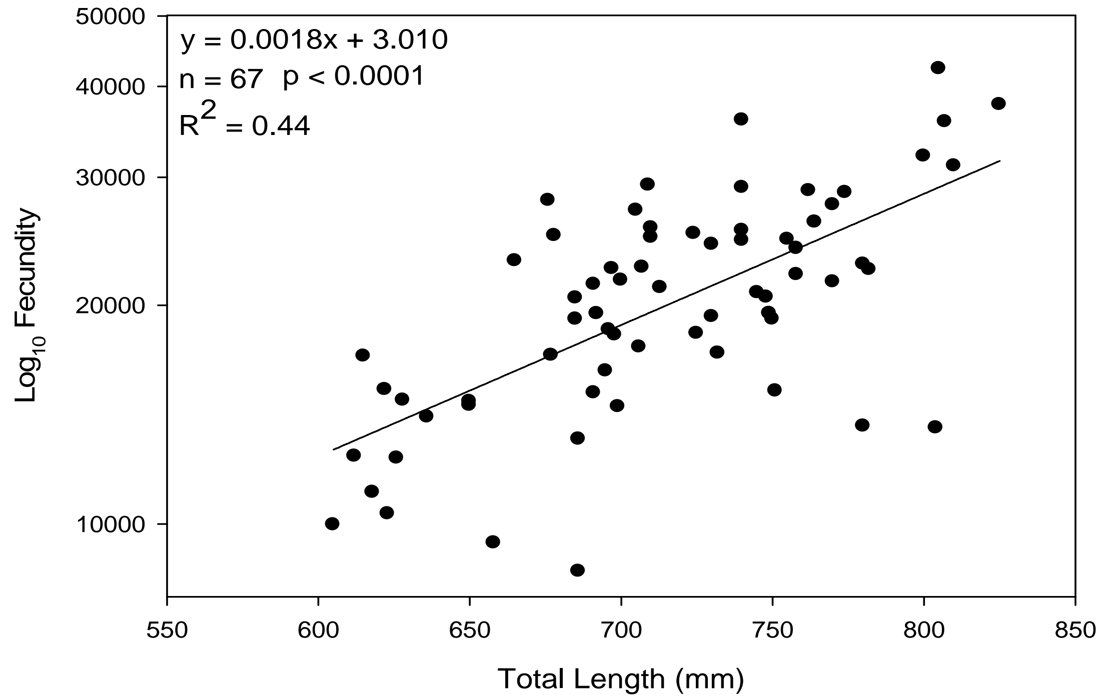


Figure 12. Total length of female and male shovelnose sturgeon with age and reproductive stage.



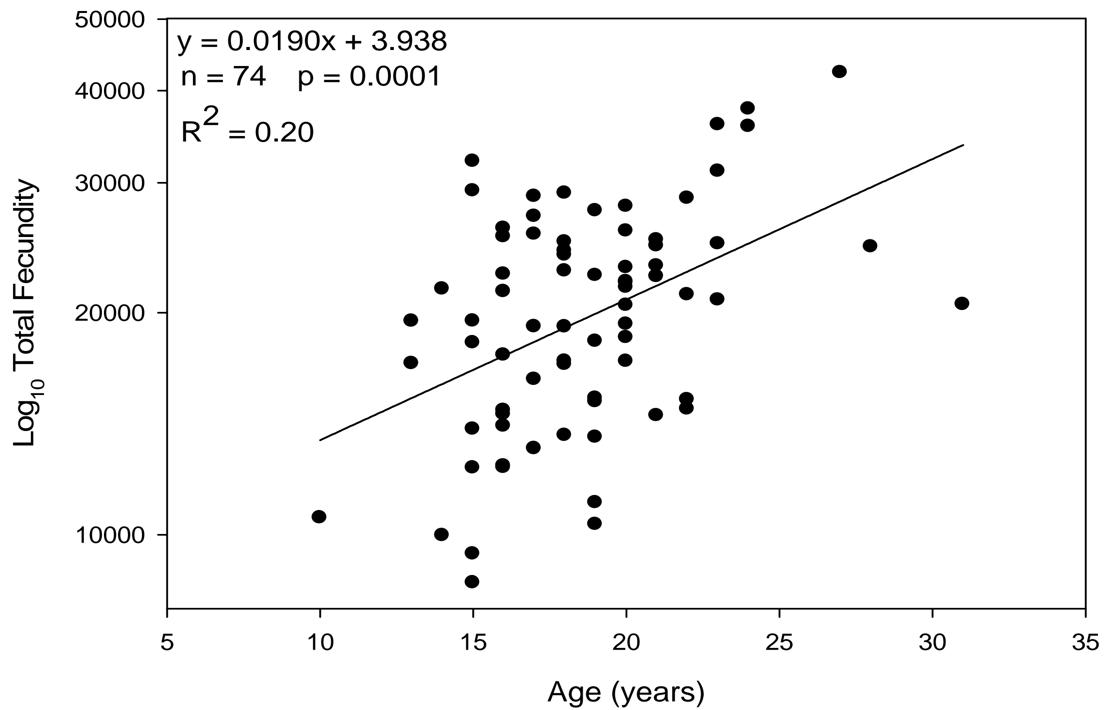


Figure 13. Fecundity (total number of advanced yolked oocytes matured per female) with length (upper panel) and total fecundity (total number of vitellogenic oocytes at any time in the ovary) with age (lower panel) of female shovelnose sturgeon.

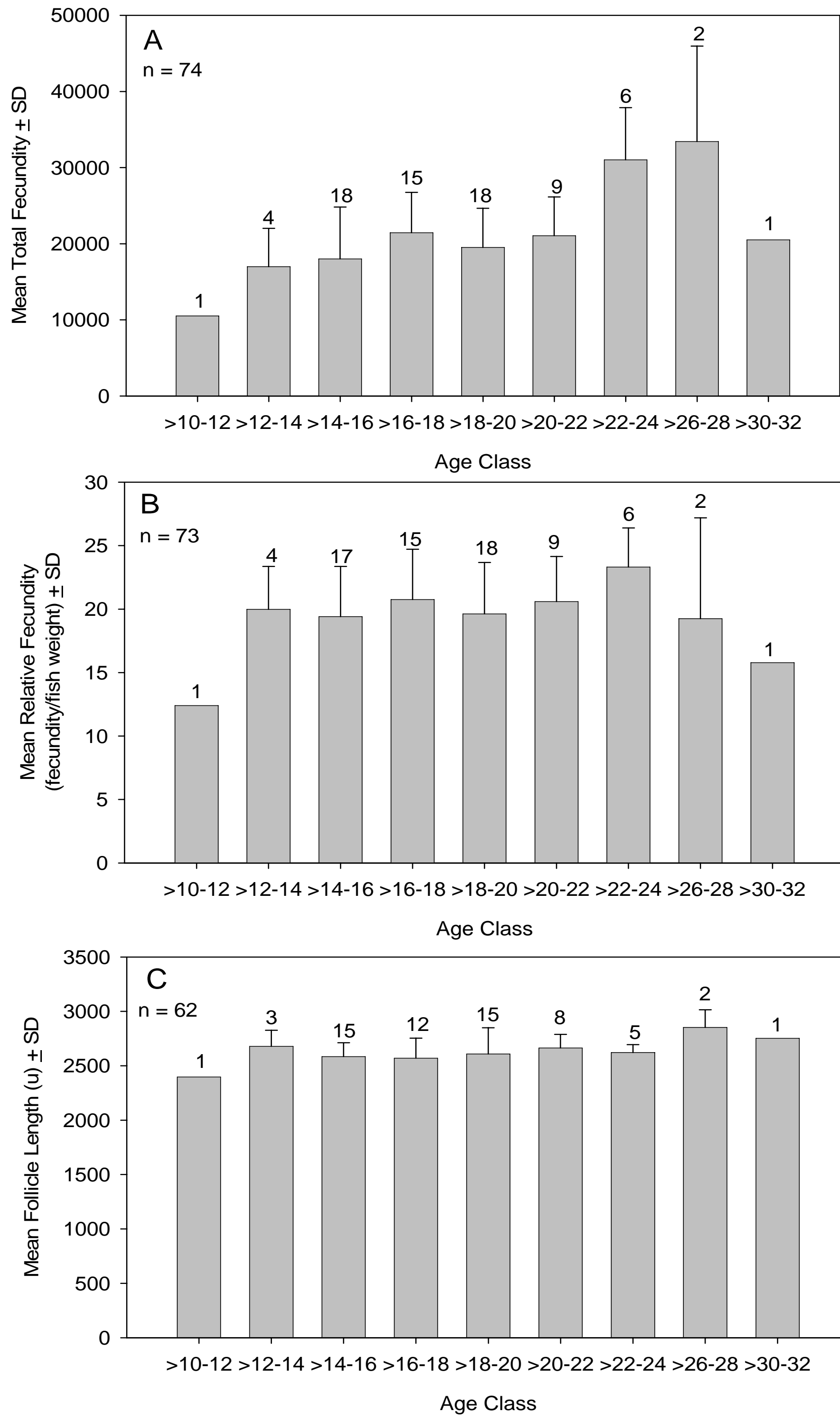


Figure 14. Mean total fecundity (A), mean relative fecundity (B), and mean follicle length (C) of shovelnose sturgeon by age class.

Table 8. Mean gonadosomatic index (GSI), sex steroids, vitellogenin, and percent fat of female and male shovelnose sturgeon at various reproductive stages. The reproductive stage takes capture date into consideration. Back transformed means, N, and log_{10}- and squareroot -transformed standard error are presented. Low Stage I fish collections may be an artifact of the 550 mm total length target size we tried to maintain throughout the study.

	GSI (%)		Fat (%)		Vitellogenin (ug/mL)		Estradiol (pg/mL)		11-Ketotestosterone (pg/mL)		Estradiol/ 11-Ketotestosterone	
Stage	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
I	2.21 (12)	2.45 (5)	0.56 (12)	9.87 (4)	10.86 (3)	0.02 (1)	31.43 (11)	41.67 (6)	336.76 (10)	1968.82 (6)	0.08 (10)	0.02 (6)
	0.173	0.185	0.422	0.224	0.132	-	0.129	0.255	0.174	0.237	0.192	0.245
II	2.64 (49)	1.19 (42)	1.13 (48)	1.57 (38)	31.87 (35)	0.04 (24)	34.78 (49)	29.64 (43)	237.79 (49)	1143.75 (43)	0.15 (49)	0.03 (43)
	0.064	0.057	0.175	0.179	0.119	0.098	0.089	0.080	0.097	0.109	0.126	0.096
III	4.96 (28)	3.68 (45)	0.71 (27)	4.10 (45)	50.47 (24)	0.07 (29)	208.44 (30)	82.59 (45)	529.87 (30)	12927.37 (45)	0.39 (30)	0.01 (45)
	0.154	0.097	0.185	0.155	0.126	0.089	0.103	0.060	0.114	0.115	0.132	0.092
IV	12.87 (22)	3.36 (33)	0.52 (22)	1.68 (32)	264.62 (15)	0.05 (24)	860.45 (22)	53.73 (33)	2375.29 (22)	10309.76 (33)	0.36 (22)	0.01 (33)
	0.111	0.093	0.169	0.186	0.071	0.073	0.120	0.083	0.107	0.140	0.129	0.136
V	19.19 (44)	3.30 (46)	0.21 (44)	1.04 (45)	186.08 (36)	0.06 (28)	336.10 (45)	44.18 (46)	1817.86 (45)	12320.20 (46)	0.18 (45)	<0.01 (46)
	0.064	0.058	0.044	0.170	0.076	0.078	0.068	0.071	0.102	0.110	0.107	0.150
VI	-	1.17 (17)	-	0.60 (17)	-	0.08 (14)	-	30.27 (17)	-	511.27 (17)	-	0.06 (17)
		0.097		0.354		0.101		0.113		0.181		0.164

Plasma Sex Steroid Concentrations

Sex steroid levels in blood samples collected immediately upon capture were not significantly different from levels in blood samples from the same fish 48 hours after transport and holding (P = 0.28 for E2 and P = 0.09 for KT; data not presented).

Concentrations of plasma sex steroids and VTG differed significantly (P < 0.0001; Table 9) among the 11 groups of sex and reproductive stage. The Bonferroni mean comparison tests revealed that plasma E2 and KT did significantly differ between some pairs of means, but there were no significantly distinct groups of means between sexes and reproductive stage (Fig. 15). Plasma concentrations of VTG were significantly higher in females compared with males, and Stage IV and V females were significantly higher than Stage I, II, or III females (Fig. 15).

Table 9. One-way analysis of variance conducted to determine whether there were differences between the $\log_{10}$-transformed sex steroids (11-ketotestosterone = KT, 17-β estradiol = E2) and vitellogenin (VTG) concentrations among the 11 groups of sex and reproductive stage.

Variable	df	MS	F	P
KT	10	14.15	28.63	< 0.0001
E2	10	6.98	26.81	< 0.0001
VTG	10	59.62	239.50	< 0.0001

Mean female E2 levels increased as oogenesis proceeded through Stage IV, then decreased (Table 8, Fig. 15). Mean male KT levels increased with spermatogenesis, plateaued at Stages III-V, then decreased (Table 8, Fig. 15). Overall, mean female KT levels were lower than males but increased steeply between Stages III and IV (Table 8, Fig. 15) and mean male E2 levels were generally lower than mean female E2 levels but increased to their highest concentration at Stage III before decreasing (Table 8, Fig. 15). In general KT levels tended to be higher in spring and summer months then tapered off into autumn, reaching lowest levels in winter months (Fig.16). 17-β estradiol levels followed a similar pattern but remained elevated through the autumn in vitellogenic females (Fig.17). The reproductive stage-specfiic mean E2/KT ratios were generally higher for females than males but there were some females at all reproductive stages that had a low ratio. Overlap predominantly occurred when both sex steroids were at relatively low levels (Fig. 18).

Sex steroid levels in the few pallid sturgeons sampled (4 females, 8 males) were comparable to those in shovelnose at a similar reproductive stage (Figs. 16 and 17). Blood was collected from 4 females and 8 males but the volume was insufficient to measure both sex steroids and VTG in all fish. Of the females, 2 were determined to be Stage V based on the polarization index (PI) (Rob Holm, pers. comm.; the PI is determined by measuring the long diameter of the oval follicle from animal to vegetal pole and dividing this into the distance between the germinal vesicle and the animal pole).and 2 were Stage I or II. Reproductive stage-specific E2 and KT values for pallids were similar to the same reproductive stage in the shovelnose. There was insufficient blood plasma to measure VTG in the Stage V females and VTG was detected in only one of the two Stage I or II pallids albeit at the low concentration similar to those found in the Stage I and II shovelnose. Reproductive stage was not determined in any of the male pallids. As a group, KT levels in male pallids were similar to that observed in

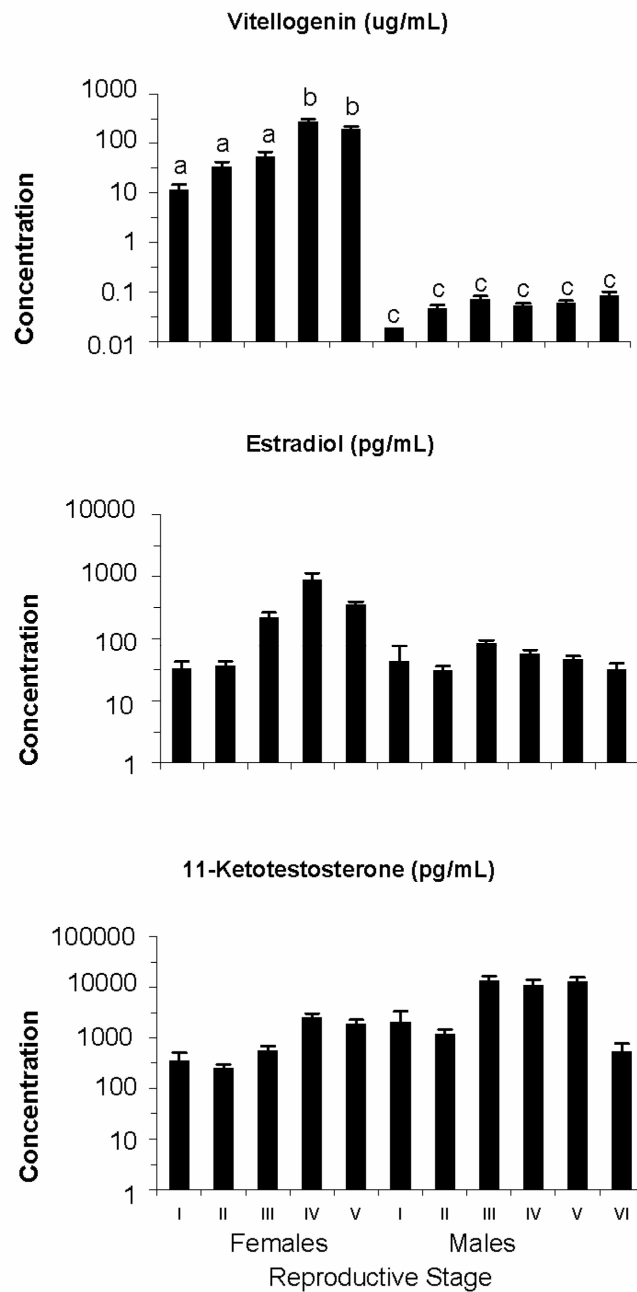
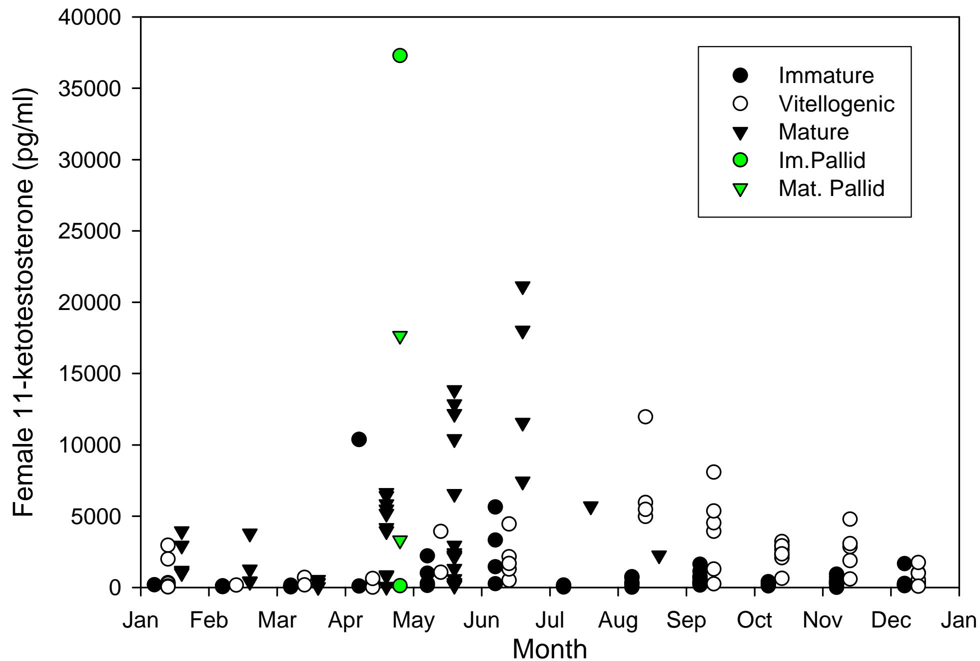


Figure 15. Mean vitellogenin (VTG), 17-β estradiol (E2), and 11-ketotestosterone (KT) levels in female and male shovelnose sturgeon with reproductive stage. The reproductive stage takes capture date into consideration. Each letter for VTG indicates a group of bars that were not significantly different from each other. For E2 and KT there were significant differences between some pairs of means, but there were no significantly distinct groups of means as was the case for VTG. Low Stage I fish collections maybe an artifact of the 550 total length target size we tried to maintain throughout the study.



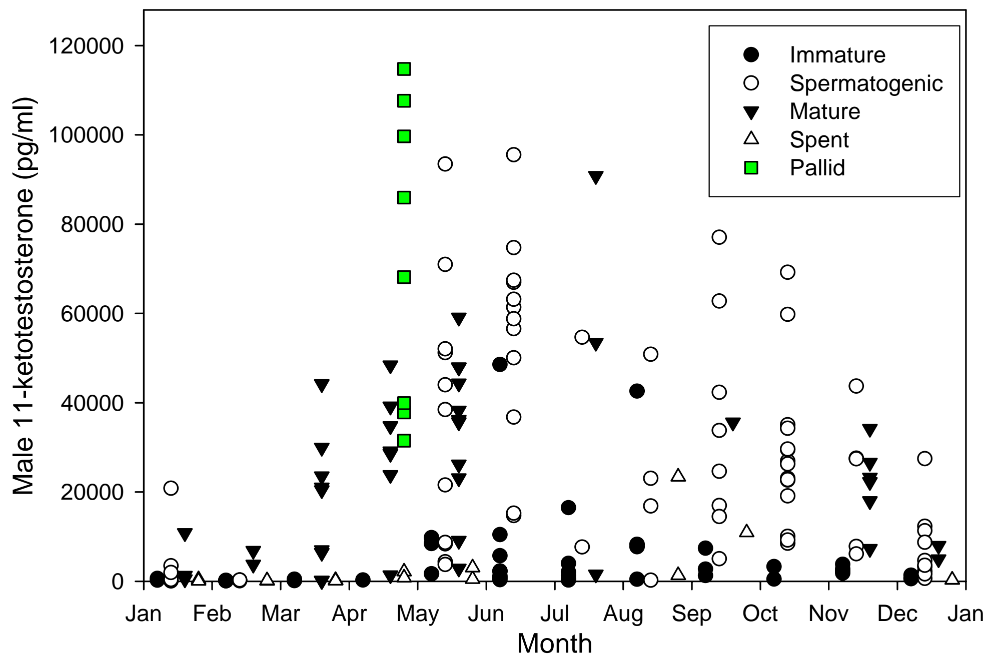


Figure 16. Mean 11-ketotestosterone levels in female and male shovelnose and pallid sturgeon by month and reproductive stage. Immature = Stages I and II, spermatogenic/vitellogenic = Stages III and IV, mature = Stage V, and spent = Stage VI. No reproductive stage determined for pallids.

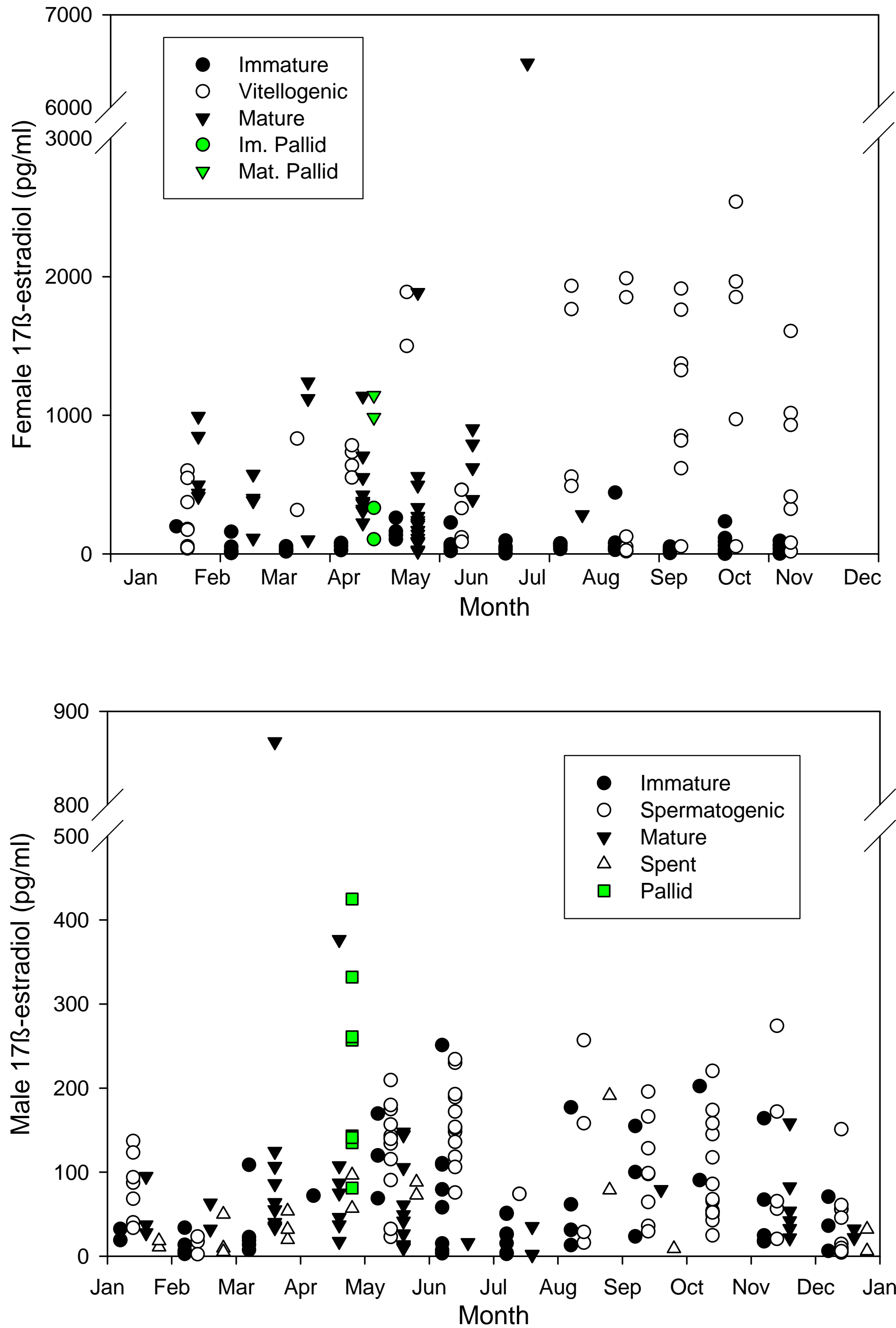


Figure 17. Mean 17-β estradiol levels in female and male shovelnose and pallid sturgeon by month and reproductive stage. Immature= Stages I and II, spermatogenic/vitellogenic = Stages III and IV, mature = Stage V, and spent = Stage VI. No reproductive stage determined for male pallids.

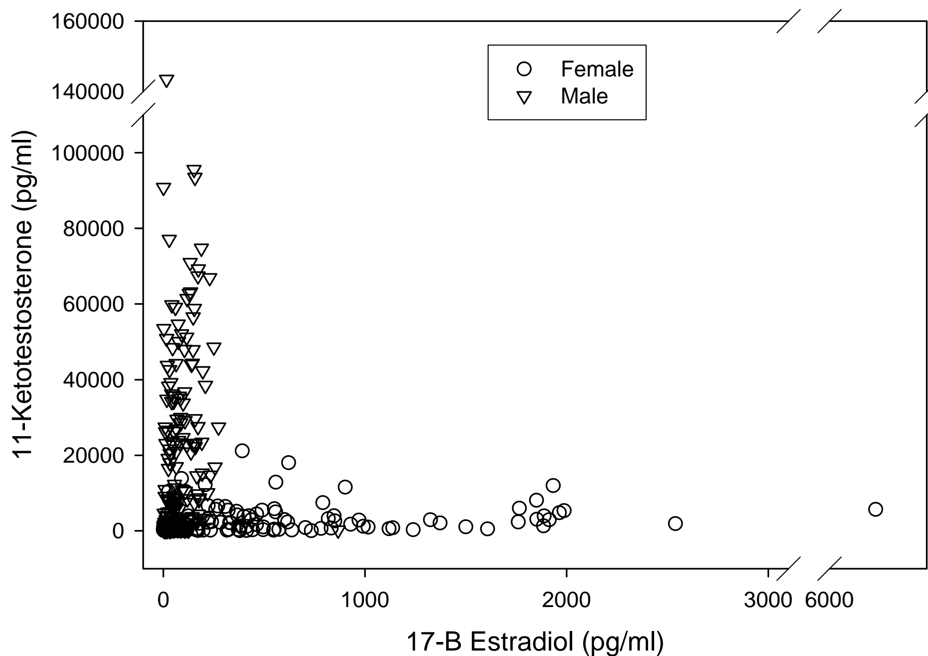


Figure 18. Relationship of 11-ketotestosterone and 17-β estradiol levels for male and female shovelnose sturgeon.

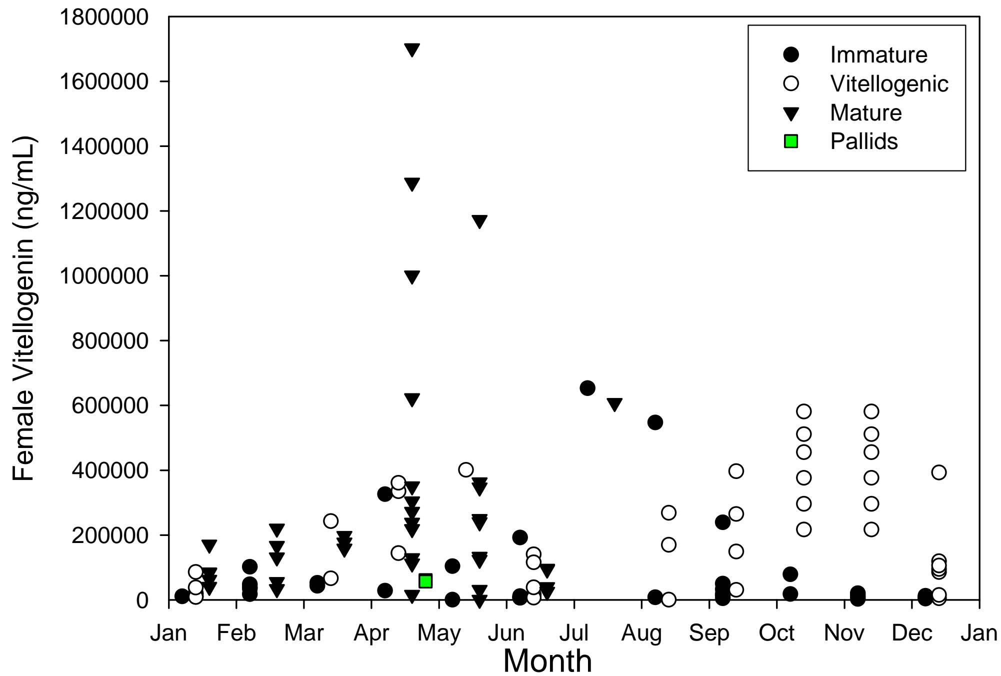
Stage II - V shovelnose males, whereas E2 levels were low and similar to those seen at all reproductive stages of shovelnose.

Plasma Vitellogenin Concentrations

Mean VTG levels in females increased with increasing reproductive stage although within a stage variability was high among individuals (Table 8, Fig. 15). Male mean VTG values were less variable both within and among reproductive stages and were generally 100- to 1000-fold lower than female values (Table 8, Fig. 15). Female VTG levels were lowest in winter months, highest in early spring and summer in Stage V fish, and then decreased but were still elevated in fish caught in the fall (Fig. 19). The VTG levels in males were not affected by seasons (Fig. 19).

Discriminant Analysis

Of the 349 total fish, we do not have the following: GSI for 11 fish (females Stage II = 2, Stage III = 3, Stage V = 1; males Stage II = 3, Stage IV = 1, Stage V = 1), E2 for one fish (female Stage II), KT for two fish (female Stage I = 1, Stage II = 1), and VTG for 109 fish (female Stage I = 8, Stage II = 15, Stage III = 6, Stage IV = 6, Stage V = 10; males Stage I = 3, Stage II = 16, Stage III = 16, Stage IV = 8, Stage V = 18, Stage VI = 3); the latter due to one processing error and 108 samples that did not contain enough plasma to run the test. The data presented are those where capture date was not used in reproductive stage determination. This means that 14 females that were Stage IV were not changed to Stage V and 4 females that were Stage V were not changed to Stage IV based on date.



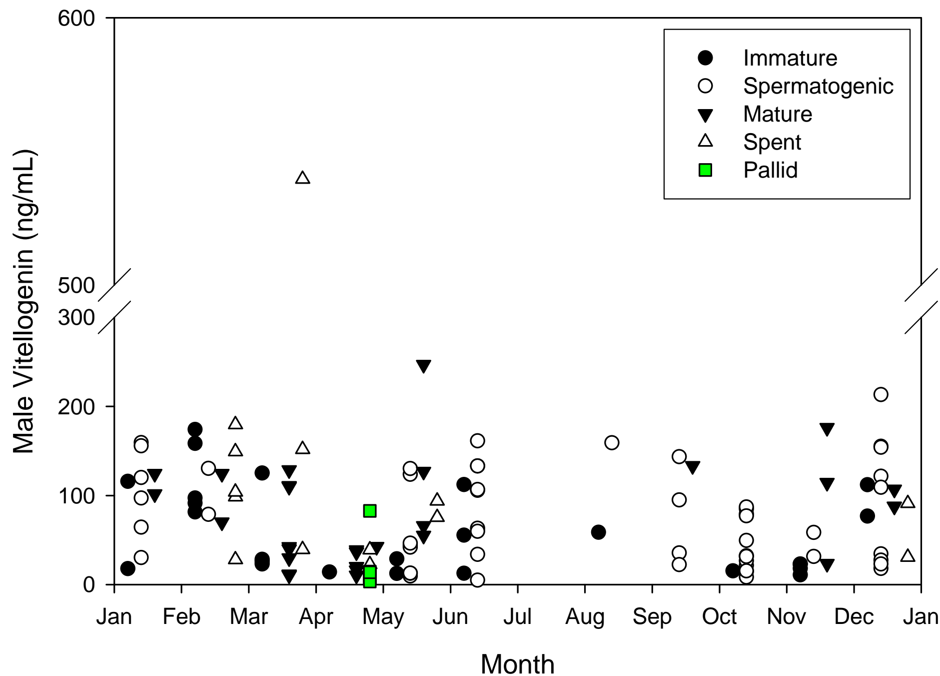


Figure 19. Mean vitellogenin levels in female and male shovelnose and pallid sturgeon by month and reproductive stage. Immature = Stages I and II, spermatogenic/vitellogenic = Stages III and IV, mature = Stage V, and spent = Stage VI. No reproducitve stage determined for pallids.

Following the same structure reported in Webb et al. (2002), concentrations of plasma sex steroids and VTG differed significantly ($P < 0.0001$; Table 10 and 11) among the four groups of sex and reproductive stage (i.e., male and female Immature and Maturing fish). The Bonferroni mean comparison tests revealed that plasma E2 and KT did significantly differ between some pairs of means, but there were no significantly distinct sets of means between sexes and reproductive maturity (Fig. 20). Plasma concentrations of VTG were significantly higher in females compared with males, and Maturing females were significantly higher than Immature females (Fig. 20). The stepwise DFA revealed that plasma concentrations of VTG and KT were the best predictors to distinguish sex of all fish (Table 12). The use of this discriminant function led to the incorrect classification of 0% of females and males, both exceeding the 50% probability of incorrectly classifying females and males by chance alone (Table 13). Due to the low amount of variance explained in the model by KT (Table 12), sex was also predicted by VTG alone with an error rate < 1% (Table 13), with an incorrect classification of 0% of females and 1% of males. The cross-validation of the model revealed the same error rates.

To distinguish fish by sex and maturity, plasma E2, KT, and VTG were found to be the best predictors in the analysis of male and female fish combined (Table 12). This derived discriminant function led to the incorrect classification of 12 – 13% of the males and females in the different maturities (Table 14). By comparison, 75% of the total number of fish would be incorrectly classified into their respective groups by chance alone. Overall, 13% of the fish were incorrectly classified into a sex and maturity by all three plasma indicators. The cross-validation of the model for sex and maturity using all three plasma indicators as the predictors revealed error rates of 12 - 16% for females and 14 - 15% for males, with a total error rate of 14 % (females: Immature = 16%, Maturing = 12%; males: Immature = 15%, Maturing = 14%). Due to the large amount of variance VTG explained in the ANOVA (Table 12), we also conducted a DFA with only VTG predicting sex and maturity, but the overall error rate increased from 13% to 31%.

When only females were used in the DFA, plasma E2, KT, and VTG were chosen as the best predictors of reproductive stage (Table 12). These variables produced incorrect classifications of 0% for Stage I, 43% for Stage II, 50% for Stage III, 15% for Stage IV, and 48% for Stage V (Table 15). Overall, 31% of the fish were incorrectly classified into a reproductive stage by the three plasma indicators. The cross-validation of this model revealed 100% (Stage I), 46% (Stage II), 63% (Stage III), 27% (Stage IV) and 48% (Stage V) error rates. All 3 (100%) of the Stage I females were misclassified as Stage III females in the cross-validation. Due to the large amount of variance E2 explained in the ANOVA (Table 12), we also conducted a DFA with only E2 predicting female reproductive stage, but the overall error rate increased from 31% to 56%.

When only males were used in the DFA, plasma KT was chosen as the best predictor of reproductive stage (Table 12). This variable produced incorrect classifications of 67% for Stage I, 98% for Stage II, 69% for Stage III, 100% for Stage IV, 41% for Stage V, and 35% for Stage VI (Table 15). Overall, 68% of the fish were incorrectly classified by sex and reproductive stage. The cross-validation of this model revealed 67% (Stage I), 98% (Stage II), 80% (Stage III), 100% (Stage IV), 57% (Stage V) and 35% (Stage VI) error rates. All 33 (100%) of the Stage IV males were misclassified as 5 Stage I, 8 Stage III, 16 Stage V and 4 Stage VI males in the cross-validation.

The quadratic classification functions derived using all fish combined to predict the sex of an unknown individual were for females, $-3.519 + 4.111\,(\log_{10}\text{VTG}) – 1.082\,(\log_{10}\text{VTG})^2$ and males, $-3.331 - 6.667\,(\log_{10}\text{VTG}) -2.666\,(\log_{10}\text{VTG})^2$. These functions may be used to predict

Table 10. Mean gonadosomatic index (GSI), sex steroids, and vitellogenin of female and male shovelnose sturgeon at various reproductive stages. The reproductive stage does not take capture date into consideration. Back transformed means, (N), and log_{10}- and squareroot -transformed standard error are presented. Low Stage I fish collections maybe an artifact of the 550 mm total length target size we tried to maintain throughout the study.

	GSI (%)		Vitellogenin (ug/mL)		17-β estradiol (pg/mL)		11-ketotestosterone (pg/mL)	
Stage	Female	Male	Female	Male	Female	Male	Female	Male
I	2.21 (12)	2.45 (5)	10.86 (3)	0.02 (1)	31.43 (11)	41.67 (6)	336.76 (10)	1968.82 (6)
	0.172	0.185	0.132	-	0.129	0.255	0.174	0.237
II	2.64 (49)	1.19 (42)	31.87 (35)	0.04 (24)	34.78 (49)	29.64 (43)	237.79 (49)	1143.75 (43)
	0.064	0.057	0.119	0.098	0.089	0.080	0.097	0.109
III	4.96 (28)	3.68 (45)	50.47 (24)	0.07 (29)	208.44 (30)	82.59 (45)	529.87 (30)	12927.37 (45)
	0.154	0.097	0.126	0.089	0.103	0.060	0.114	0.115
IV	15.06 (32)	3.36 (33)	195.46 (26)	0.05 (24)	632.59 (32)	53.73 (33)	1852.68 (32)	10309.76 (33)
	0.115	0.093	0.065	0.073	0.089	0.083	0.105	0.140
V	18.82 (34)	3.30 (46)	218.41 (25)	0.06 (28)	340.38 (35)	44.18 (46)	2113.68 (35)	12320.20 (46)
	0.074	0.058	0.098	0.078	0.088	0.071	0.112	0.110
VI	-	1.17 (17)	-	0.08 (14)	-	30.27 (17)	-	511.27 (17)
		0.097		0.101		0.113		0.181

Table 11. One-way analysis of variance conducted to determine whether there were differences between the log_{10}-transformed sex steroids (11-ketotestosterone = KT, 17-β estradiol = E2) and vitellogenin (VTG) concentrations among the 4 groups of sex and maturity.

Variable	N	df	MS	F	P
KT	346	3	43.99	85.96	< 0.0001
E2	347	3	21.03	76.50	< 0.0001
VTG	233	3	196.1	709.4	< 0.0001

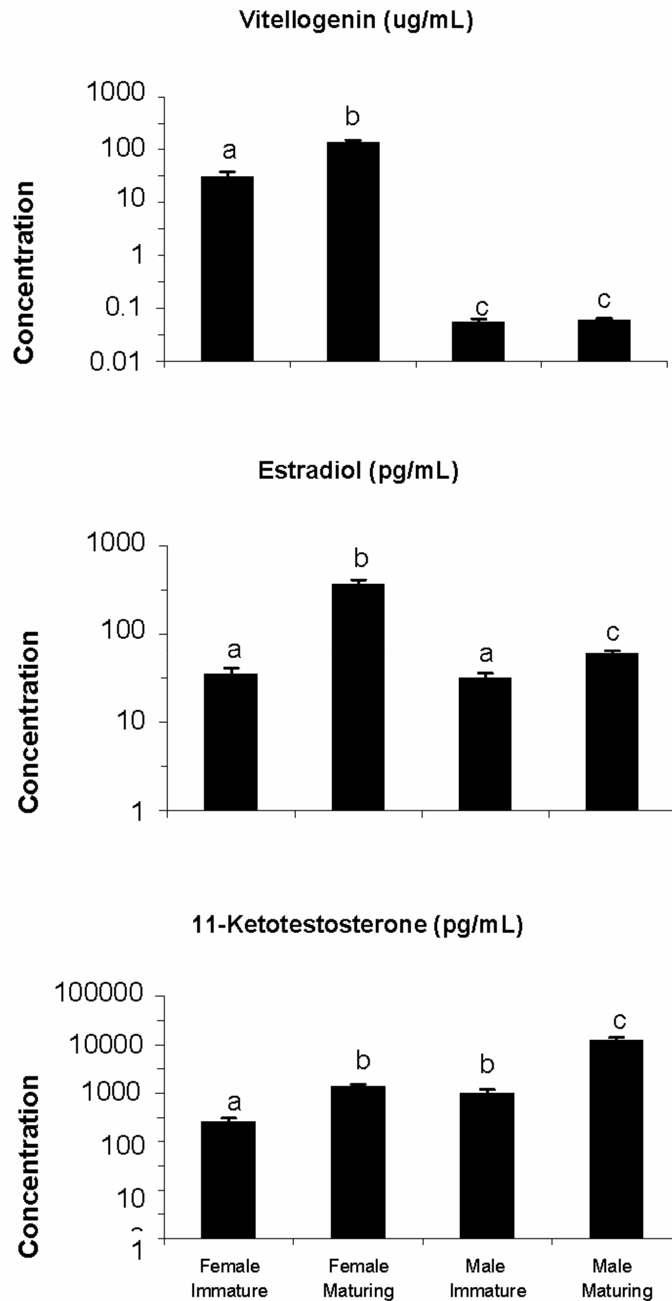


Figure 20. Mean (+ SE) plasma sex steroids (11-ketotestosterone and 17-β estradiol), and vitellogenin in shovelnose sturgeon males and females captured in the lower Missouri River. Bars having different letters are significantly different (alpha = 0.05). The maturity does not take capture date into consideration.

Table 12. Results from stepwise discriminant function analysis predicting sex only, sex and maturity, or reproductive stage by sex of shovelnose sturgeon captured in the Missouri River, which was conducted using males and females combined, male only, and female only. Blood plasma indicators used in the analysis were all log_{10}-transformed: 11-ketotestosterone (KT), 17-β estradiol (E2), and vitellogenin (VTG); P = 0.05 *, P = 0.0001 **.

Variable	Partial R^2	*F*-statistic	Wilk's lambda	Average squared canonical correlation
		Sex		
VTG	0.89	1801.02 **	0.11 **	0.89 **
KT	0.08	20.32 **	0.10 **	0.90 **
		Sex and reproductive maturity		
VTG	0.90	709.45 **	0.10 **	0.30 **
KT	0.39	49.00 **	0.06 **	0.42 **
E2	0.20	18.56 **	0.05 **	0.48 **
		Reproductive Stage; females		
E2	0.52	29.06 **	0.48 **	0.13 **
KT	0.18	5.74 *	0.40 **	0.16 **
VTG	0.12	3.63 *	0.35 **	0.18 **
		Reproductive Stage; males		
KT	0.46	19.14 **	0.54 **	0.09 **

Table 13. Classification summary for determination of sex from the quadratic discriminant function analysis for shovelnose sturgeon using log10-transformed plasma vitellogenin (VTG) and 11-ketotestosterone (KT) or only VTG as predictors. Values in bold are the percentages of fish correctly classified, whereas values not in bold are the percentages of misclassified fish: sample sizes (N) are in parentheses.

	Classified		
Actual	Female	Male	Total
	VTG and KT		
Female	**100**	0	100
	(113)	(0)	(113)
Male	0	**100**	100
	(0)	(120)	(120)
Error Rate	0	0	0
	VTG		
Female	**100**	0	100
	(113)	(0)	(113)
Male	1	**99**	100
	(1)	(119)	(120)
Error Rate	0	1	> 1

Table 14. Classification summary for determination of sex and maturity from the quadratic discriminant function analysis for shovelnose sturgeon using $\log_{10}$-transformed plasma vitellogenin (VTG), 17-β estradiol (E2), and 11-ketotestosterone (KT) or only VTG as predictors. Values in bold are the percentages of fish correctly classified, whereas values not in bold are the percentages of misclassified fish: sample sizes (N) are in parentheses.

		Classified				
		Female		Male		
Actual		Immature	Maturing	Immature	Maturing	Total
All Fish				**VTG, E2, and KT**		
Female	Immature	**87**	13	0	0	100
		(33)	(5)	(0)	(0)	(38)
	Maturing	12	**88**	0	0	100
		(9)	(66)	(0)	(0)	(75)
Male	Immature	0	0	**87**	13	100
		(0)	(0)	(34)	(5)	(39)
	Maturing	0	0	12	**88**	100
		(0)	(0)	(10)	(71)	(81)
Error Rate		13	12	13	12	13
				VTG		
Female	Immature	**74**	26	0	0	100
		(28)	(10)	(0)	(0)	(38)
	Maturing	21	**79**	0	0	100
		(16)	(59)	(0)	(0)	(75)
Male	Immature	0	0	**46**	54	100
		(0)	(0)	(18)	(21)	(39)
	Maturing	1	0	22	**77**	100
		(1)	(0)	(18)	(62)	(81)
Error Rate		26	21	54	23	31

Table 15. Classification summary for determination of reproductive stage from the quadratic discriminant function analysis for shovelnose sturgeon using the significant log_{10}-transformed plasma indicators from the stepwise selection procedure as predictors. Values in bold are the percentages of fish correctly classified, whereas values not in bold are the percentages of misclassified fish: sample sizes (N) are in parentheses.

Actual	Classified						Total
	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI	
Only Females using 17-β Estradiol, 11-Ketotestosterone, and Vitellogenin							
Stage I	**100**	0	0	0	0	N/A	100
	(3)	(0)	(0)	(0)	(0)	N/A	(3)
Stage II	0	**57**	37	0	6	N/A	100
	(0)	(20)	(13)	(0)	(2)	N/A	(35)
Stage III	0	21	**50**	25	4	N/A	100
	(0)	(5)	(12)	(6)	(1)	N/A	(24)
Stage IV	0	4	8	**85**	4	N/A	100
	(0)	(1)	(2)	(22)	(1)	N/A	(26)
Stage V	0	4	8	36	**52**	N/A	100
	(0)	(1)	(2)	(9)	(13)	N/A	(25)
Error Rate	0	43	50	15	48		31
Only Males using 11-Ketotestosterone							
Stage I	**33**	17	0	0	33	17	100
	(2)	(1)	(0)	(0)	(2)	(1)	(6)
Stage II	37	**2**	5	0	12	44	100
	(16)	(1)	(2)	(0)	(5)	(19)	(43)
Stage III	13	0	**31**	0	44	11	100
	(6)	(0)	(14)	(0)	(20)	(5)	(45)
Stage IV	15	0	24	**0**	48	12	100
	(5)	(0)	(8)	(0)	(16)	(4)	(33)
Stage V	13	0	20	0	**59**	9	100
	(6)	(0)	(9)	(0)	(27)	(4)	(46)
Stage VI	18	6	0	0	12	**65**	100
	(3)	(1)	(0)	(0)	(2)	(11)	(17)
Error Rate	67	98	69	100	41	35	68

whether new observations are female or male. For example, a sturgeon with plasma concentrations of 6.001 ug/ mL VTG has a value of -0.975 using the classification function equation for females and -10.134 using the classification function equation for males. The highest value of the two equations predicts the sex of the individual based on the discriminant analysis model (Tabachnick & Fidell, 1996). Therefore, this individual would be classified as a female.

The classification functions for the four levels of sex and maturity using all fish combined are

$-8.1708 + 2.334 (\log_{10} VTG) + 3.815 (\log_{10} KT) + 3.944 (\log_{10} E2) – 1.078 (\log_{10} VTG)^2 – 1.057 (\log_{10} KT)^2 – 1.584 (\log_{10} E2)^2 + 0.186 (\log_{10} VTG \times \log_{10} KT) – 0.023 (\log_{10} VTG \times \log_{10} E2) + 0.240 (\log_{10} KT \times \log_{10} E2)$

and

$$-7.758 - 3.731 (\log_{10} VTG) + 4.752 (\log_{10} KT) + 1.461 (\log_{10} E2) - 2.593 (\log_{10} VTG)^2 - 1.768 (\log_{10} KT)^2 - 3.362 (\log_{10} E2)^2 - 0.362 (\log_{10} VTG \times \log_{10} KT) - 0.313 (\log_{10} VTG \times \log_{10} E2) + 1.372 (\log_{10} KT \times \log_{10} E2)$$

Immature females and males, respectively, and

$$-19.683 + 0.939 (\log_{10} VTG) + 6.294 (\log_{10} KT) + 8.117 (\log_{10} E2) - 2.257 (\log_{10} VTG)^2 - 1.370 (\log_{10} KT)^2 - 2.712 (\log_{10} E2)^2 + 0.378 (\log_{10} VTG \times \log_{10} KT) + 1.191 (\log_{10} VTG \times \log_{10} E2) + 0.158 (\log_{10} KT \times \log_{10} E2)$$

and

$$-18.844 - 6.559 (\log_{10} VTG) + 5.157 (\log_{10} KT) + 6.967 (\log_{10} E2) - 2.878 (\log_{10} VTG)^2 - 0.985 (\log_{10} KT)^2 - 3.087 (\log_{10} E2)^2 - 0.231 (\log_{10} VTG \times \log_{10} KT) + 0.361 (\log_{10} VTG \times \log_{10} E2) + 0.631 (\log_{10} KT \times \log_{10} E2)$$

for Maturing females and males.

Again, it is the highest value from the four equations that determines the group of sex and maturity to which an individual would be classified.

Discussion

Females

Moos (1978) concluded that female shovelnose sturgeon are on a 2- or 3-year reproductive cycle generally passing one year in Stage II, completing Stages III and IV in the second year, and spawning the third year. His conclusion is in part based on the capture of relatively high numbers of Stage II individuals at all times of the year. Our data show the same pattern and support his findings. Because we biased our samples by targeting fish ≥ 550 mm TL (i.e., only 9 fish between 350 and 550 mm) and collected in a limited geographical area, further interpretation of the seasonal and total relative proportion of reproductive stages represented by our data must be cautioned. Nevertheless, it is interesting to note that Moos (1978) reported finding equal proportions of Stage VI and Stage V females whereas, we did not observe any spent (i.e., Stage VI) females.

Ovaries with Stage I and II oocytes tended to be very fatty and as oocytes developed the ovaries contained less fat. The female GSI values we observed were comparable to those reported by Moos (1978) and generally allowed for assignment of reproductive stage particularly when considered along with fat content.

Fecundity estimates for shovelnose sturgeon in our study ranged from 8,601 to 42,275 follicles per female depending on size. Average relative fecundity for shovelnose was 20 eggs/g. These results are similar to those reported by Christenson (1975) for shovelnose but greater than that estimated for both pallid sturgeon and white sturgeon (Keenlyne et al., 1992; Chapman et al., 1996) indicating that shovelnose tend to have smaller eggs.

Our data for shovelnose sturgeon are consistent with the observation in Atlantic sturgeon that fecundity increases with age and length then levels off or decreases at increased age (Van Eenennaam & Doroshov, 1998). Numerous reports on the use of spines to age sturgeons caution that there is a high degree of error in age assignment (Whiteman et al., 2004; Rien & Beamsderfer, 1994; Hurley et al., 2004). Shovelnose sturgeon of the same age and size as we measured have been caught from the Missouri River (Carlson et al., 1985). Although we may not precisely know the age of the fish in our study, length and fecundity co-vary supporting our age-fecundity relationship.

Males

Few Stage I and Stage VI males were captured while the other stages were well-represented in our collections. Stage I males were those that were considered young and entering the reproductive cycle for the first time and may have been underrepresented due to our size selection criteria. Stage VI testes were similar to those at Stage II but we considered testes that had almost no fat and residual sperm to be Stage VI. Our finding of spent males in late fall and early spring prior to the expected spawning season (May through July) coincides with Moos' (1978) conclusion that at least some male shovelnose do not spawn annually. The GSI was useful only for separating testes containing developing sperm from those that were pre-spermatogenic.

Sex Steroid and Vitellogenin Profiles

Sex steroids for shovelnose sturgeon follow a similar seasonal and reproductive stage-specific pattern observed in other sturgeon species (Amiri et al., 1996; Barannikova et al., 2004). Estrogen and androgen predominated in females and males, respectively with females generally showing an E2/KT ratio of greater than 0.2 and males a ratio less than 0.2. Both estrogens and androgens tended to be low in males and females prior to gamete development and post-spawn. E2 levels for females at Stages IV and V were significantly elevated compared to levels for all other reproductive stages of females and all reproductive stages of males. KT was almost ten-fold higher in males at Stages III, IV and V relative to other reproductive stages of males and all reproductive stages of females.

The VTG levels were ten- to ten thousand-fold greater in females than in males at all reproductive stages and thus may be used as an indicator of sex in shovelnose sturgeon ≥ 550 mm. As expected, VTG levels in females increased concomitantly with E2 and as gonads developed. Shovelnose sturgeon differed from a beluga-sterlet hybrid in that Stage V females maintained elevated levels of VTG (Amiri et al., 1996) but were similar to stellate sturgeon that maintained high levels of VTG during the spawning migration (Ceapa et al., 2002). A seasonal pattern was observed for VTG in females but not males. Vitellogenesis was greatest during the spring through late fall and greatly reduced during winter.

Even though VTG, KT, and E2 had significant differences between the four sex and maturity groups, VTG protein was the best single predictor of shovelnose sturgeon sex with a success rate > 99%. However, all three were needed to predict the four groups of sex and maturity with an 87 % success rate. Predicting the five female shovelnose sturgeon reproductive stages had an even lower success rate of 69%. The lowest success rate of 32 % was when we predicted the six male shovelnose sturgeon reproductive stages.

When comparing the results from the shovelnose sturgeon with the white sturgeon (Webb et al., 2002), we found similar trends between the two species with KT and E2 levels among the four sex and maturity groups. We had a higher success rate at determining sex using VTG than Webb et al. (2002) did using testosterone and E2; however, we had a lower success rate at predicting female reproductive stage. Webb et al. (2002) also found testosterone to be an important steroid in the prediction of female reproductive stage. Our lower success rate at predicting reproductive stage may be due to the lack of testosterone in our predictor models. Testosterone was not measured in this study because it was thought that KT would be a more robust indicator of male sex and reproductive stage.

Sex steroids are reportedly affected by handling stress (Pickering et al., 1987) thus it is recommended that blood be collected from fish as soon after capture as possible. In order to understand the effect that transport and laboratory holding of shovelnose sturgeon would have on sex steroid measurements we collected blood from the same fish at capture and then 48

hours later. Our finding that sex steroid measurements did not differ between the sampling times suggests that any stresses associated with transport and holding did not alter basal sex steroid measurements any more than the stresses associated with capture. Therefore, collection of blood for sex steroid analysis may be made up to 48 hours after capture without affecting results. Although we recommend that blood continue to be collected as soon after capture as possible, in those situations where this is not feasible, reasonable results may be expected.

The rarity of the pallid sturgeon has made it difficult to study in the wild and as such the shovelnose sturgeon, a congeneric, is often used as a surrogate. However, it is often argued that the biological differences inherent in these sympatric species prevent us from making inferences to pallids from knowledge gained in studies with the shovelnose. Currently, we cannot fairly evaluate this argument because so little information is available on the biology of either species. In the present study, the abundance of shovelnose sturgeon allowed us to develop temporal sex steroid and VTG profiles matched to reproductive stage, a task that would have been impossible with pallid sturgeon. The limited hormonal data we obtained from pallid females estimated to be Stage V or Stage I and II is consistent with the shovelnose data. Results for the male pallids tended to be slightly elevated but still very comparable to male shovelnose. In contrast, VTG measurements in presumably Stage V females were lower than expected for shovelnose. Our data generally suggest that pallids and shovelnose are more alike with respect to E2 and KT than either compared with another North American sturgeon species, the white sturgeon (Webb et al., 2002). Although sex steroid and VTG differences between the genera may simply be due to assay differences, we cannot reject the shovelnose as a surrogate for the pallid based on sex steroid and VTG measurements.

The reproductive cycle is controlled by sex steroids functioning along the pituitary-hypothamal-gonadal axis (Hoar 1965). Photoperiod and temperature largely regulate sex steroid activity, although many factors, both biotic and abiotic can have a modulating influence (Davies & Bromage, 2002). Data presented here provide basic biological information on the reproductive biology of the shovelnose sturgeon and limited data on sex steroid and VTG levels in pallid sturgeon that may be useful in evaluating physiological responses to environmental conditions and anthropogenic impacts.

CHAPTER 3

Visual Guide for Identifying Shovelnose Sturgeon Reproductive Stages

Abstract

Shovelnose sturgeon were collected from the Missouri River and ultrasonic and endoscopic imagery and blood and gonadal tissue samples were taken. The full set of data was used to develop monthly reproductive stage profiles for shovelnose sturgeon that could be compared to data collected on pallid sturgeon. This paper presents a comprehensive reference set of images, sex steroids, and vitellogenin (VTG, an egg protein precursor) data for assessing shovelnose sturgeon sex and reproductive stage. This reference set includes ultrasonic, endoscopic, histologic, and internal images with videos of ultrasonic and endoscopic scans of male and female gonads of shovelnose sturgeon at each reproductive stage along with complementary data on average 17-β estradiol, 11-ketotestosterone, VTG, gonadosomatic index, and polarization index. Results reported here can also be found in Wildhaber et al. (2006).

Introduction

A critical aspect for successful management for maintenance or recovery of populations of sturgeon species is to establish and validate breeding populations. To accomplish this, an understanding of the reproductive cycle of shovelnose and pallid sturgeons and the environmental factors that affect reproduction are necessary. However, shovelnose and pallid sturgeon gametogenesis and reproductive endocrinology has not heretofore been documented until this study (see Chapter 2). Sturgeons differ from most teleosts by their late puberty and biennial or longer ovarian cycles (Dettlaff et al., 1993). In fishes, gametogenesis and reproduction are controlled by a number of different steroids (Sullivan et al., 1997). Likewise, these sex steroids are controlled by numerous environmental and biological stimuli such that concentrations of the sex steroids will vary with reproductive stage, age and season (Rosenblum et al., 1987). A single steroid measurement is unlikely to differentiate between sexes and among reproductive stages (Ceapa et al., 2002; Webb et al., 2002); thus, the multi-parameter approach we took to begin to differentiate reproductive stage (see Chapters 1 and 2).

As part of this research, ultrasonic and endoscopic methods were developed to visually observe the gonads of adult shovelnose and pallid sturgeon (Wildhaber et al, 2005; Chapter 1). Concomitantly, sex steroid and vitellogenin (VTG, an egg protein precursor) profiles important in gametogenesis and gonadal maturation were measured in blood plasma (see Chapter 2). These techniques collectively allow for the direct assessment of the sex and reproductive stage of shovelnose and pallid sturgeon in the field with minimal impact to the fish. These tools are minimally invasive and sufficiently sensitive to allow investigators to track the progress of individual fish through the reproductive cycle and to determine whether spawning has occurred. In addition, histological examination was done on each fish to validate and enhance the ability of the imaging methods to identify sex and reproductive stage of shovelnose sturgeon. This chapter provides a set of reference ultrasonic, endoscopic, and histologic images for each reproductive stage of male and female shovelnose sturgeon, as defined by Moos (1978). Also

presented is a parallel set of reference internal images from fish dissections along with complementary data on 17-β estradiol (E2), 11-ketotestosterone (KT), VTG, gonadosomatic index (GSI), and polarization index (PI).

Methods and Materials

Study Area and Sampling

Fieldwork was conducted in the Upper and Lower Missouri River (Fig. 1). In the Upper Missouri River section, fish were collected in Montana and North Dakota from below Fort Peck Dam, from the Lower Yellowstone River, and from near the confluence of these two rivers. In the Lower Missouri River, fish were collected in Missouri between the confluences with the Chariton and Osage rivers (Fig. 1). Monthly from May 2001 to June 2002, attempts were made to collect up to 30 adult shovelnose sturgeon (15 males and 15 females) from the Lower Missouri River. Fish were collected using multiple gears (i.e., overnight hoopnet, trot line, stationary gillnet sets, and drifting trammel nets) depending on season or behavior of the fish. Because no Stage VI females were caught during the primary study, sex steroids and VTG protein levels were added to this study from seven Stage VI females caught from the Missouri River in Missouri in 2004. Additionally, ultrasonic and endoscopic images were collected from 14 Stage VI females caught from the Missouri River in Iowa, 2005.

Immediately after capture, shovelnose sturgeon were uniquely tagged with a Floy® tag for future reference. Tagged sturgeon were held in 378.5-L tanks constantly supplied with fresh river water until the fish could be examined on shore using a portable ultrasound unit. The shovelnose sturgeon collected in the Lower Missouri River were transported to the Columbia Environmental Research Center (CERC) where they were held at river temperatures in fiberglass flow-through circular tanks for 24-48 hrs until the sex was re-examined. In the laboratory, the sex of these fish was determined using ultrasonic and endoscopic imagery that was later verified through histological examination.

Blood Collection and Field Processing

While in the field, within 4 h after capture 6 – 8 ml of blood were collected (Fig 3) and processed to collect plasma (Schmitt et al., 1999; Fig. 4). Blood samples were kept on wet ice until centrifugation at 3500 rpms for 10 minutes (Fig. 4). Plasma was transferred to 1.8-ml cryovials and snap frozen in a slurry of ethanol and dry-ice, then transferred to CERC to be stored in a -80°C freezer until steroid extraction and analysis.

Ultrasonic Imaging

Ultrasonic imagery was collected in the laboratory using a clinical laboratory ultrasound unit (Shimadzu SDU-400 Plus with a 7.5 Mhz linear probe) or in the field using a portable ultrasound unit (Sonosite 180 Plus with a 5-10 MHz linear probe—Fig. 5). Application of ultrasound followed basic methods (Zwiebel & Sohaey, 1998) with slight modifications. Prior to scanning the body cavity of the fish, the scanning surface of the ultrasound transducer was first covered with ultrasound gel and then a plastic sheath was placed over the ultrasound transducer. During scanning, focus depth, output power, and frame rate were all kept constant. The other ultrasound settings varied slightly from primary goals of: frame averaging on, gain of 16, and preset ‘body’ settings.

In order to acquire an interpretable ultrasonic image of sturgeon internal organs, ultrasonic images had to be collected from the abdominal surface of the fish. The presence of scutes on the back and sides of sturgeons preclude the use of ultrasound on any of those body

surfaces as ultrasonic signals cannot penetrate the calcified surface of the scutes. Therefore, the fish were held ventral side up just below the surface of the water. The fish were docile enough in this position that no anesthesia was necessary. Whether in the field or in the laboratory, a full body scan along the abdominal surface was performed on each fish from vent to opercula with the ultrasound probe oriented in the transverse plane (Fig. 21). Images were recorded for later analyses. To improve on Wildhaber et.al. (2005), we also scanned the fish in the frontal view which was along the side of the fish from head to tail between the row of belly scutes and the first row of side scutes) to further refine our ability to identify sex (Fig. 22).

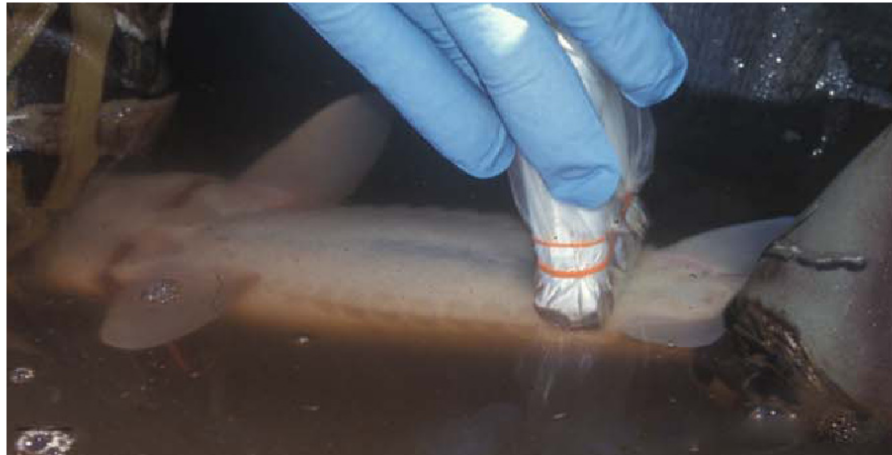


Figure 21. Collection of internal images of a shovelnose sturgeon along the transverse plane using ultrasound.

Figure 22. Collection of internal images of a shovelnose sturgeon along the frontal plane using ultrasound.

Endoscopic Imaging

The endoscopes initially used were rigid borescopes, 18 mm in length and 2 mm in diameter with a standard light source (Zibra Corporation). The first borescope had a 10-mm focal length with a view directly from the end of the scope. The second borescope had a 4-mm focal length and a view that was at a 70° angle to the end of the probe. Experience with the previous two scopes prompted having a custom built flexible borescope produced by Zibra

Corporation to meet our specifications (i.e., Milliwand flexible probe, model number P20.250.70 FMW2 with a 1-mm focal length and a view that was at a 70° angle to the end of the probe—Fig. 6) for which use began in October of 2001. For all the endoscopes used, the endoscope was covered by a glass tube that was sealed on one end in order to acquire a clearly focused image.

Using the endoscope, attempts were made to view the gonads of each fish in two ways: through the gonoduct wall and through a small ventral incision in the body cavity wall. During examination, the fish was oriented as previously described for collection of ultrasonic images. The difference in orientation from that during ultrasonic examination was the tail of the fish being raised out of the water while the head and gills were kept submerged. The result was a fish that was at a 45° angle to the water surface. This angle increased the ease of insertion of the endoscope.

The endoscope was inserted into the urogenital pore immediately posterior to the vent. The urogenital pore opens into a short sinus that branches into paired gonoducts. The paired gonoducts are located dorso-laterally between the body cavity wall and mesonephros and run from the urogenital sinus to the approximate mid-point of the body cavity (Hoar, 1969; Moos, 1978). The gonoducts terminate in a collapsible funnel-like structure, or ostia, that acts as a one-way valve to release mature oocytes from the coelomic cavity while preventing external materials from entering. The gonoducts in male sturgeon are vestigial and do not appear to have a function. To view the gonads through the gonoduct wall, the probe was inserted into the urogential pore and then directed into the right or left gonoduct.

In order to view the gonads through the body cavity wall, first a small anterior to posterior abdominal incision was made in the ventral wall of the body cavity towards the posterior end of the fish just off the midline of the body using a scalpel. The incision was approximately 1-2 cm long and was just deep enough to penetrate the body cavity wall. The endoscope was inserted through the incision and run simultaneously posterior to anterior and ventral to dorsal along the full length of the body cavity.

Measurements and Tissue Sampling

After shovelnose sturgeon were humanely euthanized, total length was measured (tip of rostrum to where uppermost ray joins caudal filament; does not include filament), and fish were weighed to the nearest 50 grams using a hanging scale (see Wildhaber et al. 2005 and Chapter 1). The body cavity was surgically opened and a voucher photo was taken of the gonad prior to removal. Gonads with associated fat were weighed to the nearest 0.05 grams (Denver Instruments, XP-1500) for calculating GSI (GSI = [gonad and associated fat weight/body weight]*100). Fat was trimmed off the gonad and the gonads reweighed to obtain an estimate of percent fat composition of the gonad. Five 1-cm^3 pieces of gonadal tissue were collected along the anterior to posterior axis. The gonad tissue was preserved in 10% neutral buffered formalin (NBF) until processing for histology. Additionally, three 5-gram pieces of ovaries containing gray to black follicles were preserved in NBF and later the PI was determined. The PI was determined by measuring the long diameter of the oval follicle from animal to vegetal pole and dividing this into the distance between the germinal vesicle and the animal pole (Dettlaf et. al., 1993).

Histological Analysis

Histological analysis was conducted on the gonads of the sturgeon to accurately determine sex and reproductive stage. Samples were preserved and processed according to methods outlined by Blazer (2002). Briefly, after an initial fixation of 48 h in 10% NBF, samples

were transferred through a series of Hepes Buffer rinses to 70% ethanol (EtOH). The samples were stored in the 70% EtOH until analysis was performed. Routine processing of tissue involved trimming into small pieces, dehydration through a series of alcohols followed by immersion in an organic solvent, then infiltration with paraffin. Paraffin blocks containing the tissues were cut into 5-μm slices. Sections were taken at three different depths to ensure the microscopic evaluations were representative of the entire tissue. These sections were placed on glass slides, allowed to dry, and then deparaffinized. The slides were stained with hematoxylin and eosin (Luna, 1968).

Light microscopy was performed to evaluate each fish as to sex and reproductive stage according to Moos (1978) without date of capture considered. Gonads of male fish were classified as Stage I- Immature: lots of fat, few spermatogonia; Stage II- Developing: germ cells consist mostly of spermatogonia in nests or singly; Stage III-Spermatogenic: lobules filled with spermatocytes, spermatids or developing spermatozoa; Stage IV- Pre-spawning: most lobules filled with spermatozoa; Stage V- Spawning: all lobules are filled with or releasing spermatozoa; Stage VI- Post-spawn (Spent): collapsed lobules empty or containing some residual spermatozoa. Gonads of female fish were classified as Stage I- Immature: large fat body, few small oogonia; Stage II- Developing: pre-vitellogenic oocytes abundant, less fat than Stage I and lamellar structure of gonad obvious (i.e., ovigerous folds); Stage III- Vitellogenesis: yolk deposition, oocytes greatly increase in size and are enveloped by follicular cells, and the germinal vesicle is near center of follicle; Stage IV- Pre-spawning: follicles enlarge, begin to turn black, and the germinal vesicle begins to shift towards the animal pole; Stage V- Spawning: germinal vesicle continues to migrate closer to animal pole where it will ultimately breakdown as mitosis resumes, follicles are black; Stage VI- Post-spawn (Spent): no to little fat, empty follicles, few atretic follicles, and early oocytes.

Sex Steroid Extraction and Analysis

Plasma samples were extracted using diethyl-ether to separate the sex steroids from the binding proteins. The steroid-containing ether phase was snap frozen and the resulting supernatant was decanted and placed in a 30°C water bath and evaporated under nitrogen. The steroid residues were then reconstituted in either Cayman Chemical's competitive enzyme immunoassay (EIA) Buffer for E2 analysis or PBSG (phosphate buffered saline pH 7.0 with 1 % gelatin) for KT. Extracted E2 samples were stored at 4° C and KT samples were stored at -20° C until assayed.

Extraction efficiency was determined by spiking the plasma with a known concentration of tritiated steroid (10,000 DPMs). The spiked sample then underwent the extraction procedure as outlined above, with a sub-sample of the ethylether supernatant taken for radioactive analyses. An additional scintillation vial containing a 10,000 DPM spike was also analyzed. The resulting radioactive count was calculated for recovery rate by comparing the recovered radioactive values to that of the known 10,000 DPM spike. A laboratory average was determined by averaging the recovery rates of several extractions to obtain an average of 95.1% for E2 and 80.0% for KT. These values were applied to the derived steroid data to correct sample steroid values for efficiency of extraction.

17-β Estradiol

The E2 concentrations were determined by EIA using a kit from Cayman Chemical (Ann Arbor, Michigan). Steroid concentration was determined through the competition between the variable amount of free steroid (plasma sample or standard) and a constant amount of steroid tracer (steroid linked with acetylcholinesterase (AChE)) available for binding to the steroid-

specific rabbit antiserum. The steroid antiserum complex (free or tracer) binds to a mouse anti-rabbit antibody attached to a 96-well microtiter plate. Following plate washing to remove unbound antigen, Ellman's Reagent was used to activate the AChE of the steroid tracer complex, producing a yellow color the absorbance of which was measured using spectrophotometer. The absorbance value is inversely proportional to the amount of free steroid in the plasma sample. The E2 concentration in a sample was determined from a standard curve of known concentrations of E2 plotted against corresponding absorbance values. Each sample was measured in duplicate and corrected for extraction efficiency.

11-ketotestosterone

The KT was assayed using the radioimmunoassay method (McMaster et al., 1992). Steroid concentration was determined through competition of the standard or sample KT and a constant volume of radiolabeled steroid for a fixed titer of antibody. Extracted samples were incubated with the antibody (anti-white sturgeon KT developed in rabbit, a gift from Dr. Tim Gross, USGS Florida-Integrated Science Center), and radiolabeled steroid (Amersham Biosciences) overnight at $4^{o}C$. The following day a chilled solution of dextran-coated activated charcoal in PBSG (0.4% dextran, 0.625% charcoal) was added and allowed to incubate before centrifugation at $0^{o}C$ for 20 minutes at 2800 rpm. A 500-µL portion of the resulting supernatant was added to a scintillation vial containing 5 mL of scintillation cocktail (Ecolume). Radioactivity was determined by scintillation spectroscopy with the resulting values indicating the amount of antibody bound to labeled steroid, which is inversely proportional to the amount of free steroid in the sample. A standard curve of a serial dilution of KT standard (Sigma-Aldrich, St. Louis, MO) was used to allow calculation of steroid concentrations in the unknown samples.

Quality Assurance Procedures and Assay Performance Characteristics

Samples in all assays were run in duplicate and included measurement of blanks which were subtracted from all absorbance values. The reported kit sensitivity for E2 was 89 pg/mL at a %B/Bo of 50%, and 9 pg/mL at a % B/Bo of 80%. KT sensitivity was 19 pg/mL and 3 pg/mL at 50% and 80% binding, respectively. Cross-reactivities of the antibodies used in these assays with other similar steroids are reportedly ≤ 17% for the E2 antiserum (Cayman Chemical, Ann Arbor, MI) and < 10% for the KT antiserum (Sepulveda et al., 2002). The E2 intra-assay variation was 1.2% and inter-assay variation was 6.6%. The KT intra-assay variation was 18% and inter-assay variation was 10%. Levels of KT were validated by verifying that serial dilutions of sample were parallel to a standard curve. A slope of 0.8 was obtained from the regression of steroid measured and concentration of steroid added to a plasma sample.

Vitellogenin Analysis

The VTG analysis was conducted using a sandwich Enzyme-Linked-Immunosorbant Assay as described by Folmar et al. (1996). Microtiter 96-well plates were coated with a mouse anti-shovelnose sturgeon VTG protein (custom made by Abraxis, LLC, Pennsylvania) and incubated overnight. After washing the plate in a wash buffer (tris-buffered saline/Tween 20) and blocking non-specific binding by incubating with bovine serum albumin, 2 dilutions of each plasma sample were added in duplicate to the plate and incubated for one hour at room temperature. A standard curve was created with serial dilutions of purified shovelnose sturgeon VTG (see Kroll, 1990) and processed in the same manner. The plate was then washed with the washing buffer before the addition of the rabbit anti-shovelnose sturgeon antibody. After one hour of incubation at room temperature, the plate was washed as before. A secondary antibody (goat anti-rabbit horseradish peroxidase) was added and incubated for an additional hour prior

to washing. A 20-minute incubation in TMB solution (Tetramethylbenzidine free base chromogen in a hydrogen peroxide-citrate buffer) allowed color development. A stop solution (2N HCl) was added prior to reading the plate at 450 nm on a spectrophotometer. As with the E2 EIA described above, the VTG concentrations were determined by quantifying the absorbance values in relation to the known values of the standard curve.

Analysis

Plasma sex steroids, VTG, and PI data were Log_{10}-transformed and GSI data were squareroot-transformed to make their distributions normal. Plots of sex steroids, VTG, GSI and PI are presented to describe the data. In the plots, the bars represent one standard error, the square represents the mean, the upper dot is the maximum and the lower the minimum, and the numbers are the number of fish and the mean. The data presented are the same as those used in Chapter 2, Table 10 where capture date was not used in reproductive stage determination and came from the Lower Missouri River shovelnose sturgeon.

Results

General Observations by Method

Ultrasound

Ultrasonic scanning through the transverse plane presented individual oocytes or follicles as a pair of light colored parabolas (Fig 8). From this view, the testes in the later stages of reproductive maturation were large and well-defined paired structures with distinct edges that were generally oval in shape that most often occurred along the entire dorsal side of the body cavity. The frontal view allowed for identification of the presence of lamella-like folds in some females in earlier reproductive stages of gametogenesis and well defined lobes with distinct edges in males in later reproductive stages of gametogenesis (Fig. 23); testes of males throughout gametogenesis lack the ovigerous folds seen in ovaries. The use of the two views in concert provided the most effective means for identifying sturgeon sex. Videos of the ultrasonic scans can be found in the reference CD (the Stage VI female ultrasonic scan video was not included due to only the use of the field ultrasound unit for this group).

Endoscope

The endoscopic view of the gonads through the gonoduct was not as clear as through the abdominal incision due to the slight opaqueness and the presence of blood vessels in the gonoduct wall (Fig. 24). For presentation purposes, we present endoscopic images collected through an incision (Figs. 25B-36B). Through the endoscope the testicular surface appeared finely textured and lacked the granular texture that the presence of oocytes and/or ovarian follicles in females created. Fat was distinguishable from testicular tissue by having a smoother surface and fewer blood vessels than testes.

Through the endoscope, the ovaries of females in the earliest stages of reproductive maturation (Stage I and II) and in the post-spawning stage contained small white pre-vitellogenic oocytes and sometimes black melanin pigment, remnants of atretic follicles from previous spawns (Figs. 25 and 26). Later reproductive stage follicles ranged from small yellow circles (early vitellogenic; Fig. 27), to medium-sized light gray circles (late vitellogenic; Fig. 28), to dark black circles in which one follicle alone filled the entire viewing area of the endoscope (maturing follicles; Fig. 29). Videos of the endoscopic scans can be found in the reference CD.

GSI, Histology, Sex Steroids, and Vitellogenin

In viewing the sequence of reproductive stages of male and female shovelnose sturgeon (Figs. 25-36) it can be seen how Stages I (Figs. 25 and 31) and VI (Figs. 30 and 36) as described by Moos (1978) can be difficult to distinguish from Stage II (Figs. 26 and 32), especially to the untrained eye. Sex-specific blood chemistry, GSI, and imaging are very similar among these stages. Distinguishing features such as fat, melanin, structural differences in arrangement of germ cells, and gross appearance of gonad are useful, but vary greatly among individual fish. While the presence of pre-vitellogenic oocytes and small dark patches of melanin clearly indicates Stage II, the absence of melanin can not be confidently used to assign the gonad to another stage. For most situations, the separation of these three stages may not be necessary and it will be sufficient to classify these fish as having pre-vitellogenic oocytes or spermatogonia. However, further classification where possible will provide additional information about relative numbers of immature (Stage I) and recently spawned (Stage VI) fish.

Female and male GSI increases were consistent with development of gametes being lowest at early reproductive stages and post-spawn (Figs. 25-27) and greatest at Stage V for females (Fig. 29) and Stage III for males (Fig. 33). The PI decreased as reproductive stage increased (Figs. 27-29). VTG in males at all reproductive stages was 10-fold lower than the lowest (Stage I) female levels (Figs. 31-36 and Fig. 25, respectively). Female E2 levels were low at Stages I and II (Figs. 25 and 26), began to increase at Stage III (Fig. 27), continued to increase through Stage IV (Fig. 28), and began to decrease at Stage V (Fig. 29) returning to Stage I and II levels at Stage VI (Fig. 30). Male E2 levels remained low for all reproductive stages (Figs. 31-36). Female KT levels increased concomitantly with E2 but, unlike E2, did not fall until post-spawn (Figs. 25-29 and Fig. 30, respectively). Male KT levels increased at Stage III (Figs. 31-33) and remained elevated until post-spawn (Figs. 34-36).

Detailed Observations

Reproductive Stage I Female Shovelnose Sturgeon (Immature; Fig. 25)

Gonads were characterized by having large amounts of fat and a slightly granular surface (Fig. 25A). Through the endoscope, ovaries of Stage I females contained small white pre-vitellogenic oocytes (Fig. 25B). In the frontal and transverse plane ultrasonic images the ovaries were small and hard to distinguish from fat (Figs. 23B and 25C, respectively). Lamella-like folds were not distinguishable in the ultrasound frontal view as in some Stage II females (Fig. 23). Microscopically, pre-vitellogenic oocytes were observed at the periphery of lamellae (Fig. 25D). Stage I ovaries are found in fish that have never spawned before; therefore, no residual black melanin pigment was ever observed. The GSI, VTG and sex steroids were low (Fig. 25E).

Reproductive Stage II Female Shovelnose Sturgeon (Developing; Fig. 26)

Gonads possessed lesser amounts of fat, the surface was more granular than in Stage I females, and, in some cases, lamellar folds could be observed (Fig. 26A). Through the endoscope, the ovaries of Stage II females contained small white pre-vitellogenic oocytes (Fig. 26B). In the frontal and transverse plane ultrasonic images, the ovaries were larger than in Stage I females but the tissue was still hard to distinguish from fat (Figs. 23D and 26C, respectively). Lamella-like folds were distinguishable in the ultrasound frontal view in some females (Fig. 23D). Stage II female ovaries were similar to Stage I ovaries in all aspects except that residual black melanin pigment was often observed and pre-vitellogenic oocytes were not organized strictly along the margins of lamellae (Fig. 26D). The GSI and sex steroids remained low but VTG increased (Fig. 26E).

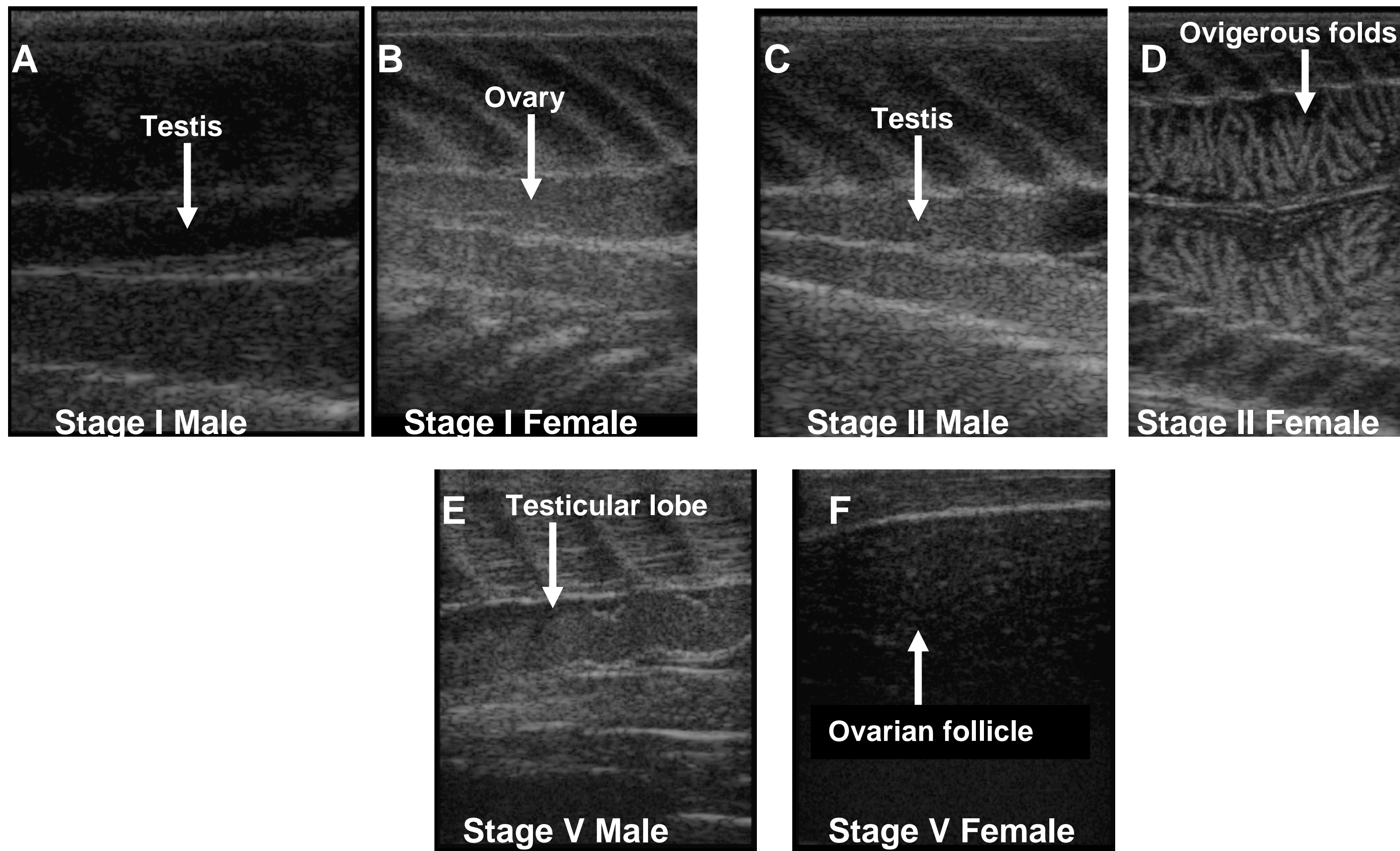


Figure 23. Frontal plane ultrasonic images of shovelnose sturgeon at different reproductive stages based on Moos (1978). The images were collected through the frontal plane from the ventral section of the side of the body cavity between the belly scutes and the first row of side scutes posterior to the air bladder.

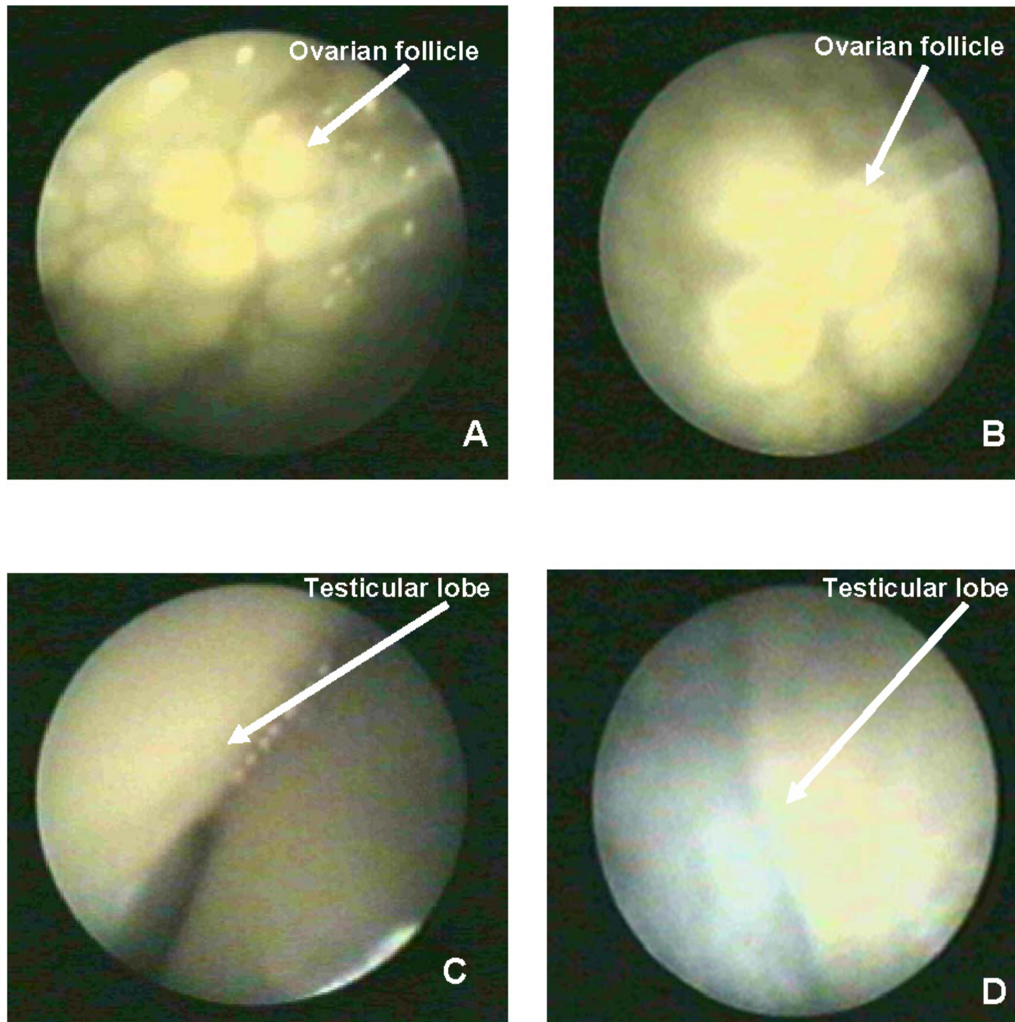
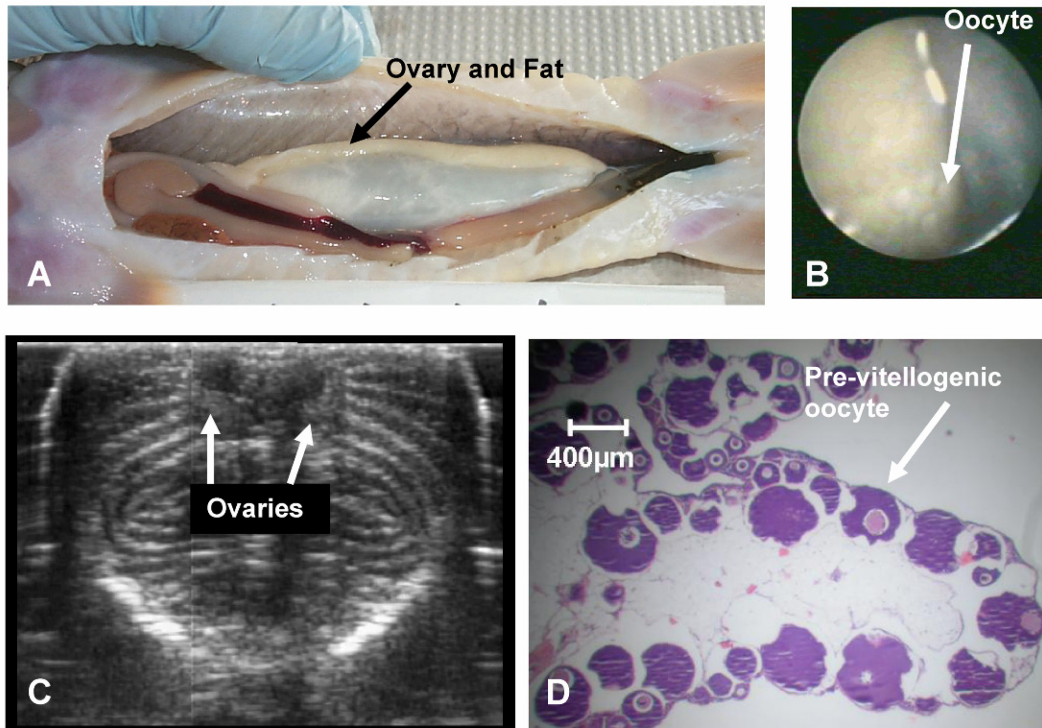


Figure 24. Endoscopic images of shovelnose sturgeon at Stage III based on Moos (1978). The endoscopic images are from a female (A, B) and male (C, D) collected through an abdominal incision (A, C) and through the gonoduct (B, D), respectively.



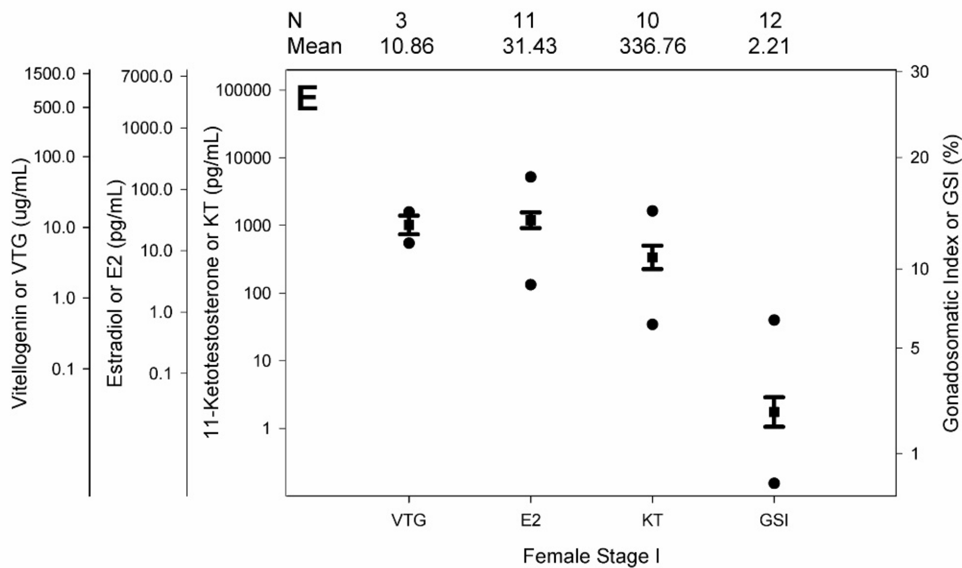
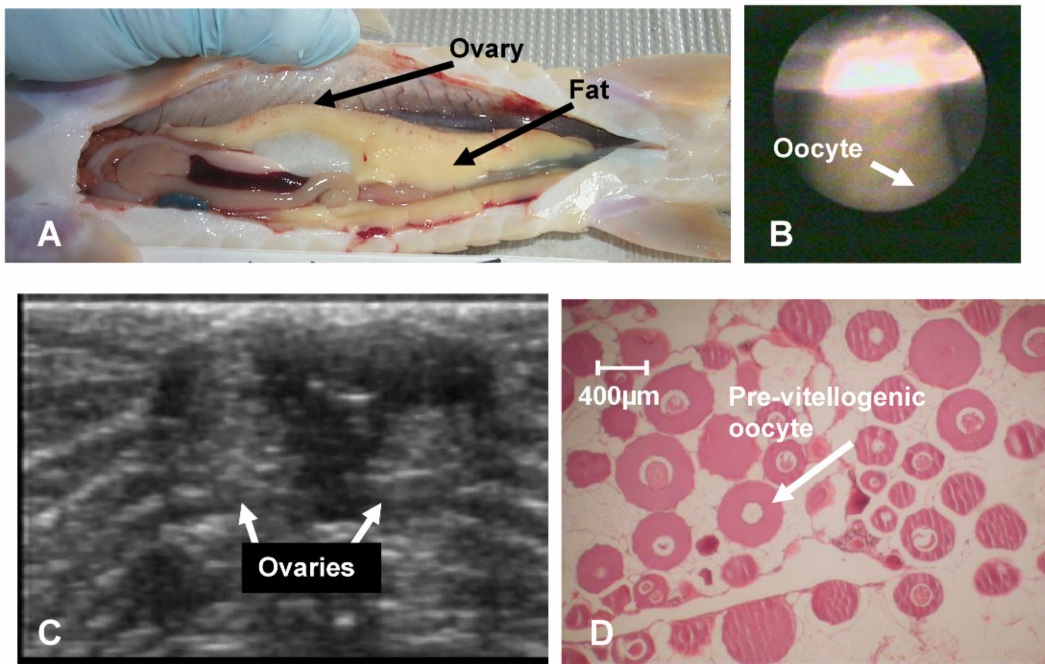


Figure 25. Four views of a Stage I female shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage I Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Female Stage I Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



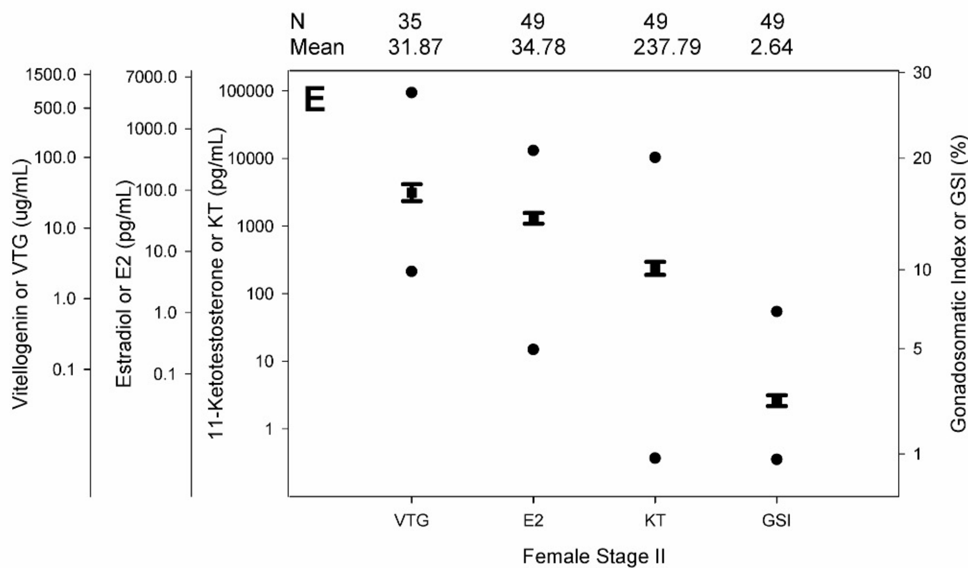
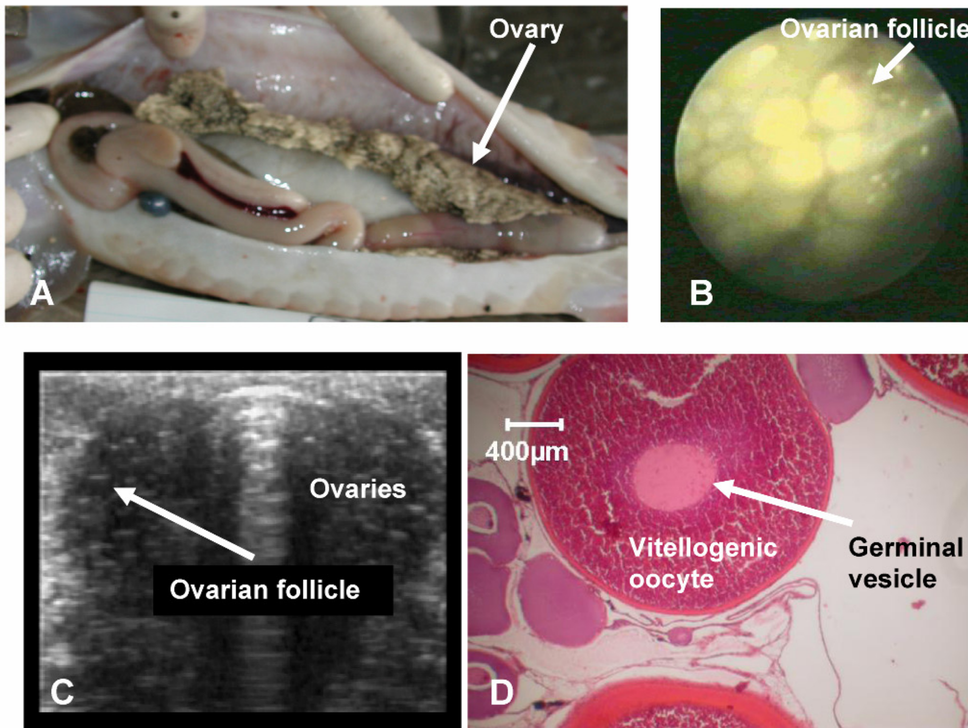


Figure 26. Four views of a Stage II female shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage II Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Female Stage II Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



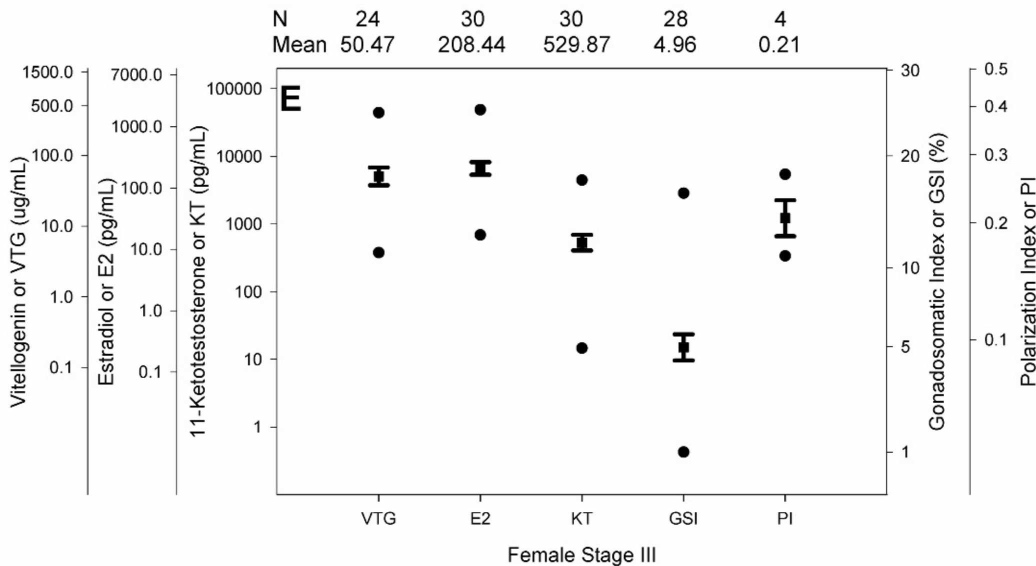


Figure 27. Four views of a Stage III female shovelnose sturgeon and blood plasma, PI, and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage III Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Female Stage III Ultrasound.avi), (D) histology, and (E) plot of blood plasma, PI, and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).

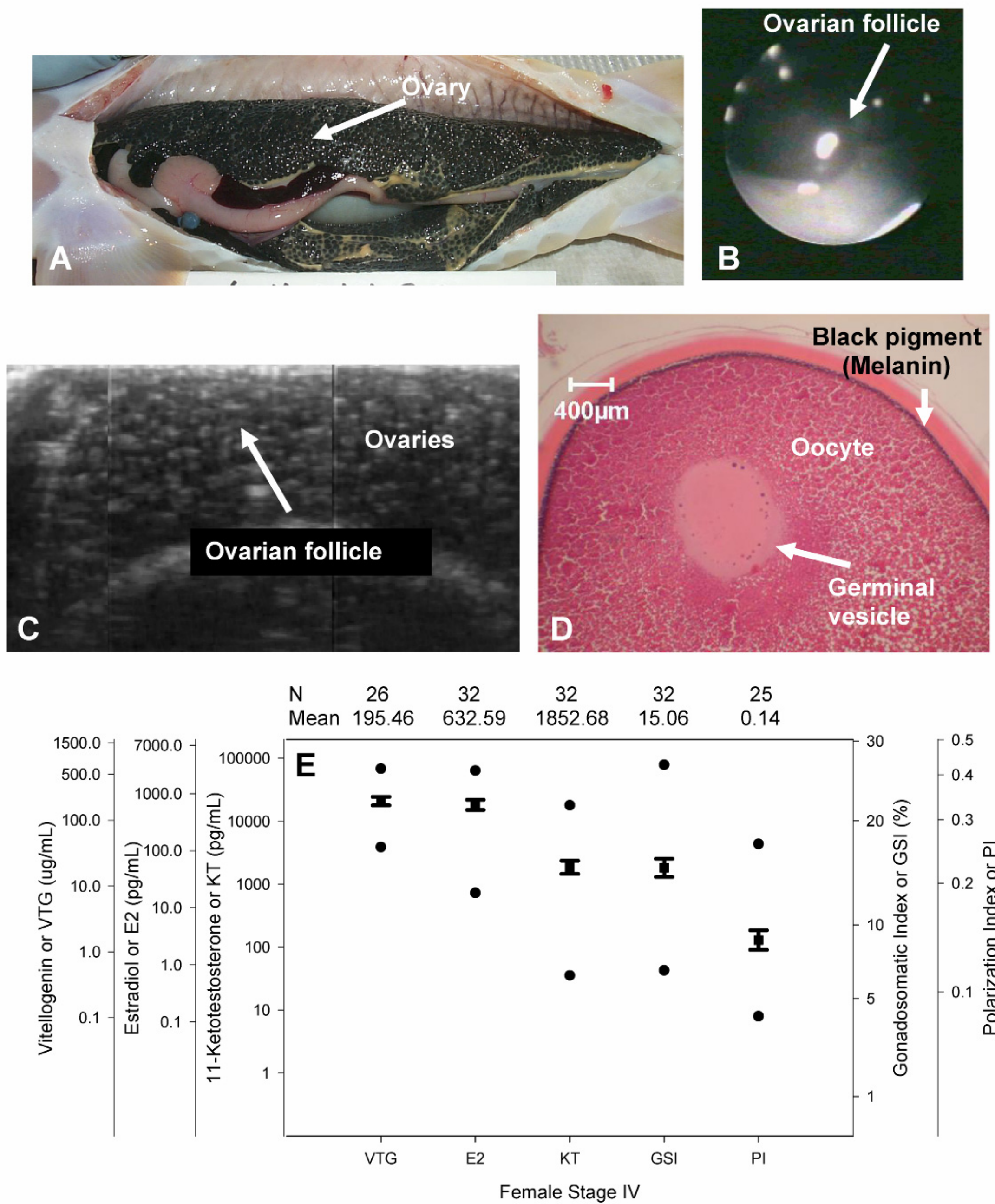
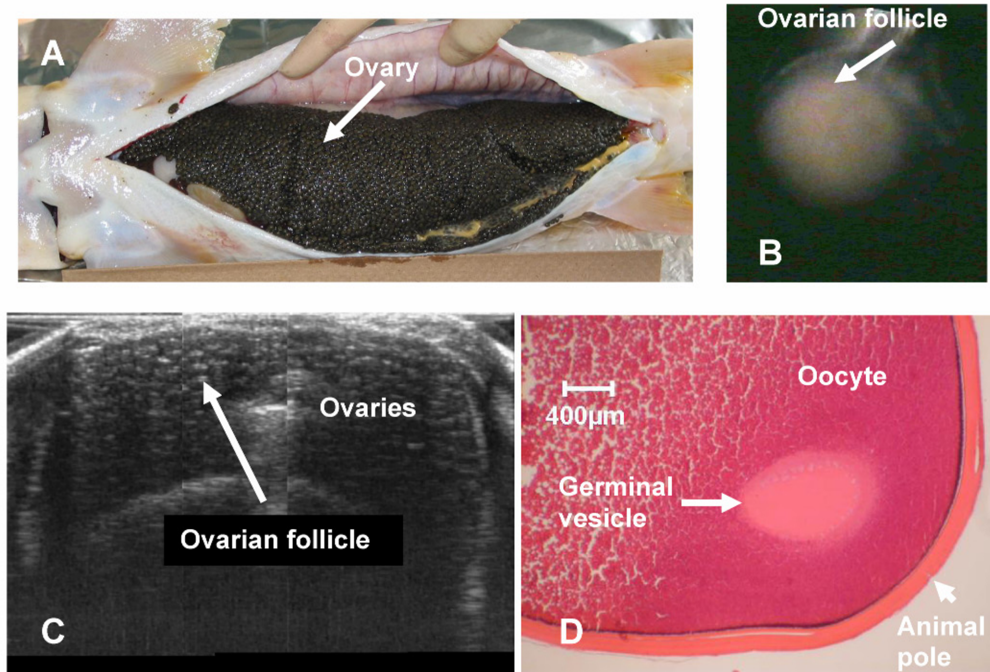


Figure 28. Four views of a Stage IV female shovelnose sturgeon and blood plasma, PI, and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage IV Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Female Stage IV Ultrasound.avi), (D) histology, and (E) plot of blood plasma, PI, and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



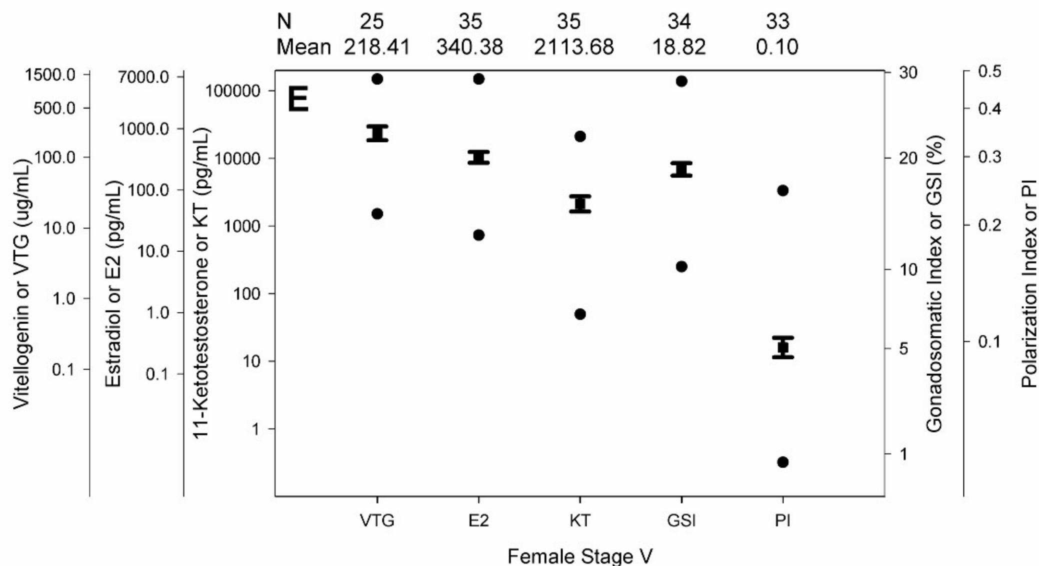


Figure 29. Four views of a Stage V female shovelnose sturgeon and blood plasma, PI, and GSI characteristics A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage V Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Female Stage V Ultrasound.avi), (D) histology, and (E) plot of blood plasma, PI, and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).

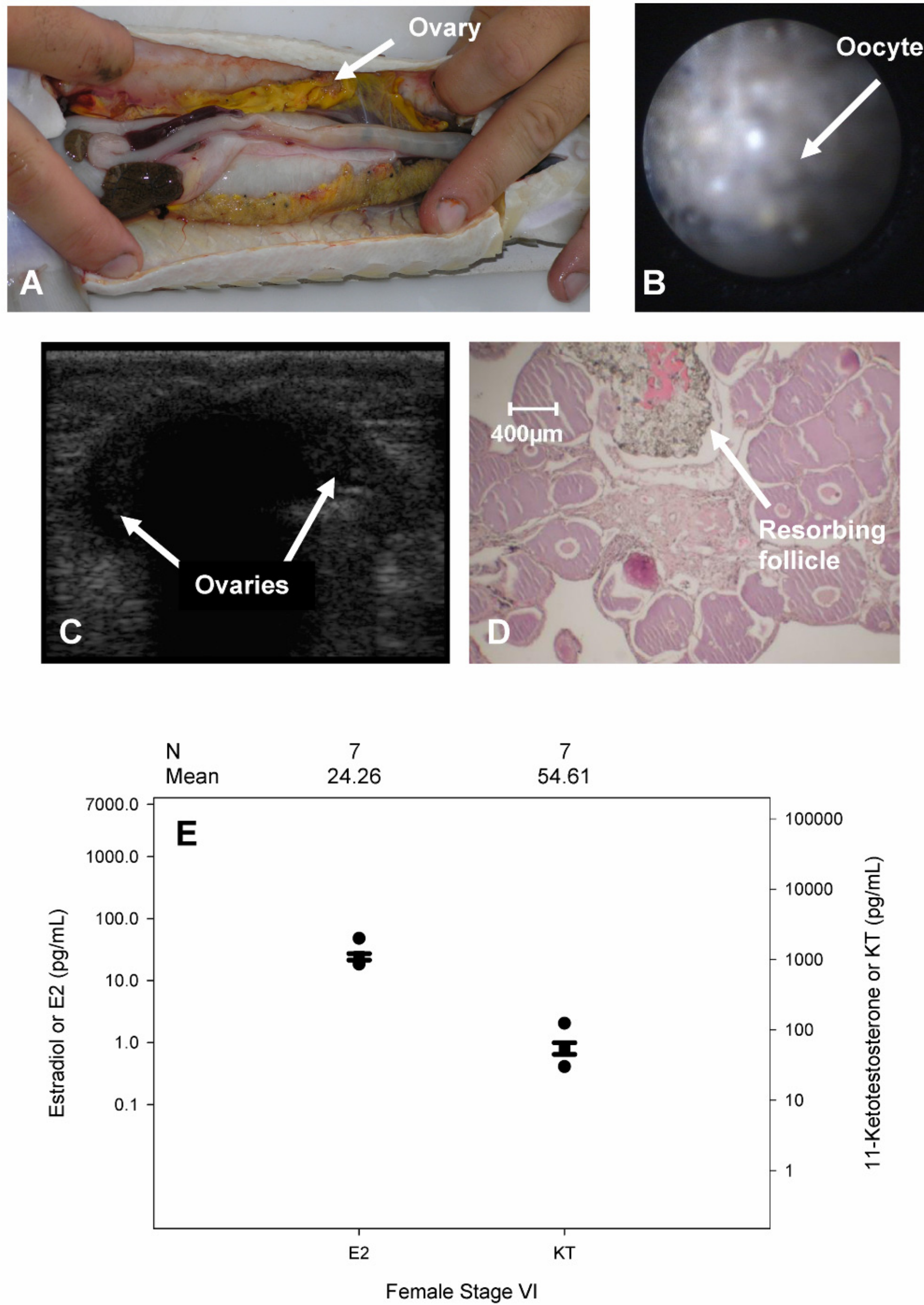
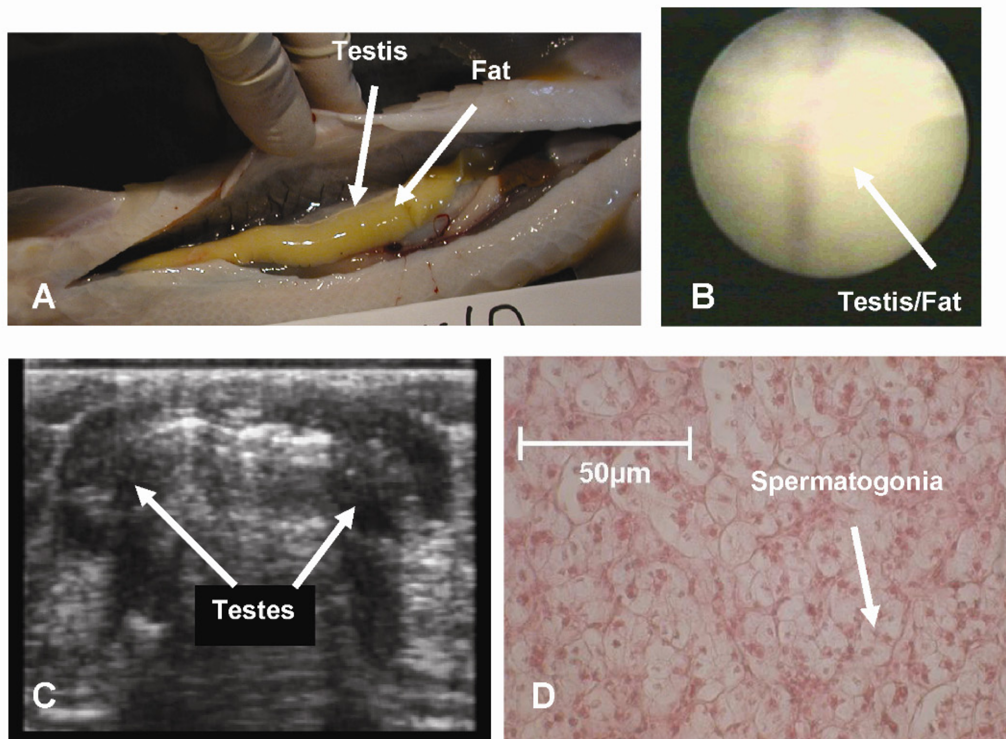


Figure 30. Four views of a Stage VI female shovelnose sturgeon and blood plasma characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Female Stage VI Endoscope.avi), (C) ultrasound transverse view, (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



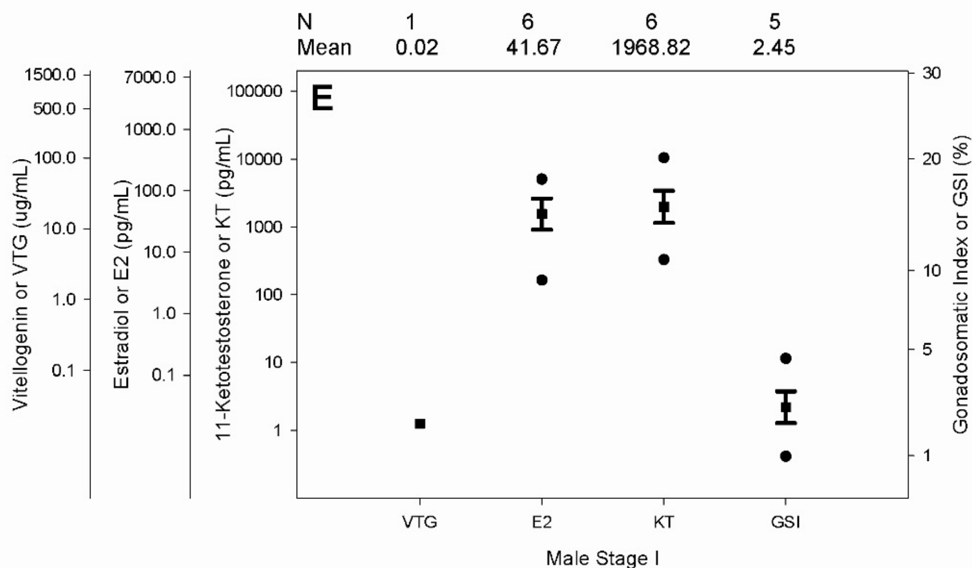
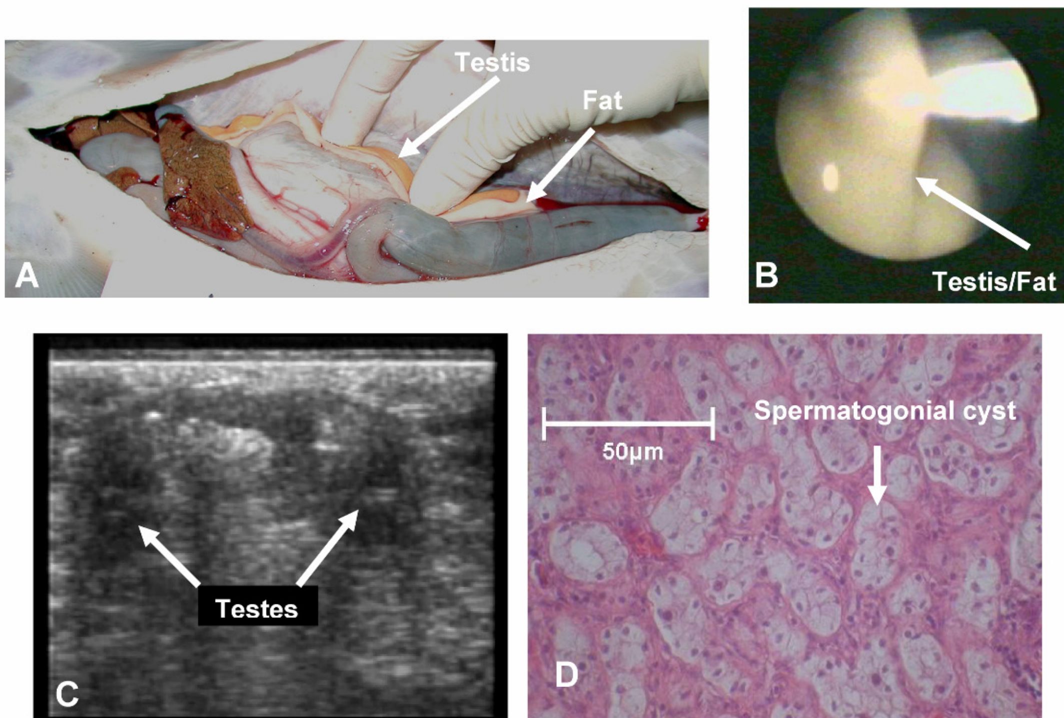


Figure 31. Four views of a Stage I male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage I Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage I Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



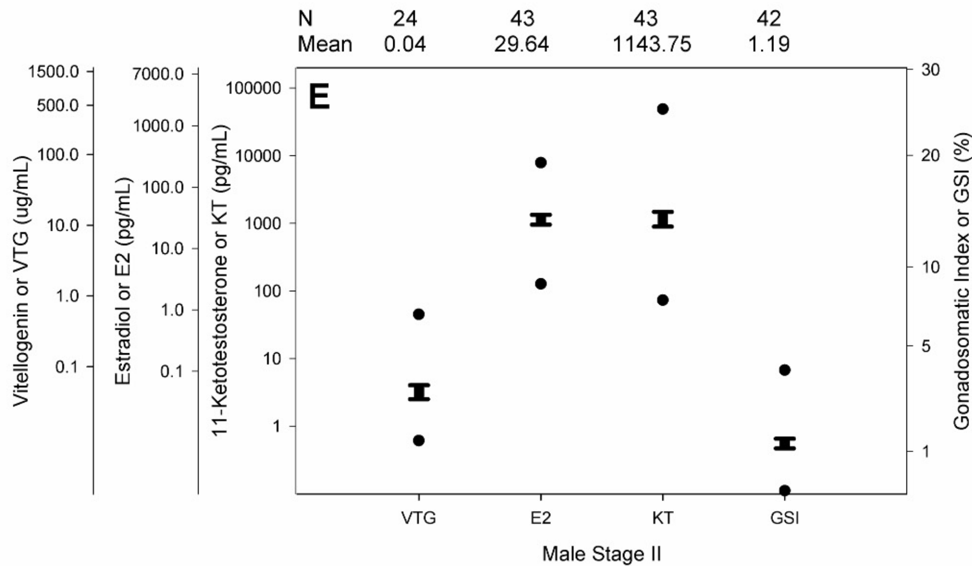
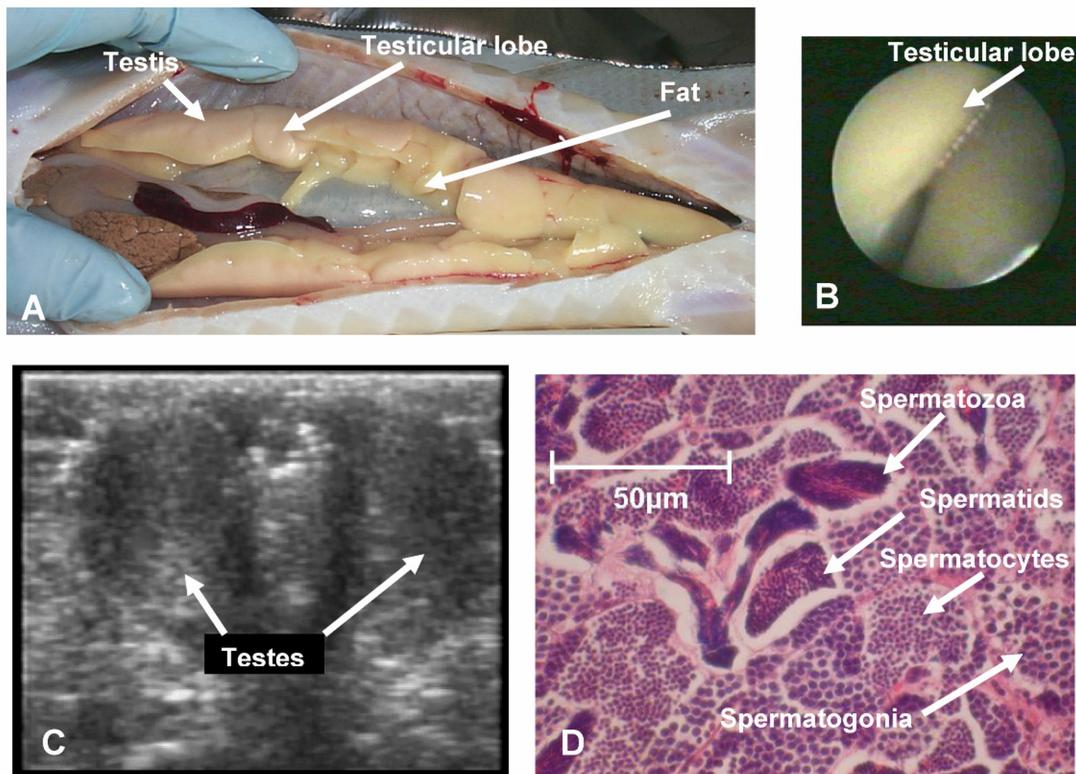


Figure 32. Four views of a Stage II male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage II Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage II Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



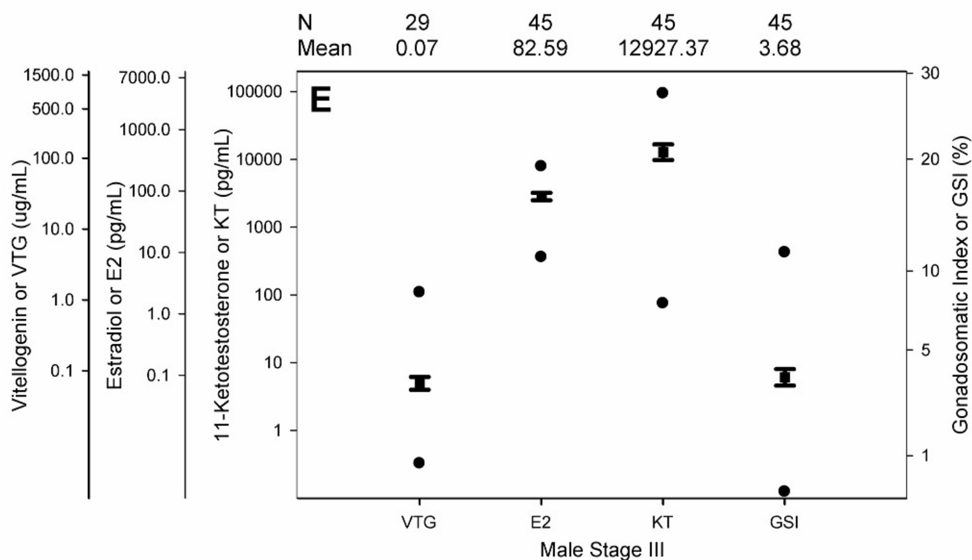


Figure 33. Four views of a Stage III male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage III Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage III Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).

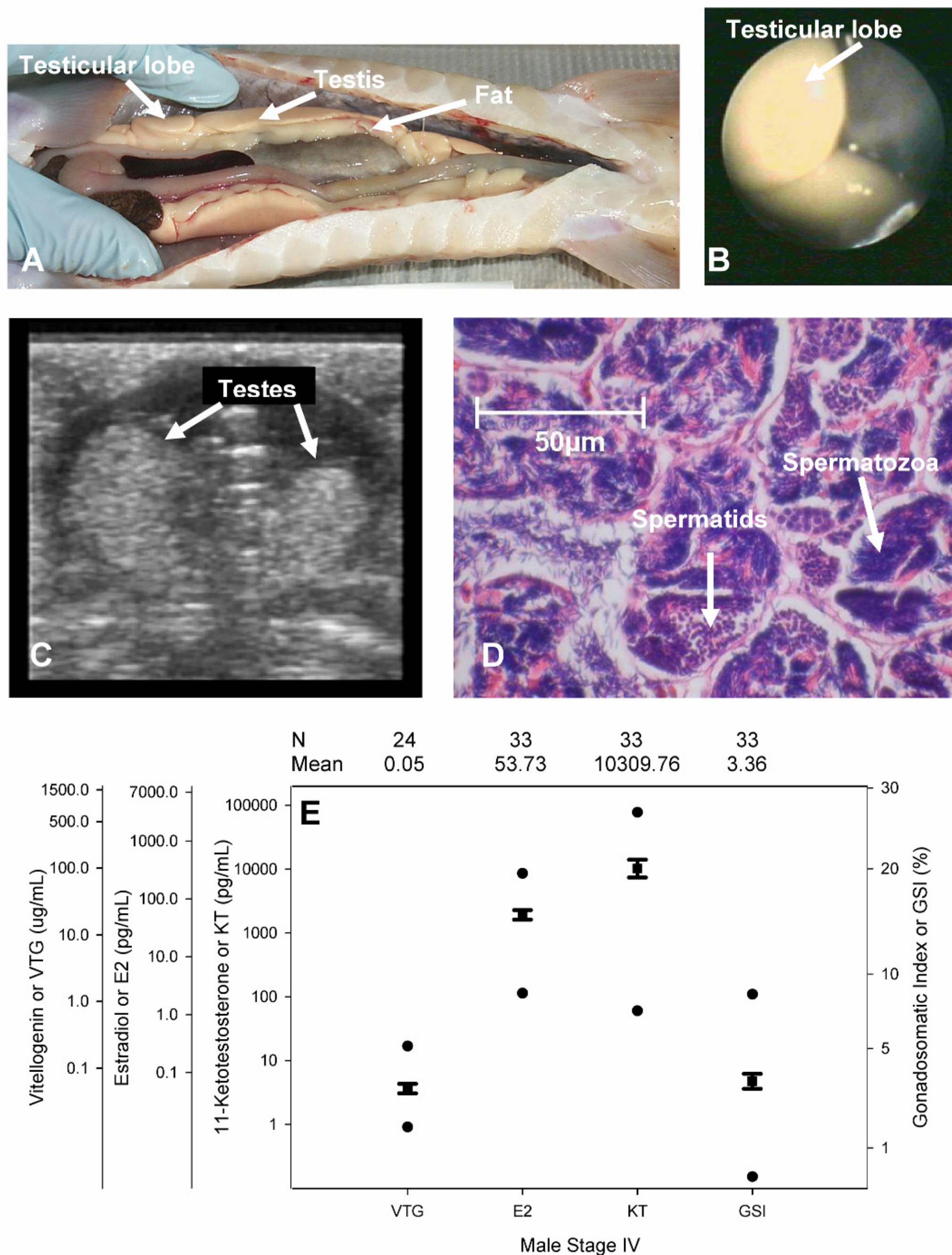
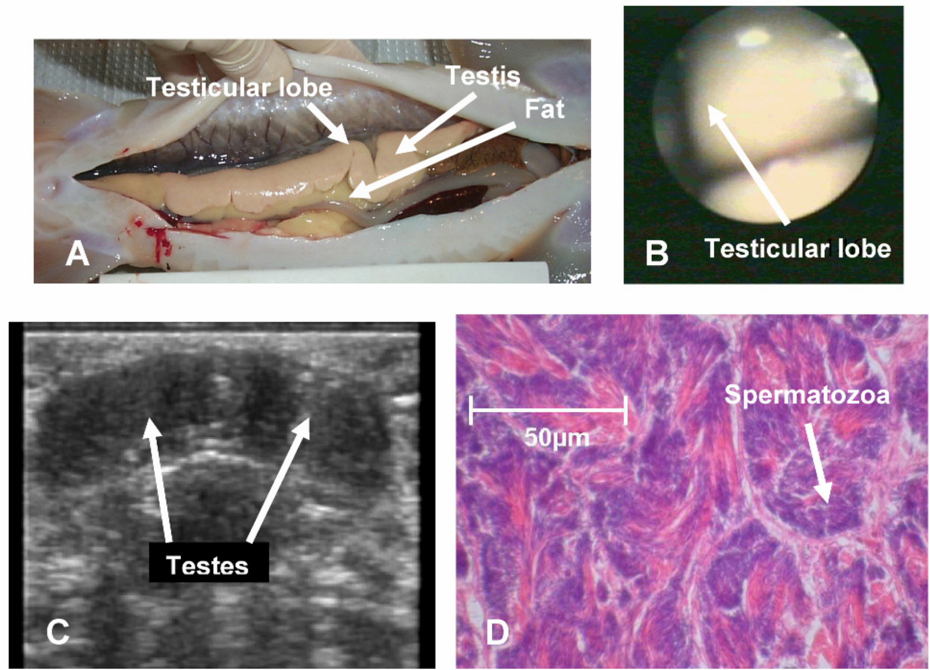


Figure 34. Four views of a Stage IV male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage IV Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage IV Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



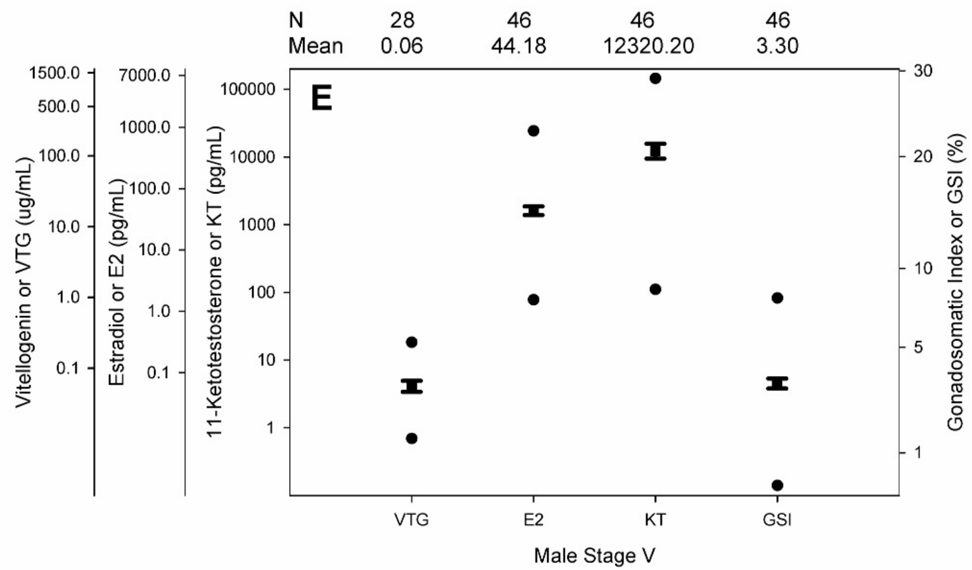
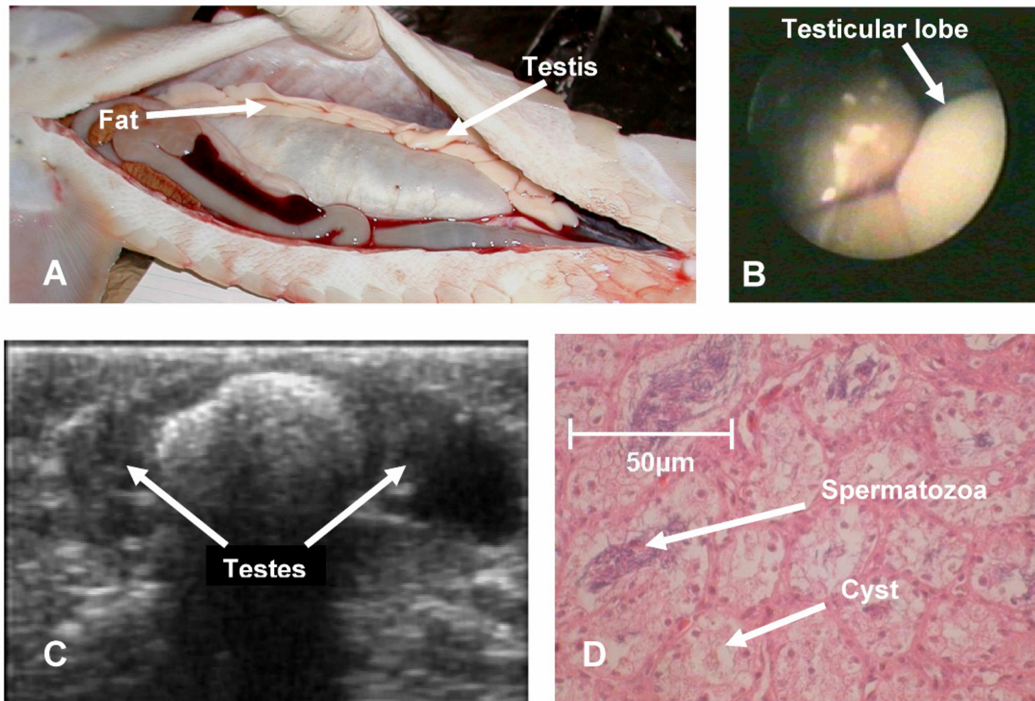


Figure 35. Four views of a Stage V male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage V Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage V Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).



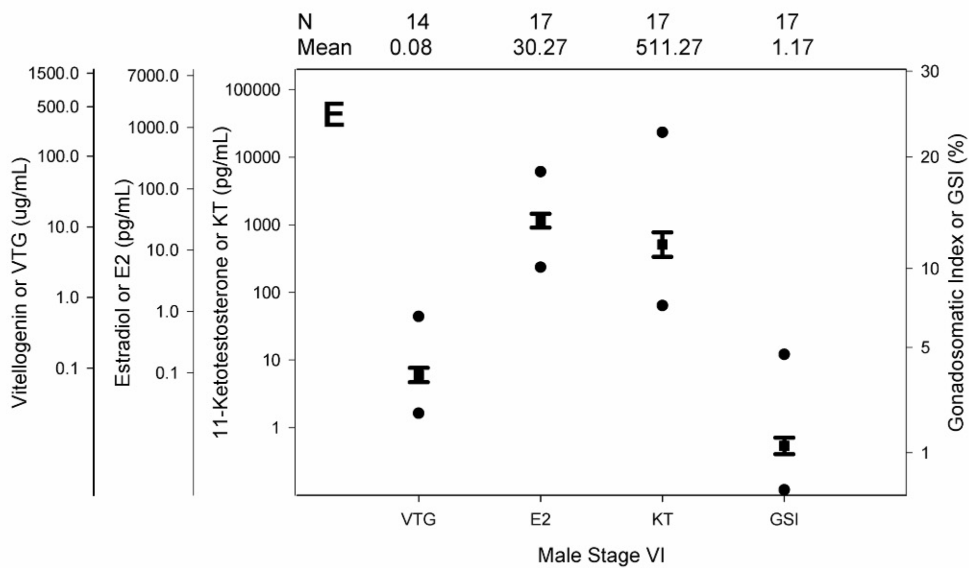


Figure 36. Four views of a Stage VI male shovelnose sturgeon and blood plasma and GSI characteristics. (A) gross anatomy, (B) endoscope through incision (video Shovelnose Sturgeon Male Stage VI Endoscope.avi), (C) ultrasound transverse view (video Shovelnose Sturgeon Male Stage VI Ultrasound.avi), (D) histology, and (E) plot of blood plasma and GSI characteristics (Square is mean; error bars are one standard error; and the upper and lower dots are maximum and minimum, respectively. The numbers above the graph are the mean and number of fish in mean—N).

Reproductive Stage III Female Shovelnose Sturgeon (Vitellogenesis; Fig. 27)

Gonads possessed lesser amounts of fat than Stage II females and yellow or grayish ovarian follicles were visible (Fig. 27A). Through the endoscope, the ovarian follicles were yellow or gray and well-defined (Fig. 27B). In the frontal and transverse plane ultrasonic images, individual ovarian follicles appeared as a small pair of light colored parabolas and occupied a large portion of the body cavity (similar to Stage V female in Fig. 23 and Fig. 27C, respectively); ovarian follicles were visible along the majority of the length of the body cavity. Stage III female ovaries contained predominantly vitellogenic follicles, the germinal vesicle near center of follicle (mean PI of 0.21; Fig. 27D). The GSI, VTG, and sex steroids were increased (Fig. 27E).

Reproductive Stage IV Female Shovelnose Sturgeon (Pre-spawning; Fig. 28)

Gonads possessed little to no fat and ovarian follicles were dark gray or black (Fig. 28A). Through the endoscope, ovarian follicles were light grey to brownish in the center with black around the edges and one ovarian follicle filled most of the endoscopic view (Fig. 28B). In the frontal and transverse plane ultrasonic images, individual ovarian follicles appeared as a more distinguishable and larger, divided pair of light-colored parabolas and the ovaries fully occupied the body cavity (similar to Stage V female in Fig. 23 and Fig. 28C, respectively); ovarian follicles were visible along the entire length of the body cavity. In late vitellogenic ovarian follicles, the germinal vesicle was off-center towards the animal pole (mean PI of 0.14) and the appearance of black melanin pigment characterized the Stage IV female ovary (Fig. 28D). The GSI in these fish increased greatly as did the sex steroids (Fig. 28E). The VTG remained elevated but not increased over levels in Stage III ovaries.

Reproductive Stage V Female Shovelnose Sturgeon (Spawning; Fig. 29)

Gonads possessed little to no fat and ovarian follicles were large and black (Fig. 29A). Through the endoscope, the ovarian follicles were black throughout and one ovarian follicle filled the entire endoscopic view (Fig. 29B). In the frontal and transverse plane ultrasonic images, the ovarian follicles appeared as a more well-defined, more divided pair of light-colored parabolas than in Stage IV females (Figs. 23F and 29C, respectively); ovarian follicles occupied the entire body cavity. Ovarian follicles in Stage V female ovaries were pre-maturational or entering the ovarian maturation phase (Fig. 29D). The germinal vesicle was close to the animal pole or breaking down (mean PI of 0.10). The GSI was at its maximum and VTG and E2 were decreasing while KT remained elevated (Fig. 29E).

Reproductive Stage VI Female Shovelnose Sturgeon (Post-spawning or Spent; Fig. 30)

Gonads possessed little to no fat, often some residual dark pigment, and early stage oocytes (Fig. 30A). Through the endoscope, the ovaries of Stage VI females contained small white pre-vitellogenic oocytes and sometimes black particles which are remnants of follicles from previous spawns (Fig. 30B). In the frontal and transverse plane ultrasonic images, ovaries were small and the oocytes were hard to distinguish from fat, similar to Stage II females (Figs. 23 and Fig. 30C). Lamella-like folds were not distinguishable in the frontal plane image as they are in some Stage II females (Fig. 23). Stage VI ovaries contained only pre-vitellogenic oocytes or atretic or empty follicles (Fig. 30D). Unlike Stage II oocytes that were generally embedded in a fat matrix, Stage VI oocytes tended to be tightly packed together in histological sections with no fat. Sex steroids were again very low (Fig. 30E).

Reproductive Stage I Male Shovelnose Sturgeon (Immature; Fig. 31)

Testes appeared as a thin thread of opaque white tissue bordered by a mass of fat (Fig. 31A). Through the endoscope, the testicular surface was finely textured and lacked the granular texture seen in ovaries (Fig. 31B). In the frontal and transverse plane ultrasonic images, the testes were small, poorly-defined, and hard to distinguish from fat compared to Stage V males (Figs. 23A and 31C, respectively). Testicular lobes were not distinguishable in the frontal plane image taken with ultrasound as they were in Stage V males (Fig. 23). Microscopically, Stage I testes were comprised of spermatogonia loosely arranged in cysts (Fig. 31D). Stage I testes are found in fish that have never spawned. The GSI was 2 or less, VTG, E2 and KT were low (Fig. 31E).

Reproductive Stage II Male Shovelnose Sturgeon (Developing; Fig. 32)

Gonads are similar in appearance to Stage I male gonads but may have less fat associated with them (Fig. 32A). Through the endoscope, the testicular surface was finely textured and lacked the granular texture seen in ovaries (Fig. 32B). In the frontal and transverse plane ultrasonic images, the testes were somewhat larger and more well-defined than Stage I male testes but they were still difficult to distinguish from fat (Figs. 23C and 32C, respectively). Testicular lobes were not distinguishable in the frontal plane image as they were in Stage V males (Fig. 23). Stage II testes were characterized by cysts with multiplying spermatogonia (Fig. 32D). The GSI remained at or below 2 % and the sex steroids remained low (Fig. 32E). Vtg continued to be low.

Reproductive Stage III Male Shovelnose Sturgeon (Spermatogenic; Fig. 33)

Gonad fat content was highly variable among fish, testicular tissue was white to off-white, opaque, and lobes could be observed (Fig. 33A). Through the endoscope, the testicular surface appeared as a set of yellow testicular lobes (Fig 33B). In the transverse plane ultrasonic image, the testes were moderately well-defined paired structures that were generally oval in shape, had a fairly uniform light coloration, and occurred along the entire dorsal side of the body cavity (Figs. 8 and 33C). Testicular lobes were generally distinguishable in the frontal plane images but were less well-defined as in Stage V males (Fig. 23 shows a Stage V as a reference). The cysts of Stage III testes were primarily filled with spermatogonia, spermatocytes, and spermatids. A few had spermatozoa (Fig. 33D). The GSI, VTG and E2 remained low, but KT was greatly increased (Fig. 33E).

Reproductive Stage IV Male Shovelnose Sturgeon (Pre-spawning; Fig. 34)

Gonads tended to possess little fat, testicular tissue was white to off-white, and testicular lobes were obvious (Fig. 34A). Through the endoscope, the testicular surface appeared as a set of large testicular lobes that encompassed more of the body cavity than in Stage III males and the testes were more vascularized (Fig. 34B). In the transverse plane ultrasonic image, testes were well-defined paired structures with distinct edges, generally oval in shape, had a fairly uniform light coloration, and occurred along the entire dorsal side of the body cavity (Figs. 8 and 34C). Testicular lobes were distinguishable in the frontal plane image as well-defined lobes that occupied less space in the body cavity compared to Stage V males (Fig. 23 shows a Stage V male as a reference). Stage IV testes were primarily filled with spermatids and spermatozoa (Fig. 34D). The GSI and KT remained elevated (Fig. 34E). Vtg and E2 remained low.

Reproductive Stage V Male Shovelnose Sturgeon (Spawning; Fig. 35)

Gonads tended to possess slightly less fat than Stage IV males but were otherwise similar in appearance (Fig. 35A). Through the endoscope, the testicular surface appeared as large lobes (Fig. 35B). In the transverse plane ultrasonic image, the testes were well-defined paired structures, with distinct edges, generally oval in shape, had a fairly uniform light coloration, encompassed even more of the body cavity than in Stage IV males, and occurred along the entire dorsal side of the body cavity (Fig. 35C). Testicular lobes were easily distinguishable as a long, sinuous tube in the frontal plane image (Fig. 23E). Stage V testes were filled with spermatozoa (Fig. 35D). The GSI began to fall as these fish are reproductively active (Fig. 35E). The KT remained elevated while VTG and E2 remained low (Fig. 35E).

Reproductive Stage VI Male Shovelnose Sturgeon (Post-spawning or Spent; Fig. 36)

Gonads tended to possess little fat, but generally were similar to Stage II males (Fig. 36A). Through the endoscope, the testicular surface was finely textured and lacked the granular texture that the presence of oocytes in females created (Fig. 36B). In the transverse plane ultrasonic image, the testes were similar to Stage II males, somewhat larger and more well-defined than Stage I male testes but still difficult to distinguish from fat (Figs. 23 and 36C). Like Stage II males, testicular lobes were not distinguishable in the frontal plane image as they were in Stage V males (Fig. 23). Stage VI post-spawn testes were similar in appearance to Stage II testes; residual spermatozoa could be seen in cysts (Figs. 32D and 36D). At Stage VI, the GSI, E2, KT, and VTG levels had returned to the low levels observed in Stage I and Stage II fish (Figs. 36E and Figs. 31E and 32E, respectively).

Conclusion

This chapter provides a reference set of images and reproductive measurements to assess reproductive status of male and female shovelnose sturgeon and potentially other congeneric sturgeon species. This reference set resulted from the work present in this report and published by Wildhaber et al. (2005). The value of this reference set lies in its usefulness for characterizing sturgeon population demographics, identifying and managing broodstock, monitoring the impact of age and sex-specific fisheries, evaluating biological responses to management actions, developing population forecasting models, and monitoring changes in populations.

Acknowledgements

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