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Reproductive biology of middle Mississippi River shovelnose sturgeon: insights from seasonal and age variation in plasma sex steroid and calcium concentrations

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Summary

Shovelnose sturgeon *Scaphirhynchus platorynchus* are endemic to the Mississippi River drainage and are commercially harvested for roe in several states. Status of shovelnose sturgeon populations throughout much of its range is unknown or declining; Mississippi River stocks are experiencing recruitment overfishing and may be at risk of collapse. Restoration of shovelnose sturgeon populations will require additional information on their reproductive biology, including age at maturity and reproductive status and cycles. The objectives of this study were to determine the effects of reproductive stage, fish age, and season on plasma 17-β estradiol (E_2) and calcium (Ca^{2+}) concentrations in female shovelnose sturgeon and plasma testosterone (T) concentrations in male shovelnose sturgeon from the middle Mississippi River (MMR). We also assessed the relationship between plasma vitellogenin (VTG) and Ca^{2+} concentrations for female shovelnose sturgeon. Plasma E_2 and Ca^{2+} concentrations in females and T concentrations in males differed among reproductive stages, consistent with results of prior research on shovelnose sturgeon and other sturgeon species. VTG and Ca^{2+} concentrations were strongly correlated in female shovelnose sturgeon, indicating that Ca^{2+} can be used as a surrogate for VTG assays for identification of vitellogenic females, as has been shown for other sturgeon species. Age at maturity was estimated at 10 years for males and 9 years for females based on T and E_2 profiles, consistent with other recent age at maturity estimates in the MMR and Wabash River determined by gonadal examination. Peaks in plasma sex steroid and Ca^{2+} concentrations during April and October possibly reflected the spring spawning season and provide additional evidence in support of recent documentation of fall spawning by shovelnose sturgeon in the MMR. Additional research is needed to elucidate fall spawning by shovelnose sturgeon in the MMR.

Introduction

The shovelnose sturgeon *Scaphirhynchus platorynchus*, one of the smallest of the North American sturgeons, is endemic to the Mississippi, Missouri, and Ohio River basins (Keenlyne, 1997). Status of shovelnose sturgeon populations throughout much of its range are unknown or declining (Keenlyne, 1997). This species has been subject to increasingly intense commercial fishing pressure in recent years due to collapse of European caviar fisheries and restrictions on importation of caviar into the United States (Keenlyne, 1997; Gnam, 1999; Auer, 2004); habitat alteration is likely another significant factor in the decline of sturgeon populations (Auer, 2004). Shovelnose sturgeon are heavily harvested from the middle Mississippi River (MMR; the portion of the river between the mouth of the Missouri River and the Ohio River confluence); strong associations between harvest and annual cohort strength suggest that Mississippi River shovelnose sturgeon are experiencing recruitment overfishing and may be at risk of collapse from overexploitation (Colombo et al., 2007a).

Knowledge of shovelnose sturgeon reproductive biology, including locations, timing, and frequency of spawning, age at maturity, and the effects of harvest and environmental factors on reproduction, is critical to efforts to restore or maintain populations of this species. Sturgeons are characterized by relatively late age at maturation and biennial or longer ovarian cycles (Dettlaff et al., 1993). Shovelnose sturgeon was, until recently, thought to reach sexual maturity at 5 and 7 years for males and females, respectively (Helms, 1974). However, recent studies have determined that shovelnose sturgeon from the MMR and Wabash River do not become mature until 9-10 years of age (Kennedy et al., 2006; Tripp, 2007). Shovelnose sturgeon spawn from late April to June (Forbes and Richardson, 1920; Eddy and Surber, 1947; Barnickol and Starrett, 1951; Christenson, 1975; Elser et al., 1977; Moos, 1978) when water temperatures are

between 16.9°C and 20.5°C (Christenson, 1975; Elser et al., 1977). Recent evidence suggests that shovelnose sturgeon may also be spawning during fall in the MMR (Tripp, 2007); fall spawning had not previously been reported for this species.

Plasma sex steroid, vitellogenin (VTG, an egg protein precursor), and calcium profiles have provided valuable insights into the reproductive cycles of sturgeon populations (e.g., Amiri et al., 1996a; Van Eenennaam et al., 1996; Barannikova et al., 2000, 2004; Ceapa et al., 2002; Wildhaber et al., 2006, 2007). Plasma VTG binds free calcium (Ca^{2+}) in fish, triggering mobilization of body Ca^{2+} reserves or increased Ca^{2+} uptake from the water for maintenance of osmotoic balance (Linares-Casenave, 1993). Indirect estimation of VTG through determination of serum Ca^{2+} concentrations has been demonstrated to be feasible for white sturgeon *Acipenser transmontanus* (Linares-Casenave et al. 2003) and Atlantic sturgeon *Acipenser oxyrhynchus* (Van Eenennam et al., 1996); whether Ca^{2+} could also be used as a surrogate for VTG in shovelnose sturgeon has not been investigated. Plasma sex steroid and VTG profiles have been successfully applied in conjunction with ultrasonic, endoscopic, and histologic image analyses to non-lethally identify sex and reproductive stage of shovelnose sturgeon from the lower Missouri River (Wildhaber et al., 2006, 2007). Seasonal changes in plasma sex steroid and VTG profiles have also been documented for Missouri River shovelnose sturgeon (Wildhaber et al., 2006). However, many population characteristics of shovelnose sturgeon are system specific (Kennedy et al., 2006); significant population structure has been detected in this species (Schrey 2007). Plasma sex steroid and Ca^{2+} concentrations have not previously been applied to investigate the reproductive cycle (including age at maturation and seasonal changes in reproductive status) of shovelnose sturgeon from the MMR.

For this study, we examined plasma sex steroid and calcium concentrations in shovelnose sturgeon collected from the MMR to gain additional insight into the reproductive biology of this commercially exploited population. The objectives of this study were to: (i) determine the effect of reproductive stage on plasma 17-β estradiol (E_2) and Ca^{2+} concentrations for female fish and plasma testosterone (T) concentrations for male fish; (ii) characterize relationships between fish age and plasma E_2 and Ca^{2+} concentrations in females and plasma T concentrations in males; and (iii) determine whether plasma E_2 and Ca^{2+} concentrations in females and plasma T concentrations in males differ among months. We also assessed the relationship between plasma VTG and Ca^{2+} concentrations for female shovelnose sturgeon to evaluate whether plasma Ca^{2+} measurements could serve as a surrogate for more complex VTG assays in this species; highly significant correlations between VTG and Ca^{2+} concentrations have been observed for other sturgeon species (Linares-Casenave et al., 2003; Van Eenennam et al., 1996).

Methods

Fish collection

Three hundred ninety-six shovelnose sturgeon ranging from 322 to 751 mm fork length (FL) were collected from the MMR between river kilometers (RKM) 201-198 near Modoc, Illinois, RKM 191-188 near Chester, Illinois, and RKM 127-124 near Grand Tower, Illinois. Fish collections were conducted monthly between October 2005 and February 2007, with the exception of July and August 2006 because of the risk involving high water temperatures and the potential for high mortality rates in sampling gear of the federally endangered pallid sturgeon occupying the sampling area. Stationary bottom set gill nets (5.08 cm bar mesh, 45.7 m long,

3.05 m deep) were used for collecting sturgeon. Gill nets were set on the tips of wing dikes parallel with the main current for 4-24 h. Collected fish were placed in an ice bath and returned to the laboratory. At the laboratory, fish were humanely euthanized by pithing (AVMA Guidelines on Euthanasia 2007).

Determination of fish age, sex and reproductive stage

Each sturgeon was weighed to the nearest 0.1 g and fork length measured to the nearest 1 mm. One pectoral fin ray was removed from each fish and dried in a coin envelope for aging. Three sections were cut from the basal portion of each fin ray using a Buhler Isomet® low speed saw. Each section, increasing in width (0.635 mm, 0.6858 mm, and 0.7366 mm) was secured to a slide using cyanoacrylate (Tripp, 2007). Cross sections were examined independently by two readers using a stereomicroscope at 7-45x magnification. Under transmitted light, a pair of opaque (growth) and translucent bands were considered an annulus (Everett et al. 2003). Annuli were counted from the origin to the apex of each section. This aging method has been validated for Atlantic sturgeon *Acipenser oxyrhynchus* (Secor et al., 1997), lake sturgeon *Acipenser fulvescens* (Rossiter et al., 1995), and white sturgeon *Acipenser transmontanus* (Brennan and Cailliet, 1989) and is widely used for shovelnose sturgeon because of the precision in terms of within- and between- reader agreement as well as coefficient of variation (Jackson et al., 2007). When readers disagreed on fish age, they examined fin ray cross sections together to reach a consensus (Tripp, 2007).

Gonads were removed from each fish and examined to determine sex and maturational stage based on criteria established by Bruch et al. (2001) and Colombo et al. (2007b) (Table 1).

Five intersex fish and two immature fish whose gender could not be determined were collected; these seven individuals were not used in this study.

Plasma steroid, vitellogenin, and calcium analyses

Within 4 hours of collection of fish from sampling gear and immediately after pithing, blood was collected from 113 male and 151 female shovelnose sturgeon via heart puncture or caudal vein using a 6-ml heparinized vaccutainer plus with HEMOGARD (Precision Glide, Franklin Lakes, New Jersey) closure. Blood samples were kept on ice and centrifuged at 4° C for 10 minutes at 390 x g. The plasma was removed and placed into a clean microcentrifuge tube and stored at -20º C until analyzed.

Available plasma 17-β estradiol (E_2) and testosterone (T) concentrations were determined by radioimmunoassay (RIA) kits DSL-4400 and DSL-4100, respectively (Diagnostic Systems Laboratories Inc., Webster, Texas). Plasma E_2 concentrations were determined for females and testosterone for males. Manufacturer's directions were used for determining E_2 concentrations except volumes of all standards, controls, reagents, and unknowns were decreased by 50%. The standard curve was prepared in duplicate (50 µl), and samples (50 µl) were pipetted into glass assay tubes. The non-specific binding (NSB) tubes contained 100 µl of the 0 pg ml^{-1} E_2 standard. I-125 radiolabeled E_2 (50 µl) was added to all tubes followed by the E_2 antibody (rabbit anti-estradiol) which was added to all except the NSB and total count tubes. All tubes were vortexed, covered with plastic wrap, and allowed to incubate at 37°C for 30 minutes. After incubating, 500 µl of precipitation reagent was added to all tubes except for total count tubes; tubes were then vortexed and incubated at room temperature (~ 25°C) for 20 minutes. After incubation, all tubes except for total count tubes were centrifuged for 20 minutes at 1500 x g.

After centrifugation, all tubes except for the total count tubes were aspirated with a single vacuum aspirator and then counted on a gamma counter. The T RIA basically followed the same sequence as the E_2 RIA. Manufacturer's directions were used except volumes of all standards, controls, reagents, and unknowns were decreased by 50%. The standard curve was prepared in duplicate (25 µl), and samples (25 µl) were pipetted into glass assay tubes. The non-specific binding (NSB) tubes contained 75 µl of the 0 pg ml^{-1} T standard. I-125 radiolabeled T (250 µl) was added to all tubes followed by 50 µl T antibody (rabbit anti-testosterone), which was added to all but the NSB and total count tubes. All tubes were vortexed, covered with plastic wrap, and allowed to incubate at 37°C for 70 minutes. After incubating, 500 µl of precipitation reagent was added to all tubes except for total count tubes, vortexed, and incubated at room temperature (~ 25°C) for 15 minutes. After incubation, all tubes except for total count tubes were centrifuged for 20 minutes at 1500 x g. After centrifugation, all tubes except for the total count tubes were aspirated with a single vacuum aspirator and then counted on a gamma counter. The lower sensitivities of the E_2 and T assays were 4.7 pg ml^{-1} and 0.05 ng ml^{-1}, respectively. The intra-assay coefficient of variation (CV) for the estradiol radioimmunoassay kits was 6.11% and the inter-assay CV was 13.4%. The intra- and inter-assay CVs for the testosterone radioimmunoassay kits were 6.65% and 6.64%, respectively.

Total plasma Ca^{2+} concentrations were measured for 151 female shovelnose sturgeon using a colormetric assay (QuantiChrom calcium assay kit DICA-500, BioAssay Systems, Hayward, California) according to the manufacturer's instructions. Duplicate 5 ul samples and diluted standards were placed into each well of a 96 well microtiter plate. 200 ul of working reagent was added to each well, mixed by plate reader for 10 seconds, and allowed to incubate for 3 minutes. Plasma Ca^{2+} concentrations were determined at 620 nm using the Multiskan Plus

plate reader (Fischer Scientific, Inc., St. Louis, Missouri) equipped with Ascent software version 2.6. The intra- and inter-assay CVs for the calcium colormetric kits were 4.92% and 5.10%, respectively.

VTG analyses were conducted on 12 female shovelnose sturgeon with at least one individual per stage using a sandwich Enzyme-Linked-Immunosorbant Assay (ELISA) as described by Folmar et al. (1996). Only 12 individuals were used for statistical analyses because the rest of the samples went through more than one freeze-thaw cycle (i.e. steroid and calcium analyses) and displayed unreliable concentrations. Vitellogenin is a temperature sensitive molecule and breaks down easily when exposed to temperatures above freezing (Mandy Annis, United States Geological Survey, *personal communication*). Microtiter 96-well plates were coated with a mouse anti-shovelnose sturgeon VTG protein (custom made by Abraxis, LLC, Warminster, Pennsylvania) and incubated at room temperature. After washing the plate with a buffer (tris-buffered saline/Tween 20) and blocking non-specific binding by incubating with bovine serum albumin, two 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 (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 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 a tetramethylbenzidine free base chromogen in a hydrogen peroxide-citrate buffer solution allowed color development. A stop solution (2M sulfuric acid) was added prior to reading the plate at 450 nm on a

spectrophotometer. VTG concentrations were determined by quantifying the absorbance values in relation to the known values of the standard curve (Wildhaber et al., 2006).

Data Analysis

Kruskal-Wallis tests were used to assess differences in plasma T among reproductive stages for male fish and plasma E_2 and Ca^{2+} concentrations among reproductive stages for female shovelnose sturgeon. Linear regression was used to relate plasma VTG and Ca^{2+} concentrations for females. Two-dimensional Kolmogorov-Smirnov (2DKS) tests (Garvey et al., 1998) were used to evaluate relationships between plasma T, E_2 and Ca^{2+} concentrations and fish age. The 2DKS procedure tests the null hypothesis that two variables are distributed independently and is useful for detecting nonrandom patterns in bivariate data, especially when relationships between controlling factors and associated response variables are complex and functional relationships are not continuous (Garvey et al., 1998). The 2DKS test also detects threshold values of the controlling factor above or below which values of the response variable are constrained (Garvey et al., 1998).

Results

Female shovelnose sturgeon

A total of 181 female shovelnose sturgeon were collected from the MMR, with all reproductive stages represented except FV. Peaks in relative abundance of mature (FIV) females in collections occurred during April and October (Table 2). Mean FIV female E_2 concentrations peaked in the spring during the month of April and also in the fall during the month of October. High concentrations also occurred during the months of September and November, but ≤ 3 individuals underwent sex steroid analysis (Fig. 1a). There were significant differences in mean

plasma E_2 concentrations among reproductive stages ($P < 0.0001$; Fig. 1b). Stages Fv, FI, and FVI had significantly lower plasma E_2 concentrations compared to other stages. Stage FIV had significantly higher plasma E_2 concentrations compared to all other stages except FIII. There was also a significant relationship between E_2 concentration and fish age ($P = 0.0066$; Fig. 2). The 2DKS test indicated a shift in the distribution of E_2 values for fish ≥ 9 years of age. Plasma E_2 concentrations > 168.97 pg ml^{-1} were present only in fish ≥ age 9 (Fig. 2). The earliest age group with FIV females was at 8 years with more individuals occurring in higher densities in older age groups, but numbers of individuals by stage or age was highly variable (Fig. 3).

A positive correlation was observed between plasma Ca^{2+} and VTG concentrations for female shovelnose sturgeon ($r = 0.801$, $P < 0.001$; Fig. 4). There was an overall significant difference in mean plasma Ca^{2+} concentrations among female reproductive stages ($P < 0.0001$; Fig. 5a). Mean plasma Ca^{2+} concentration peaked during stage FIV and was significantly higher for FIV fish compared to Fv, FI and FVI stages. There was no significant relationship between plasma Ca^{2+} concentration and age for female fish ($P = 0.186$; Fig. 5b). Mean FIV female Ca^{2+} concentrations had a very similar pattern to mean E_2 concentrations. Concentrations peaked in the spring and fall during the months of April and October. High concentrations also occurred during the months of September and November, but four or fewer individuals underwent plasma calcium analysis (Fig. 6).

Male Shovelnose Sturgeon

A total of 208 male shovelnose sturgeon were harvested from the MMR, with all reproductive stages represented. Peaks in relative abundance of mature (MII) males in collections occurred during April and October (Table 2). Mean MII male T concentrations had

a very similar pattern to monthly mean FIV female E_2 and Ca^{2+} concentrations. Concentrations peaked in the spring and fall during the months of April and November with a secondary peak occurring during the months of October and November (Fig. 7). An overall significant difference in mean plasma T concentration was observed among male reproductive stages ($P < 0.0001$; Fig. 8a). MII males had a significantly higher mean T concentration than fish of all other reproductive stages. There was a significant relationship between plasma T concentration and age for male fish ($P = 0.0086$; Fig. 8b). The 2DKS test indicated a shift in the distribution of plasma T concentrations for fish age ≥ 10; plasma T concentrations > 2289.36 pg ml^{-1} were observed only in fish ≥ age 10. The earliest age of occurrence for captured MII males was 5 years old with a higher density occurring in the older age groups, but numbers of individuals by stage or age was highly variable (Fig. 9).

Discussion

Plasma E_2 and Ca^{2+} concentrations in females and T concentrations in males from the MMR differed among reproductive stages, particularly late maturational fish from early maturational or spent individuals, consistent with results of prior research on shovelnose sturgeon and other sturgeon species. Changes in E_2 concentrations among reproductive stages similar to those we observed have been described for other species of sturgeon and other shovelnose sturgeon populations, including female shovelnose sturgeon from the lower Missouri River (Amiri et al., 1996b; Barannikova et al., 2000; Barannikova et al., 2002; Barannikova et al., 2004; Bayunova et al., 2003; Wildhaber et al., 2007; Van Eenennaam et al., 1996). Wildhaber et al. (2006) reported a similar pattern for VTG concentration in female Missouri

River shovelnose sturgeon; as we found for E_2, significant differences only occurred between early- to mid- and late-vitellogenic females. Ca^{2+} concentrations have been shown to be significantly elevated in late maturational stages of Atlantic sturgeon (Van Eenennam et al., 1996) and distinguished pre-vitellogenic from vitellogenic female white sturgeon (Linares-Casenave et al., 2003), consistent with our results for female shovelnose sturgeon. Stage Fv and FI females had higher Ca^{2+} concentrations than FVI females, likely as a result of early development or expulsion of oocytes (Love, 1970). Testosterone concentrations in male shovelnose sturgeon from the MMR differentiated ripe (MII) males from other reproductive stages. Male stellate sturgeon *Acipenser stellatus* display a very similar T cycle with low concentrations during early spermatogenesis, a sharp increase during mid-spermatogenesis, and high levels during final maturation (Barannikova et al., 2004). Although we detected differences in plasma sex steroids and Ca^{2+} concentrations among reproductive stages, our results confirm that measurements of individual steroids or metabolites do not differentiate all reproductive stages in sturgeons (Ceapa et al., 2002; Webb et al., 2002, Wildhaber et al., 2007). Multiple blood plasma indicators of reproductive stage are required to unequivocally distinguish fish in different stages of gonadal development (Wildhaber et al., 2007).

Plasma VTG and Ca^{2+} concentrations were strongly correlated in female shovelnose sturgeon. Increasing E_2 concentrations during oogenesis indirectly caused an increase in Ca^{2+} levels in female shovelnose sturgeon because E_2 stimulates the liver to produce VTG. VTG in circulation binds free calcium Ca^{2+} in the female sturgeon and they either mobilize body Ca^{2+} reserves or increase Ca^{2+} uptake from the water to maintain homeostasis (Linares-Casenave, 1993). Previous indirect measurements of VTG using plasma Ca^{2+} concentrations in sturgeon (Linares-Casenave et al., 2003; Van Eenennaam et al., 1996) resulted in strong positive

correlations and significant linear relationships. Plasma VTG and total plasma Ca^{2+} concentrations also exhibited significant positive associations in rainbow trout *Oncorhynchus mykiss* and common carp *Cyprinus carpio*, although the correlation may be obscured during the early phase of ovarian vitellogenesis (Sumpter, 1985; Tyler and Sumpter, 1990). Our results indicate that total plasma Ca^{2+} concentration can be used as a surrogate for VTG assays for identification of vitellogenic shovelnose sturgeon females, consistent with other sturgeon species (Linares-Casenave et al., 2003; Van Eenennaam et al., 1996). Measurement of total plasma calcium is less complex and can be conducted with readily available kits, in contrast to the VTG immunoassay.

Shifts in the distributions of E_2 and T concentrations and stage with fish age are likely indicative of earliest ages at maturation for female and male shovelnose sturgeon in the MMR, respectively. Age at maturation has been estimated for populations of female shovelnose sturgeon using stage at length (e.g., Kennedy et al., 2006; Moos, 1978), but no studies have used E_2 or T concentrations to estimate age at maturation for this species. We detected a significant relationship between plasma E_2 concentration and age for female fish, with E_2 concentrations > 168.97 pg ml^{-1} present only in fish ≥ age 9. Also, the earliest occurrence of FIV females was determined at the 8 years of age signifying that MMR female shovelnose sturgeon mature at the earliest ages of 8 or 9 years. The distribution of plasma T concentrations in males also shifted with fish age; plasma T concentrations > 2289.36 pg ml^{-1} were observed only in fish ≥ age 10, suggesting that MMR male shovelnose sturgeon mature at the earliest age of 10 years. Also, MII males occurred in younger age groups, but were in much lower densities than age groups greater than 8 years of age. Our ages at maturity estimates for MMR shovelnose sturgeon coincide with that of females the upper Wabash River population (9 years; Kennedy et al., 2006) and are

similar to estimates based on gonadal examination by Tripp (2007) of 10.5 years and 9 years for MMR female and male shovelnose sturgeon, respectively. Recent age at maturation estimates for shovelnose sturgeon are contradictory to previous estimates of age at maturation between 5-7 years for this species (Helms, 1974; Moos, 1978; Jackson, 2004). The contradiction in estimates of age at maturity between earlier and more recent studies may result from a reduction in growth rate across all age classes and a shift in the age-frequency distribution toward older fish in the MMR shovelnose sturgeon population between 2002 and 2006 (Tripp, 2007). A shift toward older age at maturity in MMR female shovelnose sturgeon may also be a consequence of increased harvest during recent years (Colombo et al., 2007a). Size regulations for shovelnose sturgeon did not exist in the MMR in Illinois or Missouri until 2007 (Rob Maher, Illinois Department of Natural Resources, Bob Hrabik, Missouri Department of Conservation, *personal communication*). Current regulations in Illinois and Missouri permit the harvest of shovelnose sturgeon between 609.6 mm and 812.8 mm in the MMR. Commercial fisheries may be selectively harvesting younger mature fish (Tripp, 2007), which would be expected to favor maturation at a later age and larger size (Law, 2000; Conover and Munch, 2002; Ernande et al., 2004).

Peaks in plasma E_2 and Ca^{2+} concentrations in FIV female shovelnose sturgeon and elevated plasma T concentration in MII male shovelnose sturgeon during April and September-November are reflective of the spring spawning season and may represent additional evidence of fall spawning by MMR shovelnose sturgeon. Shovelnose sturgeon are known to spawn from late April to June (Forbes and Richardson, 1920; Eddy and Surber, 1947; Barnickol and Starrett, 1951; Christenson, 1975; Elser et al., 1977; Moos, 1978) when water temperatures are between 16.9°C and 20.5°C (Christenson, 1975; Elser et al., 1977). Evidence of fall spawning by

shovelnose sturgeon is limited. However, Tripp (2007) reported that sampling in the MMR during fall 2006 produced abundant ripe egg females and milting males. A larval sturgeon was also captured in November 2006; age and growth rate estimates suggested that this individual was spawned in September 2006 (Tripp, 2007). Peaks in mean plasma E_2, Ca^{2+}, and T concentrations and relative abundances of mature male (MII) and female (FIV) fish collected during this study coincided with months in which water temperatures in the MMR were within the reported range for shovelnose sturgeon spawning (Tripp, 2007). Seasonal peaks in plasma E_2 and Ca^{2+} concentrations in female shovelnose sturgeon from the MMR may be indicative of ovulation timing. Female bester *Huso huso* × *Acipneser ruthenus*, stellate, and Atlantic sturgeons exhibit peak E_2 concentrations when gravid; E_2 concentrations drop significantly following ovulation (Amiri et al., 1996b; Barannikova et al., 2002; Van Eenennaam et al., 1996). Plasma Ca^{2+} concentration in female brook trout *Salvelinus fontinalis* increases gradually before spawning, peaks at end of the spawning period, then declines precipitously following ovulation (Love, 1970); peak plasma Ca^{2+} concentrations also occur in ovulating female Atlantic sturgeon (Van Eenennaam et al., 1996). Males of white and stellate sturgeons exhibit peak T concentrations during April and May, corresponding with the beginning of their spawning migrations; T concentrations decline precipitously at the end of their spawning runs in mid-summer (Barannikova et al., 2002; Feist et al., 2004). Collectively, seasonal changes in plasma sex steroid and Ca^{2+} concentrations observed in this study are consistent with recent evidence (Tripp, 2007) that shovelnose sturgeon may be spawning during spring and fall in the MMR. In contrast, plasma E_2 concentrations of stage IV and V female shovelnose sturgeon from the Missouri River were generally highest during spring, but were also elevated in vitellogenic (FIII) females during autumn (Wildhaber et al., 2006). Plasma VTG concentrations peaked during

spring for female Missouri River shovelnose sturgeon; no mature (stage FIV or FV) fish were collected during fall (Wildhaber et al., 2006). Relatively high mean plasma E_2 and Ca^{2+} concentrations in female shovelnose sturgeon collected during autumn in the MMR may be due to vitellogenic (FIII) fish rather than mature (FIV and FV) individuals. Spent males and females were relatively abundant in winter months during this study; however, it is unclear whether these fish spawned in autumn or during the previous spring. Spent female shovelnose sturgeon take approximately 6 months to return to the FI stage and may require 2-3 years to return to the FII stage (Moos, 1978; Roussow, 1957; Colombo et al., 2007b; Tripp, 2007). Further investigation is clearly needed to determine the frequency of fall spawning by MMR shovelnose sturgeon and the extent to which individuals hatched during autumn may contribute to the shovelnose sturgeon population in the MMR.

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Table 1. Stages of shovelnose sturgeon gonadal development (Bruch et al., 2001; Colombo et al., 2007b).

Sex	Stage	Description
Male	Mv	Virgin male; pink ribbon like testis embedded in small amount of testicular fat
	MI	Yellow tubular testis; large amount of fat
	MII	Large pink testis
	MIII	Spent; flaccid, light-colored testis
Female	Fv	Virgin female; small ovarian folds with small amount of fat
	FI	Well-ordered ovarian folds with large amount of fat
	FII	Clear to yellow, small oocytes
	FIII	Yellow to light green oocytes
	FIV	Large, black oocytes
	FV	Spawning female
	FVI	Translucent ovary

Table 2. A. Number (N) of female shovelnose sturgeon captured monthly during October 2005 to February 2007 from the middle Mississippi River in the USA by reproductive stage and percentage of the total number of individuals captured per month represented by each reproductive stage. B. Number (N) of male shovelnose sturgeon captured monthly from the middle Mississippi River by reproductive stage and percentage of the total number of individuals captured per month represented by each reproductive stage. No fish were collected during July or August.

A.

	Month																			
	Jan.		Feb.		Mar.		Apr.		May		June		Sept.		Oct.		Nov.		Dec.	
Stage	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Fv	4	19	2	12	7	23	1	5	1	34	2	13	1	17	1	4	1	7	7	27
FI	8	38	10	59	18	60	3	13	2	66	6	38	0	0	5	19	6	43	13	50
FII	1	5	2	12	2	7	1	5	0	0	3	19	2	33	4	15	2	14	0	0
FIII	0	0	1	6	0	0	3	13	0	0	1	4	2	33	1	4	0	0	0	0
FIV	0	0	0	0	3	10	14	64	0	0	2	13	1	17	12	46	5	36	0	0
FVI	8	38	2	12	0	0	0	0	0	0	2	13	0	0	3	12	0	0	6	23
Total	21		17		30		22		3		16		6		26		14		26	

B.

	Month																			
	Jan.		Feb.		Mar.		Apr.		May		June		Sept.		Oct.		Nov.		Dec.	
Stage	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Mv	5	15	3	20	2	7	0	0	0	0	2	13	1	20	2	10	4	9	1	5
MI	10	30	5	33	8	29	1	5	1	25	4	25	1	20	1	5	5	11	9	43
MII	5	15	6	40	18	64	21	95	2	50	5	31	3	60	16	80	23	52	10	48
MIII	13	40	1	7	0	0	0	0	1	25	5	31	0	0	1	5	12	27	1	5
Total	33		15		28		22		4		16		5		20		44		21	

Figure Captions

Fig. 1. (a) Mean ± SE of stage FIV female shovelnose sturgeon (n=29) plasma estradiol (E_2) concentrations collected from the middle Mississippi River (MMR) in Illinois USA during October 2005 to February 2007. Columns represent number of individuals per month and lines denote the pattern of monthly estradiol concentrations. (b) Means ± SE of plasma 17-β estradiol (E_2) concentrations in female shovelnose sturgeon (n=109) collected in MMR during October 2005 to February 2007 at different reproductive stages. Bars with different letters denote means that are significantly different from one another ($P < 0.05$).

Fig. 2. Plasma 17-β estradiol (E_2) concentrations of individual female shovelnose sturgeon (n=100) collected from the middle Mississippi River in Illinois USA during October 2005 to February 2007 versus age, encompassing multiple reproductive stages.

Fig. 3. Gonadal stage distribution of female shovelnose sturgeon (n=166) from the middle Mississippi River in Illinois USA collected during October 2005 to February 2007 by age group.

Fig. 4. Relationship between plasma calcium (Ca^{2+}) and vitellogenin (VTG) concentrations for 12 female shovelnose sturgeon collected from the middle Mississippi River in Illinois USA during October 2005 to February 2007, including at least one individual per reproductive stage.

Fig. 5. (a) Mean ± SE plasma calcium (Ca^{2+}) concentrations in female shovelnose sturgeon (n=151) collected from the middle Mississippi River (MMR) in Illinois USA and during October 2005 to February 2007 at different reproductive stages. Bars with different letters denote means that are significantly different from one another ($P < 0.05$). (b) Plasma calcium (Ca^{2+}) concentrations in individual female shovelnose sturgeon (n=140) collected from MMR during October 2005 to February 2007 versus age, encompassing multiple reproductive stages.

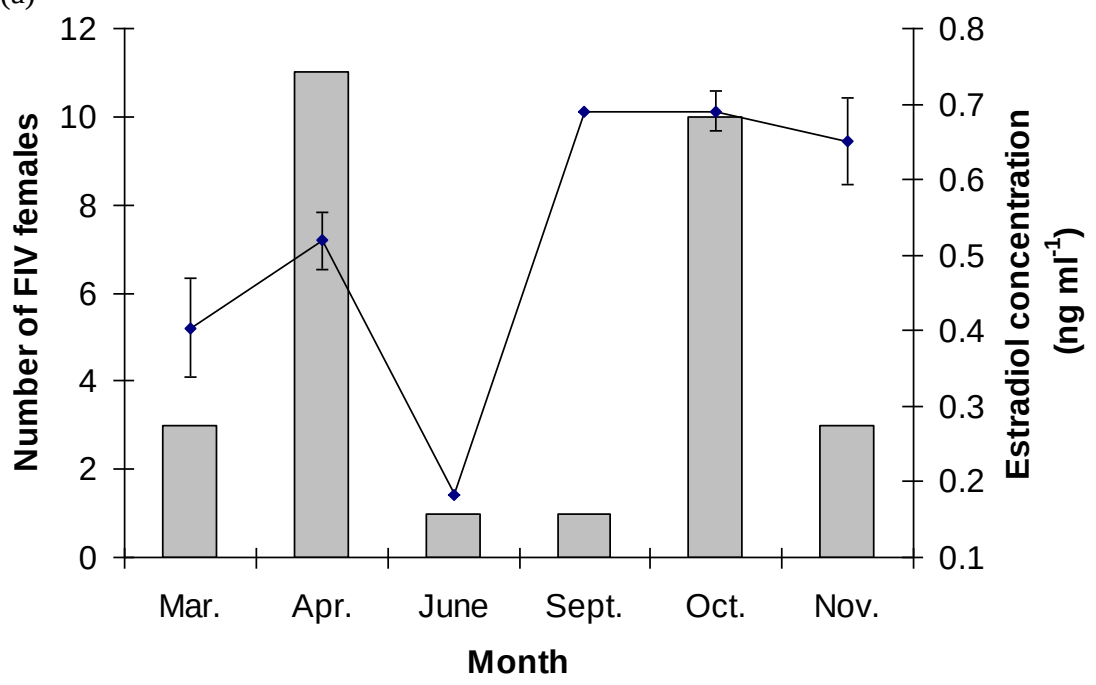
Fig. 6. Mean ± SE plasma calcium (Ca^{2+}) concentrations of stage FIV female shovelnose sturgeon (n=34) collected from the middle Mississippi River in Illinois USA during October 2005 to February 2007. Columns represent number of individuals per month and lines denote the pattern of monthly calcium concentrations.

Fig. 7. Mean ± SE plasma testosterone (T) concentrations of stage MII male shovelnose sturgeon (n=36) collected from the middle Mississippi River in Illinois USA during October 2005 to February 2007. Columns represent number of individuals per month and lines denote the pattern of monthly testosterone concentrations.

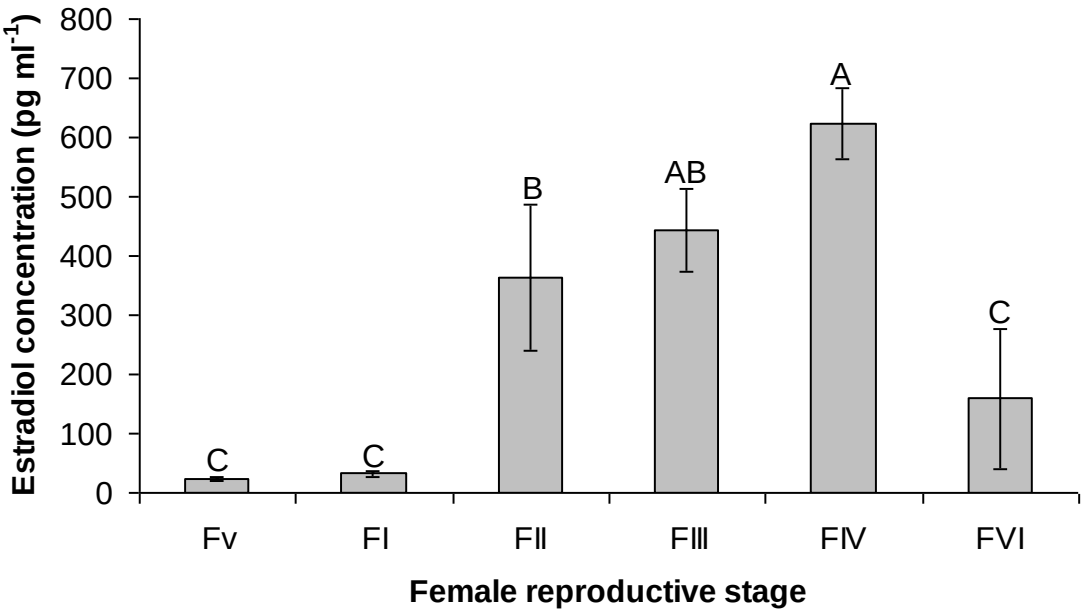
Fig. 8. (a) Mean ± SE plasma testosterone (T) concentrations in male shovelnose sturgeon at different reproductive stages (n=113) collected from the middle Mississippi River (MMR) in Illinois USA during October 2005 to February 2007. Bars with different letters denote means that are significantly different from one another ($P < 0.05$). (b) Plasma testosterone (T) concentrations of individual male shovelnose sturgeon (n=96) collected in MMR during October 2005 to February 2007 versus age, encompassing multiple reproductive stages.

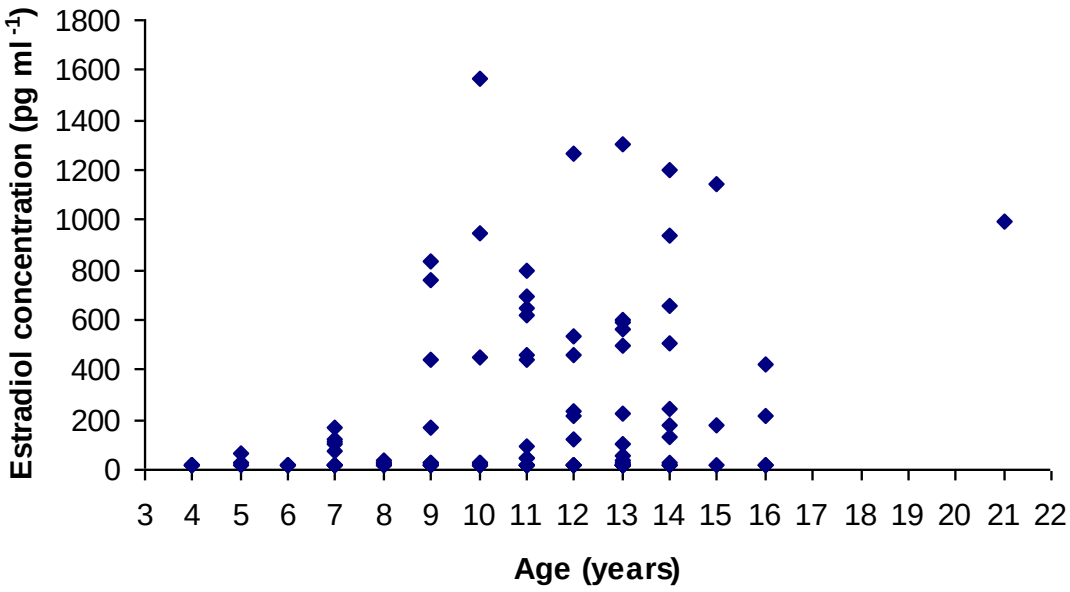
Fig. 9. Gonadal stage distribution of male shovelnose sturgeon (n=185) from the middle Mississippi River in Illinois USA collected during October 2005 to February 2007 by age group.

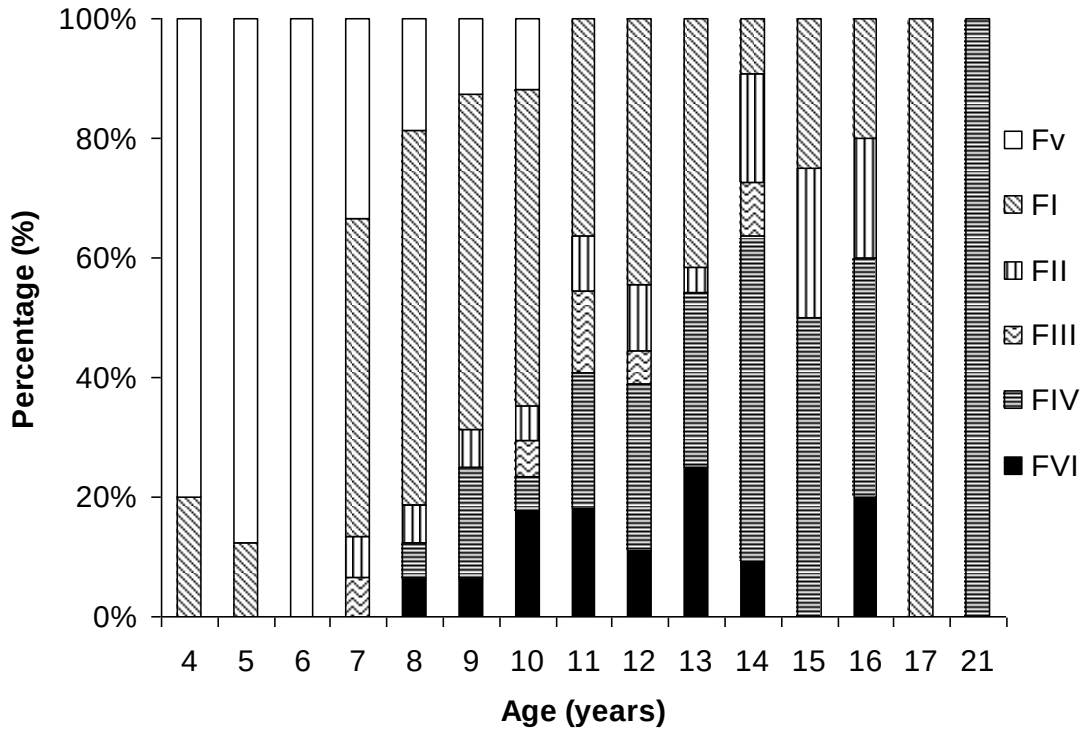
(a)

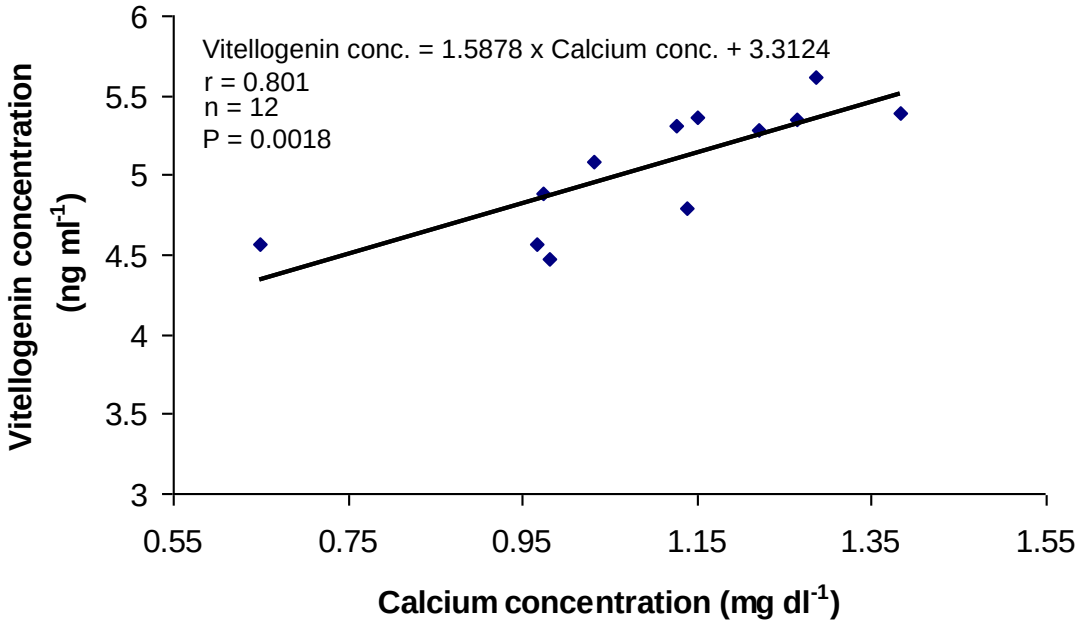

12
10
8
6
4
2
0
Number of FIV females
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Estradiol concentration (ng ml-1)
Mar.
Apr.
June
Sept.
Oct.
Nov.
Month

(b)

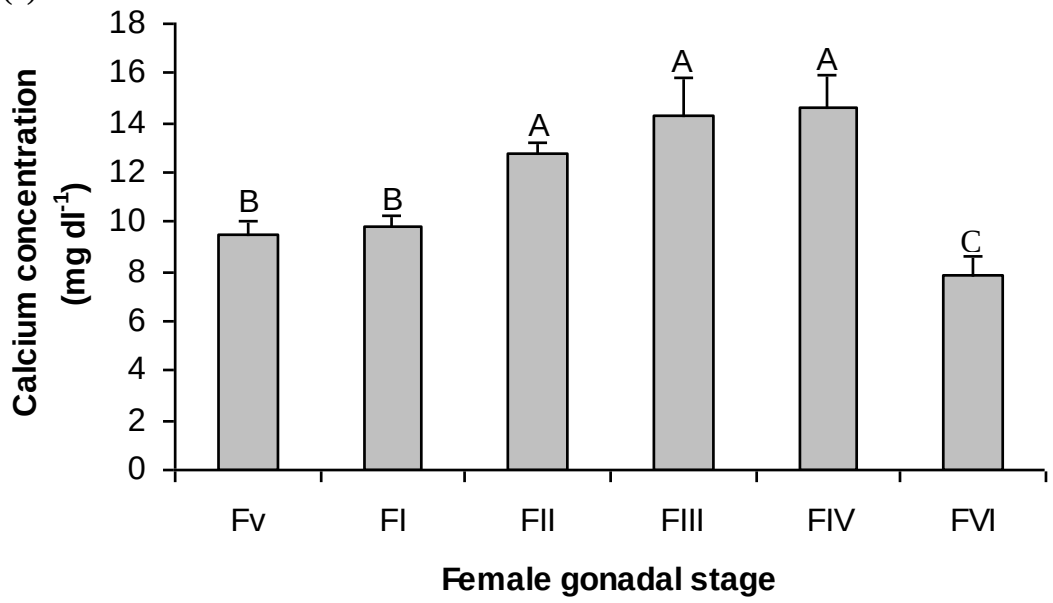

800
700
600
500
400
300
200
100
0
Estradiol concentration (pg ml-1)
C
C
B
AB
A
C
Fv
FI
FII
FIII
FIV
FVI
Female reproductive stage


Estradiol concentration (pg ml-1)
1800
1600
1400
1200
1000
800
600
400
200
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Age (years)

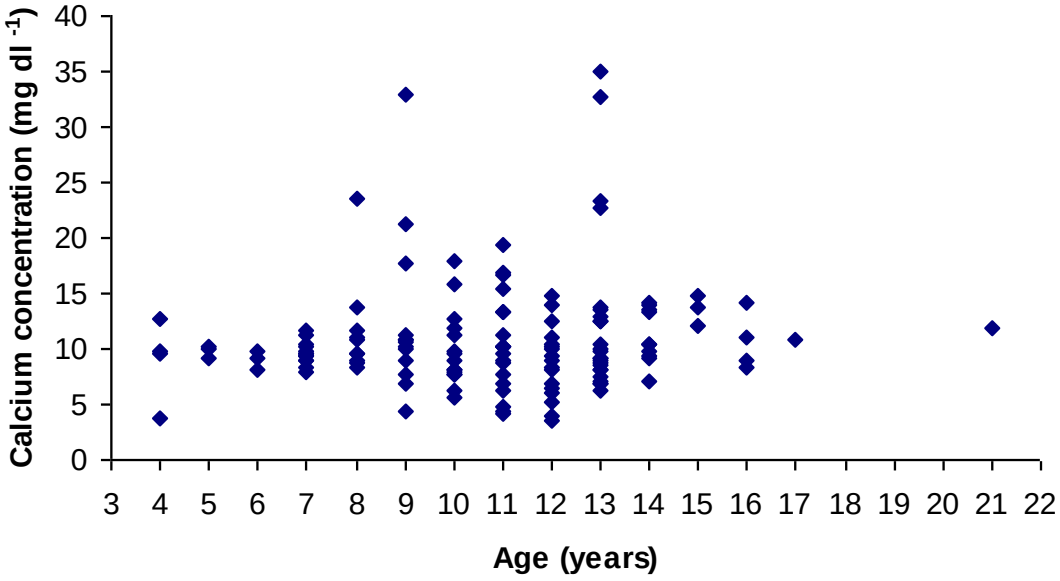

100%
80%
60%
40%
20%
0%
Percentage (%)
4
5
6
7
8
9
10
11
12
13
14
15
16
17
21
Age (years)
Fv
FI
FII
FIII
FIV
FVI

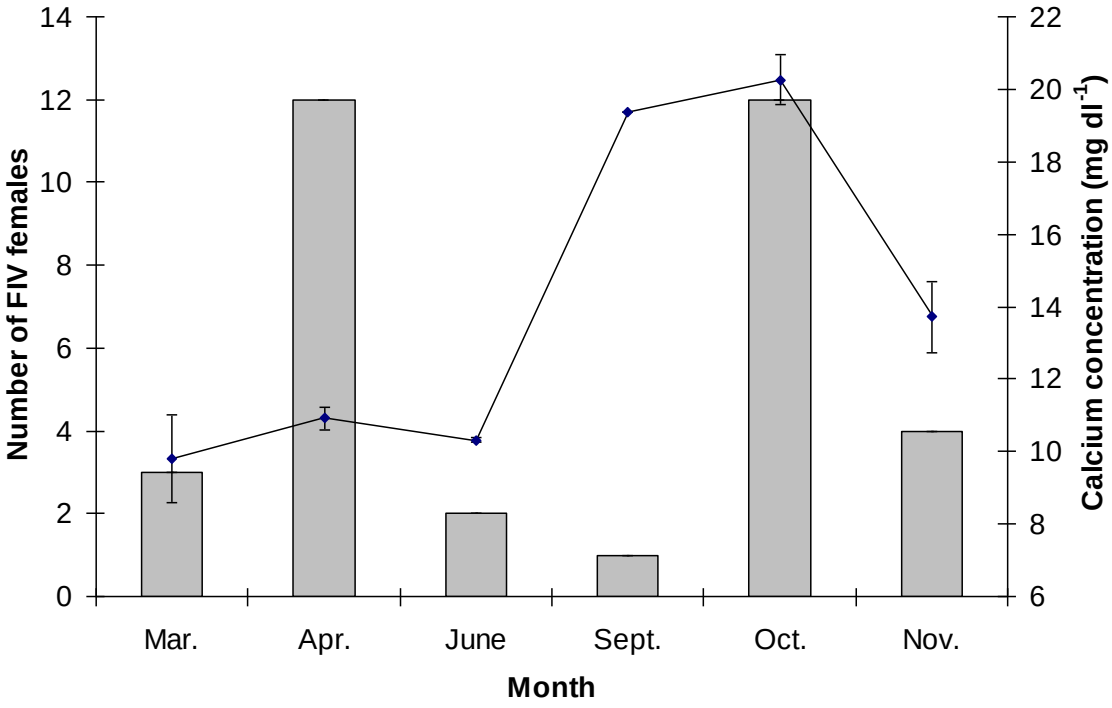

Vitellogenin conc. = 1.5878 x Calcium conc. + 3.3124
r = 0.801
n = 12
P = 0.0018
Vitellogenin concentration (ng ml-1)
Calcium concentration (mg dl-1)
6
5.5
5
4.5
4
3.5
3
0.55
0.75
0.95
1.15
1.35
1.55

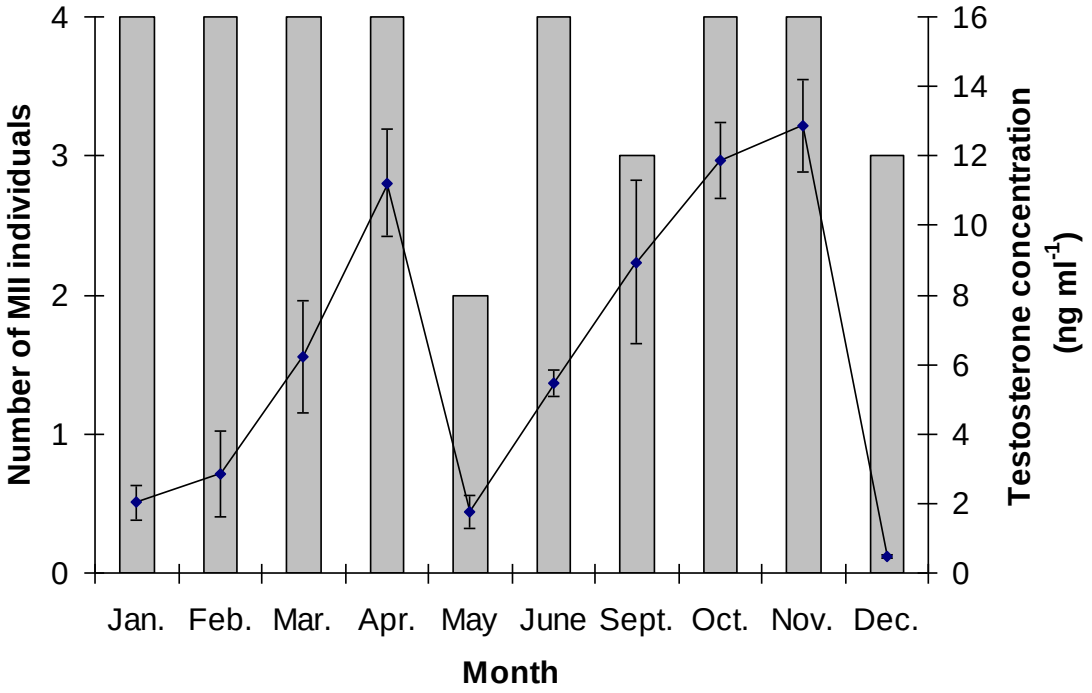
(a)


Calcium concentration (mg dl-1)
18
16
14
12
10
8
6
4
2
0
B
B
A
A
A
C
Fv
FI
FII
FIII
FIV
FVI
Female gonadal stage

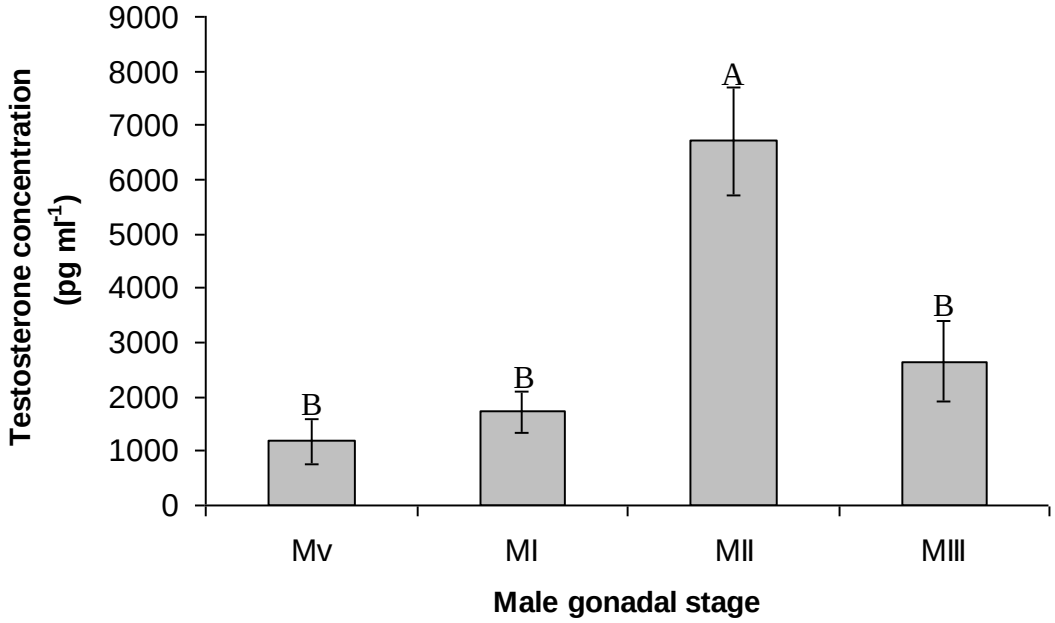
(b)


Calcium concentration (mg dl -1)
40
35
30
25
20
15
10
5
0
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Age (years)

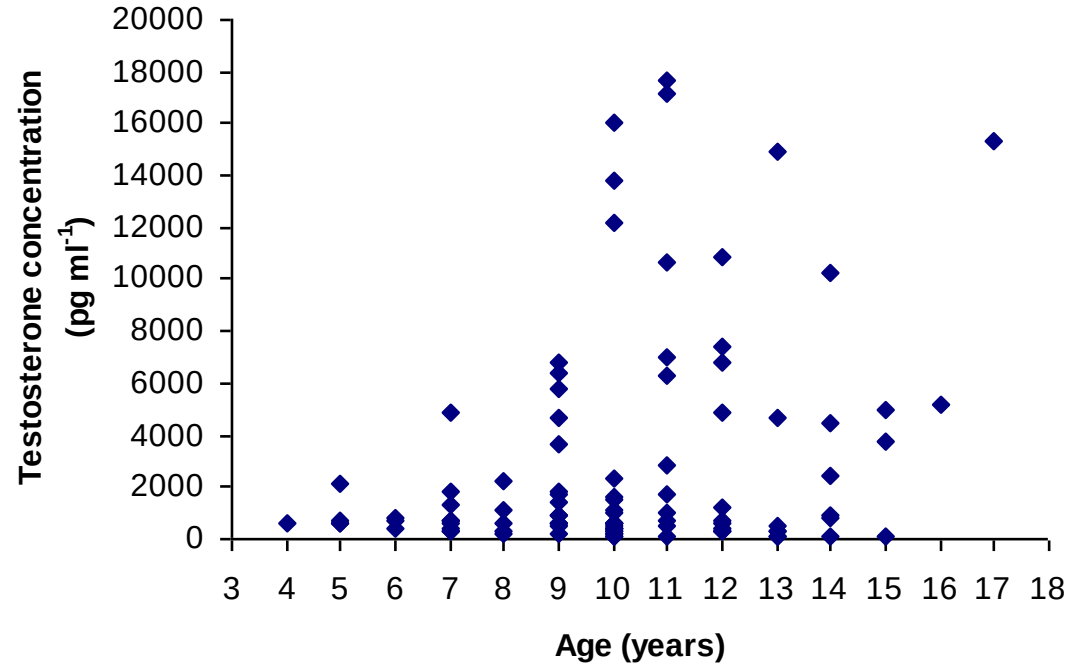

14
12
10
8
6
4
2
0
Number of FIV females
22
20
18
16
14
12
10
8
6
Calcium concentration (mg dl-1)
Mar.
Apr.
June
Sept.
Oct.
Nov.
Month

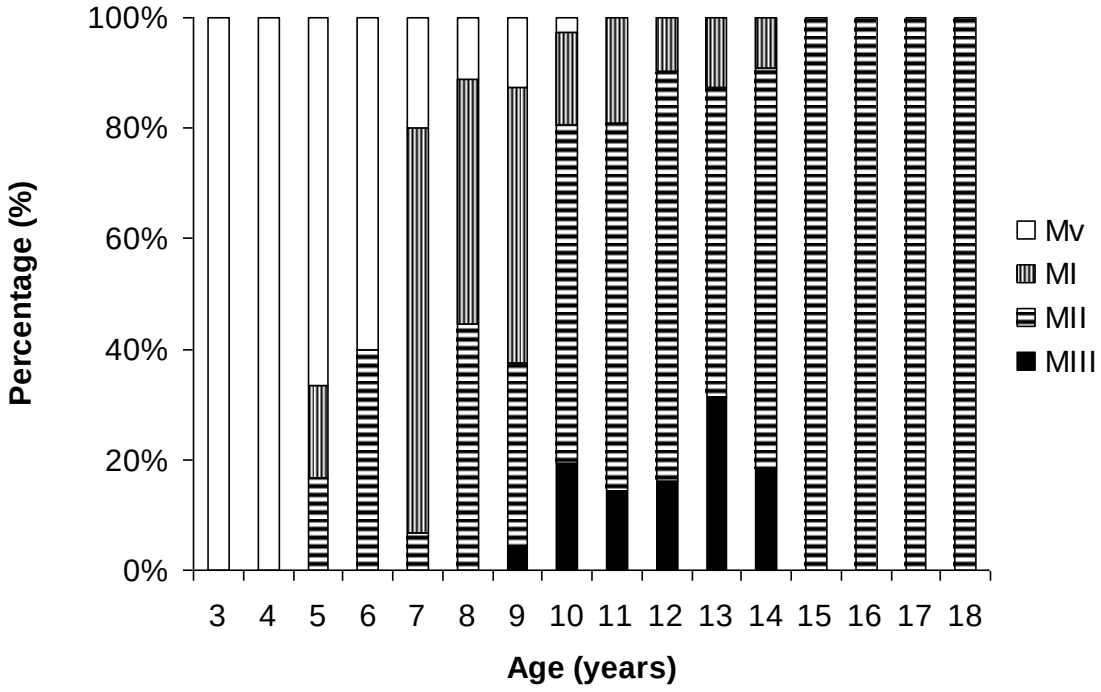

Number of MII individuals
Testosterone concentration (ng ml-1)
0
1
2
3
4
0
2
4
6
8
10
12
14
16
Jan.
Feb.
Mar.
Apr.
May
June
Sept.
Oct.
Nov.
Dec.
Month

(a)


Testosterone concentration (pg ml-1)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
A
B
B
B
Mv
MI
MII
MIII
Male gonadal stage

(b)


Testosterone concentration (pg ml-1)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Age (years)


100%
80%
60%
40%
20%
0%
Percentage (%)
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Age (years)
Mv
MI
MII
MIII