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Research Article

Gonad development and reproductive hormones of invasive silver carp (*Hypophthalmichthys molitrix*) in the Illinois River

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Abstract

AbstractReproduction is a major component of an animal's life history strategy. Species with plasticity in their reproductive biology are likely to be successful as an invasive species, as they can adapt their reproductive effort during various phases of a biological invasion. Silver carp (*Hypophthalmicthys molitrix*), an invasive cyprinid in North America, display wide variation in reproductive strategies across both their native and introduced ranges, though the specifics of silver carp reproduction in the Illinois River have not been established. We assessed reproductive status using histological and endocrinological methods in silver carp between April and October 2018, with additional histological data from August to October 2017. Here, we show that female silver carp are batch spawners with asynchronous, indeterminate oocyte recruitment, while male silver carp utilize a determinate pattern of spermatogenesis which ceases in the early summer. High plasma testosterone levels in females could be responsible for regulating oocyte development. Our results suggest that silver carp have high spawning activity in the early summer (May–June), but outside of the peak spawning period, female silver carp can maintain spawning-capable status by adjusting rates of gametogenesis and atresia in response to environmental conditions, while

males regress their gonads as early as July. The results of this study are compared to reports of silver carp reproduction in other North American rivers as well as in Asia.

Key words: atresia, fish reproduction, gametogenesis, gonadal function, gonadal steroids, oocyte development, oocyte maturation, reproductive behavior, seasonal reproduction, sperm maturation, spermatogenesis, vertebrates, non-mammalian (fish, fowl, reptiles, amphibians)

Introduction

Life-history strategies are a result of natural selection acting on a species to optimize fitness [1, 2], and reproduction is an important part of an animal's life history strategy. Fish represent the most diverse group of vertebrates, and, as a result, they also implement highly diverse modes of reproduction [3]. Fish vary widely in sex determination, gametogenesis, timing of spawning, mating, and parental behavior [4]. This variation is critical in allowing fish to successfully adapt to a wide range of habitats and environmental conditions.

Most temperate, freshwater teleost fishes are gonochoristic and fertilize gametes externally [5]. However, the timing of gamete development, the frequency of spawning, and the specific cues that initiate events in the reproductive cycle are species-specific. A semelparous fish (i.e., Salmonidae) that spawns only once in its lifetime will optimize the quality of their gametes by first generating a large store of somatic energy reserves and then devoting those energy stores specifically to gametes [6]. With only one opportunity for successful reproduction, semelparity often evolves in a species when adult survival is uncertain [7]. In contrast, an iteroparous species that spawns seasonally (i.e., Cyprinidae) will have many opportunities to produce offspring (assuming they survive between spawning periods). Iteroparous fishes must constantly assess the trade-off between reproduction and growth, and this type of spawning is often selected for when reproductive success becomes uncertain [8]. Skipping a spawning event could allow a fish to preserve energy for the following spawning season, but there is no guarantee that the fish will survive to participate in those potential opportunities. Additionally, while some iteroparous fishes release all of their gametes at once during the spawning season (total spawners, i.e., Eurasian perch [*Perca fluviatilis*] [9]), others allocate their gametes strategically across the spawning season (batch spawners, i.e., Brighteye darter [*Etheostoma lynceum*]) [10–13]. This creates additional choices that must be made between current and future reproductive potential.

While a fish's reproductive strategy is optimized for the environment in which it evolved, fishes are sometimes introduced outside of their native range. If an introduced fish is able to survive, reproduce, and spread beyond its introduction point, it is then considered an invasive species [14]. The likelihood of a species becoming invasive is actually quite low [15], because the species must have sufficient plasticity to make adjustments to its life-history strategy to match its new environment. Specifically, individuals at the invasion front (the population 'edge') generally have increased reproductive effort compared to individuals at the 'core' of the population [16, 17]. Higher gonadosomatic index at population edges has been documented in sea urchins [18] and fishes [19–21]. Other adaptations can also contribute to higher reproductive success for 'edge' populations, such as increased metabolic rate allowing more time for oviposition [22], larger body size facilitating higher fecundity [23–25], and larger or more rapidly-growing offspring [26–29]. Fishes with highly successful reproduction in a wide range of contexts are more likely to become a successful invasive species than fishes with very specific requirements for reproduction [30, 31]. Generally, higher reproductive investment (higher gonadosomatic index, larger clutch sizes) is expected at population edges such as an invasion front [17]. The African jewelfish (*Hemichromis letourneuxi*), an invasive fish in the Everglades in Florida, has been found to have higher gonadosomatic index at the edge of its invasive range compared with jewelfish at the core of the range [18], suggesting that this species can adjust their reproductive strategy based on the different pressures that arise during range expansion. Similarly, pumpkinseed (*Lepomis gibbosus*) introduced to Spain display life-history plasticity, with introduced pumpkinseed having higher reproductive investment (gonadosomatic index) than pumpkinseed in their native range [32].

The silver carp (*Hypophthalmicthys molitrix*) is a cyprinid that has been introduced in almost every continent [33], primarily due to their usefulness in aquaculture as a planktivore [34]. In their native range, which stretches from the Pearl River in southern China to the Amur River in Siberia [35], silver carp are known to display variable spawning habits. In the Amur River, silver carp have been reported to spawn between June to early August [36]; in China, they will spawn between April and July [37]; in Uzbekistan, they will only spawn from May to June [38]. It has been suggested that female silver carp have asynchronous oocyte development [39, 40], but little is known about male gametogenesis in this species.

Silver carp were introduced to the United States in the 1970s and have since escaped and spread throughout the Midwest [35]. This species has detrimental effects on the body condition of native fishes, especially planktivores, [41–43] and is known to alter fish community structure in ecosystems where they have invaded [44]. Silver carp also display jumping behavior in response to broadband sound that can interfere with boating and recreation on waterways [45]. Furthermore, silver carp appear to have different spawning habits in North America than they do in their native range. Williamson and Garvey [46], Papoulias *et al*. [40], and Coulter *et al*. [47] all noted that silver carp in the United States have a protracted spawning season compared to their native range, as mature ovaries have been found as late as October—up to 3 months after individuals in the native range. Observations have been made of partially spent ovaries in female silver carp in the Upper Mississippi River, suggesting that individual female silver carp could spawn more than once in a spawning season [46], but, to date this reproductive strategy has not been confirmed. The first recorded observation of silver carp in the Illinois River was in 2000 [35]. As they have progressed northward, there are concerns that silver carp might invade the Great Lakes [48], which would give them access to many other North American watersheds. Due to the variability in reproductive strategies used by silver carp within their native range, it is not known if silver carp in the Illinois River utilize similar or different spawning habits than silver carp in the Missouri River [40] or the Mississippi River [46]. Furthermore, there is evidence that silver carp vary in size across

their invaded range in the Illinois River, with carp at the leading edge of their range being larger than individuals from established areas [49, 50]. Body size is known to correlate positively with fecundity in fish [51], so it is possible that silver carp might display a gradient in reproductive potential in the Illinois River. If larger individuals at the edge of their range also have higher fecundity, they could be contributing disproportionate numbers of offspring to the population. As 'edge' offspring tend to have the dispersal-favoring phenotypes of their parents [17], this could further facilitate to spread.

The objective of this study was to quantify the dynamics of the reproductive cycle in silver carp in the Illinois River. Specifically, we sought to establish patterns of gametogenesis in both males and females using histological and endocrinological data, coupled with gonadosomatic index from silver carp caught from 'edge' and 'core' reaches in the Illinois River between April and October 2018. We also present histological data from silver carp collected from August to October 2017. Data gathered from this study provide insight into how silver carp in the Illinois River might differ from silver carp in other North American rivers [40, 46] as well as in their native range. Additionally, understanding the reproductive biology of this highly successful invasive species can be useful for attempts to control their spread by providing information on where and when recruitment could be occurring across this species' range.

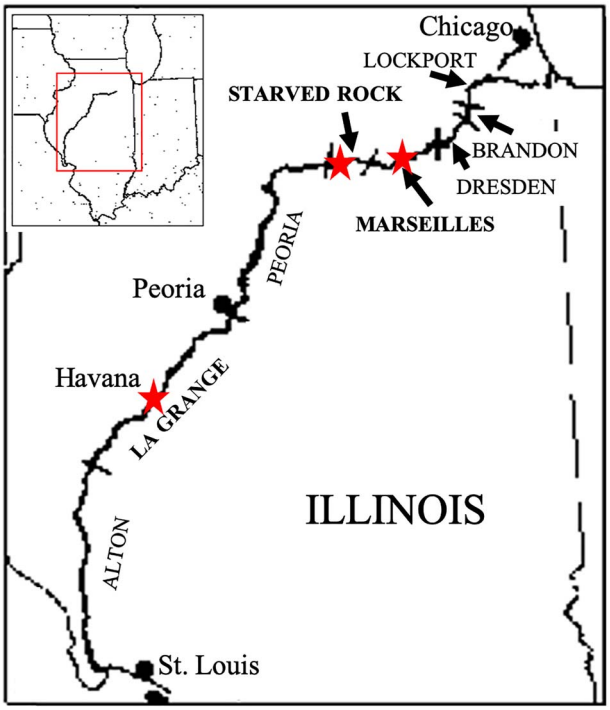

Figure 1. Reaches of the Illinois River. The Illinois River flows southwest through Illinois from the Lockport Lock and Dam near Chicago to the Alton Lock and Dam near St. Louis, Missouri. The two reaches sampled for the purposes of this study were the La Grange Reach, near Havana, and the Starved Rock Reach near the Starved Rock Lock and Dam.

Materials and methods

Study sites and sampling

The Illinois River flows southwest from its origin at the confluence of the Des Plaines and Kankakee Rivers until its confluence with the Mississippi River. The entire river is divided into reaches by a series of locks and dams (Figure 1). The La Grange Reach, which stretches from the La Grange Lock and Dam near Beardstown, Illinois, to the Peoria Lock and Dam near Peoria, Illinois, has had an established population of silver carp since the late 2000s [41, 52]. Carp from this reach were considered 'core' fish. Sampling from this reach took place between river mile (RM) 120.0 and 128.0 near Havana, Illinois, by electrofishing using standard protocols and techniques. Silver carp were also sampled from between RM 231.2 and 271.4 in the Starved Rock Reach and Marseilles Reaches, which collectively start at the Starved Rock Lock and Dam near Utica, Illinois, and end at the Dresden Lock and Dam south of Channahon, Illinois. Carp from these reaches were considered to be 'edge' fish. Silver carp in this reach were caught by commercial fishing gear, primarily short-set gill and trammel nets, through the silver carp harvest program run by the Illinois Department of Natural Resources (IDNR). The IDNR has been harvesting silver carp from the Starved Rock Reach since 2011, but the catch-per-unit-effort has generally been low in these reaches in comparison to middle and lower reaches of the Illinois River [49, 50]. The Dresden Reach was not included in this study due to the low density of silver carp [49]. The Marseilles Reach was sampled monthly between August and October 2017, the Starved Rock Reach was sampled monthly between April and October 2018, and the La Grange Reach was sampled monthly during both time periods.

Following capture, silver carp were weighed using a hanging scale (RMDS-50, Rapala USA, Minnetonka, MN) to the nearest 0.01 kg, and total length was measured to the closest mm. For fish caught in 2018, venipuncture was performed with a heparinized 3 mL syringe and 20-gauge needle inserted ventrally at the caudal vein [53]. Whole blood was transferred to a heparinized vacutainer (BD Vacuntainer, Becton, Dickson, and Company, Franklin Lakes, NJ) and stored on ice for less than 2 h prior to being spun centrifuged at 2000*xg* for 5 min (MyFuge 12 Mini Centrifuge, Thomas Scientific, Swedesboro, NJ) to separate plasma from red cells. The plasma portion was flash-frozen in liquid nitrogen and later stored at −80 °C prior to hormone analyses. Fish were then euthanized by cerebral percussion. In 2017 and 2018, both gonads were removed in their entirety and weighed to the nearest 0.1 g. A 2 cm-thick portion of gonad was removed from the medial zone of the tissue and placed in 10% neutral buffered formalin (NBF) for histological analysis. Male and female silver carp were intentionally collected in a 1:1 sex ratio.

Scales were collected dorsal to the lateral line by gentle scraping with a scalpel blade for assessment of age [54]. Scales have previously been used for determining age in Silver Carp, as one annulus is equivalent to 1 year [35], and back-calculated lengths using scales in Silver Carp are comparable to those generated using other hard parts such as otoliths and fin rays [55], though they tend to yield an underestimate of actual age. For this reason, we used scales to estimate ages solely for comparison of ages between reaches and relative comparisons, as this technique has not been validated using juvenile fish [56]. Scales were magnified under a stereoscope and annuli were counted by three independent readers to estimate age. Disagreements between readers' estimates were settled by consensus [56].

Enzyme-linked immunosorbent assays for reproductive hormones in plasma

Steroids were extracted and purified from plasma from fish caught in 2018 using diethyl ether, because reproductive hormones in plasma are frequently bound to steroid-binding proteins and must be isolated prior to measurement [57]. Diethyl ether was added to thawed plasma in a 5:1 ratio and vortexed for 2 min. Samples were then rapidly frozen at –80 °C and the liquid ether phase was collected. The diethyl ether extraction was performed twice for optimal steroid recovery. The ether was evaporated under nitrogen in a 40 °C water bath, and the desiccated steroids were stored at −20 °C until analysis.

Four reproductive steroids were measured in plasma using commercially-available enzyme-linked immunosorbent assays (ELISAs): estradiol (E_2) in females (Cayman Chemical #582251; Sensitivity 15 pg/mL; Linearity range 16.4–4000 pg/mL); 11-keto testosterone (11KT) in males (Cayman Chemical #582751; Sensitivity 1.3 pg/mL; Linearity range 3.1–100 pg/mL); and testosterone (T) (Cayman Chemical #582701; Sensitivity 6 pg/mL; Linearity range 7.8–500 pg/mL) and maturation-inducing steroid (MIS) (Cayman Chemical #498500 [Tracer], #498502 [Antiserum], #498504 [Standard]; Sensitivity 2.1 pg/mL; Linearity range 3.9–31 pg/mL) in both males and females. For measurement of E_2, 11KT, and T, steroids were reconstituted in 500 µL assay buffer for every 100 µL plasma, and then diluted again in assay buffer 1:5. For MIS, steroids were reconstituted in assay buffer as stated above but were not diluted any further. For each assay, samples were plated in triplicate. The concentration of steroid in each sample was calculated using a standard curve and adjusted for the 1:5 dilution. The steroid concentrations were normalized to mass to account for differences in fish size. Additionally, in cases where the measured steroid concentration fell below the sensitivity of the assay, the plasma steroid concentration in those fish was considered to not be statistically different from 0 pg/mL [58].

Histological preparation of gonadal tissue

Gonadal tissues from fish caught in both 2017 and 2018 were prepared for histology using standard protocols [59]. Briefly, following fixation for 72 h in 10% NBF, tissues were transferred to 70% ethanol. Tissues were then dehydrated in ethanol, cleared in xylene, and infiltrated with paraffin wax (Tissue Tek VIP). Processed tissues were embedded in paraffin wax and sectioned to a width of 8 (ovaries) or 5 µm (testes) ($n = 20$ sections/tissue).

Ovarian and testicular tissues were stained with Hematoxylin and Eosin (H&E, $n = 2$ slides/fish, two sections/slide) and Gömöri Trichrome stain (GT, $n = 2$ slides/fish, two sections/slide). All sections were randomly selected from the 20 sections generated. Testicular tissues were also immunostained for proliferating cell nuclear antigen (PCNA, $n = 2$ slides/fish, two sections/slide), which is a marker for actively dividing cells during spermatogenesis [60]. For immunostaining, de-paraffinized and rehydrated tissues were incubated in sodium citrate buffer at 99 °C for 20 min for antigen retrieval and cooled to room temperature before endogenous peroxide activity was blocked in 3% H_2O_2/Methanol for 15 min. Samples were rinsed in 1% potassium-buffered saline tween-20 (PBST) and blocked in 1% bovine serum antigen (BSA) at room temperature for 30 min. Tissues were incubated with a primary antibody for PCNA (mouse anti-PCNA; ab29, Abcam, Cambridge, MA) diluted in 1% BSA/PBST (1:5000) for 20 min at room temperature. Sections were then rinsed with PBST and incubated with a secondary antibody conjugated with horseradish peroxidase (goat anti-mouse IgG; 1:5000; ab205719, Abcam, Cambridge, MA) diluted in 1% BSA/PBST (1:5000) for 60 min at room temperature. Samples were rinsed again with PBST before being exposed to the chromagen 3,3′-Diaminobenzidine (DAB, Vector Laboratories, Inc., Burlingame, CA) for 60 s and rinsed in tap water for 5 min. Tissues were then counterstained with hematoxylin, dehydrated, and cover-slipped.

Assessment of gonadal tissue

Stained tissue sections ($n = 4$ slides/fish, two sections/slide) were digitized into high-quality images using a NanoZoomer Digital Pathology System (Hamamatsu Photonics, Hamamatsu City, Japan) using a 40× objective and NDP Scan Software and examined using NPD2.view. The H&E sections were scanned for the presence of primary growth oocytes, cortical alveolar oocytes, vitellogenic oocytes, and migrating germinal vesicles. Additionally, sections stained with GT were examined for the presence of atresia, post-ovulatory follicles (POFs), and melanomacrophages. The ovaries were then classified to developmental stages according to Brown-Peterson et al. [12]. Fish that were of reproductive size but were reproductively inactive, meaning that the gonads resembled immature gonads, were classified as "inactive." A schematic of oogenesis in the fish can be found in Supplemental Figure S1A.

For testicular tissue, entire sections were examined for the presence of proliferating spermatogonia, spermatogenesis in spermatocysts, melanomacrophages, and the presence of spermatozoa in the lumens and ducts. Testes were then classified to developmental stages according to Brown-Peterson et al. [12]. Fish of reproductive size that were reproductively inactive were classified as "inactive." A schematic of spermatogenesis in the fish can be found in Supplemental Figure S1B.

Statistical analyses

Differences between populations and sexes in total length, mass, and age were measured using two-way analysis of variance (ANOVA; main effects: sampling site, sex, and their interaction); gonadosomatic index and plasma hormone concentrations between both populations and across months were also measured using a two-way analysis of variance (ANOVA; main effects: sampling site, month, and their interaction), followed by a Tukey post-hoc test to separate means. Results were considered significant when $P < 0.05$. Differences in the proportion of fish at each gonadal stage were assessed across months using Fisher exact test, with separate tests run for each sampling site [61]. Data from 2017 to 2018 were analyzed separately. Plots of model residuals were examined to ensure accurate model fit, and data were log-transformed if residuals showed severe deviations [62]. All statistical tests were run in *R* using the base package [63], as well as multcomp [64], Rmisc [65], and ggplot2 [66].

Ethics

All animal and experimental procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana (Protocol #17118).

Results

In 2017, a total of 52 silver carp were caught ($n = 15$ females from La Grange and $n = 11$ females from Marseilles; $n = 15$ males from La Grange and $n = 11$ males from Marseilles). A total of 136 silver carp were caught in 2018 ($n = 35$ females from La Grange and $n = 33$ females from Starved Rock; $n = 35$ males from La Grange

Table 1. Demographic information of silver carp.

Year	Reach	Sex (*n*)	Total length (mm) mean ± SD (range)		Mass (kg) mean ± SD (range)		Age (years) mean SD (range)
2017	La Grange (Core)	Female (15)	525 ± 92 (380–680)	a	1.48 ± 1.19 (0.50–4.27)	a	2.94 0.68 (2–4)
		Male (15)	567 ± 62 (420–610)	a	1.75 ± 0.59 (0.70–2.26)	a	3.13 0.62 (2–4)
	Marseilles (Edge)	Female (11)	674 ± 118 (516–840)	b	4.70 ± 1.68 (2.10–7.40)	b	3.35 0.67 (3–5)
		Male (11)	653 ± 97 (518–780)	b	3.44 ± 0.70 (2.50–4.50)	b	3.17 0.58 (2–4)
2018	La Grange (Core)	Female (35)	614 ± 61 (476–672)	a	2.53 ± 0.82 (1.00–4.78)	a	3.71 0.94 (2–6)
		Male (35)	606 ± 53 (495–721)	a	2.34 ± 0.67 (0.86–4.20)	a	3.38 0.78 (2–5)
	Starved Rock (Edge)	Female (33)	617 ± 45 (517–656)	a	2.71 ± 0.59 (1.63–3.17)	b	3.72 1.21 (2–7)
		Male (33)	620 ± 58 (537–765)	a	2.67 ± 0.75 (1.65–4.84)	b	3.44 0.77 (2–5)

Length and weight data were compared between sexes and locations by two-way analysis of variance (ANOVA). Each year was analyzed separately. Lower-case letters indicate groups that were statistically similar to each other. Total lengths and masses were statistically significant between reaches (TL: $F_1 = 20.54$, $P < 0.001$; mass: $F_1 = 40.97$, $P < 0.001$) but not between sexes (TL: $F_1 = 0.35$, $P = 0.56$; mass: $F_1 = 2.27$, $P = 0.14$) in 2017. Total lengths were not different between reaches ($F_1 = 0.89$, $P = 0.35$) or sexes ($F_1 = 0.05$, $P = 0.83$) in 2018. Masses were significantly different between reaches ($F_1 = 4.20$, $P < 0.05$) but not sexes ($F_1 = 0.95$, $P = 0.33$) in 2018. Ages were not significantly different between La Grange and Marseilles Reaches in 2017 (females: $F_{1,25} = 2.58$, $P = 0.12$; males: $F_{1,25} = 0.04$, $P = 0.96$) or between the La Grange and Starved Rock Reaches in 2018 (females: $F_{1,56} = 0.001$, $P = 0.98$; males: $F_{1,52} = 0.08$, $P = 0.77$).

and $n = 33$ males from Starved Rock) across all sampling events. Sample sizes across months and locations were consistent for both sexes, with the exception of fish sampled from the Starved Rock reach in July 2018 when only four fish (two males, two females) were caught. There was no significant difference in total length between males and females ($F_1 = 0.05$, $P = 0.83$) or between sampling sites ($F_1 = 0.89$, $P = 0.35$) in 2018, but in 2017, total lengths were significantly different between sampling sites, with Marseilles fish being significantly longer ($F_1 = 20.54$, $P < 0.001$, Table 1). Mass was different between sampling sites in 2017 with fish from the Marseilles Reach weighing 61% more than fish from the La Grange Reach ($F_1 = 40.97$, $P < 0.001$), and in 2018 with fish from the Starved Rock Reach weighing an average of 11% more than fish from the core in the La Grange Reach ($F_1 = 4.20$, $P < 0.05$), but there was no difference between sexes in either year (2017: $F_1 = 2.27$, $P = 0.14$; 2018: $F_1 = 0.95$, $P = 0.33$, Table 1). Ages were not significantly different between La Grange and Marseilles Reaches in 2017 (females: $F_{1,25} = 2.58$, $P = 0.12$; males: $F_{1,25} = 0.04$, $P = 0.96$) or between the La Grange and Starved Rock Reaches in 2018 (females: $F_{1,56} = 0.001$, $P = 0.98$; males: $F_{1,52} = 0.08$, $P = 0.77$).

The intra-assay variation for the ELISA kits was 5.89% for E_2, 2.95% for 11KT, 3.36% for *T*, and 2.07% for MIS. The inter-assay variation was 3.54% for E_2, 2.41% for 11KT, 3.87% for *T*, and 7.72% for MIS.

Female reproduction

In 2018, gonadosomatic index (GSI) varied significantly between months ($F_6 = 11.27$, $P < 0.001$), but not across sites ($F_1 = 1.11$, $P = 0.30$), with female GSI in May and June being significantly higher than any other month (Figure 2A). The interaction effect between month and sampling site was not significant ($F_6 = 1.48$, $P = 0.20$).

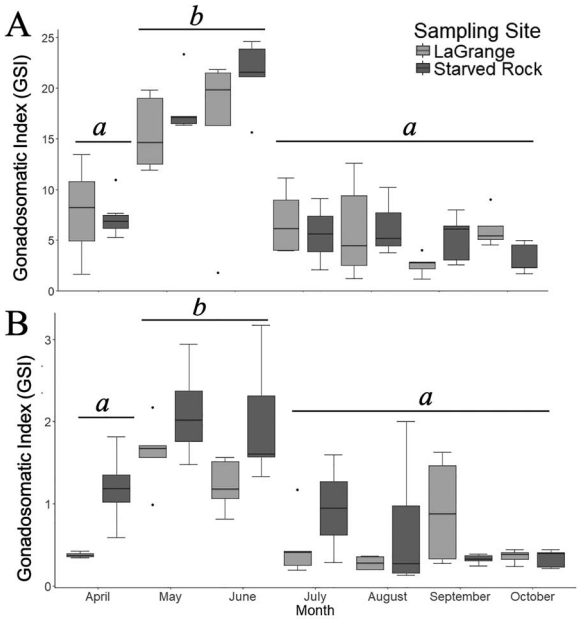

Figure 2. GSI in 2018 by month and sampling site. (A) Female and, (B) male GSI varied significantly between months (ANOVA, $P < 0.001$), but not between sampling sites. The interaction effect between month and sampling size was not significant. "a" and "b" denote months that were statistically different from each other based on Tukey post-hoc test. Females, $n = 82$; Males, $n = 81$.

In 2017, the proportion of females at each ovarian stage differed between the 3 months in the La Grange Reach, as all females caught in the La Grange Reach in September 2017 were inactive (Fisher exact test, $P < 0.05$, Figure 3A). The proportions were not

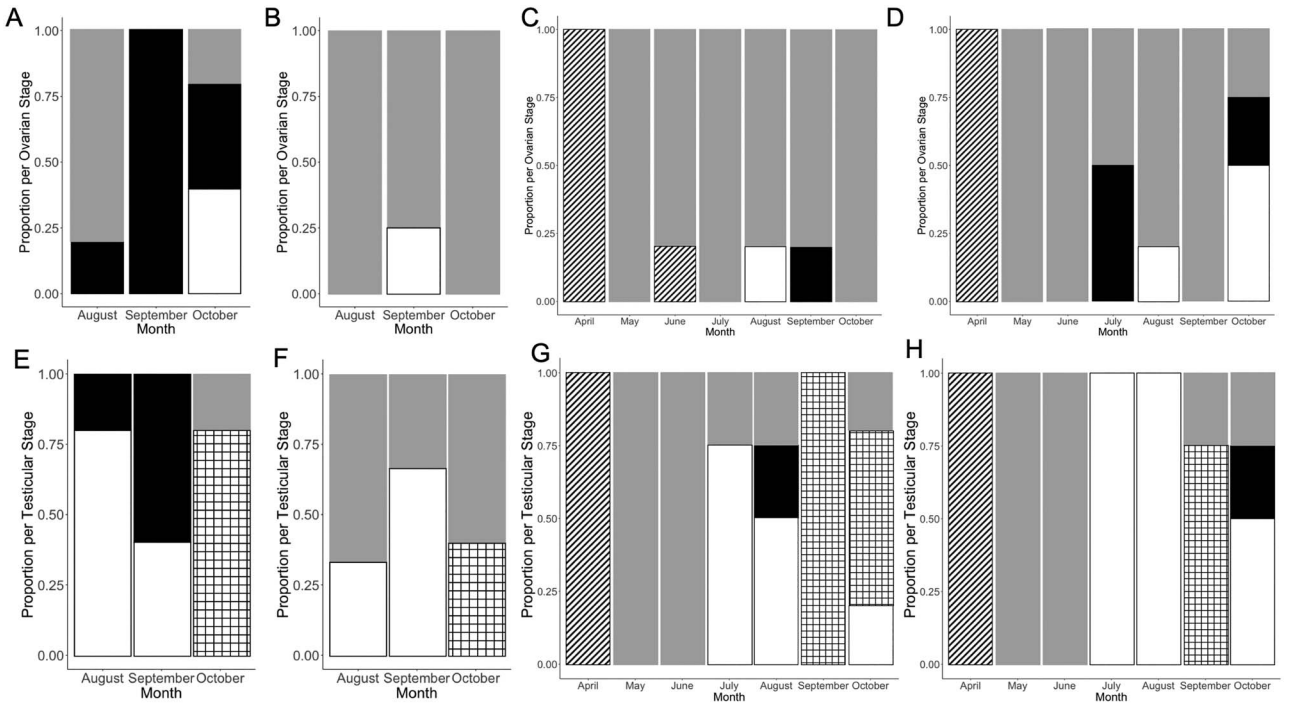

Figure 3. Gonadal stages of silver carp. Ovaries were staged using the criteria provided in Brown-Peterson et al. [12]. (A) Females from the La Grange Reach in August–October 2017; (B) females from the Marseilles Reach in August–October 2017; (C) females from the La Grange Reach in April–October 2018; (D) females from the Starved Rock Reach in April–October 2018. 2017, $n = 26$ females; 2018, $n = 64$ females. (E) Males from the La Grange Reach in August–October 2017; (F) males from the Marseilles Reach in August–October 2017; (G) males from the La Grange Reach in April–October 2018; (H) males from the Starved Rock Reach in April–October 2018.

different across months in the Marseilles Reach in 2017 (Fisher exact test, $P = 0.55$, Figure 3B), where most females were in the spawning-capable stage, identified by determining the most advanced oocyte stage present as described in Brown-Peterson et al. [12]. In 2018, the proportions of females at different stages of ovarian development differed across April through October in both the La Grange Reach and in the Starved Rock Reach (Fisher exact test, La Grange: $P = 0.003$, Starved Rock: $P < 0.001$, Figure 3C and D). Spawning-capable females were found in both sites during every month except April, in which all females from both sampling sites had a large number of atretic follicles accompanied by developing primary growth oocytes (Figure 4A). Mature tertiary vitellogenic oocytes increased during the months of May and June in both sampling sites (Figure 4B). POFs were observed in ovaries that were collected in May, June, and July (Figure 4C). Additionally, melanomacrophage centers (MMC; Figure 4D) were present in ovaries collected in all months except May and June. Although less numerous, mature tertiary vitellogenic oocytes were present in the ovaries of silver carp through October. Inactive fish were found sporadically (Figure 4E), and females in the regressing stage were found in August and October (Figure 4F).

Differences in E_2 across months in 2018 were statistically significant ($F_6 = 2.38$, $P < 0.05$, Figure 5A), with plasma concentrations of E_2 being 711% higher in April relative to October ($P < 0.05$). For E_2, sampling site ($F_1 = 0.38$, $P = 0.54$) and the interaction effect between month and site ($F_5 = 2.05$, $P = 0.09$) were not significant (average E_2 245.39 pg/mL, SD ±324.10 pg/mL). Many females had MIS levels below the level of sensitivity for the ELISA assay in April, but from May to October MIS levels were stable at between 10 and 11 pg/mL (10.90 pg/mL, SD ±5.90 pg/mL). Levels did not vary across months ($F_6 = 0.52$, $P = 0.79$) or sampling sites ($F_1 = 2.47$, $P = 0.0.13$, Figure 5B), and the interaction effect between month and site was not significant ($F_5 = 0.59$, $P = 0.71$). T levels were higher in May and June relative to August and October ($F_6 = 4.46$, $P < 0.01$, Figure 5C) but did not vary across sites ($F_1 = 0.04$, $P = 0.84$), and the interaction effect between month and site was not significant ($F_5 = 0.83$, $P = 0.53$) (average T 549.11 pg/mL, SD ±520.67 pg/mL).

Male reproduction

In males in 2018, GSI varied significantly between months ($F_6 = 13.91$, $P < 0.001$, Figure 2B), with GSI rising from 0.84% in April to 1.84% in May and 1.61% in June. There was no significant difference in GSI across sampling site ($F_1 = 2.48$, $P = 0.12$) or the interaction effect between month and sampling site ($F_6 = 2.10$, $P = 0.07$, Figure 2B).

Testicular stages in male silver carp from the La Grange Reach in 2017 differed significantly across months, as inactive and regressing males were caught in August and September 2017, but by October 2017, the majority of La Grange males had regenerating testes, though one spawning-capable male was also caught during that month in the La Grange Reach (Fisher exact test, $P = 0.003$, Figure 3E). In the Marseilles Reach in 2017, there was not a significant difference across months (Fisher exact test, $P = 0.40$), with males in the regressing and spawning capable stages making up the bulk of the sample in that reach (Figure 3F). In 2018, the proportion of males in various testicular stages from both the La Grange Reach and the Starved Rock Reach varied significantly across months (Fisher exact test, La Grange: $P < 0.001$, Starved Rock, $P < 0.001$, Figure 3G and H). In April, the testes of males

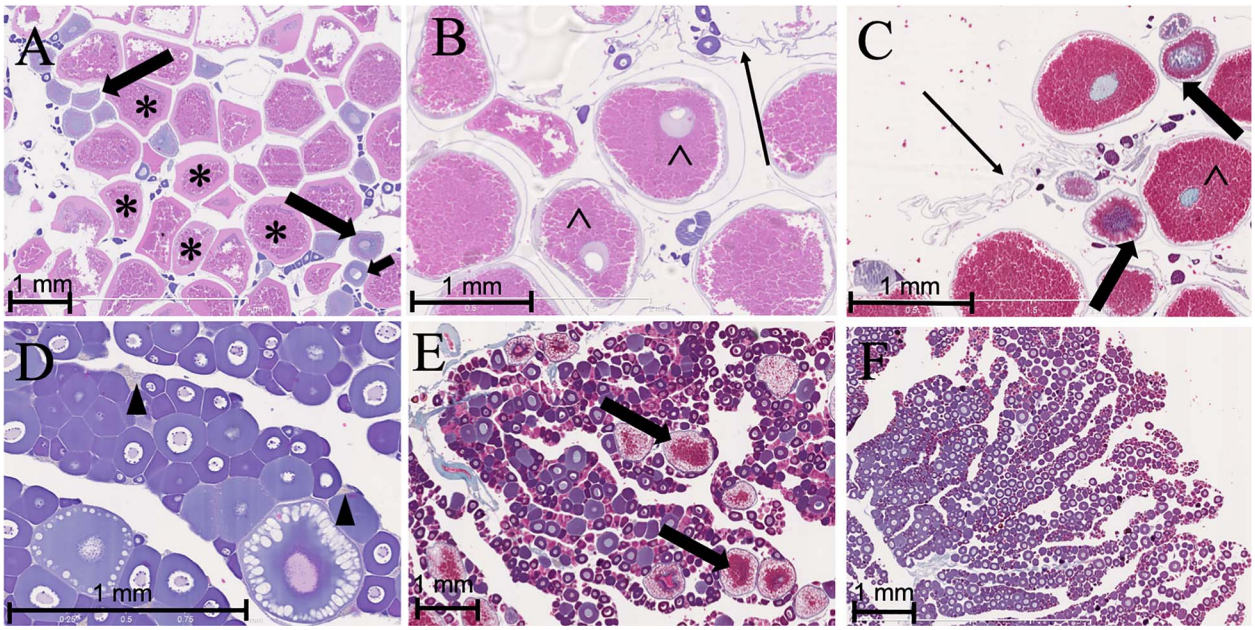

Figure 4. Ovarian histology of female silver carp. (A) Developing ovary with mass atresia (*) from a female silver carp caught in April. Cortical alveolar (thick short arrow) and primary vitellogenic oocytes (thick long arrows) were also present. (B) Spawning capable ovary from June. Tertiary vitellogenic oocytes (^) with evidence of germinal vesicle migration. Post-POFs (thin arrow) are visible at the top of the image (H + E). (C) POFs (thin arrow) among maturing (^) and vitellogenic (thick long arrow) oocytes in an ovary from May (D) MMCs (arrow heads) in a developing ovary. (E) Regressing ovary from a female caught in September. Primary growth oocytes are the predominant oocyte type, along with a few vitellogenic oocytes (thick long arrow). (F) Inactive ovary showing only primary growth oocytes. Hematoxylin + Eosin: A, B, D; Gomori's Trichrome: C, E, F.

from both the upstream and downstream location were in the developing stage and undergoing active spermatogenesis, as evidenced by spermatocysts at all stages of spermatogenesis and the presence of positive PCNA staining (Figure 6A). Spermatozoa were not present in the lumens or sperm ducts. Spawning-capable males were caught primarily in May and June. Testes of males caught in May showed active spermatogenesis as well as spermiation, with spermatozoa present in the lumens (Figure 6B). In June, spermatogenic activity had decreased in favor of spermiation, with very large sperm reserves present in the testicular lumens (Figure 6C). By July, the lumens contained spermatozoa, but spermatocysts contained only spermatogonia and no spawning-capable males were caught. From August through October, residual spermatozoa were still present in the testicular lumens and sperm ducts, but the number of spawning-capable males represented less than 25% of all males during these months. Spermatocysts in the latter stages of spermatogenesis were dispersed throughout the testis, but non-proliferative spermatogonia represented the majority of cell types in the tissue (Figure 6D). MMCs were found in fish from both sites during all months except June (Figure 6E).

Plasma concentrations of 11KT in both sampling sites in 2018 were highest from April through June and declined significantly starting in July, reaching the lowest levels in October ($F_6 = 9.08$, $P < 0.0001$, Figure 5D) (average 11KT 643.86 pg/mL, SD ±346.60 pg/mL), corresponding to a reduction in the proportion of spawning-capable males sampled from July to October (Figure 5C and D), and across all months, males from the La Grange Reach had higher plasma 11KT concentrations than those from the Starved Rock Reach ($F_1 = 7.68$, $P < 0.01$). The interaction effect between month and site ($F_5 = 1.55$, $P = 0.20$) on 11KT was not significant. MIS levels were one-third lower from April through July than from August until October ($F_5 = 6.30$, $P < 0.01$, Figure 5F), but there was no significant difference between sampling sites ($F_1 = 0.11$, $P = 0.75$) (average MIS 2.92 pg/mL, SD ±1.96 pg/mL). MIS concentrations in August were different between reaches, with males in the Starved Rock Reach having three-times higher MIS levels than males in the La Grange Reach (Figure 5G). ($P < 0.05$), but the interaction effect was not significant in other months. Concentrations of T were approximately 2.5 times higher in May and June than from July through October ($F_6 = 5.84$, $P < 0.001$, Figure 5H), and there was a significant difference between sampling sites across all months with males from the La Grange having higher testosterone than males from the Starved Rock Reach ($F_1 = 8.03$, $P < 0.01$, Figure 5I), but the interaction effect between month and site was not significant ($F_5 = 1.52$, $P = 0.20$) (average T 688.36 pg/mL, SD ±669.16 pg/mL).

Discussion

Results from this study provide evidence that silver carp from 'edge' and 'core' populations in the Illinois River do not differ in reproductive traits. As an expanding population, silver carp closer to the invasion front could be expected to have higher reproductive investment, indicated by higher GSI values. While GSI in 'edge' fish from the Starved Rock Reach was slightly higher than GSI in 'core' fish from the La Grange Reach in May and June for both males and females, this difference was not statistically significant. Additionally, GSI for female silver carp in this study reached 23% in some individuals, which is higher than the previously reported maximum values for the lower Illinois River (13%, [67]), Missouri River (13%, [40]), and Mississippi River (14%, [46]). Male GSI values are rarely

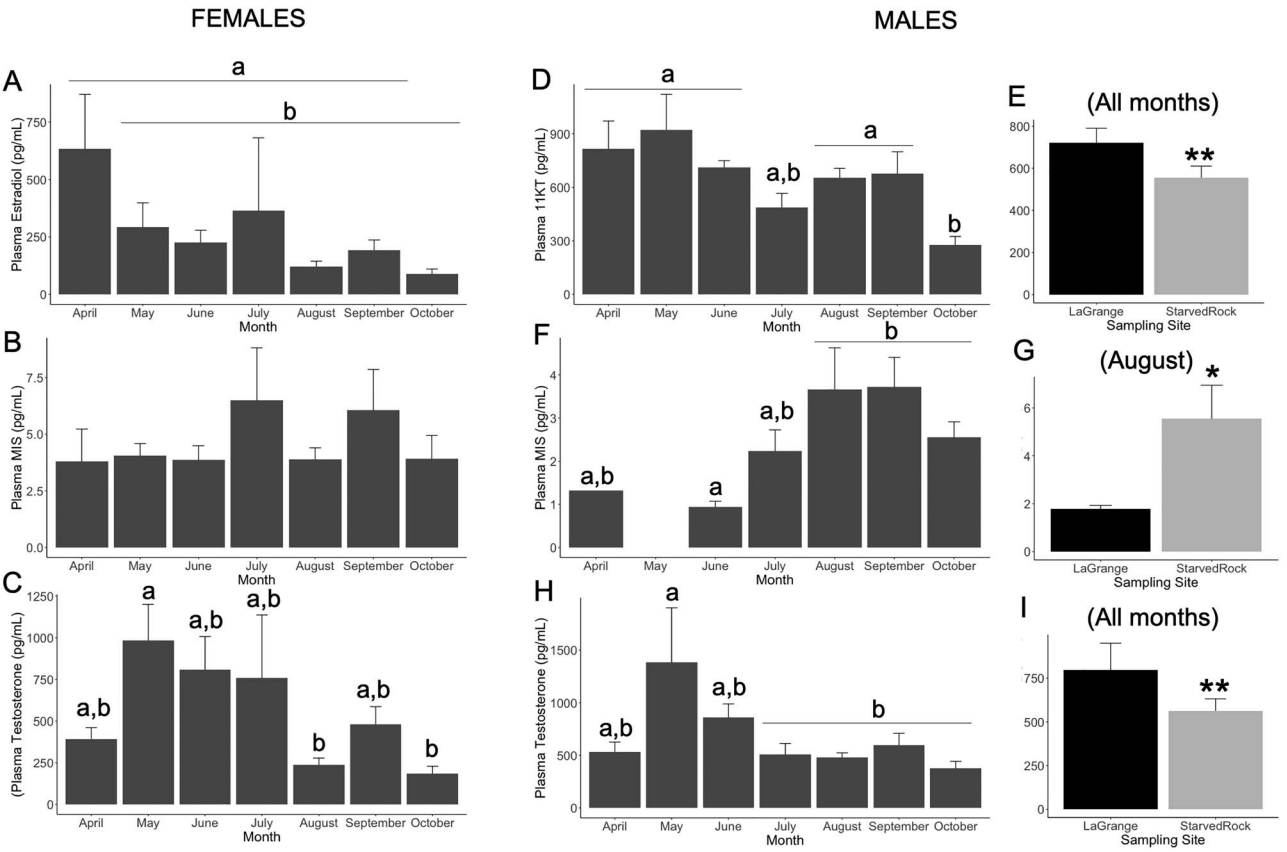

Figure 5. Plasma hormone concentrations across months (2018) in silver carp. All fish were sampled between April and October 2018 from the La Grange and Starved Rock Reaches. (A) Estradiol (E2) in females between months (B) MIS in females between months (C) Testosterone (T) in females from both sampling sites between months (D) 11 keto-testosterone (11KT) in males from both sampling sites between months; (E) 11KT in males from all months between sampling sites (F) MIS in males from both sampling sites across all months; (G) MIS in males from August 2018 between sampling sites; (H) T in males from both sampling sites between months; (I) T in males from all months between sampling sites. All statistics were analyzed using two-way ANOVA and Tukey post-hoc test. Lower-case letters denote groups that were statistically significant from each other based on Tukey post-hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$. Females: estradiol, $n = 64$; MIS, $n = 47$; testosterone, $n = 53$. Males: 11 keto-testosterone, $n = 56$; MIS, $n = 28$; testosterone, $n = 56$.

reported, but Papoulias et al. [40] reported a maximum male GSI in the Missouri River of 1.8%, which is lower than the maximum male GSI of 3.0%. Month was a much more significant driver of differences in reproductive status for both sexes, supported by morphometric data (highest GSI in May and June), hormone data (highest androgen concentrations in May and June for both sexes), and histological data (mature gametes represented majority of testicular and ovarian tissue in May and June). This suggests that the plasticity of reproduction in silver carp between native and introduced ranges that has been noted in previous studies [40, 46, 47, 67, 68] is likely a product of suitable environmental conditions rather than edge/core dynamics or differences in resources between upstream and downstream locations. Furthermore, average age estimates were not different between the locations in either year, suggesting that reproductively mature individuals within a range of ages are present at the Marseilles, Starved Rock, and La Grange Reaches. At all sampling sites, we observed reproductively mature individuals as young as 2 years, though this could be an underestimate due to the use of scales [55]. Other researchers have also documented wide variation in silver carp spawning across years, further supporting the importance of environmental conditions to spawning in this species. For example, DeGrandchamp et al. [67] saw an increase in GSI but a decrease in silver carp larvae corresponding to a drought. The exact environmental requirements for spawning in silver carp remains a mystery and requires further study, though hydrograph, temperature, and turbidity have all been proposed as predictive factors [68, 69]. Lack of an edge-core difference in spawning habits between silver carp in the Starved Rock Reach and La Grange Reach indicates that 'edge' fish are not disproportionately contributing to recruitment, as landscape-scale environmental conditions are the main driver of spawning activity in this species.

This study also supports the hypothesis that female silver carp are batch spawners, meaning that a single female can release mature gametes more than once during the spawning season [12]. There are a number of evolutionary advantages that result from a batch spawning strategy, including increased diversity of potential mates [5] and faster recovery from failed spawning events [8]. It is likely that female silver carp sampled during the current study spawned multiple times over the course of their peak spawning activity in May and June, evidenced by co-occurrence of mature vitellogenic oocytes and POFs in the ovary through July. Although the lifetime of POFs in the silver carp ovary has not been established, the presence of these structures in the ovary is considered evidence of recent spawning [10]. This is consistent with previous reports of eggs and larvae being collected from May through July in the Illinois River in 2016–2017 [70]. Our results also support the evidence provided by Konradt

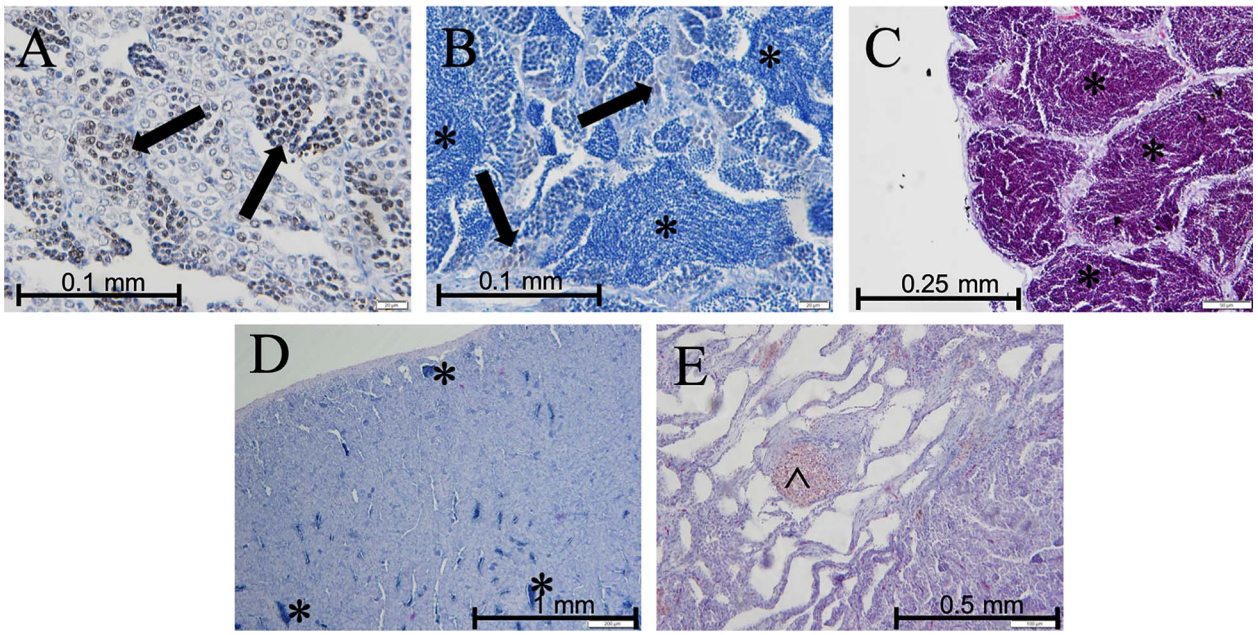

Figure 6. Testicular histology of male silver carp. (A) Developing testis from a male silver carp caught in April. Active spermatogenesis is evident by the many different cell types present, as well as positive PCNA staining (thick long arrows), which means the cells are proliferating. (B) Spawning-capable testis from male silver carp caught in May. Spermatogenesis is still active (PCNA, thick long arrow), but the lumens contain spermatozoa (*). (C) Spawning-capable testis from male silver carp caught in June. Spermatogenesis has ceased and the lumens are full of spermatozoa (*). (D) Regressing testis from a male caught in October. Some residual spermatozoa are present in the lumens (*), but the majority of the testis is comprised of non-proliferative spermatogonia. (E) Large MMC (^) in the sperm duct of a regressing testis. Immunohistochemistry (PCNA): A, B; Gomori's Trichrome: C, E; Hematoxylin + Eosin: D.

[39] and Williamson and Garvey [46] that female silver carp ovaries utilize indeterminate asynchronous oocyte development. Secondary growth oocytes developed from primary growth and cortical alveolar oocytes continuously from April through September, which is characteristic of indeterminate oocyte development [5]. This is in contrast to teleosts with determinate oocyte development, in which only a set number of cortical alveolar oocytes will develop into mature oocytes over the course of the spawning season [71]. Asynchronous oocyte growth is suggested by the fact that oocytes of multiple oocyte stages were present in the ovary at one time, as opposed to synchronous oocyte growth in which only one to two stages can be present at any given time [72]. Asynchronous indeterminate ovaries are advantageous when successful reproduction is uncertain, as new batches of mature oocytes can develop more quickly in the event of failed spawning [73], such as the mass atresia observed in April 2018. The ability of female silver carp ovaries to overcome failed spawning events using indeterminate asynchronous oocyte development has likely contributed to the success of silver carp as an invasive species.

Maintenance of high levels of plasma testosterone could explain the ability of indeterminate spawning females to have multiple oocyte populations developing at the same time. While testosterone concentrations reached a peak of about 2500 pg/mL in May and June in females, the overall concentration of testosterone in plasma never dropped below 200 pg/mL. Testosterone is an important androgen in both male and female fish. It is a precursor to both 11KT in males and E_2 in females, and as such it can increase prior to the onset of spermatogenesis and oogenesis [74]. Testosterone levels fluctuate as it is aromatized into 11KT or E2 in the gonads during gametogenesis. Testosterone in many teleosts rises prior to spawning, though the exact role of testosterone in initiation of spawning is unknown [74]. High levels of testosterone can be indicative of either E_2/11KT synthesis or spawning initiation, so plasma testosterone must be interpreted in conjunction with other hormone levels and with development of the gonads. In female fish with asynchronous ovaries specifically, high testosterone levels could be responsible for the ability to maintain multiple stages of oogenesis at once, as testosterone may be used directly for spawning initiation, and for E_2 synthesis, which is important in primary and secondary oocyte growth [74]. The three-spined stickleback (*Gasterosteus aculeatus*) is an indeterminate batch spawner that also displayed high plasma testosterone concentrations during their extended breeding season [75]. In the iteroparous rainbow trout (*Onchorhynchus mykiss*), testosterone peaks during vitellogenesis and declines during the spawning season [76]. The specific role of testosterone in female fish is not well understood, but other androgens are thought to be important, as well. While we did not measure 11KT in the female silver carp in this study, previous studies have shown that this non-aromatizable androgen might play a role in primary oocyte development in *Anguilla* spp. [77], coho salmon (*Oncorhynchus ksutch*, [78]), hapuku (*Polyprion oxygeneios*, [79]), Atlantic cod (*Gadus morhua*, [80]), and saithe (*Pollachius virens*, [81]). Studying the role of 11KT in female silver carp will likely require sampling during the winter before the onset of vitellogenesis to adequately understand the potential role of 11KT in primary oocyte growth in this species. In general, the role of androgens in female fish requires further study.

Male silver carp do not regenerate gamete stores throughout the year, and therefore have a more compressed spawning window relative to females. Though we did not measure E2 in male silver carp in this study, E2 has been shown to play a role in spermatogonial proliferation in male fish, such as in the Japonese eel (*Anguilla japonica*, [82]) and the three spot wrasse (*Halichoeres trimaculatus*,

[83]). As with 11KT in females, examining the potential role of E2 on male silver carp reproduction will likely require sampling earlier, such as during the winter, to assess the role of this hormone in early spermatogonial proliferation. Histological data suggest that spermatogenesis in male silver carp occurs primarily in the spring (April–June), and a large reserve of spermatozoa are released into the lobular lumens and sperm ducts through May. By June, almost the entire volume of the testis is comprised of spermatozoa. This reserve is then depleted as males release spermatozoa during spawning bouts until no more spermatozoa remain, as occurred in males between July and October in this study. This pattern of spermatogenesis is common in seasonal breeders, including some cyprinids such as the tench (*Tinca tinca*) [84]. These histological data were supported by levels of plasma 11KT sampled across months. 11KT is the potent androgen of teleost fishes [74], and it is considered to be the main regulator of spermatogenesis in fish, though the exact mechanisms behind how it exerts its action beyond spermatogonial proliferation are unclear [85, 86]. Male silver carp in our study had significantly higher plasma 11KT levels from April through June, when histological sections revealed active spermatogenesis. Males in the La Grange Reach also had higher plasma concentrations of 11KT overall, when adjusted for body mass. While this could indicate that La Grange males exhibit more active spermatogenesis than Marseilles males, future studies using shorter sampling intervals would be needed to confirm this hypothesis. Though 11KT was still present in the plasma through October, declining concentrations corresponded to a reduction in spermatogenesis in the testes. Atlantic cod (*Gadus morhua*), which displays similar spermatogenetic patterns to the silver carp, also shows increased 11KT levels during spermiation followed by a gradual decrease during the spawning season [87]. In contrast to female silver carp, male carp produce gametes in a determinate manner, with the total number of spermatozoa determined by the height of the spawning season. This strategy necessitates that males likely employ some form of decision-making to allocate their sperm in the most advantageous manner. Previous work in other fish species has shown that males can adjust the amount of sperm they ejaculate during spawning based on the size of the female(s) and the level of intraspecific sperm competition [88, 89]. Much of the sperm reserve in silver carp is likely used during mass spawning events, such as the one that likely occurred over May and June, due to higher sperm competition with other males that also have large sperm reserves at that time. Altogether, male silver carp implement a different pattern of gamete maturation than female silver carp.

While only 3 months of data were available for 2017, patterns observed in gonadal development and regression in August–October of 2017 were similar to those observed in August–October of 2018. Higher numbers of females in the spawning capable stage were caught in the fall of both years relative to spawning capable males. Male silver carp with regressing gonads were frequent after August, especially in 2018. The discrepancy between the timing of gonadal regression in male and female silver carp is likely due to differences between the sexes in gamete development style. Female silver carp are able to grow new oocytes toward the end of the spawning season, but males must allocate a determinate number of spermatozoa starting in the early summer. If a male silver carp engages in multiple spawning bouts throughout the summer and does not have a large reserve for additional spawning in the fall, his testes will regress. Some male silver carp with spawning capable testes were caught in the fall in both 2017 and 2018, suggesting that at least some individuals are able to maintain their sperm stores and continue spawning later in the year.

In April 2018, ovaries contained a large percentage of atretic mature oocytes, while testes showed signs of advanced spermatogenesis but without spermiation. Spermiation in fish is used to refer to the release of spermatozoa into the testicular lumens and sperm ducts and it is highly responsive to proximate environmental conditions such as temperature and photoperiod [86]. Lack of spermiation in addition to the presence of mass atresia in the ovaries suggests that environmental conditions might have prevented an anticipated spawning event in both the La Grange reach and the Starved Rock reach. Higher plasma E_2 levels in females in April, while not statistically significant, also supported the notion that female silver carp were actively engaging in vitellogenesis to replace atretic oocytes [74]. While more research is needed to determine how the environment might influence silver carp to spawn vs. resorb their gametes, there is a possibility of at least three spawning events (spring, summer, and fall) for silver carp in Illinois when conditions are appropriate.

MIS, sometimes called 17,20beta-dihydroxypregn-4-en-3-one, is the main progestin in most teleosts [74]. In both females and males, it is responsible for final maturation of the gametes. Though the mechanisms behind the switch from oocyte growth to oocyte maturation in fish are not known, a decrease in E_2 from granulosa cells and a spike in luteinizing hormone from the adenohypophysis induces production of MIS by the granulosa cells, resulting in a cascade of events leading to migration and breakdown of the germinal vesicle, oocyte hydration, and breakdown of the zona pellucida [5]. In males, MIS is synthesized by Leydig cells and is thought to control spermiation [85]. In both male and female silver carp, MIS concentrations in the plasma were often below the sensitivity of the ELISA assay. It is likely that, because MIS acts locally on the gonad by non-genomic pathways [74], the peak in MIS that occurs prior to spawning can only be observed with a shorter sampling interval, such as weekly or daily sampling. For this reason, MIS is not a useful indicator of spawning status in silver carp.

Melanomacrophage centers were observed in both the ovaries and the testes of silver carp. MMC are aggregates of pigment-containing cells and macrophages that are found in fish, reptiles, and amphibians [90]. Though they are typically found in hematopoietic tissues such as the head kidney, spleen, and, in some species, the liver, MMC have also been noted to occur in both the fish ovary and testis [91–93]. MMCs are typically used as a biomarker for disease, toxic pollutants, and environmental degradation [90], but they have also been attributed to the process of ovarian atresia [93]. It is unclear whether MMCs were present in silver carp gonads as part of the normal reproductive cycle or due to poor health. MMCs were not observed in any gonads during the months of May and June, which were the peak of the spawning season. However, histological sections of both tissues contained very little stromal area, so MMC could be present but not detectable by histology during those months. Further study into the role of MMC in normal reproductive function of fishes could allow for the use of MMC as indicators of gametic atresia or poor health in both males and females.

In conclusion, silver carp in this study did not show signs of an edge-core effect on reproduction and all animals, regardless of collection location, were investing equally in reproductive output. Potential for additional spawning activity in the spring and fall in some years cannot be ruled out, due to mass atresia observed in the spring and the presence of spawning-capable individuals in the fall. Silver carp appear to adjust gamete development in response to environmental conditions, and high testosterone levels allow them to direct the regulatory process either toward gametogenesis or

toward initiation of spawning. While much remains to be elucidated about reproduction of silver carp, especially in relation to how environmental factors influence spawning, this study has provided several clues as to what makes this species such a successful invader.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgments

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