Southeastern Fishes Council Proceedings

Number 55

July 2015

Reproductive Timing of *Erimystax insignis* (Blotched Chub) in the Flint River of North Alabama

Bruce Stallsmith
University of Alabama - Huntsville, stallsb@uah.edu

Josh Mann
University of Alabama - Huntsville, jwm0011@uah.edu

Candice Allen
University of Alabama - Huntsville, C_shell76@hotmail.com

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Abstract
Important details of the reproductive ecology of many freshwater fishes of the species-rich southeastern United States are still poorly known. One such species is *Erimystax insignis* (Blotched Chub), whose range includes the Tennessee River drainage in northern Alabama, USA. To determine timing and patterns of reproductive effort, collections were made monthly of as many as 30 individuals from August 2011 through July 2012 from a 14 km stretch of the Flint River in Madison County, Alabama. Female and male gonadosomatic index (GSI) and ovarian development data indicate that reproductive activity for the species peaks from March through May. Ripe and mature oocytes were found in females from March through June peaking in April but ripe ovaries were found only in March and April. The number of mature and ripe oocytes was larger than reported for a population in the Little River of Tennessee, and diameter of all stages of maturing oocytes was smaller than the Little River population.

Keywords
GSI, ovarian development, Tennessee River, cyprinid

Cover Page Footnote
Thanks to Kelly Hodgeskins, Patricia Debow, and Toacca Taylor for help with fieldwork and laboratory analysis.

INTRODUCTION

The southern United States supports more native species of fishes than any other area of comparable size on the continent of North America north of Mexico (Warren et al., 2000). However, the area also has a high proportion of its fishes in need of conservation action (Burr and Mayden, 1992). Important aspects of the reproductive biology of many freshwater fishes of the species-rich southeastern US are still unknown. The range of many species has shrunk in response to the loss of suitable habitat due to waterway modification, urban expansion and agricultural use (Warren et al., 2000). Reproductive schedule is an important aspect of an organism's life history; therefore, knowledge of the schedule for reproduction of any species is a necessary component of any scientifically based conservation efforts.

The global conservation status of *Erimystax insignis* is G4, apparently stable (uncommon but not rare) in the system used by NatureServe (2015). However the status of the species in specific parts of its range is more threatened: the species is S1, critically imperiled in Kentucky; S2, imperiled in Alabama and Georgia; and S3, vulnerable in Tennessee and Virginia (NatureServe, 2015). The species has already been extirpated or possibly extirpated in three locations in its known range and warrants protection from habitat destruction which could lead to further extirpation, especially in Alabama at the southern edge of its range.

Only one study has evaluated the reproductive timing or potential reproductive output of this species. Harris (1986) studied the species in the Little River in Blount County, Tennessee, in the heart of its range 100 km to the north of the southern edge of its range, which is about a full degree of latitude. The species was found to have a two month peak of female gonadosomatic index (GSI) in April and May as a measure of reproductive condition, with water temperatures of 12–15º C and river discharge in decline from a winter peak. The life history of many North American freshwater minnow species has been found to vary from the effects of both phylogeny and latitude. Spawning season was found to be shorter for a given species at higher latitude among 21 species with data compiled from the literature (Gotelli and Pyron, 1991). Hubbs (1985) found similar temporal variation for darter species, and felt that photoperiod signaled the onset of spawning and rising water temperature the cessation of spawning.

The purpose of this study was to determine the reproductive schedule of *E. insignis* by examining gonadal condition; ovarian development; both oocyte number and size by stage; and monthly changes in gonadosomatic index (GSI) measurements for both males and females throughout a one-year study period in

the Flint River of north Alabama. Mean standard length data were taken for both males and females to characterize size at reproductive maturity. If the observations of Gotelli and Pyron (1991) and Hubbs (1995) are relevant to *E. insignis* it would be expected that the species' peak reproduction would be longer and earlier in a north Alabama river compared to a more northerly river. This peak reproduction should also be related to increasing water temperature and photoperiod, and declining river discharge from an annual peak in winter.

MATERIALS AND METHODS

Collection Site and Field Collection

Erimystax insignis prefers rocky riffles and runs of clear, small to medium rivers such as the Flint River in northeast Alabama, the study site for this project (Boschung and Mayden, 2004). The Flint River watershed encompasses approximately 141,640 hectares in Madison County, Alabama, and Lincoln County, Tennessee. Fish were collected monthly from August 2011 to July 2012. No collections were made in December 2011 and January 2012 because of sustained flood pulses in the river. All fish were collected from a 14 km stretch of the Flint River in the Tennessee River watershed in northern Alabama. Fish were collected from the Three Rivers site near Winchester Road (34º 49.360' N, 86º 28.982' W) in August, September and October 2011 as well as February, March, May, June and July 2012. In an effort to utilize more easily accessible sites, fish were collected near Mt. Carmel (34° 48.328' N, 86° 28.333' W) in November 2011 and near Oscar Patterson Rd. (34° 52.832' N, 86° 28.830' W) in April and July 2012 (Figure 1).

Between 14 and 30 adult fish were taken on each collection date using either a cast net (1.8 m radius, 12.7 mm mesh) or a seine net (3.5 m length, 3 mm mesh). No effort was made to collect fish based on size or gender. Fish collected were euthanized in MS-222 (tricaine methanesulfonate) and later transferred to a 10% phosphate buffered formalin solution for transport and storage before removal and dissection of gonadal tissue.

Length and Mass Data Collection

The standard length of each fish was measured to the nearest 0.01 mm using digital calipers in August through April. Standard length was measured (0.1 mm) with a standard metric ruler in May through July due to a malfunction of the digital calipers. Fish were weighed to the nearest 0.001 g, after removal of excess surface fluid by wrapping in a paper towel. Gonadal tissue was removed and stored in formalin until it could be dissected. The gonadal mass was later recorded to the nearest 0.0001 g after excess surface fluid was removed by wrapping the

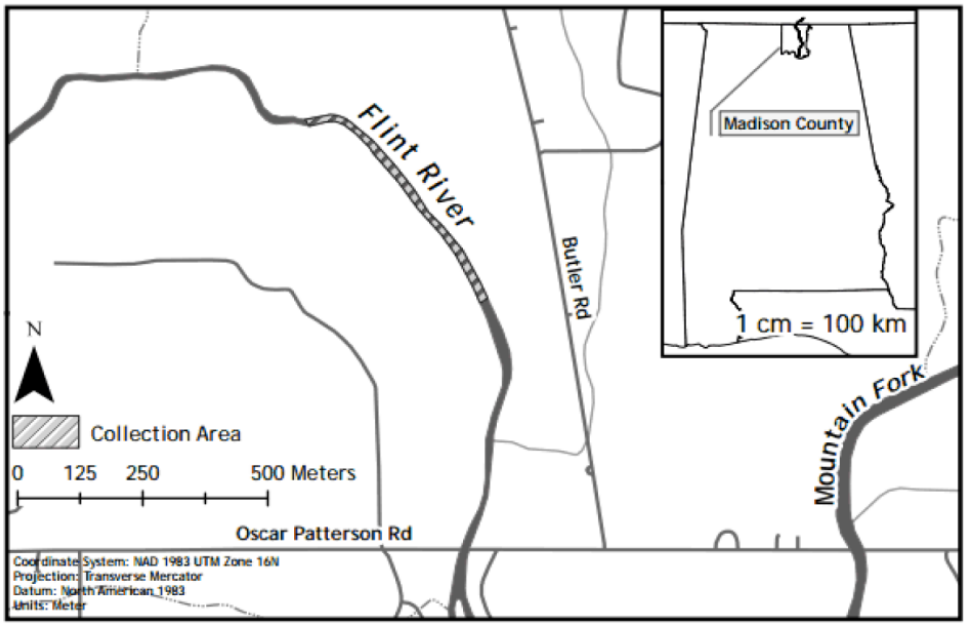

Figure 1. Map of the collecting site on the Flint River north of Oscar Patterson Road. Map courtesy of Benjamin Swan.

ovarian tissue in a paper towel. The gonadosomatic index (GSI) was calculated using the formula: (gonadal mass / (gross mass – gonadal mass)) X 100. An Ohaus Explorer balance was used for all mass data.

Collection of Reproductive Data

The status of gonadal development in all fish was visually determined by dissecting out intact gonads using an Olympus SZX7 dissecting microscope equipped with a 12 megapixel digital camera controlled with the cellSens software package. No attempt was made to examine testes beyond measuring mass. Ovaries were examined visually to assess developmental stage. If ovaries were removed intact, one ovary from each female fish was then teased apart to separate developing oocytes from surrounding ovarian tissue. The assumption was made that each ovary in an individual contained equal numbers of oocytes. If only one ovary was dissected, the number of oocytes in one ovary was multiplied by two to determine the total number of eggs per fish. Oocytes were arranged into a single layer the size of the image field. Digital photos were taken at a total magnification of 2.2x. As many as 15 images had to be taken to capture all of the oocytes in a single ovary.

Oocyte maturation stage was determined from captured images following a modification of the scheme used by Heins and Rabito (1986). The original

scheme divided oocytes into five stages: latent, early maturing, late maturing, mature and ripe. The very small latent oocytes were not measured in this study. Early maturing (stage 1) oocytes are relatively small, less than half the diameter of a mature oocyte, usually white with the nucleus obscured by yolk disposition. Late maturing (stage 2) oocytes are greater than half the diameter of a mature oocyte, more opaque and usually cream in color. Mature (stage 3) oocytes are larger than maturing oocytes, cream or yellow in color, with visible oil droplets and the vitelline membranes visibly separated from the yolk. Ripe (stage 4) oocytes are visibly larger than mature oocytes, yellow or dark yellow brown in color with the vitelline membranes well separated from a smaller yolk mass.

All oocytes that had reached at least the early maturing stage were counted and grouped according to stage using the Count & Measure Solution module available on the cellSens software package. This process marks and counts oocytes as an examiner makes stage determinations. Oocyte counts were verified by independent observers. Ovarian developmental condition was also determined from captured images. Any disagreements were resolved by reassessments until a consensus was reached.

Ovarian condition was assessed using a similar modification of the scheme also used by Heins and Rabito (1986). Early maturing (stage 1) ovaries are small in size, usually opaque, and dirty white in color. Late maturing (stage 2) are larger and expand to take up a large fraction of the body cavity, opaque and dirty white to cream in color. Mature (stage 3) ovaries are usually large enough to distend the abdomen, opaque and cream to yellow in color. Ripe (stage 4) ovaries have ripe ova ovulated and concentrated in the posterior portion of the ovary.

Once the developmental stage of all oocytes had been established, diameter measurements were taken of five oocytes per developmental stage present on each captured image using a tool available on the cellSens software. All measurement data were entered into an Excel spreadsheet in order to obtain monthly averages and averages by gender for each.

Physical Environmental Parameters

Water temperature (°C) was measured on each collection date using an alcohol thermometer. River discharge data for each collection date are reported as the mean of daily hourly values in cubic feet per second (cfs) from a gauge on the Flint River at Brownsboro, Alabama, 4–14 km downstream from the reported collecting sites. This is site USGS 035751000 of the National Water Information System (NWIS). Data were downloaded from the NWIS web site (U.S. Department of the Interior, U. S. Geological Survey, 2015). Photoperiod in hours

for each collection date was calculated using the online Daylight Hours Explorer with the collection site set as a latitude of 34.5º (UNL Astronomy). Photoperiod was also examined for this time period for the Little River in Tennessee at latitude 35.6° to determine if there was a difference in photoperiod between the collection site of Harris (1986) and the current study.

Data Analysis

Total monthly combined oocyte numbers for stages 3 and 4 were tested for any significant differences between months using one-way ANOVA and a post-hoc Tukey HSD test to pinpoint any significant pairwise differences between months. This was done with the online Statistica calculator using the algorithm of Gleason (1999) (Vasavada 2014). The counts for stages 3 and 4 are the Mature and Ripe stages that have undergone vitellogenesis and are either close to being prepared or fully prepared for spawning and as such their combined number is a good indicator of near-term spawning competence. Monthly average GSI for males and females were also tested for any significant differences through the same one-way ANOVA with post-hoc Tukey HSD test to identify significant pairwise differences between months. All test results were evaluated at $\alpha = 0.05$.

RESULTS

Oocyte and Ovarian Development

Table 1 shows the number of fish examined per month, and for females the monthly average number and range of maturing oocytes if present. Maturing ovaries with maturing oocytes were found from February through June. The average total number of all oocytes found in ovaries in the period of developed ovaries of February through June was highest in February with 1130 and steadily decreased to 403 in June. Stage 1 and 2 oocytes were found in all five months

Table 1. Number of mature fish collected per month by sex, and for females in the months of February through June with maturing ovaries the average number of maturing oocytes (stages 1–4) per fish with range in parentheses.

Month	Males	Females	Avg. # oocytes
August	17	10	
September	8	9	
October	16	14	
November	9	17	
February	8	6	1130 (677–1335)
March	12	12	782 (512–1082)
April	6	14	619 (377–944)
May	19	9	507 (381–657)
June	24	6	403 (61–548)
July	16	7	

while stage 3 and 4 oocytes were found only from March through June. Average oocyte diameters by stage for the months of maturing ovaries, February through June, were as follows: stage 1, 0.186 mm; stage 2, 0.330 mm; stage 3, 0.569 mm; and stage 4, 0.740 mm.

Figure 2 shows monthly average totals of combined stage 3 and 4 oocytes for March through Junes when these stages were present. April is the peak month, followed by March and May. A one-way ANOVA was conducted on these four monthly size distributions to determine if significant differences existed between the months. This test yielded $F(3, 36) = 3.45$ and $p = 0.026$. A post-hoc Tukey HSD test found a significant difference between April and June ($p < 0.05$) but no other significant pair-wise monthly differences.

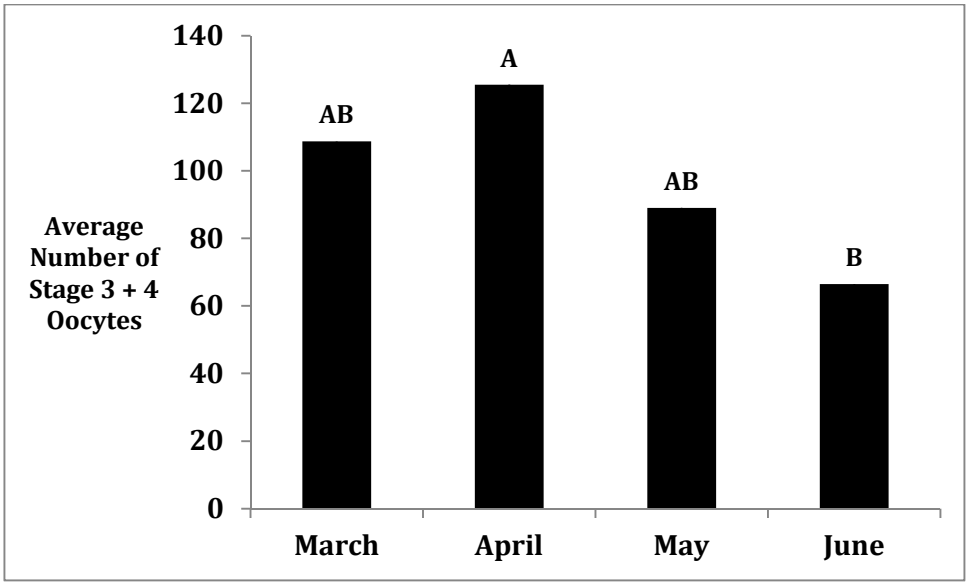

Figure 2. Monthly averages of combined total of stage 3 and stage 4 oocytes for March through June. Such vitellogenic stages are likely to be spawned within days. Letters above each bar represent groupings of similar months as determined by post-hoc Tukey HSD analysis following one-way ANOVA. April and June are significantly different from each other, $p < 0.05$, while March and May are not significantly differ from any other month analyzed.

Figure 3 depicts ovarian developmental stages by percent for months with observable development. Ovaries from August through November 2011 can be considered to be latent *sensu* Heins and Rabito (1986). Only Stage 1 ovaries were found in February. March and April were the only months with stage 4 ovaries found, with 25% of evaluated ovaries classified as stage 4 in each month.

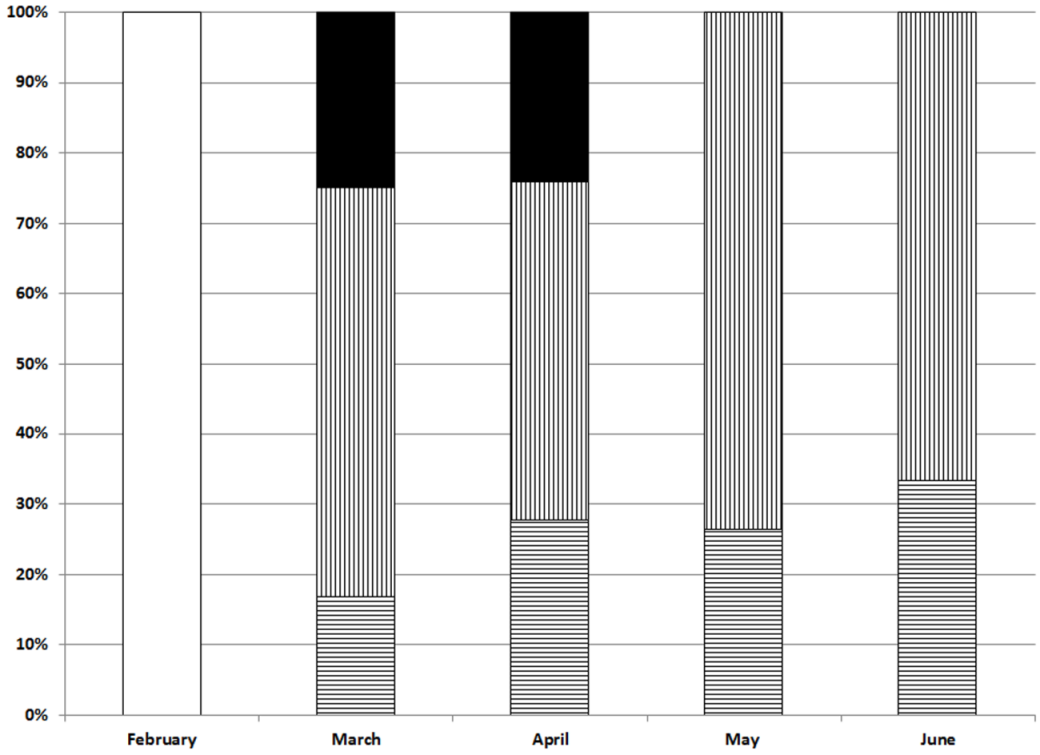

Figure 3. Ovarian developmental stages by percent for the months with observable development. White sections are stage 1, sections with horizontal lines are stage 2, sections with vertical lines are stage 3 and black sections are stage 4.

Monthly GSI Values

Monthly average GSI values for females are shown in Figure 4 and for males in Figure 5 along with clustering of months as indicated by post-hoc Tukey tests of ANOVA results between the ten months. Average GSI values for females peaked in March 2012 at 15.4. An ANOVA test of monthly average female GSI yielded $F(9,94)$ and $p < 0.001$, and a post-hoc Tukey test showed that female GSI values were statistically indistinguishable in the three months of largest GSI, March (15.4) through May (12.6), with a statistically significant drop in June to 7.7. Female GSI values were under 2 in August, September, October and November 2011 and July 2012. An increase in female GSI was observed in February 2012 preceding the sustained peak with a value of 6.2. An ANOVA test of monthly average male GSI yielded $F(9,125)$ and $p < 0.001$, and a post-hoc Tukey test showed that the largest male GSI values in the months March through June were statistically indistinguishable with a peak in April of 0.94.

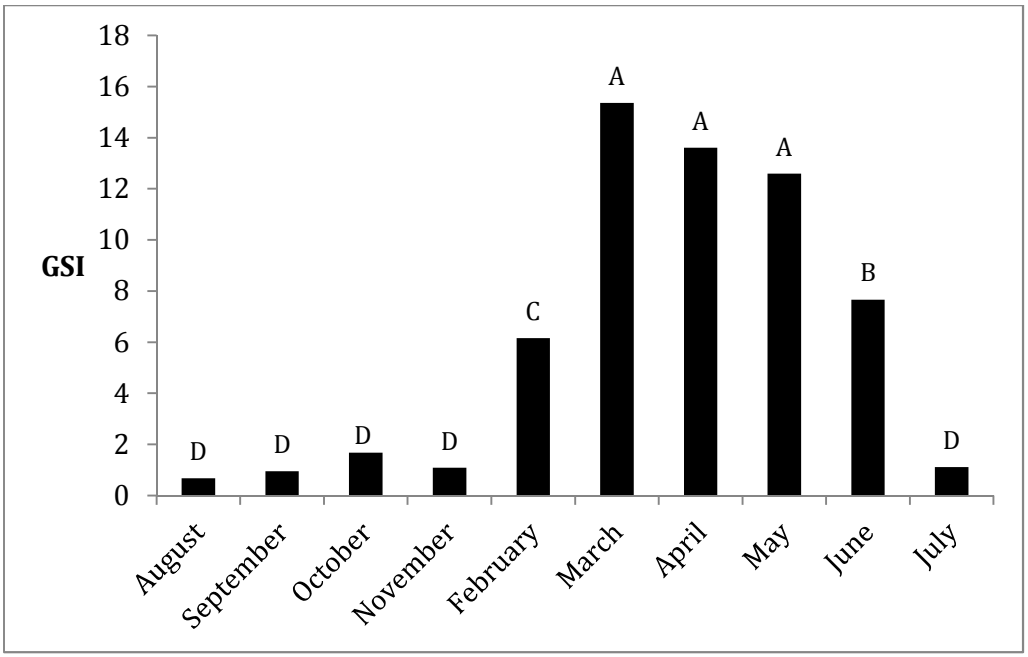

Figure 4. Monthly average gonadosomatic index (GSI) found in female fish. Letters above bar represent clusters of months that have significantly different means from other clusters as determined by one-way ANOVA and post-hoc Tukey tests.

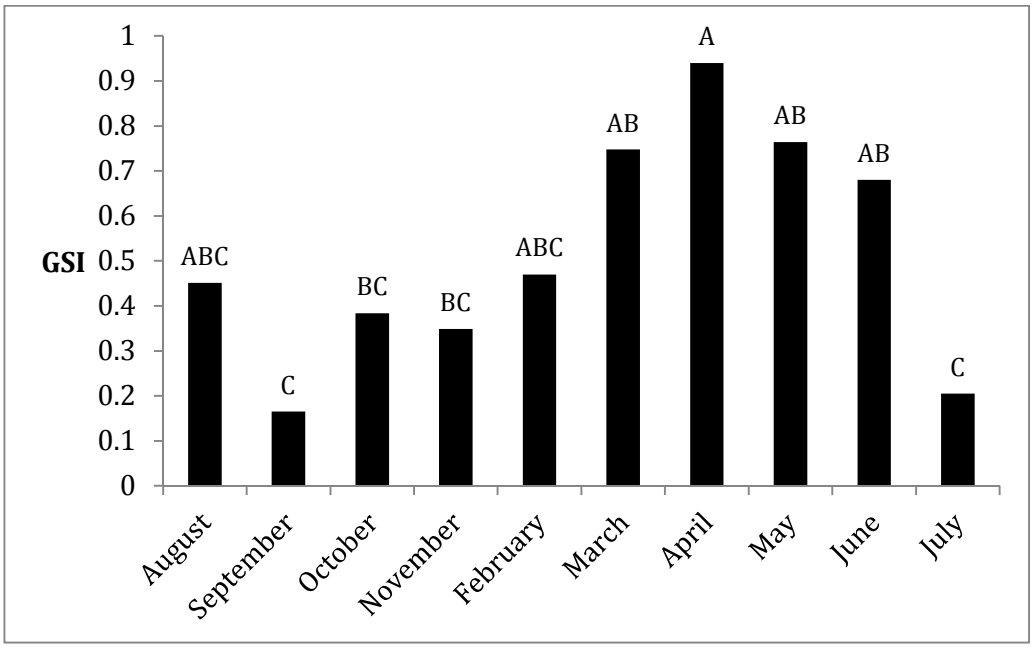

Figure 5. Monthly average gonadosomatic index (GSI) found in male fish. Letters above bar represent clusters of months that have significantly different means from other clusters as

determined by one-way ANOVA and post-hoc Tukey tests. Months with more than one letter are in two or three such clusters.

Water Temperature, River Volume and Photoperiod

Table 2 shows water temperature, river volume, and photoperiod for the days fish collections were made. Water temperature during the peak reproductive months of March through June ranged from 17°C in April to 24°C in June. Photoperiod increased from 12.1 hours in March to 14.2 hours in June. The calculated photoperiods for these days in the latitude of the Little River in Tennessee were identical. River volume throughout the observed time period was erratic depending as it does on rain events, but generally declined during the four month of peak reproductive capability from 369 to 152 cfs.

Table 2 Temperature, River Flow, and Photoperiod on Fish Collection Days.

Date	Temperature (ºC)	Flow (cfs)	Photoperiod (hours)
28 August 2011	24	96	12.9
25 September 2011	22	133	11.9
16 October 2011	19	133	11.1
9 November 2011	18	143	10.4
12 February 2012	12	602	10.7
22 March 2012	20	369	12.1
17 April 2012	17	201	13.0
10 May 2012	22	143	13.7
6 June 2012	24	152	14.2
16 July 2012	27	98	14.1

Mean Standard Length

The mean standard length of adult females was 61.5 mm with a range of 44.0–79.8 mm (n = 103). The mean standard length of males was 60.9 mm with a range of 46.3–80.0 mm (n = 134). The mean standard length of juveniles (caught only in June) was 32.0 mm with a range of 26–37 mm (n = 10).

DISCUSSION

The *E. insignis* population studied was found to spawn earlier and longer than observed by Harris (1986) and suggested by Boschung and Mayden (2004). Harris found the species' peak spawning condition in April and May at a study site further north in Tennessee. The difference between the Alabama results reported here and those of Harris (1986) for Tennessee are consistent with the findings of Gotelli and Pyron (1991) that more northerly populations of a species typically have a shorter spawning season, in this case two months versus the three months indicated by data for Alabama. The Tennessee population also initiated spawning a month later, April instead of March, at a lower water temperature, 12º

compared to 17–20º (April and March temperatures, this study). Photoperiod is the same at both sites so that does not seem to be the primary trigger for the initiation of spawning although it may well be a trigger in some synergistic fashion with rising water temperature (UNL Astronomy).

Based on the field observations of others, Boschung and Mayden (2004) infer that spawning in Alabama should occur from mid-April to late July or early August. This difference in observed spawning season could in part be attributed to the warm winter Alabama experienced in 2011–2012, as the February water temperature of 12°C seems to be adequate for inducing gonadal development as Harris found in Tennessee (1986) (Table 1, Figures 2 and 3). Data presented here show peak female ovarian development in March and April, peak female GSI in March through May, and peak male GSI in March through June (Figures 3–5).

Erimystax insignis differs from what has been found in other regional stream cyprinid species in peak spawning season. With peak spawning in March and April, *E. insignis* were relatively early spawners. Stallsmith et al. (2007) found a peak spawning season for both *Notropis asperifrons* and *Notropis stilbius* in the Sipsey River watershed of north Alabama from April to early July. Holmes et al. (2010) found a peak spawning season for *Notropis telescopus* in the Paint Rock River system of Alabama of April and May. *Notropis xaencocephalus* in the Etowah drainage of northern Georgia were found to have a narrow peak spawning season in April (Jolly and Powers, 2008). But current research (Kelly Hodgeskins, unpublished data) indicates the spawning season for a population of *Notropis photogenis,* also in the Flint River of Alabama, to be similar to that found in this study for *E. insignis*.

The oocytes of *E. insignis* found in this study were smaller at all stages than what has been reported both for *E. insignis* in Tennessee and other regional stream cyprinids, while the total number of oocytes was greater. Harris (1986) reported that mature ova of four examined *E. insignis* in the observed peak reproductive condition months of April and May had diameters of 1550 µm with 200–900 maturing oocytes in the ovaries, while in this study stage 3 oocytes had an average diameter of 569 µm and stage 4 oocytes were 740 µm and the peak reproductive months of March and April averaged 782 and 619 maturing oocytes, respectively. This compares to diameters of 1135 µm and 1430 µm, respectively, and just over 300 maturing oocytes for *N. telescopus* (Holmes et al., 2010), and 900 µm and 1300 µm, respectively, for *N. xaencocephalus* with 257 maturing oocytes (Jolly and Powers, 2008).

Why are the relatively small size and large number of mature oocytes found in this study so different from most other regional stream minnows? It is easier to explain why one might expect to find the opposite. Reproductive success of stream cyprinids is sensitive to fluctuations in stream discharge as well as water temperature, which are often negatively correlated (Nunn et al., 2003). Such variations in environmental parameters often affect the evolution of ovum size in populations of stream cyprinids, such that larger stream runoff correlates with larger ovum size possibly so that larger eggs and larvae have a better chance of survival in high flow. Such a relationship was found in *Cyprinella venusta* populations in the Pascagoula River system in Mississippi by Machado et al. (2002). The Flint River is a system with seasonally large runoff so one might expect a resident cyprinid like *E. insignis* to have relatively large ova, as was found in the Tennessee population observed by Harris (1986). It could be the case that the Flint River is subject to lesser high stream discharge events compared to other rivers. But the Flint River also supports a high diversity of fishes, 83 species (Boschung and Mayden, 2004) including numerous other cyprinid species. Another cyprinid in the Flint River, *N. photogenis*, has been found to be an early spawner like *E. insignis* and to carry as many as 10,000 maturing oocytes all less than 1000 µm in diameter (Kelly Hodgskins, unpublished data). *Notropis photogenis* is thought to be a broadcast spawner, while Harris (1986) observed apparent spawning in depressions downstream of small rocks in shallow water by *E. insignis* with no observation of whether ova produced were adherent or buoyant. The production of many small ova may be an adaptation for competing with other similar species in a high diversity community. Examination of the reproductive biology of other populations of this species may better explain this strategy of producing many small ova.

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