Reproductive biology of *Hybopsis amblops*, the Bigeye Chub, in the Flint River of Alabama

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Background. The purpose of this study was to establish a reproductive schedule and examine reproductive traits that shape fecundity of the Bigeye Chub, *Hybopsis amblops* Cyprinidae, in the Flint River system of north Alabama.

Methods. Life history traits associated with reproduction, growth, and maturation were assessed. Fish collections were made monthly from August, 2013, through July, 2014.

Results. The Bigeye Chub in Alabama primarily spawns in April and May as indicated by gonadosomatic index (GSI), ovarian condition and clutch size. Average GSI values began to rise in February, peaked in April and May at over 13% for females and 1.6% for males, and showed a steep decline from May to June for both sexes. Average clutch size was highest in April at 812. Diameter of the most mature oocyte stage averaged 0.74 mm, relatively small compared to other cyprinids found in the Flint River.

Discussion. The Bigeye Chub's relatively large clutch size as a measure of fecundity places the species intermediate between opportunistic and periodic in the trilateral life history scheme of Winemiller and Rose. The species is apparently responding to a flow regime with a defined seasonality as well as predictability of flow and resources.

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Abstract

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Introduction

Life history research is performed to study patterns of variation in key traits of the life cycle due to selection (Winemiller, 2005). These traits are associated with reproduction, growth/maturation, and survival, and are often defined as demographic factors such as size/age at sexual maturity, fecundity, clutch size, reproductive schedule, life span, senescence, and number of offspring. According to Stearns (1976), deviations in these strategies can emerge in response to intrinsic and extrinsic factors. Intrinsic factors involve the interactions of various traits, and lead to tradeoffs between them. While the level of fitness may decrease for one set of traits in response to extrinsic factors, it may increases for other traits.

Hybopsis amblops (Rafinesque), the Bigeye Chub, is a cyprinid minnow found in the Lake Ontario and Lake Erie drainages, Ohio River basin, and south to the Tennessee River drainage in northern Alabama. They are currently thought to be extirpated in the Missouri river drainage, and rarely found in segments of the Illinois River drainage. They also appear to be extirpated in the Kaskaskia and Wabash River drainages and branches of the Ohio River. The species is frequently collected over hard sand and gravel substrates in areas of low current. Bigeye Chubs attain a maximum standard length of 80 mm according to literature and anecdotally spawn during the late spring and early summer (Boschung and Mayden, 2004). This species is considered to be a lithophilic spawner, utilizing gravel and mineral substrates instead of simply releasing eggs into the sediment (Frimpong and Angermeier, 2013). Data concerning female fecundity could not be found in the literature but Frimpong and Angermeier (2013) suggest a value of 1000 eggs for maximum fecundity.

The purpose of this study was to establish a reproductive schedule and examine reproductive traits that shape fecundity of the Bigeye Chub in the Flint River system of north

Alabama, at the southern edge of its range. Life history traits associated with reproduction, growth, and maturation were assessed. Reproductive traits of North American stream cyprinids including timing, oocyte size and clutch size are known to be affected by water temperature and the volume of stream flow, so those data were collected for each monthly fish collection (Mims and Olden, 2013; Bennett et al., 2016). We also wished to compare this species to sympatric cyprinids in the Flint River which have been similarly studied including *Erimystax insignis* (Hubbs and Crowe), the Blotched Chub (Stallsmith et al., 2015), *Notropis photogenis* (Cope), the Silver Shiner (Hodgskins et al., 2016), and *Lythrurus fasciolaris* (Gilbert), the Scarlet Shiner (Stallsmith et al., unpublished manuscript). Finally, this information is essential to any conservation effort based on scientific data. The Flint River is currently relatively unaffected by anthropogenic pollution, but is at some risk as the city of Huntsville, Alabama, continues to expand west of the river.

Materials & Methods

Study site and sampling

The Flint River is 562 km long and drains approximately 141 640 hectares in Madison County, Alabama, and Lincoln County, Tennessee (Abidi et al., 2009). The Oscar Patterson Road bridge access (34˚48’24” N, 86˚28’21” W) on property owned by the Land Trust of North Alabama was the entry point used in this study (Fig. 1). The Flint River is free flowing with varying levels of discharge. Mean monthly discharge for each month of collection was obtained from the U.S. Geological Survey database (United States Geological Survey, 2014). Clear to moderately turbid water runs over exposed Tuscumbia limestone and chert. The substrate consists of boulders, large cobble, small cobble, sand, silt, and mixtures of each. Aquatic foliage grows freely on the bars and banks, with the river surrounded by mostly agricultural land.

Collections were made monthly from August, 2013, through July, 2014. All fish were collected from a 300 m length of the Flint River. Fish were netted using a seine or a cast net with the following dimensions respectively: 3.5 m length, 1.2 m depth, and a 3 mm mesh; a radius of 1.4 m and a 6 mm mesh. All fish were euthanized on site using 3 ml of (1:10) clove oil: 95% ethanol diluted with 350 ml river water and placed in 10% phosphate buffered formalin for tissue fixation and storage. Water temperature was taken during each collection with an alcohol thermometer. The average rate of river discharge for each month of collection was obtained from the U.S. Geological Survey database (Table 1) (USGS, 2014).

Laboratory analysis

Standard length (SL) was measured with digital calipers to the nearest 0.01 mm. Gross body mass was obtained to the nearest 0.0001 grams using an Explorer OHAUS digital balance after excess fluid was blotted from the fish's body. The sex of each fish was determined by excision and examination of gonadal tissue using an Olympus SZX7 dissecting microscope. After excess surface fluid was blotted away from the gonadal tissue, gonadal mass was obtained to the nearest 0.0001 g. Gonadosomatic Index (GSI) was calculated as: GSI = (body mass – gonadal mass)/body mass X 100. Images of intact gonads and oocytes were captured using an Olympus SZX7 dissecting microscope with an Olympus DP72 camera. The images were later analyzed for maturation status and number using the CellSens Standard software (Ver. 1.5) that comes with this camera. Each image was captured at 8.4X (1.6X x 4.0X) and saved as a .tiff file.

Ovarian maturation was assessed for each female using a modification of the scheme described by Núñez and Duponchelle (2009). Based on macroscopic development, ovaries were divided into five stages (Fig. 2). Immature (stage I) ovaries are small in size, usually opaque, and contain only latent oocytes. Maturing (stage II) ovaries are larger, inhabiting a larger portion of

the abdominal cavity. Maturing ovaries contain various sizes of white and cream colored oocytes. Advanced maturation (stage III) ovaries are bulkier and densely packed with oocytes. The oocytes are yellow to orange, and various sizes of vitellogenic oocytes are visible in between oocytes that are ready to be released during spawning. Ripe (stage IV) ovaries are partially ovulated and oocytes are released when squeezing the fish's sides. In stage IV the ovary has obtained maximal development, but vitellogenic oocytes of several sizes are present in between the mature oocytes due to multiple spawning. Spawned and recovering (stage V) ovaries are still relatively large and flaccid with remaining empty spaces, but contain different sizes of developing vitellogenic oocytes. This stage can occur in between spawning cycles, and is also indicative of the end of the spawning season.

Both ovaries from each female were teased apart using 21-gauge hypodermic needles to liberate developing oocytes from the ovarian tissue. All oocytes were arranged into a single layer on a Syracuse watch glass to be photographed. When the number of oocytes exceeded one frame, multiple frames were taken. Digital images were used to categorize oocytes into stages of maturation (Fig. 3) using the schematic of Núñez and Duponchelle (2009). Latent oocytes were not counted in this project. Early maturing (stage I) oocytes are previtellogenic and are distinguished by their small size which is half the diameter of a ripe oocyte. Late maturing (stage II) oocytes are in early vitellogenesis and contain small yolk granules. The diameter size is larger, and a nuclear envelope can be seen. Mature (stage III) oocytes are in late vitellogenesis, yellow in color and filled with yolk globules. The vitelline membrane is obviously divided from the yolk. Ripe (stage IV) oocytes have a larger diameter than all other oocytes and are yellow to dark yellow-brown in color with vitelline membranes that are completely separated from the yolk mass. Female fecundity was determined as average clutch size, the combined number of

stages 3 and 4 oocytes present in stage 3 or 4 ovaries. This represents the number of mature oocytes nearly or immediately ready for spawning.

All oocytes (excluding latent oocytes) were counted by stages and the total number was calculated for each female. Oocyte counts were performed using EggHelper, a custom program developed in Microsoft Visual Studio 2013, and confirmed using CellSens software. The diameters of ten oocytes per developmental stage per female were measured, and monthly averages for each stage were calculated for each female.

Statistical analysis

Average monthly GSI, total oocytes, and clutch size were evaluated for adult females from August, 2013 to July, 2014, and average monthly GSI for males. To determine if statistically significant differences existed between monthly values for GSI and clutch size, one-way ANOVAs tests were performed. Tukey HSD post hoc tests were performed on those tests showing significant P-values at $\alpha = 0.05$. All of these tests were done with the online Statistica calculator using the algorithm of Gleason (1999) (Vasavada, 2016).

Monthly average diameter measurements were calculated for stage I, stage II, stage III, and stage IV oocytes.

Results

Study site temperature and discharge data

Water temperature began to rise in February from a low of 8º C in January to a high of 24.5º C in July. River discharge was high but variable in late winter and early spring before dropping to typical summer low flow (Table 1).

Fish Collections

Twelve monthly collections of fish were made from August, 2013, through July, 2014. Few fish were collected in January and February due to sustained high river levels. A total of 81 females, 94 males and 78 juveniles were collected. Sexually mature females ranged from 47.7 mm to 77.2 mm SL with a median of 62.5 mm, and from 1.0 g to 5.9 g in body mass. Males ranged from 49.3 mm to 74.4 mm SL with a median of 61.2 mm, and from 1.5 g to 5.1 g in body mass.

Reproductive schedule

Average monthly (GSI) was evaluated for males and females from August, 2013 to July, 2014. GSI values began to rise in February, peaked in April and May at over 13% for females and 1.6% for males, and showed a steep decline from May to June for both sexes (Figs. 4A, 4B). ANOVA and post-hoc Tukey tests showed monthly GSI values for both males and females to be significantly higher in April and May ($P < 0.01$) than other months (indicated by letters over monthly bars in Figures 4A and 4B).

Ovarian development, oocyte counts, and oocyte diameters

The developmental stage of each ovary was assessed and summarized monthly (Table 2). The highest monthly average of oocytes, 1271, was found in March (Table 3). The average monthly number of stage I oocytes peaked in February at 1083, while the average number of Stage II oocytes peaked in March at 630. The average number of Stage III oocytes peaked at 754 stage III in April and the peak average number of stage IV oocytes, 58, was also found in April. The averages of the diameters of ten oocytes per maturation stage, per female, were measured for comparison. Stage I egg diameters ranged from 0.317 to 0.499 mm, Stage II diameters ranged from 0.382 to 0.675 mm, Stage III ranged from 0.501 to 1.05 mm, and Stage IV varied 0.785 to 1.19 mm. An average diameter for each stage was determined for each month of the spawning

season (Table 4). The largest average diameters for all four oocyte stages were found in April and May.

Clutch size

Females with stage III or IV ovaries carrying stage III or IV oocytes were found in March, April and May (Table 5). Average clutch size was highest in April at 812. A one-way ANOVA found a significant difference between the three months ($F = 6.55$, 2 df, $P < 0.01$) and a post-hoc Tukey test found that March, with the smallest average clutch size, was significantly different from April ($P < 0.01$) but not from May, and May was not significantly different from April. The largest individual clutches found were 1102 in April and 1340 in May. All ovaries examined in June were stage V, post-spawning, even though they still contained mature oocytes.

Discussion

The Bigeye Chub in Alabama primarily spawns in April and May as indicated by GSI, ovarian condition and clutch size. This is slightly earlier than the late spring and early summer prediction of Boschung and Mayden (2004). Oocyte size also peaked in April and May. During this spawning peak, water temperature was in the 17 – 21 °C range, and stream discharge was dwindling from the typical early spring high levels found in the Flint River (described more fully in Hodgskins et al., 2016).

Females were found with oocytes in multiple developmental stages and carrying clutches for more than two months which is good support for viewing the species as a multiple spawner *sensu* Heins and Rabito (1986). The trilateral life history framework of Winemiller and Rose (1992) establishes three end points at the corners of opportunistic, equilibrium and periodic life histories. The Bigeye Chub's relatively large clutch size, more than 800 in April, as a measure of fecundity places the species intermediate between opportunistic and periodic. As described by

Mims and Olden (2012) this implies the species is responding to a flow regime with a defined seasonality as well as predictability of flow and resources. This is consistent with our lab group's observations of conditions in the Flint River (Hodgskins et al., 2016). The position of Bigeye Chubs in the trilateral scheme based on other life history traits can also be characterized as intermediate between periodic (late maturity, low survivorship and large clutches) and opportunistic (early maturing, low survivorship, small clutches) as a small fish (< 10 cm in length) with small eggs (~1 mm in diameter), seasonal spawning and multiple bouts of spawning in a season (Winemiller and Rose 1992; Winemiller 1992).

North American stream cyprinids use a range of reproductive strategies and seasons. One uncommon strategy used by some species such as the Texas Shiner (*Notropis amabilis*, (Girard)) is a protracted spawning season, nine months in the case of the Texas Shiner, consistent with the species' utilization of spring systems on the Edwards Plateau with stable water temperatures (Craig et al., 2017). We hope our work defining some species reproductive traits of the Bigeye Chub contributes to understanding the community ecology of, in particular, the Flint River of Alabama, which is rich in cyprinid species (Frimpong and Angermeier, 2010). The rheophilic Silver Shiner in the Flint River is among the first to spawn in the river's cyprinid community, apparently stimulated to spawn by flood pulses in the late winter and very early spring as water temperature reaches about 12 °C. Females release a large number of small, buoyant oocytes into high water (Hodgskins et al., 2016). What we observed in the Bigeye Chub for spawning season is more typical of stream cyprinids, with a peak spawning season in mid-spring. A similar study of the Scarlet Shiner in the Flint River found evidence of a more protracted spawning season from April to July with a May peak, a time period typically with higher temperature and lower discharge (Stallsmith et al., unpublished manuscript). Another Flint River cyprinid, the Whitetail

Shiner, *Cyprinella galactura* (Cope), spawns in the summer, June to August (Stallsmith, unpublished data). The Whitetail Shiner is also different from the other species mentioned since they are crevice spawners with males tending the developing eggs.

A surprisingly well-studied cyprinid in the Flint River is the Blotched Chub (*Erimystax insignis),* with a relatively long spawning period of March to June with an April peak as indicated by GSI, ovarian condition and presence of clutches in females. Average clutch size in the Blotched Chub was much smaller than what we observed in the Bigeye Chub, at only 125 in the peak month of April compared to the peak April clutch size of greater than 800 we found in the Bigeye Chub. Stage 4 oocytes were also smaller, averaging 0.74 mm compared to over 1 mm in the Bigeye Chub (Stallsmith et al., 2015). Earlier captive breeding work with the Blotched Chub by Conservation Fisheries, Inc. (2001) found that the species sheds demersal eggs over coarse gravel and larvae are benthic, suggesting a need for clean substrate for successful reproduction.

Conclusion

Our data give a good indication of the timing of reproduction by the Bigeye Chub, and also fecundity. But we did not attempt to observe their spawning in nature, or keep them in aquaria to observe spawning. Various online searches lead us to conclude that all six recognized species of *Hybopsis* are apparently unknown in their spawning habits. The Fishtraits Database of Frimpong and Angermeier (2013) states that the species is likely a lithophilic spawner. The mature oocytes we observed are denser than water and spawning may be simply releasing demersal eggs into the water column over gravel or cobble, and leaving the eggs to sink to the bottom as found with sympatric Blotched Chubs (Conservation Fisheries, Inc., 2001). This remains to be determined.

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Table 1(on next page)

Monthly water temperatures and average rate of river discharge (feet^3/sec), August 2013 - July 2014.

Temperature was measured at the river during each monthly fish collection, and river discharge date are from an automated station several km downstream. Rate of discharge is reported as feet^3/sec because that is how is reported by the United States Geological Survey.

Table 1 Monthly water temperatures and average rate of river discharge (ft^3/sec), August 2013 – July 2014.

Month	Water Temperature, °C	Average Monthly River Discharge, ft^3/sec
August	25.1	819
September	22	262
October	14.4	160
November	15.3	250
December	13	1140
January	8	899
February	10.2	1248
March	15.6	539
April	17.2	1094
May	21.1	542
June	24.1	766
July	24.5	220

Table 2(on next page)

Monthly summary of ovarian maturity by stage, 2013-2014.

All ovaries observed in females from September and October were fully regressed so nothing is reported for those months, and no females were collected in January.

Table 2. Monthly summary of ovarian maturity by stage, 2013–2014.

	Stage I	Stage II	Stage III	Stage IV	Stage V
August					2
November	3				
December	10				
February		1			
March			5	2	
April			1	4	
May			1	12	4
June					6
July					3

Table 3(on next page)

Average monthly number of oocytes found per female, by developmental stage and in total.

The number of females examined per month is indicated within parentheses following the name of the month.

Table 3. Average monthly number of oocytes found per female, by developmental stage and in total. The number of females examined per month is indicated within parentheses.

	Stage I	Stage II	Stage III	Stage IV	Total
February (n = 1)	1083	164	0	0	1247
March (n = 7)	404	630	219	32	1271
April (n = 5)	253	164	754	58	1230
May (n = 16)	240	359	425	49	1073
June (n = 9)	236	275	131	41	669
July (n = 10)	72	129	574	39	796

Table 4(on next page)

Monthly average oocyte diameters at each developmental stage.

Measurements are in mm. Number of females examined is in parentheses following the name of the month.

Table 4. Monthly average oocyte diameters at each developmental stage during spawning months. Female sample size reported inside parentheses. Measurements are in mm.

	Stage I	**Stage II**	**Stage III**	**Stage IV**
February (n = 1)	0.441	0.495	n.a.	n.a.
March (n = 7)	0.455	0.583	0.768	0.997
April (n = 5)	0.470	0.616	0.911	1.052
May (n = 16)	0.471	0.620	0.928	1.035
June (n = 9)	0.436	0.577	0.744	0.940
July (n = 10)	0.3951	0.460	0.707	0.921

Table 5(on next page)

Average monthly clutch size.

The number of fish examined is in parentheses following the name of the monthly. The SE follows each monthly average in parentheses.

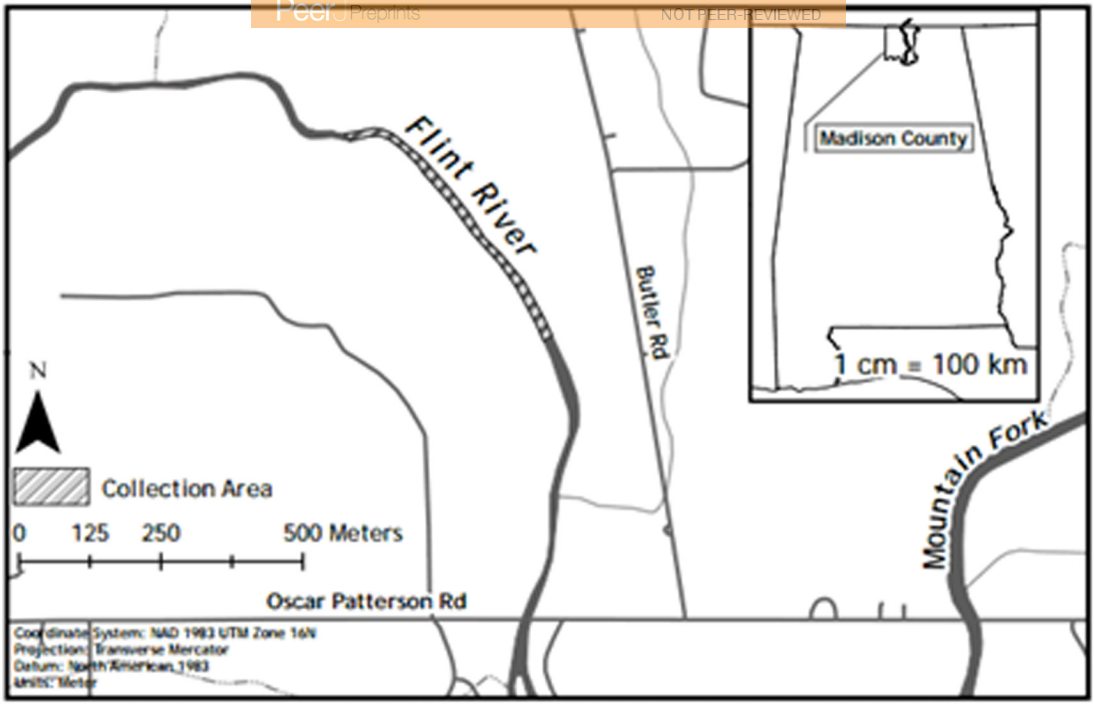
Table 5. Average monthly clutch size. Female sample size is in parentheses following month, standard error is in parentheses following average clutch.

	Average Clutch	Clutch Size Range
March (n = 7)	236 (59)	10 − 425
April (n = 5)	812 (136)	305 − 1102
May (n = 13)	541 (86)	172 − 1340

Figure 1(on next page)

Map of collection site north of the Oscar Patterson Road crossing on the Flint River in Madison County, Alabama.

All fish were collected in a 300 m stretch of the river.



Map created by Benjamin Swan using ArcGIS. Road and Political Bounday data provided by US Census Bureau; Hydrography data provided by USGS

Figure 2(on next page)

Representative photos of the five stages of ovarian maturation.

(A) Stage I, early maturing. (B) Stage II, late maturing. (C) Stage III, mature. (D) Stage IV, ripe. (E) Stage V, post-spawning.

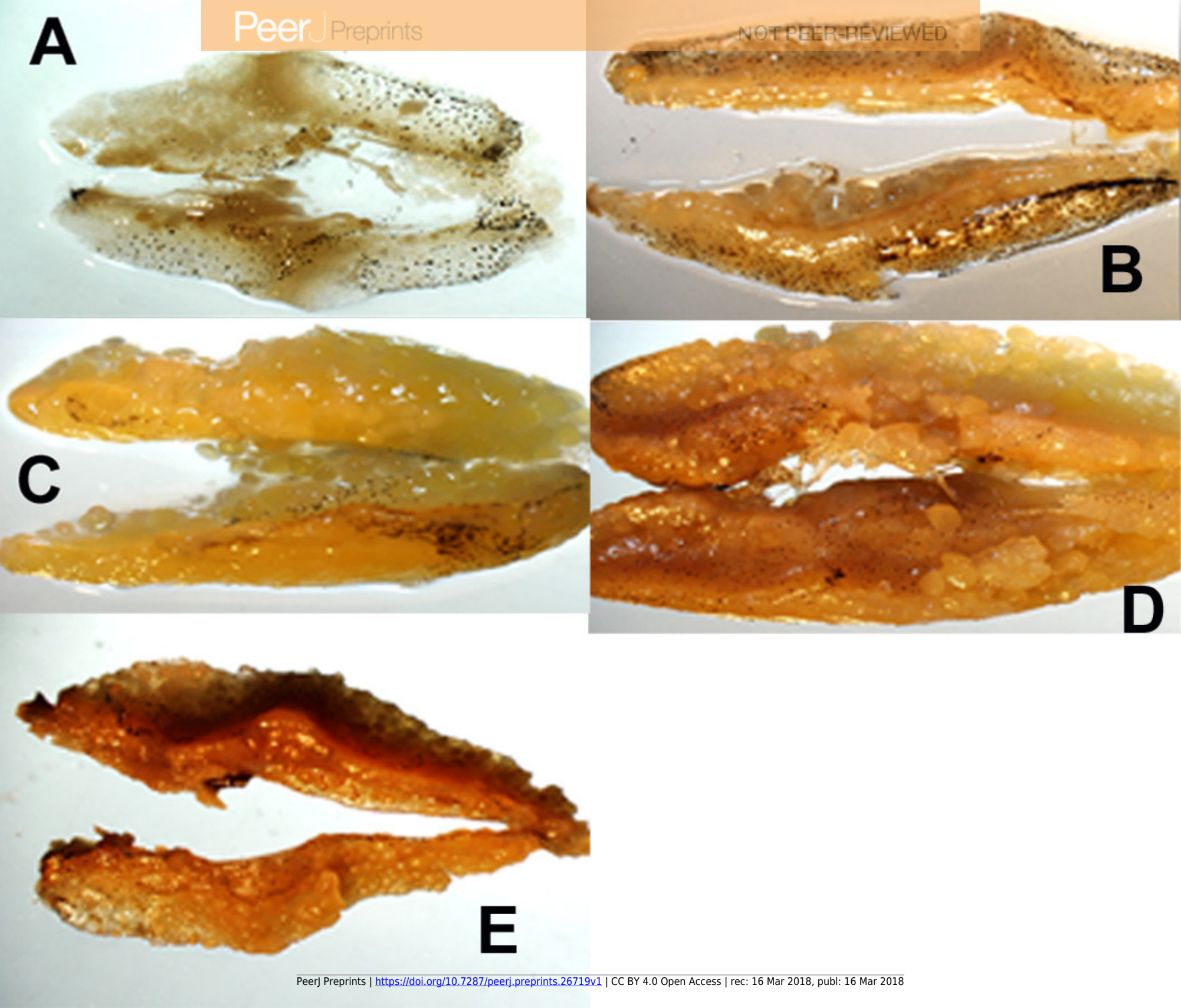


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Figure 3(on next page)

Representative images of the four stages of oocyte maturatio.

From top to bottom: stage 1, early maturing; stage II, late maturing; stage III, mature; stage IV, ripe.

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Figure 4(on next page)

Monthly GSI values of females and males.

A) Average monthly GSI of females (none collected in January). B) Average monthly GSI of males. Error bars are one SE. Letters above month indicate similar groups as indicated by post-hoc analysis of one-way ANOVA.No error bars are shown for months with two or fewer individuals collected.