

ORIGINAL ARTICLE

Ovary morphology and histological studies of the female Spotted barb *Puntius binotatus* (Valenciennes, 1842) from Agusan Marsh, Agusan del Sur, Northeastern Mindanao Islands, Philippines

Rainer P. SULARTE^{1,2*}, Joycelyn C. JUMAWAN^{2,3}

¹College of Teacher Education, City College of Bayugan, Noli, Bayugan City, Caraga Region, Mindanao Island, Philippines 8502.

²Graduate School, Caraga State University, Ampayon, Butuan City, Caraga Region, Mindanao Island, Philippines 8600.

³Department of Biology, College of Mathematics and Natural Sciences, Caraga State University.

Abstract

Agusan Marsh was a protected area located in the Mindanao Biogeographic Zone, particularly in the province of Agusan del Sur, with a unique composition of aquatic fauna. A total of 158 individuals of *Puntius binotatus* were collected from two lakes and one river in Agusan Marsh: Lake Tugno, Lake Panlabuhan, and the Agusan River. A small amount of tissue samples were taken from the anterior, posterior, and median portions of the ovaries, regardless of size, for histological examination. Results showed that female *P. binotatus* ranging from 6-11.5cm TL were captured in the study. Histological examination revealed that *P. binotatus* had mature ovaries and was actively spawning during the dry sampling month. Seasonal variation in Agusan Marsh affected the reproductive biology of *P. binotatus* in the area.

Keywords: *Puntius binotatus*, Oocyte, Reproductive cycle, Vitellogenesis

Correspondence: rainersularte2011@gmail.com

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INTRODUCTION

Agusan Marsh is a protected area in the Mindanao Biogeographic Zone, particularly in the province of Agusan del Sur. It is one of the most ecologically significant wetland ecosystems in the Philippines and was designated as a Wetland of International Importance by the Ramsar Convention (Primavera & Tumanda 2008; as cited by Sularte et al. 2015). Hubilla-Travis et al. (2007) inventoried freshwater fish in the marsh and documented 59.3% of freshwater fish, most of which were native and migratory to the area. Additionally, Davies (1991) and Oloroso et al. (2000) listed 17 fish species belonging to nine families in the lakes and swampy habitats of Agusan Marsh.

Indigenous people in the area locally call the spotted barb, *Puntius binotatus* (Cyprinidae), “banot,” and the International Union for Conservation of Nature classifies it as a species of least concern. Achille Valenciennes (1842) originally described it as *B. binotatus*; it is indigenous to Agusan Marsh and native to the Philippines. This species was a benthopelagic fish that occurred from sea level to at least 2,000m above sea level. *Puntius binotatus* inhabited medium- to large rivers, lakes, streams, stagnant water bodies (including sluggish-flowing canals), and brooks. It was found in middle to bottom depths in fairly shallow waters (IUCN 2015). It is

commonly found in Asia and distributed across several countries, including Brunei Darussalam, Cambodia, Indonesia, Laos, Malaysia, Myanmar, the Philippines, Thailand, and Vietnam, but has been introduced to Singapore and Palau (Jenkins et al. 2009; Lim et al. 2014). Meanwhile, this species was reported to be common in the Pahang River Basin, Malaysia (Khan et al. 1996), and in Singapore (Lim & Ng 2006).

However, attempts to conserve what remained in Agusan Marsh were hampered by a lack of reliable information on its biota, particularly indigenous freshwater fish species. Fish were an important biological component of the wetland ecosystem in Agusan Marsh, yet few studies had been published, particularly on the histology of native fish gonads, which could serve as baseline information for aquaculture management in the area. No previous study has examined ovary morphology and the histological components of the gonads of *P. binotatus* in this area. Therefore, this study aimed to determine the ovary morphology and gonad histology of female *P. binotatus* in selected lakes and river ecosystems of Agusan Marsh to gather baseline information on their current adaptation to agro-industrial waste-enriched water in the area and to support aquaculture management.



Fig.1. Map showing the sampling stations of *Puntius binotatus* collection stations in Agusan Marsh, Northeastern Mindanao Island, Philippines (source:stprmm.carsu.edu.phretrievedlast23october2015).



Fig.2. Lateral view of *Puntius binotatus* collected in Agusan Marsh, Northeastern Mindanao Islands, Philippines.

MATERIALS AND METHODS

Study area: Spotted barb were captured from three selected sites in Agusan Marsh (Fig. 1), particularly in Lake Tugno, Lake Panlabuhan, and the Agusan River. The study used a Global Positioning System (GPS) device to determine the sampling sites.

Sampling: Fish were collected using a cast net (5m long; mesh size 3.8cm). The captured female *P. binotatus* (n= 158) ranged from 6-11.5cm TL (Fig. 2). After capture, fish were transported to the fish laboratory for measurement and sex sorting. The collected samples were sacrificed by immersing the fish in ice for 4 hours before dissection, and sex was confirmed by examining the gonads. Standard length (SL) was measured using a measuring board to the nearest 0.1cm. Body weights were measured using a

calibrated spring balance with an accuracy of 5g and 50g. Gonads were weighed using an electronic analytical balance with 0.1g accuracy.

Dissection and microscopy of the gonads: Following Jumawan & Herrera (2014), with a few modifications, oocyte densities (number of oocytes per gram of ovarian tissue) were determined by counting the number of oocytes in a sample of ovarian tissue of known weight. After weighing the entire ovary (W ovary), 3-5 subsamples of known weight were taken from various parts of the ovary lobe. We weighed each subsample (Wi) to the nearest 0.001g, then manually dispersed it to segregate and count all vitellogenic and previtellogenic oocytes.

Ovaries were examined and assigned ovarian maturity stages using two methods: (1) a visual-based macro-

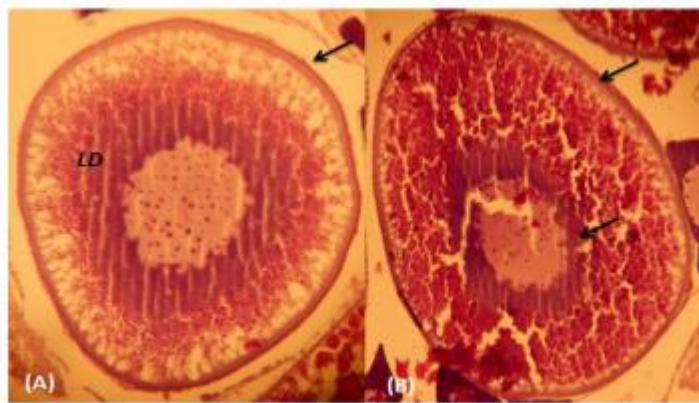


Fig.3. Primary vitellogenic follicles *Puntius binotatus*. Yolk spheres lie between the cortical alveoli. Less basophilic ooplasm was notable (Fig. 3A). The diameter of the nucleus reached to maximum diameter in this stage. B: Secondary Vitellogenic Follicles of *P. binotatus*. Zona radiata and follicle diameter reached maximum thickness. Irregular nuclear membrane (black arrow); the yolk spheres moved toward the center, and cortical alveoli and lipid droplets moved to the periphery (H&E $\times 10$).

structural scale, by which a micro-structural scale of oocyte diameter distribution was validated; and (2) histological descriptions of the ovary to characterize the stages of ovarian maturity and the sizes of developing oocytes for each stage (Lowerre-Barbieri et al. 2011; Brown-Peterson et al. 2011; Jumawan & Herrera 2014). Ovaries were classified into maturity stages based on (1) color (opalescent to yellow), (2) gross surface texture (grainy to smooth), and (3) visual estimation of the abdominal cavity occupied by the ovary (Mazzoni & Caramaschi 1997; Jumawan & Herrera 2014).

The ovaries were cut and preserved in Bouin's solution for 12-24 hours and then stored in 70% ethanol. Ethanol (75–90%) was used to dehydrate the ovary fragments, which were then embedded in paraffin and sectioned using a microtome. The 5–6 μm thin sections were stained with hematoxylin-eosin (HE) (Eagderi et al. 2013; Forsatkar et al. 2014). The occurrence of partially spent ovaries, identified by the simultaneous presence of post-ovulation follicles (POFs) and vitellogenic oocytes, was used to determine the spawning mode.

To measure oocyte size ranges and their frequency at different stages of development, at least 30 oocytes were extracted from the anterior, middle, and posterior parts of the ovaries from five freshly dissected females for each maturity stage, which were initially sorted through visual-based classification. To detect carbohydrates and proteins in vitellogenic and non-vitellogenic oocytes, a combination of Periodic Acid-Schiff (PAS)-Alcian Blue (AB) pH 2.5 (Artisan™) was used (Jumawan & Herrera 2014).

Data analysis: For all measured structures, we calculated the mean and standard deviation.

RESULTS

Stages of the reproductive cycle of *P. binotatus*

Primary vitellogenic follicles: Yolk spheres lie between the cortical alveoli. Less basophilic ooplasm was noted (Fig. 3A). The nuclear diameter reached a maximum during this period.

Secondary vitellogenic follicles: The yolk spheres (Fig. 3B) of *P. binotatus* were moved toward the center, and cortical alveoli and lipid droplets moved to the periphery. The nucleus membrane became irregular (black arrow). The zona radiata and follicle diameter reached maximum thickness.

Tertiary vitellogenic follicles: The yolk spheres of *P. binotatus* were larger, combined, and filled the ooplasm. The nucleoli gradually moved toward the center of the nucleus (Fig. 4A). B: Yolk globules start to fuse (mid stage).

Maturation and hydration stage of *P. binotatus*: The early maturation stage is characterized by yolk globule formation through the fusion of small coated yolk vesicles at the oocyte periphery (Fig. 3B). Yolk globules tend to concentrate along the outer margin of the yolk vesicle layer. The late yolk globule stage is characterized by increased oocyte size and migration of the fused/coalesced globules to the center, forming a continuous border near the zona radiata (Fig. 6C and D).

During maturation, the nucleus gradually shifted toward the animal pole. Later, the nuclear membrane disappeared (Fig. 5A and B). The primary, secondary,

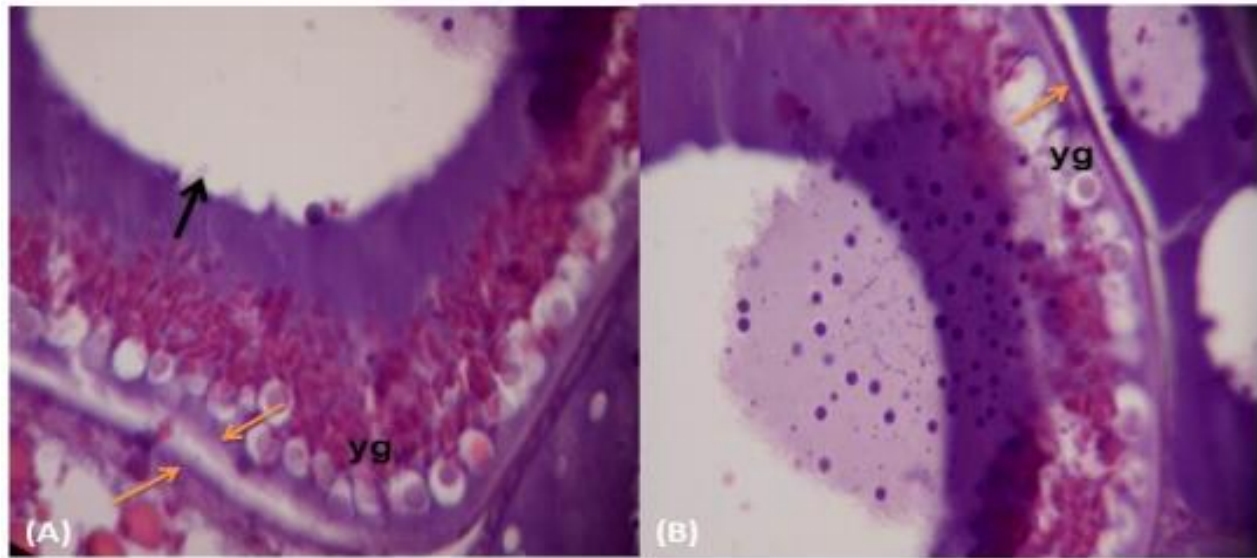


Fig.4. Vitellogenesis in *Puntius binotatus*: Zona radiata in two secondary vitellogenic follicles. A: Thick zona pellucida (orange arrows), and B: Yolk globules start to fuse (mid stage).

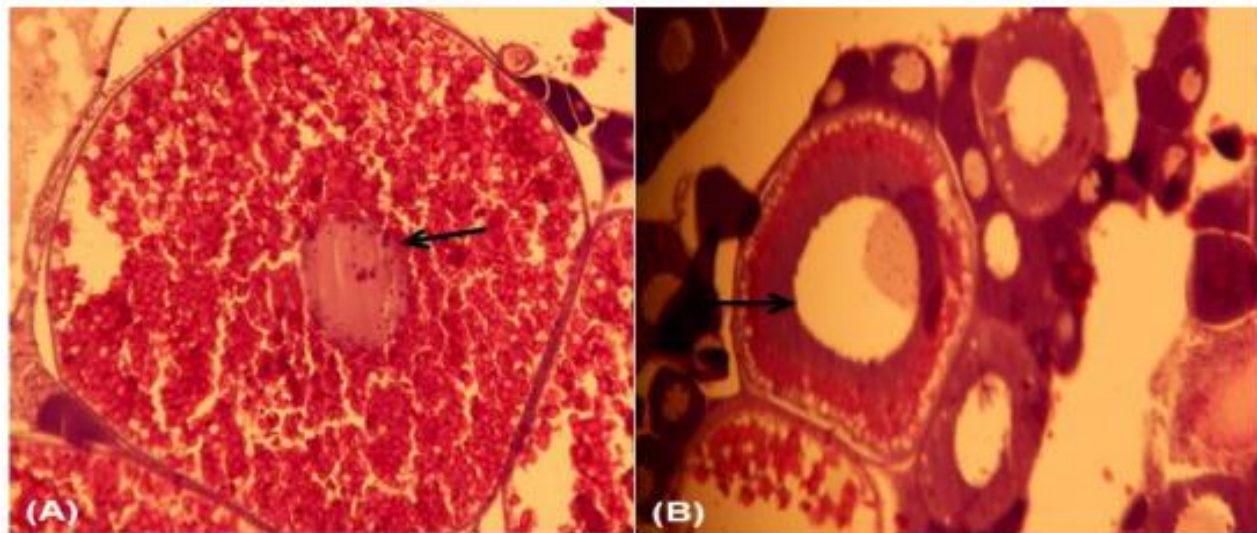


Fig.5. A: *Puntius binotatus* nucleoli started to migrate (A) to the center of the nucleus in the tertiary vitellogenic follicle (black arrow), and (B) Mature follicle of *P. binotatus*; the yolk spheres of ooplasm are completely filled (black arrow) (H&E $\times 10$).

tertiary vitellogenic, and mature follicles were considered as vitellogenic follicles. The results also showed that the reproductive cycle of *P. binotatus* can be divided into 3 stages based on the percentage of different types of ovarian follicles: previtellogenesis, vitellogenesis, and maturation. In the previtellogenesis and vitellogenesis stages, the percentage of vitellogenic follicles increased significantly from the anterior to the posterior portion ($P < 0.05$). In the maturation stage, the percentage of vitellogenic follicles was higher in the median portion than in the anterior portion ($P < 0.05$). Three follicle

types were identified in the ovigenous lamellae: chromatin nucleolus, pre-nucleolus, and early cortical alveolus follicles (Fig. 5C).

DISCUSSION

Agusan Marsh faces threats that affect its flora and fauna, particularly aquatic species such as fish. Environmental threats in the area included the continuous draining of pollutants from upstream mountains due to mining industries, the use of synthetic pesticides in agricultural farms near the peripheries of Agusan Marsh, increased fishing

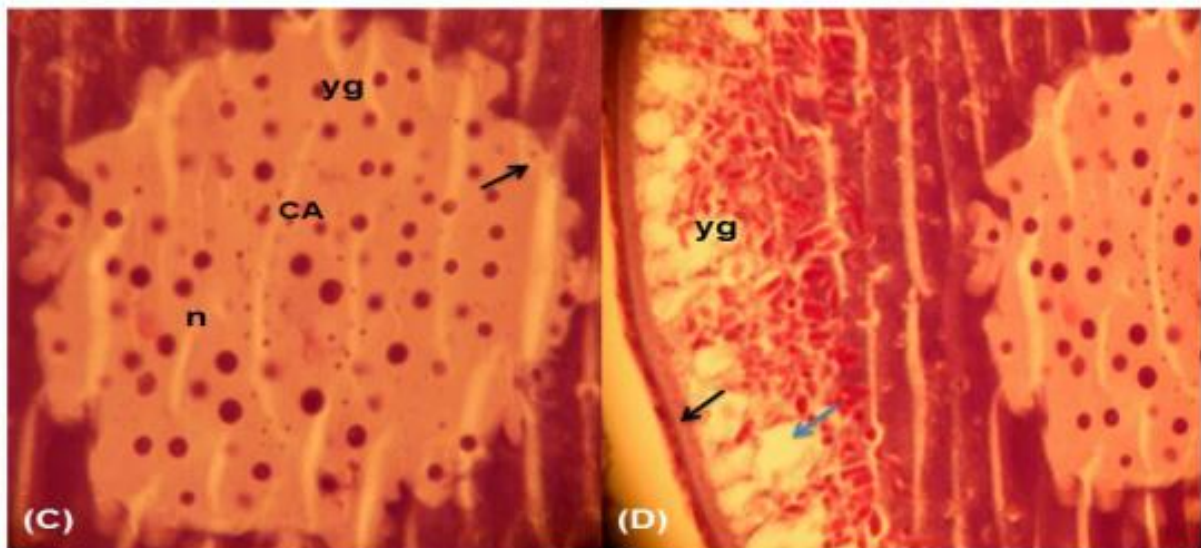


Fig.6. Maturation and Hydration stage of *Puntius binotatus*. C: Peripheral cytoplasm in early stage, Late CA stage. D: Yolk globules start to fuse (mid-stage), yolk globule coalesce with cytoplasm compacted (late stage; HE). Thicker zona radiata (black arrow), yolk spheres (blue arrow), (H&E $\times 10$).

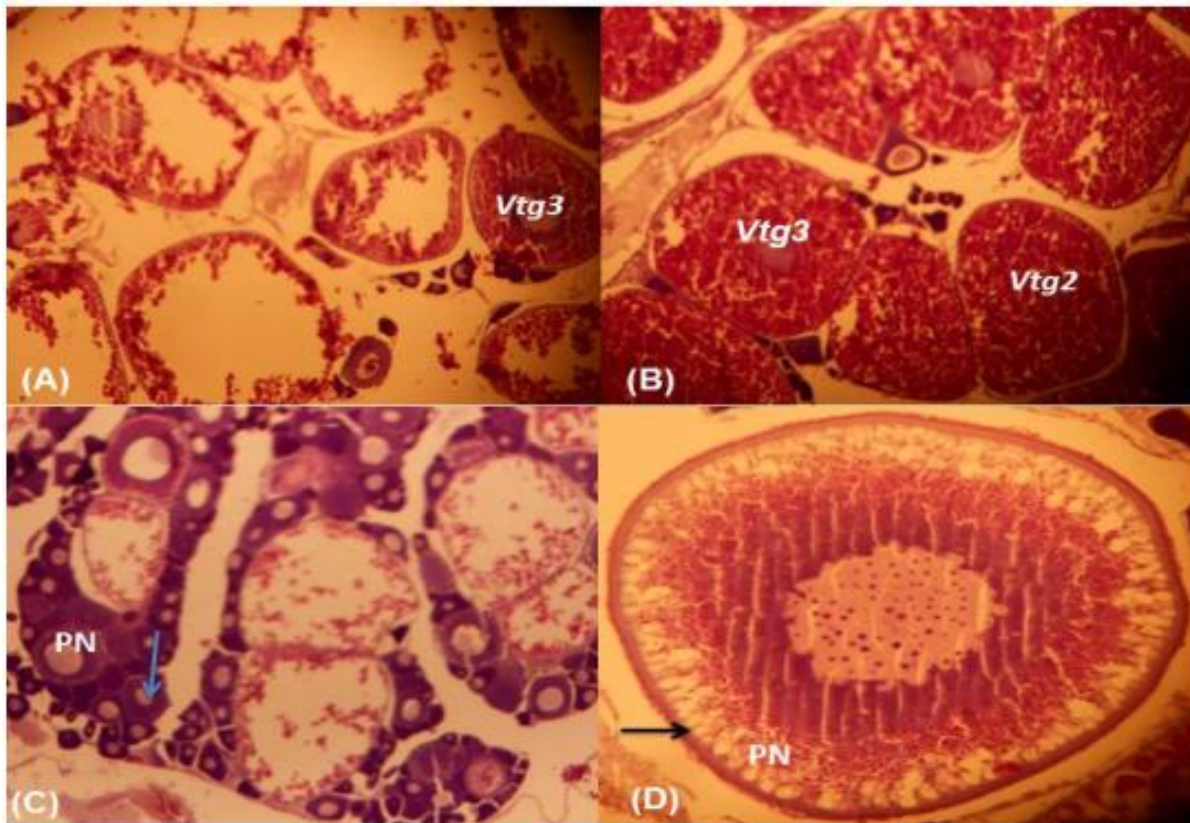


Fig.7. Stages of ovarian maturity in *Puntius binotatus*. A: Spawning capable stage characterized by presence of mostly vtg3 oocytes. B: Section of a recently spawned ovary. C: Spent ovary characterized by thick ovarian lining, ovigenous lamellae (blue arrow) D: Late regeneration stage characterized by thick ovarian wall (black arrow) and abundant PG oocytes. vtg2: migratory nucleus stage, vtg3: ripe/mature stage

activity inside the sanctuary, the presence of human settlements, overhunting of wildlife fauna, and many other activities that might have affected aquatic biodiversity in the area. Studies by Takashima et al.

(2008) and Miranda et al. (2009) found that environmental factors can profoundly affect the timing of gametogenesis, vitellogenesis, and fish maturation in aquatic ecosystems. The spawning

period of common carp typically occurred when water temperature ranged between 18-28°C (Tempero et al. 2006).

Puntius binotatus inhabits medium to large rivers, lakes, streams, stagnant water bodies (including sluggish flowing canals), and brooks, and is found in middle to bottom depths in fairly shallow waters (IUCN 2015). The ovarian structure of the Cyprinidae family changes in the annual reproductive cycle, to which *P. binotatus* belongs (Sivakumaran et al. 2003; Mabudi et al. 2010; as cited by Shirali et al. 2012). In determining the reproductive cycle in female fish of the Cyprinidae family, specifically *P. binotatus*, it was desirable to observe seasonal developmental changes in the gonads, particularly the ovary.

Histological results of *P. binotatus* showed that, in this area, the species had mature ovaries, and some samples were in the spawning stage. The yolk of *P. binotatus* was mature and regular in shape. Since the sampling period covered only one month, it was too early to conclude the reproductive cycle, particularly the spawning period of *P. binotatus* in the area. The zona radiata reached maximum thickness in secondary vitellogenic follicles, while the nucleus reached maximum diameter in primary vitellogenic follicles. We compared the reproductive cycle of *P. binotatus* with that of common carp because they belong to the same family (Cyprinidae) (Shirali et al. 2012). Shirali et al. (2012) conducted a histological study on common carp and found that some fish were in the maturation stage from April to October. They indicated that the common carp breeding season lasted about seven months, and that this species spawned from April to October. Ovarian maturation was associated with increasing temperature and photoperiod. Based on their results, they found that common carp ovulated one month earlier under long photoperiod conditions.

CONCLUSION

In conclusion, morphological and histological studies of female *P. binotatus* showed that the fish's maturation and spawning occurred during the dry months of the year. The yolk of *P. binotatus* was mature and regular in shape, ready to spawn. During this period, Agusan Marsh was in the dry season, when water temperature increased and triggered

gamete spawning.

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مقاله کامل

ریخت‌شناسی تخمدان و مطالعات بافت‌شناسی ماهی ماده بارب خال‌دار *Puntius binotatus* (Valenciennes, 1842) از تالاب آگوسان، آگوسان دل سور، جزایر میندانائو شمال شرقی، فیلیپین

رینر پی سولارتی^{۱،*}، جویسلین سی. جوماوان^{۲،۳}

^۱دانشکده تربیت معلم، کالج شهری بایوگان، نولی، شهر بایوگان، منطقه کاراگا، جزیره میندانائو، فیلیپین، ۸۵۰۲.

^۲دانشکده تحصیلات تکمیلی، دانشگاه ایالتی کاراگا، آمپایون، شهر بوتوان، منطقه کاراگا، جزیره میندانائو، فیلیپین، ۸۶۰۰.

^۳گروه زیست‌شناسی، دانشکده ریاضیات و علوم طبیعی، دانشگاه ایالتی کاراگا.

چکیده: تالاب آگوسان، واقع در منطقه زیست‌جغرافیایی میندانائو و به‌طور مشخص در استان آگوسان دل سور، یک منطقه حفاظت‌شده با ترکیب منحصربه‌فردی از جانوران آبی است. در مجموع، ۱۵۸ نمونه ماهی *Puntius binotatus* از دو دریاچه و یک رودخانه در تالاب آگوسان، شامل دریاچه توگنو (Lake Tugno)، دریاچه پانلابوهان (Lake Panlabuhan) و رودخانه آگوسان (Agusan River)، جمع‌آوری شدند. برای انجام بررسی‌های بافت‌شناسی، بدون در نظر گرفتن اندازه تخمدان، تعداد اندکی از نمونه‌های بافتی از قسمت‌های قدامی، خلفی و میانی تخمدان‌ها تهیه شد. نتایج نشان داد که ماده‌های گونه *P. binotatus* با طول کل بدن (TL) در محدوده ۶ تا ۱۱/۵ سانتی‌متر در منطقه مورد مطالعه صید شدند. بررسی‌های بافت‌شناسی نشان داد که تخمدان‌های گونه *P. binotatus* در مرحله بلوغ قرار داشته و ماهی‌ها در طول ماه نمونه‌برداری در فصل خشک، به‌طور فعال در حال تخم‌ریزی بودند. همچنین، تغییرات فصلی در تالاب آگوسان بر زیست‌شناسی تولیدمثل *P. binotatus* در این منطقه تأثیرگذار بود.

کلمات کلیدی: *Puntius binotatus*، اووسیت، چرخه تولیدمثل، ویتلوزنز (زرده‌سازی)