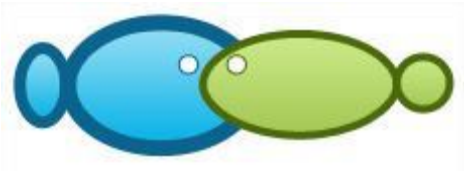

Ovarian development and spawning of Bornean endemic fish *Hampala bimaculata* (Popta, 1905)

[1]Widadi P. Soetignya, [2]Bambang Suryobroto, [3]Mohammad M. Kamal, [4]Arief Boediono

[1] Faculty of Agriculture, Tanjungpura University, Pontianak, Indonesia; [2] Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, West Java, Indonesia; [3] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, West Java, Indonesia; [4] Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, West Java, Indonesia. Corresponding author: W. P. Soetignya, fx.widadi.padmarsari.s@faperta.untan.ac.id

Abstract. A study was carried out to determine ovarian development and spawning of *Hampala bimaculata.* During the study period, 124 females of the fish samples were collected from February to October 2013 and the collection continued from July to November 2014 using gill nets and anglings. Sampling site in Betung Kerihun National Park, West Kalimantan Province, Indonesia included Embaloh and Sibau watersheds. Spawning characteristics of the fish were determined by histological investigation of ovaries and the distribution of oocyte diameter-frequency. Analysis of macroscopic and histological observations showed that the ovaries were classified into five stages: immature or resting, maturing, mature, ripe, and spawned-recovering. The spawning season was estimated to be from July to October based on seasonal changes in maturity frequency of females and monthly variations in the gonadosomatic index. Histological observation showed that the fish is an iteroparous with a group-synchronous of ovarian development. The polymodal distribution of oocyte diameter during the breeding season and the presence of cells in the vitellogenic oocytes of different sizes at the spawned stage indicated that *H. bimaculata* is a batch spawner.

Key Words: sychronous group, batch spawner, reproductive biology, spawning type.

Introduction. Fishes display diversity in reproductive strategies and linked traits such as breeding mechanism, spawning pattern, spawning season and others. In fishery biology, reproduction analysis focused on female, mainly because offspring production is limited to a greater degree by the eggs rather than sperm production (Helfman et al 1997). Therefore the ovarian maturation stages in each individual fish are very important in order to better understand the reproductive strategies.

Reviews of ovarian cycle in fish have been studied by Honji et al (2006), Nunez & Duponchelle (2009), Dorostghoal et al (2009), De Souza et al (2011) and Pereira et al (2013). Some classification of ovarian development are regarded as universal, while others have been established for certain species only. The mechanism of oogenesis and oocyte maturation among Teleosts appear to be similar in except variations in recruitment timing and oocytes maturation (Roy & Mandal 2015). In Cyprinids, Hamzaoglu et al (2015) described five ovarian development stages in *Alburnus istanbulensis*. Rahemo & Al-Shatter (2012), described six development stages of ovarian development in *Barbus luteus* and *Varicorhinuus trutta*, meanwhile Verma (2013) and Roy & Mandal (2015) reported seven stages of oocytes development in *Labeo dyocheilus* and *Labeo bata.*

Hampala bimaculata is an Bornean endemic species which belongs to the family Cyprinidae under the order Cypriniformes (Ryan & Esa 2006). Morphological character of the fish is two vertical blotches on the side, one on the anterior part of the caudal peduncle and one under the dorsal. The fish can be found in the waters in West Kalimantan and East Kalimantan (Kottelat et al 1993) and it is traditionally known as the

Arungan or Dungan. *H. bimaculata* is a valuable economic resource supporting many local people through both consume and sport fishing. Despite its great economic values, there are a little published works describing the gonad development and spawning for *H. bimaculata* in its natural environment. Soetignya et al (2016) reported dominance of females over the males and the fecundity significantly correlated to total length, total body weight, and gonade weight. Therefore, the main objective of this study was to determine ovarian development and spawning of *H. bimaculata* in the waters of Betung Kerihun National Park, West Kalimantan Province, Indonesia.

Material and Method

Samples collection. The fish samples were performed by using gillnet and angling from February to October 2013 and continued from July to November 2014. Sampling sites in the waters of Betung Kerihun National Park, Kapuas Hulu Regency, West Kalimantan Province, Indonesia included Embaloh (1°24'31.6"N-1°19'18.3"S and 112°23'44"-112°29'36.8"E) and Sibau (1°20'33.6"N-1°02'39.8"S and 112°53'23"-113°15'08.1" E) watersheds. Gill nets were composed of four panels of 20 and 30 mm mesh size each which they were positioned horizontally to the mouth of the river at 18.00 and left in place until 6.00 the following morning (a total of 12 hours). Sampling by angling was done upstream to downstream of sampling area from 8.00 to 16.00. For this study, 124 females were measured to the nearest mm for total length (TL), weighed to the nearest gram for total weight (W) using SJ-5001HS digital scale at precision 1 gram. The fish was dissected to determine the ovarian maturation stages and subsequently, ovaries were weighed to an accuracy of 0.01 g (Moslemi-Aqdam et al 2016).

The identification of the ovarian maturation stages may be done macroscopically, based on visual characters such as changes of size, colouring and shape of the ovary, transparency, degree of occupation of the abdominal cavity and the degree of visualization of the ovaries (De Souza et al 2011). Microscopically, they are made taking into consideration ovary histological characteristics (Nunez & Duponchelle 2009). After this procedure, pieces of the ovary from posterior, middle and anterior were cut and preserved fixed in 10% buffer neutral formalin solution for minimum 48 h and subsequently processed for histology following Brewer et al (2008). Each piece of tissue, embedded in paraffin, sectioned (5-7 µm) and stained with haematoxylin and eosin for histological studies of the ovarian maturation.

Gonads staged III and IV were considered as sexually mature (Kendall & Gray 2009). To estimate the size at maturity, the total length was plotted against the frequency percentage at mature individuals during the spawning season and then the length at which 50% of the total individual number was consider as the size at first maturity (El-Sayed & Moharram 2007).

To determine oocyte diameter frequency, three subsamples from a ripe stage ovaries were taken from three sites of the right lobe of the ovary (anterior, middle and posterior) (Almatar et al 2004). For subsample, 100 oocytes were measured. Ocytes ≥ 200 µm were measured using an ocular micrometer inserted in one ocular of a stereo microscope. Results were extrapolated for the entire ovary.

The gonadosomatic index (GSI) was calculated as: $GSI = 100 \times W_G \times W^{-1}$ where: W_G was the gonad weight and W, the total body weight of the fish (Koutrakis 2011; Verma 2103; Moslemi-Aqdam et al 2016). The hepatosomatic index (HSI) was also analyzed using following formula (Moslemi-Aqdam et al 2016; Keivany et al 2017): $HSI = 100 \times W_L \times W^{-1}$ where: W_L is the weight of liver and W is the total body weight of the fish. The GSI and HSI data were analyzed by Kruskal-Wallis to determine significance of each data treatment which was performed using R program. All diagrams except size at first maturity were prepared with MS Excell. Size at first maturity was performed using R program. The spawning season was determined through the analysis of the relative frequencies of gonadal maturation stages along the sampling period, GSI calculations (Gomes et al 2011; Ma et al 2012; Moslemi-Aqdam et al 2016) in samples of *H. bimaculata*.

Results and Discussion

Ovarian development. Oocytes develop within the ovary through different stages. Based on histological examinations, stages of oocytes of *H. bimaculata* were divided in four stages. Oocytes development are shown in Figure 1. Stage I, primary growth, which oocytes correspond to previtellogenic oocytes and are characterized by a small size, a basophilic homogenous ooplasm, central or sub-central nucleoli. Stage II, cortical alveoli or yolk vesicle formation stage indicate the onset of vitellogenesis and are easily distinguished from stage I oocytes by the presence, in the peripheral ooplasm, of lipid droplets, cortical alveoli and small yolk granules. Stage III, vitellogenesis is characterized by the appearance clearly visible of the chorion (or zona radiata), and the theca is generally well developed. The nucleus is still visible and located in a central position. Stage IV, maturation, the ooplasm is completely filled with large yolk globules and lipid droplets. At the end of this stage, the nucleus migrates to the periphery of the ooplasm, determining the animal pole. Four stages of the oocyte development are also similar with *Danio rerio* (Koc et al 2008). In the majority of Teleost, the oocyte development may be classified to five, six or eight stages (Gokce et al 2003).

In this study, the ovary development of *H. bimaculata* was classified into five stages: immature or resting, maturing, mature, ripe, and spawned and recovering (modified of Nunez & Duponchelle 2009; De Souza et al 2011):

A – immature or resting: the ovaries of juveniles are small, no oocytes were visible to the naked eye, occupy around 10% of the celomatic cavity, thin, varying from translucent to pink colour with incipient blood irrigation, sometime whitish. On the resting, the ovaries are similar to the immature, but usually larger, pink to red in colour. Histologically, all the oocytes in the ovary are well organized and presenting cells in the primary growth stage (chromatin and perinucleolar phase) or oocytes of stages I and II;

B – maturing: ovaries of different sizes, with a yellow colouring due to the presence of a majority of oocytes of phase III, as yet still not clearly individualised; blood irrigation is not noticeable. Histologically, they have oocytes at different phases of development, perinucleolar, lipid vitellogenesis, lipid and proteic vitellogenesis;

C – mature: the ovaries are large and occupy around 50% of the abdominal cavity, opaque, with coloring ranging from orange to reddish. Oocytes can be seen clearly through ovary wall. Three types of oocytes can be seen: the primary oocytes and the tertiary stage oocytes and oocytes entering of FOM (final oocytes maturation);

D – ripe stage: fully distended with granular surface occupying 70-80% of the abdominal cavity, are reddish brown, opaque,sack-shaped, hemorrhagic. This stage starts with the nucleus begins to leave central position and migrates toward periphery. Yolk globules fill more than two third of cytoplasm. Nucleus is observed in animal pole;

E – spawned and recovering: ovary was not full empty residual oocytes present. Flaccid and reddish, greyish in color. Ovary is occupying < 1/2 of the abdominal cavity, the ovaries are still relatively large and flaccid with remaining empty spaces, post-ovulatory follicles (POF), and new batches of developing vitellogenic oocytes.

At the end of the reproductive season, females will eventually be spawned out and their ovaries will evolve into resting stage until the next reproductive season. The resting stage is relatively similar to an immature stage, but larger, a darker pink to red in colour and at least at the beginning of the resting period, of large empty space within the ovarian lamellae. Figure 1 also displays the ovary development of *H. bimaculata*.

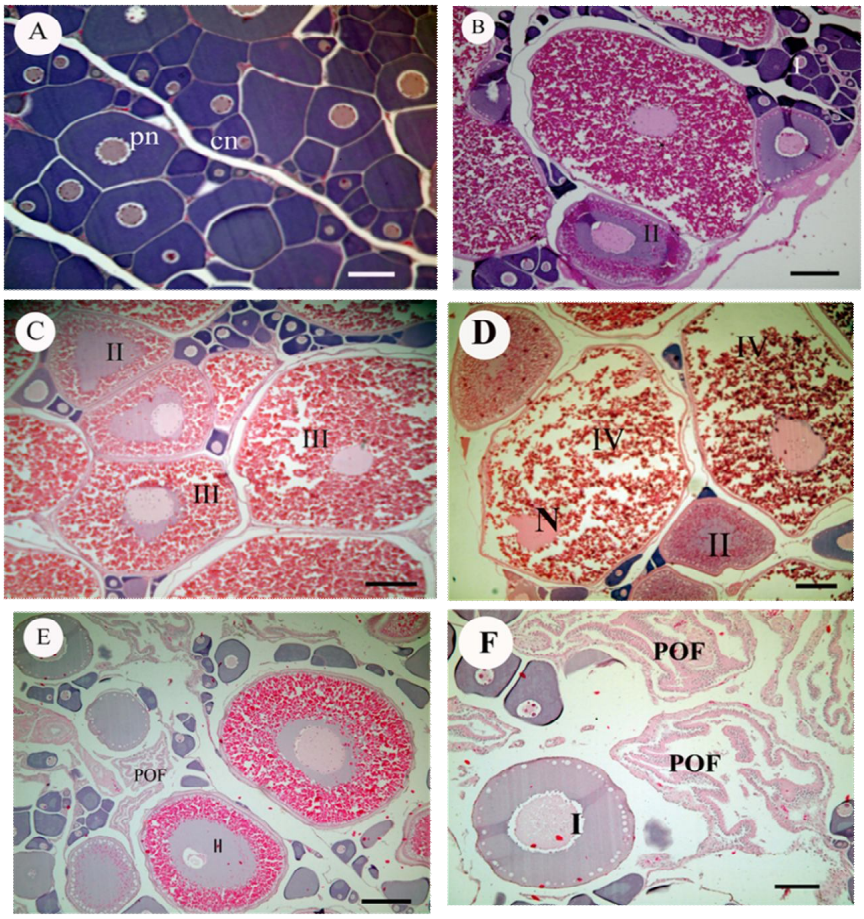

Figure 1. Photographs of histologically observed ovary sections and different maturity stages of *Hampala bimaculata* from Betung Kerihun National Park. A. Immature stage, containing germ cells, chromatin nucleolar (cn), perinucleolar (pn), scale bar = 50 µm. B. Maturing stage containing peri-nucleolus oocytes, scale bar = 200 µm. C. Mature stage, ovary containing lipid vitellogenesis oocytes, scale bar = 200 µm. D. Ripe stage ovary containing lipid and protein vitellogenesis, nucleus migrates to periphery of the ooplasm, N = nucleus, scale bar = 200 µm. E. Spawned-recovering stage containing post-ovulatory follicles (POF). F. Resting, ovary during the resting period after the breeding season, scale bar = 100 µm; I, II, III, IV = stages of oocytes development.

Gonado-somatic index (GSI) and hepato-somatic index (HSI). GSI and HSI of *H. bimaculata* females samples during the study period are presented in Table 1. The GSI of females are in the range of 0.51-8.03%. Table 1 substantially confirms that June is the month when the fish begin to breed. The GSI increased gradually and reached higher values between July to October. We observed a rapid and steady decreasing trend in the GSI value from November until May.

The GSI decreased rapidly from November and steadily until June. During July to September females GSI values were significantly different from the other months in pre-spawning season (February, March, April, May) ($p < 0.05$), but these were not significantly different from June and November.

The HSI females ranged 0.43-1.74. The trend in the females HSI were high in March and it was opposite with GSI. Female HSI value in March was significantly different from July, September and October ($p < 0.05$).

Table 1

Temporal variation in gonadosomatic index (GSI) and hepatosomatic index (HSI) of examined females of *Hampala bimaculata* from Betung Kerihun National Park

Months	*Number of females*	*Range of total length (mm)*	*GSI*	*HSI*
February	7	230-390	0.57-0.74^{c}	1.05-1.37^{ab}
March	12	225-410	0.51-0.80^{c}	1.05-1.47^{a}
April	22	220-460	0.54-0.88^{c}	0.85-1.46^{abc}
May	5	225-390	0.58-0.83^{bc}	1.06-1.19^{abc}
June	9	270-430	0.77-1.45^{ab}	0.77-1.38^{bc}
July	19	280-530	0.64-7.22^{a}	0.52-1.42^{c}
August	12	330-570	0.80-5.15^{a}	0.63-1.28^{bc}
September	16	280-620	0.63-8.03^{a}	0.43-1.74^{c}
October	8	300-480	0.75-3.02^{a}	0.70-1.50^{c}
November	14	250-490	0.73±1.04^{ab}	0.69-1.09^{bc}
Total	124			

*The mean GSI and HSI in the same column followed by a different superscript indicates significantly different ($p < 0.05$).

Length at first maturity and change in frequency maturity. Length of first maturity of *H. bimaculata* female is shown in Figure 2. The females estimated L_{50} value for length at first maturity was 450.9 mm. Meanwhile, proportion of mature females (stages mature and ripe) was 15.2% (19 of 124 individuals) during the breeding season from July to October. Monthly maturity frequency of females of *H. bimaculata* is shown in Figure 3. The immature or resting stage was encountered throughout the year. The maturing stage was first observed in June. Percentage of mature increased during July to October. The spawned and recovering stage was first observed in August which then continued until October. In November, all mature females have spawned completely, after which they have evolved into a resting stage.

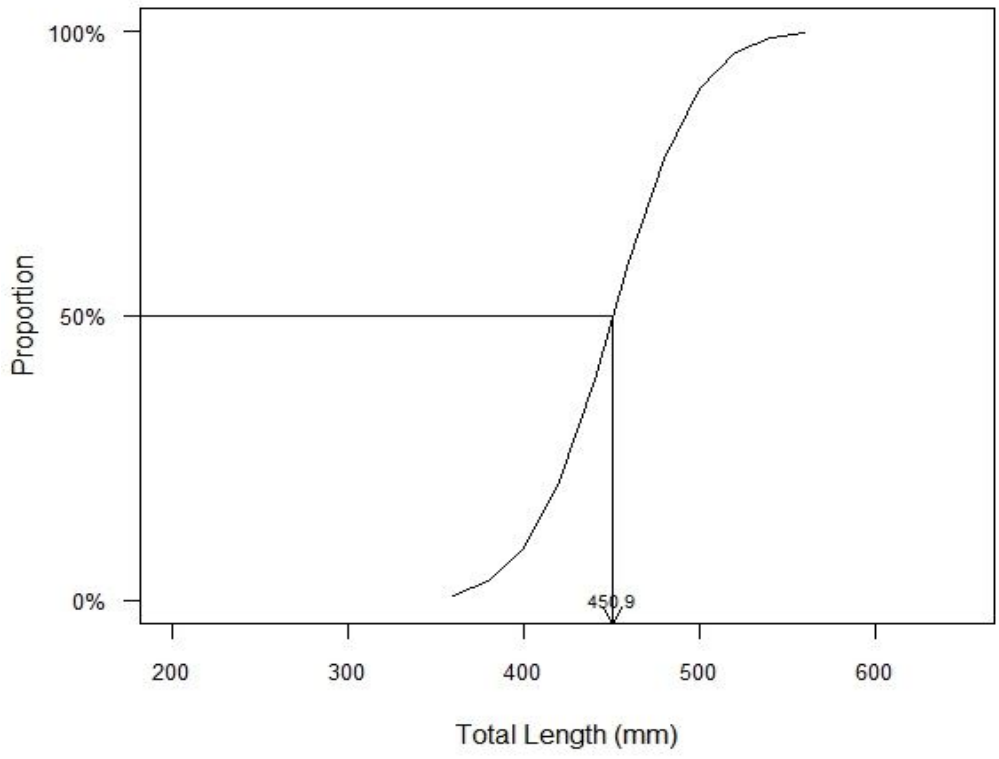

Figure 2. Relationship between proportion of maturity and mean total length of female *H. bimaculata* during sampling periods.

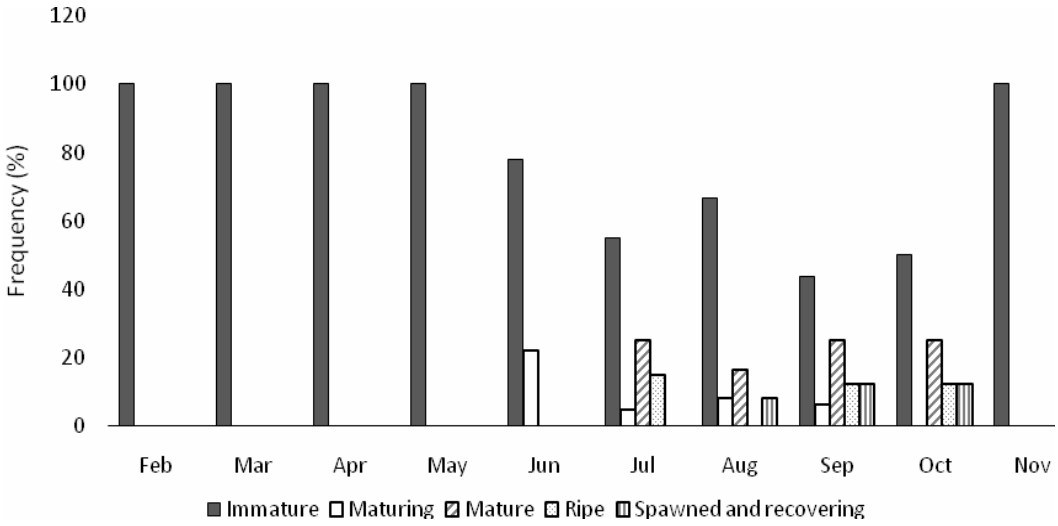

Figure 3. Monthly maturity frequency of females of *H. bimaculata* in the Betung Kerihun National Park with different ovarian development.

Oocyte diameter distribution. In this study, the frequency distribution of oocyte diameter was found to have a polymodal distribution. Oocyte diameter distributions of ripe females collected throughout the spawning season showed at least three main groups of oocytes. Changes in mean diameters (mm) of oocyte in ripe stage of *H. bimaculata* is shown in Figure 4.

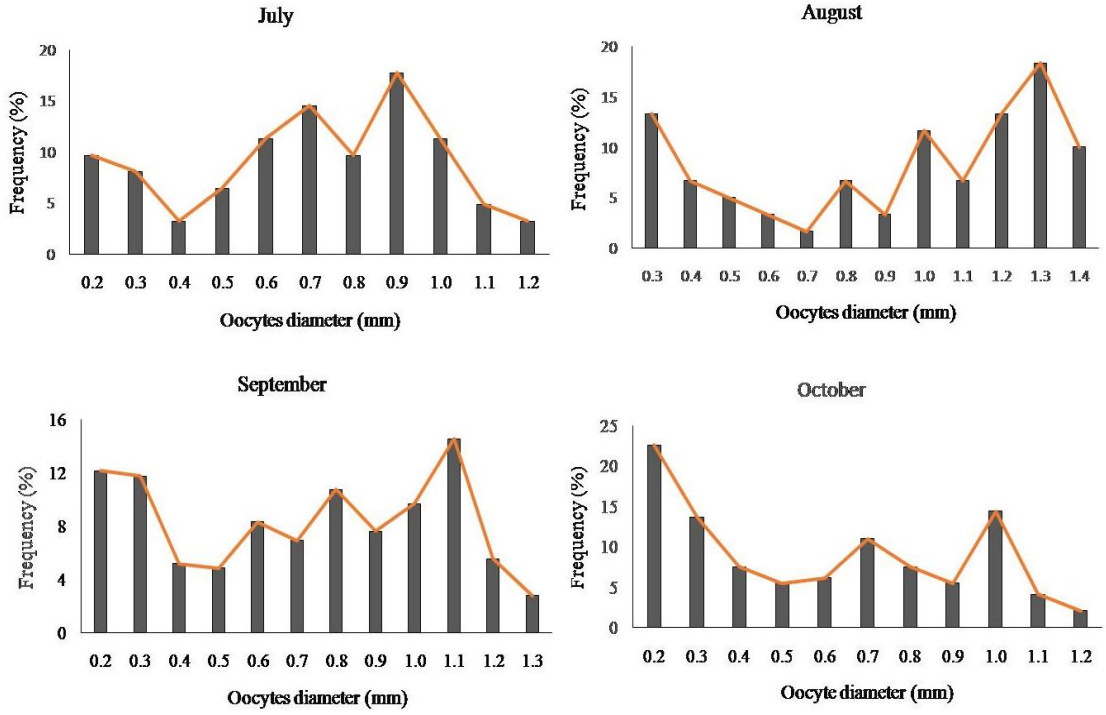

Figure 4. Monthly changes in mean diameters (mm) of oocyte in ripe stage of *Hampala bimaculata*.

Spawning season. The GSI has been extensively adopted as an indicator of the fish spawning season, but its use in reproductive biology research is more applicable when associated with other reproduction indicators for instance macroscopic and histological analysis (Santos et al 2006). Looking at the temporal variation of the GSI and the frequency of maturation stages of *H. bimaculata*, it appears that the fish go into a quiescent condition during the months November to May next year. The females have no mature stage, but only oocytes in the stage of primary growth were found. The cortical alveoli stage and the maturing stage of fish were first observed in June, which reproductive activity was started. An increase in the GSI value in July indicates that is the intensive cell growth process start before spawning. The maximum values of the GSI is a marked increase in GSI values from July to October coincides with those of high percentage of matured individuals indicating of a breeding season from July to October. First spawning activity occurred in August during the first spawned stage of female. Thus, these two methods showed that *H. bimaculata* in the waters of Betung Kerihun National Park has a prolonged spawning season, being restricted mainly from August to October, some spawning may also occur in late July.

Classification of maturity stages are important for the fishery resources assesment. Commonly, it is difficult to distinguish the immature and resting macroscopically, included *H. bimaculata.* The oocytes are not visible and only by histological analyses it is possible to identify each stage accurately. This misclassification has an impact on the estimation of the mature proportion of the stock because resting stage have already contributed to the spawning biomass of that year and are macroscopically considered immature.

In this study, histological analysis of ovarians showed two batches of oocytes in ovaries classified as ripe stage. At the ripe stage, the major part of the ovary was occupied by stage IV oocytes that comprise a synchronous population of larger oocytes, defined as a clutch. However, a large number of previtellogenic and vitellogenic oocytes (stages I, II and III) were also detected among the mature oocytes. The presence of two types of oocytes in ovaries classified as stage V suggested that *H. bimaculata* has a group of synchronous pattern in the ovarian development similar to *A. istanbulensis* (Hamzaoglu et al 2015). At least two groups of oocytes can be identified at the same time during the breeding period of synchronous group (Albieri et al 2010; Pereira et al 2013).

The spawning type is how females release mature oocytes in a reproductive period. Histological observations of the spawned-recovering stage showed that, the post-ovulatory follicles occurred together with a heterogenous population of secondary developing oocytes in the ovaries of the reproductively active females. This strongly suggests that *H. bimaculata* spawns multiple times in a season. According to Honji et al (2006), Chen & Tzeng (2009), Nunez & Duponchelle (2009), the presence of development oocytes of different sizes in spawned stage characterizes the beginning of one or more new spawning cycles until the end of the breeding season which differs at multiple spawners from single spawners. During the breeding season the polymodal distribution of oocyte diameter was found in ripe stage, this may validate *H. bimaculata* being a batch spawner.

Batch spawning is also encountered in other species, *Acrossocheilus fasciatus* (Yan et al 2009), *Luciobarbus capito* (Eagderi et al 2013), and *Alburnus mossulensis* (Keivany et al 2017). Batch spawning can suggest a way of increasing total fecundity in cases where body size might limit the number of mature oocytes a female could hold at any one time. Batch spawning might also understood a strategy to ensure some reproductive success under dynamic environmental conditions (Burt et al 1988). We proposed that batch spawning of *H. bimaculata* should be an adaptive strategy to the habitat conditions, where its habitat in running waters has quite dynamic environmental factors.

Ovarian development in *H. bimaculata* is different compared with the *H. macrolepidota* observation (Abidin 1986). The finding in the previous study on ovarian development for *H. macrolepidota* was similar in the most tropical Teleostei, which oogenesis occurred continuously. The mature female of *H. macrolepidota* was observed

throughout the year, meanwhile this was only observed during spawning season for *H. bimaculata.*

Conclusions. Ovarian development of *H. bimaculata* in Betung Kerihun National Park was classified to five stages. Histological observation indicated that the fish is an iteroparous with a group-synchronous of ovarian development. The spawning season was estimated to be from July to October based on seasonal changes in frequency maturity of females and gonadosomatic index. The polymodal distribution of oocyte diameter during the breeding season indicated that *H. bimaculata* is a batch spawner. This study records new data on reproductive traits of the natural history of *H. bimaculata*. The results may be used to study the management strategies and conservation of the species.

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Authors:
Widadi Padmarsari Soetignya, Tanjungpura University, Faculty of Agriculture, Indonesia, Pontianak, Prof. Hadari Street, Postal Code: 78124, e-mail: fx.widadi.padmarsari.s@faperta.untan.ac.id
Bambang Suryobroto, Bogor Agricultural University, Faculty of Mathematics and Natural Sciences, Department of Biology, Indonesia, West Java, Bogor, IPB Dramaga, Agathis Street, Postal Code: 16680, e-mail: suryobroto@ipb.ac.id
Muhammad Mukhlis Kamal, Bogor Agricultural University, Faculty of Fisheries and Marine Sciences, Department of Aquatic Resources Management, Indonesia, West Java, Bogor, IPB Dramaga, Agathis street, IPB Dramaga, Postal Code: 16680, e-mail: m_mukhliskamal@yahoo.com
Arief Boediono, Bogor Agricultural University, Faculty of Veterinary Medicine, Physiology and Pharmacology, Department of Anatomy, Indonesia, West Java, Bogor, IPB Dramaga, Agathis street, Postal Code: 16680, e-mail: aboedi@yahoo.com

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