Histological investigation on gonad maturation of cultured short fin eel, *Anguilla bicolor* (mcclelland, 1844) in captivity.

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Abstract. *Anguilla bicolor* or commonly known as a shortfin eel is a subspecies of eel in the genus of Anguilla and widely distributed in South East Asia. Present study reveals the first report of the early gonad development of cultured *A. bicolor* in captivity via histological analysis. The total length and body weight of *A. bicolor* were 511 to 614 mm and 209 to 420 g respectively. The result showed all specimens were female with no sign of male or intersexual stage. Through histological analysis, identified female were classified in 1) immature (n=3) by the presence of oogonia (O) and primary germ cell (PG) and also 2) developing stages (n=11) by the presence of previtellogenic-stages oocyte (PVO), oogonia (O) and cortical alveolar oocyte (CAO). Meanwhile, the fin difference index (FDI) and eye index (EI %) ranged from 0.55 to 3.89 % and 1.64 to 4.56% respectively. This study concludes smaller size (511 to 614 mm) of cultured *A. bicolor* is immature, while gonad maturation was already in developing stage for larger size (565-614 mm), hence these range of *A. bicolor* are still unable to be used as broodstock

1. Introduction

Anguillid eel species are generally distributed in tropical and temperate seas. To date, 15 species of Anguilla have been reported and seven subspecies alone can be found in Asia including Malaysia and Indonesia [1]. *Anguilla bicolor* or known as shortfin eel is among the most abundant and common species can be found. This species have an excellent organoleptic qualities and often attain high demand in many parts of the world. In Japan, *A. bicolor* is more preferred for consumption as “unagi kabayaki”, a renowned cuisine of eel rather than other species of Anguilla after *A. japonica*, *A. Anguilla* and *A. rostrate* [2]. It is actually boned and filleted fish, grilled with a sweet Japanese kabayaki sauce, whereby only eel suits to produce this delicacy. Because of the continuous high demand of unagi kabayaki, there is many kabayaki processing factories has been expanded in South East Asia. However, the supply of *A. bicolor* are still highly depending on wild stock, since its artificial breeding techniques is not yet firmly established anywhere. The collection of wild *A. bicolor* has been banned since 2009 right after they are listed under Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2009. Hence, eel has now becoming new emergence of aquaculture species to suppress the exploitation of wild stock [1].

In aquaculture, study of gonad stages of maturation has become increasingly important in fish production, notably in induced spawning purposes. Hence, this study aims to investigate the sex and gonad maturation of *A. bicolor* in captivity in order to serve as primary data for its seed production in the future. There are plenty of studies have been carried out to understand gonad development of eels done including those by [3,4,5,6]. However, the findings on the development pattern of gonad remain a matter of debate among the researches around the world. Contradict to a study done by [7], sex differentiation of eel was related with size of eel (total length) rather than age. Findings by [4] Colombo and Grandi (1996) had revealed body size of *A. anguilla* are relatively smaller than 20 cm was actually undifferentiated, and those beyond 20 cm was female with no clear sign of intersexual
stage and transformation into male stage. However, there is still a lack of study on gonad development, sex determination and relation with body size on tropical eel. Thus, the present study was aimed to investigate on gonad development and sex determination of A. bicolor through histological study and its relation with body size and other morphological characteristics. Fish reproduction information is important as primary guideline not only for aquaculture but to fisheries management as well.

2. Materials and Methods

2.1 Specimens Collection
A total of 14 specimens of cultured A. bicolor were collected alive from eel farming and processing factory, Pt. Iroha Sidat at Bali, Indonesia. All the specimens were identified and categorized in yellow stage where the pectoral fins of specimens was white or grey, green or grey fin and yellow or white belly. Prior to measurement and analysis, the specimens were transferred into laboratory and anesthetized with cold water method.

2.2 Morphological Analysis
Morphological data were recorded including total length (TL), standard length (SL), head length (HL), body weight (BW), body round (BR), horizontal eye diameter (HED), vertical eye diameter (VED), inter-orbital width (IOW), preorbital length (POL), prepectoral length (PPL) and preanal length (PAL) to the nearest 1 mm similar as [8]. Fin Difference Index (FDI,%)

Another index calculated was Eye Index (IE,%)

Where A is the horizontal eye diameter, B is the vertical eye diameter and L is the total length.

2.3. Gonad Maturation and Fecundity
Gonad maturation and fecundity were described according to method of [9], whereby it include measurement of gonad length (GL) in both left and right side. After measurement, weight of gonad (GW) was recorded. The Gonad Somatic Index (GSI) was calculated as follows:

Gonad Somatic Index = (gonad weight/body weight) x 100

2.4. Morphological Study
The eel gonads were initially fixed in Bouin’s solution for 24 hours and later dehydrated in series of ethanol. After that, sample was embedded in paraffin, sectioned to 7 µm thickness and then stained with Haematoxylin and Eosin. Gonad samples were mounted in glass slide and observed under light microscope (Nikon, Eclipse E600, Japan). Gonad maturity of eel was classified based on description developed by [10].

3. Results and Discussion

3.1. Morphological, Fin Index, Eye Index and Gonado Somatic Index
The FDI % ranged from 0.55 to 3.89%, with mean ± SD of 2.40±0.88% (Table 1). [1] found that FDI% A. bicolor bicolor in Peninsular Malaysia within the range of -1.93 % to 2.63 %, mean±SD of 1.06±1.20%. The value was in the range compared to previous study (range; -3 – 3.9% in A. bicolor bicolor by [11]. Therefore, those specimens could be easily identified as the A. bicolor.
Meanwhile, IE% was ranged from 1.64 to 4.56%, with mean±SD of 0.89 ± 0.57% (Table 1). According to [12], eye index or known as ocular index (OI) is very powerful indicator of transformation degree in gonadal development of eel. [13] revealed eye index of *A. bicolor* in range of 5.72 to 6.02% were high as it is approaching sexual maturity. Fully matured *A. bicolor* possess of eye index in range of 12.31% above and ready to leave freshwaters for spawning. The alteration of external morphology of the silver phase eel has been suggested as part of the preparation for the adaptation to the marine setting before their spawning migration occur [14].

### Table 1. Value of indices for gonad somatic index (%), eye index (%) and fin difference index (%) of *A. bicolor* in captivity.

| No | Gonad Somatic Index (%) | Eye Index (IE, %) | Fin Difference Index (FI, %) |
|----|-------------------------|------------------|-----------------------------|
| 1  | 0.47                    | 3.16             | 3.89                        |
| 2  | 0.36                    | 2.85             | 0.55                        |
| 3  | 1.12                    | 2.69             | 1.44                        |
| 4  | 0.31                    | 2.91             | 2.37                        |
| 5  | 0.49                    | 4.56             | 2.56                        |
| 6  | 0.45                    | 2.80             | 1.95                        |
| 7  | 2.23                    | 2.52             | 3.62                        |
| 8  | 1.32                    | 3.30             | 1.61                        |
| 9  | 0.89                    | 3.26             | 2.64                        |
| 10 | 0.85                    | 1.89             | 0.67                        |
| 11 | 2.00                    | 1.64             | 2.14                        |
| 12 | 1.38                    | 2.28             | 2.40                        |
| 13 | 0.88                    | 2.33             | 2.35                        |
| 14 | 0.25                    | 3.00             | 2.68                        |

The TL and BW of *A. bicolor* in the present study was range from 511 to 614 mm and 209 to 420 g respectively. Other morphological characteristics were provided in Table 2. Mean value of Gonado Somatic Index (GSI, %) in the present study was 0.89±0.57. GSI values of female *A. japonica* collected in the East China Sea ranged from 1.3 to 3.5 [15] meanwhile [16] reported GSI value of females *A. japonica* collected from Mikawa Bay and Amakusa Islands ranged from 1.0 to 4.3. Both studies showed that female *A. japonica* were in developing and maturation stages. Compare to the present study, these GSI values were relatively higher. [17] claimed that GSI value of female *A. bicolor bicolor* that collected from the Waters of Segara Anakan ranged from 0-3% and believed that the immature female and they still require longer time to develop as sexually mature adults before spawn in the ocean. Therefore it could be suggested that, GSI value of immature and developing stage of female *A. bicolor* ranged from 0-3%.
3.2. Histological Analysis- Gonad Maturation

Histological observation revealed that the samples in the present study were ovary because of the presence of oocytes. Based on gonadal classification by [10], the present female specimens were classified in immature and developing stages. The TL of immature specimens was 511 to 565 mm. Based on histological observation, the immature specimens were defined as presents of oogonia (O) and primary germ cell (PG) (Figure 1a,b). Similar with [10] Tongnunui et al. (2016), the immature A. bicolor in the coastal waters of Thailand was in range of 302 – 742 mm (SL). This stage also identified as oogonia and chromatin nucleolar stage where the ovary was composed of bunches of oogonia, oocytes which were believed to further development of ovarian sexual cells.

Oogonia (marked as arrow in Figure 1a,b) are the smallest germ cells with no noticeable boundaries, whereby the cells found either solitary or in bunches of lamellae adjacent to the germinal epithelium and was used to describe the gonadal development. [18] found that gonads of A. australis in the range of 75 – 100 mm in TL were consisting of two to six primordial germ cells. Meanwhile in 100 – 200 mm A. australis, gonads shift into a lamellar shape containing 5 – 50 primordial germ cells which will again increase to bunches. As eels larger in size (75 – 150 cm) the gonads tend to expand to lamellar shape and the density of primordial germ cells will eventually rise. In 150 – 200 mm eels several primordial germ cells will increase to bunches of primordial germ cells and later lamellae also will increases in size.

Meanwhile the developing stage was well-defined by ovaries that possessed previtellogenic-stages oocyte (PVO), with oogonia (O) and cortical alveolar oocyte (CAO). The TL of developing specimens were normally between 565 to 614 mm. [10] found that A. bicolor in developing stages was 421 to 885 mm. At this time, the ovaries were enlarging because of the deposition of trophic substance such as yolk and fat. Early stage of ovaries were characterized by lamellae comprising double rows of previtellogenic oocytes at the perinucleolus stage, running from the vascular to the germinal region by a thin mesogonadium [4]. Figure 1e showed the oocytes in the previtellogenic stage, which comprising a large central round nucleus (or germinal vesicle), multiple nucleoli, and ample cortical alveoli that completely filled the cytoplasm. As the size of gonad upsurge, the cortical alveoli oocytes (CAO) are

| No. | TL (mm) | SL (mm) | HL (mm) | BW (g) | BR (mm) | HED (mm) | VED (mm) | IOW (mm) | POL (mm) | PPL (mm) | PAL (mm) | PDL (mm) |
|-----|---------|---------|---------|--------|---------|----------|----------|----------|----------|----------|----------|----------|
| 1   | 511     | 493     | 61      | 227    | 88      | 4.9      | 5.0      | 2.2      | 9.4      | 68       | 232      | 229      |
| 2   | 514     | 495     | 61      | 243    | 85      | 4.2      | 4.6      | 1.2      | 8.5      | 62       | 230      | 193      |
| 3   | 532     | 518     | 70      | 331    | 100     | 5.2      | 5.5      | 2.0      | 9.4      | 68       | 239      | 218      |
| 4   | 535     | 524     | 62      | 282    | 92      | 4.9      | 5.0      | 3.0      | 8.1      | 64       | 226      | 193      |
| 5   | 542     | 524     | 66      | 209    | 83      | 8.5      | 8.7      | 4.3      | 20.0     | 76       | 241      | 226      |
| 6   | 542     | 530     | 67      | 352    | 103     | 6.3      | 6.6      | 3.1      | 10.1     | 81       | 270      | 260      |
| 7   | 555     | 542     | 68      | 387    | 110     | 4.2      | 4.4      | 2.4      | 9.1      | 71       | 259      | 235      |
| 8   | 560     | 547     | 61      | 327    | 100     | 3.4      | 3.7      | 2.0      | 7.0      | 57       | 197      | 189      |
| 9   | 565     | 554     | 64      | 420    | 112     | 3.3      | 3.5      | 1.8      | 7.0      | 64       | 237      | 233      |
| 10  | 592     | 583     | 71      | 378    | 105     | 4.3      | 4.6      | 1.6      | 9.4      | 75       | 255      | 241      |
| 11  | 593     | 581     | 69      | 354    | 108     | 5.2      | 5.5      | 2.7      | 10.0     | 78       | 257      | 245      |
| 12  | 601     | 587     | 73      | 335    | 106     | 4.2      | 4.5      | 2.4      | 9.7      | 72       | 241      | 222      |
| 13  | 614     | 604     | 74      | 370    | 107     | 6.6      | 6.3      | 2.3      | 8.1      | 66       | 249      | 223      |
| 14  | 615     | 603     | 67      | 394    | 108     | 2.8      | 3.0      | 2.5      | 5.1      | 77       | 240      | 228      |

Table 2. Morphometric characteristics of A. bicolor in captivity.
increased in number and size. Yolk globules were visibly can be observed because of intensively stained.

![Figure 1](image)

**Figure 1.** Photomicrograph of gonad sections of *A. bicolor* in captivity. (A) Gonad section of *A. bicolor* in immature stage. Large number of Oogonia (O), primordial germ (PG) chromatin nucleolus (marked as arrowhead) with nucleoli extrusions in the ovary of *A. bicolor*. (B) Gonad section of *A. bicolor* in immature showing Oogonia (O), chromatin nucleolus (marked as arrowhead) and perinucleolus oocytes (C) Gonad section of *A. bicolor* in developing stage. Ovarian lamella shaped able to observe. Oocytes in the chromatin-nucleolus phase exhibiting a large nucleolus in the nucleus known as previtellogenic oocyte (PVO). (D) Oocyte in the perinucleolar phase possessing chromatin and a small nucleolus. (E) Vitellogenic oocyte (VO) in the cortical alveoli phase with cortical alveoli in the cytoplasm and around the nucleus. (F) Vitellogenic oocyte (VO) showed the liquefaction of the yolk sphere with large vacuoles. (G) The number of liquefaction VO was increase. (H) Yolk vesicle oocyte with central nucleus (marked as asterisk) in developing of *A. bicolor* gonad. Scale bar: 2.0 mm.

4. **Conclusion**

In conclusion, cultured *A. bicolor* in the captivity with total length and body weight at 511 to 615 mm and body weight 209 - 420 g respectively were female (immature and developing stages). There are no sign of intersex and male stage was observed. In the next study, variation of size is required to reveal the process of gonad development, sex differentiation and its relation with body size should be carried out.

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