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Early Sex Differentiation of Climbing Perch (*Anabas testudineus* Bloch.): A Pathway to Feminization

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Abstract

The phenomenon of sexual dimorphism in climbing perch, which shows that female fish grow faster than males, underlies the development of mono-sex culture. Female mono-sex culture is more applicable for farmers by crossing neo-male fish with normal females. The timing of sexual differentiation in climbing perch is still unknown. It is very useful in sex reversal procedures to produce neo-male climbing perch. This study revealed the time and status of climbing perch sexual differentiation. Ten samples of climbing perch from the spawning of five pairs of parents were taken from the nursery pond at 10–29 days post-hatching (dph). Samples were prepared through a histology preparation procedure. Observations of the structure and characteristics of the gonads were carried out using a light microscope and analyzed histologically. The results indicated that gonad samples aged 10, 11, 12, 13, 15, and 16 dph showed primordial germ cells surrounded by somatic tissue forming genital ridges and mitotic division. Meanwhile, the gonads begin to differentiate as ovaries found at 18 dph with the presence of oogonia and ovarian cavities. Gonads aged 20–21 dph increasingly showed single oogonia cells (size 20–37.5 µm), germ cell cysts, genital ridges, oocytes undergoing the vitellogenesis process, perinucleolar oocytes, and the formation of the ovarian cavity. Sex differentiation of climbing perch was predicted from 18–21 dph. This conclusion underlies that the sex reversal procedure in climbing perch must be carried out before 18 dph.

Keywords: sex differentiation, sex reversal, climbing perch

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INTRODUCTION

Climbing perch is a local-specific fish distributed in India and most of Southeast Asia (Ahmad *et al*., 2019). This fish has become a commercial commodity farmed traditionally in cages and ponds (Udin *et al*., 2016; Sarker *et al*., 2014; Mondal *et al*., 2010), as well as intensively in tanks with a bio floc system (Ahmadi *et al*., 2021). Climbing perch culture has been constrained by sexual dimorphism related to growth, which affects the size diversity of harvested fish and farmers' income.

Studies on the growth of wild climbing perch by estimating age show that the growth rate of female climbing perch is higher than that of males (Nagris, 2010; Behera *et al*., 2015a). This is in line with Hidayat *et al*. (2016), stating that female climbing perch is 1.42 times the growth of male climbing perch. This is different from tilapia, where male tilapia grows faster than female (Chakraborty and Banerjee, 2010; Bhatta *et al*., 2012b). Furthermore, the production level of mono-sex male tilapia culture is around 10% higher (Nguyen and David, 2000) than mixed male-female tilapia. The female mono-sex culture of goldfish in Central Europe had a significantly higher harvest weight of 29.7% compared to mixed-sex populations (Kocour *et al*., 2005).

The phenomenon of female climbing perch growing faster than males is the basis for developing broodstock that can produce mass mono-sex female climbing perch. Mass production of female mono-sex fish can be carried out using gynogenesis techniques (if the female sex chromosomes are homogametic XX/WW) (Nakamura, 2013), directing sex differentiation or sex reversal using the estrogen, and crossing neo-male male fish (XX fish that produce sperm) with normal female (XX) (Piferrer, 2001). Technically, the third method is more applicable to farmers. Functional males can be produced

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through sex reversal using the hormone 17α-methyltestosterone (MT) through immersion or oral administration.

Sex reversal involves a hormonal process to direct fish sex during sexual differentiation. Sex reversal can be done because sex determination in fish is a flexible process (Penman and Piferrer, 2008) during the differentiation period. However, it is difficult, even impossible, to do after the differentiation period (Nakamura *et al*., 2003). Sexual differentiation is transforming undifferentiated gonads into testes or ovaries, which is influenced by genetic information, the environment, or both so phenotypically produces male or female individuals (Piferrer, 2011; Hidayatulloh *et al*., 2021). In this case, induction of sex reversal is easy to do by giving hormones to the fish during the sexual differentiation period. Therefore, differentiation in climbing perch was important to investigate the timing of fish sex differentiation, which is useful as a basis for determining the age of the fish. In addition, the differentiation was to determine the correct method for administering the MT hormone in the sex reversal process (oral or immersion) in the context of developing female mono-sex climbing perch farming.

MATERIALS AND METHODS

Ethical Approval

This study was conducted under following the ethical consideration and the approval of the Faculty of Fisheries and Marine Sciences IPB University (Letter of Assignment from Vice Dean No. 168/IT3/PP/AP/2014, No. 386/IT3.3/PP/AP/2015, and No. 462/IT3.3/PP/AP/2015).

Study Period and Location

This study was conducted from June to July 2014 at the Freshwater Aquaculture Development Center Mandiangin (Banjar Regency, South Borneo, Indonesia).

Fish Preparation

Climbing perch fish were obtained from spawning five pairs of female green variety climbing perch (females' weight of 89.21 ± 18.37 g, and males' weight of 60.27 ± 7.56 g). Before spawning, male and female fish are kept separately for two days and are not given food during this process. The broodstock were injected with Ovaprim® (Syndel Laboratories Ltd., Canada), at a dose of 1 mL/kg fish weight (Rahmadi *et al*., 2021) of the broodstock intramuscularly, then each pair of broodstock was spawned in a continuously aerated 60×40×50 (l×w×h) cm^3 aquarium. After spawning, the female was moved to a broodstock rearing tank, and the eggs are incubated in the same aquarium for 18–20 hours at a temperature of 28–30ºC. Furthermore, 2-day-old larvae are stocked in 5×2×1 m^3 nursery tanks (water depth = 60 cm). Preparatory management for larval rearing was carried out by adopting natural food growing procedures as a food source for the early stages of larvae by spreading dolomite at a dose of 50 g/m^2 and fertilizing with chicken manure at 0.75 kg/m^2 (Bhuyan *et al*., 2018), contains macro and micro elements such as nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), and manganese (Mn). Along with eating natural food available in the nursery pond, larvae were trained to eat commercial crumble food (Matahari Sakti, Indonesia) with a protein content of > 40%. Fishes were fed three times a day with a feeding rate of 10% of the total body weight. Initially, fishes were fed with a crumble feed size < 0.4 mm at the age of 5 days post-hatching (weight 0.0038 ± 0.0003 g/individual) until 20 dph (weight 0.1023 ± 0.0165 g/individual), then fed with a crumble feed size 0.4–0.7 mm until the age of 26 dph. Water changes of 10% of the nursery water volume were carried out every 10 days.

Experimental Design

This study used a descriptive research design to determine the initial time of sexual differentiation in climbing perch. Observations on the timing of sexual differentiation were carried out on fish from the larval to juvenile stages during the nursery process. This study used seeds aged 10–26 days post-hatching taken from traditional nursery ponds with a sample size of 10 individuals per day. Fish samples at this age to be classified in the flexion and post-flexion phases

aged 5–19 dph to juveniles aged 19–35 dph (Morioka *et al*., 2009) were determined by considering the sexual differentiation scheme in teleost fish at the age of 5–40 dph (Kobayashi, 2010). Fish body weight was measured using a digital scale (smallest scale = 0.0001 g) and total length using a caliper (smallest scale = 0.05 mm). Samples were prepared through a histology procedure on the part containing the gonads, according to Genten *et al*. (2009).

Sex Differentiation Analysis

The structure and characteristics of the gonads are known by observing the gonads histologically (Nakamura, 2013; Genten *et al*., 2009; Pandian, 2013). Observations were carried out using a light trinocular microscope (Olympus® CX41, Japan), with magnifications of 40×, 100×, 400×, and 1000×. The results of observations of the gonads of test fish in sex differentiation study were analyzed histologically. Histologically, undifferentiated gonads only show primordial germ cells (PGCs) in small numbers and were initially isolated among the somatic cells scattered throughout the gonad. Furthermore, differentiated gonads showed changes in gonadal tissue morphology; as ovaries wee characterized by the formation of the ovarian cavity and the occurrence of germinal cell meiosis, as testes were characterized by the formation of testicular ducts and testicular lobules (Mazzoni *et al*., 2017). The gonadal differentiation rate (%) was calculated by comparing the number of differentiated gonads with the number of samples for each fish age observed.

RESULTS AND DISCUSSION

Histological identification of gonads was successfully carried out on climbing perch aged 10, 11, 12, 13, 15, 16, 18, 20, 21, 23, and 26 dph, determining the differentiation status, as shown in Table 1. Histological analysis of fish gonad samples aged 10–16 dph shows 100% undifferentiated status, and aged 18–20 dph shows 20%–40% differentiated gonads. The climbing perch gonads are fully differentiated starting at the age of 21 dph.

The description of the fish gonads for each individual and age shows different gonad sizes, ranging from 6.6 μm wide × 15.4 μm long to 48.4 μm wide × 80.9 μm long. Sexual differentiation is determined by observing the development of the number of PGCs, somatic genital ridges, and the appearance of the ovarian cavity as a characteristic of female status.

The position of the gonads (Figure 1) shows that the gonads are in the body cavity, hanging from the dorsal peritoneal wall, are a small part of the contents of the body cavity, are located on the ventral side of the gas bladder, around which are the liver and intestines (Figure 1A). There are a pair of gonads supported by mesogonium (Figure 1B).

Histology of tissue sections on gonads aged 10, 11, 15, and 18 dph shows that the gonads are supported by mesogonium, have single PGCs or cyst cells surrounded by somatic tissue forming a genital ridge, and have blood vessels and show mitotic division (Figure 2). Morphologically, PGCs of climbing perch have a round and large shape, with a core size of 1.1–2.4 μm and varying numbers (2–11 pieces). Gonads aged 15 dph (Figure 2C) showed the least number of PGCs (2 pieces), the size tended to be larger (5.8 μm) compared to gonads aged 10, 11, and 18 dph (2.4–2.9 μm). This difference in size and number of PGCs shows the characteristics of germ cells (GC) at an early stage as oogonia or spermatogonia. However, sexual differences cannot yet be known with certainty, so they are interpreted as undifferentiated status.

Figure 3 presents a cross-section of the gonads that have differentiated as ovaries. Differentiation in fish aged 20, 21, 23, and 26 dph shows gonads with soft and connective tissues. Gonads aged 20 and 21 dph (Figure 3A and Figure 3B) begin to have single oogonia cells (20–37.5 μm) and GC cysts undergoing prophase division and changes in the characteristic genital ridges that develop laterally. The justification for GC morphology as the large number of GCs characterizes an initial stage called oogonia and shows the beginning of the ovarian cavity (OC).

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Oocytes undergoing vitellogenesis were found in gonads aged 23 and 26 dph (Figure 3C and Figure 3D). The egg yolk was stored in the cytoplasm, which is protected by a chromatin layer, besides, oocytes are found in the perinucleolar phase. These gonads indicate the occurrence of gametogenesis, indicated by the appearance of meiotic divisions, and the development of the size of the oogonia (2.0–3.7 μm). The appearance of the OC in Figures 3C and 3D is increasingly clear, indicating the morphology as an ovarian cavity.

Study on sexual differentiation in climbing perch was successful in revealing the timing of the sexual differentiation based on the histological characteristics of the gonads. Histology of the gonads of climbing perch aged 18 dph (Figure 1) shows the position of a pair of gonads hanging from the dorsal peritoneal wall into the body cavity, supported by thin somatic cells (mesogonium) (Nakamura, 2013). The undifferentiated gonads are characterized by PGCs in the form of single nuclei and large spheres surrounded by somatic cells (Bhatta *et al.*, 2012a; Tong *et al.*, 2010; Yön and Akbulut 2015) and indicate the presence of mitotic division of GCs (Okuthe *et al*. al., 2014). This is by the histology of the gonads in fish samples aged 10–18 dph (Figure 2) which are still classified as undifferentiated. Undifferentiated gonads are divided into two types based on the number of GC gonia, namely GCs with greater numbers are ovaries while GCs with small quantities tend to differentiate into testes (Pandian, 2013). However, in loach fish (*Misgurnus anguillicaudatus*, Cypriniformes: Cobitidae), gonadal dimorphism can be seen through gene expression methods in gonads with few GCs, which are gonads with differentiated status as ovaries (Fujimoto *et al.*, 2010).

Table 1. Identification of the gonad status of climbing perch aged 10–26 dph

Fish age (dph)	Total length (cm)	Weight (mg)	Status		Diff.[1] rate	Gonads type
			Diff. (individual)	Undiff. (individual)		
10	0.5 ± 0.05	4.35 ± 0.82	0	10	0%	-
11	0.6 ± 0.06	5.16 ± 0.96	0	10	0%	-
12	0.8 ± 0.05	5.42 ± 0.94	0	10	0%	-
13	0.8 ± 0.05	5.83 ± 0.97	0	10	0%	-
15	0.7 ± 0.19	9.27 ± 2.45	0	10	0%	-
16	1.0 ± 0.11	13.80 ± 4.94	0	10	0%	-
18	1.1 ± 0.13	22.59 ± 10.16	2	8	20%	ovary
20	1.3 ± 0.11	38.39 ± 9.42	4	6	40%	ovary
21	1.4 ± 0.10	47.45 ± 12.07	10	0	100%	ovary
23	1.5 ± 0.14	66.74 ± 16.17	10	0	100%	ovary
26	1.7 ± 0.22	107.85 ± 33.66	10	0	100%	ovary

dph= days post-hatching, Diff= Differentiated gonads, Undiff= Undifferentiated gonads.

The histology of the gonads of climbing perch aged 20, 21, 23, and 26 dph (Figure 3) shows that the oogonia are in the gonad tissue, associated with somatic cells, or remaining isolated. This is in line with Mazzoni *et al*. (2017), stating that the characteristics of oogonia cells are oval and have large and round nuclei with one or more clear nucleoli, showing little cytoplasm with granulation. Meanwhile, oocytes have a more basophilic nucleus than oogonia, and there is a decrease in the number of granules in the cytoplasm. The development of the genital area, a greater number of GCs, meiotic division occurring in oocytes, a perinucleolar phase, and the presence of an ovarian cavity structure describe clear criteria for the initial phase of gonad development as ovaries (Arezo *et al.*, 2007; Nakamura, 2013; Cho *et al.* al., 2014, Ye *et al.*, 2019). Sex differentiation occurs flexibly because PGCs have a high level of elasticity toward sexual bipotency (Okutsu *et al.*, 2006). At the next stage of gonad development, they develop into differentiated gonads at a more mature age. Apart is controlled through hormonal activity (GnRH)

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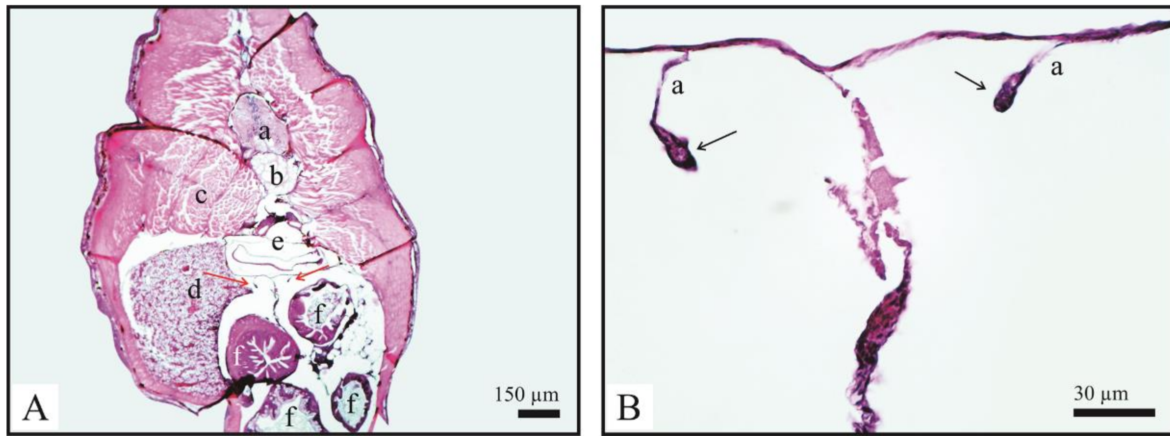

Figure 1. Histology of climbing perch aged 18 dph, yellow arrows indicate the position of the gonads. (A) Cross section of the fish body; (a) spine, (b) blood vessels, (c) muscle tissue, (d) liver, (e) swim bladder, and (f) intestines. (B) a pair of gonads hanging from the dorsal peritoneal wall of the body cavity, (a) connected by the mesogonium.

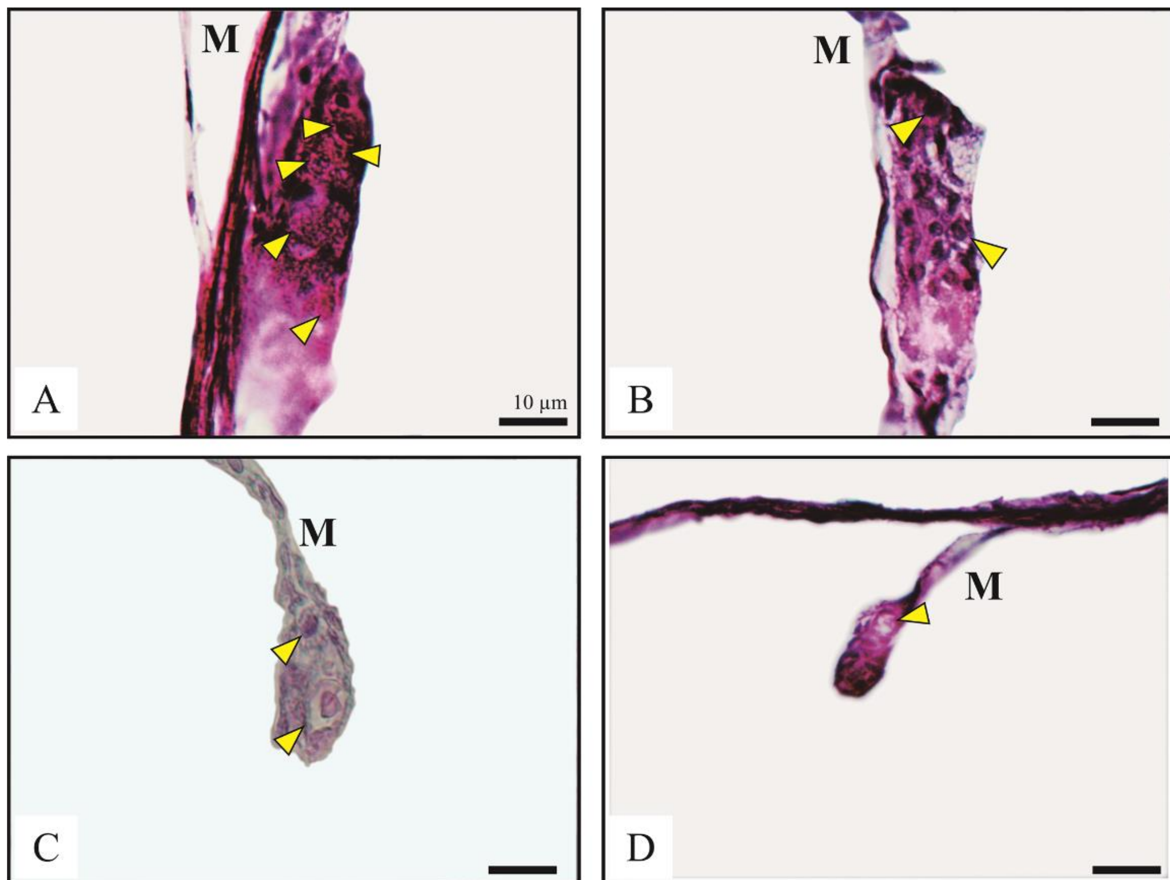

Figure 2. Cross section of undifferentiated gonads of climbing perch, aged (A) 10 dph, (B) 11 dph, (C) 15 dph, and (D) 18 dph. M = mesogonium, arrow = primordial germ cell. Scale = 10 µm.

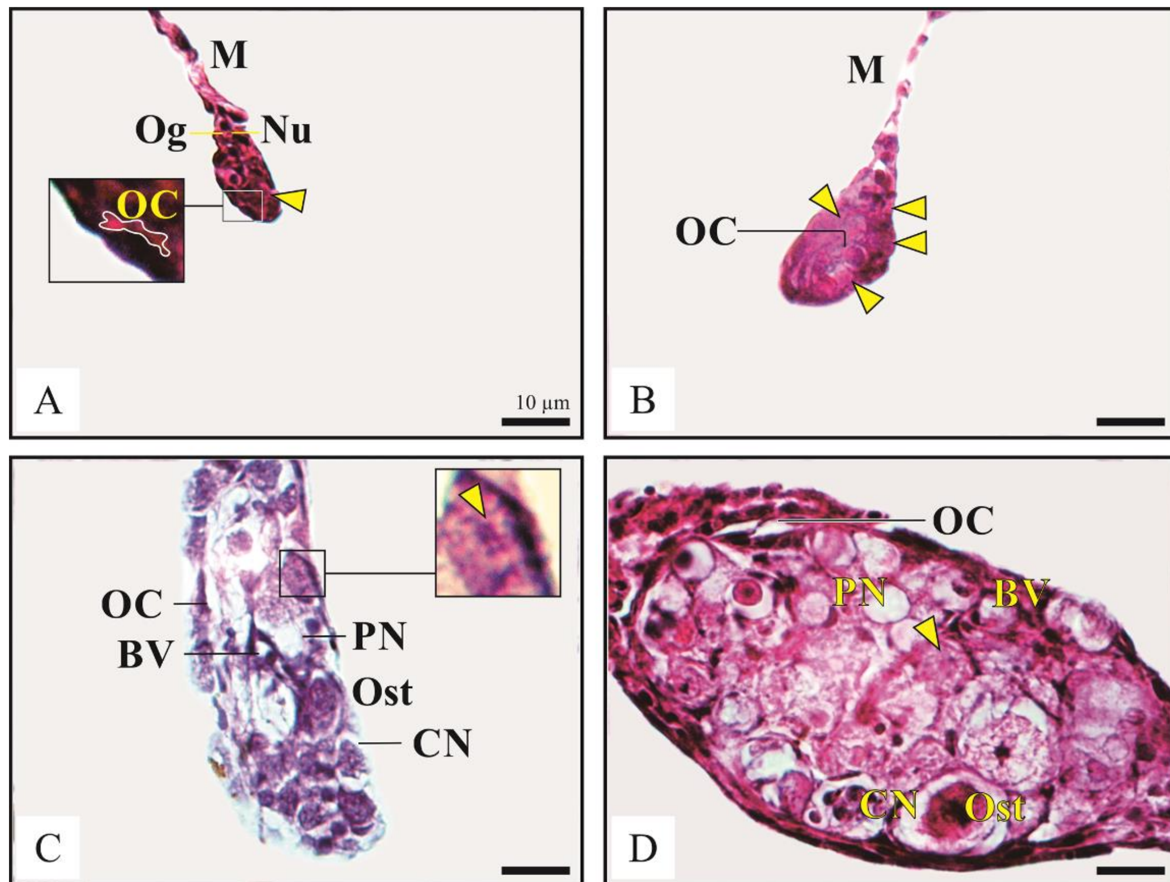

Figure 3. Cross section of the differentiated climbing perch gonads, aged (A) 20 dph, (B) 21 dph, (C) 23 dph, and (D) 26 dph. M = mesogonium, Og = oogonia, Ost = oocyte, OC = ovarian cavity, N = nucleolus, CN = nucleolar chromatin, PN = Perinucleolus, BV = blood vessels. Arrows indicate meiotic division. Scale = 10 µm.

by the pituitary gland (gonadotropins) from being influenced by genetic and environmental factors, the differentiation process and neuroendocrine glands (Heule *et al*., 2014) which are ontogenically present in the early stages of larval development (de Jesus *et al*., 2013).

The critical period of sexual differentiation during gonad development in gonochoristic fish is influenced by the ratio of the absolute amounts of estrogen and androgen, as well as the type of steroid receptor (Piferrer and Guiguen 2008), where endogenous estrogen induces the formation of ovaries. Meanwhile, the lack or absence of estrogen determines the formation of testes (Nakamura *et al*., 2003). Steroids (estrogens and androgens) are produced from the synthesis process in steroid-producing cells (SPC), which are in the theca layer surrounding the oocyte (ovary), or Leydic cells in the interstitial tissue between spermatogenic GC cysts (testes) (Murata *et al*., 2011; Sakai *et al*., 2008).

Studies on sexual differentiation have been carried out in several types of fish. Grayling fish (*Thymallus thymallus*) showed completely undifferentiated gonads at the age of 51 days post-fertilization (dpf), and differentiation as testes was found at the age of 79 dpf (Maitre *et al*., 2017). Furthermore, initial gonad differentiation of Mexican snook (*Centropomus poeyi*) was reported to occur at 178–213 dph and was fully differentiated at 355–367 dph. Spermatogenesis was found at 355–367 dph (Vidal-López *et al*., 2018). Histology of the gonads of medaka fish (*Oryzias latipes*) aged 10 dph showed female and male differentiation, revealing the presence of germline cysts resulting from oogonium division in the gonad. Ovarian cavities and sperm were visible at the age of 50 dph (Zhang *et al*., 2013). Morphological differentiation of Atlantic salmon

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(*Salmo salar*) ovaries, as indicated by the formation of germ cell cysts, was visible starting at the age of 52 dph. Moreover, the ovarian phenotype was fully visible at the age of 79 dph (Brown *et al*., 2022). Sexual differentiation in *Tachysurus ussuriensis* catfish occurs more quickly in female fish. The ovarian cavity begins to form at 21 dpf and is complete at 25 dpf. Meanwhile, mitosis of germ cells and blood vessels appears as a marker for gonads as testes at the age of 30 dpf. In general, this shows that the gonads are differentiated as ovaries or testes at 17–21 dpf (Pan *et al*., 2017). The period for catfish gonad differentiation is like that of the climbing perch in this study.

Currently, studies on sexual differentiation in several types of fish have been carried out using molecular markers. The critical time for sex determination in goldfish (*Cyprinus carpio*) was known based on the expression of female marker genes (cyp19a1a and foxl2) and male markers (amh, dmrt1, and sox9b), which increased significantly at 30–40 dph (Jiang *et al*., 2019). Vizziano *et al*. (2007) reported that the gonads of rainbow trout (*Oncorhynchus mykiss*) differentiated after 35 dph based on the detection of markers sox9a2, dmrt1, cyp11b2.1, amh as testes and foxl2a, foxl2b, hsd3b1, inha as ovaries. However, there was a gonad sample aged 35 dph which histologically showed undifferentiated characteristics, still in the form of somatic cell structures with a few germ cells. Studies on sexual development in zebrafish (*Danio rerio*) aged 2–40 dph show the involvement of dmrt1 in the timing of sexual differentiation at the early stages of the gonad as evidenced by the first significant peak of gene expression at the age of 10 dph (Jørgensen *et al*., 2013). Dmrt1 detects testicular differentiation in medaka fish (Zhang *et al*., 2008). Dmrt1 indicates testicular differentiation, and P450arom detects ovarian differentiation in tilapia (Nagahama, 2005; Kobayashi *et al*., 2008). In addition, the expression of foxl2 in the ovaries and Dmrt1 in the testes occurs during early gonad differentiation (5–6 dph) of tilapia and becomes a critical period for undifferentiated gonads (Ijiri *et al*., 2008). Foxl2 expression is directly influenced by temperature (Baroiller *et al*., 2009) and activating cyp19a1a, which characterizes the ovary (Guiguen *et al*., 2010). In addition, sexual differentiation in zebrafish is known through the expression of the ftz-f1 gene (Hofsten and Olsson 2005), or cyp19a1a, which marks ovarian differentiation, and the expression of the sox9a and amh genes which mark testicular differentiation (Siegfried and Nüsslein-Volhard 2008).

The description of sexual differentiation of climbing perch aged 10–26 dph in this study is important information in the sex reversal procedure. Although the study results can only provide differentiated gonads of females starting at 18–21 dph, this can provide an initial picture of the timing of sexual differentiation in climbing perch. Study has not found differentiated male gonads until the age of 26 dph, or there is a suspicion that sexual differentiation in climbing perch occurs more quickly in female fish. Suppose more in-depth study is needed regarding sex differentiation. In that case, sex-related molecular markers can be applied to climbing perch to produce a clearer picture of sex differentiation. Nakamura *et al*. (2003) stated that induction of sex reversal is easy to do by giving hormones to target fish during the differentiation period but is very difficult, even impossible to do after the differentiation period has passed. This is caused by GC and somatic cells losing their sexual bipotential capabilities after the gonads have differentiated into ovaries or testes. In this case, information on the eating habits of climbing perch must also be known to determine the sex reversal induction strategy. Amornsankum *et al*. (2005) stated that climbing perch larvae aged more than 11 dph (average total length 5.51 mm) only consumed Moina. Then, larvae aged 14–15 dph (average total length 7.34–12.60 mm) consumed Moina and artificial feed. After 16 dph, the climbing perch completely consumes artificial feed. This pattern occurs in controlled climbing perch nurseries. Therefore, administering hormones orally or by immersion for sex reversal in climbing perch is considered the initial stage of feeding with powdered pellets in the climbing perch hatchery procedure. Generally, this study provided an early important piece of information

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on the development of female mono-sex climbing perch culture technology. Based on this study which can only reveal the differentiation of female gonads, further investigation is necessary to find out when male differentiation occurs.

CONCLUSION

The gonads of climbing perch differentiate at 18–21 days post-hatching, histologically marked by the development of genital ridges as the number of PGCs increases and the formation of the ovarian cavity. Therefore, hormone induction treatment for sex reversal of climbing perch through oral administration must be carried out before the age of 18 dph. Further study needs to be done to determine the right hormone dose to produce neo-male broodstock.

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AUTHORS' CONTRIBUTIONS

All authors have contributed to the final manuscript. RH devised the main conceptual ideas, collected the data, drafted the manuscript, and designed the figures. Meanwhile, OC and AA improved conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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