ELUCIDATING THE GONADAL DEVELOPMENT AND SEX DIFFERENTIATION OF THE RICE PADDY EEL *Monopterus albus* (Zuiew, 1793), BASED ON GONADAL HISTOMORPHOLOGY AND STEROID HORMONE LEVELS

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ABSTRACT The establishment and survival of a species population in their breeding ground can be attributed to reproductive behavior and adaptive capacity. The rice paddy eel *Monopterus albus* is an introduced fish in the Philippines. It is a protogynous hermaphrodite which has a natural ability for sex reversal. This study described the histomorphology of the different gonadal maturation stages and measured the concentration levels of the steroid hormones 17ß-estradiol (E2) and testosterone (T) in the blood plasma during sexual development. This paper aimed to better understand the gonadal development and sex differentiation of the rice paddy eel as a hermaphroditic fish. Samples were collected randomly from farm ponds located in three provinces within the island of Luzon, Philippines. Then, the fishes were segregated by sex by observing the presence or absence of ovarian and testicular tissues. Histological analysis of the gonad showed that immature, maturing and mature female gonad exhibited oocytes at different stages. The immature ovaries consisted mainly of perinucleolar oocyte clusters in the previtellogenic stage, whereas the maturing ovaries contained oocytes in the cortical alveoli stage and early vitellogenesis phase. Meanwhile, the oocytes in the mature ovaries were mostly in the advanced vitellogenic stage with a few cortical alveoli and perinucleolar stages. On the other hand, the intersex gonad showed the existence of both ovarian and testicular tissues. The male gonads contained spermatogenic cells and testicular lobules. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration levels of gonadal steroid hormones. Differences in the concentration levels of the two hormones were evident across all stages of gonad maturation, although there were no significant differences between the groups. The functional role of these hormones might be in vitellogenesis and spermatogenesis that lead to the development of sex-changing gonads. This reproductive characteristic may contribute to understanding the reproductive success of this hermaphroditic fish.

Keywords: 17ß-estradiol, histomorphology, intersex, *Monopterus albus*, testosterone.

1. INTRODUCTION

Monopterus albus, commonly known as the rice paddy eel, is native to Asian inland waters (Rosen & Greenwood, 1976; Rainboth, 1996; Cheng et al., 2003). It belongs to the Synbrachidae family with an elongated body, short tail and tapering to a point (Rainboth,

1996). It has no scales, and its fins are reduced to skin folds (Kottelat et al., 1993) with a wide mouth and small rounded eyes (Cheng et al., 2003) (Figure 1). Guerrero (2011) has reported this invasive species in some rice paddies areas in Luzon, Philippines. This exotic fish is a popular dish in most Asian countries.

M. albus exhibited no sexual dimorphism (Liem, 1963) and is classified as a protogynous hermaphrodite; a sequential species that develops first as female and changes to male naturally during its life cycle (Liem, 1963; Chan 1967; Matsumoto et al., 2011; Angelis et al., 2015). Common marine fishes that experience sex change are wrasses, anguillid eels and groupers, born females and later change to males during reproductive maturation (Gailard et al., 2004). Devlin & Nagahama (2002) stated that sex transformation in fish has evolved to provide maximum reproductive output such as increased egg quantity and quality, enhanced competitive fertilization and survival progeny under parental care.

Several publications have reported on the reproductive characteristics of *M. albus*, including the structure of its gonad during sex reversal (Chan & Phillips, 1967), sex reversal as a natural process (Liem, 1963), mating system and the size advantage of male (Matsumoto et al., 2011). Furthermore, since *M. albus* is a protogynous hermaphrodite, it is a suitable fish species for studies related to sexual differentiation (Zhou et al., 2002).

Gonad differentiation in fish requires changes in steroidogenesis, where steroid hormones act as natural inducers of sexual development (Yamamoto, 1969). This hormone is essential in various reproductive processes such as gametogenesis, sex differentiation, embryonic development and reproductive behavior regulation in fishes. Estrogen is a sex steroid hormone produced by the ovary in teleost fish that regulates oocyte growth, while androgen is produced by the testis and involved in spermatogenesis regulation (Nagahama, 1994). Several studies have identified the roles of these hormones in the gametogenesis regulation of teleost fish. However, to date, no study has yet to elucidate the gonadal differentiation of *M. albus* as it undergoes sexual maturity based on levels of sex hormone during differentiation.

To better comprehend the gonadal development and sex differentiation of the hermaphroditic fish *M. albus*, this study described the gonadal histomorphology at different gonad maturation stages along with the plasma concentration levels of the steroid hormones 17ß-estradiol (E2) and testosterone (T). Furthermore, this study will provide valuable information on *M. albus'* reproductive capacity to understand its life cycle and invasive capability better. In addition, these findings may help implement effective management measures involving this potentially invasive fish.

2. METHODS

2.1 Sampling collection and identification

Fish samples were collected in July 2018 from farm ponds located on the island of Luzon, Philippines, which includes provinces of Nueva Ecija (Latitude 15^{o} 13' 27.8N; Longitude 120^{o} 55' 13.06 E), Bulacan (15^{o} 8' 3.72 N; 120^{o} 59' 22.7 E) and Laguna (14^{o} 13.376^{o}N; 21^{o}19.758'E) (Figure 1). An electrofishing backpack gear that produces a 12 V electric current was used to stun and immobilize the fish temporarily. The electrofishing technique is accepted as the most effective way to collect fish species (Mazzoni et al., 2000).

Samples identification was verified via voucher specimens sent to the Philippine National Museum. Then, the samples were measured from snout to tail using a measuring tape for the total body length (TL) expressed in centimetres (cm).

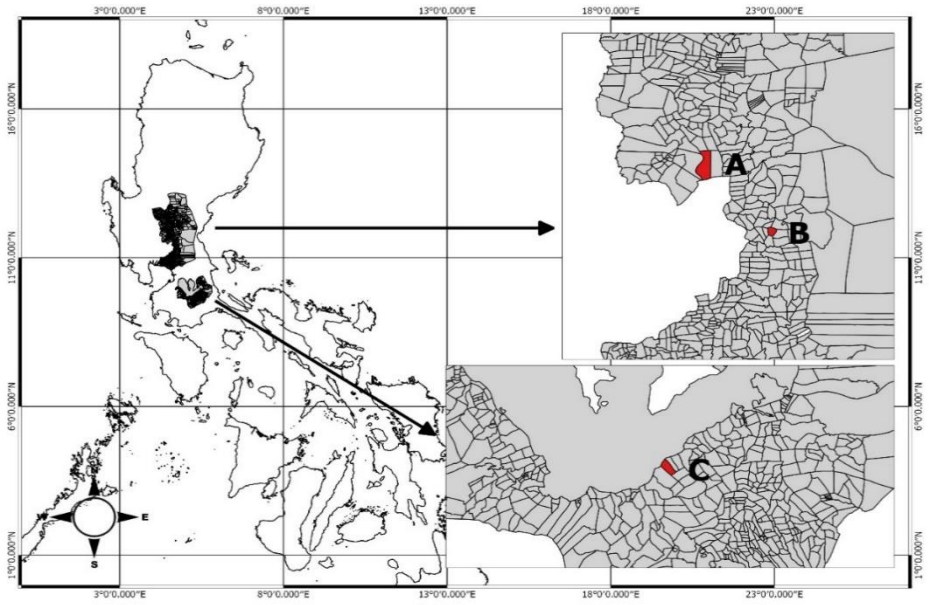

Figure 1. QGIS map of the selected rice paddies as collection sites of *M. albus*
A. Nueva Ecija (Latitude 15º 13' 27.8N; Longitude 120º 55' 13.06 E),
B. Bulacan (15º 8' 3.72 N; 120º 59' 22.7 E) and Laguna (14º 13.376ºN; 21º19.758'E).

2.2 Determination of sexual identity and gonad maturation stage

Fish samples were placed in an icebox filled with crushed ice. Upon their arrival at the laboratory, the individual fish samples were immediately dissected, and the gonads were removed by detaching them from other organs held by thin membranes. After that, their sex and gonadal maturation stages were identified based on macroscopic examination of gonadal characteristics and histomorphological structures according to the method and classification criteria by Murua et al, (2003). Meanwhile, fish samples with gonadal tissues that were difficult to identity were evaluated using the histomorphological description of Wallace & Selman (1981) and Ravaglia & Maggese (2002).

For further verification of the sex and maturity stage, the fish gonad samples were prepared for histological assessment. First, 10% formalin solution was used to fix whole gonad tissues for 24 h and then transferred to 75% ethanol for another 24 h for the dehydration process. Secondly, approximately 1 cm of tissue samples was submitted to Hi-Precision Diagnostic Center (Manila, Philippines) for embedding, sectioning and hematoxylin-eosin staining procedures. After histological processing, prepared slides of the gonadal tissues were examined under the Eclipse E200 Nikon microscope with a magnification power of 40X to 400X**.** Finally, the sex classification and gonad maturation stages were determined based on Murua et al. (2003).

2.3 Extraction of blood plasma samples

Blood samples (2-3 cm^3) were collected using a disposable 3 ml syringe with a hypodermic needle. The needle was inserted into the muscle perpendicular to the ventral surface along the midline, anterior of the anus. The caudal vein is located ventrally from the overlying spine. Extracted blood samples were transferred immediately into heparinized tubes (Sterilab Co. Philippines) and allowed to stand for 2 h at room temperature and later stored at -4.0 ^oC overnight. Then, the tubes were centrifuged at 12500 rpm for 15 min for plasma separation from the whole blood samples. The plasma was then aspirated and transferred to a 2 ml microcentrifuge tube and kept frozen at -20^oC for short term storage until hormone assays.

2.3 Quantification of hormone concentration levels using ELISA

Steroid hormone levels (ng/ml) were determined from the plasma using commercially available, solid phase immunoenzyme assay kits (Cusabio Technology LLC, China) designed for fish species. The process was carried out according to the manufacturer's protocol.

Optical density (OD) was measured at a wavelength of 450 nm for 10 min on a BioTek microplate reader (ELx800, U.S.A.). Absorbance measurements were recorded, and Curve Expert software was used to obtain the standard curve. Data were linearized by plotting the log of hormone concentrations versus the log of the OD. The best fit line was then determined by regression analysis.

3. RESULTS

3.1 Description of the collected specimen

M. albus collected from farm ponds sites (Figure 1) were identified based on the external morphology of the fish (Figure 2). The collected fish samples profiles were similar to the verified collection from the Zoological Division of the Philippine National Museum (Figure 3).

Since the fish did not exhibit sexual dimorphism based on morphology alone (Figure 2), the gonad samples were observed for macroscopic differentiation. Out of the 11 samples, three were immature, four maturing to mature and four with undetermined gonads.

The total length of immature females ranged from 53.55 to 57.93 cm. Each specimen has a small ovary but with no visible eggs (Figure 3A). Meanwhile, maturing and mature females had total lengths ranging from 51.16 to 61.31 cm. Each specimen has a medium-sized ovary occupying ¼ to ¾ of the body cavity along the intestine with prominent opaque whitish to yellowish eggs. Furthermore, the maturing female ovary has eggs about 1-2 mm in diameter (Figure 3B), while the ovary of mature females, which is more gravid, contains yellowish to orange-colored, 3-4 mm diameter eggs (Figure 3C). The gravid ovary with blood capillaries contains a mixture of both developing and advanced stages of oocytes.

The large fish samples have total lengths ranging from 69.33 to 76.99 cm. The gonads appeared flaccid and purple in color,

located in the body cavity alongside the intestine and connected by a thin membrane. Nevertheless, the gonads were assumed to be intersex (Figure 4D) or male (Figure 3E) due to the absence of visible eggs. The identity of the gonads was confirmed based on the histomorphology characteristics observed under the microscope.

Figure 2. Physical profile of *M. albus* (**A.**) Total length of 60.2 cm (**B.**) Lateral view showing a dome-shaped head and (**C.**) Ventral part of caudal end showing single anal and urogenital orifice. Scale bar = 5 cm

Figure 3. Macroscopic images of gonads of *M. albus* (**A.**) Immature ovary without visible oocytes (TL of 403.27 mm). (**B.**) Maturing ovary with visible opaque oocytes (TL of 405.60 mm). (**C.**) Mature ovary with yellow to orange oocytes. (TL of 467.50 mm). (**D.**) Intersex gonad (TL of 486.14 mm). (**E.**) Male gonad (TL of 601.87 mm.) TL = total length. Scale bar = 1cm.

3.2 Histomorphology at different gonad aturation stages

Histological analysis of the gonads revealed three sexual identities in the fish samples: females (immature, maturing and mature), intersex, and males.

Immature females had ovaries with oocytes in the previtellogenic stage, which is the initial stage of oocyte growth (Wallace & Selman, 1981; Ravaglia and Maggese, 2002). At this stage, the ovary consisted of chromatin-nucleolar and perinucleolar oocytes in which the nucleus was surrounded by a thin layer of cytoplasm and contained nucleolus (Figure 4A).

A maturing female ovary contained fewer oocytes in the perinucleolar stage. Most oocytes were in the cortical alveoli stage, marked by the appearance of oil vesicles in the cytoplasm and the presence of follicle layers. In addition, some oocytes appeared to be in the early vitellogenesis phase in which the yolky oocytes with eosinophilic yolk granules and oil vesicles (Figure 4B). Besides, most mature females had gravid ovaries in the advanced vitellogenic stage, characterized by yolky and large oocytes, with several oocytes in the cortical alveoli stage and a few oocytes in the perinucleolar stage (Figure 5C).

The intersex stage gonad or the ovotestis was observed to have few ovarian follicles, some vitellogenic oocytes, cortical alveoli cells coexisting with many testicular lobules and spermatogenic cells (Figure 5A). At this stage, the fish undergoes sex reversal, transitioning from a mature female to an adult male.

The male gonads mainly have testicular lobules and spermatogenic cells (Figure 5B).

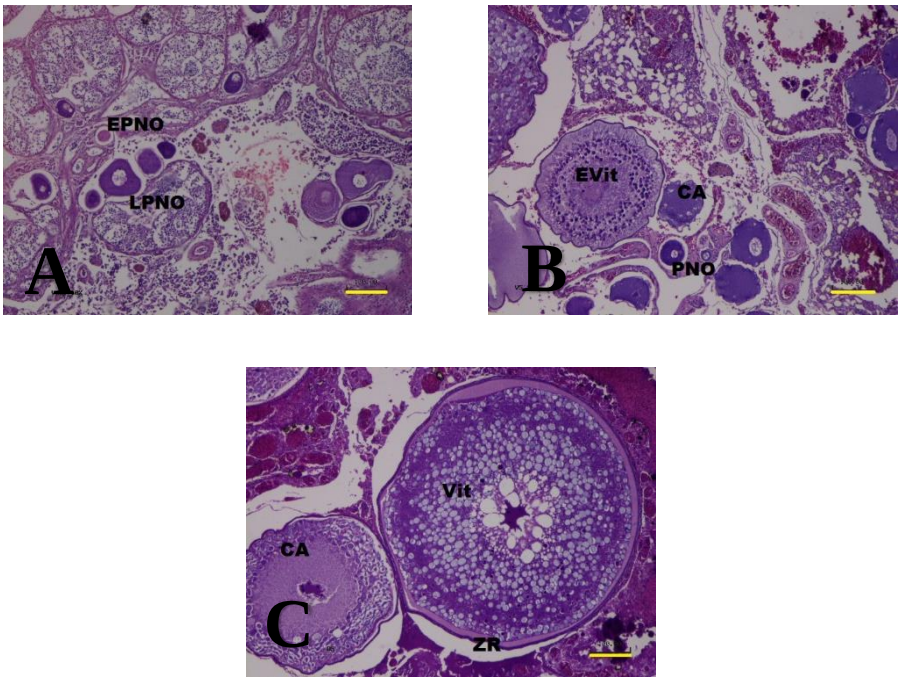

Figure 4. Micrograph image of a serially sectioned (**A.)** Immature female gonad showing clusters of early perinucleolar oocytes (EPNO) and late perinucleolar oocyte (LPNO) 100X. (**B.)** maturing female gonad showing perinucleolar oocyte (PNO), cortical alveoli (CA) and early vitellogenic oocyte (Evit) 100X. **C.** mature female gonad showing cortical alveoli oocyte (CA), vitellogenic oocyte (Vit) with yolk globules and zona radiata (ZR) 40X. Scale = 100 µm

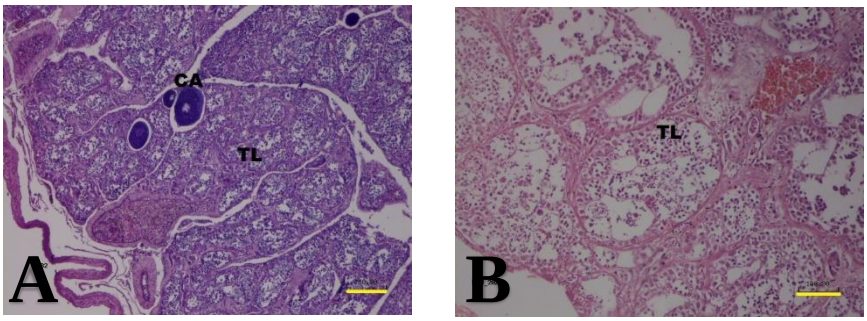

Figure 5. (**A.)** Micrograph image of serially sectioned intersex gonad showing clusters of cortical alveoli oocyte (CA) and testicular lobules (**B.)** male gonad showing testicular lobules (TL) 100X. Scale = 100 µm

3.3 Concentration of the steroid hormones' levels

The mean concentration levels of the steroid hormone 17ß-estradiol (E2) were similar for immature females and intersex *M. albus*, while the concentration level was markedly higher for mature females from 0.06 to 0.08 ng/ml. Despite that, there were no significant differences between the different stages at $p > 0.05$. The mean concentration levels of testosterone (T) were closely similar between immature and mature females, which were noticeably higher than the intersex stage from 0.20 to 0.26 ng/ml. Nevertheless, the differences in steroid hormone concentration between groups were not significant ($p>0.05$).

Table 1. Concentration of the gonadal steroid hormones' levels from blood samples (*ng/ml*)

Maturation stage & sexual status	Range E2 level	Mean E2 level	Range T level	Mean T level
Immature females (*n=3*)	0.95-1.13	1.030[a]	1.03-1.66	1.31[a]
Maturing-matured females (*n=4*)	0.94-1.27	1.110[a]	1.20-1.45	1.37[a]
Intersex (*n=4*)	0.89-1.22	1.040[a]	0.99-1.20	1.11[a]

n = sample size
Values of the same letters are not significantly different from each other at ($p > 0.05$)

4. DISCUSSION

Histomorphological findings in the gonadal development of *M. albus* demonstrated distinct ovarian tissues, testicular tissues, and both ovarian follicles and testicular lobules. These observations indicated the gonadal maturity stage and sexual status of this protogynous hermaphroditic fish that exhibited no sexual dimorphism based on morphology (Figure 2).

Immature gonads, in general, only have distinct ovarian tissue. As shown in Figure 5A, rounded cells were surrounded by a follicular layer. The oocytes were in their primary growth or perinucleolar stage. Oocytes in the maturing and mature ovaries were characterized by the copious yolk globules and the thick vitelline envelope, especially in mature ovaries (Figures 4B and 4C). In maturing and mature ovaries, the primary process responsible for significantly increasing

the size and volume of oocytes is called vitellogenesis (Ravaglia & Maggese 2002).

Considering the mean concentration levels of the steroid hormones of both 17ß-estradiol (E2) and testosterone (T) that were markedly higher in mature females than those of immature and intersex despite the absence of significant differences (Table 1), this might suggest that both plasma steroid hormones play a crucial role in the sexual maturation of the ovaries particularly in oocyte growth and vitellogenesis. 17ß-estradiol (E2) therefore, is vital for vitellogenesis to proceed. Similar results were reported in the marine fish *Merluccius australis* (Alvarado et al., 2015).

The ovarian histomorphology of mature females (Figure 4C) was asynchronous. The gravid ovary comprises oocytes in varying developmental stages, with few oocytes in the previtellogenic stage, several in the cortical alveoli stage, and numerous in advanced vitellogenic stages. This characteristic is commonly seen in batch spawner fishes.

A gravid female *M. albus* with an asynchronous ovary is a common feature in batch spawners with a low concentration of steroids. For example, *Gobio gudgeon* and *Verasper variegatus* undergo asynchronous gonadal development with a low concentration of steroid hormones (Rinchard et al., 1993; Koya et al., 2003).

In addition, low concentration levels of sex hormones in *M. australis* were observed due to an extensive spawning period for partial spawners with asynchronous ovaries (Alvarado et al., 2015). Thus, the concentration levels of sex steroid hormones in fish are dependent on the gonad development stages and spawning behavior.

Furthermore, testosterone levels varied in different *M. albus* groups (immature, mature and intersex) but were markedly higher in mature females (Table 1).

Alam et al. (2005) have reported that the protogynous grouper *Epinephelus merra* ovarian cells synthesized androgens such as 11-ketotestosterone (11-KT). This hormone may have a significant role in regulating oocyte development and restructuring of the gonad during the sex reversal. In addition, androgens may also contribute to sex differentiation and sex change in hermaphroditic fish (Cardwell & Liley, 1991). A significant amount of this circulating hormone in the blood of female teleost suggests its vital role in oogenesis (Lokman et al., 2002).

Natural sex reversal occurs commonly in rice field eels. For example, Chan et al. (1972) biopsy examinations of protogynous *M. albus* revealed anatomical changes from an ovary through an intersexual or transitory stage to testis as it matures. On top of that, Chan & Phillips (1969) observed that *M. albus* undergoes natural sex reversal that involves structural and physiological changes in the reproductive function of the gonad.

The intersex stage is a post nuptial event in which both ovarian and testicular tissues are present in the gonad structure but remains inactive reproductively (Chan & Phillips, 1967). The histomorphology of *M. albus* intersex (Figure 5A) exhibited that the gonad consisted of fewer oocytes dispersed in testicular tissue alongside testicular lobules.

However, the noticeably lower concentration levels of both hormones, 17ß-estradiol (E2) and testosterone (T) in intersex (Table 1), contributed to the inactivity of the fish to function as a reproductive female or male. Thus, both sex steroid hormones might play a crucial role in oogenesis and spermatogenesis.

5. CONCLUSION

Histological analysis of this study illustrated the structures present in each developmental stage of gonadal maturity for *M. albus.* The different ovarian tissues were observed in the female gonads, whereas the ovotestis was present in an intersex gonad. On the other hand, the testicular lobules were found in males. The differences in the concentration levels of hormones estradiol and testosterone were not significantly different across all the stages of gonad maturation. However, it does not rule out their functional roles in vitellogenesis and spermatogenesis to the natural sex-changing gonads process. In conclusion, the reproductive characteristics elucidated in this study help understand the reproductive success of this introduced hermaphroditic fish.

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