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Differential gonad histomorphology and steroid hormones in rice paddy eel *Monopterus albus.*

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Abstract: *Monopterus albus* known as the rice paddy eel is an introduced freshwater fish distributed in some areas in Luzon rice paddies. The establishment and survival of the population of species in their breeding ground can be attributed to the species' reproductive characteristic and capacity. The *M. albus* is a protogynous hermaphroditic fish which has a natural ability for sex reversal. The species developed first as female then into intersex stage and finally, transitioning into male. This study describes the histomorphology of the different stages of gonadal maturation and determines the blood plasma concentration levels of the steroid hormones 17ß-estradiol (E_2) and testosterone (T). Fish samples were collected randomly from selected farm ponds in Bulacan, Nueva Ecija and Laguna. The gonads were categorized into immature, maturing, mature ovary, intersex and male based on the presence of ovarian and testicular tissues. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration levels of steroid hormones of immature female, mature females and unidentified (intersex or male) gonad. The histological analysis of the gonad showed that the intersex gonad exhibited the presence of both an ovarian and testicular tissues. Based on ELISA, the mean concentrations in ng/ml: (1) for immature females E2 is 1.031 and T is 1.312, (2) for mature females E2 is 1.111 and T is 1.368 and (3) for undefined gonads E2 is 1.045 and T is 1.07. The differences in the concentration levels of the two hormones are apparent across all the stages of gonad maturation. The functional role of these hormones in vitellogenesis and spermatogenesis leads to the development of this sex-changing gonads. This kind of reproductive characteristic contributes to our understanding of the reproductive success of this hermaphroditic fish.

Key Words: estradiol; gonad; hermaphrodite, *Monopterus albus*; steroid hormones

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1. INTRODUCTION

The *Monopterus albus* or the rice paddy eel exhibits sexual amorphism (Liem 1963) and is classified as a protogynous hermaphrodite, a sequential species which changes sex during their lifetime that develop first as females and change to males (Liem 1963, Chan and Philips 1967, Matsumoto 2011, Angelis 2015). These special characteristics have made the rice field eel fit for sexual differentiation studies (Zhou et al., 2002).

Sexual determination plasticity is a result of the genetic or environmental factors such as temperature, pH, or social cues that define the fate of the gonad (Angelis 2015). However, the detailed mechanism of sex reversal of this species needs to be investigated.

According to Yamamoto (1969), sex steroids are the natural inducers of gonadal differentiation in teleost, which require changes in steroidogenesis, preceding or coinciding with gonadal differentiation. It is widely accepted that steroid hormones play an important role in processes such as gametogenesis, sex differentiation, embryonic development and in regulating reproductive behaviour and development in fish.

Estrogen have been identified in several studies on gametogenesis regulation in teleost fish. This sex steroid hormone is produced by the ovary to regulate oocyte growth, while androgens are produced by the testis to regulate spermatogenesis (Nagahama, 1994, 1997).

Hence, to better understand gonadal development and sex differentiation of the rice paddy eel as a hermaphroditic fish, this study aimed to describe the gonadal histomorphology and to determine the steroid hormones 17ß-estradiol (E_2) and testosterone (T) concentration levels in plasma using the enzyme-linked immunosorbent assay (ELISA) of the different gonadal maturation stages of the fish.

2. METHODOLOGY

2.1 Sampling

Fish samples were collected in July 2018 from farm ponds located in three sites: (1) San Isidro Nueva Ecija (15° 13' 27.8N; Longitude 120° 55' 13.06 E), (2) San Miguel, Bulacan (15° 8' 3.72"N; 120° 59' 22.7") and Victoria Laguna (14° 13.376°N; 21°19.758'E). An electrofishing back pack gear which produces 12 Volts electric current was used to temporarily stun and immobilize the fish.

2.2 Gonad maturation stage identification

The fish was sacrificed and dissected to remove the gonad. The sexual identity and maturation stage was macroscopically identified based on gonad structures according Murua (2013) maturity classification criteria. Gonad was preserved in a 10% formalin for 24hrs then transferred to 75% ethanol for another 24hrs until further processing. About two cm gonad cross section was process with dehydration and clearing technique. A thickness about 5 µm was sectioned in a microtome and stained in hematoxylin-eosin. Microscopic examination of the gonad was performed to assess and verify the sex and maturity stage of the fish according to Murua (2013) classification.

2.3 Blood plasma extraction

Aliquot of blood was drawn from the fish by caudal venepuncture using a disposable 3 cc syringe with hypodermic needle. The sample was then immediately transferred to a red top vacutainer or green top heparinized tube and was left standing for 2 hours at room temperature or overnight and kept at -4 °C. The tube with the sample was set for centrifuged at 12500 rpm for 15min after which the plasma portion was aspirated. The extract was kept frozen at -20°C for short term storage until hormone assays.

2.4 Quantification of hormone concentration levels using ELISA

Steroid hormone levels (ng/ml) were estimated in plasma using commercially available

solid-phase immunoenzyme assay kits and followed the manufacturer's instruction. Optical density (OD) was measured on a microplate reader ELx800 model and absorbance was measured at a wavelength of 450nm within 10min.

The Curve Expert software was used in creating a standard curve. The data are linearized by plotting the log of hormone concentrations versus the log of the OD and the best fit line was determined by regression analysis.

3. RESULTS AND DISCUSSION

A total of 11 *M. albus* fish were selected and grouped according to reproductive stages. The basis of grouping was based on gonadal characteristics and histomorphological structures. The groups included three immature fish, four mature fish, four undefined gonad identity.

3.1 Histomophology of the gonadal stages of M. albus.

Immature female has an ovary which is not yet prominent. The histology of immature female gonads showed the presence of oocytes in the previtellogenic stage consisting of chromatin-nucleolar and perinucleolar oocytes (Figure 1A).

Maturing or mature female has an ovary with prominent yellowish eggs were visible. The micrograph shows to have the most advanced oocytes in the cortical alveoli and undergoing a vitellogenic stages (Figure 1B and 1C).

Gonads without visible ovary and found to be difficult to identify the sex can be assumed to be an intersex or a male gonad. Intersex stage gonad was observed to have coexisting ovarian follicles and testicular lobules (Figure 1D) while the male gonad appeared to have testicular lobules and spermatogenic cells solely (Figure 1E).

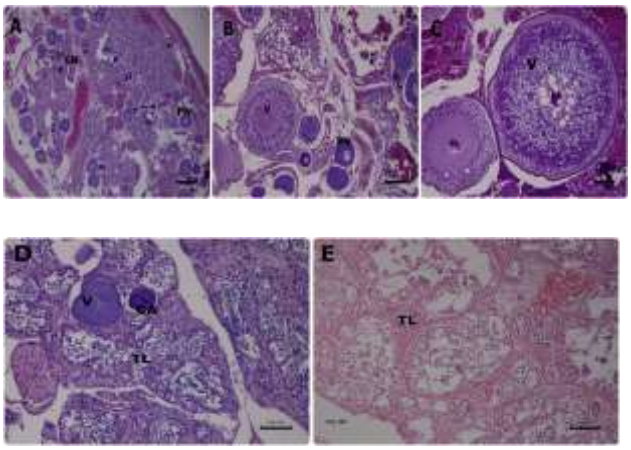

Figure 1. Micrograph image of M. albus at different sexual maturity stages. A. Immature female gonad (4x). B. Maturing female gonad (10x). C. Mature female gonad (4x). D. Intersex gonad tissue (40x). E. Male gonad tissue (200x). chromatin nucleolar (CN), perinucleolar oocytes (PN), cortical alveoli (CA), vitellognic yolk (V), testicular lobules (TL). Scale 100µm

2.3 Estradiol and testosterone level

The mean concentration level steroid hormones of both estradiol and testosterone are found lower in immature female and higher in value compared to mature gravid female fish. The data shows that there was an increase in the average level of concentrations of both estradiol and testosterone as the ovaries mature by the process of oogenesis. Suggesting that the presence of both plasma steroid hormones are playing a crucial role in sexual maturation of the ovaries of young female individuals.

All gonad samples of varying stages contain certain amount of testosterone but found higher in concentration in both immature and mature female. As observed in the study of Alam (2005) on protogynous grouper *Epinephelus merra,* ovarian cells biosynthesize 11-ketotestosterone and probably have a physiological role in in oocyte growth and gonadal restructuring during the sex change of the fish. Androgens such as 11-ketotestosterone play

roles in sex differentiation and sex change in protogynous hermaphrodites (Cardwell and Liley 1991) and a significant amount of the hormone in the blood of female teleost suggest its functional role in oogenesis (Lokman et al. 2002)

Meanwhile, it was also evident in the data that there was a decrease in the average amount of both sex steroids from mature ovaries to undefined gonads. This may describe a short period leading to sexual inactivity of individuals under intersex stage of development right after the maturation stage of the ovaries performing its role as females.

In addition, the undefined gonad stage has a higher mean concentration level of testosterone compared with that of the mean concentration level of estradiol. This may be an indicative that the gonads were in transitory stage of changing from female to male stage. A higher concentration level of testosterone at spermatogenesis stage compared to other maturational stages, demonstrates the importance of this hormone in the process of spermatogenesis (Alvarado et al, 2015).

Table 1. Sex steroid hormone level concentration in *M. albus* samples

Maturity stages	Total TL (cm)	Total BW (g)	Mean E2 (ng/ml)	Mean T (ng/ml)
Immature female (n=3)	55.13	152.83	1.031	1.312
Mature female (n=4)	55.74	170.58	1.111	1.368
Undefined gonad (n=4)	72.36	370.8	1.045	1.107

4. CONCLUSIONS

This study attempted to present histological identification and characterization of sex steroid hormones particularly the estradiol and testosterone of this sexually amorphic, protogynous hermaphroditic fish the rice paddy eel (*Monopterus albus*). Understanding the fish reproductive biology of is important in fisheries and aquatic management.

Estradiol and testosterone hormones are both necessary for the processes of vitelogenesis and spermatogenesis in the sexual maturation and differentiation of the *M. albus* species. Further studies are still needed to be able demonstrate and evaluate the physiological role of other hormones in the sexual maturation and reproductive behaviour of *M. albus*.

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