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Annual Dynamics of the Plasma Sex Steroid Hormones of the Malaysian Walking Catfish *Clarias batrachus* (Linnaeus 1758)

[1]L.A. Argungu, [1,2]A. Christianus, [1]M.S.N. Amin, [3]S.K. Daud and [1]S.S. Siraj
[1]Department of Aquaculture, Faculty of Agriculture,
[2]Institute of Bioscience,
[3]Department of Biology, Faculty of Science, Universiti Putra Malaysia, Selangor, Malaysia

Corresponding Author: A. Christianus, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, 43400, Malaysia Tel: +603-89474884 Fax: +603-89408311

ABSTRACT

Malaysia is one of the countries in Asia with higher scarcity of *Clarias batrachus*. Consequently, to arrive at better ways of handling the situation the reproductive endocrinology of the species was investigated. Testosterone (T), 11-ketotestosterone (11KT) and 17β-estradiol (E_2) were the plasma sex steroid hormones monitored monthly throughout the reproductive cycles. Several peak levels were observed in the annual profiles of all the steroid hormones, implying that *C. batrachus* is a non-seasonal breeder, signifying that the species could spawn several times during the reproductive cycle. Most of the scholars who earlier worked on the breeding of this fish concentrated on a particular period (May to August) assuming that was the only season successful induced breeding of the species could be achieved. The present study has enhanced the understanding of the reproduction of *C. batrachus*. It has provided a platform for the optimization of reproduction and breeding program of the species.

Key words: *Clarias batrachus*, reproductive cycle, plasma sex steroid hormones

INTRODUCTION

Clarias batrachus is a notable indigenous air breathing walking catfish in Asian continent and one of the most popular in aquarium business and aquaculture among the Asian species (Ng and Kottelat, 2008) It is confirmed to be a leading Asian catfish in the area of terrestrial migration to nearby water bodies (Ahmad *et al*., 2012). It holds promise for high density culture for the maximization of product per unit area due to its ability to survive water bodies with depleted water quality condition (Areerat, 1987). It is also cherished as a medicinal fish (Hossain *et al*., 2006; Debnath, 2011). However, abuse of agrochemical applications in paddy fields and habitat loss as a result of natural disasters and human activities have deepened the hopelessness of relying on the wild as a significant source for the seeds of *C. batrachus* (Das, 2002; Ahmad *et al*., 2012). The species is threatened (Hossain *et al*., 2006; Ahmad *et al*., 2012) and vanishing in some part of Asia (Binoy, 2010) as such vulnerable to extinction (Wiecaszek and Krzykawski, 2010) and in captivity, unless fish are induced to reproduce, hatchery-bred fish do not reproduce spontaneously (Rottmann *et al*., 1991). In an attempt to arrest the dwindling condition of this economically important fish, many scholars had worked on the induced breeding of *C. batrachus* (Manickam and Joy, 1989; Das, 2002; Sahoo *et al*., 2003; Mahapatra, 2004; Sahoo *et al*., 2005; Hossain *et al*., 2006; Sahoo *et al*., 2007, 2009; Sharma *et al*., 2010; Srivastava *et al*., 2012). The pioneer breeding trial

on this species in Malaysia was carried out by Cheah *et al.* (1990). Breeding trials, although quite important in improving the plight of *C. batrachus* but is not sufficient to provide the adequate platform for the revolutionary approach needed in the culture of this species to arrest it dwindling towards extinction. To achieve this, the reproduction of the species needs to be understood through characterization of the spawning pattern of the fish. This will pave way for the optimization of the reproduction of the fish. The spawning patterns of fish species are understood when the dynamics of the reproductive hormones across the annual reproductive cycles of the fishes are investigated (Callard *et al.*, 1991; Tan-Fermin *et al.*, 1997; Roberts *et al.*, 1999; Barcellos *et al.*, 2001; Lee and Yang, 2002; Manosroi *et al.*, 2003).

Sex steroids are the regulators of reproductive physiology, sexual differentiation and the development of sexual characteristics (Nelson, 2005). Gonadotropin-releasing hormone is released by the hypothalamic-pituitary-gonadal axis from the hypothalamus, which stimulate the anterior pituitary to secrete gonadotropin GTH I and II Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)]. These initiate gametogenesis and steroidogenesis in the gonads (Wierman, 2007). 17β estradiol, Testosterone and 11-ketotestosterone are among the major sex steroids produced via the gonads. These hormones are involved in the control of reproductive process in fish (Kime, 1993), leading to ovulation and spermiation in female and male individuals respectively (Adebiyi *et al.*, 2013). The 17β-estradiol is the most effective estrogen in female teleosts and is a principal steroid in vitellogenesis and oocyte maturation (Fostier *et al.*, 1983) while testosterone and 11-ketotestosterone have been reported to control reproductive behavior and morphology of the male fish (Fostier *et al.*, 1987). The 11-ketotestosterone appears to be physiologically more potent androgen than testosterone (Borg, 1994).

Malaysia is a one season climatic environment that is facing higher scarcity of *C. batrachus* and since environmental cues are believed to exert tremendous influence on the reproductive behavior of fishes (Rottmann *et al.*, 1991) to achieve better understanding of its reproduction for the enhancement of the breeding program there is the need to determine its spawning pattern in the climatic peculiarities of the Malaysia ecology. This was the motivation for the present study.

MATERIALS AND METHODS

Experimental fish and management: The study was carried out between February 2012 and January 2013 at the Universiti Putra Malaysia, Aquaculture Research Station in Puchong, Selengor, Malaysia. A total of 72 brood stock fish (3 males and 3 females every month for 12 months) were used for this experiment. The body weight of the specimens used was in the range of 80-210 g and total length was in the range of 21-33 cm. The fish were obtained from a local fish supplier at Negeri Sembilan. They were conditioned to captivity condition for a minimum of one month in 2 t fiberglass tanks prior to the experiment. The fish were fed with pelleted artificial feed containing 35% crude protein once in a day *ad libitum*.

Measurement of fish body weight, length and water quality parameters: The specimens were captured in the morning with suitable hand nets. They were sedated using clove oil solution (ethanol and clove oil, 5:1) at 2 mL of the clove oil solution and 5 L of water in 3 min. The body weights were determined using a spring balance and digital scale balance with 0.001 sensitivity was used to measure the gonad weights. Total and standard lengths were measured using one-meter measuring board. Water temperature, dissolved oxygen and pH were monitored using multi probe water analysis YSI 556 and were in the ranges of 27.0-28.0°C, 6.2-6.9 mg L^{-1} and 7.1-7.8, respectively.

Sampling of blood: Blood was sampled by severing the caudal peduncle with the sterilized surgical blades. The blood was collected in the 1.5 mL eppendorf tubes containing EDTA (Ethylenediaminetetraacetic acid) as anticoagulant. The sampled blood was kept in ice pending the time it was centrifuged at 6000 rpm per 4 min to obtain plasma. The plasma was kept in fresh eppendorf tube and kept under -80°C until assayed.

Measurement of sex steroid hormone levels: Commercial ELISA (Enzyme Linked Immunosorbent Assay) kits from Caymen chemical company, USA was used to determine the levels of the sex steroid hormones according the manufacturer's directives. The assay standards for the Testosterone, 11-ketotestosterone and 17 β-estradiol were prepared accordingly. The plates were set-up and by the protocol, 50 µL of the samples and standards was added to each well of the 96 wells. The 50 µL of antiserum was added to the samples and standard wells. The plate was incubated at room temperature for 2 h. The plate was washed five times using wash buffer. Two hundred milliliter of AChE (acetylcholinesterase) substrate was added and the plate was incubated for 60-90 min for colour development in dark. The plate was read at 405 nm in micro plate reader. The concentration of the sex steroid hormone of each standard and Optical Density (OD) was used to plot the standard curve and calculation of hormone concentration in each samples using software JMP 9 (SAS, Cary, NC, USA).

Statistical analysis: JMP 9 (SAS, Cary, NC, USA) statistical software was used for the analysis. Data was analyzed using ANOVA (analysis of variance) and mean variations were determined using Turkey Honestly Significant difference (HSD). Pairwise correlations were used to analyze the relationship between the steroid hormones. Data was expressed as Mean±SEM (standard error of mean). Significance was determined at $p<0.05$.

RESULTS

Testosterone: The mean value of the plasma testosterone in the male *C. batrachus* was lowest (26.67±0.98 pg mL^{-1}) in the month of September 2012. The value was not significantly different ($p>0.05$) from the values obtained in the months of February and May 2012. The highest mean value (209.21±19.49 pg mL^{-1}) of the hormone was recorded in October 2012, which similarly did not exhibit significant difference ($p>0.05$) from the values recorded in December 2012 and January 2013. Peak levels of the hormone were observed in the months of April, June, August, October 2012 and January 2013 (Fig. 1a).

The testosterone in the female *C. batrachus* exhibited lowest mean value (7.02±0.06 pg mL^{-1}) in February 2012, which was not significantly different ($p>0.05$) from the mean values obtained in the months of May, June, November and December 2012. Mean value of 49.99±2.17 pg mL^{-1} represents the highest mean value of female testosterone recorded in October. This value was not significantly different ($p>0.05$) from the mean values presented by the months of April and August 2012. Peak levels in this hormone were found in the months of April, August and October 2012 (Fig. 1b).

11-ketotestosterone: 11-ketotestosterone is another androgenic steroid hormone that was investigated in the present study. The monthly profile of 11-ketotestosterone indicated 6.61±0.24 pg mL^{-1} as the lowest mean value of its concentration recorded in the month of September 2012. The value did not show significant difference ($p>0.05$) from the mean values

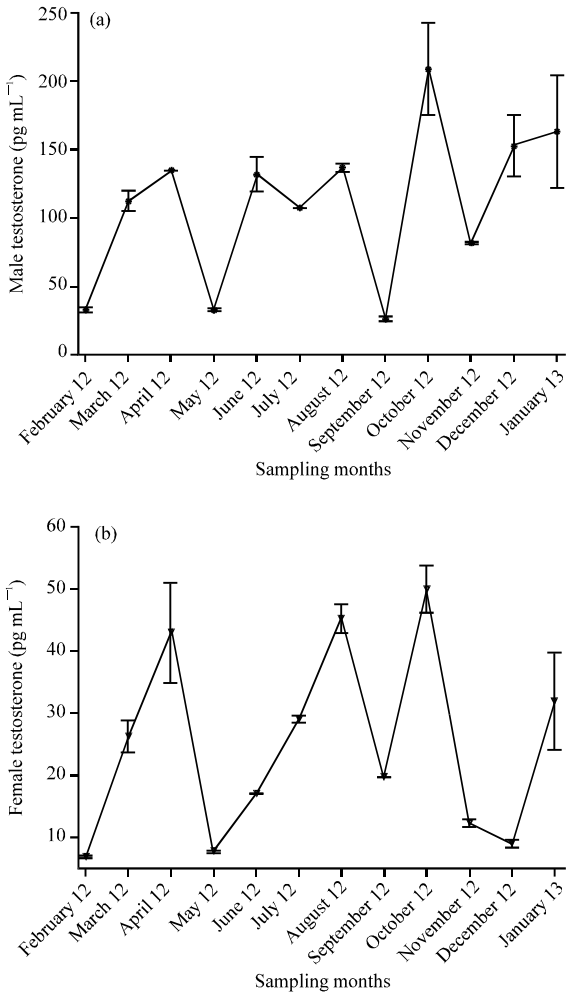

Fig. 1(a-b): Monthly changes in the concentrations of testosterone for (a) Male and (b) Female *C. batrachus* during the study period

obtained in the months of February, March, May and June 2012. Likewise, the highest mean value for the hormone was 80.27±5.29 pg mL^{-1} observed in the month August 2012, which was not significantly different ($p>0.05$) from the mean values observed in January 2013. The peak levels for this hormone were noted in the months of April, Augus and October 2012 (Fig. 2). The androgenic hormones (male testosterone and 11-ketotestosterone) were observed to exhibit positive correlation ($r = 0.6045$) (Fig. 3).

17β-estradiol: 17β-estradiol is a major estrogenic hormone that was studied under the present work. The month of December 2012 was found to produce the lowest mean value of 111.68±3.62 pg mL^{-1} while the highest mean value of 695.30±8.94 pg mL^{-1} was presented by the month of October 2012. The mean values obtained in the month of January 2013 was however not significantly different ($p>0.05$) from what was obtained in December 2012 while the mean value observed in August was not significantly different ($p>0.05$) from the highest value recorded in

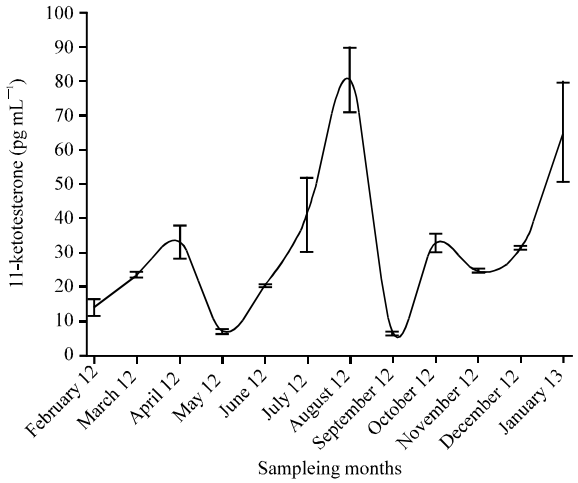

Fig. 2: Monthly changes in the concentrations of 11-ketotestosterone for male *C. batrachus* during the study period

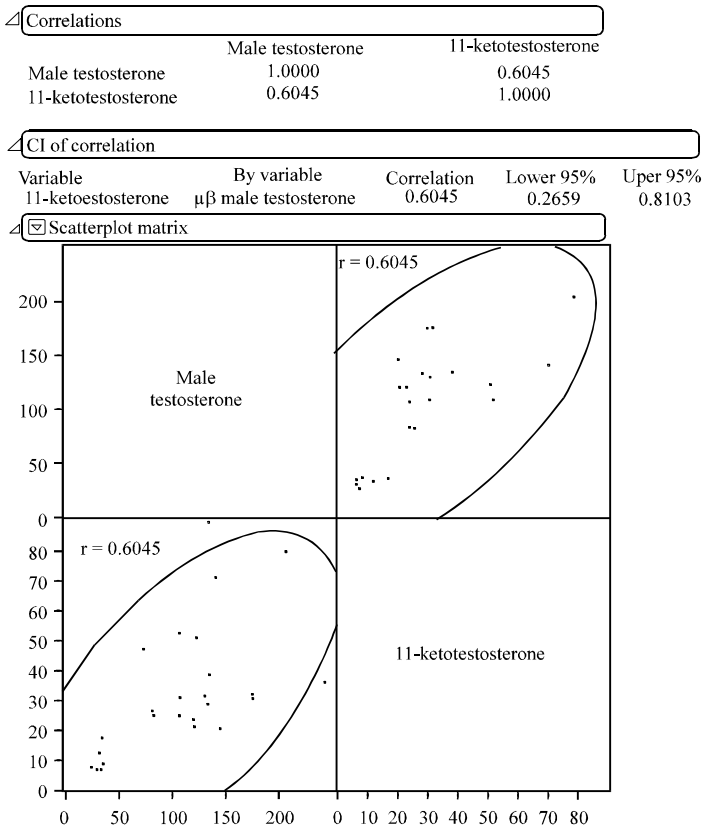

Fig. 3: A scatterplot matrix showing a positive correlation between testosterone and 11-ketotestosterone of the male *C. batrachus*

October 2012. The peak values of estradiol were recorded in the months of April, August and October 2012 (Fig. 4). The estrogenic hormones (female testosterone and 17β-estradiol) exhibited positive correlation (r = 0.8261) (Fig. 5).

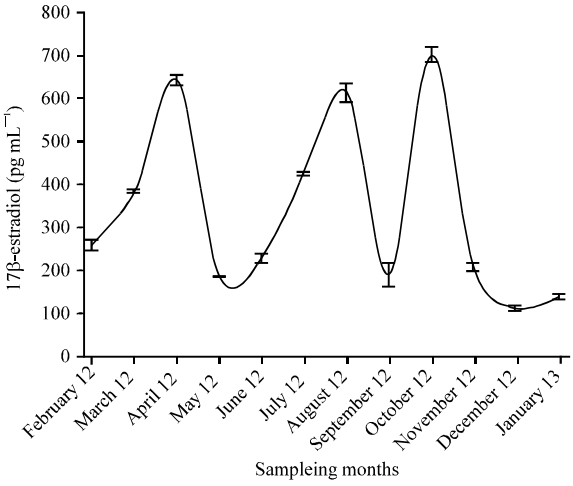

Fig. 4: Monthly changes in the concentrations of 17β-estradiol for female *Clarias batrachus*

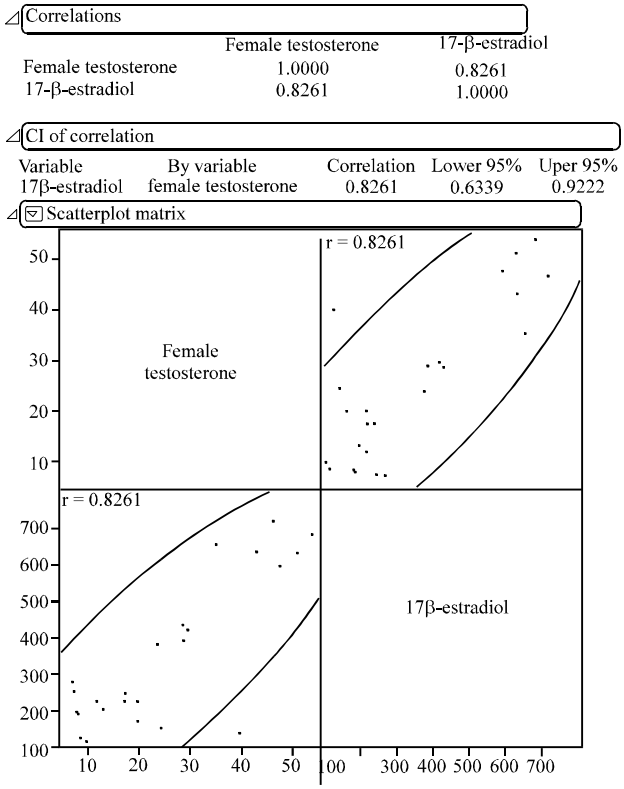

Fig. 5: A scatterplot matrix showing a positive correlation between 17β estradiol and testosterone of the Female *C. batrachus*

DISCUSSION

Testosterone: In the present study the testosterone from male *C. batrachus* exhibited peak levels in the months of April, June, August and October 2012 during the experimental period indicating that the male fish can readily be utilized for the purpose of milt production for induced breeding

several time during the annual period. It is also suggestive of the ability of the fish to observe spermatogenesis in captivity. The onset of spermatogenesis and spermiation is associated with rising level of androgenic hormones (Harmin and Crim, 1992). In male *C. macrocephalus* investigated in Philipines, the serum testosterone profile was observed to peak in November, February, April and August (Tan-Fermin *et al.*, 1997). Similar trend was also reported by Adebiyi *et al.* (2013) on *Hemibagrus nemurus*. The testosterone from the male fish in this study also presented lower levels in its profile in the months of February, May, September and November 2012, which may represent the dominance of spermatogonea, indicating a resting phase in fish (Weltzien *et al.*, 2002). It may also signify the presence of undetermined metabolites (Cornish, 1998). It is also indicative of the ability of the fish to observe spermiation in captivity. Fostier *et al.* (1983) noted that it is typical of testosterone in male teleosts to become elevated during spermatogenesis and then subsides at the beginning of spermiation.

The testosterone profile exhibited by female *C. batrachus* in this study generated peaks in the months of April, August and October 2012, confirming the capacity of the fish to breed several time in a year, implying that it is a non-seasonal breeder. Adebiyi *et al.* (2013) also observed peaks in male testosterone in the months of October, November, February, June and August in *H. nemurus*. The levels of testosterone in female fish (49.99±2.17 to 7.02±0.06 pg mL^{-1}) in the present study were much lower then what was recorded in the male fish (209.21±19.49 to 26.67±0.98 pg mL^{-1}). Similar findings were noted on *H. nemurus* (Adebiyi *et al.*, 2013). This agrees with the observation of Davis and Marler (2003) that the amount of testosterone in most vertebrates is higher in males then females.

Water temperature, dissolved oxygen and pH in this study were in the ranged of 27.0-28.0°C, 6.2-6.9 mg L^{-1} and 7.1-7.8, respectively. These values are consistent with what was described as optimum in tropical fish production by Boyd and Tucker (1998). In this study, the range of water temperature during the experimental period was 27-28°C. The performance of the testosterone and other sex steroid hormones can be attributed to this level of temperature throughout the period of the experiment. Temperature was suspected to be the environmental cue responsible for the testosterone to peak which brings about reproductive maturity to the gonads and resultant gametes (Taghizadeh *et al.*, 2013). Cornish (1998) also implicated higher dam water temperature for the resultant increase in the level of the plasma testosterone.

11-Ketotestosterone: The annual profile of 11-ketotestosterone, which is an androgenic steroid hormone, was observed to have peak levels in the months of April, August and October (2012) similar to what was demonstrated by testosterone, a male hormone discussed earlier. In its cousin brother, *C. macrocephalous*, 11-ketotestosterone was reported to exhibit four peaks (November, February and March, June and September) in its profile (Tan-Fermin *et al.*, 1997). In the case of *H. nemurus*, the peak levels on the profile of 11-ketotestosterone were recorded in the months of October, February and June (Adebiyi *et al.*, 2013). While in the present study, the concentration of 11-ketotestosterone (80.27±5.29 to 6.61±0.24pg mL^{-1}) is much lower than male testosterone (209.21±19.49 to 26.67±0.98 pg mL^{-1}) in *C. macrocephalus* the concentrations of 11-ketotestosterone (159-434 ng mL^{-1}) is much higher than that of testosterone (15-25 ng mL^{-1}) (Tan-Fermin *et al.*, 1997).

The implication of this is that while in *C. macrocephalus*, 11-ketotestosterone is the major androgen, in the case of *C. batrachus* it is the testosterone. Generally, the behavior of 11-ketotestosterone in this study is indicative of active spermatogenesis in captivity and the

non-seasonal breeding capability of *C. batrachus*. The finding was similar to what was reported in *Tor tambroides* (Ismail *et al*., 2011) and *Hemibagrus nemurus* (Adebiyi *et al*., 2013). Cavaco *et al*. (2001) reported the ability of 11-ketotestosterone to have triggered spermatogenesis while the acceleration of pituitary gonadotroph development was believed to be caused by testosterone. Komatsu *et al*. (2006) also noted the relevance of 11-ketotestosterone in the development of sperm during spermatogenesis. The control of male reproductive behavior, development of secondary sexual characteristics and spermiation in majority of teleost species was believed to be the responsibility of 11-ketotestosterone (Fostier *et al*., 1987; Borg, 1994).

17β-estradiol: In all vertebrates studied so far, 17β-estradiol has been established as the principal estrogen controlling critical physiological activities in female reproductive cycles (Taghizadeh *et al*., 2013). It is a formidable sex steroid hormone in vitellogenesis and oocyte maturation (Fostier *et al*., 1983). The annual hormonal profile of 17β-estradiol in this study demonstrated peaks in the months of April, August and October. Thus, several peaks of 17-β-estradiol were indicative of *C. batrachus* undergoing vitellogenesis and oocyte maturation in the captive Malaysian climatic environment. The peaks also suggest the potentiality of *C. batrachus* to breed several times during the reproductive cycle. Similar pattern of hormonal behavior was reported in *Tor tambroid* (Ismail *et al*., 2011) and *H. nemurus* (Adebiyi *et al*., 2013). This however, presented a deviation from what was observed in female *C. batrachus* in India (Singh and Singh, 1987) a scenario that could be attributed significantly to the influence of the environmental cues which were obviously in wide disparity between the two experimental ecological zones. This development has presumably brought to the fore the greater influence the environmental factors particularly temperature have on the reproductive physiology of this species than the internal reproductive mechanism. The present study had shown a positive correlations ($r = 0.8388$) between testosterone and 17β-estradiol which suggest the aromatizing relationships between testosterone and 17β-estradiol in vitellogenic activities and oocyte maturation. The concentrations of 17β-estradiol were greater than that of the testosterone throughout the period of the study which is becoming a characteristic of majority of the non-seasonal spawners. Similar trend was reported by Manosroi *et al*. (2003) on *Pangasianodon gigas* and Adebiyi *et al*. (2013) on *Hemibagrus nemurus*. Higher concentrations of 17β-estradiol than testosterone in female fish indicative of efficient aromatization of testosterone into 17β-estradiol in the ovary (Pellegrini *et al*., 2005).

Interestingly, the seasonal breeders are noted to have concentrations of 17β-estradiol lower than that of the testosterone (Barcellos *et al*., 2001; Kumakura *et al*., 2003; Richter *et al*., 1987; Tan-Fermin *et al*., 1997). This is a crucial discovery as far as the optimization of the reproduction of this species is concern. It shows that to reproduce *C. batrachus* in Malaysia and similar environments artificially, scholars and culturists do not have to wait until a particular period of the year (May-August) as has been the practice with most of the scholars that have worked on the induced breeding of the species earlier (Sahoo *et al*., 2003, 2005; Hossain *et al*., 2006; Sahoo *et al*., 2007, 2009; Sharma *et al*., 2010; Srivastava *et al*., 2012).

CONCLUSION

Malaysia is one the countries in Asia with higher scarcity of *C. batrachus*. It is therefore interesting to state that the present study has widened the knowledge about the reproductive biology of *C. batrachus*. The study has confirmed that *C. batrachus* is a non seasonal spawner,

which could spawn several times in the year. With this information the idea of multiplying the population of this species cannot longer be left under the mercy of the season in an effort to arrest the extinction of *C. batrachus*.

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