CHANGES IN PLASMA LEVELS OF STEROID HORMONES DURING SEXUAL MATURATION OF MALE HELICOPTER CATFISH (*WALLAGO ATTU*) IN CAPTIVITY

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Abstract

In order to provide reliable indicators of the spawning season of captive helicopter catfish (*Wallago attu*), *this study evaluated the temporal variation in gonadosomatic index (GSI), plasma levels of testosterone (T), and 11-ketotestosterone (11-KT) in male broodstock in captivity. GSI was estimated as the percentage of the relative weight of testis to total body weight. Plasma levels of sex steroids were determined by enzyme-linked immunosorbent assay (EIA). Testis samples were dehydrated and embedded in paraffin, then sectioned at 5 µm thickness. The highest level of T (402.1 ± 16.7 pg/mL) was found in June, followed by a peak in 11-KT level (76.9 ± 4.7 pg/mL) in May. Testes containing the highest concentrations of spermatozoa were observed from June to August. The GSI of males increased significantly from January to June and peaked in July (2.14%). Taken together, we conclude that the spawning season of captive helicopter catfish occurs from June to August. These results will contribute to the basic knowledge of the reproductive biology of helicopter catfish, which can be useful in artificial breeding.*

Keywords: 11-Ketotestosterone; Reproductive cycle; Testis; Testosterone; *Wallago attu*.

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1. INTRODUCTION

The helicopter catfish (*Wallago attu*) is a new potential fish for aquaculture in Vietnam. It is a large freshwater fish species of the family Siluridae. *Wallago attu* is fast growing and has good market demand as a food fish having high nutritional value and high protein content in its flesh (Lilabati & Vishwanath, 1996). Irrespective of the wide distribution of this species in Asian countries, populations of *Wallago attu* have rapidly decreased due to overfishing, environmental pollution, and lack of suitable management (Patra et al., 2005).

The success of artificial breeding of this species in captivity has been outlined previously (Gupta et al., 1992; Parameswaran et al., 1988). However, scientific data on the reproductive cycle and maturation of *Wallago attu* broodstock in captivity are very limited, although Prasad and Desai (2020) recently studied wild *Wallago attu* collected from the Bhadar reservoir in Gujarat, India. Controlling the artificial spawning process of fish in cultured conditions is an important factor in producing seed for sustainable aquaculture development (Mylonas et al., 2010).

Understanding endocrine regulation and gonad development stages is crucial in manipulating the spawning of teleost fishes and predicting condition factors in artificial propagation (Hyeon et al., 2019; Manosroi et al., 2003). Several previous studies have suggested using gonadosomatic and spermatogenic maturity indices as indicators of development stages of testicular spermatogenesis (Hajirezaee et al., 2012; Nunes et al., 2011). In fish, testosterone (T) is produced by Leydig cells in the testes and stimulates testicular development and sexual behavior in males (Zohar et al., 2010). According to Assem et al. (2016), testosterone has an important role in various aspects of male fish reproduction. Spermatogenesis and male fertility depend upon the presence of testosterone in the testis. Testosterone is a precursor of 11-ketotestosterone (11-KT), which is the predominant androgen in fish (Chauvigné et al., 2016; Schulz et al., 2010).

However, no data are available related to the reproductive development of this species in captivity. Until now, temporal changes in the levels of plasma T and 11-KT in *Wallago attu* have remained undescribed. It is necessary to elucidate the changes in sex steroid levels during the different stages of maturation (Mylonas et al., 2010). In the present study, the annual reproductive cycle of male *Wallago attu* was observed by the changes in gonadosomatic index (GSI), sex steroid hormones of testosterone, 11-ketotestosterone, and testicular development stages. The results of this study are expected to contribute to advances in the control of reproduction to further the sustainable development of *Wallago attu* farming in Vietnam.

2. MATERIALS AND METHODS

2.1. Animals

Two hundred male *Wallago attu* (2+ years of age, body weight 1.3-1.7 kg, and total length 45-56 cm) were purchased from a local *Wallago attu* farm in Quang Tri

Province, Vietnam. The fish were then cultured in an outdoor earthen pond (16º49′51″ N, 107º04′02″ E) located in the same province. The dimensions of the pond were 20×30×1.5 m in width, length, and depth, respectively. Partial water exchanges of about 30% were made each week. Fish were fed daily with 3% of their body weight with a commercial pellet having an approximate composition of 43% protein, 6% lipid, 14% ash, and 3% fiber (Nutrilis, Ocialis).

2.2. Sampling protocols

Ten male fish were randomly sampled for gonad, blood, and biometrics by seine net from the pond at monthly intervals from January 2020 to December 2020. Fish were euthanized in 25 mL/m^3 Aqui-S® (Bayer Company, Vietnam). Subsequently, the total length and weight of the fish were measured prior to collecting blood (3 mL) from the caudal vein using a needle (21 gauge) and syringe. The blood was placed in 1.5 mL centrifuge tubes containing 5 µL of 200 mg/mL ethylenediaminetetraacetic acid to prevent the blood from clotting and stored in ice-boxes. The blood was centrifuged at 4º C and 1,000 g for 10 min. The supernatant was carefully collected, and the blood plasma was aspirated, aliquoted, and stored frozen at -80º C in a BioUltra UL570 freezer until sex steroid analysis. Testes were removed from each fish and weighed. The gonadosomatic index was calculated as a percentage (%) of the relative weight of gonad to total body weight.

The temporal variation in temperature and light intensity during the rearing period from January to December was recorded using automatic light/temperature data loggers (HOBO Pendant® Temperature/Light 64K Data Logger). This equipment is placed in the culture pond at a depth of 1 m and set to record data every 2 hours continuously, 24 hours/day. The pH was measured using a pH meter (model: HI 98127; manufacturer: Hanna Instruments, USA). The dissolved oxygen (DO) was measured using a DO meter (model: YSI 550A; manufacturer: Yellow Spring Instrument Company, Ohio, USA). Water samples were collected daily at 7:00 h and 16:00 h from the earthen ponds to determine $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$. Water quality parameters were as follows: pH 7.7 ± 0.4 (mean ± SD), dissolved oxygen 5.2 ± 0.3 mg/L, ammonia and nitrite nitrogen < 0.1 mg/L and 0.06 mg/L, respectively.

2.3. Histological processing

Small portions from three parts of the testis (anterior, middle, and posterior) were fixed in 5% buffered formaldehyde solution. These samples were dehydrated in gradually increasing ethanol solutions before being embedded in paraffin. Histological sections were sectioned at 5 µm thickness using a Leica RM 2245 microtome and stained with Harris's hematoxylin and eosin. Testicular development was classified into four periods (immature, pre-spawning, spawning, and post-spawning) based on criteria comparable to those outlined by Guerriero et al. (2005) and Xie et al. (2020).

2.4. Assay for plasma T and 11-KT quantification

The concentrations of T and 11-KT in plasma samples were measured by enzyme-linked immunosorbent assay (EIA) kits purchased from Cayman Chemical Company, as previously described (Chauvigné et al., 2016; Pham & Le, 2020). Briefly, the plasma samples were mixed well with 5 ml diethyl ether. The aqueous portion was then frozen using an ethanol/dry ice bath. The lipophilic phase was poured into a clean tube and the ether phase was evaporated overnight at room temperature using a vacuum centrifuge. Subsequently, 300 µl of EIA buffer was used to reconstitute extracted samples. Enzyme immunoassays were then performed following the manufacturer's instructions with a development time of 75 min. Data were quantified against a standard curve that was linearized using a logit transformation of B/B0 (bound sample/maximum bound). Serially diluted plasma competed with the tracer for antibody binding sites in a manner similar (parallel) to the standard curve (data not shown). Due to the lack of space to analyze all samples in a single assay, samples were run in two assays. The intra- and inter-assay coefficients of variation were 4.4% (n=2) and 7.2% (n=2) for T, 9.8% and 11.5% for 11-KT, respectively. The sensitivity of testosterone was 6 pg/mL, and that of 11-KT was 1.3 pg/mL.

2.5. Statistics

The data analyses from different sampling months were performed by analysis of variance (one-way ANOVA test) (multi-comparisons Tukey-Kramer HSD post-hoc test) at a significance level of $p < 0.05$, using SPSS software version 20.0. Data were expressed as mean ± standard error (SE).

3. RESULTS AND DISCUSSION

3.1. Water temperature and light intensity

Figure 1 shows the monthly average water temperature and light intensity, which ranged from 24.5-30.2^{o}C and 621-5,068 lux, respectively, during the experimental period. The reproductive cycles of the fish closely followed the seasonal fluctuations of temperature and light intensity.

These two factors are often the most important and are directly impacted through sense organs and other hormone-producing glands, which produce the appropriate physiological or behavioral responses (Mommsen & Walsh, 1988). According to Soletchnik (1984), the role of environmental factors in releasing reproductive hormones in fish is strongly associated with light. Since GSI values peaked in June and July when the water temperature and light intensity in the pond rose to a maximum and spawning occurred, water temperature is found to be one of the important factors in the reproductive cycles of fish (Lam, 1983). These results suggest that higher water temperatures may increase hormonal activity in male *Wallago attu*.

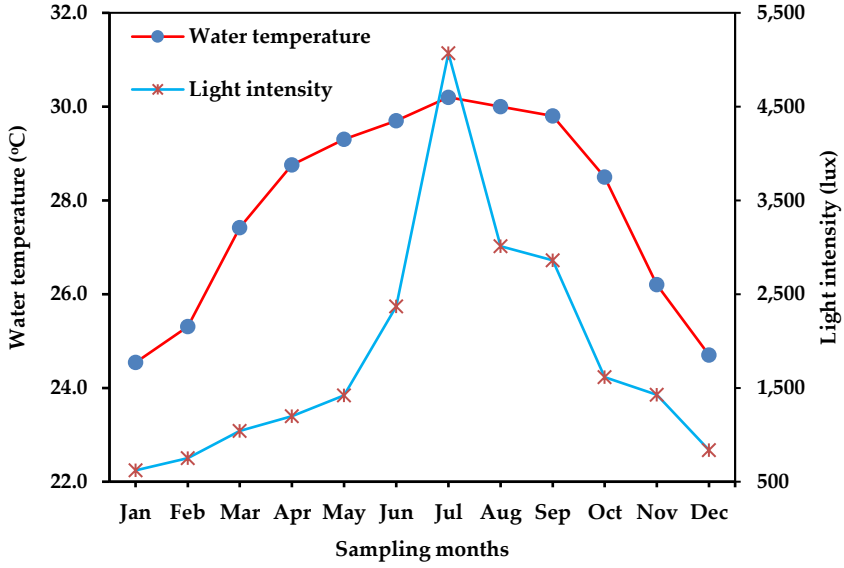

Figure 1. Temporal changes in temperature and light intensity

3.2. Gonadosomatic index

The GSI of males sampled increased significantly, from 0.31% in January to a peak in July of 2.14% (Figure 2). The GSI was still high in August (1.73%) but dropped significantly ($p < 0.05$) in September (1.23%). The average GSI collected from November to December remained below 1.0%.

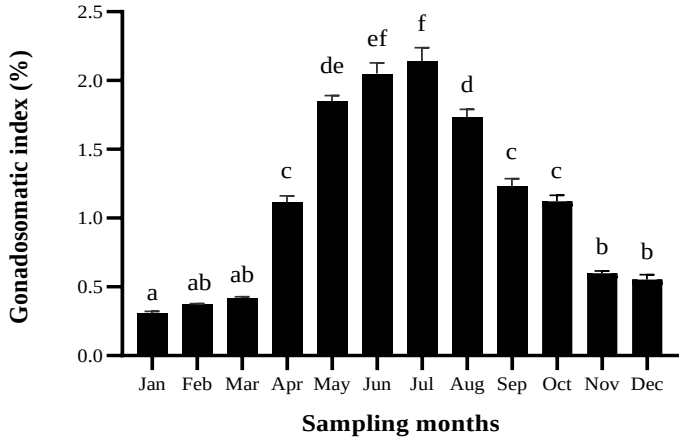

Figure 2. Temporal changes in gonadosomatic index of male *Wallago attu*

Notes: Each value represents mean ± SE. Different letters above the bars denote significant differences in GSI between sampling times.

Based on the GSI values, our findings suggest that the maturation of male *Wallago attu* started in May and lasted until August. Among indicators of the stage in the reproductive cycle of teleost fishes, the GSI value has been used widely to represent

gonad development in terms of maturation and spawning (Shendge et al., 2010). These data are in agreement with results from wild *Wallago attu* collected from Maharashtra, India, in which the minimum and maximum GSI were recorded in January and August, respectively (Shendge et al., 2010). Previous studies have considered similar levels of GSI as an indicator that can be used to predict the degree of maturation and energy reservation in fish (Schulz et al., 2010; Zutshi & Murthy, 2001). Moreover, GSI can be considered an index to identify testicular development in male *Wallago attu*. According to Shendge et al. (2010), the gonadosomatic index showed a peak in reproduction during the breeding season. Therefore, it is probable that the spawning period of *Wallago attu* males cultured in captivity in the central part of Vietnam occurs from June to August. In India, spawning of wild *Wallago attu* chiefly occurs during the monsoon season from May to September, with the peak of spawning varying regionally (Gupta, 2015).

3.3. Testicular development

From January to March (immature period), gonad histology of the testes showed spermatogonia and spermatocytes. During this period, all males were observed to be immature. Seminiferous tubules contain all the spermatogenic cells. The number of spermatocytes steadily increases with time, but spermatids and spermatozoa are absent (Figure 3a). From April to May (pre-spawning period), the seminiferous tubules contained a high concentration of spermatocytes, spermatids, and some spermatozoa. The seminiferous tubules are filled with spermatozoa at the end of this stage (Figure 3b). The testis reaches its largest size from June to August (spawning period) when the testis contains the highest concentration of spermatozoa. A large amount of milt was expressed from the testes and sperm duct during this period (Figure 3c). From September to December (post-spawning period), residual spermatozoa were still present in the sperm duct and the seminal lobules. Gonad histology of the testes showed that testis in regression is characterized by empty tubules with connective tissue and primary and secondary spermatocytes (Figure 3d).

Moreover, in Vietnam, *Wallago attu* broodstock cannot naturally release gametes or fertilize in captivity. Therefore, it is particularly important to understand the timing of sperm production, which requires a thorough understanding of spermatogenesis and will also help improve the guidance on broodstock breeding and gamete production of *Wallago attu*. The process of spermatogenesis goes through several stages, including spermatogonia undergoing proliferation and differentiation, developing to spermatocytes and then spermatids, and finally, maturing into spermatozoa (Mylonas et al., 2010; Palma et al., 2019). From our data, reproductive maturity is considered to be the spermiation in male *Wallago attu* and is divided into four periods: immature, pre-spawning, spawning, and post-spawning. This assumption is reinforced by the histological examination of testis samples collected during this study and the observation that the majority of testes sampled in June had reached the highest concentration of spermatozoa. On the other hand, testicular histology was observed to be variable at sampling times and well-correlated with variations in GSI.

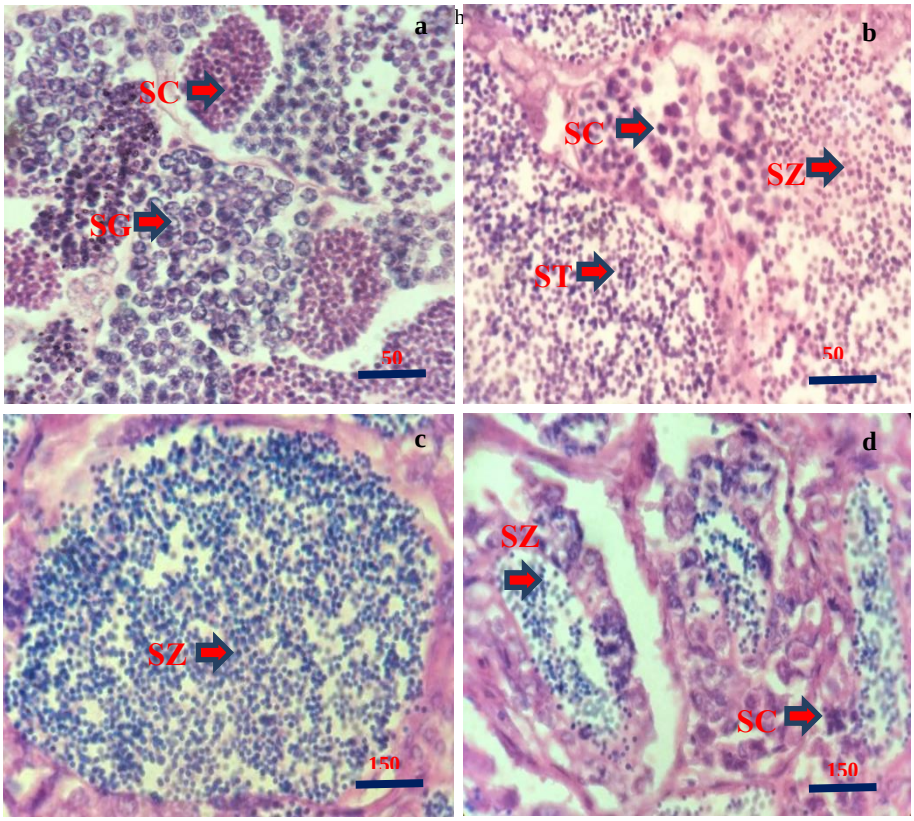

Figure 3. Histological observations of *Wallago attu* testes at different stages of spermatogenesis

Notes: SG: spermatogonia; SC: spermatocytes; ST: spermatids; SZ: spermatozoa.

3.4. Plasma levels of T and 11-KT

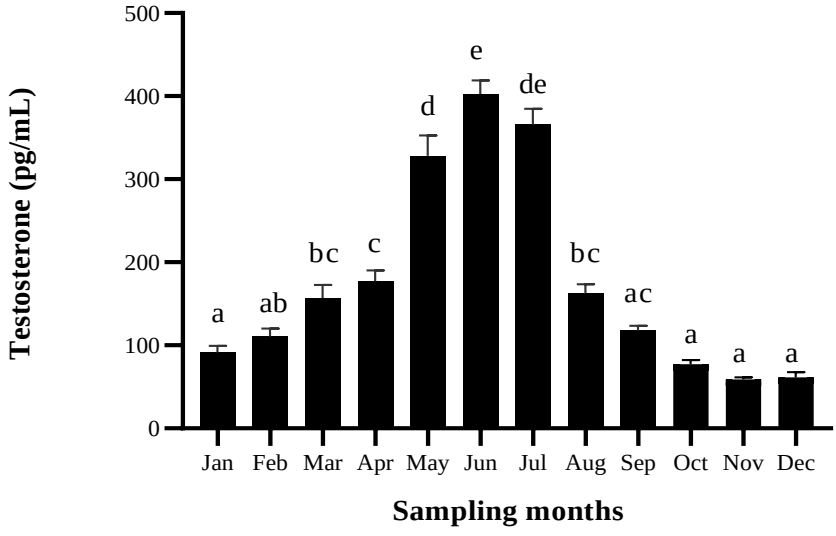

Figure 4. Temporal changes in plasma T of male *Wallago attu*

Notes: Each value represents mean ± SE. Different letters above the bars denote significant differences in T between sampling times.

In this study, the plasma T levels showed monthly fluctuations. The T levels were low during the period of immaturity from January to March, then increased rapidly from April to May, and reached a peak in June (402.1 ± 16.7 pg/mL). In contrast, T level concentrations decreased significantly from August to December, with the lowest value (58.8 ± 2.5 pg/mL) in November. No significant differences were observed in plasma T levels among the monthly samples from October to December (Figure 4).

Significant differences in the levels of plasma 11-KT between sampling months were observed ($p < 0.05$). The concentrations of 11-KT were low and no significant differences were found from January to March. The levels of plasma 11-KT increased significantly and reached a peak in May (76.9 ± 4.7 pg/mL). This is attributed to the fish undergoing spermiation with the advance of active spermatogenesis. The concentration of 11-KT in the plasma of fish in June and July started to decrease slightly but were not significantly different ($p < 0.05$) from May. The lowest plasma 11-KT levels (10.8 ± 0.9 pg/mL) were found in November (Figure 5).

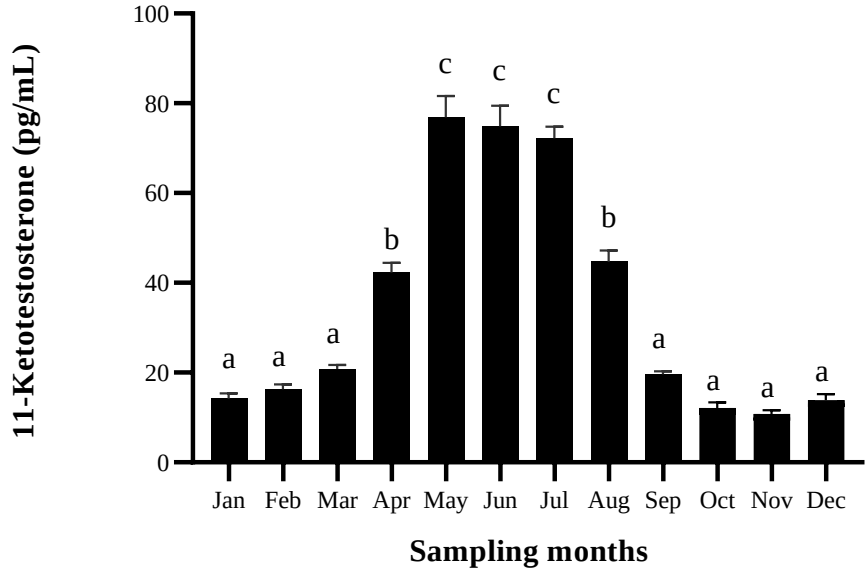

Figure 5. Temporal changes in plasma 11-KT of male *Wallago attu*

Notes: Each value represents mean ± SE. Different letters above the bars denote significant differences in 11-KT between sampling times.

The temporal changes in plasma T concentrations followed the increasing water temperature and light intensity, and the gonadosomatic index entered the rapid growth stage. Conversely, lower levels of plasma T in the winter from October to December could be related to lower water temperature and light intensity that may reduce the hormonal processor of fish.

Thus, testicular development is related to increases in plasma T concentration. This shows that T influences some steps of spermatogenesis, such as spermatogonial multiplication and spermatocyte formation (Billard et al., 1982). Our results are consistent

with the findings by Pham and Le (2020) and Borg (1994). Previous studies on the same species demonstrated that plasma T levels simultaneously increased during the pre-spawning period and rose markedly during the spawning period (Adebiyi et al., 2013; Ismail et al., 2011). In several fish species, the mature testis contains not only T but also 11-KT, which are the main testicular steroids functioning in the final maturation process (Nagahama et al., 1994; Schulz et al., 2010). In the present study, the strong seasonal pattern of circulating concentration of T and 11-KT in captive *Wallago attu* confirms findings from previous studies in other freshwater catfish (Cavaco et al., 2001; Manosroi et al., 2003). Plasma 11-KT were at low levels during the immature period and post-spawning period. In this study, the fluctuation of plasma 11-KT is evidence that it plays an important role in the testicular activity of *Wallago attu* in captivity. According to Lintelmann et al. (2003), high levels of 11-KT suggest active spermatogenesis and spermiation and fall during reproductive quiescent periods in fish. From our data, we can conclude that there is a relationship between plasma levels of steroid hormones and stages of testicular development and GSI in male *Wallago attu.*

4. CONCLUSION

In our study, temporal variations of GSI, histological observations, and plasma levels of T and 11-KT were measured to find reliable parameters to predict the maturation stage of male *Wallago attu* in captivity. Taken together, these data suggest that the onset of the spawning season of captive male *Wallago attu* in the central part of Vietnam ranges from June to August. This research is expected to facilitate the future development of *Wallago attu* farming in Vietnam by providing the first definitive data regarding this species' reproductive cycle, which, in turn, help determine the type and time point of hormonal administration to induce spawning by *Wallago attu* in captivity.

5. THE CERTIFICATE OF ANIMAL ETHICS APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were approved by the Hue University Animal Ethics Committee (Permission No. 449/QĐ-DHH-HUVN0006).

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