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Steroid Profiles of Wild Terror Green (*Andinoacara rivulatus*) Associated with Gonadal Histology in the Baba River, Ecuador

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***Abstract*— Native cichlids in Ecuador are represented by *Andionoacara rivulatus* and *Cichlasoma festae* mainly. These aquatic species are a fundamental part of the food chain of rural communities in the western subtropical region of Ecuador. For the first time, a detailed study on the gonadal development in *A. rivulatus* males and females was carried out, and the characterization of sex hormones present in their blood plasma from May 2019 to January 2020. Fish samples were captured in the multipurpose Baba River dam upstream of the Guayas River, Ecuador. Gonad and blood samples were collected for histological analysis and assaying of steroidal hormones such as 17β-estradiol and 11-ketotestosterone. The sex steroids were quantified by enzyme immunoassay (ELISA). As a result, the fish samples have asynchronous characteristics and show all development stages in testes and ovaries. The serological fluctuations show statistical differences ($p < 0.05$) between the development stages, with low concentrations of estradiol and 11-KT. These results are somewhat related to the hydrological period of capture, where the temperature, luminosity, and rainfall play a fundamental role in the oogenesis and spermatogenesis in the captured fishes. The highest concentration of E2 is detected in female vitellogenesis stages and the highest concentration of 11-KT in the male spermatogenesis stage. These results confirm the fundamental role of these hormones in the key periods of gonadal development. In future studies, it is important to monitor the maturational hormone 17α, 20β-DHP, and the vitellogenin concentrations and thus understand the reproductive physiology of *A. rivulatus*.**

***Keywords*— *Andinoacara rivulatus*; green terror; Gonad maturation; sex steroids; Baba river; Ecuador.**

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I. INTRODUCTION

Terror green (*Andinoacara rivulatus*) is a freshwater benthopelagic cichlid fish, locally known as "*vieja azul*" in Ecuador. The genus *Andinoacara* is originated from north-western South American species found between Esmeraldas, Ecuador, and Tumbes, Peru [1], [2]. The global landings of *A. rivulatus* caught and harvested from aquaculture had a historical peak of about 329 tons between 2000 and 2018. However, the catches were intensely decreasing in later years due to overexploitation, environmental pollution, and loss of habitats and refuges for spawning [3]-[5].

Few studies related to nutritional behavior and taxonomy were conducted for green terror [1], [6], [7]. However, many relevant questions about reproductive biology, especially about the cycle of sexual maturation and reproductive endocrinology, remain undisclosed [6]. The hypothalamic-pituitary-gonad (HPG) axis is the central paradigm in the study of the reproduction biology, which can be affected by environmental, endocrine, paracrine, autocrine, and intracrine factors [8]-[10].

Wild cichlids can naturally spawn in a tank when environmental conditions are favorable. However, several fish species are inhibited from reproductive processes during captivity [11]. In general, the inhibition of reproductive processes occurs in females and is associated with final oocyte maturation, ovulation, and spawning [11]. These dysfunctions are attributed to a combination of captivity-induced stress, a suitable environment for natural spawning, and social stimulation of a compatible mate [12]. Endocrine regulation strategies have been widely employed to synchronize fertility and improve reproductive processes in fishes. However, their success varies by species. In this regard, the carp pituitary extracts, and gonadotropin-releasing hormone (GnRH) analogs were widely used in the critical stage of fish reproduction [13]. However, these treatments are not effective in the quality of reproduction in many species of

cichlids. They can affect the quality of oocytes due to different structures of the glycosidic residues in the heterodimers that constitute follicle-stimulating hormone (FSH), especially in the β subunit, which alters the response of the steroidogenic signal for binding to its specific membrane receptor. In farming green terrors, an alternative for reproductive management is the use of tilapia pituitary, which has a homology and provides interesting results in *in vitro* and *in vivo* cultures in tilapia [10]. Another is the use of fortified foods to achieve high productivity and controlled spawning of captive wild breeders [6].

Thus, the steroid levels in blood have been analyzed to find the optimal time for inducing hormonal spawning in fishes. This analysis can help to obtain high-quality gametes and strong larvae. It is also viable for production and prevents over maturation and follicular atresia of gametes [14]. Strictly, the quantitative analysis of steroidal hormones in blood to predict the maturation stage of green terror requires less manipulation of the reproducers than the conventional invasive method of gonadal biopsies, which requires a large number of samples. On the other hand, understanding of the concepts of reproductive organization, such as the maturation cycle, reproductive endocrinology, and gonadal development, is unclear for green terror. To improve small-scale aquaculture with a focus on the sustainability of local fisheries. Thus, study on native cichlids in Ecuador is essential. Therefore, the objectives of this study are to recognize the developmental stages of gametes and to characterize the plasma sex steroidal hormones during the maturation cycle of wild green terror.

II. Materials and Method

A. Biological Samples

A. rivulatus specimens were captured by shortline gear in 2-3 m depth at the multipurpose Baba River dam upstream Guayas River (0° 39' 08.47'' S; 79° 25' 06.90'' W, Buena Fé, Ecuador). Fishing activities were carried out from May 2019 to January 2020. Throughout this period, fishes were collected every three or four weeks, depending on weather conditions. Immediately upon reaching the surface, fishes were sacrificed, and the samples (blood and gonads) were collected and stored for further examination.

B. Gonadal Histology

For histological analysis, we collected 43 samples consisting of 23 females and 20 males of *A. rivulatus* at different maturation stages, as shown in Fig. 1. After extracting gonads from the cultured fishes, the tissues were fixed in 5% formalin for 24 h. The fixed tissues were then subsequently dehydrated and embedded in paraffin blocks. The embedded tissues were longitudinally sectioned (7 μm) and stained with hematoxylin and eosin. The fish sections were examined by using an optical microscope and classified according to their maturation status, as immature, proliferation, growth, maturation and spawning. After determining the maturation stage, the samples were correlated with the levels of serological steroid.

C. Analysis of Hormonal Steroids

Blood was collected from caudal vein of each fish with a heparinized syringe. The collected blood samples were placed on ice, where they were allowed to clot for 3-6 h. Blood samples were later centrifuged for 15 min at 1500 g (Scilogex Centrifuge, D3024 R) and serum stored at -80°C for further sex steroid hormone analysis.

An enzyme-linked immunoassay technique (ELISA) was adopted by using commercial kits, protocols in 11-ketotestosterone (11-KT) and 17 β-estradiol (E2) (Cayman Chemicals Company, MI, USA). ELISA analysis was performed for all samples in duplicates and a separate standard curve was run for each ELISA plate. Different serial dilutions of the steroid were prepared, and their levels were validated by running in parallel to the relevant standard curve. The detection limit of steroid was 10 pg/mL by estradiol and 11-KT.

D. Statistical Analysis

Data from steroid samples were monitored for checking similarity of alteration and familiarity with the Levene and Shapiro-Wilk tests, respectively. The analysis of variance (ANOVA) was conducted to determine the existence of significant differences among treatments. Tukey´s test determined differences in mean values. In addition, data were analyzed by employing the Kruskal-Wallis nonparametric test followed by the Mann-Whitney test. The results were considered as significant if $p \leq 0.05$. The data were expressed in mean ± SEM with Info-stat 5.0 statistical software.

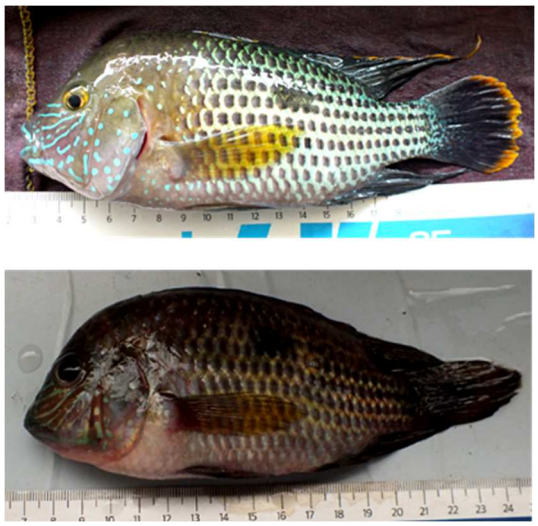

Fig. 1 *Andinoacara rivulatus* "vieja azul" (a) male; (b) female.

III. Result and Discussion

The average temperature from May to October in 2019 was 23.92 ± 0.69 °C and from heliophany are November 2019 to January 2020 was 25.75 ± 0.49 °C, respectively. Corresponding rainfall 34.10 ± 54.56 and 376.31 ± 265.69 mm, and 62.96 ± 25.93 and 69.04 ± 37.11 hours, respectively. Under these environmental conditions, the detected natural spawning frequencies were different ($p < 0.05$).

A. Gonad Morphology and Histology in Male Fish

The testes of *A. rivulatus* males are pairs, of similar size and color. The organ has a lobular with the same

morphological characteristics throughout the tissue and is located next to the swim vesicle of the fish. Histological analysis for the testes shows the presence of all stages of gonadal development, as demonstrated in Table 1.

TABLE I
HISTOLOGICAL CLASSIFICATION OF GONADAL MATURITY STAGE OF WILD TERROR GREEN (*A. RIVULATUS*)

Stages	Females (n=23)	Males (n=20)
Immature	3	7
Proliferation (spermatogenesis)	8	2
Growth (spermiogenesis)	4	2
Maturation (spermiation)	2	4
Spawning	6	5

The longitudinal and width of testes extracted from seven immature individuals are 18 ± 1 mm and 2 ± 0.3 mm, respectively. Germ cells exist in testicular lobes and primary spermatogonia. In addition, sertoli cells are bordering the spermatogonia, spermatocytes, and fibroblasts in the interlobular interstitial tissues (Fig. 2A). Two individuals are in the spermatogenesis stage. The presence of different generations of spermatogonia and spermatocytes in the secondary spermatogonia that proliferate mitosis is observed. The length and width of the testis are 20 ± 2 mm and 2 ± 0.3 mm, respectively. Each spermatogonium gives rise to a clone of isogenic germ cells, which in turn convent to spermatozoon spermatocytes (Fig. 2B).

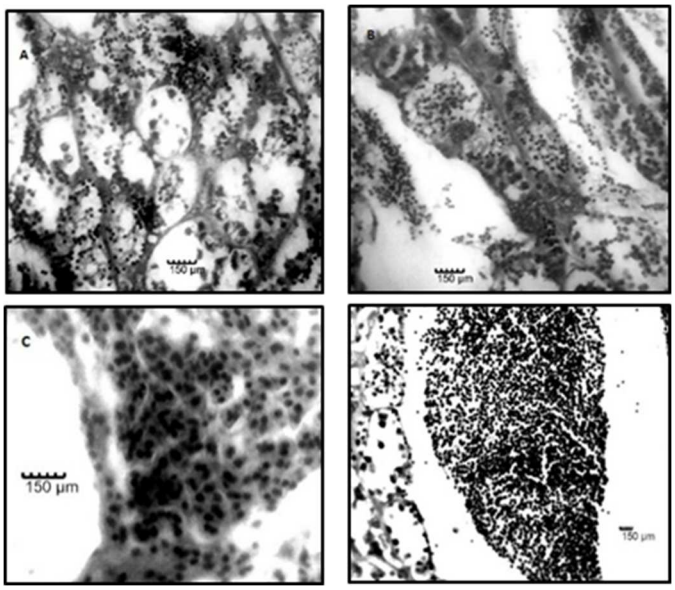

Fig. 2 Cross sections of *A. rivulatus* testes showing different maturity stages. Testes in (A) immature stage (40x), (B) proliferation stage (40x), (C) growth stage (40x), (D) maturation stage (40x).

Two individuals are found in the spermiogenesis stage, with a dominance of spermatids and a considerable quantity of sperm, spermatocytes, and spermatogonia (Fig. 2 C). The length and width of testis are 25 ± 2 mm and 4.00 ± 0.2 mm, respectively. Four individuals are in the spermiation stage, with exclusive presence of free semen in the lumen of lobular testicle (Fig. 2D) with a testis length of 30 ± 3 mm, width 6 ± 0.2 mm. Finally, five fish are in the spawning stage with a small amount of free sperm and occasional spermatogonia in the lumen of the testis.

B. Gonad Morphology and Histology in Female fish

The ovaries of *A. rivulatus* are elongated paired organs with bilobed sacs, located around the swim vesicle. The wall of ovaries is transparent and thin and contains oocytes at different stages of development, characteristics of an asynchronous fish (Fig. 3).

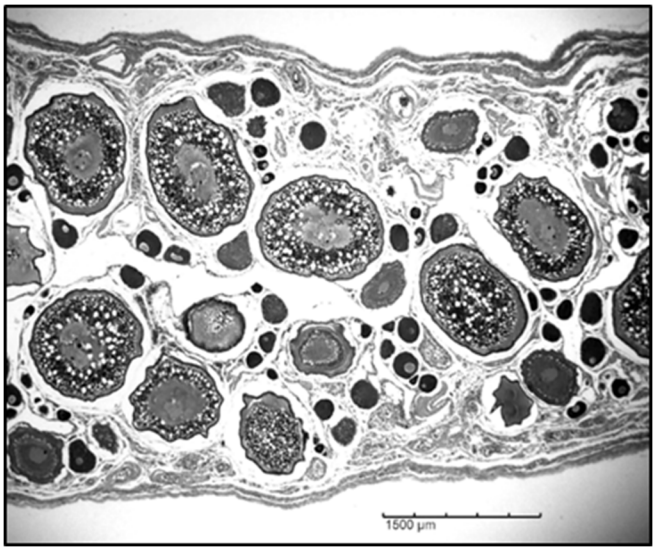

Fig. 3 Asynchronous ovary of *A. rivulatus* (4x) (1500 µm).

The ovaries are fused with an extended and short oviduct, which is directed towards the urogenital pore. The ovaries at the early-stage exhibit yellowish color, their coloration increased with the advance of development stage. The absolute fecundity per fish is 1884 ± 250 oocytes. Histological analysis shows that the ovaries of all fish samples are at different stages of development (Table 1).

Three individuals are in the immature state with the formation of follicular cells, where ovarian lamellae are observed with a fold of germinal epithelium that covers the internal part of the ovary. In addition, the tunica albuginea and various oogonia immersed in the ovarian stoma are observed, giving rise to perinuclear follicles (Fig. 4A). Eight individuals are in the proliferation, the first growth stage. They show the presence of previtellogenic oocytes at different stages of primary follicular development and the beginning of the secondary growth. The visualized ovarian cavity has several groups of oogonia, follicular layers, and zona radiata (Fig. 4B).

Four individuals are in the early vitellogenesis phase, which contains oil droplets and cortical alveoli. The second growth phase follicles with lipid inclusions are also detected in the ooplasm, located around the nucleus with numerous nucleoli. The cortical nucleoli arranged in the peripheral ooplasm, below the zona radiata are observed (Fig. 4C).

Two individuals are in the maturation stage, with notable increase in size due to hydration, with lipid inclusion and yolk granules distributed in a crown shape in the peripheral ooplasm, with the area radiata thickened and surrounded by follicular layers (Figs. 4D and 4E). Finally, six fish are in the spawning phase. They have a large number of post-ovulatory follicles, atretic oocytes, and oocytes in the early stages of oogenesis (Fig. 4 F).

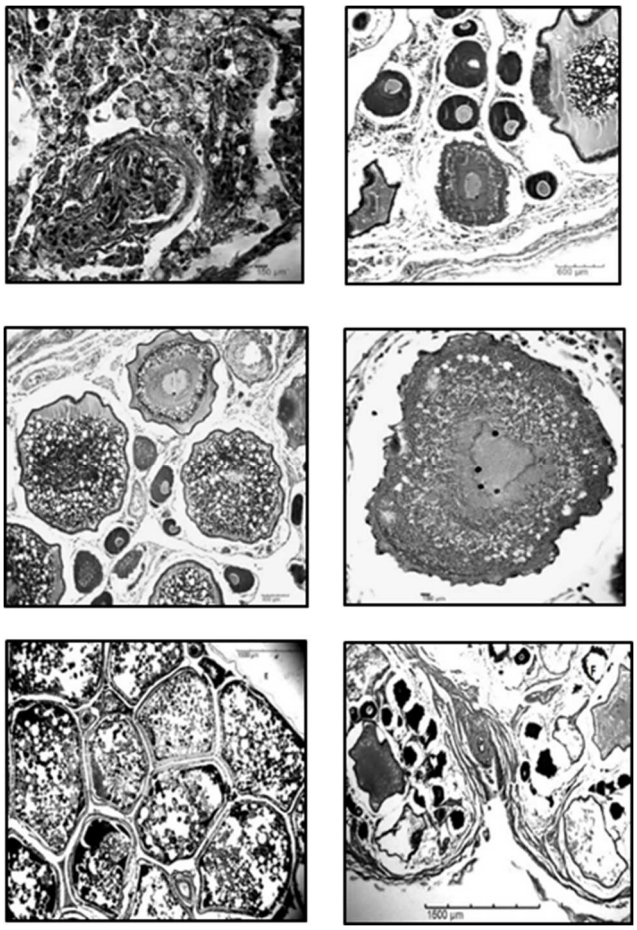

Fig. 4 Cross sections of *A. rivulatus* ovaries showing different maturity stages. Ovary in (A) mature stage (40x), (B) proliferation stage (10x), (C) vitellogenesis stage (10x), oocite in (D) vitellogenesis stage (40x), ovary in (E) maturation stage (4x), (F) spawning stage (4x).

C. Plasma sex Steroid Hormonal Profile 11-keto-testosterone

The concentrations of 11-KT in plasma show significant differences ($p < 0.05$) between the males found in the proliferation stage and other developmental stages. The average size of the male samples was 20.54 ± 2.04 cm with a bodyweight of 214.00 ± 64.33 g.

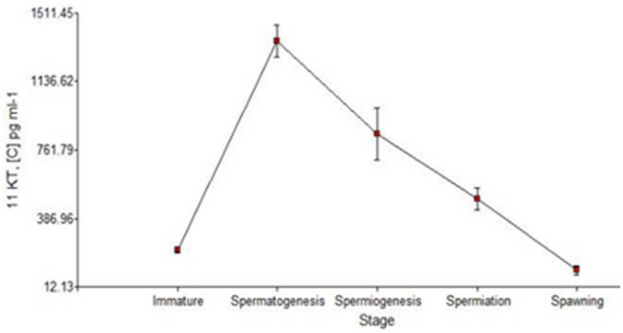

Fig. 5 Plasma steroidal profiles of *A. rivulatus* males in a maturation cycle (mean ± standard deviation) ($P < 0.05$).

In the immature stage, the 11-KT in plasma of collected fish samples maintain an average of 215.75 ± 39.48 pg/mL. However, in the period of spermatogenesis, the levels of this hormone are increased significantly ($p < 0.05$), reaching a high level up to 1357.2 ± 121.75 pg/mL. As the gonadal develops, the concentration of 11 KT begins to decrease progressively from 849.56 ± 200.98 pg/mL, 464.28 ± 120.30 pg/mL, and 103.33 ± 51.55 pg/mL in the spermatogenesis, spermiation, and spawning stages, respectively (Fig. 5).

D. 17β-estradiol

The estradiol concentrations in *A. rivulatus* plasma show significant differences according to the development of gonadal stages ($p < 0.05$). These differences occur in the developmental phases and the vitellogenesis period. The average length and bodyweight of the female ovaries are 13.13 ± 1.45 cm and 171.12 ± 40.9 g, respectively.

The immature gonadal stage maintains a concentration of 94.99 ± 5.10 pg/mL and the proliferation stage with a concentration of 124.78 ± 16.48 pg/mL. Thereafter, the concentration in the vitellogenesis stage is significantly increased ($p < 0.05$), reaching to 668.68 ± 436.93 pg/mL. Subsequently, the estradiol levels are decreased largely ($p < 0.05$) to concentrations of 58.76 ± 2.31 pg/mL and 36.91 ± 8.56 pg/mL at the maturation and ovulation stages, respectively (Fig. 6). On the other hand, the estradiol concentrations in the plasma of immature males fluctuate from 10.4 to 32.75 pg/mL, and according to the histological samples analyzed (Fig. 2A).

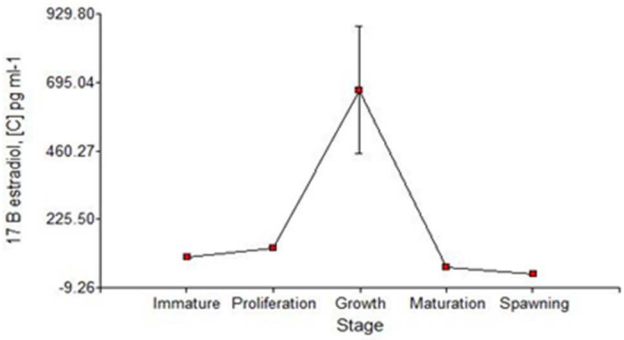

Fig. 6 Plasma steroidal profiles of *A. rivulatus* females in a maturation cycle (mean ± standard deviation) ($P < 0.05$).

The Cichlidae family is common on the Ecuadorian coast. It includes several species such as *Andinoacara rivulatus, Cichlasoma festae, Aequidens sapayensis* [15], *Aequidens coeruleopunctatus* [16,17], as well as exotic species such as Nile tilapia (*Oreochromis niloticus*), Tilapia hybrids (*Oreochromis* sp.) among others [18].

Studies on the reproductive anatomy and physiology are important to understand the reproduction of these species in natural environments. This study represents histological details of the gonadal development at different stages of development and the profile of sex steroids related to the reproduction of *A. rivulatus*.

The histological evaluation shows that germ cells exist in all developmental stages, clearly determining the asynchronous ovary and testicular development. The histology of testes shows the four relevant stages, including spermatogenesis, spermiogenesis, spermatocytes, and release of sperm into the lumen. The morphological and physiological changes in the testicle are observed. These changes are directly associated with spermatogenesis having the characteristics described at each stage of development [19]. In the majority of organisms, sperms are released in the tubular lumen more to the vas deferens. This implies that the

organisms are in a continuous spawning process. The presence of estrogens in male plasma samples is related to the renewal of primary spermatogonia, but not to spermatogonial proliferation [20,21]. Therefore, the test samples analyzed in the immature stage of *A. rivulatus* exhibit these characteristics. However, the low concentrations of 11-KT would be an incidence of primary spermatogonia as in the proliferation phase [22].

The androgen 11-ketotestosterone is the most representative hormone. It regulates the behavior of spermatogenesis from spermatogonial proliferation to spermiogenesis [23], [24]. The concentration of 11-KT in the spermatogenesis phase is high compared to the other stages of development. These demonstrate the importance of this hormone at this phase of development. In other fish, such as Neotropical cichlids, there is a direct effect on the behavior of spermiogenesis, and the concentrations are different [25]. However, it should be noted that the samplings were conducted in cold seasons. Thus, the low concentrations of steroid in *A. rivulatus* might be affected by the temperature during the fish sampling [26].

The gonadal development and histology for *A. rivulatus* females are similar to commercial cichlids, such as tilapia zilli and tilapias [27], exhibiting the same characteristics at each stage of oocyte development with asynchronous characteristics. Relative fertility studied by Jamali *et al.* [6] was ranged from 8.5 to 28.6 oocytes/g of female. This result is comparable with those from 9.54 to 12.4 oocytes/g female obtained in *A. rivulatus* under natural conditions.

Studies on the reproductive cycle of commercial cichlids show parallel fluctuations in the FSHβ and LHβ promoter genes after spawning and ovulation. This suggests that the FSH and LH play an important role in asynchronous fishes, both for vitellogenesis and for maturation and spawning [8,9]. In the present study, the concentrations of E2 are elevated in vitellogenesis of *A. rivulatus*. However, it is much smaller than commercial cichlids ranging from 7.7-9.7 to 20.8-23.1 ng/mL [28,11]. In this sense, it can be speculated that the response generated in follicular cells of ovary does not depend only on the availability of circulating peptide hormones, but also on the nearby factors such as light and temperature. In addition, it would affect the seasonality and concentration of E2, and thus the concentrations of Vitellogenin (Vtg) in plasma, prolongation of gonadal development during this period [10]. Consequently, these aspects are related to the low concentrations detected in *A. rivulatus*.

The monitoring of environmental parameters, such as temperature, oxygen, pH, salinity, light and conductivity, affects the reproductive cycles of fishes. The number of oocytes per laying is increased. Thus, the steroidogenic behavior occurs in asynchronous fishes. Furthermore, it should be considered that the enzymatic machinery in asynchronous fishes directly affects gonadal maturation and steroidogenesis [29].

It was reported that *A. rivulatus* has a buccal incubation and presents a specific gonadal structure in the spawning stage, primary oogonial formation, and previtellogenic proliferation. Baroiller *et al.* [30] found that the enzymatic activity 3β-hydroxysteroid dehydrogenase was over-expressed in buccal incubation periods in commercial cichlids, in addition to the differences in enzymatic activities of cytochrome P450c17, 17α hydroxylase, and 17,20 lyase, after spawning and before ovulation. These enzymes are important because they regulate the pathways for E2 synthesis and the maturational hormone (DHP), directly affecting the proliferation, vitellogenesis, maturation, and spawning stages in asynchronous fishes.

IV. Conclusion

This study examined the serological fluctuations under natural conditions of estradiol and 11-ketotestosterone during the gonadal maturation of *A. rivulatus* caught in the Baba River reservoir. These hormones are responsible during the periods of oogonial proliferation and vitellogenesis in adult females and the processes of spermatogenesis, spermiogenesis, and spermiation in males, respectively. It is a kind of asynchronous characteristic where the gonadal development is closely related to the environmental conditions and where temperature affects the gonadal development under natural conditions. Studies to determine maturational hormone concentrations in the near future and to detect plasma vitellogenin for gonadal development would be carried out. This will provide basic evidence on the reproductive physiology of *A. rivulatus*. This information would be useful for effective management of this species' reproduction, focusing on the conservation and sustainability of local fisheries.

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