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Dispersion of hooks on the anal fins of primary and secondary males in *Brycon orbignyanus* (Characiformes: Bryconidae): a secondary sexual trait for breeder selection

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Brycon orbignyanus exhibits seasonal reproduction where males can be distinguished from females by the roughness present in the anal fin of the specimens that represents a secondary sexual characteristic known as hooks. This study aimed to describe the appearance and morphology of hooks on the anal fin in *B. orbignyanus* and relate them to the phases of the reproductive cycle of these animals to determine a parameter that can be used for the selection of suitable males for use in induced reproduction. Monthly male specimens of *B. orbignyanus* of different ages were collected (n = 50 total; n = 10/month) and the specimens were euthanised, biometrically measured, and the testes and anal fins were collected. As sample were processed according to the typical techniques for light microscopy, stereomicroscope, diaphanization and scanning electron microscopy. Except for specimens in the testicular stage of Regenerating, hooks were observed in all other stages of the reproductive cycle in *B. orbignyanus* males; however, specimens that possess six or more rays with hooks are considered Spawning Capable and can be selected for breeding. No differences were observed in hooks development patterns as related to secondary sexual characteristics among intersex individuals, primary males, or secondary males.

Keywords: Characiformes, Fish reproduction, Piracanjuba, Sexual dimorphism, Testicular development.

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Brycon orbignyanus apresenta reprodução sazonal onde os machos podem ser diferenciados das fêmeas pela aspereza presente na nadadeira anal dos exemplares, que representa uma característica sexual secundária conhecida como espículas. Este estudo teve como objetivo descrever a morfologia das espículas na nadadeira anal de *B. orbignyanus* e relacioná-los com as fases do ciclo reprodutivo desses animais para determinar um parâmetro que possa ser utilizado na seleção de machos adequados para reprodução induzida. Foram coletados mensalmente machos de *B. orbignyanus* de diferentes idades (n = 50 no total; n = 10/mês), no qual foram eutanasiados, biometricamente mensurados e tiveram os testículos e nadadeiras anais coletados. As amostras foram processadas segundo as técnicas típicas de microscopia óptica, estereomicroscópio, diafanização e microscopia eletrônica de varredura. Com exceção dos espécimes na fase testicular de Regeneração, foram observadas espículas em todas as outras fases do ciclo reprodutivo em machos de *B. orbignyanus*; entretanto, espécimes que possuem seis ou mais raios com espículas são considerados Aptos a Espermiar e devem ser selecionados como reprodutores. Não foram observadas diferenças nos padrões de desenvolvimento das espículas relacionadas às características sexuais secundárias entre indivíduos intersexo, machos primários ou machos secundários.

Palavras-chave: Characiformes, Desenvolvimento testicular, Dimorfismo sexual, Piracanjuba, Reprodução de peixes.

INTRODUCTION

In teleosts, females and males are distinguished based on their primary and/or secondary sexual characteristics (Wootton, Smith, 2014). The primary characteristics refer only to the presence of sexual organs: ovaries and testes. On the other hand the secondary sexual characteristics are associated with other physical characteristics and represent a form of sexual dimorphism based on morphological differences during the reproductive period of fish (Reis, Malabarba, 1987; Rapp Py-Daniel, Fernandes, 2005).

These morphological features can be easily visible and include characteristics such as size, colouration and gibbosities (Theis *et al*., 2017; Longoni *et al*., 2018) or they can be discrete to the point that they are only noticeable based on touch. Such characteristics include the development of hooks on anal fins or gill glands (Malabarba, Weitzman, 2003; Gonçalves *et al*., 2005; Vieira *et al*., 2016).

The development of these characteristics is involved in the reproductive physiology of the species and is dependent upon the hormonal cascade that triggers the development of the gonads. Therefore, even in fish that undergo sexual inversion or reversal, secondary sexual characteristics follow the phenotype presented in the gonads (Papoulias *et al*., 2000). For example, in the characiform *Astyanax lacustris* (= *A. altiparanae*) (Lütken, 1875) males hooks on the anal fins are present regardless of the season due to the various reproductive peaks that the species undergoes throughout the year; however, these hooks become much more promenent as the testes reach the reproductive stage of Spawning Capable, and this is likely due to the release of androgens that occurs at this point in the reproductive cycle (Siqueira-Silva *et al*., 2020).

In Cichlids, it is common for males to present a post-occipital protuberance (gibbosity) throughout the reproductive period; however, this protuberance is absorbed within a few days after spawning and disappears completely (Chellappa *et al*., 2003; Ronco *et al*., 2019). In Siluriformes, the dimorphism is evident based on size, as in most species of this order the males are smaller than the females. This characteristic alone can allow for the identification of the sexes of these specimens. Additionally, in species of the Doradidae family, during the reproductive period males develop hooks on the dorsal fin that are accompanied by an extension of the first ray of this fin or even the development of odontodes on the sides of the head, thus becoming an adornment for the reproductive members of these species (Rapp Py-Daniel, Fernandes, 2005; Oliveira, Oyakawa, 2019).

The Neotropical region encompasses the greatest diversity of fish species worldwide, and understanding the differences and similarities among the morphological, behavioural, and physiological characteristics of the reproduction of these species is essential to promote production plans for species of commercial interest in captivity (Vari, Malabarba, 1998; Reis *et al*., 2016). *Brycon orbignyanus* (Valenciennes, 1850) that is also known as piracanjuba, is a relatively late sexual maturation characiform (Ganeco *et al*., 2001; Zardo *et al*., 2021) and undergoes sexual differentiation as a gonochoristic species; however, this species does develop secondary males within the population that originate from the sexual inversion of females (Quirino *et al*., 2022).

In addition to being a species of great economic interest, *Brycon orbignyanus* is classified in the "Red Book of Endangered Brazilian Fauna" as Critically Endangered (CR) (A2c), according to the classification established internationally by the International Union for Conservation of Nature (IUCN) (MMA, 2022), due to the low genetic variability of natural populations (Ashikaga *et al*., 2015), and anthropogenic changes in natural environments preventing the reproduction of the species from occurring (Oliveira *et al*., 2017).

Piracanjuba is a species of seasonal reproduction with a reproductive period that occurs between September and January, and during this time period, males can be distinguished from females according to rough present in the anal fins that are termed hooks (Ceccarelli *et al*., 2010). However, despite being present during the reproductive period, little is known regarding the synergy between the emergence of hooks and testicular development in this species.

Thus, this study aimed to analyse the dynamics of the appearance of hooks, a secondary sexual characteristic in males of *B. orbignyanus*, relate them to the phases of the reproductive cycle of these animals to determine a parameter that can be used for the selection of males that are suitable for use in induced reproduction procedures in fish farming. Additionally, we sought to verify if there are differences between secondary sexual characteristics in primary and secondary males.

MATERIAL AND METHODS

Obtaining the animals. Male specimens of *Brycon orbignyanus* (n = 50) of different ages (1–3 years), initially donated by AES Tiête, and later kept in the Experimental Greenhouse of the Neotropical Ichthyology Laboratory (LINEO) – UNESP/FEIS. Voucher specimens were deposited in the Coleção Ictiológica de Três Lagoas, UFMS/Campus Três Lagoas (CITL 1021).

Sampling of fins and testes. Between September and January (2020–2021) that encompasses the reproductive period of *B. orbignyanus* (Ceccarelli *et al*., 2010), monthly collections (n = 10/month) of the testes and anal fins were performed. The animals were euthanised in a benzocaine solution (0.05%), and biometry was subsequently performed to assess body mass (g), total and standard length (cm), total fin length (cm), and height of the first, second, and last ray (cm). Additionally, the number of total rays and those with hooks was determined, and the number of hooks per ray was assessed. Finally, the anal fins and testes were surgically removed by ventral incision of the specimens. All sampled biological material was fixed in 4% paraformaldehyde and 2% glutaraldehyde in Sorensen phosphate buffer (0.1M at pH 7.2). The testes were processed for light microscopy, and the fins were analysed using a stereomicroscope and later processed for diaphanization and scanning electron microscopy.

Light microscopy and stereomicroscopy. After fixation (24h), the testes were sectioned transversally and subjected to dehydration in an increasing series of ethyl alcohol (70%–95%). They were later embedded in Historesin (glycolmethacrylate) Leica Biosystems®. Subsequently, the encased testes were cut into 3µm-thick sections using a Leica® RM 2245 semiautomatic microtome. The slides were stained following the standard Hematoxylin/Eosin (H.E.) protocol. Subsequently, they were analysed and photographed using a Zeiss® AxioScope-A1 light microscope. The stages of the reproductive cycle in the species were classified according to the macroscopic and microscopic characteristics of the testes according to the method proposed by Brown-Peterson *et al*. (2011), and the identification of primary and secondary males was achieved based on the classification system of Quirino *et al*. (2022).

Anal fins were maintained in 4% paraformaldehyde and 2% glutaraldehyde in Sorensen phosphate buffer (0.1M at pH 7.2) for seven days and then transferred to a 70% ethyl alcohol solution. Subsequently, they were photographed and analysed using a Motic® SMZ168 stereomicroscope. All fins had their rays analyzed one by one, so that, when present, all hooks were quantified.

Diaphanization. The protocol proposed by Potthoff (1984) was adapted according to the size of anal fin samples. After they were initially placed in a fixation solution, the fins were transferred to a 70% ethyl alcohol solution for at least two days. They were then dehydrated in an increasing series of ethyl alcohol solutions (80, 90, and 100%) for 3 h each and then with xylene for 24 h. Next, they were stained with alcian blue solution (30%), bathed in bleaching solution and later placed in trypsin to dissolve the musculature. They were then stained with alizarin red solution (30%) and placed in a bleaching solution. The diaphanized fins were preserved in increasing glycerin solutions (25, 50, and 75%) and stored in a 100% glycerin + thymol solution. The times established for each solution were determined according to the size of the samples and thus exhibited slight variations according to sample size.

Scanning electron microscopy. After fixation (seven days), the anal fins were fragmented to obtain individual rays. The rays were then subjected to dehydration in an increasing series of ethyl alcohol solutions (70, 80, 90, 95, and 100%) for 15 min at each concentration, and complete dehydration was performed in a critical point

device with liquid CO_2 (Critical Point Leica® CPD300). After these processes, the rays were metallised with gold-palladium ions using a Leica® Metallizer MED 010. All biological materials were examined and electron micrographed using a Scanning Electron Microscope (EVO LS15, Carl Zeiss ®).

Statistical analysis. To determine the gonadosomatic index (GSI), the formula GSI = (gonad mass/body mass) × 100 was used as described by Vazzoler (1996). Pearson's correlation coefficient was used to analyse the correlations between variables (numerical data was established to classify the stages of testicular development, being: 0 – Immature specimens, 1 – Regressing, 2 – Regenerating specimens, 3 – Developing specimens, 4 – Spawning Capable specimens). Data are expressed as mean ± standard deviation.

RESULTS

Anal fin and hook morphology. The anal fins can be divided into cranial, medial and caudal regions and are 6.4 ± 3.1 cm long and possess 25 ± 3 rays (Figs. 1A, B, D). A first soft ray of 0.73 ± 0.4 cm in height develops in the cranial region, while the second ray that is the first hard ray is 1.5 ± 0.3 cm in height. The remaining rays possess a mean height of 0.8 ±0.2 cm (Figs. 1B, D).

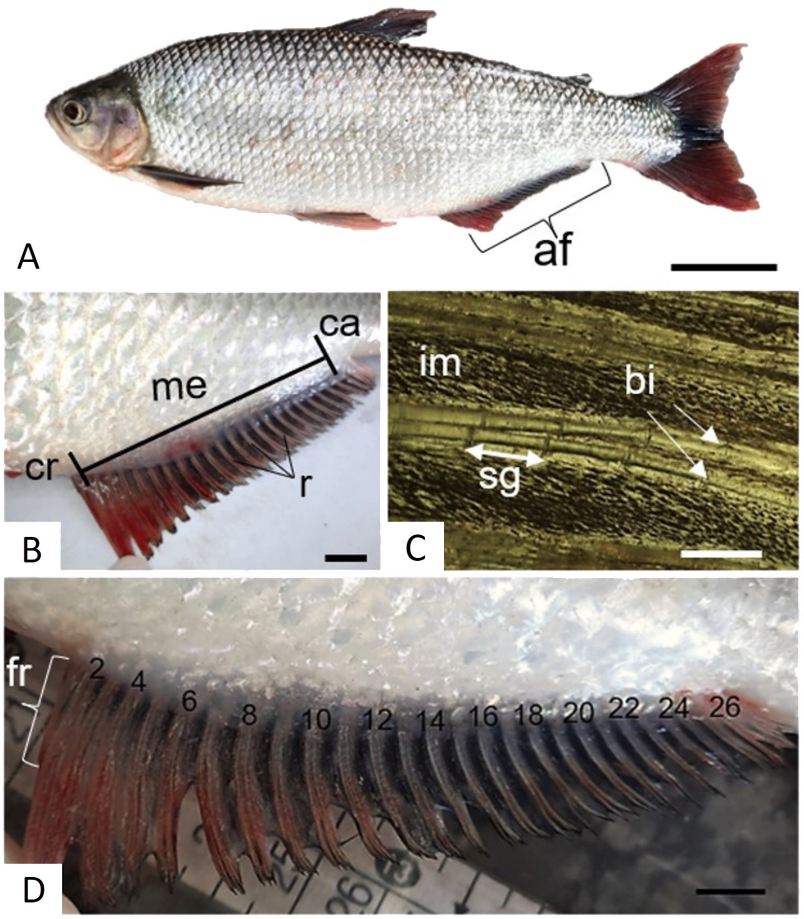

FIGURE 1 | Anal fins in *Brycon orbignyanus*. **A.** Specimen of *B. orbignyanus*. **B.** Anal fin regions. **C.** Rays (r). **D.** Anal fin rays. af: anal fin. bi: bifurcation of rays. ca: caudal region. cr: cranial region. fr: first ray. im: interradial membrane. me: medial region. sg: radius segment. Scales: **A.** 5 cm; **B** and **D.** 1 cm; **C.** 200 µm.

The anal fin of *B. orbignyanus* possesses dark red rays and black interradial membranes (Figs. 1A–D, 2A). Each ray is formed by segments joined by small joints that may or may not contain hooks (Figs. 1C, 2A, B). All rays are covered by an extension of the inter-radial membranes (Fig. 1C). Each ray undergoes an initial bifurcation, and as it approaches the final portion, there is a new terminal bifurcation (Figs. 1C, 2A).

In non-breeding males, the ray segments were smooth and exhibited no traces of hooks development (Figs. 2C, E, 3A–C). However, these animals may undergo the formation of a callosity in the region of articulation between one segment and another (Figs. 3B, C), thus giving the false impression that the animal possesses hooks when, in fact, there are none. Calluses are perceived as sporadic elevations and differ from hooks in that there is no sensation of roughness to the touch.

With the naked eye, the hooks can be observed as small elevations along the rays, and they are best observed in the central region of the fin, as the hooks rays decrease in quantity as they approach the caudal region of the animal (Fig. 2B). Additionally, the hooks were located from the medial portion towards the terminal portion of the rays, and reached the terminal bifurcations, and they were absent in the initial portion located closer to the stomach of the animal (Figs. 2B, 4A, B). Therefore, the sensation of roughness when touching the fins is intense in these regions.

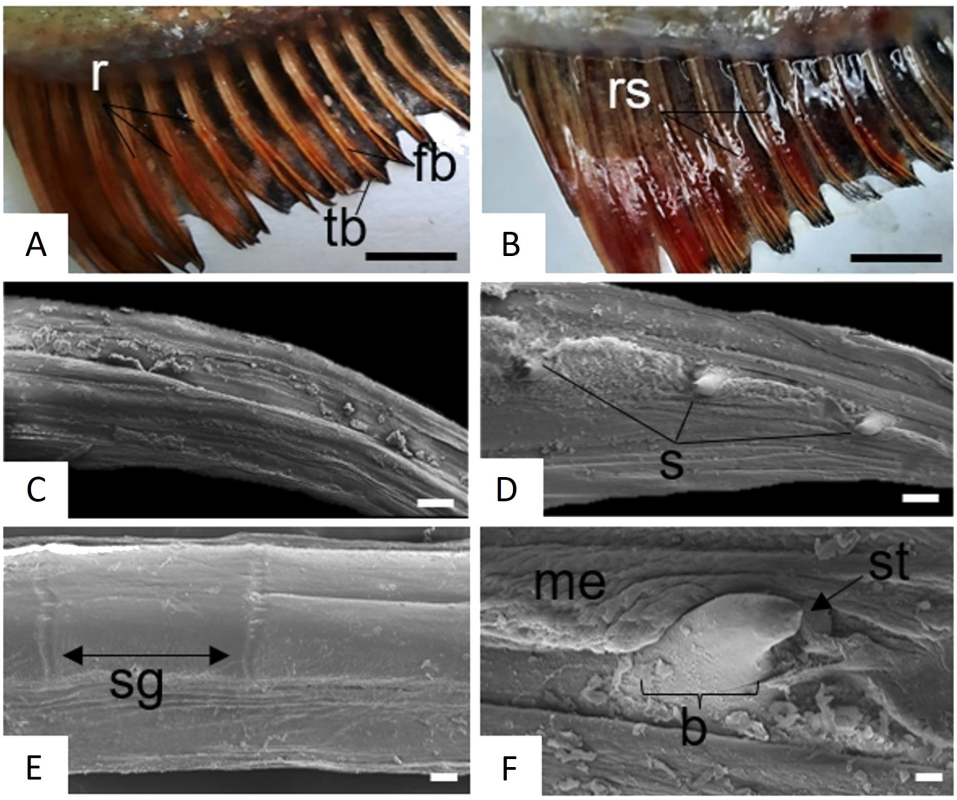

FIGURE 2 | Details regarding the fins of *Brycon orbignyanus*. **A, C** and **E.** Rays without hooks. **B, D** and **F.** Rays with hooks. b: base. fb: first fork. r: rays. rs: rays with hooks. s: hooks. sg: rays segment. st: hooks cusp. tb: terminal bifurcation. Scales: **A** and **B.** 1 cm; **C** and **D.** 200 µm; **E.** 100 µm; **F.** 50 µm.

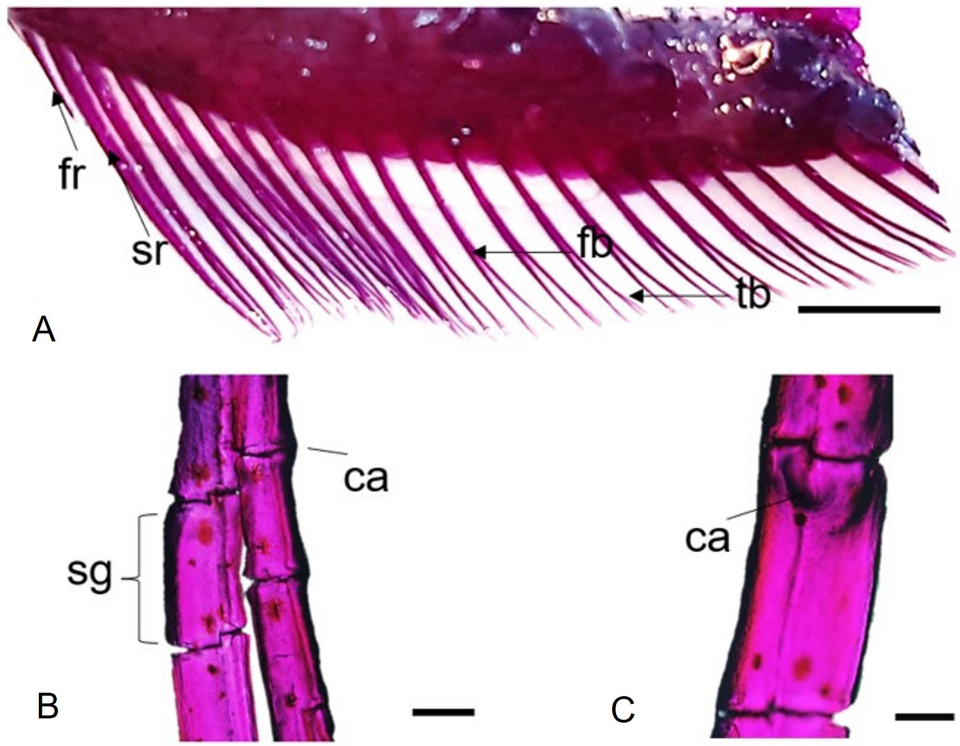

FIGURE 3 | Anal fin of *Brycon orbignyanus* without hooks. ca: callosity. fb: first fork. fr: first ray. sg: rays segment. sr: second ray. tb: terminal bifurcation. Scales: **A.** 0.5 cm; **B.** 200 µm; **C.** 100 µm.

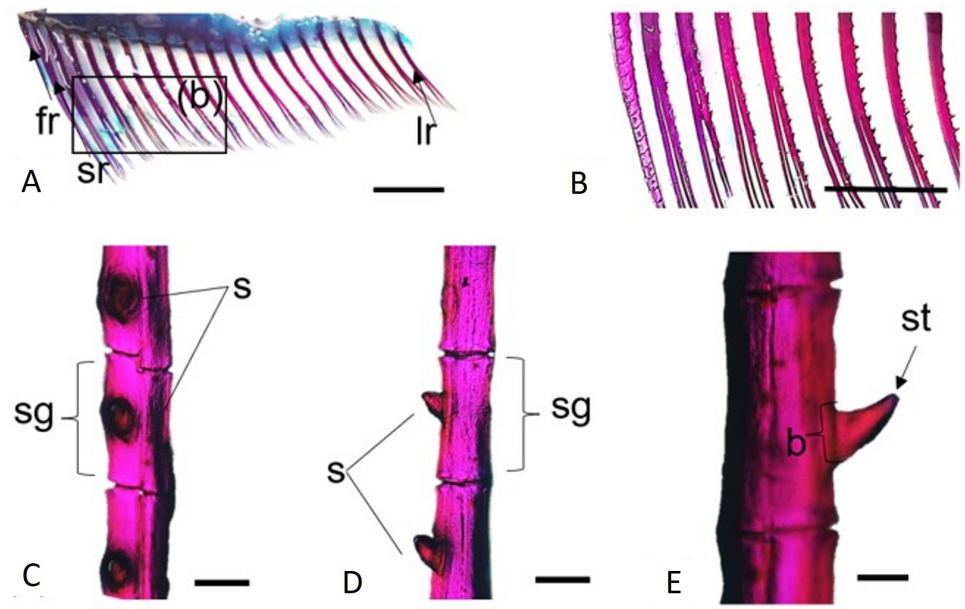

FIGURE 4 | Anal fin of *Brycon orbignyanus* with hooks. b: base of the hook. fr: first ray. lr: last ray. s: hooks. sg: rays segment. sr: second ray. st: hook cusp. Scales: **A** and **B.** 1.0 cm; **C** and **D.** 200 µm; **E.** 100 µm.

Furthermore, the hooks were located from the medial portion towards the terminal portion of the rays, and reached the terminal bifurcations, and they were absent in the region located closest to the stomach of the animal (Figs. 2B, 4A, B). Hooks always emerge in the central region of the ray segment, and therefore, they can be felt from the first signs of growth (Fig. 4C). As they develop, these hooks are characterised by small projections that exhibit the shape of keels with a wide cylindrical base and a thin terminal portion that is similar to a hook-shaped tip facing the stomach of the animal (Figs. 2D–F, 4D, E). They are arranged from the second to the sixteenth ray and contain only one hook per segment (Figs. 4D, E), even after bifurcation. As the rays are cylindrical and each segment possesses only one hook, the hook will only be observed or felt on one side of the fin. Thus, the left or right side of the fins will present or feel differently (Fig. 4B).

Relationship between testicular development and the presence of hooks on the anal fin. According to the classification of the phases of the reproductive cycle proposed by Brown-Peterson *et al*. (2011), the analysed specimens of *B. orbignyanus* were classified as Immature (Figs. 5A, B), Regressing (Fig. 5C), Regenerating (Fig. 5D), and Spawning Capable (Figs. 5E, F) (Tab. 1). Except for specimens in Regenerating, in all other stages of the reproductive cycle in *B. orbignyanus* males with hooks were observed; however, specimens that were Spawning Capable exhibited a greater number of rays that developed hooks (Tab. 1). Two individuals that were considered intersex (Immature) (Fig. 5B; Tab. 1) and two secondary males (Spawning Capable) (Fig. 5F) were identified (Fig. 5F). Both categories (intersex and secondary male) showed the same hooks growth pattern as the other males (Tab. 1).

There was a positive correlation ($p = 0.0001$; $R^2 = 0.78$) between the phases of testes maturation and the number of rays with hooks in primary and secondary males in *Brycon orbignyanus* (Fig. 6). Thus, animals that possess more than six rays with the development of hooks are all considered Spawning Capable (Fig. 6). There was a weak correlation between length and rays with hooks ($p = 0.001$, $R^2 = 0.40$) (Fig. 7).

TABLE 1 | Association of the development of hooks on anal fins with reproductive cycle in males of *Brycon orbignyanus*. TL = Total length; TM = Weight of specimens; PRH = Position of the rays with hooks; GSI = Gonadosomatic index; N = Number of specimens.

Reproductive Phase	N total	N with hooks	PRH	TL (cm)	TM (g)	GSI
Immature	26	1	2–3°	21.5 ± 3.1	126.5 ± 86.5	0.05 ± 0.2
Immature/Intersex	2	1	2–3°	22.5 ± 1.06	135 ± 17	0.019 ± 0.021
Regressing	4	3	2–3°	33 ± 6.06	571 ± 243	0.046 ± 0.04
Regenerating	6	1	2–3°	31.6 ± 5.4	452.2 ± 235.6	0.009 ± 0.009
Spawning Capable/ primary male	10	10	2–15°	32.5 ± 3.5	726.58 ± 184.7	0.917 ± 0.67
Spawning Capable/ secondary male	2	2	2–10°	21.5 ± 0.7	114.28 ± 1.01	0.10 ± 0.10
Total of specimens	50					

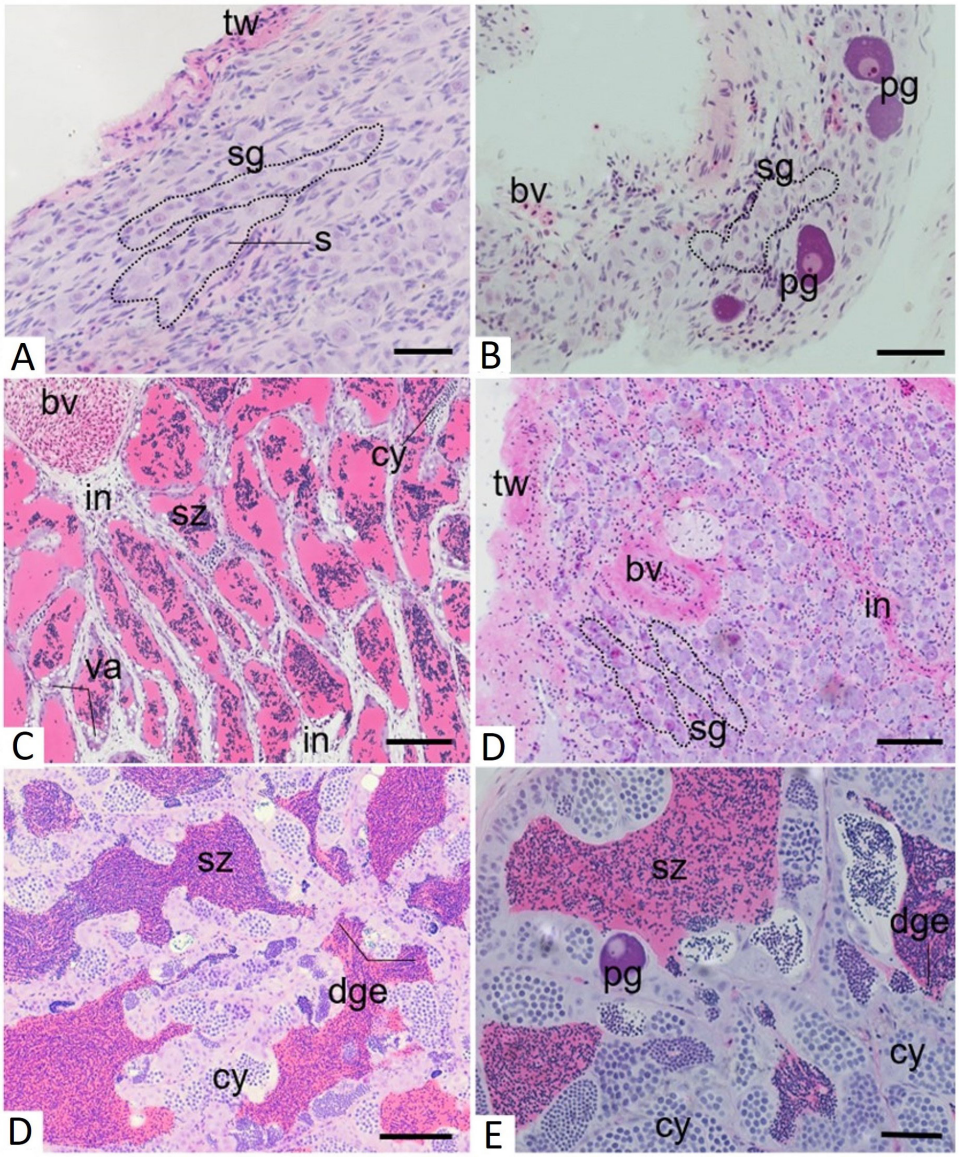

FIGURE 5 | Phases of testes maturation in *Brycon orbignyanus*. **A.** Immature. **B.** Immature intersex. **C.** Regressing. **D.** Regenerating. **E.** Spawning Capable (primary male). **F.** Spawning Capable (secondary male). bv: blood vessels. cy: germ cell cysts. dge: discontinuous germinal epithelium. in: interstice. pg: primary growing oocyte. sg: spermatogonia. s: Sertoli cell. sz: sperm. tw: testis wall. va: vacuoles. Scales: **A**, **C**, **D**, **E.** 20 µm; **B**, **F**. 50 µm. Staining: Hematoxylin and Eosin.

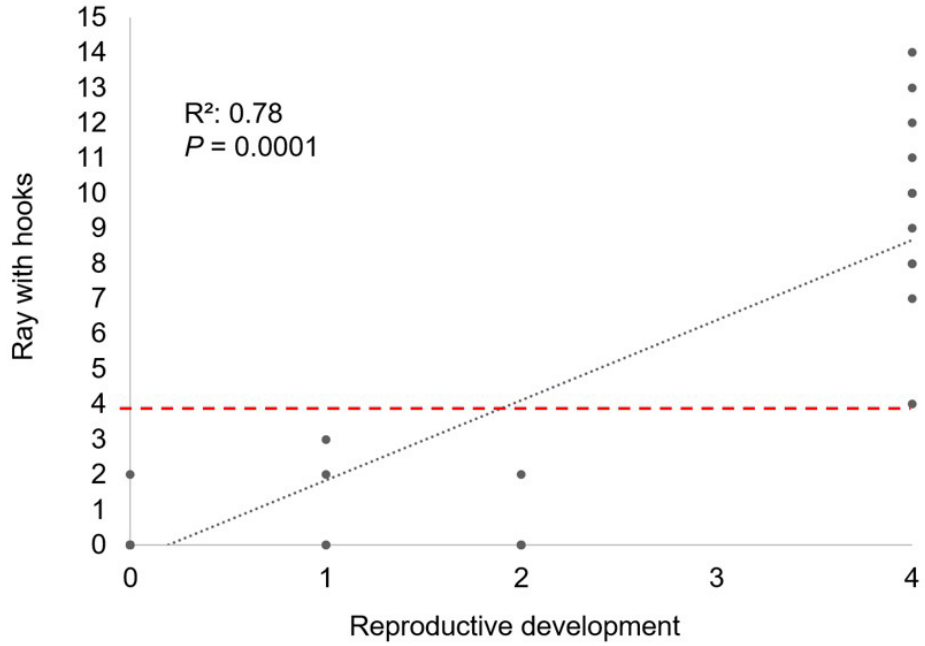

FIGURE 6 | Correlation between stages of the reproductive cycle and the number of rays with hooks in males of *Brycon orbignyanus*. X axis: Stages of the reproductive cycle, being, 0 – Immature specimens, 1 – Regressing, 2 – Regenerating specimens, 3 – Developing specimens, 4 – Spawning Capable specimens. Y axis: number (n°) of anal fin rays that developed hooks.

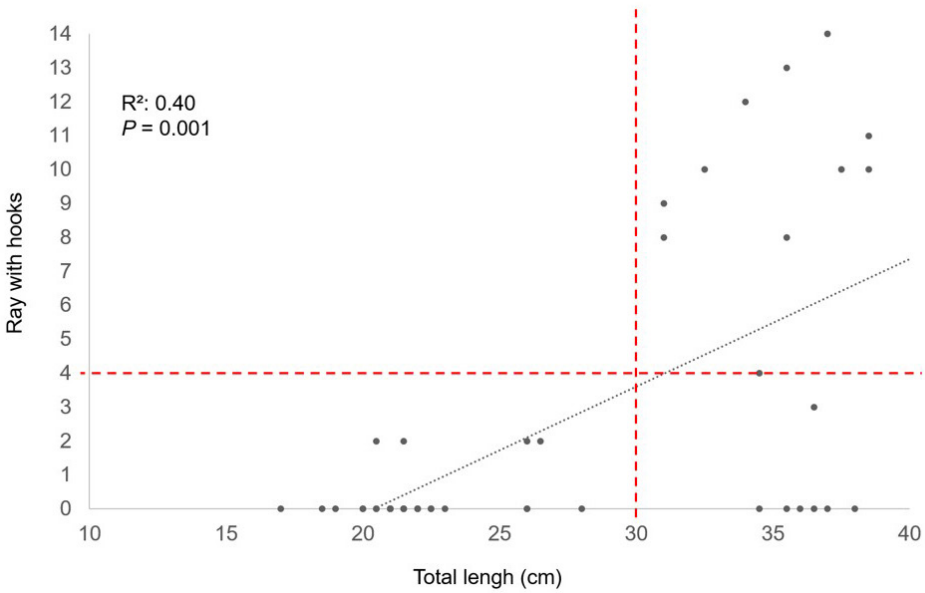

FIGURE 7 | Correlation between total length and number of rays with hooks in males of *Brycon orbignyanus*. X axis: total length in cm. Y axis: number (n°) of anal fin rays that developed hooks.

DISCUSSION

The selection of good breeders is essential for induced reproduction in production systems, and the selection of males occurs based on the observation of semen release after abdominal pressure or secondary sexual characteristics that are developed during the breeding season (Ceccarelli *et al*., 2010; Siqueira-Silva *et al*., 2020). In this study, we demonstrated that the presence of hooks on anal fins in *Brycon orbignyanus* is a secondary sexual characteristic that is present in males and that can be effectively used to choose sexually active males.

The genus *Brycon* comprises species that arouse great commercial interest in aquaculture due to their exponential growth in the first months of life, and these species include *B. amazonicus* (Agassiz, 1829) (Carvalho, Urbinati, 2005; Santos *et al*., 2015), *B. opalinus* (Cuvier, 1819) (Gomiero *et al*., 2007) and *B. orbignyanus* (Gomiero *et al*., 2009). Additionally, the species of this genus are morphologically very similar, and this can cause difficulty in regard to differentiate among these species, as they even exhibit similar characteristics in regard to anal fin length and ray numbers (Lima, 2017). Although the number of rays for *B. orbignyanus* is estimated and exhibits low variation, we observed that the length of the anal fin is directly related to the length of the animal, thus indicating that the spacing between the rays is altered.

The presence of hooks in anal fins is a common secondary sexual characteristic of species of the order Characiformes, and the hooks exhibit different growth and dispersal patterns regardless of the family to which they belong (Teixeira, 2016). In *Prionobrama paraguayensis* (Eigenmann, 1914) and *Microschemobrycon casiquiare* Böhlke, 1953 (Characidae), a single ray segment may possess two to three hooks (Teixeira, 2016), and in species of the genus *Characidium* (Crenuchidae), hooks emerge at the articulation between segments (Teixeira, 2016; Teixeira, Melo, 2021). Species of the genera *Astyanax* (Baird & Girard, 1854) (Malabarba, Weitzman, 2003; Siqueira-Silva *et al*., 2020), *Moenkhausia* (Eigenmann, 1903) (Benine *et al*., 2009; Zanata *et al*., 2009) and *Hyphessobrycon* (Durbin, 1908) (Teixeira *et al*., 2019; García-Alzate *et al*., 2020), all of which are also members of the Characidae family, form the bone processes that give rise to the hooks in the centre of the ray segment from which only one hook per segment emerges as described for *Brycon orbignyanus* in this study. This species was removed from Characidae and relocated to Bryconidae.

Recently, the characiform *Moenkhausia andrica* Reia, Oliveira & Benine, 2021 was described as a new species, and it was observed that both sexes develop hooks on anal fins during the reproductive period but differ in the frequency of rays that possess hooks (Reia *et al*., 2021). In this study, we determined that males of *Brycon orbignyanus* possess hooks as secondary sexual characteristics, while females do not; however, it should be noted that females that undergo sexual inversion and give rise to secondary males may possess hooks that are present from the first signs of gonadal remodelling.

Typically, species with rheophilic habits or that undergo seasonal reproduction develop secondary sexual characteristics only during their reproductive periods (Malabarba, Weitzman, 2003; Ohara *et al*., 2019). In species of the order Cichlidae, fat accumulation that forms a cephalic protuberance (gibbosity) occurs in males that are able to reproduce, and this fat is rapidly absorbed after reproduction (Chellappa *et al*., 2003; Muñoz *et al*., 2006). In contrast, in Characiformes such as *Astyanax lacustris* the

presence of hooks in the anal fins of males occurs regardless of the time of year due to the continuous reproduction of the species and becomes more pronounced when males are able to release sperm. (Siqueira-Silva *et al*., 2020). Piracanjuba differs from lambari in that it reproduces seasonally (Cassel *et al*., 2017; Zardo *et al*., 2021); however, it has in common with *A. lacustris* the occurrence of hooks in males at different times of the reproductive cycle. Based on this, the presence of hooks alone is not an indication of gonadal maturation, and instead the frequency (quantity) of rays possessing hooks must be assessed.

Finally, this study demonstrated that the occurrence of hooks on anal fins in *Brycon orbignyanus* is a secondary sexual characteristic that is present in both primary and secondary males. Although the occurrence of hooks is not indicative of sexual maturity based on their ability to emerge at any time during the reproductive cycle, the number of rays that possess hooks must be assessed to determine sexual maturity. Males possessing eight or more rays with hooks are characterized as Apt to Sperm and may possibly yield better fertilisation results when selected as captive breeders. Furthermore, we did not identify any differences in the growth pattern of the hooks in primary and secondary males.

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ETHICAL STATEMENT

The tank maintenance, handling, and collection of animals were all performed according to the CEUA 0012/2017 and SISGEN A069C6E protocols.

COMPETING INTERESTS

The author declares no competing interests.

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