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Use of B-mode ultrasonography for sex determination and maturation monitoring in *Prochilodus brevis* (Steindachner 1875)[1]

Carminda Sandra Brito Salmito-Vanderley[2]*, Pedro Emidio Leite Moraes Ferreira[3], Mirley Barbosa de Souza[3], Guilherme Melo Madeira[2], Bruna Farias Brito[3], Lívia Correia Magalhães[4], Carlos Henrique Sousa de Melo[4], Assis Rubens Montenegro[3]

ABSTRACT - The Brazilian bocachico is a rheophilic fish from the northeast region of Brazil that holds great economic and scientific interest. The absence of evident sexual dimorphism and the invasive character of sexing methods and evaluation of gonadal maturation are obstacles to its artificial reproduction. In this scenario, ultrasonography emerges as a non-invasive method for sexing. The objectives of this study were to establish an ultrasound sexing method for Brazilian bocachico and select equations to estimate the gonadosomatic index (GSI), gonad weight (GW), and gonad volume (GV) of live bocachico. A total of 32 bocachico fish (18 females and 14 males) were analyzed ultrasonographically and morphologically. The association of morphometric and ultrasonographic parameters was investigated using Pearson Correlations. The following prediction equations were selected using multiple regression analysis: GSI = 16.22 × transvarea; GW = 30.3 × transvarea; and GV = 0.001260036 × pixels. A model capable of predicting the sex of the animals based on the ultrasonographic parameters was obtained using partial least-squares discriminant analysis with principal component analysis. The significance level adopted was $P < 0.05$. In conclusion, ultrasonography is a useful tool to perform sexing and evaluate the gonads during the breeding season, contributing to artificial reproduction in captivity.

Key words: Fisheries. Brazilian Bocachico. Native fish. Gonadosomatic Index. Ultrasound.

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*Author for correspondence
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[1]This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq)
[2]State University of Ceará (UECE), Fortaleza-CE, Brazil, sandra.salmito@uece.br (ORCID ID 0000-0002-2011-2021), guilhermemelomadeira@gmail.com (ORCID ID 0000-0001-5325-3018)
[3]Graduate Program in Veterinary Sciences, State University of Ceará (UECE), Fortaleza-CE, Brazil, pedro_emidio@hotmail.com (ORCID ID 0000-0002-8842-7804), mirley.souza@gmail.com (ORCID ID 0000-0002-4386-1447), britobf@live.com (ORCID ID 0000-0002-5190-024X), assismontenegro@yahoo.com.br (ORCID ID 0000-0001-9304-3461)
[4]Veterinary Medicine, INTA University Center (UNINTA), Sobral-CE, Brazil, livia.magalhaes@uninta.edu.br (ORCID ID 0000-0001-6285-2004), carlos.melo@uninta.edu.br (ORCID ID 0000-0001-7848-6184)

INTRODUCTION

The Brazilian bochachico is a reophilic fish of the order Characiformes and the family Prochilodontidae. It is endemic to the semiarid region of northeast Brazil and highly appreciated by the local population, mainly due to the consumption of its spawn, which holds notorious scientific and economic interest (BOMFIM *et al*., 2015). This species is in decline due to predatory fishing and the construction of dams to perpetuate intermittent rivers, where it naturally occurs, as these constructions prevent their reproductive migration, known as 'piracema' (NASCIMENTO *et al*., 2012). Currently, some wild fish populations are challenged by the negative impacts of generalized environmental variations caused by humans, which can lead to phenotypic changes with significant ecological and evolutionary consequences that ultimately put the survival of these animals at risk (JENKINS *et al*., 2021).

Due to this threat, research is being carried out to enhance the biotechnologies used in the reproduction of this species (ALMEIDA-MONTEIRO *et al*., 2020; TORRES *et al*., 2022). According to Bomfim *et al*. (2015), males and females of Brazilian bochachico reach all stages of maturity (immature, maturing, mature, and in regression) even in captivity, with mature individuals possibly being found after 247 days after hatching (DAH).

However, some obstacles are encountered in the captive breeding of this species, e.g. the sexing of breeding stock and the evaluation of the gonadal maturation stage of the available specimens (CASTAÑEDA-CORTÉS; FERNANDINO, 2021; NASCIMENTO *et al*., 2012), which makes it difficult to separate breeding stock in lots of the same sex. Moreover, because it is a reophilic species, it is not able to spontaneously spawn in these conditions due to the absence of environmental stimuli, requiring the application of pituitary extract.

Existing methods for sexing and for the evaluation of gonadal maturation, such as the calculation of the gonadosomatic Index (GSI), ovarian biopsy, and hormonal measurements, among others, are considered invasive, requiring the sacrifice of part of the animals in the school or harming their welfare and health (COLOMBIER *et al*., 2015). Thus, adequate sexing and effective monitoring of gonadal maturation in males and, mainly, females, will enable the development of aquaculture and species preservation (CASTAÑEDA-CORTÉS; FERNANDINO, 2021; ZOHAR, 2021).

In this context, ultrasound appears as an alternative non-invasive method for assessing fish gonads. This method has the advantage of using portable equipment, having a shorter execution time, and the optional use of anesthesia in some species (NOVELO; TIERSCH, 2012). In addition to sex identification, ultrasonography can also be used to distinguish immature from mature specimens. The evaluation of gonadal structure is important for estimating gamete quality and gonadal morphology.

In view of this and the existing variations in hormonal induction protocols for reproduction regarding the dose of hormone to be used for each sex (PIRES *et al*., 2018), as well as the proportion between males and females to be allocated in each tank (SHIMODA *et al*., 2015), an effective and non-invasive method must be adopted for sexing and for evaluating gonadal maturation. Despite the use of ultrasonography to identify both sex and maturation stage in other fish species, the evaluation of gonadal morphology in *Prochilodus brevis* by ultrasound has yet to be reported in the literature.

Therefore, the objectives of this study were: (1) to establish an ultrasound sexing method for Brazilian bocachico fish; and (2) to select equations to estimate GSI, gonad weight (GW), and gonad volume (GV) in non-anesthetized live Brazilian bocachico.

MATERIAL AND METHODS

This project was approved by the Ethics Committee on Animal Experimentation at the State University of Ceará (approval no. 6699198/2017). Its execution was divided into two stages, considering the breeding season of the species. The first was in February 2018 and the second in August of the same year, during the breeding and non-breeding seasons, respectively.

A total of 32 Brazilian bocachico fish were selected at random from a school of 55 animals collected from a reservoir in the municipality of Canindé-CE. These were identified individually through the intramuscular implantation of a microchip and kept for at least two months in a permanent-recirculation tank of approximately 7,000 L at the State University of Ceará, located in Fortaleza.

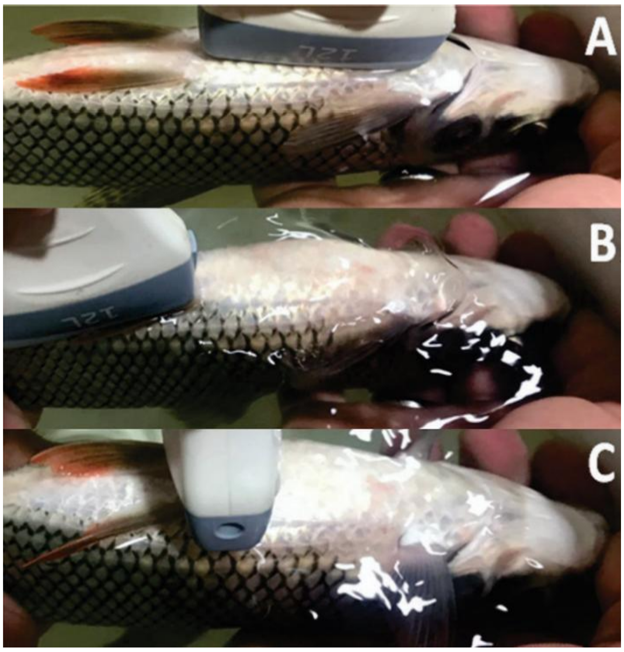
The fish were fed a commercial maintenance feed. The temperature remained at approximately 28 °C degrees, ranging from 26.5 to 29.5. The pH remained stable, at approximately 7.0. Ammonia and nitrite were systematically monitored and water was exchanged so as not to compromise the welfare of the animals, always keeping the levels of these substances below 0.25 ppm.

In the two experimental stages, a fishing net was used to remove the Brazilian bocachico from the tanks. Then, the animals were handled carefully so as to cause the least stress and subjected to ultrasound examination.

In February, during the breeding season, 11 females and five males were analyzed ultrasonographically. The fish were manually contained in the supine position, completely submerged in water, without sedation. They were evaluated along the abdominal surface, in the transverse plane, from the cranial to the caudal region. Two longitudinal and one cross-sectional image were captured, totaling three images per animal.

In August, in the non-breeding season, seven females and nine males were analyzed ultrasonographically. Due to the difficulty in obtaining the images, it was necessary to perform the ultrasound analysis outside the water. Therefore, the animals were sedated with 1 mL Eugenol (Shanghai Medical Instrument Co., Ltd) for each 10 mL of absolute alcohol for each 10 L of water. During this analysis, an appropriate gel based on carboxymethylcellulose was used and the examination sequence was similar to the first step. The longitudinal and cross-sectional images were captured digitally and stored in JPEG format. The total ultrasound length of the gonad was measured by viewing its extremities, using the pelvic fins as a reference (Figures 1A, B, and C), with the first image captured with the caudal region of the transducer positioned on the ventral fin and the second image with the cranial region of the transducer on the same fin, thus allowing full coverage of the gonad.

Figure 1 - Transducer placement during the ultrasound examination of *Prochilodus brevis* in the supine position using the ventral fins as an anatomical reference. (A) longitudinal cranial, (B) longitudinal caudal, and (C) transverse



For both sexes, cross-sectional images were taken at half the length of the gonad, following the methodology used by Colombier *et al*. (2015). After ultrasound examinations, the fish were euthanized with an overdose of Eugenol (Shanghai Medical Instrument Co., Ltd) to confirm sex and morphometry and thus validate the use of ultrasound to calculate GSI, GW, and GV.

In the first stage, ultrasound analyses were performed using portable equipment (Logic E, General Eletric); and in the second stage, fixed equipment (Mylab 40, Esaote). Both were coupled to linear 12-MHz transducers. The signal amplification (gain) was adjusted to obtain quality images and the B-mode two-dimensional ultrasound was performed in the resolution mode.

The images were evaluated using ImageJ® software, and the following gonadal ultrasound parameters were measured: length (uslength), width (uswidth), height (usheight), cross-sectional area (transvarea), and number of pixels in the cross-sectional area (pixels).

Pearson's correlation was used to investigate the association between morphometric and ultrasound parameters and multiple regression analysis was performed to obtain prediction equations for IGS, GW, and GV, depending on the ultrasound parameters evaluated. The equations were selected by the values of the coefficient of determination (R^2) and the corrected Akaike criterion (AICc), weighting the predictive capacity and the parsimony of the models.

Discriminant analysis was performed by the partial least squares (PLS-DA) and principal component analysis (PCA) methods to develop a model capable of predicting the sex of animals using ultrasound parameters. Statistical analyses were carried out using R software (R CORE TEAM, 2018). The hypothesis tests were considered significant for $P < 0.05$.

RESULTS AND DISCUSSION

Based on the ultrasound analyses, the following images were obtained as representative of the patterns found in the breeding and non-breeding seasons, in longitudinal section (Figures 2A, B, E, and F) and in cross-section (Figures 2 C, D, G, and H). The application of ultrasound in the study of animal reproduction makes it possible to acquire knowledge of reproductive biology in different species (KAUFFOLD *et al*., 2019; MANTZIARAS; LUVONI, 2020; MEDAN; EL-ATY, 2010; NAEVE *et al*., 2019).

The best images were obtained using the maximum frequency of 12 MHz, corroborating the study by Colombier *et al*. (2015), which used frequencies between 11 and 15 MHz. In other studies (CREPALDI *et al*., 2006; GUITREAU *et al*., 2012; KUCHARCZYK *et al*, 2016; NAEVE *et al*., 2018), different frequencies were used, probably due to the different body dimensions of the species. Brazilian bocachico are known to be smaller than the other fish species studied, which showed better results at lower frequencies. Based on the images, it was observed that, in the breeding season, the ovaries and testes showed greater measurements when compared with the non-breeding season, with greater changes observed in the size of the ovaries. These results are in accordance with the reproductive biology of the bocachico species, corroborating Bomfim *et al*. (2015) and Nascimento *et al.* (2012). Similar occurrences are seen in other species, such as stellate sturgeon (*Acipenser stellatus*), shovelnose sturgeon (*Scaphirhynchus platorynchus*), pallid sturgeon (*Scaphirhynchus albus)*, Neosho madtom (*Noturus placidus*), Murray cod (*Maccullochella peelii*), and others (NOVELO; TIERSCH, 2012).

In the breeding season, the ovaries were subjectively more echogenic than the testes, and it is possible to observe the level of association between the ultrasound parameters and the morphometric parameters analyzed (Table 1).

Figure 2 - Ultrasonographic aspect of longitudinal and cross-sectional scans of *Prochilodus brevis* ovary in the breeding season (A and E) and in the non-breeding season (B and F). Ultrasonographic aspect of longitudinal and transverse scans of *Prochilodus brevis* testes in the breeding season (C and G) and in the non-breeding season (D and H). Images obtained using linear 12-MHz transducers

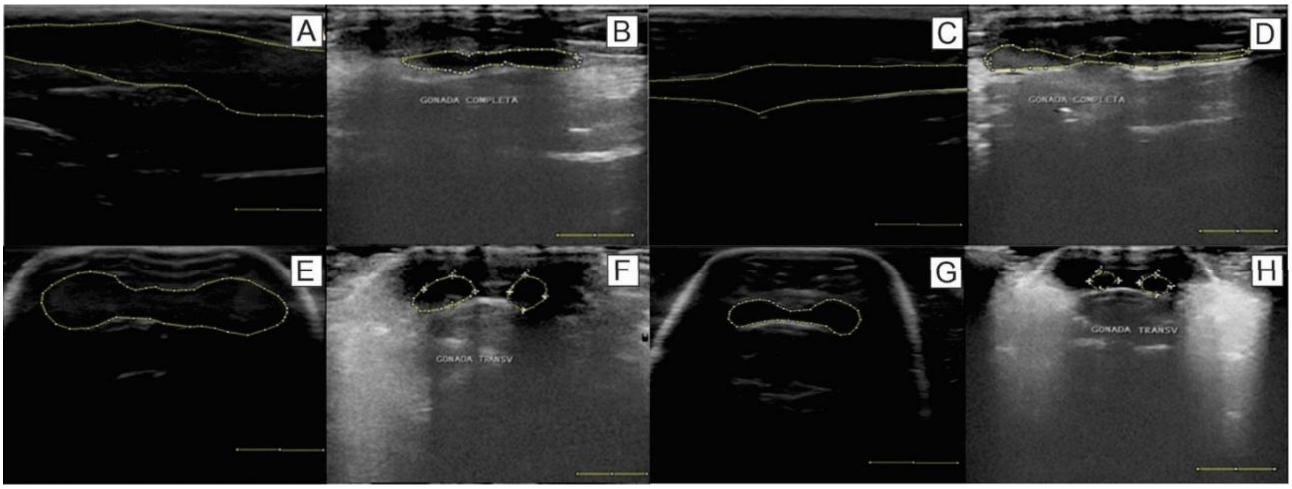


Table 1 - Correlations between the analyzed parameters

	uslenght	uswidht	usheigh	transvarea	pixels	GSI	glenth	gwidht	gheight	gwight	gvol	weight	tleght	slenght	width	depth	abdcirc
uslenght	1	0.12603	0.20371	0.1829	0.18239	-0.8735	-0.27877	0.19337	-0.06333	-0.17733	-0.38626	-0.23126	-0.45265	-0.44264	0.08251	-0.04971	-0.33109
uswidth	0.677	1	0.83602	0.69366	0.69328	0.67465	0.59656	-0.10722	0.71501	0.61326	0.44137	0.08323	-0.08162	-0.01202	0.64251	0.14055	0.24978
usheight	0.4849	0.0002	1	0.63312	0.63241	0.66029	0.49298	0.16069	0.71783	0.59334	0.43931	-0.14575	-0.36947	-0.27307	0.42202	0.18419	-0.00162
transvarea	0.5314	0.0059	0.0151	1	0.99999	0.81588	0.80652	0.01613	0.85552	0.82022	0.70162	0.26853	-0.04297	0.04131	0.77328	0.3946	0.33636
pixels	0.5326	0.006	0.0152	< 0.0001	1	0.81569	0.80666	0.16213	0.85521	0.82011	0.70143	0.26934	-0.4219	0.04322	0.77402	0.39437	0.33689
GSI	0.5213	0.0081	0.0102	0.0004	0.0004	1	0.88342	0.02429	0.97172	0.98937	0.87987	0.27572	-0.00377	0.08247	0.78181	0.47715	0.4805
glenth	0.3345	0.0243	0.0733	0.0005	0.0005	< 0.0001	1	0.12841	0.83057	0.89575	0.8043	0.49345	0.24166	0.27297	0.6759	0.47901	0.5937
gwidht	0.5077	0.7152	0.5831	0.5815	0.5797	0.9343	0.6618	1	0.0234	0.01793	-0.19565	-0.19737	-0.36737	-0.2042	-0.1141	-0.25111	-0.43149
gheight	0.8297	0.004	0.0038	< 0.0001	< 0.0001	< 0.0001	0.0002	0.9367	1	0.96515	0.85653	0.3124	-0.00456	0.09066	0.82774	0.55669	0.48613
gweight	0.5442	0.0197	0.0253	0.0003	0.0003	< 0.0001	< 0.0001	0.9515	< 0.0001	1	0.9061	0.36812	0.0623	0.13819	0.78776	0.55441	0.55594
gvol	0.1725	0.1141	0.1160	0.0052	0.0052	0.001	0.0005	0.5027	< 0.0001	< 0.0001	1	0.40965	0.19606	0.21547	0.62346	0.65822	0.64743
weight	0.4263	0.7773	0.6191	0.3533	0.3518	0.3400	0.0730	0.4988	0.2768	0.1953	0.1458	1	0.86336	0.82264	0.45865	0.7289	0.79179
tlenght	0.1041	0.7815	0.1935	0.8840	0.8861	0.9898	0.4052	0.1963	0.9877	0.8324	0.5017	< 0.0001	1	0.91993	0.18852	0.45073	0.5896
slenght	0.1130	0.9675	0.3449	0.8885	0.8834	0.7793	0.3450	0.4838	0.7579	0.6376	0.4594	0.0003	< 0.0001	1	0.34129	0.38645	0.55087
width	0.7792	0.0132	0.1328	0.0012	0.0012	0.0010	0.0080	0.6977	0.0003	0.0008	0.0172	0.0990	0.5186	02324	1	0.46961	0.55156
depth	0.8660	0.6318	0.5285	0.1626	0.1629	0.0845	0.0831	0.3865	0.0387	0.0396	0.0105	0.00031	0.1058	0.1723	0.0902	1	0.74132
abdcirc	0.2476	0.3891	0.9956	0.2397	0.2389	0.820	0.0266	0.1234	0.0780	0.0390	0.0123	0.0007	0.0265	0.0412	0.0409	0.0024	1

* uslenght: ultrasonographic gonad lenght; uswidht: ultrasonographic gonad width; transvarea: cross-sectional gonad area; pixels: number of pixels of gonad area; GSI: gonadosomatic index; glength: gonad length; gwidth: gonad width; gheight: gonad height; gweight: gonad weight, tleght: total length; slength: standard length; width: animal width; depth: animal depth, abdcirc: abdominal circumference. Above the diagonal ($R^2 = 1$) are the values of R^2 and below it, the values of P

The ultrasound images obtained are compatible with the ultrasound findings described in other species, such as in salmon (*Salmo salar*), by Naeve *et al*. (2018), who observed that ultrasound parameters are highly correlated with GSI and ovarian weight (R = 0.99, P < 0.001), and can be a non-invasive alternative for estimating GSI, detecting females in advanced maturation stage even before detection through hormonal dosages, eliminating sacrifice, reducing stress, and improving the welfare of breeding stock.

The difficulty in sexing varies between fish species and between maturation stages; however, it is usually based on the echotexture, grayscale, and visibility of ovaries and testes in ultrasound images. Monitoring fish maturation generally depends on the estimation of gonad diameter or volume, but there are still few studies involving the ultrasound estimate of gonad weight for calculating GSI. The total gonad length and/or cross-sectional area at different locations is estimated and then used for prediction, either using linear models or associating these measurements with an early representation of the gonad shape (COLOMBIER *et al*., 2015).

In this study, it was only possible to subjectively sex fish in the breeding season. Thus, a predictive model was created based on PLS regression, which classifies males and females based on two principal statistical components (Figure 3). To obtain a prediction equation for GSI, GW, and GV (Figures 4 A, B, and C) based on longitudinal and cross-sectional gonad ultrasound during the breeding season, all morphometric and ultrasound parameters were analyzed statistically and then a model with maximum RSQ and minimum AICc, based on a single variable: GSI = 16.22 × transvarea, GW = 30.3 × transvarea, GV =11 0.001260036 × pixels.

Figure 3 - Partial Least Squares (PLS) analysis used to develop a statistical model for sexing *Prochilodus brevis*

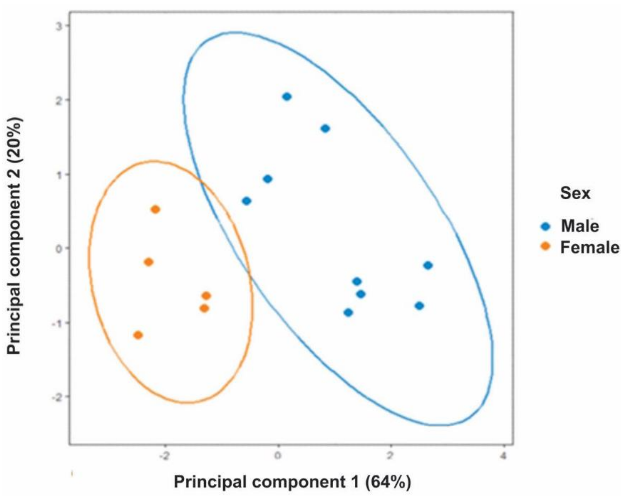
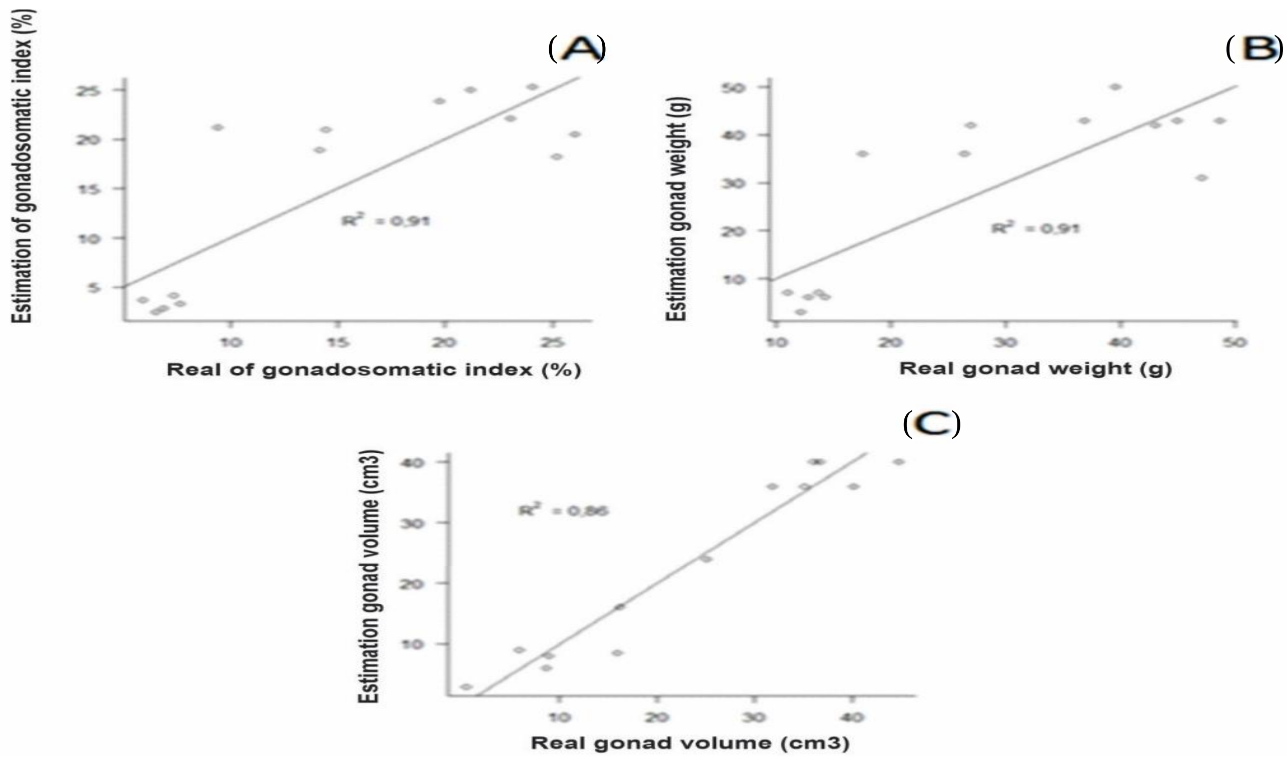


Figure 4 - Estimation of gonadosomatic index (A), gonad weight (B), and gonad volume (C) in *Prochilodus brevis* from ultrasound measurements, using Pearson's Correlation



The histological slides of ovaries and testes, obtained by conventional processing and hematoxylin-eosin staining (Figures 5 A, B, C, and D), revealed the stage of maturation at which the animals were on each occasion.

The ovaries showed a great histological difference in the obtained images (Figures 6 A, B, C, and D). In the breeding season, type-V (larger) and type-II (smaller) oocytes were observed, demonstrating that the females were in the mature stage. In the non-breeding season, type-II, -III, and -IV oocytes were found, indicating that the animals were in the maturing stage.

In the testes, a large amount of sperm was present in the seminiferous tubules, with higher values found in the breeding season. These results are in agreement with the study by Bomfim *et al*. (2015), who performed a histological evaluation of the gonads of this species.

Reproductive ultrasound proved to be an effective, noninvasive tool for determining the sex of *Neosho madtoms* and performing repeated fish examinations without causing injury or appreciable stress (BRYAN; WILDHABER; NOLTIE, 2005), and its use has already been reported in some catfish species (GUITREAU *et al*., 2012). However, there are still no reports for the species *P. brevis* or even for the order Characiformes.

Ultrasonography showed to be an effective technique, as it allows for sexing and accurate gonad evaluation and is a non-invasive procedure, since it causes little stress and can be performed with the animal submerged in water. Additionally, it has a low cost and the equipment is easy to acquire, given the availability of ultrasound devices used in cattle farming, a very common activity throughout Brazil, and whose use in fish breeding started over 30 years ago (MARTIN *et al*., 1983).

Therefore, further studies are clearly warranted, since ultrasound can improve the selection of breeding stock and the monitoring of maturation, making it possible to maintain animals with superior growth characteristics alive in the herd for scientific, conservational, or commercial purposes. In addition, it may also be used in future works to determine the exact time of hormonal induction in the species (CREPALDI *et al*., 2006).

Figure 5 - Histological sections of *Prochilodus brevis* ovary in the breeding season (A) and in the non-breeding season (C). Histological sections of testes of *P. brevis* in the breeding season (B) and in the non-breeding season (D). Hematoxylin-Eosin. 40x magnification

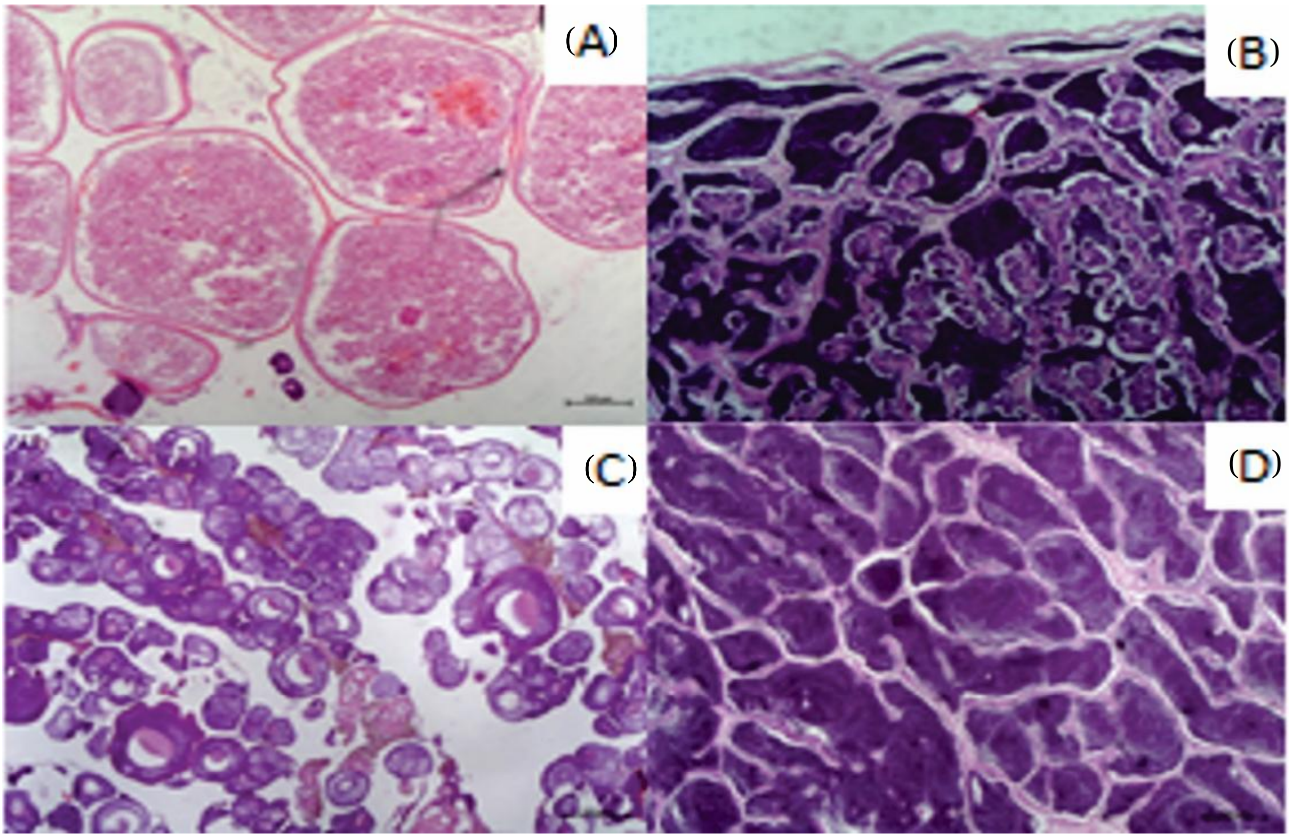
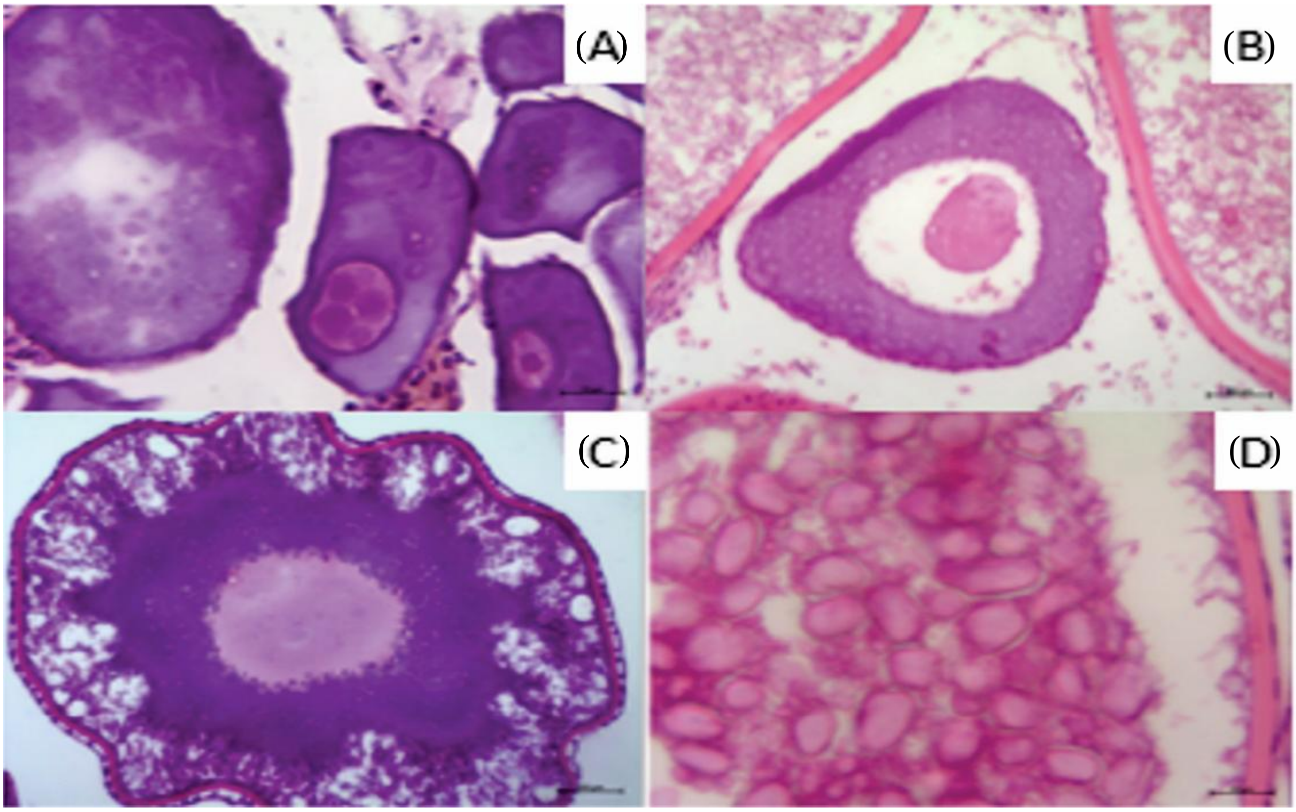


Figure 6 - Histological sections of *Prochilodus brevis* ovary at different stages, showing type-II oocytes (A), type-III oocytes (B), type-IV oocytes (C), and yolk granules in type-V oocytes (D). Hematoxylin-Eosin. 400x magnification. The mature stage contains types II and V. The maturing stage contains types II, III, and IV



CONCLUSION

Ultrasonography is a useful tool to perform sexing and estimate the gonadosomatic index, gonad weight, and gonad volume of *Prochilodus brevis* during the breeding season in a non-invasive manner, causing as little stress as possible and without harming its reproduction.

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REFERENCES

ALMEIDA-MONTEIRO, P. S. *et al*. Sperm vitrification of *Prochilodus brevis* using Powder Coconut Water (ACP-104) in association with different cryoprotectant concentrations. **Aquaculture Research**, v. 51, n. 11, p. 4565-4574, 2020.

BOMFIM, A. C. *et al.* Reproductive biology and variations in the gonadal development of the fish Curimatã (*Prochilodus brevis* Steindachner, 1875) in captivity. **Biota Amazônica**, v. 5, p. 57-60, 2015.

BRYAN, J. L.; WILDHABER, M. L.; NOLTIE, D. B. Examining Neosho madtom reproductive biology using ultrasound and artificial photothermal cycles. **North American Journal of Aquaculture**, v. 67, n. 3, p. 221-230, 2005.

CASTAÑEDA-CORTÉS, D. C.; FERNANDINO, J. I. Stress and sex determination in fish: from brain to gonads. **The International Journal of Developmental Biology**, v. 65, n. 4-5-6, p. 207-214, 2021.

COLOMBIER, S. B. e*t al.* Ultrasonography as a non-invasive tool for sex determination and maturation monitoring in silver eels. **Fisheries Research,** v. 164, p. 50-58, 2015.

CREPALDI, D. V. *et al*. A ultra-sonografia na piscicultura. **Revista Brasileira de Reprodução Animal**, v. 30, n. 3/4, p. 174-181, 2006.

GUITREAU, A. M. *et al*. Fish handling and ultrasound procedures for viewing the ovary of submersed, nonanesthetized, unrestrained channel catfish. **North American Journal of Aquaculture,** v. 74, p. 128-187, 2012.

JENKINS, M. R. *et al*. Natural and anthropogenic sources of habitat variation influence exploration behaviour, stress response, and brain morphology in a coastal fish. **Journal of Animal Ecology**, v. 90, n. 10, p. 2446-2461, 2021.

KAUFFOLD, J. *et al*. Principles and clinical uses of real-time ultrasonography in female swine reproduction. **Animals**, v. 9, n. 11, p. 1-17, 2019.

KUCHARCZYK, D. *et al*. Use of an ultrasound device to determine sex and to perform gonad biopsy in the European eel Anguilla Anguilla. **Brazilian Journal of Veterinary Research and Animal Science**, v. 53, n. 2, p. 199-206, 2016.

MANTZIARAS, G.; LUVONI, G. C. Advanced ultrasound techniques in small animal reproduction imaging. **Reproduction in Domestic Animals**, v. 55, n. 2, p. 1-9, 2020.

MARTIN, R. W. *et al*. Ultrasonic imaging, a potential tool for sex determination of live fish. **North American Journal of Fisheries Management**, v. 3, n. 3, p. 258-264, 1983.

MEDAN, M. S.; EL-ATY, A. M. A. Advances in ultrasonography and its applications in domestic ruminants and other farm animals reproduction. **Journal of Advanced Research**, v. 1, n. 2, p. 123-128, 2010.

NAEVE, I. *et al*. Ultrasound as a noninvasive tool for monitoring reproductive physiology in female Atlantic salmon (*Salmo salar*). **Physiological Reports**, v. 6, p. e13640, 2018.

NAEVE, I. *et al*. Ultrasound as a noninvasive tool for monitoring reproductive physiology in male Atlantic salmon (*Salmo salar*). **Physiological Reports**, v. 7, n. 13, p. e14167, 2019.

NASCIMENTO, W. S. *et al*. Length–weight relationship for seven freshwater fish species from Brazil. **Journal of Applied Ichthyology**, v. 28, p. 272-274, 2012.

NOVELO, N. D.; TIERSCH, T. R. A review of the use of ultrasonography in fish reproduction. **North American Journal of Aquaculture**, v. 74, n. 2, p. 169-181, 2012.

PIRES, L. B. *et al*. *Colossoma macropomum* females can reproduce more than once in the same reproductive period. **Animal Reproduction Science**, v. 196, p. 138-142, 2018.

R CORE TEAM. R: A language and environment for statistical computing. **R Foundation for Statistical Computing**, Vienna, Austria. URL https://www.R-project.org/. 2018.

SHIMODA, E. D. *et al*. Influência da presença da fêmea sobre as características seminais do curimbatá (*Prochilodus marggravii* Walbaum, 1972). **Revista Brasileira de Ciência Veterinária**, v. 4, n. 1, p. 39-42, 2015.

TORRES, T. M. *et al*. Sperm cryopreservation of *Prochilodus brevis* using different concentrations of non-permeable cryoprotectants. **Animal Reproduction**, v. 19, n. 1, p. e20210083, 2022.

ZOHAR, Y. Fish reproductive biology: reflecting on five decades of fundamental and translational research. **General and Comparative Endocrinology**, v. 300, p. 1-15, 2021.