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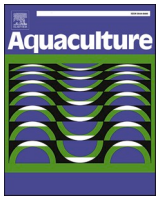

Mass synchronization of gonadal maturation in banded knifefish broodstock (*Gymnotus* cf. *carapo*)

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ABSTRACT

The banded knifefish *Gymnotus* cf. *carapo* (Pisces, Gymnotidae), also known locally as tuvira, is a natural resource of great importance to the Pantanal biome that has been used as live bait in sport fishing. This study investigated the mass synchronization of gonadal development of banded knifefish in captivity by subjecting them to different water electrical conductivity at different time intervals (pre and post conductivity change) and also evaluated the use of ultrasound imaging to identify the sex and phases of ovarian recrudescence of the captive broodstock. Fish were exposed to three different combinations of time intervals (Group 1: 40 + 30 days; Group 2: 45 + 25 days; Group 3: 50 + 20 days) and water conductivity (high: 180 μS/cm, followed by low: 15 μS/cm conductivity, respectively). The control group was kept in low water conductivity throughout the 70-day trial. Based on the results, 93%, 94%, and 86% of mass gonadal maturation of *Gymnotus* cf. *carapo* could be achieved by keeping the fish in high water conductivity (180 μS/cm) for 40, 45, or 50 days, and subsequently in low conductivity (15 μS/cm). The sexing and maturation assessment of the fish using ultrasound imaging was fundamental for effectively evaluating the mass gonadal maturation protocol. The combined use of these techniques proved safe in reproductive studies with banded knifefish.

1. Introduction

The Pantanal is one of the main South American biomes, mainly composed of floodplains, covering approximately 150,000 km^2 in the central area of South America, including part of the Upper Paraguay River basin, of which 93% is in Brazil (Alho, 2020; Fernandes et al., 2019; Pereira et al., 2012; Tomas et al., 2019). It was recently recognized as a Ramsar site (Alho, 2020), an area with high fish occurrence (Britski et al., 2007), including different species of Gymnotidae (Crampton et al., 2005; de Sousa et al., 2017; Fernandes et al., 2005). The genus Gymnotus has the broadest geographic distribution among all Gymnotiformes, occurring in the plains of South and Central America, from the Salado River in the Argentinean Pampas to the San Nicolas River in Mexico, and in the Caribbean island of Trinidad (Albert and Crampton, 2003).

The banded knifefish *Gymnotus* cf. *carapo* (Pisces, Gymnotidae), also locally known as tuvira, is a natural resource of great importance to the Pantanal (de Sousa et al., 2017), supporting the sport fishing activity in the region. The species constitutes >50% of the baits captured in this biome (Moraes and Espinoza, 2001; Silva, 2009), and such preference can generate significant ecological losses (Theodoro, 2003). Furthermore, banded knifefish are preferred by live bait collectors in the Pantanal and beyond (Castro et al., 2014) because the species uses the modified gas bladder as an accessory structure for air-breathing (Liem et al., 1984), which allows the fish to survive during extended transportation and storage periods, permitting it to be sent as live bait to farther regions (Alho, 2020; Rotta, 2004). According to the International Union for Conservation of Nature's Red List of Threatened Species (IUCN Red List) (IUCN, 2022), from the 25 species of the genus Gymnotus registered, next to 25% of the species are classified as under some threat. In this sense, developing captive breeding programs could help increase the knowledge of the reproductive biology of such threatened

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fishes (Bryan et al., 2005). This can promote the production of this fish bait in aquaculture facilities, a situation that was not achieved until now, as stressed by Sussel (2022), who has followed the Brazilian fishing sector for the last 20 years.

Environmental factors and physicochemical water parameters directly influence fish reproduction (Welcomme, 1985; Zaniboni Filho and Weingartner, 2007), and many of them are complex or impossible to replicate in captivity, leading to the use of hormonal stimulation for maturation and spawning (Almeida, 2013). In confined environments, banded knifefish reproductive trigger cues have been identified as changes in water conductivity (Kirschbaum and Schugardt, 2002; Kirschbaum and Wieczorek, 2002; Souza and Andrade, 1984). Additionally, sexing is one of the biggest bottlenecks for developing captive breeding programs for this species since they do not exhibit distinct secondary morphological characteristics between males and females (Crampton and Hopkins, 2005). Among the procedures available for sexing, two feasible methods are established, e.g., invasive (Thomaz et al., 2019) by abdominal puncture or non-invasive (Rotta et al., 2007) through ultrasound imaging.

Given the information available on the reproductive biology of banded knifefish, it is necessary to search for alternatives that can optimize the assisted reproduction process of the genus Gymnotus for aquaculture purposes. Thus, this study aimed to assess the mass synchronization of the gonadal development of *Gymnotus* cf. *carapo* in captivity by changing the electrical conductivity of the water in set time intervals and identify the phases of ovarian recrudescence in captive broodstock by using ultrasound (US) imaging, permitting the development of future protocols for this fish bait commercial production.

2. Materials and methods

2.1. Fish acclimatization

The experiment was conducted in a commercial fish farm in Campo Grande/MS countryside, Brazil (UTM coordinates: 774657.30 m E; 7,718,488.73 m S; alt. 510 m). A total of 2200 fish were purchased from the Pantanal plains fisherman (in the Corumbá region) and kept for 30 days in an earthen pond (with 1200 m^2 and 600 m^3) for acclimatization. Fish were fed a commercial feed (32% CP, 4 mm) mixed with ground beef liver (0.8%) to increase palatability and facilitate feeding training at a 3% biomass/day feeding rate. Water quality parameters (temperature, dissolved oxygen, pH, conductivity, alkalinity, and transparency) were monitored weekly. The electric conductivity in these thanks was usual for the water in the region, ranging around 15 μS/cm.

2.2. Fish measurements

First, the fish's total length was measured with an ichthyometer (Rotta, 2003), tanking the direct measure by reading the scale inside the equipment after the fish was well positioned. Next, the fish volume was measured with a graduated cylinder named "ichthyotube" (Rotta and Gonda, 2004), from which the individual weight of the fish was calculated using the average density for the species (established before the study) multiplied by each fish volume. Finally, the Fulton's factor (K) was used to calculate the fish condition factor, which is the weight in grams divided by the cubic of the standard length in millimeters, according to Williams (2000) and Froese (2006).

2.3. Sex and gonadal maturity determination

Following initial fish measurement, they were anesthetized using a 5% alcohol and clove oil solution composed of 95% ethyl alcohol and commercial clove oil in volumetric dilution of 1:20 (Pádua et al., 2012), i.e., 5 mL of solution per liter of fresh water (250 mg/L) in a bath of 3–5 min to reach very fast the anesthetic stage 3, when the fish do not react to touch stimuli (Coyle et al., 2004). Next, the abdominal cavity was individually analyzed in the anesthetized fish to assess sex and gonadal maturation (Rotta et al., 2007) before their distribution in the tanks in the proportion of 2 females for each male. For it was used a portable ultrasound (US) equipment (ALOKA C.O. LTD. Model SSD-500) with a 5 MHz linear rectal transducer (10 cm image maximum depth) for veterinarian purposes. The assay was done inside a plastic tray (low border with 6 L of volume) full of natural freshwater, where the fish were placed with the belly facing upwards and the transducer placed in the fish lateral (almost sagittal) along with its belly, with the end of the transducer just behind the fish jaw and the image plane of the transducer longitudinal with the body but 4–6 mm above the anal fin insertion, as presented in Fig. 2. No conductive gel was used for the procedure since the water contained in the tray proved to be a very efficient medium for transmitting the ultrasound signal. To capture the best image was carried out smooth movements (slight rotation) were made with the transducer (usually positioned in the right hand) in its longitudinal plane to reach a point that was possible to obtain a clearer image and do the sexing and ovary classification, as exemplified in Fig. 3, which presents the four possible categories with the US equipment used in this study.

The images generated with US have a proper interpretation depth of 2 cm (the fish thickness in the transducer plane), permitting analysis of the thickness and shape of the anechoic/echoic tissue (below the abdominal wall) to determine these two sexual aspects (Fig. 3) and was established by the consensus of two observers.

2.4. Sex and gonadal maturity confirmation

After the initial sex identification at the beginning of the experiment, 50 fish were randomly picked from the studied group and euthanized to confirm the sex and gonadal maturity identification by macroscopic analysis after dissection. The maturation stage determined by US has been confirmed with the classifications system established by França (2010) and González et al. (2001) to the macroscopic analysis of gonadal maturation in Gymnotiformes, which consider five stages of gonadal development according to a combination of macroscopic and microscopic morphological characteristics (Table 1). The euthanizing procedure was taken using the same method used for anesthetizing, but the time in the bath was at least 10 min to reach the anesthetic stage 1 V, when the fish did not breathe and the heart stopped beating (Coyle et al., 2004). Initially, the fish was measured and weighed again. In sequence, the sex was determined, and, in females, the gonad maturation stage was established using a US image. After that, the fish's celomatic cavity was opened with surgical scissors to reach the gonads. At this moment, the confirmation of the sex and ovary maturation was done utilizing just macroscopic characteristics.

In the same way, at the end of the experiment, 50 fish from each treatment and the control group were sampled, totaling 200 fish. Fish were anesthetized, euthanized, and analyzed as described previously, and the accuracy of sexing and gonadal stage obtained by US imaging was assessed and rechecked with the visual analysis of reproductive organs obtained by dissection, utilizing just macroscopic characteristics. All the US and macroscopic interpretations were made by the consensus of two observers.

2.5. Gonadosomatic index

To establish the relationship between ovarian maturation stage and Gonadosomatic Index (GSI), calculated as the percentage that the gonads represent of the individual's total weight (Vazzoler, 1996), the maturation stage determined by US was linked to the classifications established by França (2010) and González et al. (2001). To this end, at the end of the study, 50 males and 50 females already measured, euthanized, and classified about the maturation stage had their individual GSI calculated once the ovary and testis were excised and individually weighted on a small electronic scale (TS model TS500). With

Table 1
Morphological classification system for Gymnotiformes maturation stage using macroscopic analysis of gonadal characteristics as indicators of microscopic histological features.

Classification	Morphological characteristics	
	Macroscopic	Microscopic
Stage 1 - immature (regressed phase)	Ovary small and elongated, well attached to the swim bladder, translucent, with a pale pink hue. No oocytes are observed at simple sight.	The females in this gonadal maturation stage presented in the ovarian lamellae nests containing proliferating oogonia, early prophase oocytes, and previtellogenic oocytes.
Stage 2 - on maturation (stage of ovarian development)	The ovary is pinkish, from intense pink to light vine, but it has poor irrigation, and the oocytes are barely visible.	As the ovary advances in the reproductive cycle, part of the follicles develop, and the oocytes form the cortical alveoli layer.
Stage 3 - mature (phase suitable for spawning)	The ovaries increase in volume, occupying the entire abdominal cavity. They have a cylindrical-conical shape with a yellow-orange or yellow-gold coloration, and the oocytes are very visible and large.	The ovarian lamellae in this stage are full of vitellogenic oocytes, and many are at the end of yolk incorporation, and it is possible to observe maturing oocytes, which have already fully incorporated the yolk.
Stage 4 - partially unemployed (active spawning phase)	The ovaries in this state are more flaccid than in stage 3 and decrease in size and weight due to the removal of mature oocytes. The blood vessels are more dilated, and the yellow-gold color is less intense than the mature ovary.	The lamellae have numerous dilated and spherical structures resulting from the release of mature oocytes from their follicles, remaining the so-called post-ovulatory complex in the ovary.
Stage 5 - spawned (regression phase)	The ovaries are of well-reduced size with an irregular surface and with a sanguinolent appearance.	Oocytes in advanced stages of development, maturing, vitellogenic, intermediate, and not spawned, suffer atresia. Occurrence of follicular cell layer hypertrophy, followed by liquefaction of the yolk granules and rupture of the zona pellucida.

Adapted from França (2010) and González et al. (2001).

this association, it was possible to confirm the relation of US image analysis results with female gonadal development and GSI, as it was possible to distinguish the three first stages of ovary development.

2.6. Fish breeding sites

Water hyacinth (*Eichhornia crassipes*) was placed in all ponds since the beginning of the experiment (groups of 16 m^2 within floating bamboo frames) in order to maintain the well-being of the fish, mimicking the natural breeding environment, as banded knifefish uses this substrate as shelter and spawning place (Kirschbaum and Schugardt, 2002; Pereira and Resende, 2006; Resende et al., 2006).

2.7. Water quality management

The usual water conductivity reported in Pantanal waters (Oliveira and Ferreira, 2003) before the natural banded knifefish time reproduction is 180 μS/cm. However, in studies with freshwater electric fish, the electrical conductivity used was >2000 μS/cm (Kirschbaum, 1987), making the level of this study far from a possible upper limit. Therefore, electrical conductivity was chosen as a primary parameter to calculate the respective salt inclusion and establish the salt application. Formerly the salt application, an assay was fulfilled to understand how the coarse salt changes the conductivity in distilled and tank water. An amount of 160 g of coarse salt per cubic meter was necessary to raise electrical conductivity in 1.78 μS/cm per kg of coarse salt.

The conductivity was gradually increased by adding 100 kg/pond (average per tank) of coarse salt (NaCl 98%) during the first week of the experiment, corresponding approximately to a water salinity of 0.4‰, achieving the conductivity of 180 μS/cm. There is no established upper limit for water salinity for this species without any restriction about the kind of salt used, while some studies were done with a mixture of salts such as NaCl, CaCl, and MgSO (Kirschbaum, 1979, 1987). The salt was deployed around the water body perimeter, and the conductivity was measured the next day to permit total salt dissolution. This slow increase was to avoid any unnecessary stress to the fish by a sudden change in conductivity (Wynne and Wurts, 2011).

Following the initial period at high conductivity in the treatment tanks (Group 1, Group 2, and Group 3), conductivity was gradually reduced over five days by exchanging the pond water until it reached 15 μS/cm. During the study, the water in the ponds was not exchanged to maintain the water conductivity established for each treatment. When conductivity was altered due to rain, the coarse salt was added to the ponds until the electrical conductivity was adjusted back to 180 μS/cm.

Besides conductivity monitoring, the following water quality parameters were measured every five days (between 8:00 and 9:00 AM) during the experiment: temperature and dissolved oxygen (oximeter with thermometer); pH and conductivity (digital pH and conductivity meters); alkalinity (titrimetric analysis) and transparency (Secchi disk).

2.8. Experimental design

Two weeks before the start of the experiment, seven earthen ponds with uniform soil characteristics and an average area and volume of 1220 m^2 and 990 m^3, respectively, were prepared. Ponds were drained, limestone was applied at a dose of 3 t/ha (360 kg/pond) and fertilized with 24 kg of rice bran (200 kg/ha), 30 kg/ha of urea (3.5 kg/pond), and 25 kg/ha of triple superphosphate (3 kg/pond). The experimental ponds were filled with water from a reservoir until the normal operating level was reached. When necessary, the fertilization process was repeated whenever the water transparency reached a value >50 cm to increase primary production (achieve transparency between 40 and 20 cm), but keeping the water electrical conductivity near 15 μS/cm to receive the fish with the same conductivity level from the acclimatization tank.

After 30 days of acclimatization to captivity, 1400 fish fit for reproduction, with a minimum total length of 24 cm (Fronk et al., 2019; Resende et al., 2006), were selected for the experiment. Fish were randomly distributed in the seven experimental ponds at a density of 1 fish per 6 m^2 of water surface (Souza and Andrade, 1984), at a female: male ratio of 2:1 in conformity with the sex identification previously done. The broodstock was fed daily with a commercial feed (32% CP, 4 mm) at a 3% biomass/day feeding rate during the experimental period.

Fish were subjected to distinct patterns of water conductivity changes over a period of 70 days. Initially, was applied a high conductivity (180 μS/cm) for some days (40, 45, or 50), followed by low water conductivity levels (15 μS/cm) at three combinations of time intervals (treatments) to reach 70 days in the conductivity variation procedure (Group 1: 40 days high +30 days low; Group 2: 45 days high +25 days low; Group 3: 50 days high +20 days low), as presented in Fig. 1. The treatments were designed to evaluate how the recrudescence works in a 70 days interval as spawn occurs between 30 and 50 days after the manipulation of water conductivity (Kirschbaum, 1984, 1987). The three treatments were done in duplicate tanks, and the control group (70 days low) was one tank, in which there was no change in electrical conductivity over the study period.

With the proposed protocol, the following questions should be

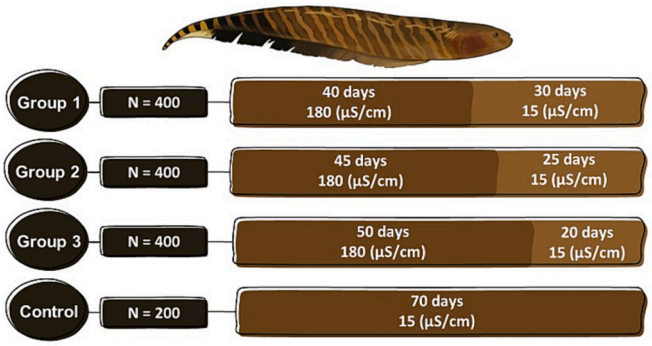

Fig. 1. Experimental design of the mass gonadal development induction protocols for banded knifefish (*Gymnotus* cf. *carapo*), tested for 70 days with different water conductivity levels.

answered: a) what is the proportion of fish in large groups that reach ovarian readiness (stage 3 of maturity) when induced by the change in water conductivity? b) is the 40 days of high conductivity sufficient to synchronize the maturation in banded knifefish broodstock? c) is the 20 days of low conductivity sufficient for the banded knifefish to start breeding? and d) is it possible to utilize ultrasound images to assay the sex and stage of ovary maturation in banded knifefish broodstock?

2.9. Statistical analysis

Data were subjected to descriptive statistical analysis and expressed as mean ± standard deviation. In addition, a chi-square analysis of the percentage values was performed to evaluate the effect of the change in water electrical conductivity on the gonadal development stage, specifically among the treatments with conductivity change. Statistical analysis and graphs were performed using GraphPad Prism 7.00 statistical software.

2.10. Ethical statement

The ethical aspects used for the procedures of fish handling, anesthetizing, and euthanizing were the best at the moment of the study (which at that time did not demand ethics authorization), and were conducted in a way that attended *ex-post* Castro (2013) premises.

3. Results

The procedures for measuring the total fish length and weight and identifying the gonadal development stage, according to the image standards presented in Fig. 3, were successfully performed with an accuracy compatible with the method and without causing injuries to the fish. The length and weight values of the broodstock and the condition factor calculated in the initial (single population sample of 50 fish) and final moment (multiple samples of 50 fish per treatment/control group) of the experiment are presented in Fig. 4. At the beginning of the study, no fish mortality was identified in the tanks. At the end of the study, the fish presented a mean mortality of 15.21% in the period, reaching survival rates of 87.25%, 79.75%, 88.50%, and 82.50% for groups 1, 2, 3, and control, respectively.

Concerning sex and gonadal maturity determination, these same samplings were used to analyze via US image and, posteriorly, confirm the interpretation by macroscopic visualization of the gonads after dissection. The initial group had 100% of accuracy, were was identified 20 males and 30 females without any error in the sex and maturations stage, and the final group was identified 75 males and 125 females, with just three mistakes (1.5%), with which two females in stage 1 and one female in stage 2 were erroneously classified as male.

The procedure for classifying the maturation stage of females and its relation with GSI presented effective ($p < 0.004$) to differentiate means from stages 1 (1.13% ± 0.29%) and 2 (2.94% ± 0.52%) from stage 3 (7.94% ± 2.09%), as is possible to see in Fig. 5. In males, the GSI mean ± SD was 0.14% ± 0.04%.

Documented nest sites were done on the breeding island, with the occurrence of four egg masses in one pond of group 1, to which the broodstock was subjected for a more extended period under low conductivity (30 days). The eggs found had adherence to each other but not to other substrates because their shells were completely clean at the time of collection, showing no particulate material adhered (organic particles were expected at the breeding island roots). Furthermore, the egg mass did not stick to the macrophyte roots, detaching easily from the nest site, and could sink and get lost in the pond bottom.

The measures in water quality parameters for the different treatments and control groups of banded knifefish broodstock are presented in Table 2. Given the rain events (three occurrences with high rainfall), the transparency was adjusted by applying half of the organic and chemical fertilizers used in the tank preparation each time. The same occurred for water conductivity adjustment when 20–25 kg of coarse salt was applied in each event to maintain conductivity near 180 µS/cm.

About a change in the proportion of maturation stages in females, it was possible to observe an increase in stage 1 females in group 3 and the control group. In contrast, a decrease was observed in group 1 and group 2, as presented in Fig. 6. A reduction in the percentage of stage 2 females was observed in all treatments, with percentage points varying between 76 pp. and 77 pp., while the control group ranged from just 26 pp. The percentage of females in stage 3 increased in all treatments, confirming the stimulus of sexual maturation of the broodstock when applied water conductivity variation. Depending on the treatment, this increase ranged from 64 pp. to 80 pp. in the fish submitted to electrical conductivity manipulation. However, in the control group, only 21 pp. migrated to stage 3, demonstrating the direct effect of the electrical conductivity in the sexual maturation of the banded knifefish.

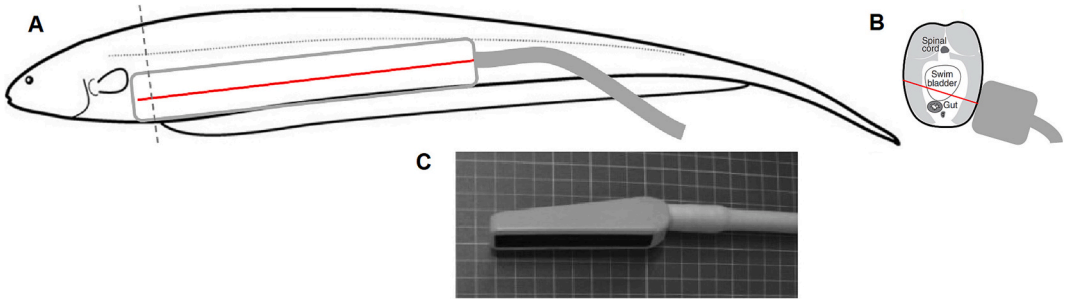

Fig. 2. Banded knifefish figure where it is possible to identify: (A) the appropriate longitudinal position of the linear transducer for US analysis to identify sex and maturation stage (4–6 cm above the anal fin insertion); (B) the appropriate transversal position of the linear transducer for US analysis to identify sex and maturation stage (with the rotation of the transducer to capture a better image); and (C) an example of linear rectal transducer used for veterinarian purposes. Figures adapted from Castelló et al. (2009) and Malabarba and Carvalho (2020).

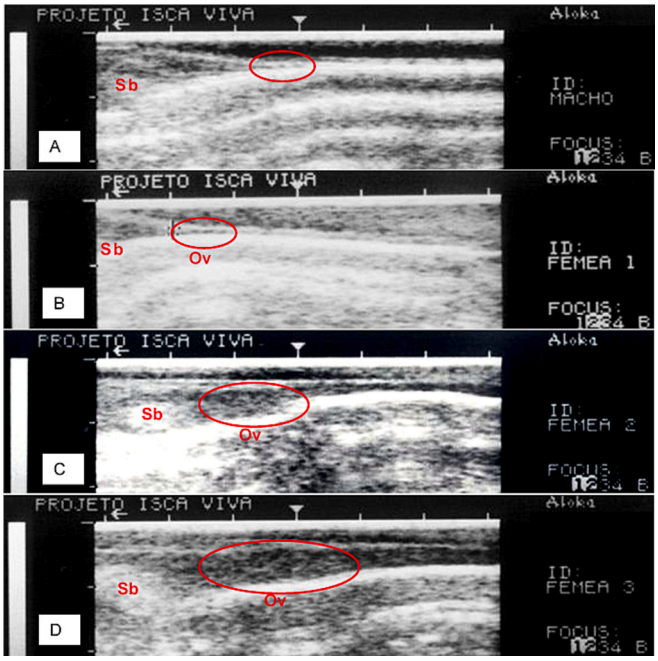

Fig. 3. Longitudinal ultrasound image (anterior left and ventral above) of the belly of banded knifefish specimens (*Gymnotus* cf. *carapo*) with distinct sex and ovarian maturation stage, where is possible identify the swim bladder (Sb) and the ovary (Ov) regions in females: (A) Male with the absence of anechoic tissue (dark region) connecting anterior and posterior celomatic cavity (circle stressing the presence of a thin echoic (light) tissue splitting celomatic cavity, a situation that can be misinterpreted as a female not sexually mature); (B) Female in stage 1 (S1) of maturation evidencing a small anechoic tissue at ovary site (circle) showing continuity of this dark region in both anterior and posterior celomatic cavity (without echoic tissue splitting the celomatic cavity); (C) Female in stage 2 (S2) of maturation showing a developed anechoic tissue at the ovary site (thicker dark area in the region posterior to stomach); (D) Female in stage 3 (S3) of maturation showing a well-developed anechoic tissue in the ovary region (thickest dark area in the posterior region of the swim bladder and that continues to the posterior celomatic cavity). Source: Adapted from Rotta et al. (2007).

When we evaluated the association of the gonadal development stages of the female fish with the manipulation of the electrical conductivity by chi-square, we observed that the females in (i) stage 1 in the initial ($p = 0.1959$) and final ($p = 0.3715$) identifications, in (ii) stage 2 in the initial identification ($p = 0.5475$) and in (iii) stage 3 in the initial identification ($p = 0.2538$) were not affected by the treatments with the electrical conductivity change. On the other hand, the females in stages 2 and 3 identified at the end of the experiment (calculated $p < 0.0001$) showed an association with the treatments with change in electrical conductivity. Furthermore, the females subjected to the effect of electrical conductivity went from stage 2 to 3, whereas in the control group, around 20% evolved to stage 3, significantly less than the treatments (calculated $P < 0.0001$).

4. Discussion

For the first time, it was possible to set up an experiment with a group of banded knifefish where the correct sex ratio was established (via US imaging), and the synchronization and gonadal maturation in females was assessed by changing the electrical conductivity of the water. Although fish did not have a massive spawn during the experiment, as the conditions of the study showed that females might need more time (at least 30 days) in low conductivity to trigger spawning, the information obtained in this study allows significant advances in the reproductive management of the species in captivity.

The measurement and handling procedures taken in the study were adequate for the fish's well-being and health, as no mortality was verified at the beginning of the study. Furthermore, the water parameters were maintained at a good quality level for all study periods (Table 2), reinforced by the absence of drastic changes in the water quality given the low initial biomass (about 160 kg/tank or 13 g/m^2). The final mortality of the experiment (15.21%) was similar to those observed in field conditions, with the live bait fishermen reporting a mean mortality of 14% (Moraes and Espinoza, 2001). The mortality observed in the present study occurred randomly, and the sex, treatments, or reproductive stage of the fish appeared not to influence it.

In the present study, fish sexual maturity (98.9%) was observed in fish with a total length >26 cm (Fig. 4), above the range of 24–26 cm suggested by Resende et al. (2006) for banded knifefish reach full gonadal maturity (100% frequency) in the Pantanal. Fronk et al. (2019) corroborate this observation by reporting that *G. carapo,* with a maximum total length of 20.8 cm, is still sexually immature. On the other hand, maturation (50% frequency) in female banded knifefish occurs when they reach a total length between 24.8 and 25.6 cm (Barbieri and Barbieri, 1983). As for the weight of females (52.9 to 173.4 g) and males (61.7 to 201.7 g), these were higher than those reported in the literature for mature individuals (13.9 to 63.9 for females and from 14.6 to 70.8 g for males) (Fronk et al., 2019).

The sexing accuracy obtained in all assessment procedures (initial and final) was 98.5% at the end of the experiment (3:200 error), confirming the effectiveness of the US identification method (98% of accuracy postulated by Rotta et al., 2007). It is important to note that the present study accurately determined the sex of fish in the genus Gymnotus before the beginning of the experiment, unlike other studies with banded knifefish in a confined environment (Kirschbaum and Schugardt, 2002; Kirschbaum and Wieczorek, 2002; Souza and Andrade, 1984). Furthermore, the same accuracy of sex determination is applied to identify the maturation stage in the study, as the fish misinterpreted in the sexing assessment were just the ones with similarity in the gonad structure (male and immature females).

The results of GSI and its relation with the maturity stage in females were established (Fig. 5), permitting the US image analysis to correctly determine a distinct GSI to stage 3 compared to stages 1 and 2. Stage 3, with a GSI mean ± SD of 7.94% ± 2.09% (interval between 3.5 and 12.1), is similar to other studies with Gymnotiformes in the reproductive cycle peak, as found by Giora et al. (2014) to *Brachyhypopomus gauderio* females with a GSI mean of 6.1 (interval between 4.6 and 8.9) and by Vanin (2015) for *Gymnotus* sp. with a GSI mean of 10.1 (interval between 7.7 and 12.4). For males, the mean GSI was 0.070 ± 0.002% in the reproductive period of *Gymnotus carapo* (Vergílio et al., 2013), a value similar to that obtained in this study. These GSI values of the ovaries and testis show that the values measured in this study are compatible with wild fish in natural conditions, with higher mean GSI when conductivity is lower (Cognato and Fialho, 2006) and can be related to the reproductive stages. The results are consistent with the development of the gonads throughout the reproductive cycle, with the increase in the percentage of gonads in the animal's body occurring concomitantly with the development of the ovaries and testis in the reproductive stages, and this development stage can be correctly established by US image interpretation.

This reproductive dynamic of the species is extensively covered in the classical fish reproductive concept, which is stimulated and regulated by external environmental factors and internal mechanisms of the organism, allowing abiotic factors to trigger internal, usually hormonal mechanisms (Almeida, 2013). The internal mechanism that controls the reproduction process in fish is the brain-hypothalamus-pituitary axis (Rottmann et al., 1991), in a very synchronic way in all endocrine events involved in gonadal function, including suppressing it if necessary (unfavorable conditions for reproduction) (Almeida, 2013). These exogenous (abiotic) factors, such as photoperiod, temperature, water properties (e.g., electrical conductivity), or food supply, are somehow

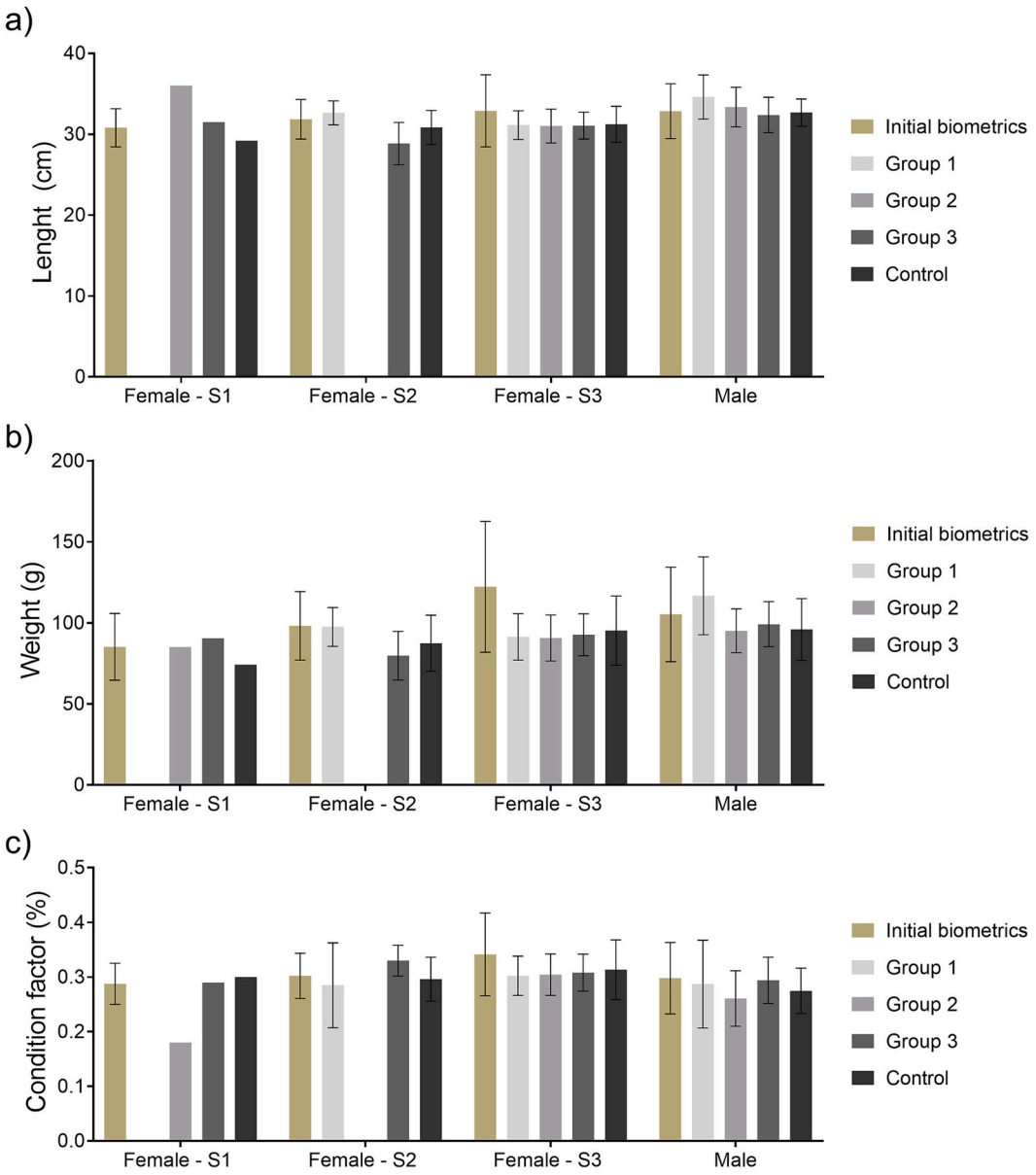

Fig. 4. Body condition factor of banded knifefish (*Gymnotus* cf. *carapo*) broodstock for different sexes and levels of female sexual maturity (stages of development - S1, S2, and S3) in each treatment and control group: a) Total length (cm); b) Total weight (g); c) Condition factor (%). Initial measurements: FS1 ($n = 4$), FS2 ($n = 19$), FS3 ($n = 7$) and M ($n = 20$); Treatment 1: FS1 ($n = 0$), FS2 (n = 2), FS3 ($n = 27$) and M ($n = 21$); Treatment 2: FS1 (n = 1), FS2 (n = 0), FS3 (n = 27) and M ($n = 22$); Treatment 3: FS1 (n = 1), FS2 (n = 2), FS3 ($n = 32$) and M ($n = 15$); Control: FS1 (n = 1), FS2 ($n = 23$), FS3 ($n = 9$) and M ($n = 17$).

perceived by the organism and trigger neuromodulations in the brain-pituitary-pituitary axis that initiates sexual maturation (Almeida, 2013). The reproduction of fish in captivity can be controlled by environmental manipulation, such as water temperature and quality, photoperiod, or the presence of shelters and nests for mating and spawning (Taranger et al., 2009). Thus, adapting species-specific induced spawning protocols through environmental alteration or hormonal induction would be ideal (Almeida, 2013). This adaptation would allow for maximizing productivity or optimizing the duration of the reproductive process.

Environmental factors control the periodic fish reproduction in nature and are closely related to the occurrence of water quality change in the rainy season, particularly for Gymnotiforms (Kirschbaum, 1984). Several environmental factors, like salinity, regulate the hormone activity of the fish (Mishra and Sarkar, 2013). Many South American fish are halophobic or alkaliphobic, even with a low salt content, inhibiting gonadal development (Kirschbaum, 1979). Lam (1983) suggested that the effect may be attributable to enhanced osmoregulatory expenditure, thereby reducing energy resources for ovarian development. When conductivity decreases, it provokes gonadal recrudescence, equivalent to a salinity decrease (Kirschbaum, 1979). In many fishes, this affects osmoregulation and increases the release of the appropriate hormone, prolactin (Blüm, 1977, cited by Kirschbaum, 1979). Paralactin (the teleost homolog of prolactin) has been implicated in the parental behavior of certain species (Liley and Stacey, 1983), promoting maturation and reproductive cycling (Whittington and Wilson, 2013), and osmoregulation as prolactin controls the activities of gills whose primary function is ion and water exchange (Saha et al., 2021).

Experiments with Gymnotiforms (Kirschbaum, 1975) showed that gonadal recrudescence could be provoked by a continuous decrease in conductivity and pH, increased water level, and simulated rainfall. In some cases, the reduction of conductivity alone can induce gonad maturation in Gymnotidae, which is corroborated by the fact that gonadal regression is caused only by the continuous increase of

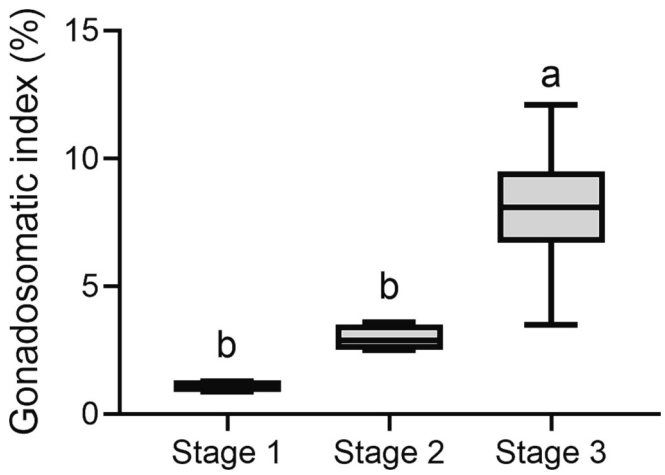

Fig. 5. Classification of the ovarian development stages (Groups 1, 2, and 3) and its relation with GSI of banded knifefish (*Gymnotus* cf. *carapo*) females ($n = 50$) after induction to gonadal maturation by the change in water conductivity. Groups followed by the same letter have no statistically significant difference ($p < 0.004$).

electrical conductivity (Kirschbaum, 1979). This characteristic was later confirmed (Kirschbaum and Schugardt, 2002), since only the decreased conductivity can induce the gonadal maturation in different Mormyrids and Gymnotids. Corroborating with that, the constant and considerable conductivity increase led the Mormirid *Pollimyrus isidori* to gonadal regression, not showing refractory behavior to post-spawning reproduction (Kirschbaum, 1987).

It has already been demonstrated in the laboratory that the spawning trigger in Gymnotidae is solely provoked by decreasing in water conductivity (Kirschbaum, 1975; Kirschbaum and Schugardt, 2002; Kirschbaum and Wieczorek, 2002; Schugardt and Kirschbaum, 2004) and that maturation occurs between 30 and 50 days after the onset of conductivity decrease (Kirschbaum, 1984, 1987). However, information on field tests managing water quality to simulate the natural environment and induce mass reproduction of banded knifefish is scant in the literature.

Understanding this process under captivity conditions that can be reproduced in a fish farm is fundamental to assessing the effectiveness of induction via electrical conductivity with numerous broodstock fish. Furthermore, it would be possible to evaluate the distribution of reproductive stages within the studied population and plan the synchronized spawning of large groups of fish for production in captivity. In this study, electrical conductivity variation triggered mass gonadal development in banded knifefish. The other water physical-chemical factors were kept stable (Table 2) and within the variation range of water physicochemical characteristics that were 22.5 to 35.0 °C for temperature, 5.0 to 6.8 for pH, 27 to 1300 mg $CaCO_3$/L for alkalinity, and 0.9 to 9.7 mg/L for dissolved oxygen, usual in Pantanal rivers (Resende et al., 2006).

Keeping banded knifefish for a minimum period of 40 days in high conductivity (180 μS/cm) was enough to block gonadal development. Consequently, the fish gonads synchronized, and maturation occurred after the conductivity decreased to 15 μS/cm for at least 20 days. Knowing the minimum time in high conductivity that allows the regression of the gonads and, subsequently, the time required for the gonad recrudescence for the fish to start breeding is relevant information for the breeding management of this species on a large scale. The intervals observed in the present study were shorter than those reported for the synchronization of another Gymnotid fish (*Eigenmannia virescens*). For such species, fish were kept for four months in high conductivity for ovarian regression and subsequent spawning synchronization, and after 33 days in low conductivity, they were mature (Kirschbaum, 1979).

In this study, it was evident that female individuals improved their reproductive condition, moving from stages 1 and 2 (immature or maturing eggs) to stage 3 (mature eggs), as shown in Fig. 6, by manipulating the electrical conductivity of the water. Although at the beginning of the experiment, <20% of females were in stage 3, after 70 days, independent of the treatment, >85% of the banded knifefish were mature (stage 3) and ready to spawn, demonstrating that the procedure was adequate for the mass synchronization of banded knifefish in a shorter period than previously reported. Furthermore, it was observed that 25 to 30 days after the decrease in conductivity (groups 1 and 2) might be sufficient for recrudescence since, at these terms, the percentage of females in S3 was between 93 and 94%, slightly higher than group 3 at 86%.

Previous studies have demonstrated that the ovaries of *E. virescens* can recrudesce after 30 days in low conductivity (Kirschbaum, 1984), and their spawning occurs 70 days after this decrease (Kirschbaum, 1975). However, the minimum period of 30 days (group 1) in this high and low conductivity protocol was insufficient to allow a significant spawning rate of females. Another critical factor to be highlighted is recrudescence, triggered by a decrease in conductivity, which does not seem to be influenced by the variation of the absolute conductivity value or the ionic composition in the water. In *P. isidori* (Osteoglossiformes), Kirschbaum (1987) observed that maturation was completed after about 50 days of the decrease in water conductivity. Based on such information, it would be possible to trigger regression, and subsequent maturation using shorter time and lower conductivity values (bringing an operational and input use advantage) and optimize future spawning that continues to occur in these species even after the first recrudescence.

The treatments mean value of 91.25% of females in stage 3, which is more than double the mean value (39.42%) in the control group after 40 days of the experiment, could be double-confirmed from US imaging analysis and by dissection of 50 fish per group at the end of the study. Banded knifefish in gonadal development stages 3 and 4 (Table 2) are similar and challenging to determine via US imaging (França, 2010; González et al., 2001). However, after dissection to confirm the stages observed by US, no females in stage 4 were observed.

Another important observation in this study was the presence of four spawning events in one pond of group 1, where the banded knifefish were subjected for the most prolonged period in low conductivity (30 days). Although the egg occurrence was an exception, this corroborates the observation by Kirschbaum and Wieczorek (2002) that banded knifefish need to remain exposed to low electrical conductivity for at least 50 days to spawn. Notably, when Gymnotidae are subjected to high and later to low conductivity, the variation of environmental factors is no longer necessary to maintain mature gonads and the occurrence of consecutive multiple spawning (Kirschbaum, 1975, 1979, 1984, 1987). The spawn interval in this genus is nearly 60 days for multiple spawning maintained in low water conductivity (Kirschbaum and Schugardt, 2002).

The presence of eggs in this study in the macrophyte roots matches field observations by Crampton and Hopkins (2005), that the banded knifefish release egg masses close to the water hyacinth roots. In Pantanal, the macrophytes *Eichhornia azurea*, *E. crassipes,* and *Pontederia lanceolata* are predominant in different environments (Silva and Pinto-Silva, 1989), usually where banded knifefish occur naturally (Pereira and Resende, 2006). The installation of islands of *Eichhornia crassipes* since the beginning of the experiment in all ponds to mimic the natural environment contributed to the necessary shelter and well-being of the broodstock and, consequently, may have favored the four spawning events that occurred in one of the ponds. It is relevant to notice that the eggs were fertilized as they hatched into viable larvae after incubation in the laboratory.

With these two techniques (US sexing and water conductivity management) being used together, it will be possible to conduct reproductive studies in banded knifefish in a safe and no invasive way. It also

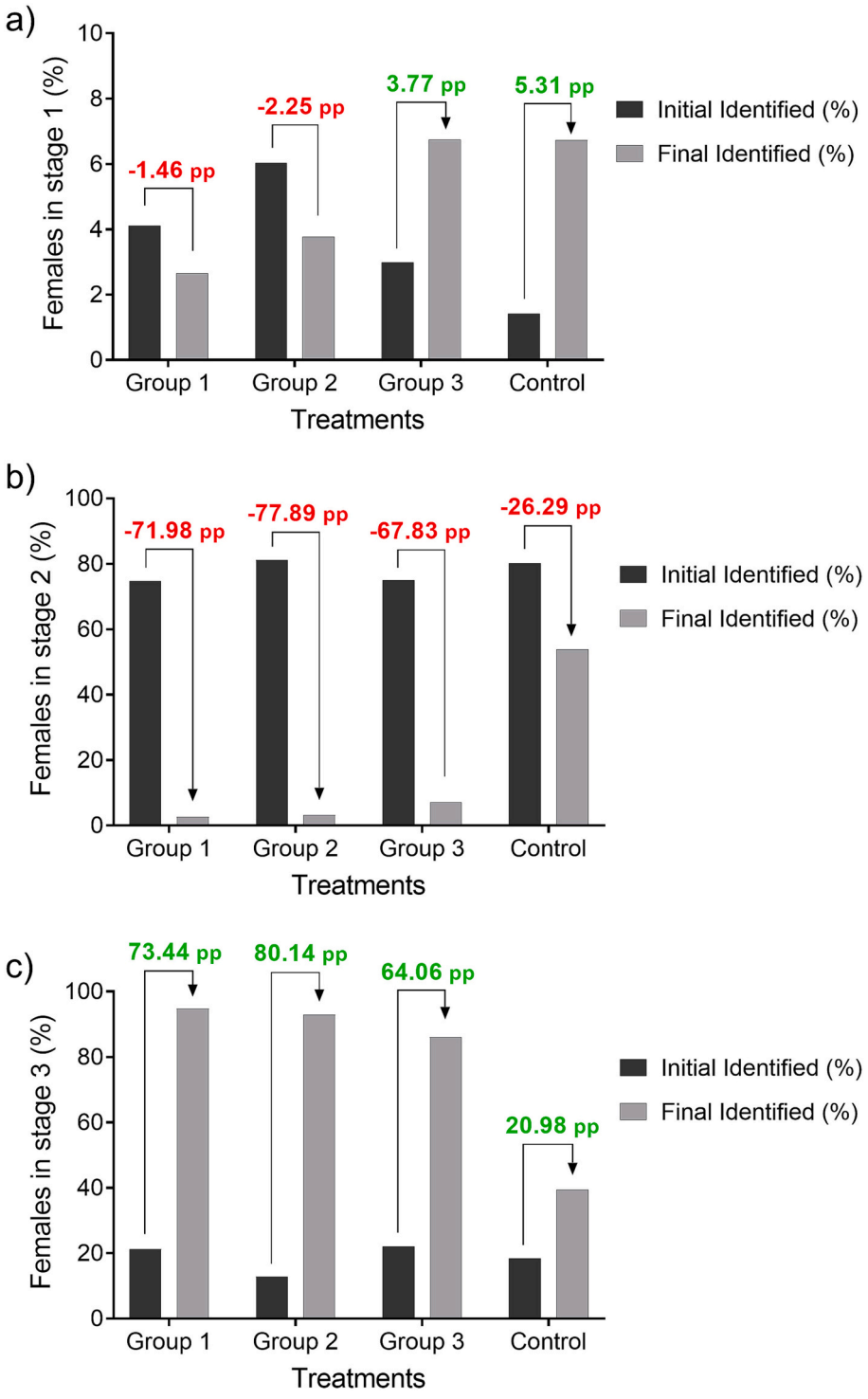

Fig. 6. Mean percentage of banded knifefish (*Gymnotus* cf. *carapo*) females in different stages of ovarian development after 70 days of the experiment, indicating the initial and final percentage and the difference in percentage points found between the two analyses. a) females in stage 1; b) females in stage 2; c) females in stage 3. Values in percentage points on the bars indicate the difference between the final and initial evaluation in the total percentage.

helps the development of technologies and protocols for reproducing this species without the use of hormones, which will allow bred the species in captivity for a commercial purpose and promote the aquaculture fish bait business to decrease the fishing pressure on the wild population.

Further studies that assess banded knifefish spawning in periods longer than 30 days in low water conductivity are warranted to understand the species spawning triggers to mass production in captivity.

5. Conclusions

The synchronization protocol of mass gonadal maturation of *Gymnotus* cf. *carapo* was achieved by subjecting the fish to high water electrical conductivity (180 μS/cm) for 40 days and subsequently exposing them to low electrical conductivity (15 μS/cm). In addition, the sexing of banded knifefish using ultrasound imaging to correlate with the reproduction stage and GSI was fundamental for the efficacy of the mass

Table 2
Water quality parameters (mean ± SD) for the different mass induction treatments of banded knifefish (*Gymnotus* cf. *carapo*) by a change in water conductivity.

Variables	Treatment			
	Group 1	Group 2	Group 3	Control
Temperature (°C)	27.19 ± 0.45 (26.6–27.95)	27.24 ± 0.51 (26.50–28.15)	27.23 ± 0.46 (26.50–28.15)	27.22 ± 0.41 (26.50–28.15)
Dissolved oxygen (mg/L)	5.17 ± 0.44 (4.30–5.90)	5.05 ± 0.42 (4.40–5.75)	5.13 ± 0.43 (4.40–5.80)	5.18 ± 0.38 (4.50–5.80)
pH	6.89 ± 0.31 (6.25–7.40)	6.84 ± 0.27 (6.35–7.25)	6.94 ± 0.26 (6.50–7.40)	6.94 ± 0.23 (6.50–7.30)
Conductivity (μS/cm)	177.84 ± 7.16 (14.15–190.00)	176.60 ± 6.20 (15.00–184.50)	174.55 ± 6.72 (14.5–185.0)	15.16 ± 1.00 (14–18)
Alkalinity (mg $CaCO_3$/L)	33.50 ± 3.01 (27–38.5)	33.87 ± 3.54 (28–40.15)	33.80 ± 3.52 (25.5–39.5)	33.80 ± 3.60 (28–41)
Transparency (cm)	54.07 ± 10.84 (42.5–86.5)	54.34 ± 11.49 (38.5–87.5)	54.44 ± 10.59 (39.5–83.5)	54.00 ± 12.80 (39–90)

Mean ± Standard deviation; Minimum and maximum values in parentheses.
Group 1: high conductivity (180 μS/cm) for 40 days + low conductivity (15 μS/cm) for 30 days.
Group 2: high conductivity (180 μS/cm) for 45 days + low conductivity (15 μS/cm) for 25 days.
Group 3: high conductivity (180 μS/cm) for 50 days + low conductivity (15 μS/cm) for 20 days.
Control: Low conductivity (15 μS/cm) for 70 days.

gonadal maturation protocol. By understanding these factors, it will be possible to promote the aquaculture of banded knifefish and decrease the fishing pressure on the wild population, ultimately contributing to their conservation. However, further research is needed to establish the optimal spawning conditions and refine the induced spawning protocols for this species, which came with testing, practice, and eye calibration.

Contribution statement

The authors' contributions in this paper were: conceptualization and methodology, M.A.R.; formal analysis, M.A.R., R.B.R., and D.P.S.Jr.; investigation, M.A.R., M.F.P., and L.C.A.F.; resources, M.A.R.; writing - original draft preparation, M.A.R., R.B.R., and D.P.S.Jr.; writing - review and editing, M.A.R., R.B.R., J.A.F.d.L., and D.P.S.Jr.; visualization, M.A.R., and R.B.R.; supervision, M.A.R.; project administration, M.A.R., and J.A.F.d.L.; funding acquisition, M.A.R.. Finally, all authors discussed the results, commented on the manuscript, and read and agreed to the published version.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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