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Ultrasonography for sex determination of Lebranche mullet

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ABSTRACT

Ultrasonography has been increasingly used in aquaculture breeding programs for non-invasive determination of sex and the gonad maturation stage in fish. For the first time, this image exam was performed on a mugilid (Lebranche mullet). Nineteen male and 12 female fish were submitted to this evaluation, which were selected because they were identified to be in the final gonad maturation stage, which was previously confirmed through spermiation by abdominal pressure and ovarian sampling biopsy (oocytes of 626 ± 12 µm), respectively. The ultrasound images were obtained after anesthesia, using a linear multifrequency probe at the frequency of 8 MHz in females and 10 MHz in males, which revealed significant differences in echogenicity between the gonads. In males, the testes presented a hypoechoic and homogeneous aspect, while in females the ovaries had a hyperechoic appearance and granular echotexture, compared to adjacent body structures. The scanning time for sexual identification and characterization of reproductive system structures in each fish was 2.58 ± 1.68 seconds for males and 2.40 ± 1.05 seconds for females, there was no significant difference ($p > 0.05$) in relation to sexing time. Thus, the identification of the sex of the Lebranche mullet (*Mugil liza*) through ultrasonography proved to be an effective, fast and non-invasive method when applied to fish with mature gonads during the species' reproductive period.

Keywords: Gonads; Marine fish; Mugilidae; Reproduction; Ultrasound.

Ultrassonografia para determinação do sexo da tainha

RESUMO

A ultrassonografia tem sido cada vez mais utilizada em programas de melhoramento de aquicultura para determinação não invasiva do sexo e do estágio de maturação das gônadas em peixes. Pela primeira vez, esse exame de imagem foi realizado em um mugilid (tainha de Lebranche). Dezenove peixes machos e 12 fêmeas foram submetidos a essa avaliação, os quais foram selecionados por estarem em estágio final de maturação das gônadas, fato previamente confirmado por espermiação por pressão abdominal e biopsia ovariana (oócitos de 626 ± 12 µm), respectivamente. As imagens ultrassonográficas foram obtidas após a anestesia, utilizando uma sonda linear multifrequencial na frequência de 8 MHz em fêmeas e 10 MHz em machos, que revelaram diferenças significativas na ecogenicidade entre as gônadas. Nos machos o teste apresentou aspecto hipoecoico e homogêneo, enquanto nas fêmeas os ovários apresentaram aspecto hiperecogênico e ecotextura granular, em comparação às estruturas corporais adjacentes. O tempo de varredura para identificação sexual e caracterização das estruturas do sistema reprodutivo em cada peixe foi de 2,58 ± 1,68 segundos para machos e 2,40 ± 1,05 segundos para fêmeas, não houve diferença significativa ($p > 0,05$) em relação ao tempo de sexagem. Assim, a identificação do sexo da tainha Lebranche (*Mugil Liza*) por meio da ultrassonografia se mostrou um método eficaz, rápido e não invasivo quando aplicado a peixes com gônadas maduras durante o período reprodutivo da espécie.

Palavras-chave: Gônadas; Peixes marinhos; Mugilidae; Reprodução; Ultrassom.

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INTRODUCTION

Currently, there are several methods to determine the sex of fish. One of them is the determination of sex through blood hormones (Masoudifard et al., 2023). However, these methods generate stress to fish, require advanced devices to assess and determine the number of hormones, are time-consuming, expensive, and dangerous (Masoudifard et al., 2023). The use of techniques such as ventral wall cutting is one of the most traditional. However, it is more invasive, slower and creates a risk to fish health and calf performance, including death (Bahadir, 2023; Blythe et al., 1994; Kucharczyk et al., 2016). They are considered aggressive methods, especially in the case of Mugilids that are sensitive fish (Masoudifard et al., 2023). In this sense, the work points to less invasive methods and promotes the use of the ultrasound technique as an alternative to this problem.

An ultrasound image (USG) results from the emission of sound waves whose frequencies exceed 20,000 MHz. It provides a real-time view of the internal tissues of fish, such as bones, muscles, tendons, ligaments, joints, ovaries, testes, and the material within them (Jennings et al., 2012). The echogenicity refers to a tissue's ability to reflect or transmit sound waves. This property determines the intensity of the signal that returns to the transducer and, consequently, the appearance of the image displayed on the monitor (Carvalho, 2014; Papaléo & de Souza, 2019). Hyperechoic tissues reflect more waves, appearing brighter, while hypoechoic tissues reflect fewer waves, resulting in darker images (Carvalho, 2014; Papaléo & de Souza, 2019; Peixoto et al., 2010).

In 2012, a review work reported the use of ultrasound for various purposes in studies with fish, highlighting its application in studies about reproductive biology for more than 30 years (Novelo & Tiersch, 2012). This review mentions 17 studies regarding the use of USG for identifying sex and/or gonadal development stage in several species of commercial importance. Among the mentioned studies about marine and diadromous species, there was successful sex identification registered for Pacific herring (*Clupea pallasii*) (Bonar et al., 1989), Atlantic cod (*Gadus morhua*) (Karlsen & Holm, 1994; McEvoy et al., 2009), barfin flounder (*Verasper moseri*) (Matsubara et al., 1999), Atlantic halibut (*Hippoglossus hippoglossus*), winter flounder (*Pleuronectes americanus*), yellowtail flounder (*Limanda ferruginea*), haddock (*Melanogrammus aeglefinus*) (Martin-Robichau & Rommens, 2001), Atlantic salmon (*S. salar*) (Mattson, 1991), striped bass (*Morone saxatilis*) (Blythe et al., 1994; Jennings et al., 2005), hybrid striped bass (*M. saxatilis* × *M. chrysops*) (Blythe et al., 1994) and red hind (*Epinephelus guttatus*) (Whiteman et al., 2005).

A compilation of the studies published after 2010 regarding the use of USG for sex identification and/or assessment of maturation stage in marine and diadromous fish is presented in Table 1. In aquaculture, the work involving this technology is more recent,

Table 1. Studies of the use of ultrasound in the reproduction of marine and diadromous fish since 2010 until 2024*.

ID	Species	Objective	Reference
1	*Acipenser gueldenstaedtii* (Acipenseridae)	SI-1 RI-1	Memis et al., 2016
2	*Acipenser persicus* (Acipenseridae)	RI-1	Vajhi et al., 2013
3	*Acipenser sinensis* (Acipenseridae)	SI-2 RI-2	Du et al., 2017
4	*Anguilla anguilla* (Anguillidae)	SI-1, RI-1	Kucharczyk et al., 2016
5	*Anguilla anguilla* (Anguillidae)	S1-2, RI-2	Colombier et al., 2015
6	*Anguilla anguilla* (Anguillidae)	RI-1	Müller et al., 2015
7	*Arothron manilensis* (Tetraodontidae)	SI-1	Doi et al., 2024
8	*Cyclopterus lumpus* (Cyclopteridae)	RI-2	Mlingi et al., 2023
9	*Dicentrarchus labrax* (Moronidae)	SI-1	Macri et al., 2013
			Frost et al., 2014
10	*Hippoglossus stenolepis* (Pleuronectidae)	SI-1	Loher and Stephens, 2011
11	*Huso huso* (Acipenseridae)	SI-1	Masoudifard et al., 2011
12	*Mugil cephalus* (Mugilidae)	SI-1, RI-1	Masoudifard et al., 2023
13	*Oncorhynchus nerka* (Salmonidae)	SI-1, RI-1	Frost et al., 2014
14	*Polyprion oxygeneios* (Polyprionidae)	SI-1	Kohn et al., 2013
15	*Salmo salar* (Salmonidae)	RI-1	Næve et al., 2018

*This list includes the study identification number (ID), species, objective, and references. The objective of each study is listed as sex identification (SI), reproductive indexes (RI) or both (SI, RI). Sex identification and reproductive indexes were divided into data based only on ultrasonography (SI-1, RI-1) and those derived from ultrasonography and other methods (SI-2, RI-2).

but no less important. The USG involves work in the areas of disease diagnosis, carcass evaluation and reproduction (Crepaldi et al., 2006). It has increasingly been used to non-invasively determine the sex and gonadal maturation of fish in reproduction programs in aquaculture, it is considered a safe technique, with no harmful biological effects for the patient or the operator (Preston & Shaw, 2001). It is particularly useful for fish species without apparent sexual dimorphism (Bahadir, 2023; Masoudifard et al., 2023; Novelo & Tiersch, 2012). USG enhances induced spawning protocols by accurately identifying the optimal timing for induction (Crepaldi et al., 2006). It offers benefits to aquaculture, such as increased larval production, optimized nutritional management, and control over fish sales during gonadal development (Moghim et al., 2002). Studies report nearly 100% efficacy in sex differentiation, high survival rates, minimal invasiveness, and faster procedures in species such as sturgeon (*Acipenser ruthenus*) (Golpour et al., 2021), lamprey (*Lampetra fluviatilis*) (Kujawa et al., 2019), and mullet (*Mugil cephalus*) (Masoudifard et al., 2023).

The mullet Lebranche (*Mugil liza*, Valenciennes, 1836) presents behavioral, physiological, and morphological characteristics similar to those of other species of the genus (Durand et al., 2012; Masoudifard et al., 2023; Menezes et al., 2015; Siccha-Ramirez et al., 2014). Mugilidae are present on all continents, distributed in coastal, estuarine, and freshwater regions, from tropical to subtropical zones (Menezes, 1983; McDowall, 2007; Menezes & Figueiredo, 1985). They have expressive commercial value and importance and are widely studied to support their reproduction and cultivation (Masoudifard et al., 2023). They are fish that are not sexually dimorphic, which aggregate and migrate in large groups to spawn and have large male and female gonads. Currently, the sexual differentiation of mugilids can only be performed during the period in which the gonads are developing for reproduction, through ovarian sampling biopsy in females and abdominal pressure to verify spermiation in males. For the selection of females, the procedure is considered invasive and can cause internal injuries to the oviduct and gonads, it is slow and potentially stressful for the animals, and can delay and prevent ovulation (Blythe et al., 1994; Kucharczyk et al., 2016). Thus, the objectives of this study were to evaluate the USG technique in mugilids and verify its efficiency in identifying the sex of fish as a non-invasive and fast method.

MATERIAL AND METHODS

The fish were kept at the Marine Aquaculture Laboratory (LAPMAR) in a 40-m^3 outdoor raceway tank, with a continuous flow (rate 150–200%) of sea water pumped from the Mozambique beach, Florianópolis, SC, Brazil (27°34'S and 48° 25'W) and aerated by a radial blower coupled to a microporous hose ring (Aquadrop Air, São Paulo, Brazil). Water temperature (maximum 30.3°C in January and minimum 15.3°C in August) and natural photoperiod (at latitude 27°S, maximum 13h53min, and minimum 10h23min) varied according to the seasons. Feeding was conducted four times a day until apparent satiety (Calixto et al., 2020) with commercial feed (45% crude protein and 8% ethereal extract) of 2.6 mm. Maximum fish biomass was 1.5 $kg \cdot m^{-3}$ in the gonadal maturation phase.

Nineteen males and 12 females of *M. Liza* were used, between 3 and 5 years old, with body weight between 555 and 2,800 g, and total length from 39.1 to 56.5 cm. The fish selected met the basic characteristics of mature specimens and were analyzed in June and July, during the reproductive period of the species in captivity (Magnotti et al., 2020). To identify the sex and degree of maturation of gonads, fish were anesthetized by benzocaine immersion at 50 $mg \cdot L^{-1}$ (Braz et al., 2017). The analysis of maturation of females was performed through ovarian biopsy, with a non-toxic PVC probe (tracheal probe no. 04) inserted in the oviduct and aspiration of an oocyte sample (Magnotti et al., 2020). Fifty oocytes were measured with a stereoscopic loupe (Leica EZ4HD) and LAZ EZ 2.1.0 software (Leica, Switzerland). Females were considered mature and suitable for reproduction when average oocyte diameter was greater than 600 µm (Cerqueira et al., 2017). Males were considered mature and able to reproduce when they released semen after abdominal massage (Castro et al., 2019; Magnotti et al., 2018a; Magnotti et al., 2018b). Subsequently, the fish were sent for ultrasound examination. All procedures used in this study were approved by the Ethics Committee for the Use of Animals, under protocol number 3102220419.

For the ultrasonographic analysis, a portable GE device (General Electric Company), model Logiq *e* Veterinary, was used, coupled to a multifrequency linear probe (8–13 MHz). Images of the gonads with more detail and clarity were obtained at the frequency of 10 MHz for males and 8 MHz for females, for both the longitudinal and transverse planes. The linear probe captured 3.00 cm deep rectangular images with a 3.86 cm long horizontal plane that were frozen on the screen, allowing measurement of structures and the addition of texts for archiving and later analysis. For image optimization, adjustments to the overall gain (Gain), time gain compensation control (TGC), focus and depth (Depth) were performed during the exam. For brightness control, the overall gain

was set between 65 and 90 dB. The time gain compensation (TGC) buttons helped to control the sound wave attenuation, decreasing or increasing the gain in specific areas, such as the most distal region of the ovary. The position of the focus and the depth maximized the visibility of the organs of interest. The images obtained were recorded in the ultrasound device and transferred to a computer with a USB device.

To perform the USG, the anesthetized fish were positioned in dorsal recumbency and held at the head and tail by an operator, with the head, back, and tail partially submerged in a container with salt water. Water-based gel (Condu Gel, Contato Industrial) was used to conduct the ultrasound, without submerging the probe. The probe was first positioned longitudinally, on the midline of the ventral abdomen, with its cranial end facing the fish's head and the caudal end over the anus orifice, which served as an external reference point. From there, longitudinal, caudal and cranial, sagittal and transverse scans were performed. The fish was then positioned in right and left lateral recumbency, allowing a complete scan of the body cavity and subsequent identification of the structures. Comparison between males and females in each scanning time was performed by Student's t test ($p < 0.05$) by the program GraphPad Prism 6.

RESULTS

The fish selected had a degree of total maturation of gonads and were able to spawn. Males showed spermiation after light abdominal pressure, and females, oocytes with a mean diameter of 626 ± 12 um.

At the initial moment of USG, the final portion of the bowel was identified with the probe positioned over the anal orifice, considering that it is located longitudinally in the body cavity between the male and female reproductive organs. In this reproductive phase, both the ovaries and the testes were presented as elongated structures, extending from the cranial portion, from the pectoral fins externally and from the liver and gastric cavity, internally, to the most caudal region, adjacent to the final segment of the bowel. In both sexes, the reproductive organs are positioned side by side, on the right and left sides of the body cavity, and gradually decrease in width in the terminal portion, until they reach the genital orifice. The echogenicity of the gonads was determined based on the adjacent tissues and structures, externally delimited by a thin hyperechoic line. The testes showed a hypoechoic and homogeneous aspect, with a fine echotexture (Figs. 1a, 1b and 1c). The ovaries presented a

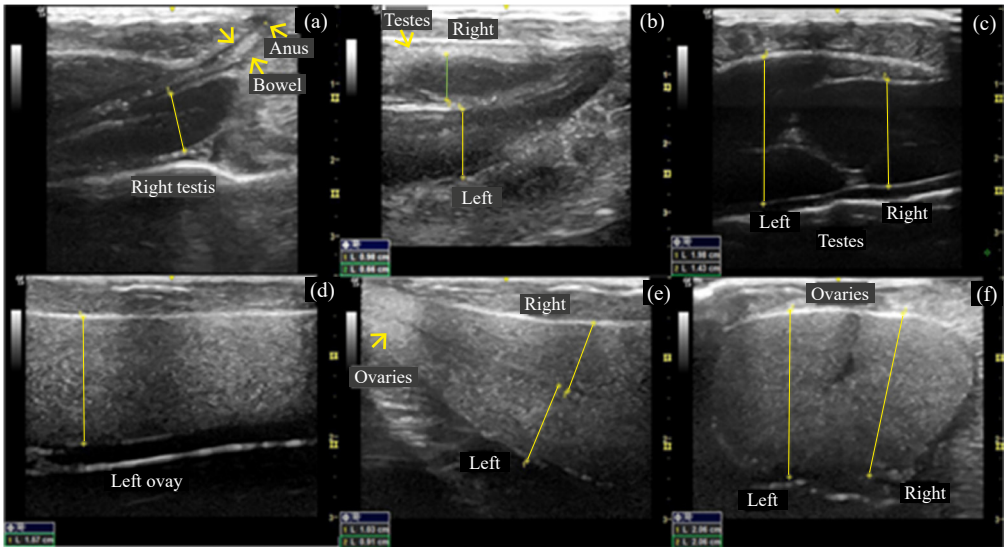

Figure 1. Ultrasonographic image of the gonads of Lebranche mullet (*Mugil liza*) during the complete maturation phase. (a, b and c) Images of the testes presenting a hypoechogenic and homogeneous aspect in relation to the adjacent tissues. (a) Longitudinal image of the final portion of the bowel to the anal orifice (arrows), filled with hyperechogenic fecal content. Bowel in direct contact with the final portion of the right testis, which serves as an internal anatomical reference; (b) longitudinal plane image of the narrowing of the final portion of the right and left testes; (c) transverse image of the right and left testes at their maximum diameter. (d, e and f) Images of the ovary showing a hyperechogenic aspect and granular echotexture in relation to adjacent structures. (d) Longitudinal image of the left ovary; (e) longitudinal image showing the narrowing in the final portion of the right and left ovaries; (f) transverse plane image of the right and left ovaries at their maximum diameter. Images obtained with a multifrequency linear probe (8–13 MHz), at the frequency of 8 MHz for females and 10 MHz for males.

hyperechoic sonographic appearance and a granular echotexture (Figs. 1d, 1e and 1f), which caused attenuation of the sound wave in the most distal region of the organ. The measurements obtained for the width of the organ at its maximum diameter were 1.98 cm for the left testis and 1.43 cm for the right testis. The maximum diameter measured of the right and left ovaries was a width of 2.06 cm in the fish studied. The scanning time for sex identification and characterization of structures adjacent to the reproductive system in each fish was 2.58 ± 1.68 s for males and 2.40 ± 1.05 s for females. No significant difference ($p > 0.05$) between them in relation to sexing time was seen. All fish were kept alive and returned to the tank after full anesthetic recovery.

DISCUSSION

The gonads were easily identified and differentiated, considering that during the final maturation phase they occupy most of the body cavity (Novelo & Tiersch, 2012). As in this study, frequencies ranging between 3.5 and 13 MHz are used to identify sex and reproductive indices for other species with protocols already defined (Novelo & Tiersch, 2012). However, with the 8–13 MHz linear transducer, it was not possible to identify the degree of maturation and measure the diameter of the Lebranche mullet oocytes.

Measurement of the diameter of the oocytes is essential data in marine fish that need hormonal induction to stimulate the final maturation of the oocytes and/or spawning, in order to identify the female capable of receiving the hormone (Magnotti et al., 2020). Marine fish species with pelagic spawning have smaller oocytes and are suitable for hormonal induction when females present: > 600 µm for Lebranche mullet (*M. liza*) and > 400 µm for common snook (*Centropomus undecimalis*) (Cerqueira et al., 2017); > 325 µm for dusky grouper (*Epinephelus marginatus*) (Kerber et al., 2012); > 400 µm for Brazilian flounder (*Paralichthys orbignyanus*) (Sampaio et al., 2008); among other species. This group of fish, and others with small oocytes, will have the same limitation with USG when this probe frequency is used. Alternatives such as obtaining high frequency transducers (18–22 MHz) may resolve this issue in the future. The 20-MHz frequency is already used to correlate ultrasound and histological examinations of human skin (Barcaui et al., 2015; Jasaitiene et al., 2011), generating images with greater detail and sharpness at a maximum depth of 1 cm. It is of considerable interest that more detailed studies be carried out correlating the size of the gonads through imaging (the gonadossomatic index) with the size of oocytes collected by ovarian biopsy or histology, to standardize the method and allow identifying the evolution of gonadal maturation in each species studied. A strong positive correlation between these parameters has already been found for Atlantic salmon (*S. salar*), validating this possibility (Næve et al., 2018).

Sexual identification tests on live fish may have an average time of less than 1 minute, as reported for the species: Atlantic cod (*G. morhua*) (Karlsen & Holm, 1994; McEvoy et al., 2009), Stellate sturgeon (*Acipenser stellatus*) (Moghim et al., 2002) and Striped bass (*M. saxatilis*) (Blythe et al., 1994). In the study conducted in *M. cephalus* by Masoudifard et al. (2023), the time of sex identification was less than 30 s. However, no study or USG method showed to be able to identify the sex of each fish in less than 10 s for males and females of *M. liza*. Although technology has become more accessible over time, the use of high-quality ultrasound equipment can still be costly (over 26,170 USD), and it is not always available in all research facilities (Novelo & Tiersch, 2012). However, its efficiency, accuracy, long-term benefits, and minimal invasiveness justify the investment. Sex identification is obtained immediately, saving time and costs (Masoudifard et al., 2023), making it a strategic tool for aquaculture and the conservation of native species. The high percentage of precision in the identification of sex and minimal contact with the body of the fish are one of the most important advantages of this technique, because in USG the fish is immobile in water with an anesthetic, and ultrasound is performed with a minimum of touch (Masoudifard et al., 2023), generating a high impact on the health and well-being of fish, avoiding methods of catheterization and biopsy for sexing in species that do not present dimorphism (Bahadir, 2023; Novelo & Tiersch, 2012).

USG offers significant benefits but has limitations when applied to fish with varying body characteristics and reproductive strategies (Barroso and Ueda, 2014; Novelo & Tiersch, 2012). In species with small or hard-to-distinguish gonads, image quality may be insufficient for accurate maturation identification (Novelo & Tiersch, 2012). In larger species or those with dense tissues, limited ultrasound penetration hinders the visualization of internal structures (Novelo & Tiersch, 2012). Species with hermaphroditism or complex gonadal anatomies require specialized approaches, and rapid gonadal changes in species with short reproductive cycles may not be captured accurately (Novelo & Tiersch, 2012). These challenges underscore the need to adapt USG to species-specific biological traits for improved reproductive diagnostics.

In this study, the image generated by the mature ovary caused attenuation of the sound beam. This attenuation is generated by the interaction of the sound wave with the tissues, causing a progressive loss of the intensity of the initial pulse, with a consequent reduction in the resolution of the contrast in the deeper areas (Penninck & d'Anjou, 2017). Adjusting the focus and depth in the region of interest, concentrates the intensity of the sound beam in a small area and increases the contrast resolution, resulting in image optimization. By adjusting the overall gain and the TGC, the amplification of the echoes can be changed to compensate for tissue attenuation. Moreover, a low frequency transducer can be used, which can correct this signal loss. In the current study, changing the frequency from 10 to 8 MHz and adjusting the TGC buttons in the deepest region of the ovaries efficiently compensated for the loss of signal strength.

In an evaluation of the effectiveness of USG in sexing Atlantic cod (*G. morhua*) in the spawning period, Karlsen and Holm (1994) obtained the same results when compared with the females of *M. liza* in the present study, the mature ovaries showed granular appearance and were considered as hyperechogenic. On the contrary, the testicles presented hypoechogenic aspect and could be observed and identified at the highest frequency, with an optimized image. The hyperechogenic and hypoechogenic aspect of the sexual structures of fish, regardless of being females or males, will depend on the degree of maturation in which the fish are at the time of analysis. For example, according to Masoudifard et al. (2023), specimens of *M. cephalus* males presented gonads with hyperechogenic structure of oval shape, of small diameter and single layer, and the females presented gonads with parenchyma completely homogeneous, hypoechogenic and identifiable wall in three layers, because the beginning of their maturation. Although there are morphological differences between species, the ultrasound images of the gonads of Lebranche mullet (*M. liza*) were similar to those of cod (*G. morhua*) and grey mullet (*M. cephalus*).

The most favorable place for obtaining the sonographic images in both sexes was the region anterior to the anus orifice, which coincides with that reported by Masoudifard et al. (2023); noting that, for a quick and easy identification of sex in *M. cephalus*, the best place to place the transducer is on the ventral surface of the fish in the caudal part of the coemic cavity (between the ventral and anal fins). The longitudinal image of the final portion of the bowel served as an internal anatomical reference, because, in addition to maintaining direct contact with the reproductive organs, it is present in fish of both sexes. In the longitudinal plane, the total length of the reproductive organs in both sexes could not be obtained directly by USG, because it was not possible to acquire an image of the cranial and caudal limits in the same slice. However, it is possible to obtain the total length of the organ indirectly, by identifying and observing the cranial margin of the gonad by USG and later measuring the external surface of the fish with a ruler, up to the region of the urogenital pore (Novelo & Tiersch, 2012).

USG has great potential to improve sustainable practices in aquaculture by providing a precise, non-invasive method for sex identification and gonadal maturation assessment. Despite challenges like high costs and limited availability of high-frequency probes, USG offers benefits such as reduced stress on fish and increased production efficiency, making it a valuable investment. As technology advances, USG's role in aquaculture is expected to grow. Future research should focus on developing affordable high-frequency transducers, integrating USG with tools like the gonadosomatic index, and optimizing imaging protocols for different species to enhance its accuracy and expand its applicability in aquaculture.

CONCLUSION

The identification of the sex of Lebranche mullet (*M. liza*) obtained through USG examination was an effective, fast, and non-invasive method when applied to fish that had mature gonads, in the species' reproduction period. Significant differences in the images acquired of the gonads of males and females were characterized and described. However, complementary studies should be conducted to categorize the degree of gonad maturation and oocyte size.

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTIONS

Conceptualization: Santarosa, I.M.; **Methodology:** Santarosa, I.M., De Castro, M.A., Dos Santos, M.C., Magnotti, C.; **Writing – original draft:** Santarosa, I.M., Dos Santos, M.C., Riofrio, L.C.P., Magnotti, C.; **Formal Analysis:** Santarosa, I.M., Dos Santos, M.C.; **Investigation:** Santarosa, I.M., De Castro, M.A., Dos Santos, M.C., Riofrio, L.C.P., Magnotti, C.; Brum, A.; **Data curation:** Dos Santos, M.C.; **Writing – review &**

editing: Riofrio, L.C.P.; **Resources:** Brum, A., Magnotti, C.; **Supervision:** Brum, A., Magnotti, C.; **Funding acquisition:** Magnotti, C. **Final approval:** Riofrio, L.C.P.

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