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NOTE

In vivo determination of sex and reproductive status of *Octopus tehuelchus* (Cephalopoda: Octopodidae) by ultrasound techniques

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ABSTRACT. In recent years, studies on the cultivation of various species of cephalopods have grown with the aim of diversifying the supply of aquaculture products in the world market. Ultrasound images constitute a non-invasive method that has been utilized to determine sex and maturity stages in fish, being a useful tool for the management of captive broodstocks. The objective of this work was to evaluate the effectiveness of ultrasound techniques for the identification of sex and reproductive status of the small Patagonian octopus *Octopus tehuelchus*. Observations were carried out with a portable ultrasound machine (Sono Site Ultrasound System) and a transducer (10-5 Mhz) in eight anesthetized individuals. In ventral view, the testis had a spherical, homogeneous and compact appearance, while the spermatophore complex appeared as a hyperechoic structure with respect to the testis, highlighting the presence of spermatophores in mature individuals. In females, the dorsal view allowed to identify a granular-appearance ovary with anechogenic areas, indicating a maturing ovary. Ultrasounds allowed unequivocal sexing. In this way, the ultrasound technique was a practical and non-invasive method for determining sexes and gonadal maturation in live specimens of *O. tehuelchus*.

Key words: Ultrasound, octopus, sex determination, sexual maturity.

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Determinación *in vivo* del sexo y estado reproductivo de *Octopus tehuelchus* (Cephalopoda: Octopodidae) mediante técnicas de ultrasonido

RESUMEN. En los últimos años, los estudios sobre el cultivo de diversas especies de cefalópodos han crecido con el objetivo de diversificar la oferta de productos acuícolas en el mercado mundial. Las imágenes de ultrasonido constituyen un método no invasivo que se ha utilizado para determinar el sexo y los estados de madurez en peces, siendo una herramienta útil para el manejo de reproductores en cautiverio. El objetivo de este trabajo fue evaluar la efectividad de la técnica de ultrasonido para la identificación del sexo y el estado reproductivo del pulpo patagónico *Octopus tehuelchus*. Las observaciones se realizaron con un ecógrafo portátil (Sono Site Ultrasound System) y un transductor (10-5 Mhz) en ocho individuos anestesiados. En vista ventral, el testículo presentó un aspecto esférico, homogéneo y compacto, mientras que el complejo espermatóforo apareció como una estructura hiperecogénica respecto del testículo, resaltando la presencia de espermatóforos en individuos maduros. En las hembras, la vista dorsal permitió identificar un ovario de aspecto granular con áreas anecogénicas, indicando un ovario en maduración. Las ecografías permitieron determinar el sexo de manera inequívoca. De esta manera, la técnica ecográfica resultó un método práctico y no invasivo para la determinación de sexos y maduración gonadal en ejemplares vivos de *O. tehuelchus*.

Palabras clave: Ultrasonido, pulpos, determinación del sexo, madurez sexual.

The routine assessment of sexual maturity in aquatic animals often involves a macroscopic or microscopic determination by visual examination of gonads, involving the sacrifice of animals. Ultrasound imaging is a useful tool for studying the internal structure in living animals. This methodology, currently used for the determination of sex and maturity stages in fish, began to be utilized for fisheries management (Martin-Robichaud and Rommens 2001) and is regularly applied as a non-invasive method for the identification of mature salmonids (Evans et al. 2004), sturgeons (Colombo et al. 2004; Wildhaber et al. 2005), and several flatfish species in aquaculture (Peleteiro et al. 1995; Martin-Robichaud and Rommens 2001; Stephens 2009; Radonic 2011). This fast and easy technique with minimum stress has become relevant in the context of animal care. In cephalopods, this technique was applied to observe the morphology of the mantle, muscles, brain and arms (Davenport 1993; King et al. 2005; Grimaldi et al. 2007; Margheri et al. 2011; Ponte et al. 2017). Ultrasounds can be performed with or without sedation, as in *Sepia officinalis* (King et al. 2005; King and Adamo 2006) or *Octopus vulgaris* (Grimaldi et al. 2007), respectively.

Cephalopods represent 5% of the total volume of catches in the world and 4% of the total volume in the global fisheries trade (FAO 2024). The interest in the consumption and cultivation of various species of cephalopods has increased, since they have a high nutritional value and a lower fat content compared to fish (Vidal et al. 2014; FAO 2018). Octopuses offer numerous advantages for commercial aquaculture, particularly because of their rapid growth (> 15% of body weight per day) and high feed conversion rates (Martínez et al. 2014; Rosas and Martínez-Yañez 2015; Tercero et al. 2015; Caamal-Monsreal et al. 2016; Gallardo et al. 2020).

The Patagonian octopus *Octopus tehuelchus* d'Orbigny (1835) is one of the few species of coastal octopods living in the Argentine Sea. It has been studied in terms of its ecological, biological, and fishing aspects (Ré 1998; Iribarne et al. 1991; Storero and Narvarte 2013; Fassiano et al. 2017; Berrueta and Desiderio 2019). Its ability to adapt to captivity, the direct development of a benthic juvenile and its acceptance in the regional market have generated great interest in the cultivation of this species (Iribarne 1990a; Klaich et al. 2006, 2008; Berrueta et al. 2019, 2020a, 2020b; Bocco et al. 2019; Braga et al. 2021). This small octopus is endemic to South America. It is distributed in the Atlantic Ocean from southern Brazil (16° S) to northern Argentine Patagonia (44° S), inhabiting intertidal zones up to 100 m deep (Ré 1998; Narvarte et al. 2006; Storero et al. 2010; Ré and Ortiz 2011). *O. tehuelchus* is a holobenthonic species, presenting a small adult size (up to 150 g) and a seasonal reproductive cycle (Ré 1998; Storero et al. 2010). This species is classified as a simultaneous terminal spawner with low fecundity, releasing a maximum of 220 eggs per clutch. Their relatively large eggs are attached to rocks or mollusk shells, giving rise to large benthic juveniles (Pujals 1986; Iribarne 1990b; Ré 1998; Storero et al. 2012). Its life cycle is short, approximately 18 months.

Sex determination in octopods relies on the analysis of secondary sexual characteristics. In smaller individuals, where the hectocotylized arm is not evident, dissection is typically required for identification (Pujals 1986). The objective of this work was to evaluate the effectiveness of ultrasound techniques for the identification of sex and reproductive status of the small Patagonian octopus *O. tehuelchus*.

Eight *O. tehuelchus* specimens born at the Mariculture Station of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP, Argentina) were kept individually in 30 l aquaria connected to a recirculating aquaculture system (RAS). Shells of marine gastropods were used as natural shelters. Octopuses were fed shrimps and mussels to satiety once a day in the morning. The photoperiod was set at 12 L:12 D (light: dark). Water quality parameters were recorded daily: temperature 15.5 ± 1.1 °C (Earth Industries heater/cooler, Japan), pH 7.7 ± 0.2 (Oakton pH/temperature Serie 110 RM232), salinity 34 ± 1 (Tanaka NewS-100 light

refractometer), and total ammonium concentration 0.0 ± 0.5 mg NH_4^+ l^{-1} (Merck colorimetric kit). A portable ultrasound machine (Sono Site Ultrasound System) and a transducer (10-5 Mhz) were used to determine sex and identify maturation stages in octopuses aged 8 to 12 months. Once a month, individuals were anesthetized by immersion in a tray with a 2% alcohol solution in seawater until total relaxation (Fiorito et al. 2015; Butler-Struben et al. 2018), and subsequently transferred to a second tray filled with filter seawater for ultrasound examinations. The transducer was located 2 cm above the mantle of the octopus in order to obtain a correct ultrasound image (Davenport 1993). Additionally, mantle length (ML) and total weight (TW) were recorded for all individuals. Males were then recovered from anesthesia and returned to their respective tanks. Once the reproductive stage was identified, females were sacrificed by deep anesthesia and dissected. Stages of maturation and corresponding gonadal morphometrics of *O. tehuelchus* followed the description of Pujals (1986). The accuracy of ultrasound-derived sex determinations was verified by visual examination of eviscerated octopuses. The type of image format obtained with this ultrasound machine corresponded to mode B (brightness), e.g. the more intense the echo reflected by a certain structure the brighter it will appear in the image. Ultrasound observations were carried out in both dorsal and ventral areas of specimens, scanning with the transducer in a posterior-anterior direction of the mantle with the gonad being the first structure to be identified (Figure 1).

The following gonadal structures were identified. Females: the dorsal view allowed the identification of the ovary with a granular appearance with increased anechogenic areas indicating its maturation. Early maturity ovary (M1), 2.2 cm transverse diameter, appeared granular with incipient anechogenic zones (Figure 2 A-B). Medium maturity ovary (M2) presented abundant hyperechoic areas with a circular appearance and 2.5-3.0 cm transverse

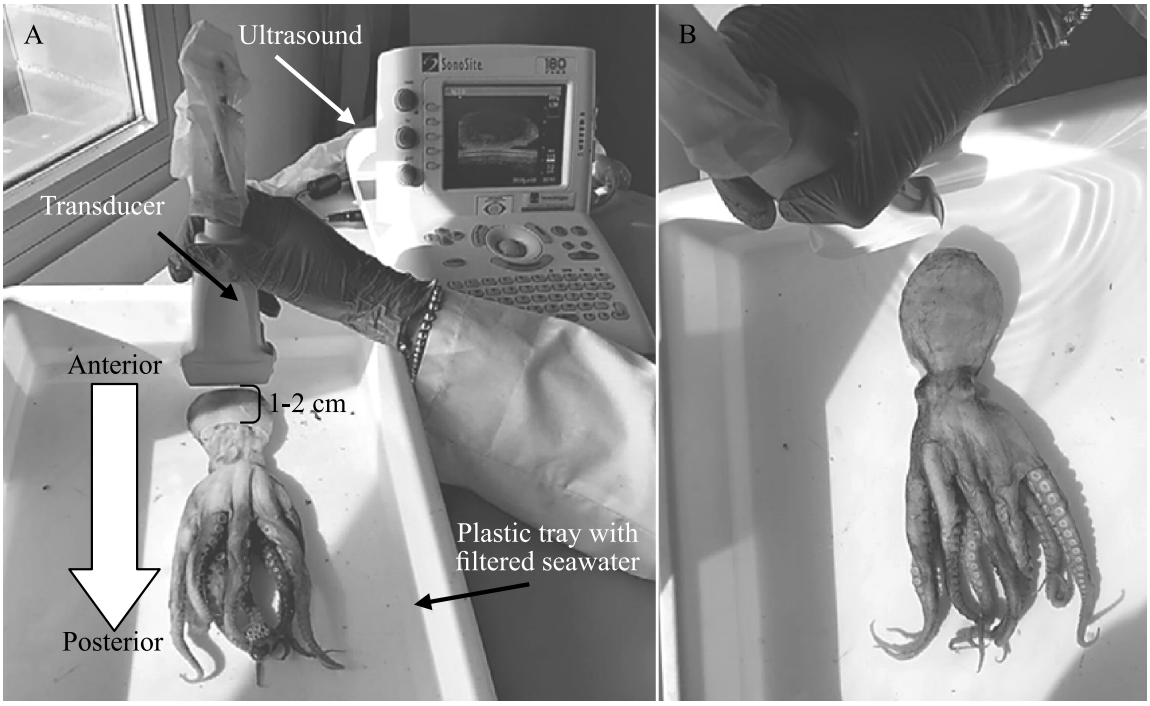

Figure 1. Equipment and technique used in *Octopus tehuelchus* for ultrasound observations. A) Ventral view. B) Dorsal view.

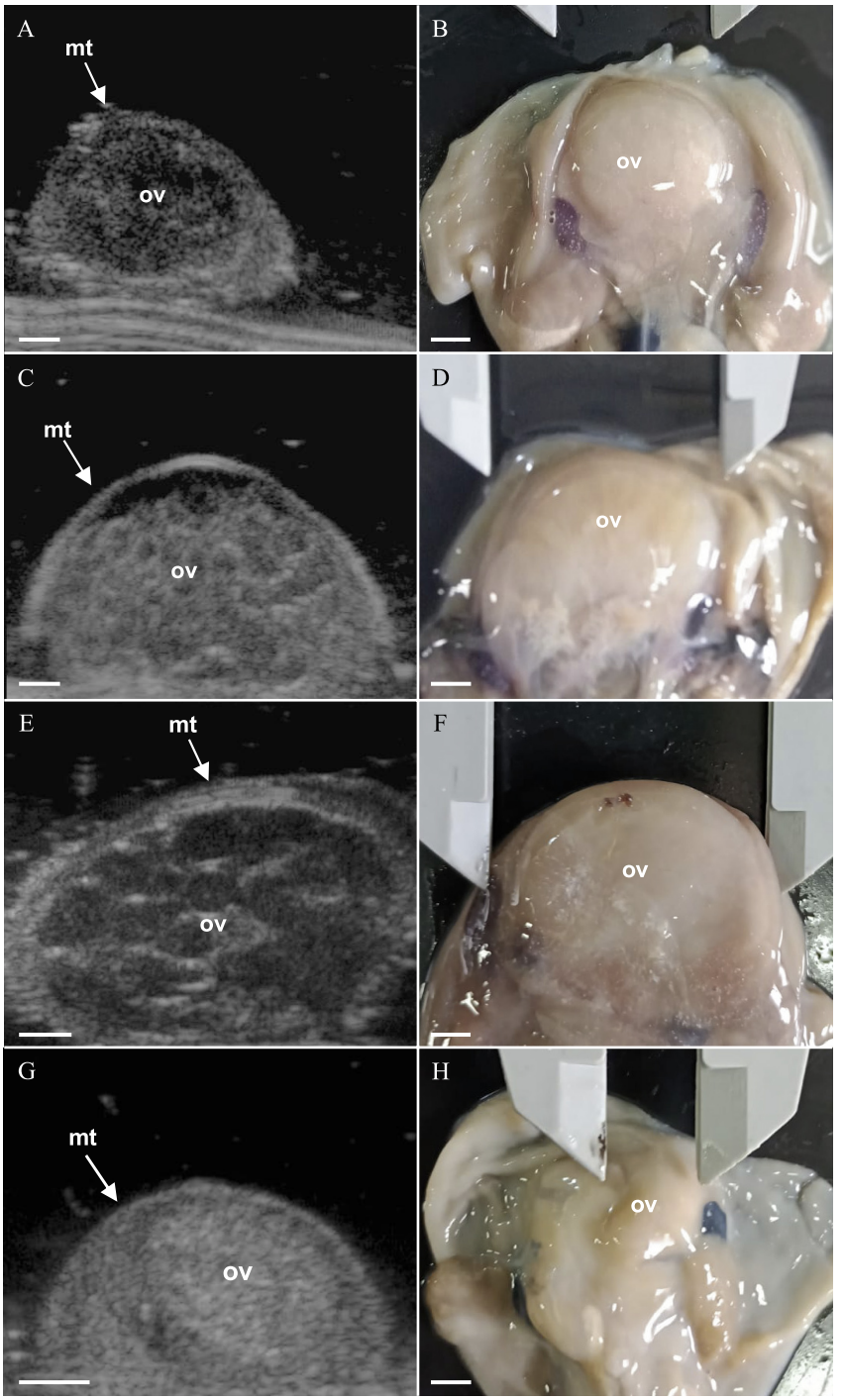

Figure 2. Detail of the ultrasound (left) and eviscerated (right) images of *Octopus tehuelchus* ovary. Early maturity stage M1 (A-B). Average maturity stage M2 (C-D). Advanced maturity stage M3 (E-F). Spawned stage M4 (G-H). OV: ovary; mt: mantle. Scale bar: 5 mm. Maturity stages from Pujals (1986).

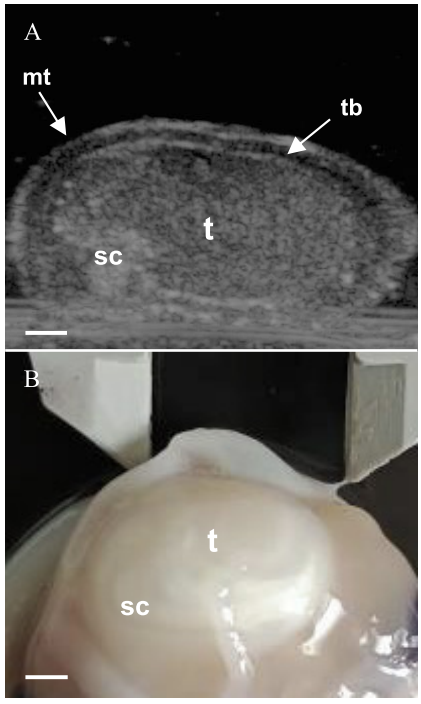

Figure 3. Detail of the ultrasound (A) and eviscerated (B) images of *Octopus tehuelchus* a mature (M2 stage) testis. t = testis; sc = spermatophore complex; tb = testicular bag; mt = mantle. Scale bar: 5 mm. Maturity stage from Pujals (1986).

section (Figure 2 C-D). Advanced maturity ovary (M3), ≥ 3 cm diameter, presented anechogenic circular zones occupying the entire volume of the gonad (Figure 2 E-F). Post-spawning (M4) hyperechoic and flaccid ovary measured less than 1 cm in diameter, without anechogenic zones (Figure 2 G-H). The immature ovary (≤ 6 mm diameter) could not be clearly identified either *in vivo* or by ultrasound technique. Males: the ventral view of the mantle was the most appropriate for observing male gonads. They were made up of the testicle and the spermatophore complex. The mature testicle (M2 stage) was spherical, homogeneous, compact in appearance and 1.8-2.0 cm transverse section. The spermatophore complex extended on the left side at the base of the testicular sac as an image reflecting a hyperechogenic structure with respect to the testis (Figure 3 A-B).

Total weight and mantle length of females per reproductive stage were, respectively: early maturity ovary (M1) 46.74 ± 6.59 g TW and 3.15 ± 0.21 cm ML; medium maturity ovary (M2) 76.5 ± 18.5 g TW and 4.25 ± 0.3 cm (ML); advanced maturity ovary (M3) 135.8 ± 5.2 g TW and 5.6 ± 0.3 cm ML. Post-spawning (M4) 64.27 ± 0.8 g TW and 3.3 ± 0.4 cm ML. For males, the only stage observed was maturity (M2) with 38.6 ± 13.9 g TW and 3.2 ± 0.7 cm ML.

Ultrasound technology enabled the rapid acquisition of high-resolution images quickly, resulting in precise gonad recognition. This reinforces the usefulness of ultrasound as a powerful, non-invasive tool for examining biological structures in living organisms while preserving the integrity of the animal. Given that octopus are semelparous organisms (Boyle and Chevy 1992) with seasonal reproductive periods and little generational overlap (Boyle and Rodhouse 2005), implementing protocols allowing monitoring the maturation of broodstocks without the need to sacrifice them would allow programming egg production according to the demand. Thus, results of this work could be applied in future reproductive conditioning programs, particularly for the study and definition of parameters that modulate the timing of reproduction, in the framework of animal care and well-being required for this type of organisms. Results confirm that ultrasound is a powerful tool for assessing the reproductive status of *O. tehuelchus* using a non-invasive method, which is particularly useful in species where sexual dimorphism is not easily recognizable.

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Author contributions

Mercedes Berrueta: investigation; conceptualization; project administration; formal analysis; methodology; writing-original draft; writing-review and editing. Andrea V. López: investigation; conceptualization; supervision; writing-review and

editing. Mariela Radonic: investigation; writing-review and editing. Eddie Aristizabal: visualization; writing-review and editing. Bárbara Gorriti Goroso: writing-review and editing.

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