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ENDOCRINE AND MORPHOLOGICAL CORRELATES OF REPRODUCTION IN THE PACIFIC SHARPNOSE SHARK (*Rhizoprionodon longurio*) FROM LA PAZ BAY, B.C.S., MEXICO

TESIS

PARA OBTENER EL GRADO DE

MAESTRÍA EN CIENCIAS EN MANEJO DE LOS RECURSOS MARINOS

PRESENTA

ADRIA BOSCH SOLER

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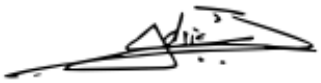

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Resumen

Rhizoprionodon longurio es un tiburón costero que se distribuye desde California (EEUU) hasta Perú. Es un tiburón que, pese a no tener una fecundidad elevada, su temprana madurez y a su corta longevidad le permiten resisitir una pesca que, durante cierta parte del año, es dirigida. No obstante, se encuentra como "*data deficient*" dentro de la IUCN. Entender la biología de las especies de tiburones capturados comercialmente es de gran importancia para su manejo sustentable, siendo la reproducción el proceso biológico más importante en la propagación exitosa de las especies. La examinación visual de las gónadas es la metodología mayormente empleada para evaluar los patrones reproductivos (estadio gonadal), sin embargo, esta metodología requiere que el organismo esté sin vida. En este trabajo se implementó un método no letal, para determinar el estado reproductivo del tiburón *R. longurio*, basándose en la medición las hormonas reproductivas 17β-estradiol (E_2) y Testosterona (T) junto con observaciones macroscópicas para determinar el estatus reproductivo. Las concentraciones plasmáticas de las hormonas se midieron mediante la técnica RIA (radioinmunoensayo). Adicionalmente, se calculó para ambos sexos, el IGS (Índice Gonadosomático) y el IHS en hembras (Índice Hepatosomático) y se determinó la $L_{50\%}$. Se recolectaron un total de 38 muestras de sangre y 113 muestras de gónadas recolectadas de noviembre de 2018 a julio de 2020 , con una proporción de sexos 1.27M:1H (p = 0.18), y una representación de tallas de 46 a 123cm longitud total (LT). En hembras, los niveles de T y E_2 se correlacionaron con el diámetro folicular máximo (DFM) (r = 0.64, p < 0.01 y r = 0.54, p = 0.03, respectivamente). En machos, tanto el ancho testicular (tw) como el largo de los gonopterigios estuvieron significativamente correlacionados con los niveles de T (r = 0.61, p < 0.001 y r = 0.52, p = 0.03), pero solamente el tw presentó una correlación significativa con E_2 (r = 0.79, p < 0.001). En hembras el IGS alcanzó su máximo en julio y en machos en mayo, con 0.7% y 1.7% respectivamente, llegando a su mínimo en agosto con valores de 0.2% y 0.05%. De igual forma, el folículo de mayor diámetro se observó en julio (20.5 mm Ø), seguido de un declive para el mes de agosto (aprox. 5mm Ø) en hembras con foliculos postovulatorios. El IHS no presentó diferencias significativas entre meses, aunque sí estuvo cerca de presentarlas entre las hembras maduras preñadas y no preñadas (ANOVA, F = 3.63, p = 0.07). La $L_{50\%}$ hembras fue de 90 cm LT y para machos fue de 83 cm, encontrándose una relación lineal (R^2 = 0.87; p < $2.2e^{-11}$) entre la LT y el gonopterigio. Las observaciones hormonales y macroscópicas permitieron conocer que la temporada de alumbramiento ocurre entre mayo y junio y el apareamiento en julio. Se puede afirmar que, para esta especie, la veda protege tanto el apareamiento como la expulsión de neonatos. Por otro lado, se detectó una correlación significativamente negativa entre la temperatura superficial del mar (TSM) y los niveles de E_2, T (r = -0.80, p < 0.001; r = -0.62; p = 0.01). En machos,

no se encontró ninguna correlación con los niveles hormonales, pero sí con las medidas morfométricas, encontrando una correlación significativamente negativa entre tw e IGS con la TSM (r = -0.63, $p < 0.001$; r = -0.69; $p < 0.001$). Consecuentemente, se puede concluir que el uso de las hormonas esteroides reproductivas es un buen método para conocer el ciclo reproductivo y la talla de madurez sexual para *Rhizoprionodon longurio*, teniendo en cuenta que, la temperatura es un factor ambiental que puede desenacadenar los procesos reproductivos.

Palabras clave: hormonas reproductivas, madurez sexual, ciclo reproductivo, temperatura.

Abstract

Rhizoprionodon longurio is a coastal shark distributed along the Pacific coast from California (EEUU) to Perú. Is suggested that this species can handle directed fisheries although having low fecundity, because it is a short-lived organism that reaches sexual maturity rapidly. However, it is cataloged as “data deficient” within the IUCN. Understanding the biology of commercially-caught shark species is important for their sustainable management, being reproduction a crucial biological process for successful propagation of any species. Visual examination of the gonads is the most widely methodology used for reproductive assessment (gonadal stages), however, this methodology requires the organism to be killed. In this work, a non-lethal method based on the reproductive hormones 17β-estradiol (E_2) and Testosterone (T) together with morphological observations was used to determine the reproductive status of the *R. longurio*. Plasma hormone concentrations were measured by RIA (radioimmunoassay) technique. Additionally, GSI (Gonadosomatic Index), HSI (Hepatosomatic Index) and the size at 50% maturity was determined. A total of 38 blood samples and 113 gonad samples were collected from November 2018 to July 2020, with a sex ratio of 1.27M: 1H ($p = 0.18$), with sizes going from 46 to 123cm total length (TL). In females, T and E_2 levels were correlated with maximum follicle diameter (MFD) (r = 0.64, $p < 0.01$ and r = 0.54, $p = 0.03$, respectively). In males, both testicular width (tw) and clasper length were significantly correlated with T levels (r = 0.61, $p < 0.001$ and r = 0.52, $p = 0.03$), but only tw presented a significant correlation with E_2 (r = 0.79, $p < 0.001$). Females GSI reached its maximum in July and males in May with 0.43% and 1.7% respectively, reaching both their minimum in August with values of 0.05% and 0.2%. Similarly, the largest follicles were observed in July (20.5mm Ø), with a decline observed in August (approx. 5mm Ø) in females with postovulatory follicles in ovary. The HSI did not show significant differences between months (ANOVA, $p = 0.13$) and neither had statistical

differences when comparing mature non-pregnant females and pregnant females (ANOVA, F = 3.63, p = 0.07). Female's $L_{50\%}$ was 90 cm TL and for males was 83 cm, finding a linear relationship (R^2 = 0.87; p = $2.2e^{-11}$) between TL and clasper length. Hormonal and macroscopic observations allowed us to know that birth season occurs between May and June and mating season occurs around July. With this, we can confirm that, for this species, ban period is protecting both mating and parturition period. Additionally, a significantly negative correlation was detected between Sea Surface Temperature (SST) and E_2 and T levels (r = -0.80, p < 0.001; r = -0.62; p = 0.01). In males, no correlation was found with hormonal levels, but with morphometric measures, finding a significantly negative correlation between tw and GSI with SST (r = -0.63, p < 0.001; r = -0.69; p < 0.001). Consequently, we can conclude that the use of reproductive steroid hormones is a good method to know the reproductive cycle and the size of 50% maturity for *R. longurio*, with temperature as an environmental factor that triggers the reproductive processes.

Key words: Reproductive hormones, reproductive cycle, size at maturity, temperature.

1. Introduction

The elasmobranchs (sharks, rays and skates) are one of the most vulnerable of aquatic species, with increasing numbers being listed as endangered or threatened over the last two decades (Dulvy et al., 2014). The group is characterized by a combination of biological traits (long lived, slow growth and producing few offspring) that might result in a low potential capacity to recover from increasing anthropogenic stressors, being harvested as bycatch (discarded after capture) in the world's fisheries which target teleost species the main cause of population declines (Galván-Magaña et al., 2019; Stevens et al., 2000). In the last 50 years, worldwide artisanal fisheries (which represent the highest percentage of the total worldwide catches) began to expand due to the rapid increase of fishing efforts, expansion of fishing areas and the diversification and intensification of fishing gears (Selgrath et al., 2018). It is estimated that 90% of fishermen are part of the small-scale fisheries, contributing of 70% of total world catch which is used primary for local consumption (Kolding et al., 2014; Mills et al., 2011). In recognition of the vulnerability of elasmobranch species, an International Plan of Action for the Conservation and Management of Sharks (IPOA-Sharks) was adopted by the 23rd session of the United Food and Agriculture Organization's in 1999 (FAO, 2017).

Similar to artisanal fishing trends worldwide, elasmobranch landings in Mexico are increasing year after year due to increasing in fishing effort, areas and technologies (Arreguin-Sánchez *et al*., 2004; CONAPESCA, 2017). Historical elasmobranch landings data in Mexico are grouped in three categories ("*cazón*", "*tiburón*" and "*raya*") without distinction by species (Ramírez-Amaro & Galván-Magaña, 2019; Ramirez-Amaro et al., 2013). Consequently, 33% of elasmobranchs that inhabiting Gulf of California waters are cataloged in UICN (International Union for Conservation of Nature) Red List as "data deficient" (Galván-Magaña et al., 2019). Currently, the *Norma Oficial Mexicana* NOM.029-PESC-2006 regulates the sustainable use of sharks and rays, as well as contributing to the conservation and protection of elasmobranch and other species that are caught incidentally. Moreover, since 2012 elasmobranch fisheries are closed every year from 1st May to 31st of July along the Pacific coast of Mexico, mainly to protect pregnant females approaching

to the coast during spring-summer (northern hemisphere) (DOF, Diario Oficial de la Federacion, 2012).

Along the Mexican Pacific coast, the Pacific sharpnose shark, *Rhizoprionodon longurio*, (*tiburón bironche* or *hormita* in Spanish) is one of the 14 species of shark reported to be inhabiting the area (Galván-Magaña et al., 2019). The species distributes from California (USA) to south Perú (Compagno et al., 2005) (Figure 1).

Figure 1. Rhizoprionodon longurio female (123 cm TL) captured in La Paz Bay, México. Birds-eye view (A) and lateral view (B).

The species belong to the order Carcharhiniforms, the Carcharhinidae family and the *Rhizoprionodon* genus. The genus comprises seven species distributed around the world (Compagno et al., 2005). It is a small shark that don't usually exceeds 120 cm inside the GC waters (Márquez-Farias et al., 2005). Conversely to other shark species, *Rhizoprionodon* species are short lived organisms, with fast growth and reach the maturity relatively early (Lessa et al., 2009; Simpfendorfer, 1999), and for it, their populations are continuously renewing. Differences were observed between previous works as one suggests *R. longurio* reaches sexual maturity at 80 and 82 cm TL (Mejía-Salazar, 2007) while the other suggests 92.4 and 100.5 cm TL with

age at maturity of 1.5 and 2.4 years for females and males respectively (Corro-Espinosa, 2011; Corro-Espinosa et al., 2011; Márquez-Farias et al., 2005).

The species have paired reproductive organs excepting the ovary, which only the left one is present and like others species within the genus, the reproductive strategy of the *R. longurio* is matrotrophic, placentatrophy, where the early embryonic nutritional demands are supported by the yolk sac during approximately eight weeks (Castro & Wourms, 1993), after which, a placenta-like connection between the mother and the embryo is fully developed (Hamlett et al., 2005). The species developed an annual reproductive cycle with the ovarian cycle occurring in parallel with the gestation cycle with parturition occurring later spring and summer (Motta et al., 2007; Parsons, 1983). Studies suggest the gestation period last between 10 to 12 month, showing a litter size of seven embryos (Corro-Espinosa et al., 2011; Márquez-Farías et al., 2005). The species shows a seasonal directed fishing from November to the closure beginning on the northern and southern Gulf of California (GC) in Sinaloa and Sonora (Corro-Espinosa et al., 2011). Furlong-Estrada et al. (2015) hypothesized that *R. longurio* could handle intense exploitation, as they productivity is high enough to resist directed fishery. This hypothesis might be correct, however, highly directed exploded species with “data deficient” status in IUCN are terms that do not match in fisheries when trying to reach proper management plans a thus, conservation of the species.

These management plans are built by gathering information and understanding several aspects of the life history traits (Dulvy et al., 2017; Dulvy & Forrest, 2010). Within these life-history traits, reproduction in one of the most important events, as is the primary requirement for successful propagation of any species and their individuals (Awruch, 2015). Thus, understanding the species reproductive strategies (e.g. mating, parturition, ovulation and gestation/incubation times) is essential to effectively delineate management and conservation policies (Baum et al., 2003). Traditionally, to obtain reproductive information on elasmobranch species, macro and microscopical examinations of the gonads after killing the animals was required. However, about 15-20 years ago, non-lethal methods to address the reproductive biology in several elasmobranch species began to be explored, such as uterine

endoscopy (Carrier et al., 2003; Murray, 2010), ultrasound examinations of the reproductive tract (Anderson et al., 2018; Sulikowski et al., 2016) and reproductive hormone analysis (Awruch, 2013; Henningsen et al., 2015; Maruska & Gelsleichter, 2011). Nowadays, it has been well proved that measuring circulating concentrations of reproductive hormones can provide accurate information on the reproductive cycle (Koob & Callard, 1999; Mull et al., 2010) and size maturity (Awruch et al., 2008; Sulikowski et al., 2005).

The role of reproductive hormones in elasmobranch has been increasingly studied in the last 20 years. In elasmobranch females higher E_2 concentrations occurs during follicle development playing a main role in regulating hepatic vitellogenin synthesis resulting in vitellogenesis and follicular growth (Callard & Koob, 1993; Gelsleichter & Evans, 2012). This steroid has also been linked in regulating protein secretion by oviducal gland controlling the passage of a fertilized egg into the uterus in viviparous species (Awruch, 2013; Callard et al., 2005). Progesterone (P_4) plays a role in regulating the ovulation process raising close to the ovulation time while inhibiting hepatic vitellogenin synthesis (Prisco et al., 2008), thus P_4 and E_2 have antagonist behavior (Heupel et al., 1999; Tricas et al., 2000). It has been reported that as E_2 is decreasing, P_4 levels increase and remain elevated during the initial pregnancy stages in viviparous species (Mull et al., 2010). Testosterone appears to be the main androgen in elasmobranch females, however, the role that T plays in regulating the reproductive processes remains poorly understood. Androgens may serve as precursor of E_2 synthesis as endogenous concentrations of seems to rise during follicular development closely follow E_2 levels (Callard et al., 2005; Koob & Callard, 1999). Circulating T concentrations have been reported to increase during the mating period in several viviparous elasmobranch females, suggesting a role in mediating copulatory behaviors (Mull et al., 2010; Tricas et al., 2000).

In elasmobranch males, testis are the main source of reproductive hormones, which are synthesized mainly in the Sertoli cells, followed by Leydig cells (Engel & Callard, 2005). Testosterone displayed higher concentrations during mid to late spermatogenesis stages in *Squalus acanthias* (Callard *et al.*, 1985; Cuevas & Callard, 1992), *Scyliorhinus canicula* (Sourdaine & Garnier, 1993), *Dasyatis sabina*

(Tricas et al., 2000) suggesting a sperm maturation role (Awruch, 2013; Gelsleichter & Evans, 2012). 17β-estradiol, despite is not always measured, has been associated with early to mid-spermatogenesis stages, although its role remains poorly understood because the lack of changes during major reproductive events (Awruch, 2015). Similarly, P_4 has not always being measured in males, but it has been reported to increase with testicular development (Manire & Rasmussen, 1997).

Few of studies have investigated the reproduction hormones patterns on *Rhizoprionodon* species. Prohaska et al. (2013) reported high T concentrations and low E_2 and P_4 titers in *Rhizoprionodon terraenovae* preovulatory females, followed by an increase in P_4 values during early pregnancy stages and E_2 values from mid to late gestation. Waltrick et al. (2014) observed a diapause period on *Rhizoprionodon taylori*, in which P_4 levels remained high suggesting that this hormone could have a non-development embryonic role. E_2 circulating levels started to increase from late diapause reaching the maximum around parturition and remained low during early diapause. In males, Hoffmayer et al. (2010) compared the reproductive cycle in *R. terraenovae* with previous work (Parsons, 1983) by using both plasma steroid concentrations and reproductive tract morphometric indices in which T and E_2 values trend coincided with GSI.

As any other vertebrates, in elasmobranch, is well proved that reproduction is regulated by environmental factors as water temperature, day length (photoperiod) or food availability (Awruch, 2015; Gelsleichter & Evans, 2012). In females, Waltrick et al. (2014) reported in *R. taylori* a positive significant correlation between SST and day length with E_2 circulating concentrations while in the zebra shark *Stegostoma fasciatum* a negative significant correlation between SST and E_2 levels has been reported (Nozu et al., 2018). However, E_2 levels and temperature were not correlated in *Hemiscyllium ocellatum* and *Urobatis hallery* females (Heupel et al., 1999; Mull et al., 2010). On the other hand, non-linear correlation between reproductive hormones and temperature was reported in *Mustelus schmitti* (Elisio et al., 2019), observing that T together with P_4 triggered ovulation. In males, E_2 and T concentrations (together with GSI) were positively correlated, observing high concentrations during colder months and low concentration during warmer months, suggesting an inverse

correlation between reproductive hormones and temperature (Hoffmayer et al., 2010).

In this context, this project investigates the reproductive biology of *R. longurio* by correlating macroscopic changes in the reproductive tract with plasma levels of gonadal steroids and SST. In this case, we will take advantage of the shark landings from a small fishing camp were artisanal fishery takes place and were not many sharks are caught per day, which final product is a local protein intake. That way, we benefit from those landings having the opportunity to compare gonadic stages with reproductive hormones by *in-situ* blood extraction on fresh looking landed sharks.

2. Objectives

Main objective

- To validate the use of reproductive hormones as a non-lethal methodology to determine the reproductive status of *R. longurio.*

Specific objectives

- Explore the correlations between morphological observations of the reproductive tract and reproductive hormone concentrations.
- Describe the *R. longurio* size at maturity and reproductive cycle by using a combination of macroscopic observations of the reproductive tract and reproductive hormone concentrations
- Evaluate temperature effects on *R. longurio* reproductive biology.

3. Materials and methods

3.1. Sampling and data collection

A total of 125 *R. longurio* individuals (55 females and 70 males) were obtained from *Bahía de La Paz* (LPB, Mexico), 113 of which were from a fishing camp called "*El Saladito*" (24º 26'N, -110º 41'W; Figure 2) and 12 were obtained from the southern BLP.

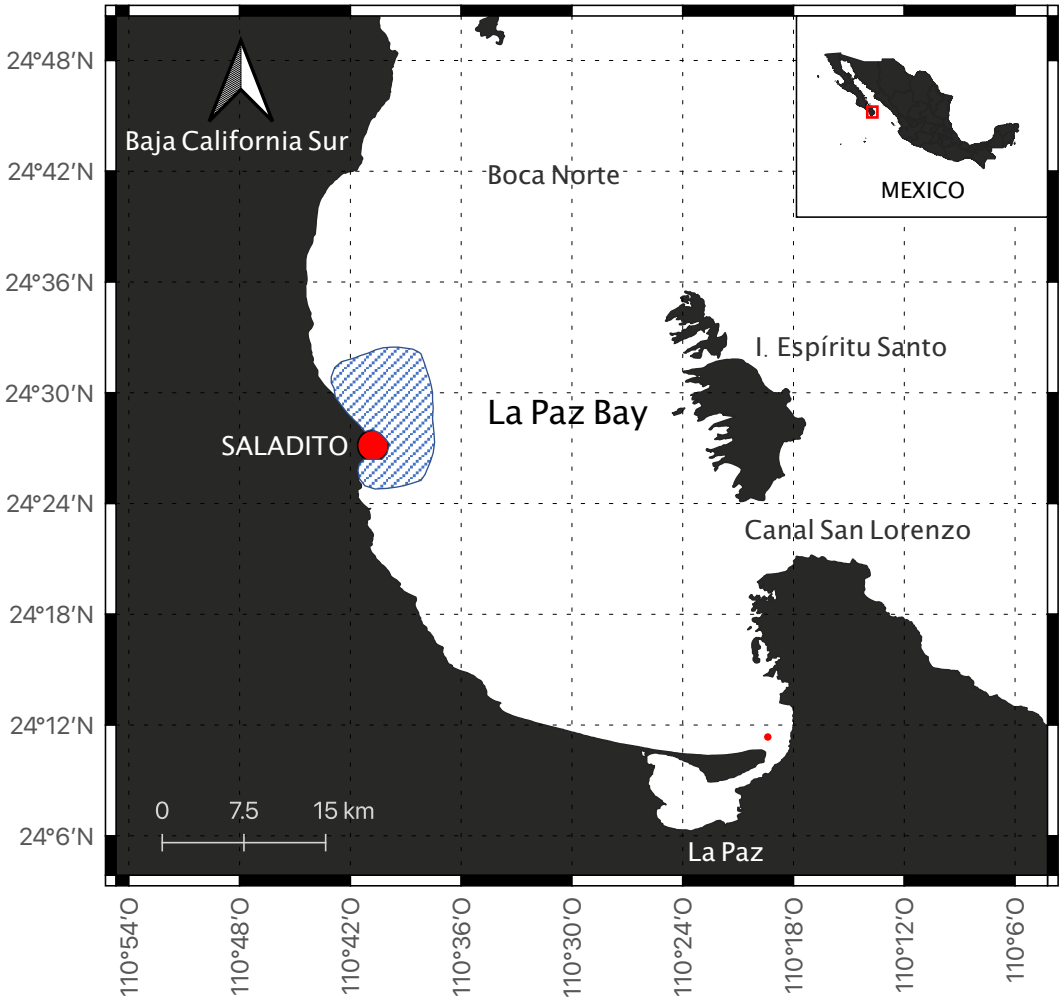

Figure 2. Sampling sites for *R. longurio* with the major fishing area represented in blue.

Sharks in "*El Saladito*" were caught by bottom long-line and gillnets between 30 to 80 m depth from November 2018 to July 2020, while sharks in southern BLP were caught using a single fishing line. Sharks were caught by artisanal fishermen and brought to port between six to 10 hours after being caught. Total length (TL, cm), precaudal length (PCL, cm), total weight (TW, g) and liver wet weight (LW, g) were

measured in both sexes. The following measurements were recorded in males: clasper total length (CL, cm), clasper calcification assessed by hand (0: non calcified, 1: slightly calcified and 2: fully calcified), riphiodon condition (open or closed), presence or absence of seminal fluid were recorded. For morphometrical analysis, the reproductive tract of both sexes was removed and fixed in formaldehyde (10%) and conserved in alcohol (70%). From 21 females and 17 males that looked very fresh after landing, blood samples (3 ml) were taken by caudal venipuncture using 22G syringes, transferred to heparinized tubes (*Sodium Heparin*^{N}; BD Vancuntainer®) and placed on ice for 2-3 hours, after which, samples were centrifuged (10.000 rpm for 5 minutes) and plasma was stored at -20ºC for reproductive hormone analysis.

3.2. Steroid hormone measurements

Plasma levels of E_2 and T for both sexes were measured by radioimmunoassay (RIA). Within the CICIMAR-IPN facilities (Mexico), plasma samples (400µl) were extracted twice with diethyl ether in a 1:5 ratio. The mixtures were vortexed for 1 min and freeze for an hour for the two phases (aqueous below and organic above) to separate perfectly. In each extraction, the aqueous phase was transferred onto 15 ml glass tubes which was placed in an immersion bath at ≤ 37 ºC, under extraction fume hood, for solvent evaporation. Reproductive hormones were measured following protocols previously reported by Ravaglia et al. (1997) at the INTECH (CONICET-UNSAM-Argentina) facilities. For the RIA, samples were resuspended with double RIA buffer of the initial plasma volume. Extraction efficiency determined by recovery of 3H-steroid added to plasma was >95%. The antisera for the two steroids were provided by Dr. Niswender (University of Colorado, USA). All samples measurements for each steroid were performed in a single assay. The lower detection limits were 0.072 ng ml^{-1} for E_2 and 0.05 ng ml^{-1} for T. The intra-assay coefficients of variance were 13% for E_2 and 4% for T.

3.3. Size at 50% maturity (L_{50})

Length at 50% maturity (L_{50}) was calculated for both sexes using the equation proposed and reformulated by Walker (2005) in order to provide parameters more biologically meaningful:

$$P = P_{MAX} \cdot \left(1 + e^{-\ln(19)\left(\frac{L-L_{50}}{L_{95}-L_{50}}\right)}\right)^{-1}$$

where P is the proportion of the population mature at a given TL, P_{MAX} is the maximum proportion of individuals in mature condition, L_{50} and L_{95} are the lengths at which 50% and 95% of the maximum proportion of individuals in mature condition. It is based on a binomial logistic method where immature and mature organisms are classified as 0 and 1, respectively. Through macroscopic visualizations and plasma steroid concentrations, females and males were considered immature or mature when presenting features described in Table 1 and Table 2.

Table 1. Maturity assessment in *R. longurio* females

		Mature	**Immature**
Females	Morphological	Yellowish follicles	Pale follicles < 5 mm
		Highly irrigated oviducal gland	Small pale oviducal gland
		Differenced irrigated uterus	Whitish uterus hard to distinguish
		Mature Pregnant	-
		Having mating scars or with distended cloaca and uterus	-
	Hormonal	T ≥ 0.25 ng ml^{-1} during follicular growth	T < 0.25 ng ml^{-1} during follicular growth
		E_2 ≥ 2.50 ng ml^{-1} during follicular growth	E_2 < 2.50 ng ml^{-1} during follicular growth

Table 2. Guidelines on maturity assessment in *R. longurio* males.

		Mature	Immature
Males	Morphologica	Fully calcified clasper	No fully calcified clasper
		Clasper rotation	Clasper with no rotation
		Riphiodon opens	Riphiodon doesn't opens
		Irrigated testes	Whitish testes with no irrigation
	Hormonal	T ≥ 2.00 ng ml^{-1} during active season	T < 1.00 ng ml^{-1} during active season
		E_2 ≥ 1.00 ng ml^{-1} during active season	E_2 < 1.00 ng ml^{-1} during active season

3.4. Indices and Measurements

Gonadosomatic index (GSI), hepatosomatic index (HSI) and epididysomatic index (ESI) were calculated by dividing ovary, testis, epididymis or liver weight by TW and multiplicated by 100. That way, it's possible to know the percentage that these organs occupy within the total body weight. The scale precision was 1 g and 0.01 g for TW and organs respectively.

GSI	HSI	ESI
$\frac{Ovary/testis\ weight\ (g)}{Body\ weight\ (g)} \cdot 100$	$\frac{Liver\ weight\ (g)}{Body\ weight\ (g)} \cdot 100$	$\frac{Epididymis\ weight\ (g)}{Body\ weight\ (g)} \cdot 100$

In females, number of vitellogenic follicles present in uterus were counted, Maximum Follicle Diameter (MFD, mm) was measured using a digital Vernier and uteri length and width (UL, mm; UW, mm) and oviducal glands width (OGW, mm) were measured, while in males, testis length (tl, mm) and width (tw, mm) were measured to the nearest millimeter.

Due to the small sample sizes in some months, those were grouped depending on the female follicular stage (Table 3).

Table 3. Classification of follicular stages for *R. longurio* females.

Stage	Ovary characteristics	Uteri content
Early	Vitellogenic follicles 5–7 mm	Mid-term embryos or empty
Mid	Vitellogenic follicles 7–15 mm	Late-term embryos or empty
Preovulatory	Vitellogenic follicles 15–20 mm	Terminal embryos or empty
Postovulatory	Previtellogenic 3 – 6 mm	Yolk sacs with no visible embryo

3.5. Statistical analysis

One-way analysis of variance (ANOVA) and subsequent Tukey's multiple comparison tests (Zar, 1999) was used to determinate if there was significant differences in plasma steroid concentrations, MFD, GSI, and HSI between months. Mature sharks showed a normal distribution size (Shapiro Wilk-test). Spearman's correlations were used to explore the relationship between sex steroid concentrations (E_2 and T) and morphological measurements (MFD, tw, clasper length, GSI, HSI). The influence of SST on E_2, T, MFD, GSI and tw was analyzed by Spearman's correlation. Sea Surface Temperature data was downloaded from NOAA–ERDDAP (https://coastwatch.pfeg.noaa.gov-/erddap/index.html).

All statistics were done with RStudio program Version 1.2.5033. The significance level was set at $p \leq 0.05$ for all analysis.

4. Results

4.1. Size composition

A total of 125 individuals, 55 females and 70 males, were captured in this study. Sizes ranged from 46 to 123 cm TL, being individuals between 90 to 115 cm TL the most represented (Figure 3). No significative difference was found in sex proportions 1.27M:1F ($\chi^2 = 1.8$, $p = 0.18$). As expected, catches were more frequent before and after the ban, as fishermen incomes highly depend on sharks catches (Figure 4). Sharks caught during ban season (May, June and July) were by-catch of the grouper *Hyporthodus acanthistius* (*baqueta* in Spanish) or sampled with special fishing permits (Nº PPF/DGOPA-024/20, SAGARPA, CONAPESCA), which allow fishing sharks and rays always with research purposes.

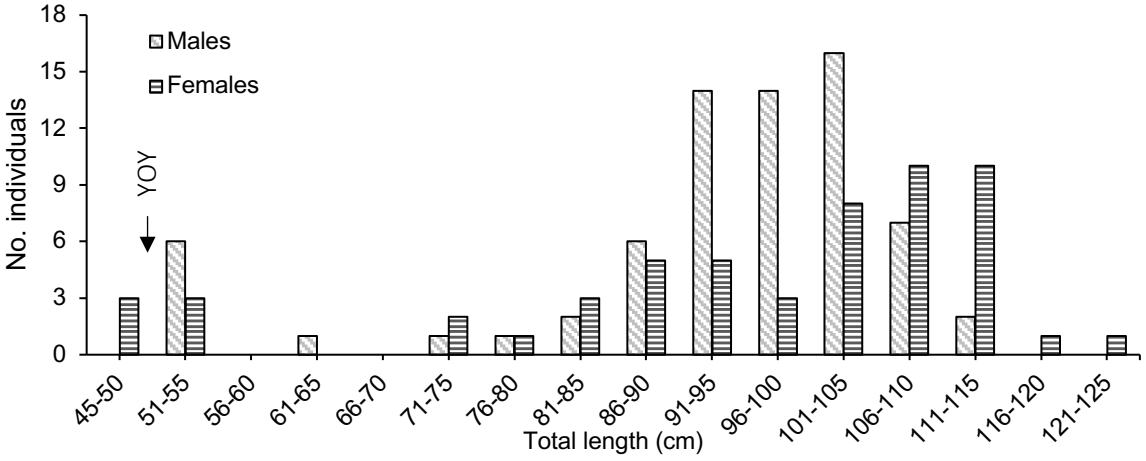

Figure 3. Rhizoprionodon longurio size distribution by sex.

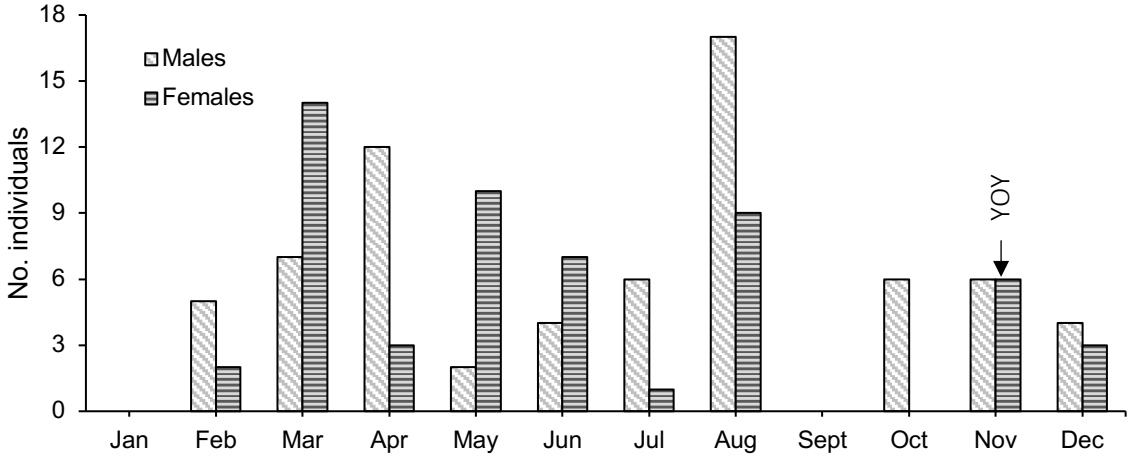

Figure 4. Rhizoprionodon longurio catches by month.

Both sexes showed similar size-weight relationships (T-Student, p = 0.49 for sizes; p = 0.18 for weights), a slight positive allometric relationship with weight increasing faster than length (exponent values of 3.19 and 3.13 for females and males respectively) (Figure 5). Embryos found in pregnant females throughout the sampling were included in the size-weight relationship but not in the T-Student test.

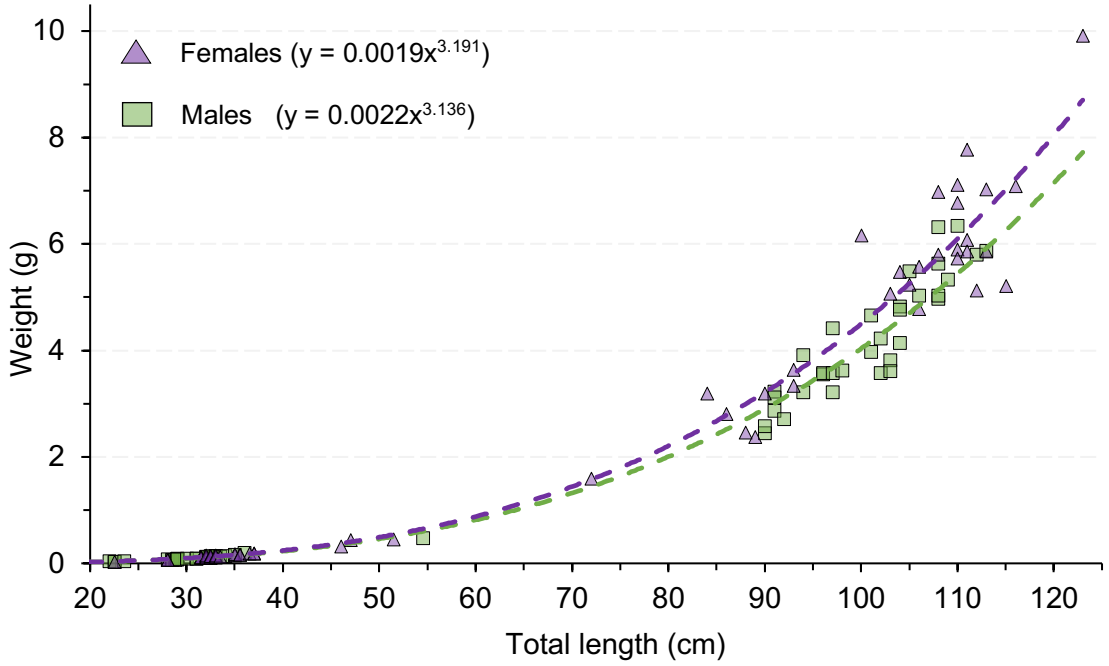

Figure 5. Size-weight relationship for 179 *R. longurio* individuals (55 females, 70 males and 27 female embryos and 27 male embryos).

Adult females were found from February through August, not being targeted likely for six months from September to January while juveniles were caught in June and December showing statistical differences between lengths (One-way ANOVA, F = 7.18, p = 1.3e^{-6}). Contrarily, males appeared throughout all year within the catches with a few juveniles during June and December (One-way ANOVA, F = 7.85, p = 5.71e^{-7}) (Figure 6).

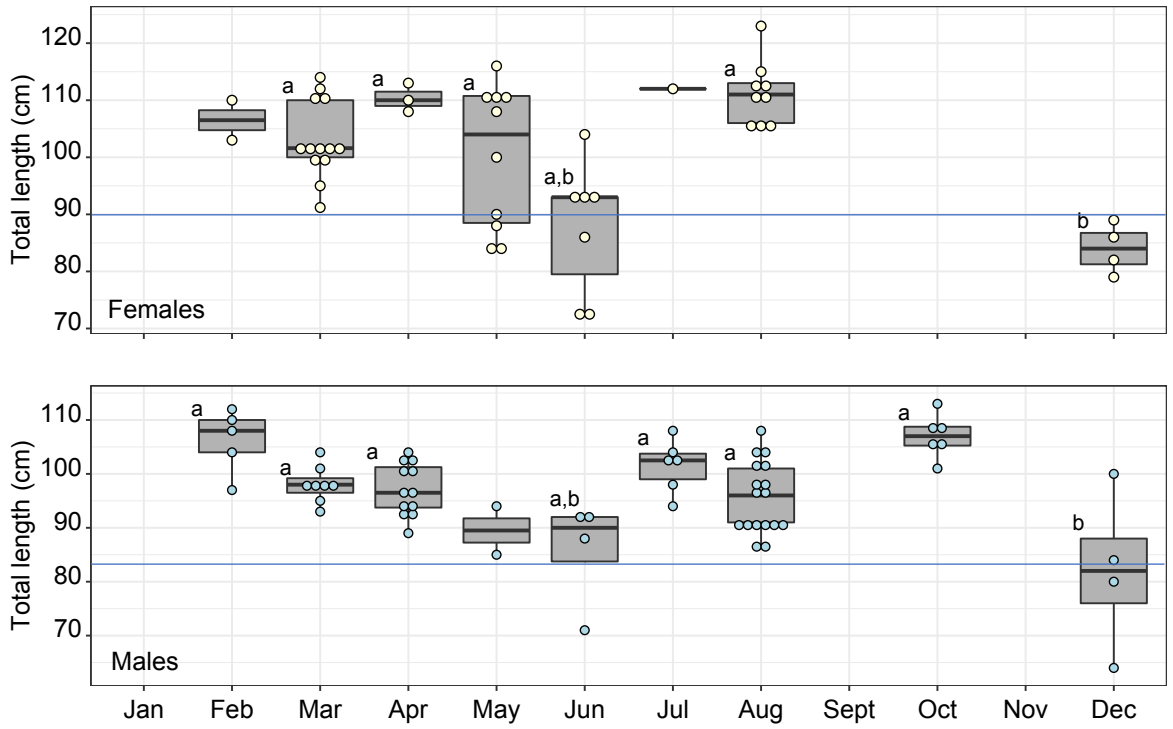

Figure 6. Total lengths observed throughout the year. Blue line showing size at 50% maturity. Letters show statistical differences between months using Tukey test in months with more than two observations.

4.2. Endocrine and morphological correlations

Females

Rhizoprionodon longurio female reproductive system is attached by connective tissue beneath the vertebral column in the middle of the body cavity. The system is composed by single external left ovary embedded within the epigonal, an ostium, a pair of oviducal glands and a pair of uteri followed by the cloaca (Figure 7).

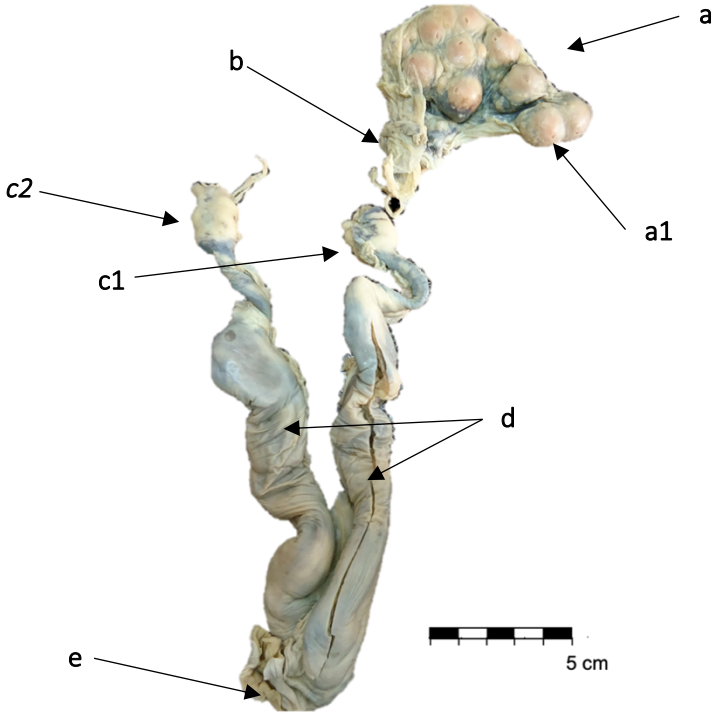

Figure 7. Rhizoprionodon longurio female reproductive tract with left ovary (a), visible vitellogenic follicles (a1), epigonal organ (b), left and right oviducal glands (c1, c2), uteri (d) and cloaca (e). Due the way fishermen remove the coelomic cavity, ostium is not present in this figure.

Adult females were defined when a clutch of four to twelve follicles of similar sizes started a vitellogenesis process by acquisition of yolk. Follicles reached ovulation size at about 20 mm diameter, after which three to ten follicles are ovulated into the coelomic cavity and the oocytes passed through the ostium to the oviducal gland (were fertilization occurs) to finally reach the uterus for embryo development. Non Vitellogenic follicles remaining in ovary undergo atresia (~5 mm diameter). After distinguishing immature and mature females, measurements were taken and represented in Table 4.

Table 4. Measurements obtained from *R. longurio* female reproductive tract.

Status	MFD (mm)	OGW (mm)	Uterus length (mm)	Uterus width (mm)
Immature	< 6	11.00 ± 2.12	84.16 ± 24.93	5.5 ± 3.92
Mature	5* – 20.5	15.40 ± 5.49	143.97 ± 54.12	38.72 ± 30.37

* Females having postovulatory follicles while eggs or yolk sacs on the way or inside the uteri.

The relationship between maturity stages and hormone levels showed marked differences between sexually immature and mature females (Figure 8). Significant differences were found in E_2 ($F = 4.85$, $p = 0.04$), but not in T ($F = 2.25$, $p = 0.15$). Maturing/mature females ≥ 80 cm TL were considered as mature because of the high concentrations of both E_2 (6.84 ± 0.48 ng ml^{-1}) and T (0.22 ± 0.04 ng ml^{-1}). Variation within mature females indicates the different stages founded during the study throughout the year.

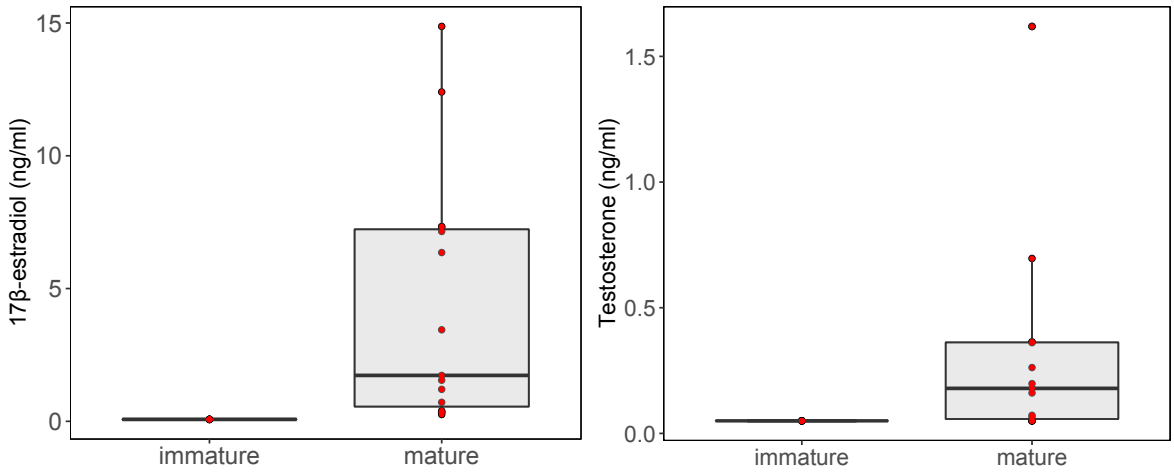

Figure 8. Circulating hormone concentrations in mature (n = 15) and immature (n = 7) *R. longurio* females.

There was a significant positive correlation between the MFD and E_2 and T, higher levels of both hormones had high MDF values (E_2 Spearman's correlation, $r = 0.67$, $p = 0.006$; T Spearman's correlation, $r = 0.54$, $p = 0.03$) (Figure 9).

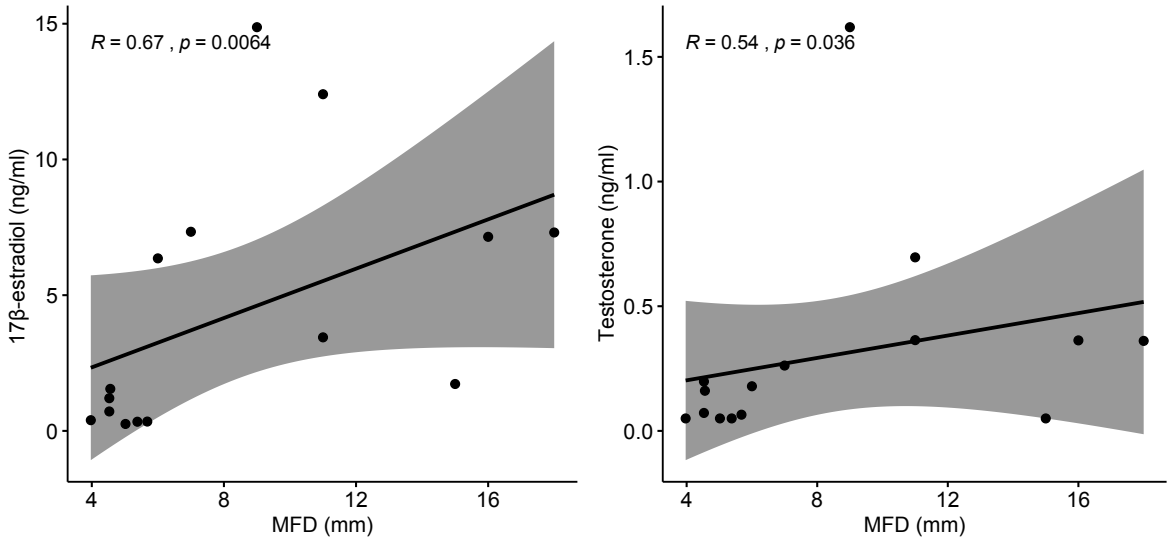

Figure 9. Correlations between MFD and reproductive hormone concentrations in *R. longurio* females (n = 15).

Sexually immature females (45 to 55 cm TL) showed the lowest reproductive hormone circulating levels (< 0.07 ng ml^{-1} for E_2 and < 0.05 ng ml^{-1} for T). An increment in E_2 (6.84 ± 0.48 ng ml^{-1}) and T (0.22 ± 0.04 ng ml^{-1}) levels started to be visible in maturing/mature females ≥ 80 cm TL. The higher E_2 (14.87 ng ml^{-1}) and T (1.62 ng ml^{-1}) values were found in fully sexually mature females ≥ 105 cm TL. The range in E_2 (0.25 – 14.87 ng ml^{-1}) and T (< 0.05 to 1.62 ng ml^{-1}) concentrations in sexually mature females are an indication that different sexual reproductive stages (reproductively active and inactive) are found in *R. longurio* females at any giving size (Figure 10).

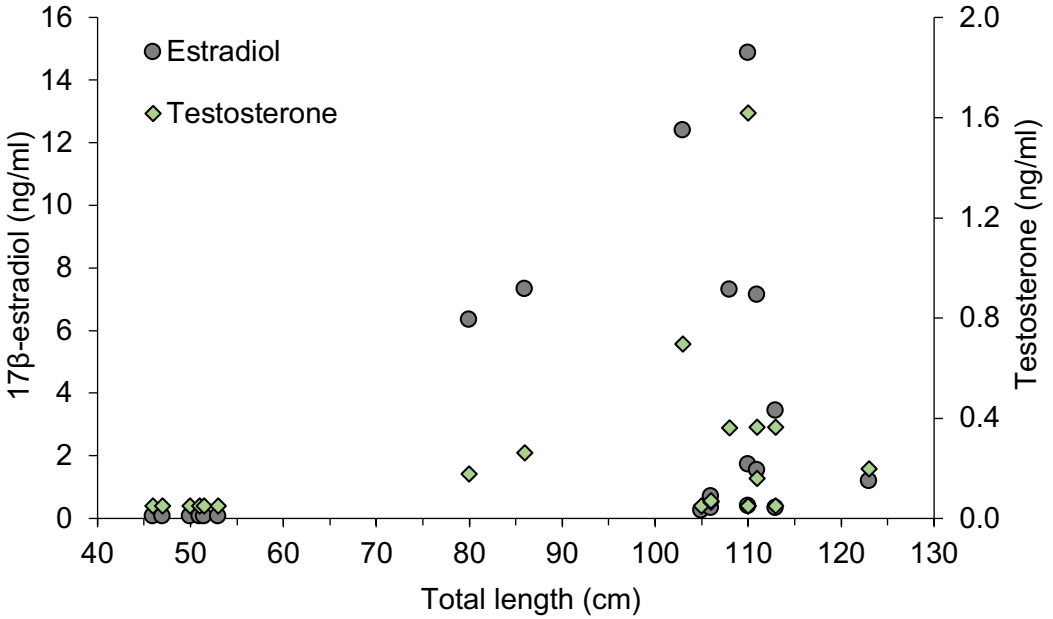

Figure 10. Relationship between total length and reproductive hormone concentrations in *R. longurio* females (n = 21).

Males

The organization of *R. longurio* male reproductive tract is composed by a pair of testes and epididymis located in the anterior part of the body attached beneath the vertebral column by connective tissue and associated to the epigonal organ. The epididymis, are connected through the ductus deferens, attached beneath both vertebral column and kidney, to the seminal vesicles (Figure 11).

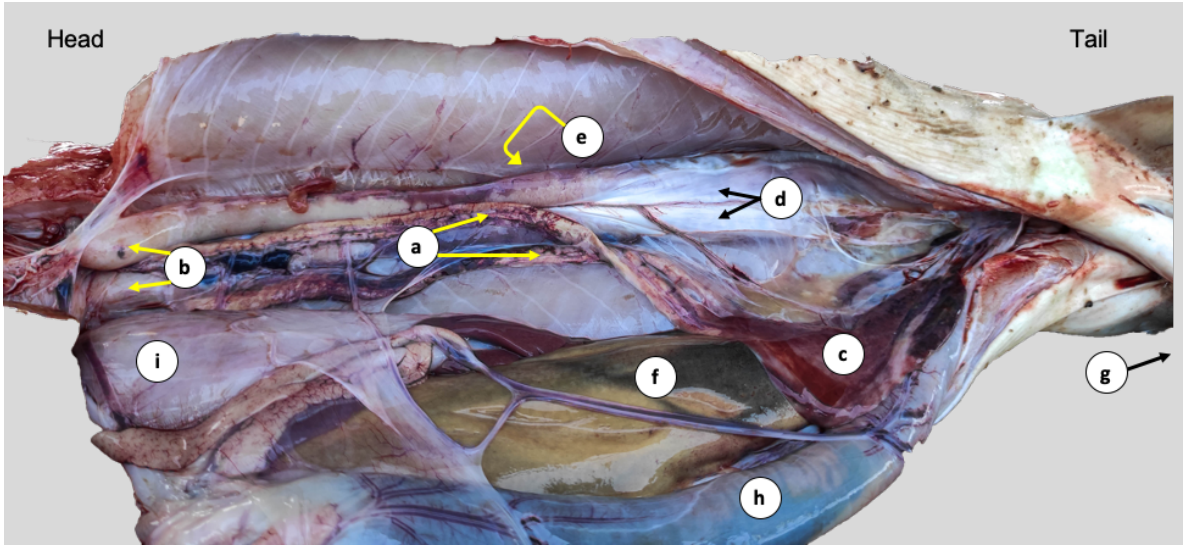

Figure 11. *Rhizoprionodon longurio* male coelomic cavity. Reproductive tract composed by paired testis (a), paired epididymis (b), epigonal organ (c), paired seminal vesicles (d) where ductus deferens and claspers (g) are not visible. Also, other organs as kidney (e), liver (f), spiral valve (h) and stomach (i).

Claspers are the copulatory organs, which varied from 2.5 cm length on immature individuals to 13 cm length on mature ones. Adults, contrary to immature individuals always had an evident testicular irrigation. Just before mating, testes suffer regression as sperm moves towards epididymis and sperm sac, increasing their volume inside of body cavity (Figure 11 and Figure 29). Once mating occurred, epigonal organ occupies the majority of the reproductive tract, with testis, epididymis and sperm sac reduced in size (Figure 29). After distinguishing immature and mature males, measurements were taken and represented in Table 5.

Table 5. Measurements obtained from *R. longurio* male reproductive tract.

Status	CL (cm)	Calcification	tl (mm)	tw (mm)
Immature	< 8	0, 1	55 to 140	4 to 11 (non-irrigated)
Mature	≥ 8	2	84 to 200	4 to 25 (irrigated)

The relationship between maturity stages and hormone levels showed visible differences between sexually immature and mature males (Figure 12). Comparing mature individuals, males showed lower in E_2 and higher T values than females.

However, no significant differences were found in both E_2 (F = 4.06, p = 0.06) and T (F = 2.68, p = 0.12) concentrations between status. But, as females, there is a marked variation within mature males circulating concentrations, showing the different stages (reproductively active and inactive) founded during the study. One maturing 80 cm TL male was considered as immature although having the highest concentration of T (5.89 ng ml^{-1}) reported in this study and high E_2 (1.24 ng ml^{-1}) concentrations, because it was below L50% size estimation (Figure 16).

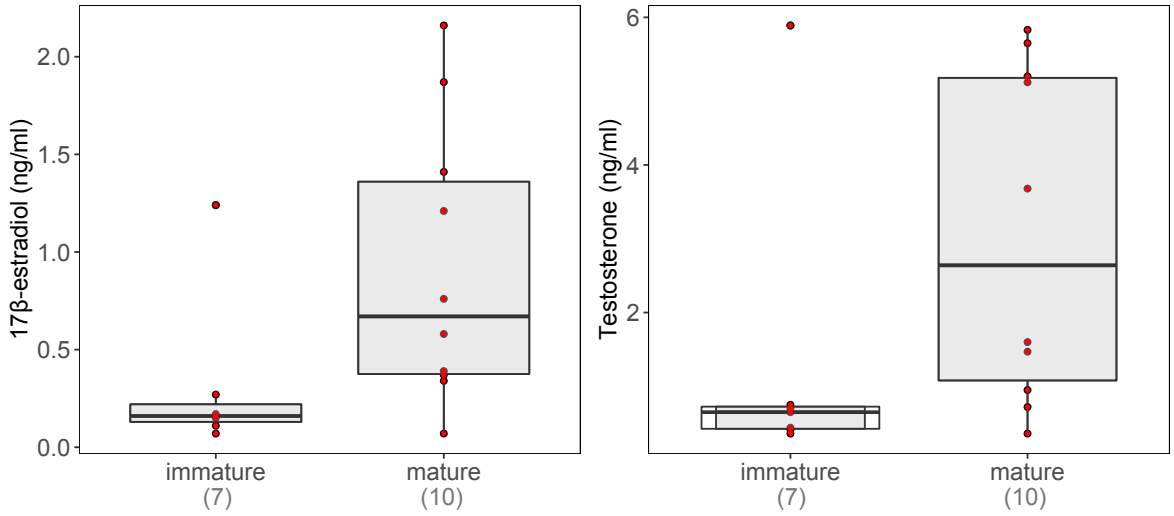

Figure 12. Circulating hormone concentrations between mature (n = 11) and immature (n = 6) *R. longurio* males.

There was no significant correlation between both T (Spearman's correlation, r = 0.21, p = 0.55) and E_2 (Spearman's correlation, r = 0.51, p = 0.11) concentrations and tw (Figure 13). Because no significant correlations were seen, plots were rebuilt with theorical missing tw values from immature individuals (50 – 60 cm TL) knowing that testis won't be wider than 4-5 mm (*). With that, testosterone (Spearman's correlation, r = 0.61; p = 0.009) and 17β-estradiol (Spearman's correlation, r = 79; p = 0.0001) were correlated with tw. Clasper length showed a significant correlation with T (Spearman's correlation, r = 0.52; p = 0.03) but not with E_2 (Spearman's correlation, r = 0.46, p = 0.06).

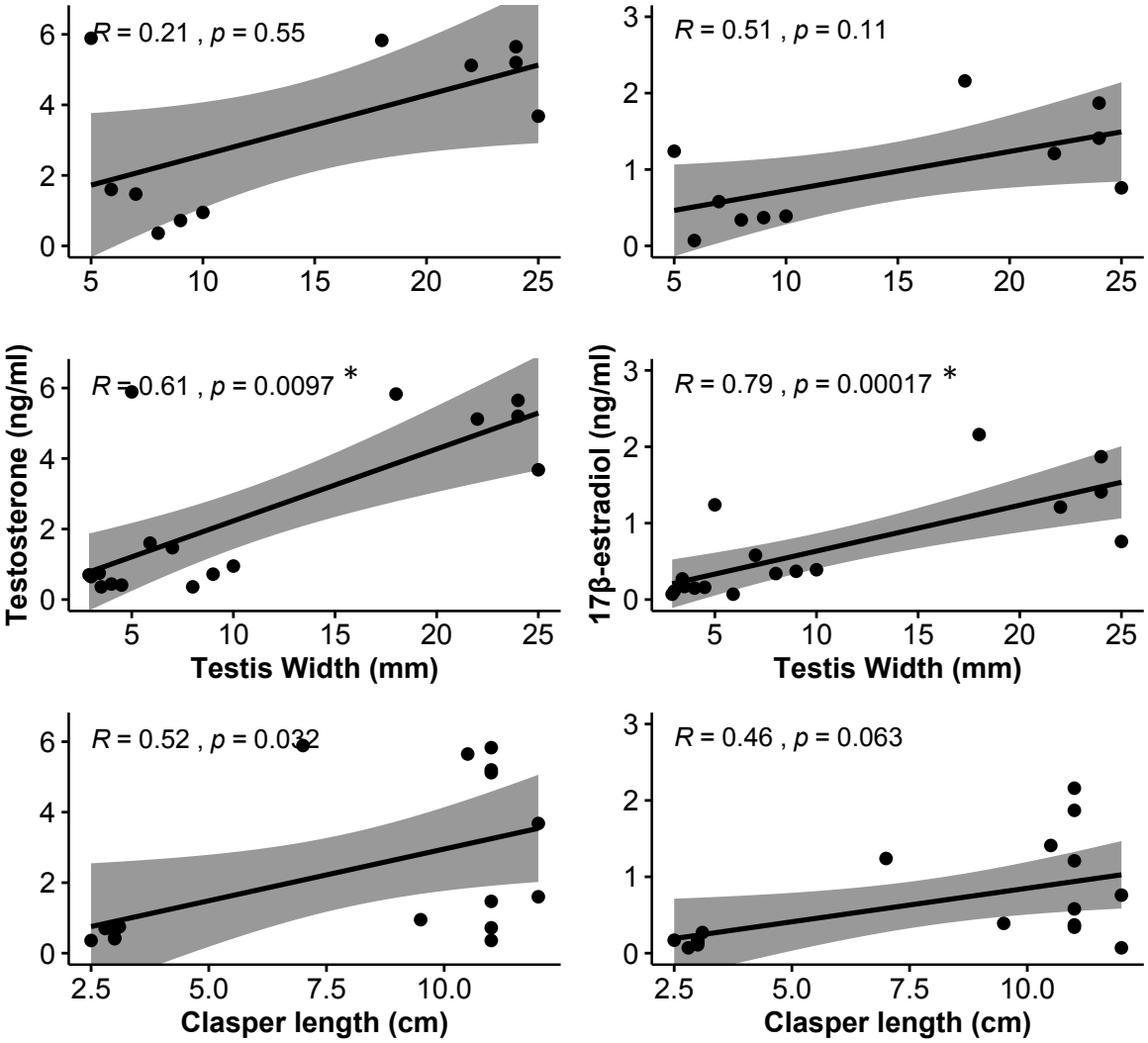

Figure 13. Correlations between reproductive hormones (T and E_2) and morphological features (tw and CL). Second row represents the first row with the extra theorical tw values from immature individuals (*).

Sexually immature males (50 to 60 cm TL) showed low T (0.36 – 0.75 ng ml^{-1}; mean 0.55 ± 0.15 ng ml^{-1}) and E_2 (< 0.07 – 0.27 ng ml^{-1}; mean 0.15 ± 0.06 ng ml^{-1}) concentrations. The higher T (5.89 ng ml^{-1}) value was observed in a maturing individual 80 cm TL with non-fully calcified claspers and morphologically immature. 2 reproductive stages are found in *R. longurio* mature males at any giving size (Figure 14).

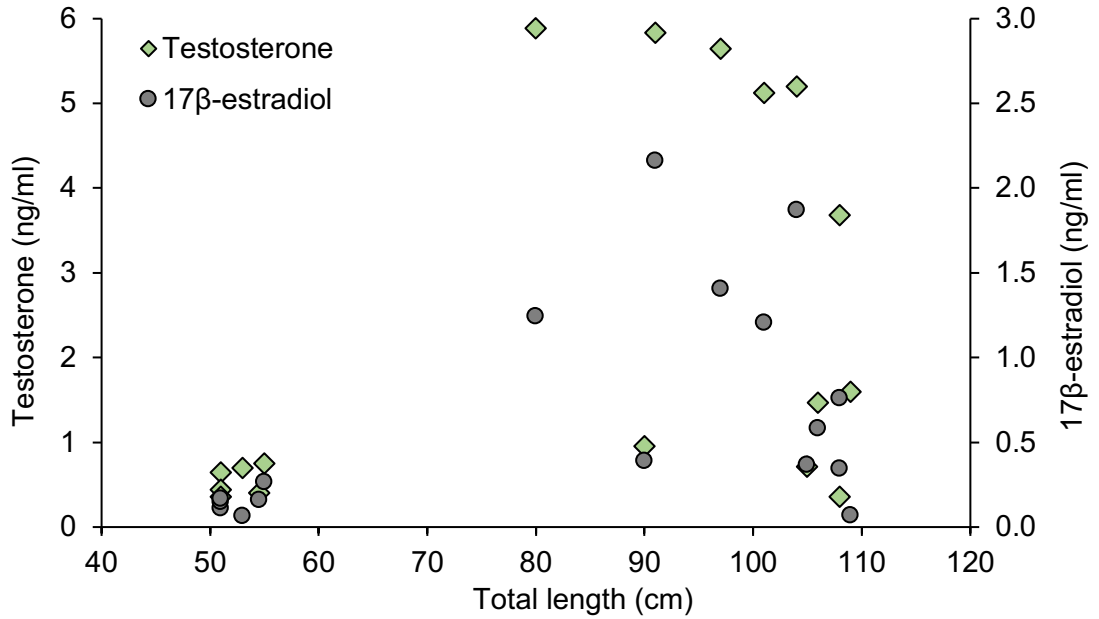

Figure 14. Testosterone and 17β-estradiol concentrations with TL in males (n = 17).

Looking at clasper length, there is an evident growth when comparing against TL and T levels, founding larger claspers in bigger individuals and higher T circulating concentrations. Males above macroscopic L_{50} length had at least 10 cm CL and higher T concentrations during the reproductively active months (Figure 15). Thus, this can be an indicator of T role in clasper development, with males being physiologically mature but not yet morphologically, and being in a transitional stage.

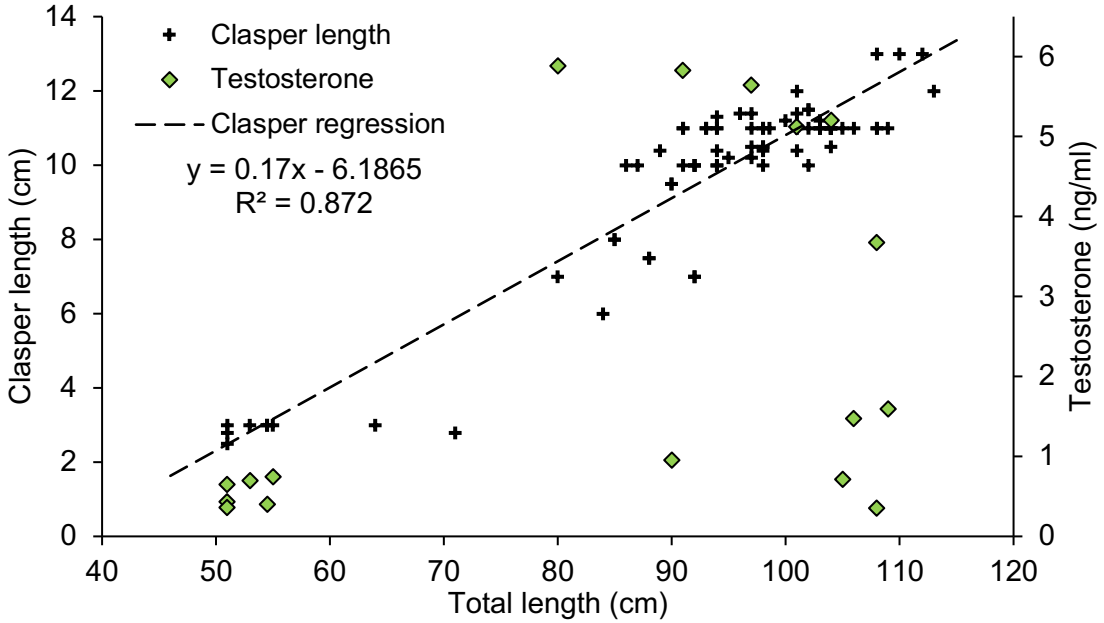

Figure 15. Relationship between CL, TL and T concentrations in *R. longurio* males.

4.3. Size at 50% maturity

Using visual examinations, females reached L_{50} at 93.7 cm TL with values for 95% confidence intervals of 89.9 to 97.6 cm TL, and males at smaller sizes, 86.3 cm TL with values for 95% confidence intervals of 81.7 to 91 cm TL (Figure 16). Using hormone plasma concentrations together with macroscopical observations, L_{50} changed to 89.9 and 83.2 cm TL for females and males, respectively. With this, hormone concentrations brought size at maturity to smaller sizes, always within 95% confidence intervals. For the rest of the work, we used the results obtained by the combination of both methods.

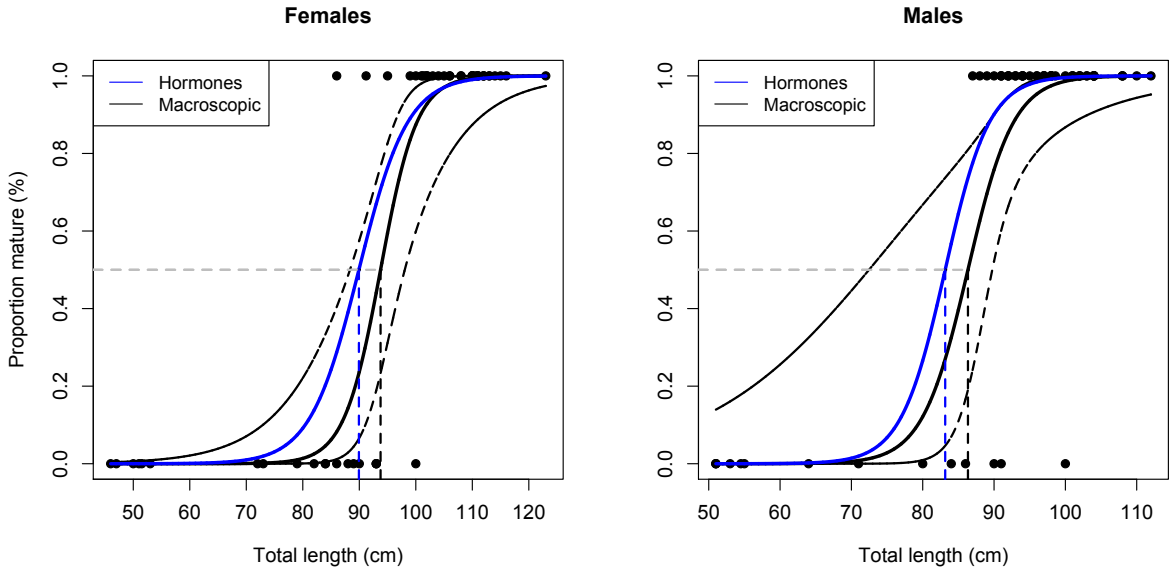

Figure 16. Size at 50% maturity for *R. longurio* calculated using morphological visualizations and hormone concentrations. Slashed curved lines representing 95% confidence interval.

4.4. Reproductive cycle

Females

It was not possible to obtain adult females throughout the whole year due to several reasons, no females were caught in January, September, October and November. However, event with low sample sizes (n = 55), by looking the reproductive hormone levels, macroscopic examinations of the reproductive tract and mating scars, it was possible to address the reproductive cycle in *R. longurio* females. The MFD seems to begin to increase in December (6.5 ± 0.7 mm) and continues growing throughout February (10 ± 1.41 mm), March (11.69 ± 1.7 mm), having a significative increase in April (15.17 ± 2.73) and May (17.33 ± 1.37 mm). The MFD from two mature females continued to increase in June and July (18.5 and 20.5 mm, respectively) before dropping significantly in August, showing atretic ovaries in new pregnant females (Figure 17 and Figure 18A).

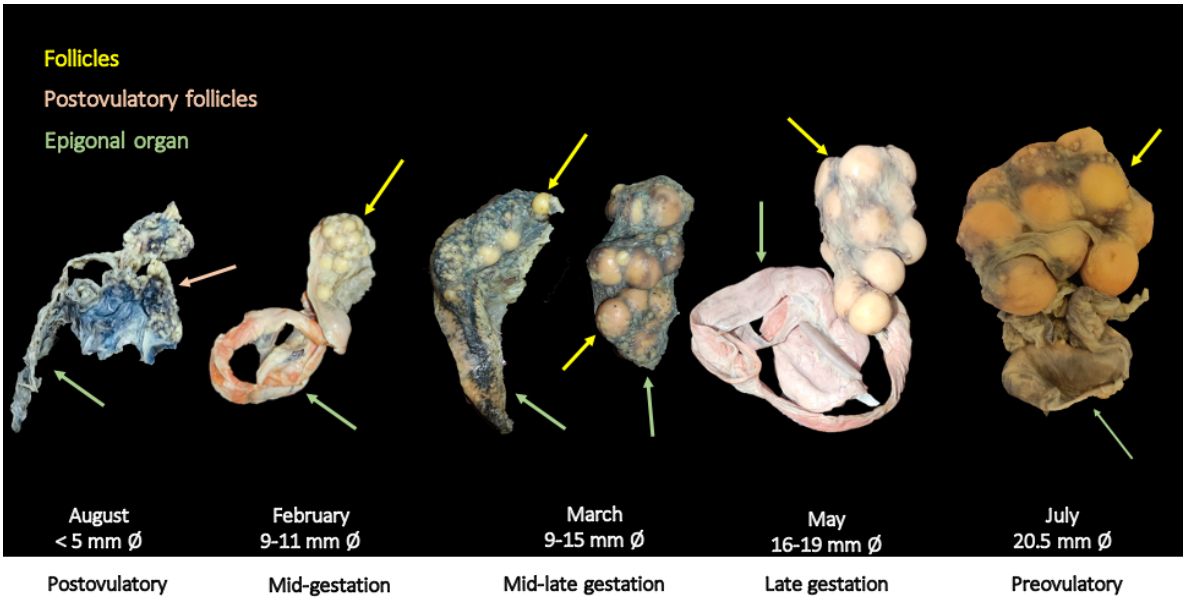

Figure 17. Follicular evolution throughout one-year cycle in *R. longurio* females.

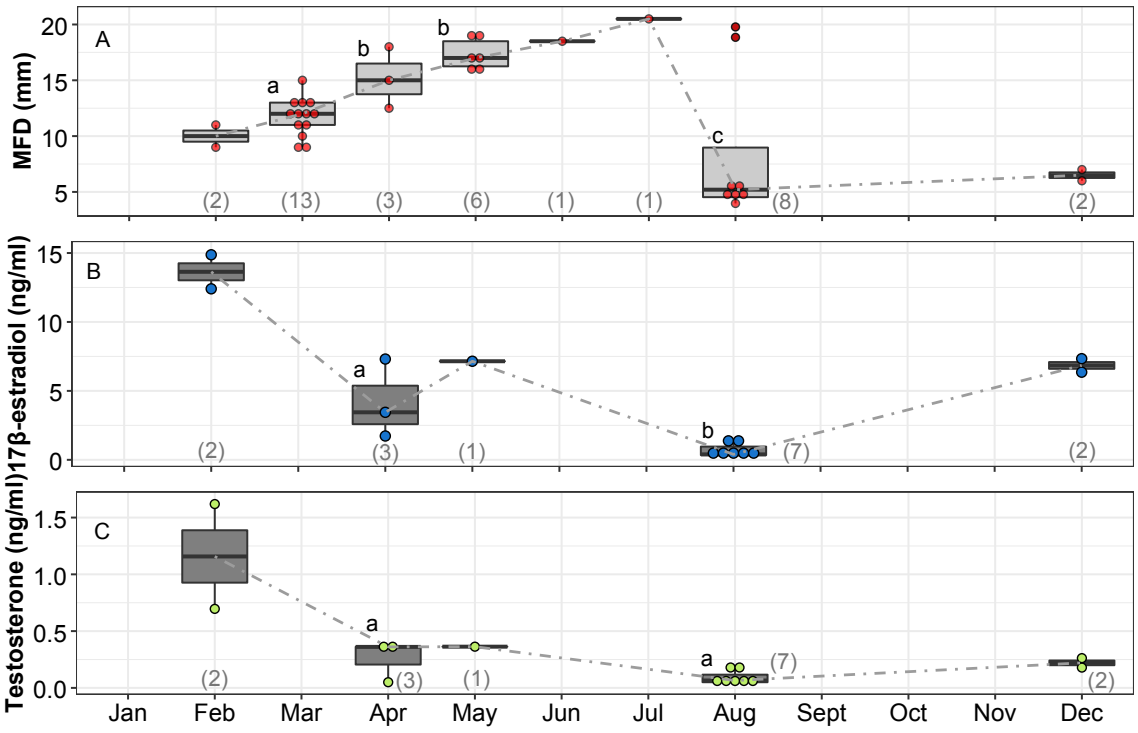

Figure 18. Monthly variation of reproductive hormone concentrations in and maximum follicle diameter (MFD) in *R. longurio* females. Mating scars are represented by "*". Letters show statistical differences between months using Tukey test in months with more than two observations.

Circulating plasma concentrations throughout the year showed a peak in E_2 in February (13.64 ± 1.23 ng ml^{-1}) decreasing (4.91 ± 2.40 ng ml^{-1}) just prior and during the first month of the ban (April-May) to reached the lowest levels in August (0.69 ± 0.47 ng ml^{-1}). Although no data is available from Sept-Nov as no females were caught, E_2 titers raised again in December raised again (6.85 ± 0.69 ng ml^{-1}) (Figure 18B). Similar trend can be seen in T (Figure 18C), with higher concentrations in February (1.16 ± 0.28 ng ml^{-1}), decreasing in April-May (0.28 ± 0.14 ng ml^{-1}) to reach the lowest levels in August after the ban (0.09 ± 0.06 ng ml^{-1}), and increasing again in December (0.22 ± 0.06 ng ml^{-1}).

Significant differences in oviducal gland width between months were seen (One-Way ANOVA, F = 2.77; p = 0.02) but, after using Tukey Post hot analysis no significant differences appeared likely due to small sample sizes in some months (Figure 19A). There is similar trend in oviducal gland size when comparing with MFD, showing a peak in June-July with a decrease in August and December. Significant differences

were observed when looking the oviducal gland size in relation to follicular stages (One-Way ANOVA, F = 5.98; p = 0.002), reaching the wider sizes in preovulatory follicles in comparison with other follicle stages (Figure 19B).

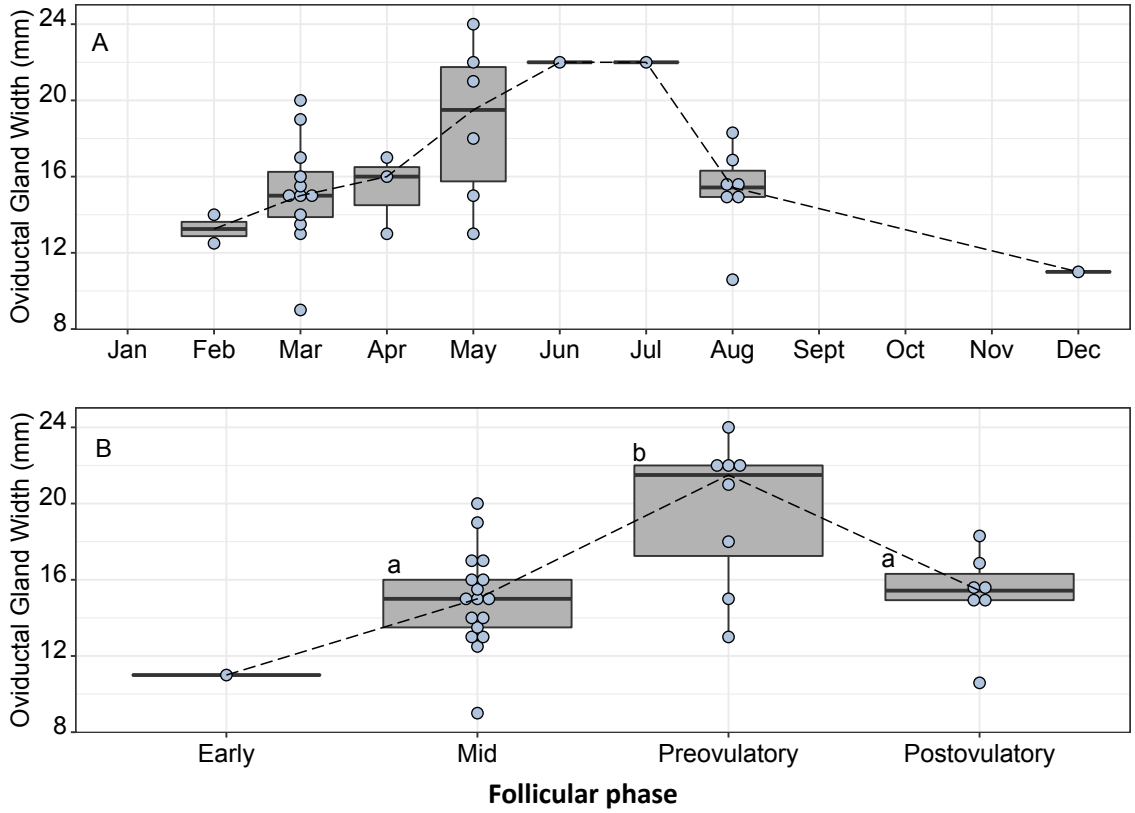

Figure 19. Monthly variations in oviducal gland width in *R. longurio* female (A) and classification by follicular stages (B). Letters show statistical differences between months using Tukey test on those months with more than two observations.

Morphological indices

The GSI showed a strong significant correlation with MFD (Spearman's correlation, r = 0.91, $p < 0.0001$) (Figure 20). The GSI had statistical differences between months throughout the year (One-Way ANOVA, F = 36.61, $p < 0.0001$) and it began to increase in December to reach a peak in July followed by a significant decline in August after the ban (Figure 21A).

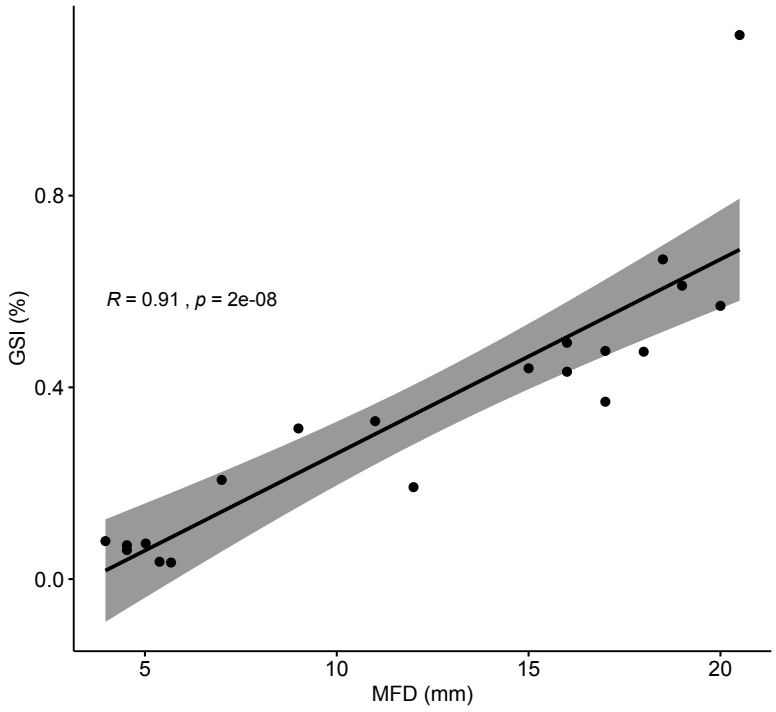

Figure 20. Spearman's correlation between maximum follicle diameter (MDF) and gonadosomatic index (GSI) in *R. longurio* mature females (n = 20).

The HSI did not show statistical differences between months (One-Way ANOVA, F = 2.13, $p = 0.11$), however, there is a trend just before and during ban season (excepting in June) were the lower HSI were found coinciding with the higher GSI values (Figure 21B).

Due to the small sample sizes, no significant differences were observed when comparing the HSI between pregnant females and mature but not-pregnant females (One-Way ANOVA, F = 3.63; $p = 0.07$) however, can be noticed that pregnant females had lower HSI in all months due to extra energy demand not in follicular growth but also in embryo nourishment during terminal gestation (Figure 22).

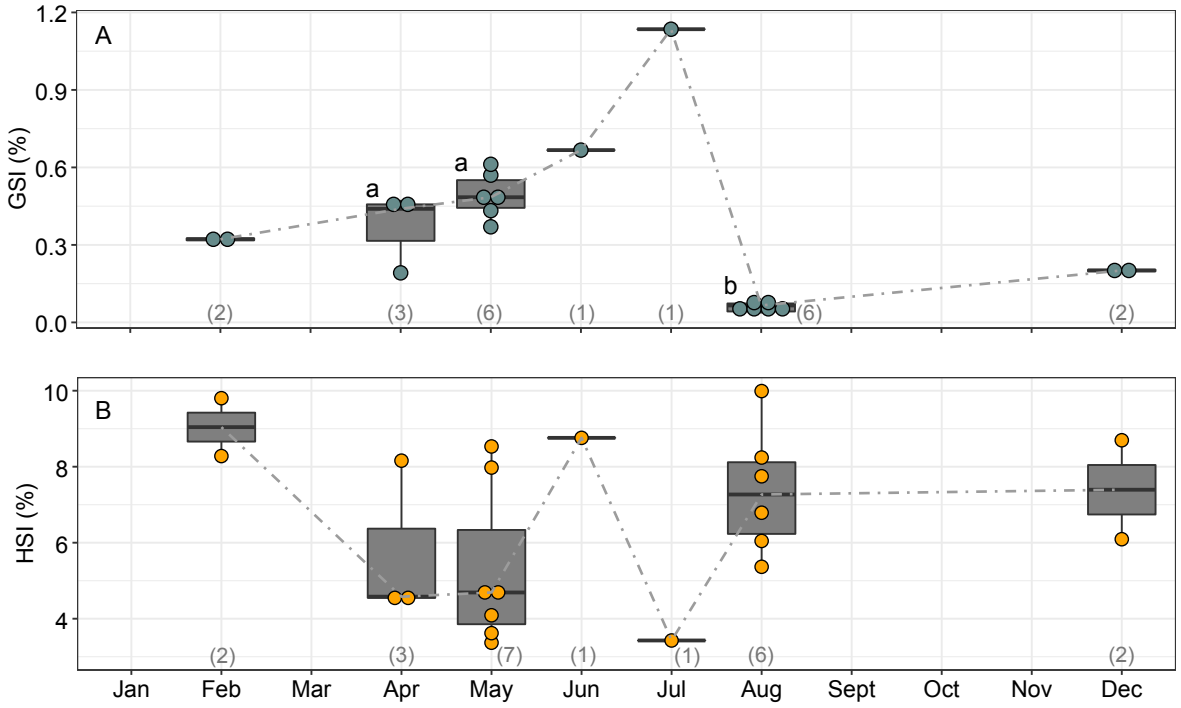

Figure 21. A) GSI variation during one-year cycle; B) HSI variation during one-year cycle. Letters show statistical similarity between months using Tukey test on those months with more than two observations.

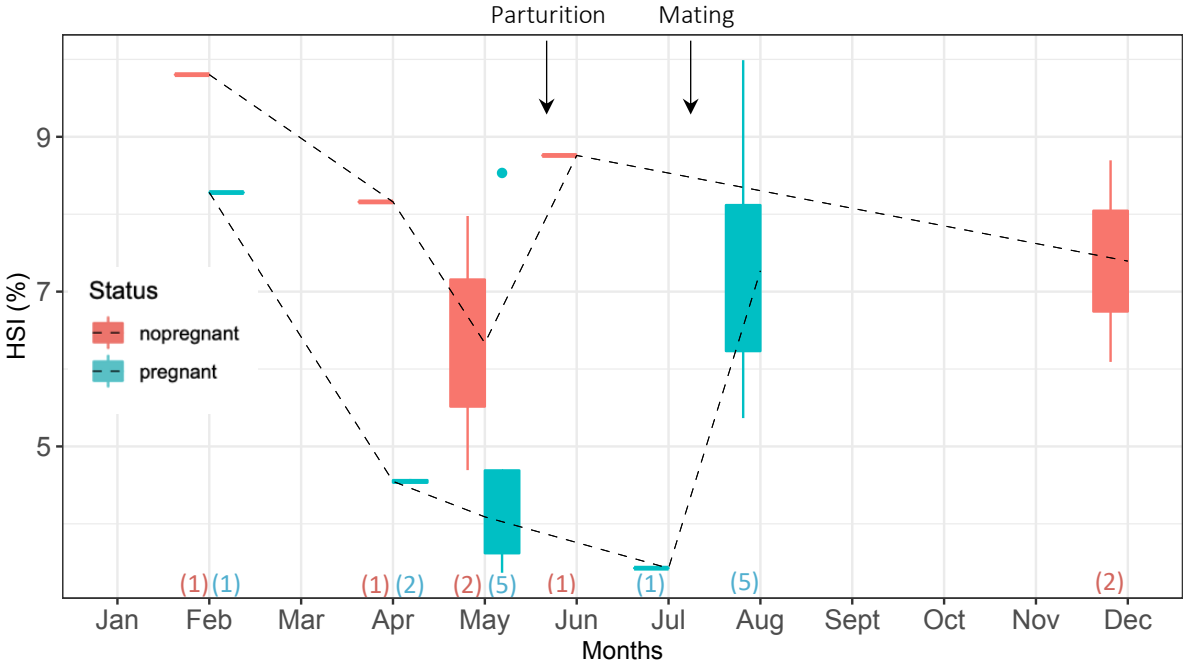

Figure 22. Monthly comparison in Hepatosomatic index between pregnant and non-pregnant *R. longurio* females.

4.4.1. Embryo development

A total of 9 females carrying 54 embryos and 6 females carrying 27 eggs with no embryo development yet were analyzed during all sampling period, with a sex proportion of 1F:1M (27 females – 27 males) and a mean of six embryos por female (Table 6).

Table 6. Number of embryos per pregnant female by gender. *Embryo sex in August Non-Determined.

Month	Females	Males	Total
August	ND	ND	ND
February	1	4	5
March	2	3	5
April	0	3	3
	0	3	3
May	4	5	9
	7	1	8
	5	5	10
	4	0	4
	4	3	7
Total	**27**	**27**	**54**
Mean	**-**	**-**	**6**

Pregnant females caught in August (n = 6) carried eggs in uterus with no visible developed embryos and the ovary containing post-ovulatory follicles. No pregnant females were caught from September through January. Throughout February to May, all pregnant females carried fully developed embryos increasing in length from February (22.6 ± 0.48 cm ETL) towards May (33.09 ± 1.83 cm ETL), suggesting May-June as parturition time (Figure 23).

During embryo development, length increases faster during early pregnancy, while weight increases during late pregnancy, tripling its values from February (37.4 ± 2.5 g) through May (136 ± 27.8 g). The biggest and heaviest embryos were found in May with 37 cm ETL and 186 g, and 36 cm ETL and 196 g, respectively (Appendix I: Embryos), while the smallest embryo was found in a pregnant female caught in April, apparently stunted with 13 cm ETL and 6.42 g.

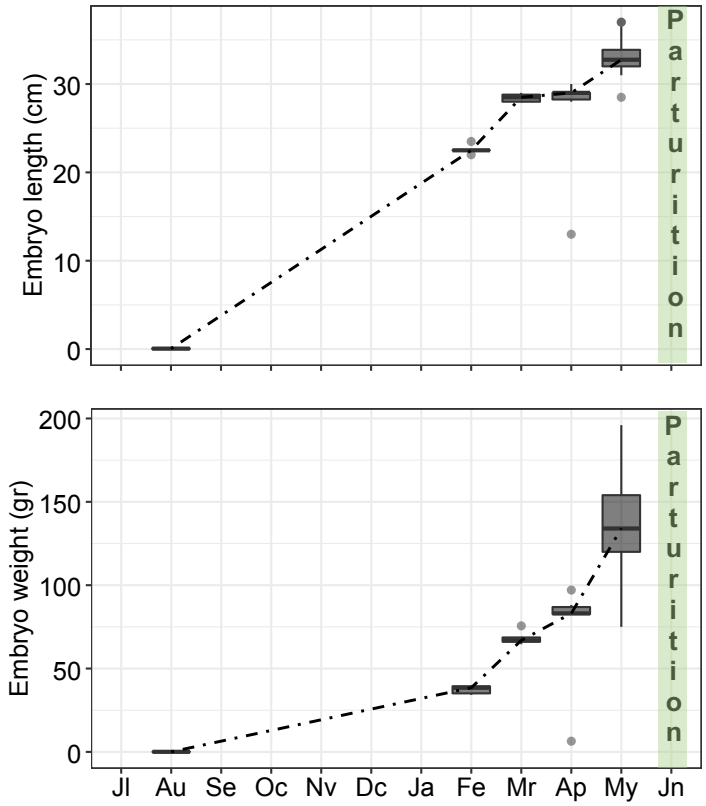

Figure 23. Monthly embryo total length (ETL) and embryo weight (EW) with estimated parturition in green.

Throughout February to May, all pregnant females caught had parallel follicular development, with MFD increasing from February (9 mm) through May (17.6 ± 1.3 mm). This strongly suggest one-year reproductive cycle. In addition, one likely post-partum female was caught in June and another was caught in July showing 18.5 mm and 20.5 mm MFD, respectively, being the one in July with follicles ready to ovule and with mating scars all along the body (Figure 24).

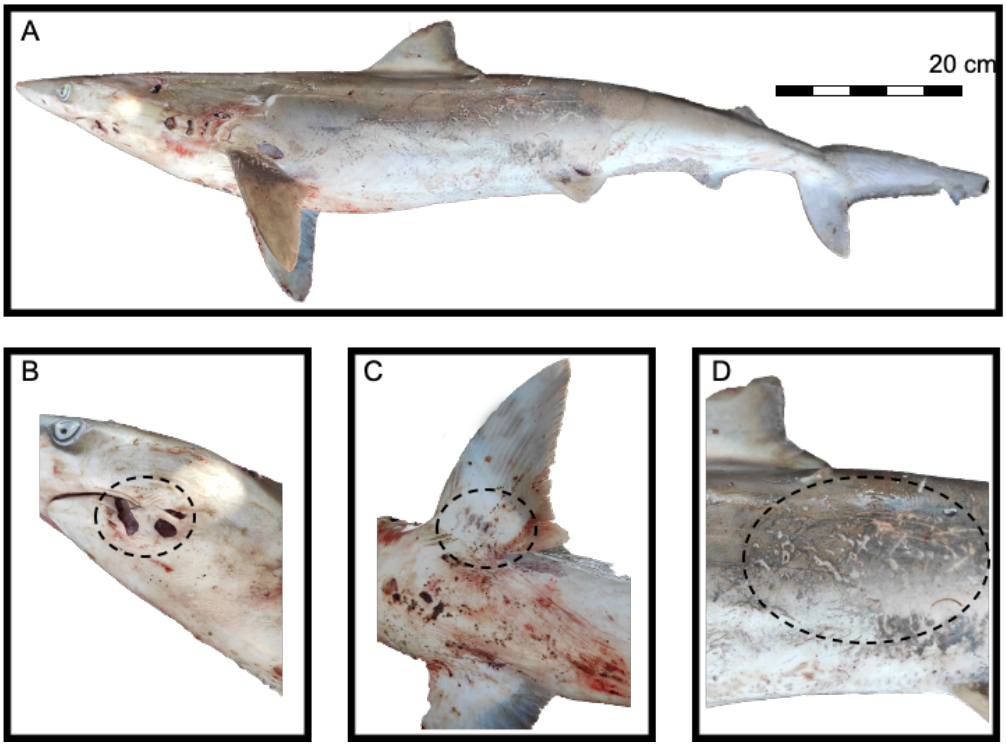

Figure 24. Mating scars in *R. longurio* females with scars all along the body: jaw (B), pectoral fins (C) and in the dorsal area of the body (D), caused during courtship.

Briefly, with all this information, it can be strongly suggested that females show an annual reproductive cycle with pregnancy and follicular cycle occurring in parallel finding both large embryos and follicles during spring (March-June). Mating scars in females through July and August with preovulatory follicles in July (20.5 mm) and post-ovulatory follicles in August with eggs in uteri, suggest ovulation occurs in between July and August. Therefore, *R. longurio* females have ten months gestation period with less than two months of resting period between one pregnancy and another (Figure 25; Figure 39). Ban period protects both parturition and mating periods.

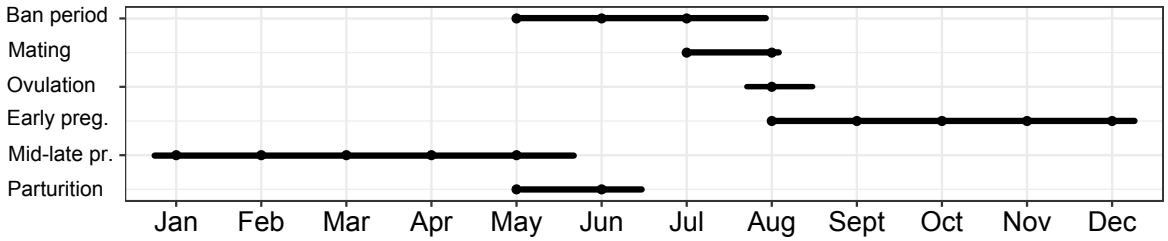

Figure 25. Rhizoprionodon longurio females reproductive cycle timeline throughout one-year.

Males

In males, testis width (tw), testis length (tl) and GSI showed significant differences between active and inactive reproductive seasons (One-Way ANOVA; F =281.4; $p < 0.001$; F = 64.56; p < 0.001; F = 168.4; $p < 0.001$) (Figure 26). The active reproductive season takes place in the first half of the year from January through May, while the inactive reproductive season takes place once the ban finished (August) through December. June and July are transition months in which testes suffer regression as sperm movers towards the seminal vesicle and its values are not represented in Figure 26. Thus, the ban does protect the mating period because when it finishes, males are already inactive. During the active season, testes were larger and wider (168.42 ± 43.15 tl mm; 22.09 ± 2.36 tw mm; n = 21) than during the non-active season (109.37 ± 24.59 tl mm; 8.19 ± 2.96 tw mm; n = 21). Gonadosomatic index had the same pattern higher values on active season (1.66 ± 0.47 %; n = 21) and lower (0.20 ± 0.18 %; n = 21) in non-active season (Figure 26). Sizes from juvenile male testes (not represented in the graph) were 85.75 ± 16.17 mm tl and 4.5 ± 2.88 mm tw but comparison can be observed in Figure 27.

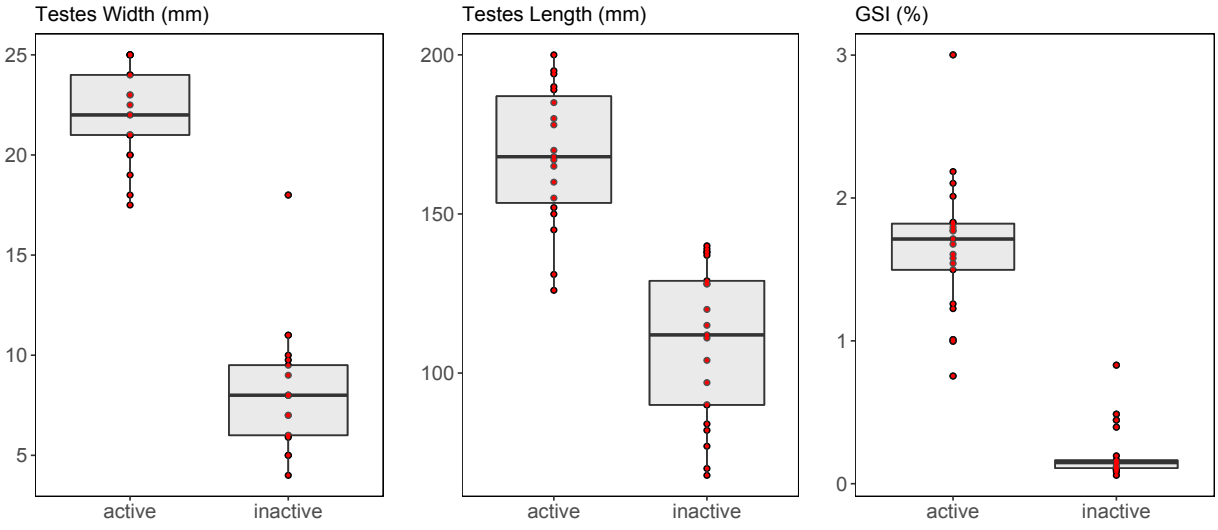

Figure 26. Testes width, testes length and GSI values throughout the active and inactive reproductive season in *R. longurio* males. Ban period separates both seasons. Active season represents February, March and April and May, while inactive season represents August, October and December, with June and July being transition months (not represented).

In mature individuals, even during the inactive reproductive season and regardless of the small size in comparison with the body, testes were highly irrigated (Figure 27).

Figure 27. Rhizoprionodon longurio male reproductive tract from a mature during active season (A), mature during inactive season (B) and immature (C) individuals.

Significant differences were observed in both tw and GSI in *R. longurio* males throughout one-year reproductive cycle (One-Way ANOVA, $F = 38.91$, $p < 0.0001$; $F = 31.92$, $p < 0.0001$, respectively). Testis width showed an increase from February (21 ± 2.34 mm) through May (25 mm) and then significantly collapsed in June-July (10.5 ± 3.1 mm) and kept low through December (Figure 28A). Very close to tw trend, GSI increased significantly from February (1.21% ± 0.4%) through April-May (1.89% ± 0.48%) and significantly decreased during June-July (0.43% ± 0.26%) keeping low through December (Figure 28B).

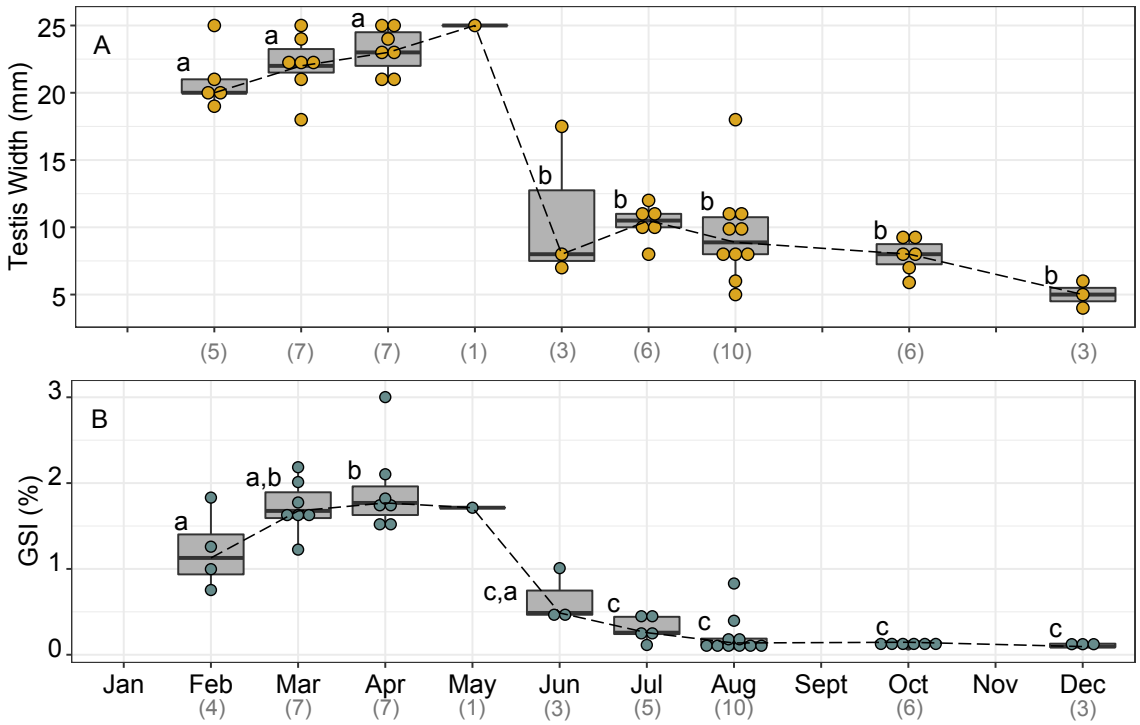

Figure 28. Annual variation in testes width (A) and GSI (B) in *R. longurio* males. Letters show statistical differences between months using Tukey test.

During one-year cycle, it was possible to distinguish the movement of sperm through the reproductive tract, by the decrease in testis size and consequent increase in epididymis and sperm sac weight (Figure 29). In July, epididymis (38.14g ± 8.89g) were three times heavier than testis (12.88g ± 5.11g), which was reflected in both ESI (Epididysomatic Index) and IGS (0.89% ± 0.17% and 0.30% ± 0.14%, respectively).

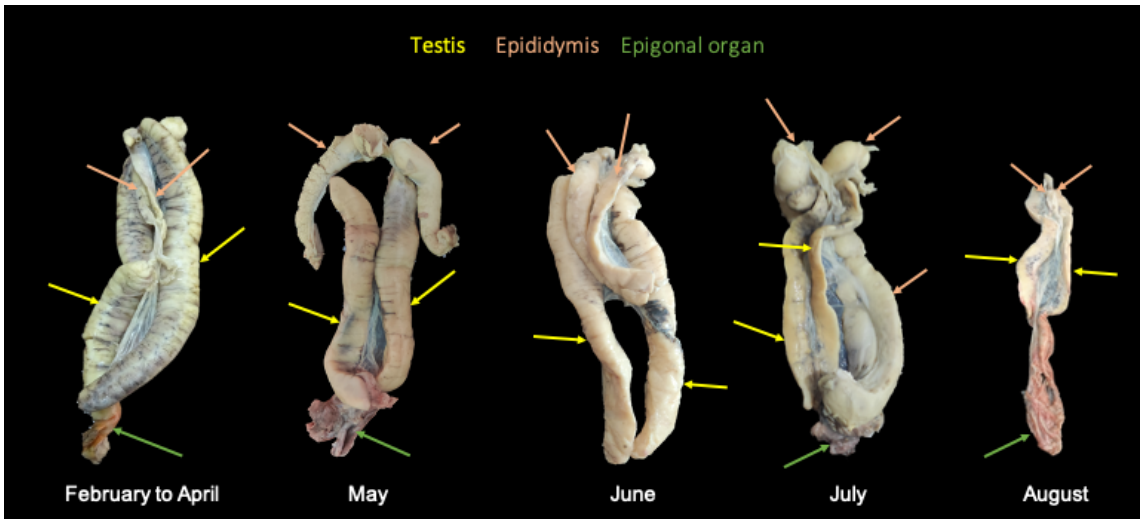

Figure 29. Reproductive tract comparison between months in *R. longurio* males.

Although small sample sizes, endocrine correlates of reproductive hormones and morphological characteristics can be observed. 17β-estradiol and T levels are relatively higher from December to February (Figure 30). The reproductive hormones decreased in August at the beginning of the inactive season (0.65 ± 0.29 ng ml^{-1} T; 0.36 ± 0.02 ng ml^{-1} E_2), while one individual remained active (T = 5.83 ng ml^{-1}; E_2 = 2.16 ng ml^{-1}). During October, both hormones showed a large range of values from 1 ng ml^{-1} and 5 ng ml^{-1} can be observed. E_2 had the same pattern of dispersed values.

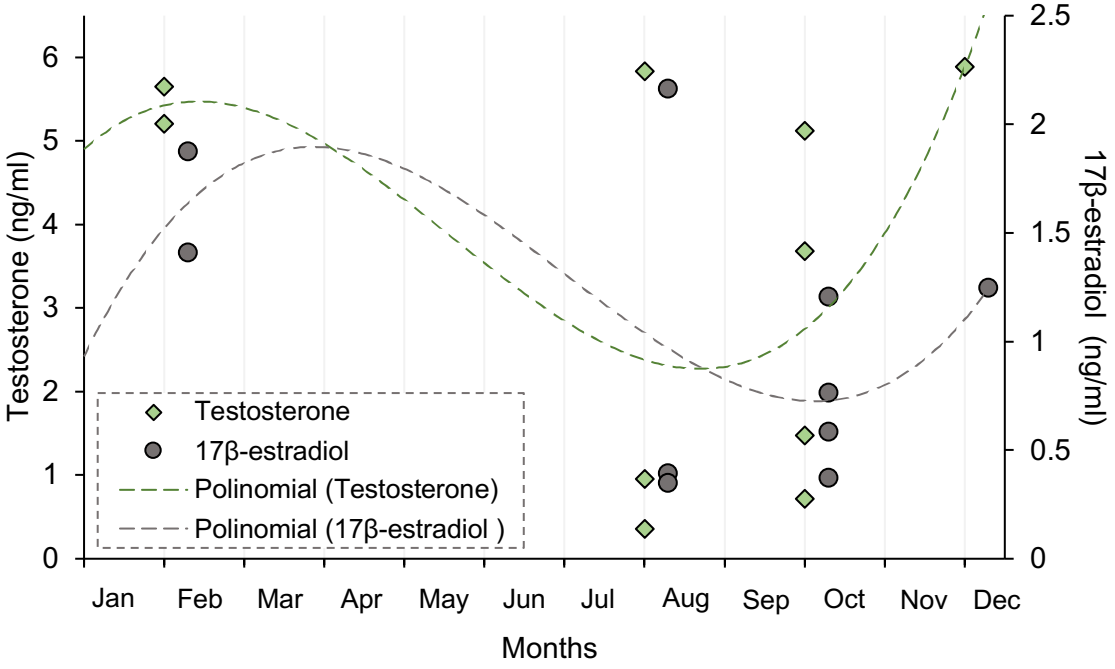

Figure 30. Testosterone and 17β-estradiol values represented in one-year cycle with numbers in x axis representing month number.

4.5. Temperature correlations

Sea surface temperature (SST) in La Paz Bay has a great variation during all year round, from minimum temperatures of approximately 21 ºC in February to more than 30 ºC in September. Mean temperature in the study area was 26.07 ± 2.90 º.C (Figure 31).

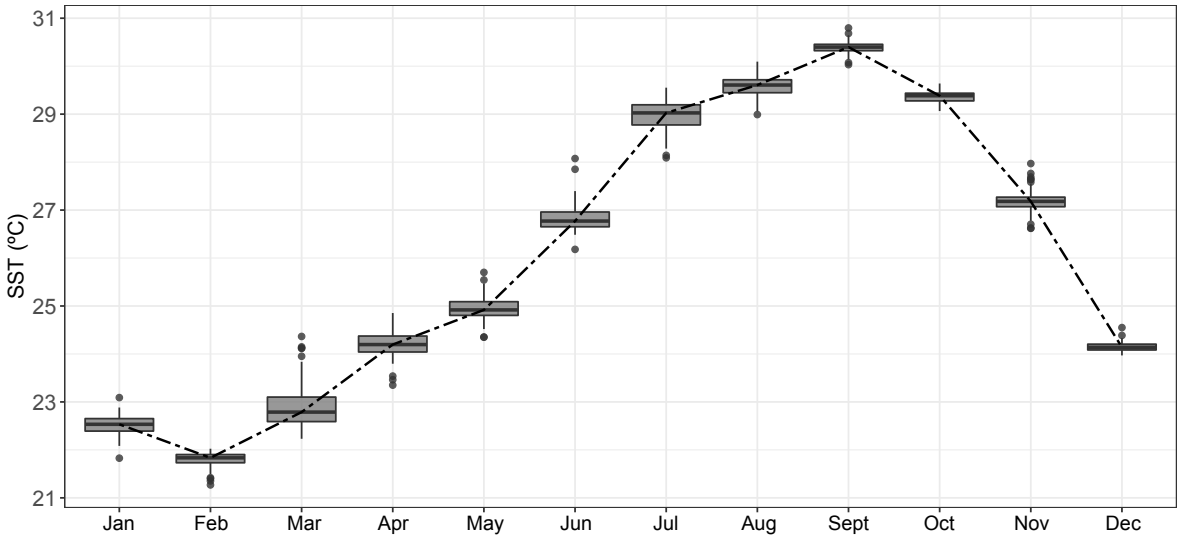

Figure 31. Sea Surface temperature (SST) represented in one year-round in La Paz Bay. Source Aqua Modis, ERDDAP, NOAA.

Females

When looking the relationship between SST and the reproductive parameters in *R. longurio* females, a significative inverse correlation was found between E_2 (Spearman's correlation; r = -0.80, $p < 0.001$) and T (Spearman's correlation; r = -0.63, $p = 0.01$) and SST (Figure 32). However, a non-significative inverse correlation was found between MFD and SST (Spearman's correlation; r = -0.12, $p = 0.49$) as both large and small sized follicles were seen during the highest temperatures (> 28ºC).

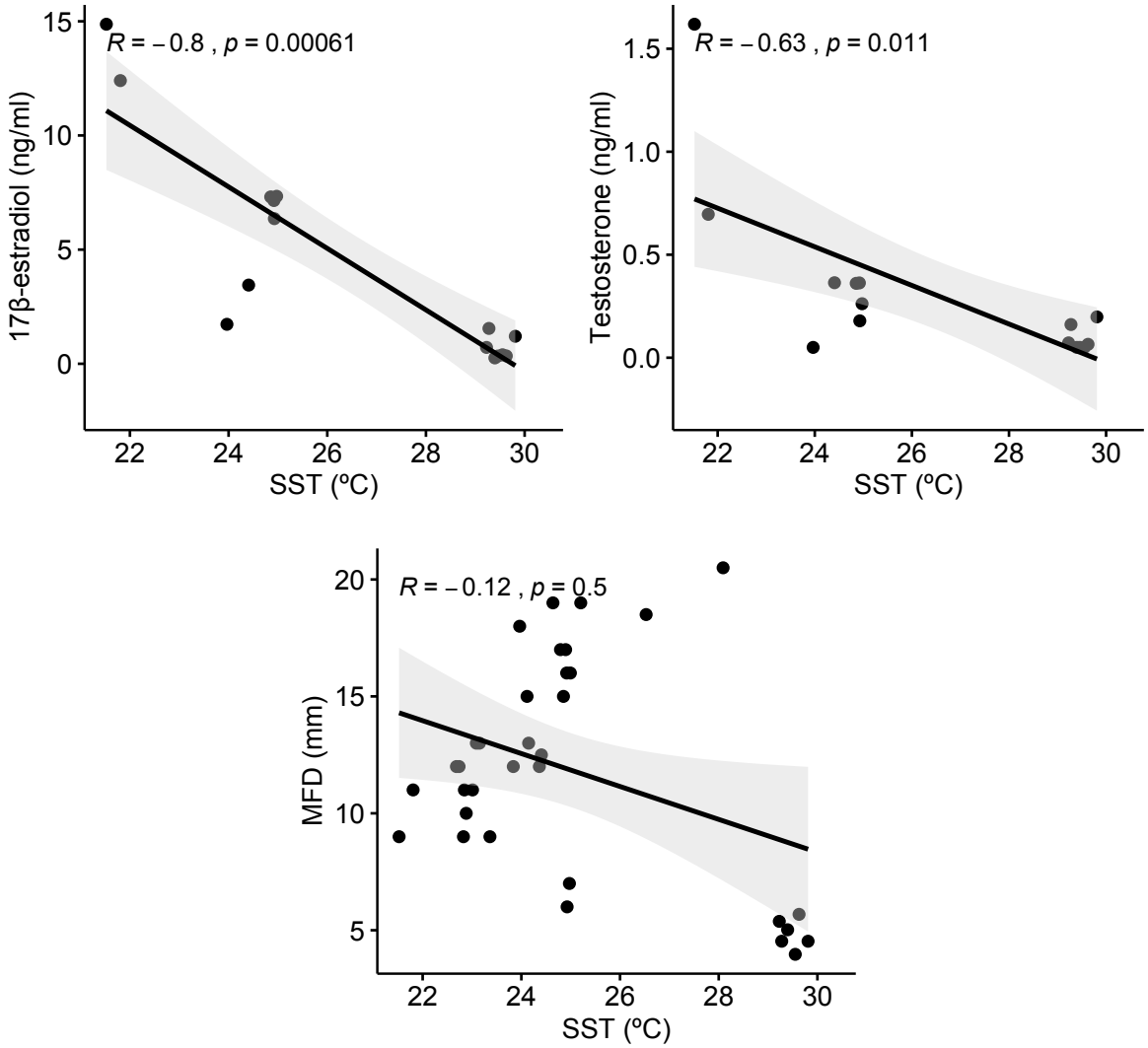

Figure 32. Sea surface temperature correlated with reproductive hormones and maximum follicle diameter (MFD) on *R. longurio* females.

Males

No significant correlations were observed between reproductive hormones and SST in males, but it could be due to the low sample size (Spearman's correlation for T; r = -0.27, *p* = 0.49, and Spearman's correlation for E_2; r = 0.46, *p* = -0.28). Both testes width and GSI showed a significant inverse correlation with SST (Spearman's correlation; tw: r = -0.63, *p* < 0.001; GSI: r = -0.69, p < 0.001) (Figure 33).

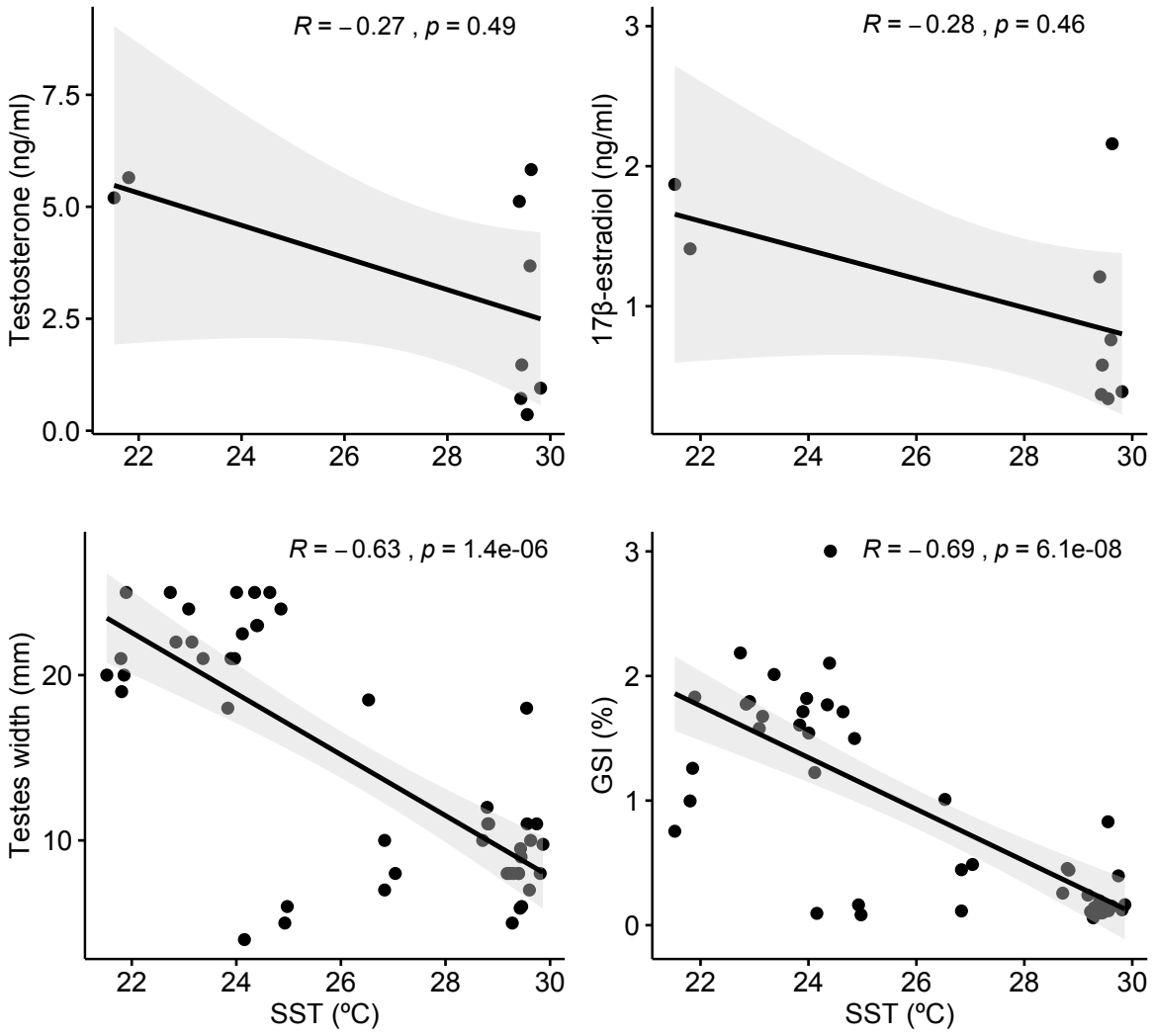

Figure 33. Sea surface temperature correlated with reproductive hormones and morphometric measurements on *R. longurio* males.

5. Discussion

This is the first study correlating sex steroid hormone levels with macroscopic observations of reproductive tract in *R. longurio* from the Pacific coast of Mexico. Although these correlations were done on small number of sex steroid hormone samples, it was possible to assess the reproductive cycle and size at maturity. Furthermore, non-lethal endocrinological tools seem to provide accurate results when assessing this species' reproductive status.

5.1. Size composition

In total, 75% of individuals collected throughout this study were adults, however by removing the 12 immature individuals takes from southern La Paz Bay, 85% of the total catches in "*El Saladito*" fishing camp (main study area) were adult, in concordance with the pattern previously reported for the entire Gulf of California (Corro-Espinosa, 2011). These results represent a higher presence of adults than previously reported within La Paz bay area: 29% of adults were reported by Mejía-Salazar (2007) and no presence of adults were recorded by Trejo-Ramírez (2017). However, the main reason for these differences might be due to differences in sampling areas (either location or depth) within the BLP. While previous studies within the bay took their samples from both southern and central part of the bay (Mejía-Salazar, 2007) and only in the south (Trejo-Ramírez, 2017), this study was carried out in the central/northern area of the bay at depths between 50 to 80 meters. The southern part of the bay, considered a nursery area for this species (Trejo-Ramírez, 2017), is sandy and shallow (< 30 m depth), with depth increasing and the continental slope getting steeper towards the north of the bay (Del Monte-Luna et al., 2005). Thus, it is reasonable to suggest a pattern of *R. longurio* distribution inside BLP where YOYs and juvenile individuals are distributed in the southern and shallower areas while adults occupy the northern deeper areas.

In total, no significant sex ratio differences were found for the two and a half years sampling. Pregnant females were captured during first pregnancy stages in August, after which no pregnant females were captured until February, suggesting these

females are going to deeper or offshore waters not being targeted neither with bottom longline nor with gillnets near the shore.

Results in the present study are similar to previous work suggesting *R. longurio* is a migratory species inhabiting the shallower coastal areas between Winter – Spring months (January to May) but migrating to deeper offshore waters during late Summer – Autumn (September-December) (Smith et al., 2009). From our results seems clear that *R. longurio* have sexual and size segregation with large adult females migrating to deeper waters during gestation while juveniles and adult males remain in the shallows. Similar observation were reported for other *Rhizoprionodon* species, such us *R. terraenovae* from Mississippi waters and *R. lalandii* from Brazilian waters (Parsons & Hoffmayer, 2005; Motta et al., 2005) which denoted sexual and size segregation with males being more vulnerable to shallow fishing gears than females remaining in deeper waters during the gestation period.

5.2. Reproductive tract description

Rhizoprionodon longurio females showed a single left ovary with paired oviductal glands, oviducts and uteri. Similar reproductive structures have been reported for *R. terraenovae* and *R. acutus* being characteristic of the genus (Parsons, 1983; Shaaban et al., 2018). The ovary carries a clutch of four to twelve vitellogenic follicles reaching a MFD 20 mm at the time of ovulation coinciding with previous reports on the same species (Corro-Espinosa, 2011) and genus (Parsons, 1983; Prohaska et al., 2013).

Rhizoprionodon longurio males have paired testes, epididymis and seminal vesicles, with testes varying significantly in shape throughout the year. Both epididymis and seminal vesicles increased sizes during the copulatory months in June and July.

5.3. Endocrine correlations of the reproductive tract

Females

By correlating sex steroids with morphological observations of the reproductive tract it was observed that E_2 levels increased with ovarian growth reaching a peak in females carrying vitellogenic follicles, while lower E_2 levels were found in females

carrying previtellogenic, atretic or post-ovulatory follicles. Similar correlations between MFD and E_2 have been registered within the same genus in *R. terraenovae* (Prohaska et al., 2013) and *R. taylori* (Waltrick et al., 2014). 17β-estradiol roles in follicular development preparing females for ovulation has been demonstrated in wild species (Awruch, 2013) and *in-vitro* studies in sharks, *Scyliorhinus canicula* and *Squalus acanthias* (Craik, 1978b; Ho et al., 1980) and rays, *Torpedo marmorata* (Prisco et al., 2008). Thus, it can be concluded that E_2 is a good indicator of vitellogenic processes in *Rhizoprionodon* species.

Testosterone was also correlated with MFDs, however this correlation was weaker than E_2. Similar pattern was observed for *R. taylori* (Waltrick et al., 2014) while T was not correlated neither with MFD nor ovary mass in *R. terraenovae* (Prohaska et al., 2013). Some studies found that androgens serve as precursors in E_2 synthesis (Koob & Callard, 1999; Manire et al., 1995) arguing that T was also correlated with ovary development. In the viviparous lemon shark *Negaprion brevirostris* and in the smooth hammerhead shark *Sphyrna tiburo* T levels increased during mating and preovulatory periods (Manire et al., 1995; Rasmussen & Gruber, 1993), while T highest concentrations in *R. terraenovae* and *R. taylori* were observed during early to mid-pregnancy stages (Prohaska et al., 2013; Waltrick et al., 2014). In the current study, T maximum concentrations coincided with 7 to 10 mm follicles in ovaries during early vitellogenesis suggesting T could act as a precursor of E_2 synthesis triggering follicular growth, and therefore regulating some reproductive phases.

Males

Although small sample sizes for males, testes development and sex steroid concentrations were correlated. Large testis showed high T and E_2 levels, while small testis showed variable T and E_2 values. Although no testes were extracted and measured in males beneath 60 cm TL, undetectable T and E_2 levels were registered in these immature individuals with apparently non-developed testis. Similar observations were seen in *R. terraenovae*, where T levels were highly correlated with larger testis sizes (Hoffmayer et al., 2010). For elasmobranch species, androgens have been observed to be associated with spermatogenesis in both continuous and seasonal sperm producers (Awruch, 2015). Testosterone is the main

androgen in males, *in-vitro* studies in *S. acanthias* demonstrated the role of T on spermatocyst maturation (Cuevas & Callard, 1992). Similar findings were also reported in the skate *Leucoraja ocellata* and the ray *Dasyatis sabina* (Sulikowski et al., 2004; Tricas et al., 2000). Although no other androgens were measured in the current study, it has been suggested that different androgens might play a role in males, for example, dihydrotestosterone (DHT), 11-ketosterone (11-KT), or 11-ketoandrostenedione (11-KA) seems to be involved in *S. canicula* and *S. tiburo* spermatogenesis (Garnier et al., 1999; Manire et al., 1999), while 11-KT was not present in *C. laticeps* and *N. cepedianus* (Awruch et al., 2008, 2014). 17β-estradiol levels are not usually measured in males, but in this work were significantly correlated with testis size. Similar observations were found in *R. terraenovae*, were E_2 high levels coincided with high GSI values and vice versa (Hoffmayer et al., 2010). Therefore, for this work, it can be strongly suggested that T and likely E_2 play a role in testes development in *R. longurio*.

5.4. Using non-lethal methodology to address size at maturity and the reproductive cycle

During the past 15 years, in order to improve ethical investigations in Chondrichthyans while providing reproductive information necessary to delineate management plans in response to Chondrichthyan population declines, non-lethal sampling approaches have been increasingly used to study reproductive parameters into this vulnerable group of marine animals (Awruch, 2013; Hammerschlag & Sulikowski, 2011). Non-lethal assessments of size at maturity and reproductive cycle, by using reproductive steroid hormones and more recently by combining ultrasound methodologies (Anderson et al., 2018; Sulikowski et al., 2016), have been done in sharks (Awruch et al., 2008, 2009), skates (Sulikowski, Kneebone, et al., 2005; Sulikowski, Tsang, et al., 2005), and chimaeras (Barnett et al., 2009, 2019).

The use of reproductive hormones is particularly important in females, as do not have external structures to be able to distinguish mature individuals from sexually immature ones. In the case of males, clasper calcification is an external distinguishing feature that allows to distinguish sexual maturity in chondrichthyans.

However, it has been demonstrated that even males carrying partially calcified clasper the gonads are fully mature producing sperm. Thus, obtaining size at maturity by using sex steroids could provide more accurate information, which has important implications for conservation management policies.

5.4.1 Size at maturity

Size at maturity estimates were slightly different depending on the method employed, macroscopic observations or hormone levels. However, although smaller size at maturity estimates were obtained by reproductive hormones 4.3% (from 94 to 90 cm TL) for females and 3.5% (from 86 to 83 cm TL) for males, these results felt within 95% confidence interval of the L50 obtained only by visual examinations.

Comparisons between morphological and hormonal methodologies have been reported in several elasmobranch species. Size at 50% maturity differed between methods in the Patagonian broadnose sevengill shark *Notorynchus cepedianus* (Irigoyen et al., 2018; Lucifora et al., 2005), the spotted ratfish *Hydrolagus colliei* (Barnett et al., 2009), and the thorny skate *Amblyraja radiata* (Sulikowski et al., 2006). However, no difference were observed in the maturity sizes between the methods in the draughtboard shark *Cephaloscyllium laticeps* (Awruch et al., 2008).

Previous maturity estimates within the Gulf of California showed variable results. Corro-Espinosa *et al*. (2011) stablished 93 cm TL and 100 cm TL L50 for females and males respectively by using reproductive organs macroscopic visualizations, while Mejia-Salazar (2007) estimated 80 cm TL and 82 cm TL in females and males respectively, using histology of the reproductive organs. The current results showed similar trend when compared with previous information, with individuals maturing at smaller sizes when using physiological or histological methodologies than using morphometrical ones. These are not surprising as it is well known that sexual hormones trigger reproduction processes, which is first reflected microscopically (histology) and later macroscopically (Pankhurst, 2008).

Contrary to previous studies done before inside the Gulf of California, *R. longurio* females in this work matured at larger sizes than males (Corro-Espinosa, 2011;

Corro-Espinosa et al., 2011; Márquez-Farias et al., 2005; Mejía-Salazar, 2007). Females maturing at larger sizes than males have been reported in the Brazilian sharpnose shark *R. lalandii* (Motta et al., 2007), in the Atlantic sharpnose shark *R. terraenovae* (Parsons, 1983) and in the Grey sharpnose shark *R. oligolinx* (Purushottama et al., 2017), while females maturing at smaller sizes than males have been reported before in the milk shark *R. acutus* (Henderson et al., 2006; Shaaban et al., 2018). However, size at maturity can also differ between areas within the same species, for example *R. taylori* grows faster in Papua Guinea Gulf than in Australian waters, reaching maturity at smaller sizes (Baje et al., 2018; Simpfendorfer, 1992). This intraspecific differences could be explained because of the reproductive plasticity of this species (Hoffmayer et al., 2013). In this case, could be possible that *R. longurio* size at 50% maturity differs from one place to another, but the greatest differences would come from the methodology of maturity assessment between studies (macroscopical, histological, hormonal).

Assessing size at maturity in season sperm producers is crucial to have enough samples throughout the entire reproductive cycle, identifying important events as spermatogenesis, mating, resting period and clasper growth. However, in any case, for this species the idoneal moment to assess size at maturity is during late Winter and Spring (December to June), when they are reproductively active. Otherwise, sampling and measuring reproductive steroid hormones during Summer and Autumn without previous knowledge of the reproductive cycle, could cause errors finding low concentrations in mature but non-reproductively individuals.

Additional information on the relationship between clasper length and TL as an indicator of sexual maturity showed a linear relationship. Looking previous studies in *Rhizoprionodon* species, it is possible that the relationship may not necessarily be linear, fitting better in a logistic as claspers experienced a rapid growth and calcification after 70 cm TL in *R. terraenovae* (Loefer & Sedberry, 2003; Parsons, 1983). The same rapid enlargement trend was observed in *R. lalandii* (Motta et al., 2007). Therefore, should be necessary to fill the lacking information of clasper size within 70 to 85 cm TL to ensure the relationship between clasper size and TL

Determine size at 50% maturity of elasmobranchs has a crucial importance when management plans are built in order to propose minimum fishing sizes in fisheries, stock evaluation and population dynamics predictions and demographic studies (Corro-Espinosa et al., 2011).

By using physiological variables to address site at maturity we can infer more accurately when an organism is not only reproductively capable, but also when its system begins to behave such as one (Awruch et al., 2014; Sulikowski et al., 2006; Sulikowski, Tsang, et al., 2005), puberty development is considered critical for sharks as they prepare to be sexually active (Gelsleichter et al., 2002). In this study, while *R. longurio* females and males between 80 to 90 cm TL looked macroscopically maturing, the reproductive hormone levels indicated that these individuals are sexually mature for the following breeding season and might need to be protected. These smaller sizes should be the ones to be considered in fisheries management as they are close to became part of the reproductive population.

5.4.2. Reproductive cycle

Females

Observations in *R. longurio* females throughout the year showed an annual reproductive cycle in which the ovulatory and gestation cycle occur in parallel, making *Rhizoprionodon longurio* a continuous breeder (Castro, 2009; Koob & Callard, 1999). Similarly to previous work done in other *Rhizoprionodon* species, parturition is followed really close by mating, evincing a really short time (one to two months) of resting between cycles (Ba et al., 2013; Capapé et al., 2006; Henderson et al., 2006; Hoffmayer et al., 2013; Parsons, 1983; Shaaban et al., 2018; Simpfendorfer, 1992).

Females were observed carrying fully developed follicles ready to be ovulated at the time of parturition in May. Although, no females were captured during September to November, enough information was obtained to be able to address the reproductive cycle. The presence of fresh mating scars in the only adult female caught during July carrying preovulatory follicles together with the presence of females captured in

August with healing mating scars and depleted ovaries (minimum GSI registered), E_2 and T levels near undetectable levels, and uterine eggs is an indication that ovulation occurred between July – August period. It is then concluded that pregnancy started in between July and August, in agreement with previous work (Corro-Espinosa, 2011; Mejía-Salazar, 2007). Although no females were caught in September-November period, December mature non-pregnant females showed high E_2 and T levels with small developing follicles, indicating a resumption of the ovulatory cycle.

Low concentrations during beginning of pregnancy have been reported before in the same genus (Prohaska et al., 2013; Waltrick et al., 2014). In the current study, pregnant females in February showed mid-term embryos with both highly increased E_2 and T concentrations and developing follicles, coinciding with Waltrick et al. (2014) after *R. taylori* embryonic diapause. However, *R. terraenovae* showed low E_2 and high T values (Prohaska et al., 2013). Increased E_2 after ovulation is likely showing that pregnancy first stage ended as P_4 has stopped inhibiting E_2 synthesis making follicles grow again. Highest T levels in *R. terraenovae* and *R. taylori* studies occurred during early-mid embryo development, so we cannot infer if levels in this study are high or low as we do not have represented plasma concentrations during early to mid-pregnancy females (September – January period). For this, more samples would be needed for the present study in order to fill the gap information during these months

According to Márquez-Farias et al. (2005), April and May pregnant females had late and terminal embryos respectively, indicating parturition. Those females showed reduced E_2 and T concentrations when comparing against February but still being elevated. This coincides also with Trejo-Ramírez (2017) thesis, who concludes that the southernmost part of La Paz Bay is a nursery area and birth season peaks in May-June and with Corro-Espinosa (2011), who found newborns from May to August. Inside GC, embryos were found to measure 2 cm in August, 5.3 cm in September, 10.5 cm in October and 17.5 cm in December (Mejía-Salazar, 2007), filling the gaps on this study. Moreover, parturition occurring during spring raises the probability of

survival of newborns as this season its characterized of high primary productivity (Castro, 2009; Valdez-Holguin et al., 1995).

Our results reflect the role of E_2 in inducing vitellogenesis by the liver, remaining low until the vitellogenesis cycle recommence in parallel with early pregnancy stages. Similar results with high E_2 levels during follicular development and low values after ovulation have been reported many elasmobranch species such *Prionace glauca, Hemiscyllium ocellatum*, *Raja eglanteria*, *D. sabina*, *S. tiburo* (Fujinami & Semba, 2020; Heupel et al., 1999; Manire et al., 1995; Snelson et al., 1997; Sumpter & Dodd, 1979). In *R. terraenovae*, T imitated P_4 concentrations in all stages having a small peak in preovulatory females, but highest levels where found during minimum MFD after ovulation (Prohaska et al., 2013). However, *R. taylori* T concentrations were highly variable throughout all reproductive cycle being low during ovulation, increasing in most part of diapause and peaking and triggering embryo development, reducing again after mating season (Waltrick et al., 2014).

On the other hand, while GSI was increasing throughout the ovulatory cycle, HSI decreased collapsing in females near mating season. Similar HSI observations were seen in the same genus during maximum MFD and GSI (Ba et al., 2013; Shaaban et al., 2018). In addition, females carrying late or terminal embryos showed nearly significant lower HSI values than non-pregnant females carrying large follicles, suggesting embryo nourishment demands more energy than follicle development. Waltrick et al. (2014) suggested that *R. taylori* females induce embryonic diapause mainly to recover liver weight to ensure next follicular cycle as diapause period coincided with HSI increase. Therefore, we suggest low HSI values during April-June could be explained a as consequence of high plasma E_2 concentration during follicle development and embryonic nourishment. Moreover, HSI seems to be recovering in August, with females energetically ready to start a new gestation or follicle development period.

Fecundity

Females showed a slightly lower fecundity, of six embryos, than previously reported from females caught in the east coast of the Gulf of California showing a fecundity of seven embryos (Márquez-Farias et al., 2005) but similar to *R. terraenovae* in the Gulf of Mexico (Driggers et al., 2020). In the present study, all pregnant females carrying visible embryos (at least 20 cm ETL) also carried at least one egg without embryo development. Similar uterine eggs were found in *R. longurio* females caught along different areas of the southern Gulf of California including La Paz bay (Mejía-Salazar, 2007). Previous work in *R. terraenovae* suggest that eggs provide energy for the first eight weeks of development with embryo reaching 10 to 15 cm TL (Castro & Wourms, 1993). Thus, it can be stablished that *R. longurio* fertilized eggs do not always developed into full term embryos. The fact of no developing embryos from a fertilized egg could be related with energetic condition or environmental and anthropogenic stressors. In bony fishes has been demonstrated a reduction of egg number when suffering from food limitation during early cycle phases (Luquet & Watanabe, 1986). Furthermore, it is known that stress can produce, depending of the duration and nature off the stressor, disturbances from molecular (homeostasis perturbation) to hole organism / population status and performance like changes in growth or reproduction (Skomal & Mandelman, 2012).

Comparing the present study with a work done on females from Santa Rosalia, village located inside the Gulf of California and 400 km northeast from La Paz, the fecundity and number off fertilized eggs without visible embryo development were different. Females caught in Sant Rosalia had more fecundity than the present study (mean = 7.2, mode = 8) but only 10% of the females (7 of 71) had eggs with no embryo development (Chavez *et al*., *unpublished data*). It would be great to know what is happening by comparing energetic condition between locations, possible stressors, pollutants or available food incomes.

Males

In general, elasmobranch males displayed two types of reproductive cycles, with seasonal sperm production like *H. ocellatum* or *R. terraenovae* (Heupel et al., 1999; Hoffmayer et al., 2010) or continuous sperm producers (Awruch et al., 2009).

Although small plasma sample sizes, observations of the GSI and reproductive hormone levels throughout the year are indicative of one-year reproductive cycle. Males showed a resting period towards the summer (August) to resume the reproductive cycle in early winter (December) with a strong elevation on T and E_2 levels. February, had both elevated reproductive hormones levels and GSI but, through the ban period, testis had a 90% mass decline. Additionally, after ban period (August), both reproductively inactive males carrying low T and E_2 circulating plasma levels and reproductive active males with T and E_2 concentrations were observed. 17β-estradiol role in males remains unclear, with few studies founding high levels in early spermatogenesis and others having no variation during important reproductive events (Awruch, 2015). Thus, given the macroscopic and hormonal observations in mature males, a clearly sperm production seasonality is proposed for *Rhizoprionodon longurio*.

Similar trend in GSI decreased was observed in Corro-Espinosa (2011) who found a decrease from 1% in May to less than 0.2% in June. Gonadosomatic index decreasing during summer's beginning season (June) were reported in other *Rhizoprionodon* species (Ba et al., 2013; Shaaban et al., 2018; Simpfendorfer, 1999). In elasmobranch males, including *R. terraenovae*, showing reproductive seasonality, an increase in circulating T levels is associated with sperm maturation during breeding season (Hoffmayer et al., 2010; Wyffels et al., 2020).

5.5. Temperature and hormonal correlations

The correlation between the reproductive hormones, morphometric parameters and SST analyzed in this study suggest that water temperature fluctuations might play a role in *R. longurio* reproduction. In females, an inverse significant correlation between E_2, T and SST was observed. The MFD increase in parallel with water temperature with ovulation occurring just before reaching higher temperatures, as both E_2 and T had near undetectable levels coinciding with females carrying post-ovulatory follicles. Therefore, declined MFD, E_2 and T values coincided with high water temperatures. It can be suggested that SST elevations (from 21 to 29 ºC) increase E_2 and T concentrations, but higher temperatures (from 29 to 31 ºC) stots vitellogenesis, reducing E_2 and T plasma levels.

Similar results were reported on the Australian sharpnose shark *R. taylori*, where E_2 was correlated both daylength and temperature (Waltrick et al., 2014). Previous work reported in the zebra shark *Stegostoma fasciatum*, under regulated environmental conditions in aquarium, a significative inverse correlation between SST and E_2 concentrations and ovarian regression (Nozu et al., 2018). Similar results were observed in plasma E_2 levels in the smooth-hound shark *Mustelus schmitti*, increasing first in parallel with increasing SST, however as the water temperature raised circulating E_2 concentrations decreased, reaching the lowest concentrations during highest temperatures, while T levels had an inverse pattern (Elisio et al., 2019).

In the case of males, no correlation was observed between reproductive hormone concentrations and SST, most likely due the small sample size. However, using morphological features as testis width (tw) and GSI, which are a result of hormone levels, significant inverse correlation was found between both tw and GSI. Reproductively active males were present solely in cold waters during winter (21 to 25 ºC) while inactive males were found during the warmer seasons were water SST was above 27 ºC. This has been observed in previous studies where warmer waters reduce both androgens and sperm production in *H. ocellatum* and *S. canicula* (Heupel et al., 1999; Garnier et al., 1999). Hoffmayer et al., (2010) observed *R.*

terraenovae males in the Mississippi waters (USA) maturing earlier than previously observed males within the same area by comparing GSI cycle (Parsons, 1983), suggesting that increased temperatures between the two sampling periods induced GSI to raise and decrease before previous study. Similar results were reported in sand tiger shark *Carcharias taurus* captivity studies, where, an increase in water temperature resulted in changes on the reproductive cycle months, as all events happened out of date (Henningsen et al., 2008, 2015). Therefore, as with females, it can be concluded that SST clearly affects the reproductive cycle in males.

5.6. La Paz Bay as a key area for Rhizoprionodon longurio reproduction

There is a need of considering LPB as a crucial area for this shark species as pregnant *R. longurio* females arrive to the bay months before parturition suggesting they use these nearshore waters as feeding and shelter areas from predators (Branstetter, 1990). Parturition occurs between May and June and it has been demonstrated that neonates and juveniles stay for more than a year in southern LPB, and mature/maturing organisms were re-captured 10 months and 1 year and 7 months later near BLP area (Trejo-Ramírez, 2017). Contrarily, tagged *R. terraenovae* juveniles from Upper Gulf of Mexico left the study site in a few days and were never recorded again (Carlson et al., 2008). In this case, we suggest, contrary to Trejo-Ramírez (2017), that BLP should not be only considered a nursery area for this species but also as a reproductive/mating area

Although not yet enough evidence, the results from pasts and current studies showed that *R. longurio* used the bay as a reproductive and nursery grounds. These results are in coincidence with previous reports suggesting that both immature and mature sharpnose shark females con occupy same regions and habitats (Knip et al., 2010). Moreover, the evidence off recaptured or tagged *Rhizoprionodon* species returning years later to the same area (Carlson et al., 2008; Trejo-Ramírez, 2017) opens a possible philanthropy behavior (site fidelity and residency) in this species according to Chapman et al. (2015) definitions.

6. Conclusions

In the present work, we provided detailed information about *Rhizoprionodon longurio* reproduction using both hormonal and macroscopical analysis. Reproductive hormones were used together with macroscopical observations to assess size at 50% maturity, denoting that the use of endocrine tools can provide accurate information, bringing maturity to smaller sizes. However, besides the small plasma sample size, measuring steroid reproductive hormones seem to be good for this species. We denoted one-year reproductive cycle with mating and ovulation occurring in July-August period, follicular development running in parallel to pregnancy, and parturition occurring in May-June. Additionally, temperature seems to be a crucial environmental factor, triggering and regulating reproductive events in this species, with higher temperatures inducing mating and ovulation in females, and low temperatures stimulating testicular growth in males. The ban interval effectively protects both mating and parturition periods.

Knowing that traditional and artisanal fisheries in Mexico are linked to a large amount of different elasmobranch species, combining macroscopical visualization with reproductive hormone profiles can be a notable source of information that would be useful in future studies in near threatened or endangered similar species around the globe. Moreover, this methodology can be used in those protected species within NOM-029-PESC-2006 and also applied during ban period.

Future Pacific Sharpnose shark sampling efforts should be focused during Summer/Autumn, in order to fill the gaps from September to January. Moreover, the existing evidence of the importance of the bay in this species could be used as a potential refuge area where *R. longurio* could be protected if some decline population or change in the IUCN status occurs.

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Appendix I: Embryos and schematic summary

Figure 34. Litter from one *R. longurio* female during February. Mean ETL = 22.6 cm.

Figure 35. Litter from one *R. longurio* female during March. Mean ETL = 28.5 cm.

Figure 36. Litter from one *R. longurio* female during April. Mean ETL = 29 cm.

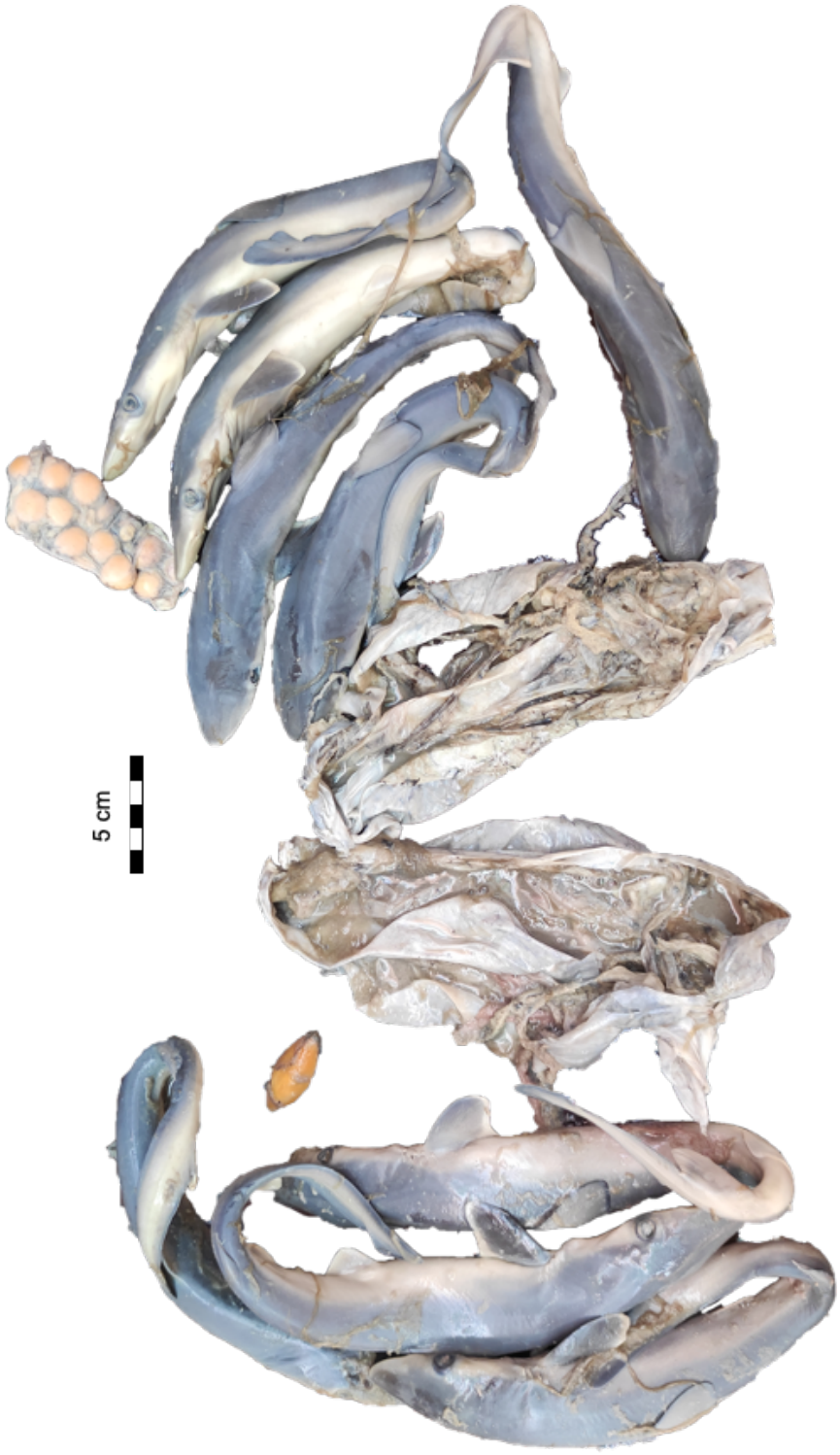

Figure 37. Litter from one *R. longurio* female during May. Mean ETL = 33.1 cm.

Figure 38. Largest embryo registered in this work during May 2020. ETL = 37 cm.

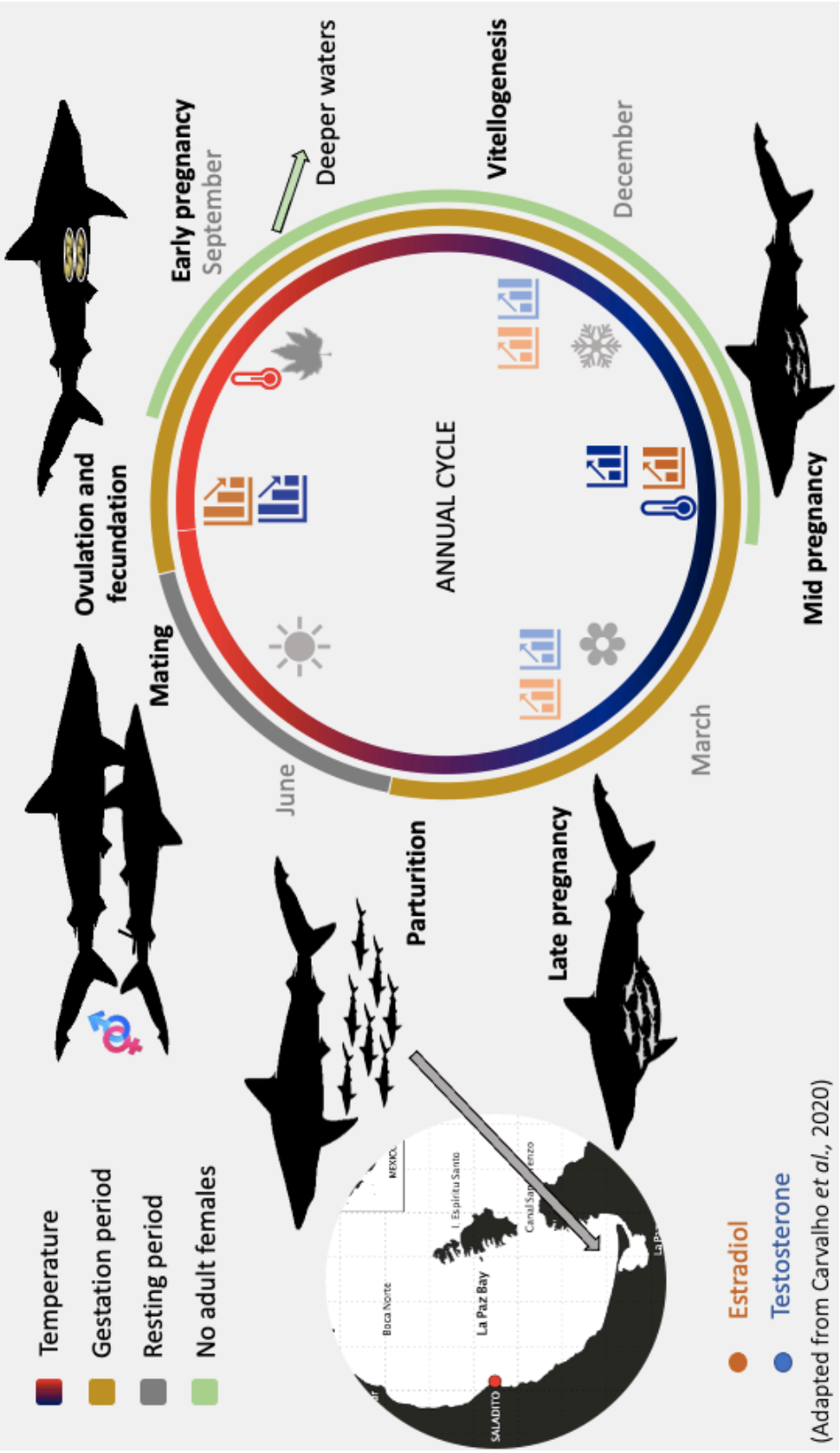

Figure 39. Schematic overview of *R. longurio* reproductive cycle.