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RESEARCH ARTICLE

Gonadal Organogenesis and Histoarchitectural Configuration of Eastern Little Tuna (*Euthynnus affinis*) - Initial Findings on Reproductive Apparatus Profile in Southern Philippines

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ABSTRACT

Organogenetic alterations described over the stages of gonadogenesis at the gross and microscopic scale can be used to determine spawning behavior in iteroparous fishes and provide finer details of the reproductive functions of commercially important species. This information can be used as the basis for stock assessment and maintenance of wild populations. In this study, the gross morphology of Eastern Little Tuna (*Euthynnus affinis*) gonads from Sarangani Bay, Davao Gulf, Moro Gulf, and Sulawesi Sea collected in 2020–2022 was investigated along with its microanatomy through histotechniques. We determined four different size classes (FL): size 1 (≤ 30 cm), size 2 (30.1–40 cm), size 3 (40.1–50 cm), and size 4 (> 50 cm), and six stages for reproductive maturity: Immature, Onset Maturation, Developing/Maturing, Spawning, Spent, and Recovering/Resting. We observed that at the Spawning stage of the testis, the gonad appears creamy-white in color and soft in texture, with the contents freely released. The ovary is characterized by conspicuous blood vessels, a large ovum on the surface, and a pink-orange color. Histoarhitecture of the same stage showed the presence of lobular and tubular tissues, spermatozoa, primary and secondary spermatocytes, and proliferation of spermatids in the testis. In the female, the ovary is populated by secondary vitellogenic oocytes and yolk granules, indicating that the follicles are fully mature. Spawning was observed to commonly occur at Size 3 (40.1–50cm FL) in both males and females. Our paper is the first study that relates the gonadal sexual maturity of *Euthynnus affinis* in the southern Philippines to its fish length.

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1. INTRODUCTION

Fish have a wide array of reproductive methods. In addition to normal male-female patterns, there are self-fertilizing hermaphrodites, seasonal and continuous breeders, single-spawners, multi-spawners, and sex-reversing fishes. This also includes mouth breeders, live-bearers, and fish that disperse their eggs (Breder and Rosen 1966; Balon 1975, 1981; Potts and Wooten 1984). As for scombrids, such as the Eastern Little Tuna *(Euthynnus affinis)*, reproduction is external, whereby sperm and eggs, which develop internally, are discharged in the event of reproduction (Nishimura and Tanaka 2014).

In the Philippines, tuna is a much-utilized resource used not only as a food source but as a commercial export product. However, the reproductive maturation cycle of this species has yet to be investigated with sufficient scientific rigor. To improve the conservation and management of the species, the impacts of climate change and seasonal fluctuations on the reproductive biology of tuna species must be assessed (Dell'Apa et al. 2018; Stéquert et al. 2001).

Determining the size at which reproductive maturity occurs is carried out by examining whole fish gonads. This is a well-established technique used in fisheries science research. Observations at the histological level define the physiological characteristics and reproductive biology of tuna species and serve as the foundation for creating suitable management plans and preserving a sustainable fishery resource. Although the relevance and significance of histology approaches in reproductive research have been contested (Alonso-Fernández et al. 2011), length-stage data, spawning season, size and age at sexual maturity, sex ratio, annual/batch fecundity, and gonadosomatic index are some of the parameters employed and are best understood when coupled with identified reproductive physiological characteristics.

A thorough comprehension of these and the precise methods for producing objective estimations can help enhance stock management (Zudaire et al. 2010), especially since Eastern Little Tuna is a valuable domestic and international commercial resource.

To date, most of the studies on the reproductive biological characterization of tuna are focused on the most commercially significant species, such as yellowfin, skipjack, and bigeye (Digal et al. 2017; FAO 2014; Miyake et al. 2010), and the majority of these studies were conducted in major oceanic regions like the Indian (Grande et al. 2014; Liu et al.2014; Zudaire et al. 2014; 2010; Nootmorn 2004) and Pacific oceans (Grande et al. 2014; Sun et al. 2013; Zhu et al. 2011) and some other regions like the Gulf of Mexico (Brown-Peterson et al. 2013), Atlantic Ocean (Zudaire et al. 2015), Arabian sea (Nissar et al. 2015), and Taiwan (Chiang et al. 2011). Studies conducted in the Philippines mainly focus on major landing sites, such as General Santos City (Macusi et al. 2015). Furthermore, current data on sexual maturity is based only on fish size in the classification presented by the International Council for the Exploration of the Sea (ICES) in their 2018 Report of the Workshop for Advancing Sexual Maturity Staging in Fish. In this study, sexual maturity is defined through the morphological and histological appearance of the gonads.

Hence, on this premise, this research aimed to provide a much-needed update and more comprehensive data on the length of sexual maturity of Eastern Little Tuna *(Euthynnus affinis)* through gonadal organogenesis. This study aimed to (1) evaluate the gonadal morphogenesis of *Euthynnus affinis*, first in evaluating the gonad morphology and subsequently in assessing its histological characteristics, and (2) establish at which fish length the sexual maturity of *E. affinis* occurs, as shown in its gross (macro) and histoarchitecture (micro). This report will also provide inputs suitable for consideration in efforts concerning fish management, especially of neritic tuna in the Southern Philippines.

2. MATERIALS AND METHODS

2.1. Field sampling

This study was conducted in collaboration with the National Stock Assessment Program of the Bureau of Aquatic Resources (NSAP-BFAR) Region XI and XII, private tuna industries using their ships or vessels, and local fisherfolk around the sampling areas. If sampling was unfeasible or impossible because of unfavorable weather conditions, we employed fishery-independent sampling methods. Tuna specimens were collected every month for one year around the major bodies of water around Mindanao: Sarangani Bay, Davao Gulf, Moro Gulf, and Sulawesi Sea, as strategically designed and identified by the regional offices of BFAR XI and XII (Figure 1). Sampling collection utilized fishery-dependent and -independent sampling techniques.

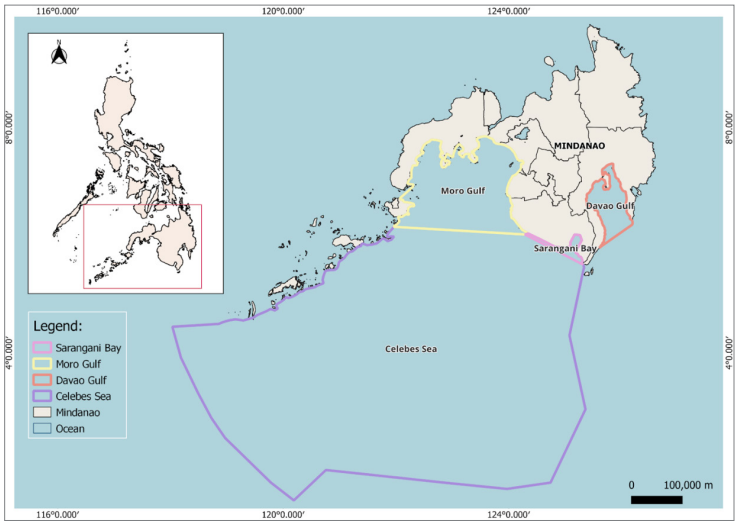

Figure 1. Collection sites for Eastern Little Tuna *(Euthynnus affinis)*. Sampling was conducted throughout the main large waters of Mindanao, particularly where fishing grounds for tuna species are well-established, specifically in Sarangani Bay (5.9656° N, 125.1929° E), Davao Gulf (6813° N, 125.8280° E), Moro Gulf (6.8014° N, 123.4384° E), and Sulawesi Sea (3.6121° N, 122.2998° E). Legend indicates where the fish were taken in the sampling sites. (Content created from QGIS)

Extraction of gonads and other subsequent analyses, such as histology and microscopy, were conducted at the Histopathology Laboratory at Mindanao State University in General Santos City.

2.2. Morphological diagnostic of Eastern Little Tuna

Morphological characteristics of collected Eastern Little Tuna *(Euthynnus affinis)* **samples were identified based on the description given by Collette and Nauen (1983) (Figure 2).**

2.3. Length and weight measurement

Measurement of the length of gonads was determined using vernier calipers (0.01 mm accuracy), weight was measured with the use of an electronic weighing scale balanced to the nearest 0.001 g in accuracy, and the lengths of fish samples were taken using a ruler (1 mm accuracy), following the protocol by Akter et al. (2019).

2.4. Sexual maturity

Determination and classification of maturity stages were done primarily through visual assessment of ovaries (yellow, granular) and the testis (smooth, predominantly white, especially in the latter stages). The histological stage of each gonad was determined through histological analysis. The general criteria provided by McBride et al. 2004 were followed to determine the sexual maturity stage. First, we size-classified the fish based on their Fork Length (FL) before exposing the gonads for ocular inspection. We then profiled the sexual maturity of the gonads based on their gross morphology. Afterward, we randomly selected gonads representing each sexual stage to undergo histology profiling. We separated gonadal (macro) development and histological (micro) assessments due to the subjectivity and obscurity of the ocular gross identification method, which may lead to critical misclassification of the fish's reproductive status. In comparison, histology examinations are more precise and allow the identification of clear and specific alterations in the cellular and tissue structures.

It must be noted that in this study, we identified two types of fork length: (1) the FL of the fish sample based on which stage the (macro) gonad morphology was, and (2) the FL of the fish sample based on the (micro) histological characteristics present within its gonad. All gonad samples for a particular maturity stage underwent gross morphological analysis; the minimum and maximum values for FL and gonad length were identified. Subsequently, gonads that underwent histological analysis were selected randomly from the same stock of gonad samples of each stage. This can be observed through the fact that the FL range for micro is within the FL range of macro.

2.5. Histology of gonads

Approximately 1 cm thick cross-sections across the middle portion of the gonads were taken and fixed in a 10% buffered formaldehyde solution. Gonads were then dehydrated in increasing alcohol concentrations, cleaned in xylene, and embedded in paraffin wax afterward. Tissue thicknesses of 5–10 µm were then taken from the gonad tissue block using an automated microtome. Hematoxylin and eosin stains were used. We have designated at least three (3) samples for histological examinations per stage of gross gonadal development and integrated serial sectioning. The present study followed the previous reports by Bahou et al. (2016) and Bahou et al. (2017) on determining the histological variations among the samples.

2.6. Morphological diagnostic of gonads

Phases of maturity were identified according to the methods standardized by the General Fisheries Commission for the Mediterranean (GFCM) macroscopic maturity scale for bony fish (ICES WKASMSF 2018), while the ovarian and testicular identification followed was characterized and described by Bahou et al. (2016) and Bahou et al. (2017).

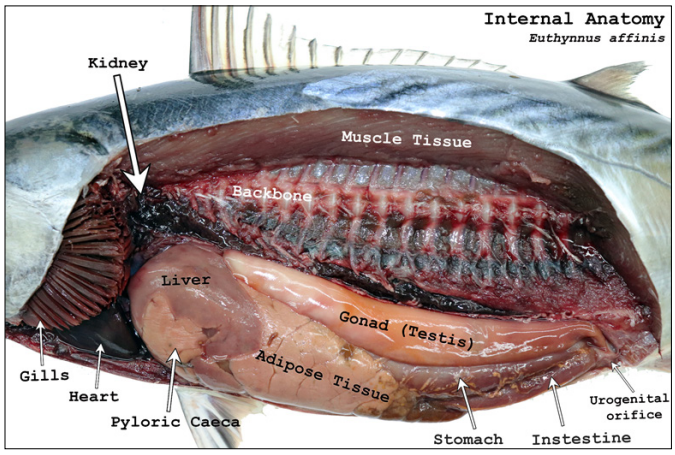

Figure 2. Internal anatomy of *E. affinis* as represented by the male. Photo by Entia JCD and Nabre NMB.

3. RESULT

3.1. Biological characteristics

Eastern Little Tuna (*Euthynnus affinis)* is characterized by its narrow interspace at the dorsal section and the black spots at the pelvic fin region. The study developed a scheme for categorizing the sizes of Eastern Little Tuna (*Euthynnus affinis)*: Size 1 at ≤ 30 cm, Size 2 at 30.1 – 40cm, Size 3 at 40.1–50 cm, and Size 4 (> 50 cm) (Figure 3).

3.1.1. Female

At Stage 1 (Immature), the ovary length was at 3.00–16.25cm, with the corresponding fork length of the fish measuring at 14.0–44.80 cm. The appearance of the gonad at this stage was dark pink in color, with an ovary shorter than one-third of the body cavity and no eggs (granules) visible to the naked eye. At the tissue level, histoarchitecture of the female at this stage revealed the presence of previtellogenic oocytes (pr) within the cytoplasm (Cyt) surrounded by the oocyte membrane (OM). The intercellular matrix was primarily occupied by the interstitial connective tissue (ICT). FL of fish samples with this reproductive stage at the histological level was identified to be at 20.40–36.0 cm (min. - max.).

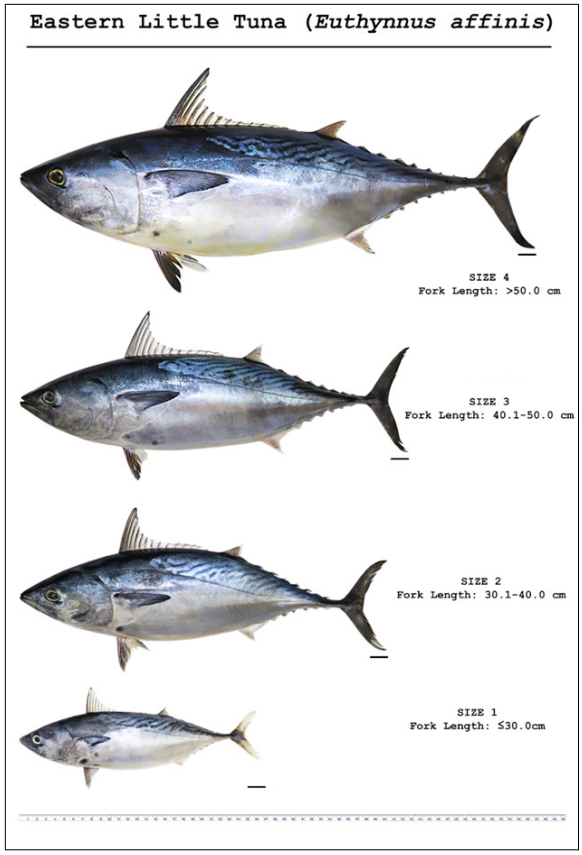

Figure 3. Determined fish size classes of *E. affinis*: Size 1 (≤ 30 cm), Size 2 (30.1–40.0 cm), Size 3 (40.1–50.0 cm), Size 4 (> 50.0 cm). Bar = 2.0 cm. Photo by Entia JCD and Nabre NMB.

In Stage 2 (Onset Maturation), the color of the ovary was observed to lighten, where the color was changing from red to orange (pink or yellow). Venation was also initially visible at this phase. The length of the ovary at this stage was identified at 9.30–14.35 cm, belonging to fish samples of FL 25.30–47.0 cm. per histoarchitecture, while previtellogenic oocytes (pr) were still present, primary vitellogenic oocytes (Ost-I) were observed to dominate the ovarian space, with its much enlarged, prominent nucleus (N). Supporting structures pertinent to the maturation of the oocytes, such as the ICT and the oocyte membrane, were also still visible at this phase. Fish size of *E. affinis* samples of this reproductive maturity (histology-wise) was identified at 21.70–27.40 cm (FL).

In Stage 3, appropriately called the Developing or Maturing phase, the size of the lobes increased significantly. Eggs were more visible to the naked eye through the ovarial tunica, which was not translucent. However, although visible at this point, the eggs did not freely escape under light pressure. At Stage 3, the length of the ovary occupied about two-thirds of the body cavity, identified to be at 10.65–15.85 cm. The FL of *E. affinis* samples of this gonadal maturity stage was measured at 38.50–48.30 cm. Subsequently, upon examination of histomicrographs, the landmark histological feature of the ovary at this point was the secondary vitellogenic oocyte (Ost-II), the necessary successor to Ost-I. At this stage, along the edges of the oocytes, the zona radiata (ZR) is apparent, along with the theca (TQ) and the alveoli (alv). Yolk granules (YG) begin to appear in this stage. Stage 3 *E. affinis* samples possessing these histological traits were measured to be at 38.50–41.70 cm (FL).

Stage 4 (Spawning) is when the fish is actively mid-breeding. At this stage, the fullness of the gonad was more advanced, and the contents freely escaped under light pressure. The color was predominantly orange rather than pink, and the blood vessels were much more conspicuous on the surface. The ovary length at this stage was identified at 11.55–19.80 cm, with the corresponding fork length of the fish samples at 41.50–49.70 cm. At the tissue level, the ovaries of this stage showed the presence of a mature follicle (FO) containing yolk granules (YG), with a distinguishable follicular epithelium (FE) at the border of the follicle. Fish samples at this stage of reproductive development (tissue-wise) measured 41.50–56.70cm in FL.

At Stage 5 (Spent), the gonad showed a dramatic decrease in size, evidently due to the release of the contents of the ovaries. The color of the ovaries was dark red, with the walls flaccid, containing remnants of disintegrating opaque and translucent eggs. Ovaries at this stage measured between 14.15–14.20 cm, belonging to fish samples of fork length measuring 45.10–49.70 cm (FL). This atrophy of the gonads was supported by the numerous "holes" observed upon microscopic examination: vacuoles (vac) dominated the space, with empty follicles (EF) and post-ovulatory follicles (POF) also remaining, visible evidence of the post-spawn phase. Also present in the interstitial space were numerous invaginated follicular epithelia (invaFE) and remaining mature follicles (FO). Meanwhile, the fish length of *E. affinis* collected falling under this stage in sexual maturity at this histological level was measured at 49.0–58.60 cm (FL).

In Stage 6 (Recovering or Resting), the ovarian walls, although still visibly flaccid, had acquired a comparable gain in mass, along with the apparentness of venation that was also beginning to reappear. The color of the ovaries also lightened from red to pink. The ovary length at Stage 6 was 14.35 cm, belonging to a 47.0 cm (FL) fish sample. Upon microscopic examination of tissues, we observed that the ovary of a recovering post-spawner displayed atretic follicles (AF), follicles in resorption state (FR), reconstitution of the interstitial connective tissue (recICT), and most notably, previtellogenic oocytes (pr). Collected samples of E. *affinis a*t this stage of reproductive development were estimated at 52.0–64.70 cm (FL).

3.1.2. Male

The lobes of the testis in Stage 1 (Immature) were thin and pale in color, with red-to-brown pigmentation on the surface, comparably more opaque than Stage 0. The length of the testes at this stage measured at 3.0-16.25 cm, belonging to fish samples of between 20.50-44.60 cm (FL). Histological investigation of this stage revealed the occurrence of tubules (Tb) and lobules (Lb), however they were difficult to distinguish due to the cortical and medullary regions being mostly eosinophilic at this stage. Fish samples in Stage 1 of maturity were determined to be between 13.70–16.40 cm (FL).

The red pigmentation was still observable by Stage 2 (Onset Maturation), although the lobes by this point had developed to become elongated and symmetrical, occupying almost half of the body

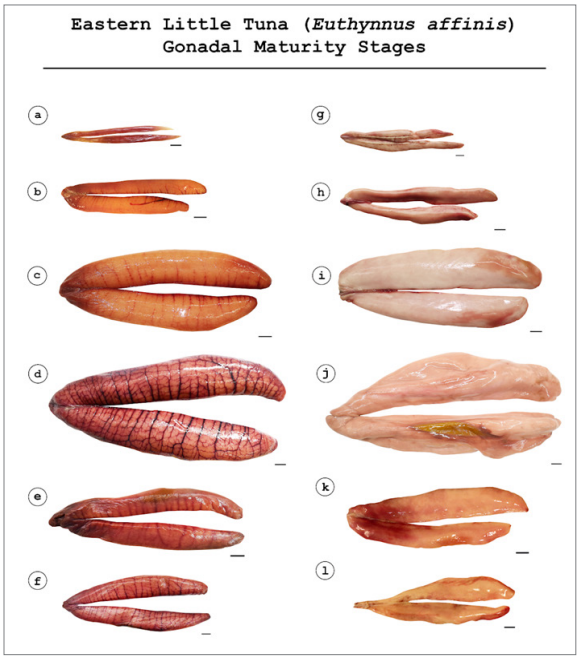

Figure 4. Ovarian (left) and testicular (right) development of Eastern Little Tuna *(E. affinis*). (a) Stage 1 (Immature) ovary; (b) Stage 2 (Onset Maturation) ovary; (c) Stage 3 (Developing/Maturing) ovary; (d) Stage 4 (Spawning) ovary; (e) Stage 5 (Spent) ovary; (f) Stage 6 (Recovering/Resting) ovary; (g) Stage 1 (Immature) testis; (h) Stage 2 (Onset Maturation) testis; (i) Stage 3 (Developing/Maturing) testis; (j) Stage 4 (Spawning) testis; (k) Stage 5 (Spent) testis; (l) Stage 6 (Spent) testis. Bar = 2.0 cm. Photo by Entia JCD and Nabre NMB.

cavity, measuring 7.80–13.95 cm. FL of fish samples of this gonadal stage was identified at 24.10–40.0 cm. For its histology, the medulla and cortex were more distinguishable, with the medullary region showing a higher hematoxylin (purple) stain. There was a higher occurrence of tubules (Tb) and lobules (Lb) as well. Stage 2 (at the histology level) *E. affinis* samples were 24.50–26.0 cm in fork length.

At Stage 3 (Developing or Maturing), the color of the lobes was fully opaque and white, with limited red-orange pigmentation at the end of the lobes. At this stage, the testis occupied almost two-thirds of the body cavity as the lobes gained more mass and thickness in size. Despite this, the contents were not immediately expelled when light pressure was applied. The length of the testes ranged between 13.0–17.75 cm, belonging to fish samples with fork lengths of 38.0–44.80 cm. Investigation of the histoarchitecture of the male gonad at this stage revealed the occurrence of primary spermatocytes (Sp-I), simultaneous with secondary spermatocytes (Sp-II), spermatids (Sptd), and the sperm duct (SPD). Tubules (Tb) were also observable at this stage. Male *E. affinis* samples at this histological reproductive stage were 23.2–27.0 cm in size (FL).

Stage 4 (Spawning) testes were fully white and wrinkled in texture, with the length spanning from two-thirds to the entire body cavity of the fish. At this stage, the lobes were fully replete, indicating ripeness, with their contents dispersing freely, suggesting advancement towards the spawning stage. Testis lengths of Stage 4 samples were at 14.50–26.05 cm, found in fish samples with fork lengths ranging from 42.50 cm to 64.70 cm. On the tissue level, Stage 4 shared characteristics with Stage 3, with the addition of the main sperm duct (MSD), spermatozoa (Spz), and lobular lamina (LL). *E. affinis* samples at this stage were measured at 26.40–28.30 cm (FL).

In Stage 5 (Spent), the loss in mass was also the most noticeable attribute of the gross morphology of the testis. The lobes of the testis regained their red-orange pigmentation at this stage and smoothness in appearance. Also, the length of the testis measured 14.05 cm, belonging to samples measuring 44.70 cm. Following the loss in mass, the histoarchitecture also showed less occurrence of spermatozoa (Spz). The fish size of *E. affinis* at this histological stage was 31.80–33.0 cm (FL).

The color of the testes in Stage 6 (Recovering or Resting) was noticeably lighter than in the previous stage—more orange compared to the strong red color observed in Stage 5. As sperm regeneration occurs at this stage, similar to Stage 3, the lobes in this stage were observed to be increasing in size (the testis were 16.0 –17.0 cm in length, belonging to fish samples of 55.70 cm in fork length). However, unlike in Stage 3, the texture of the lobes was flaccid and atrophied in appearance, with the length occupying only one-third of the body cavity. Examination of histomicrographs revealed the occurrence of residual spermatozoa (RSpz) and the widespread presence of the sperm duct (SPD). The size of *E. affinis* at this stage of reproductive maturity was 28.0–33.0 cm (FL). A comprehensive outline of the histology photomicrographs of female (Figure 5) and male (Figure 6) and reproductive

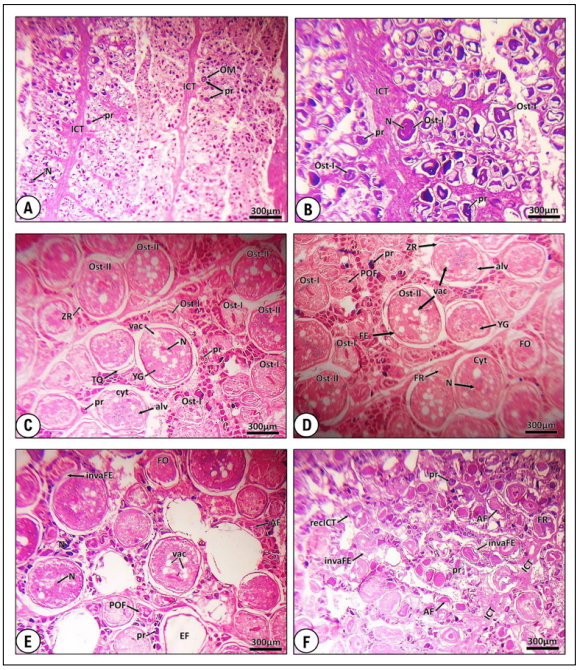

Figure 5. Histoarchitecture of the ovary of Eastern Little Tuna (*E. affinis*). (A) Stage 1 (Immature): pr = previtellogenic oocytes, ICT = interstitial connective tissue, OM = oocyte membrane; (B) Stage 2 (Onset Maturation) Ost-I = primary vitellogenic oocyte, N = nucleus; (C) Stage 3 (Onset Maturation): Ost-II = secondary vitellogenic oocyte, ZR = zona radiata, alv = alveoli, cyt = cytoplasm, TQ = theca, (D) Stage 4 (Spawning): FE = follicular epithelium, YG = yolk granules, FO = mature follicle; vac = vacuoles (E) Stage 5 (Spent): invaFE = invagination of the follicular epithelium; POF = post-ovulatory follicle, EF = empty follicle, AF = atretic follicle; (F) Stage 6 (Recovering/Resting): recICT = reconstituting interstitial connective tissue; FR = follicle in resorption state. M x 400. (H&E). Bar = 300µm. Photo by Entia JCD and Nabre NMB.

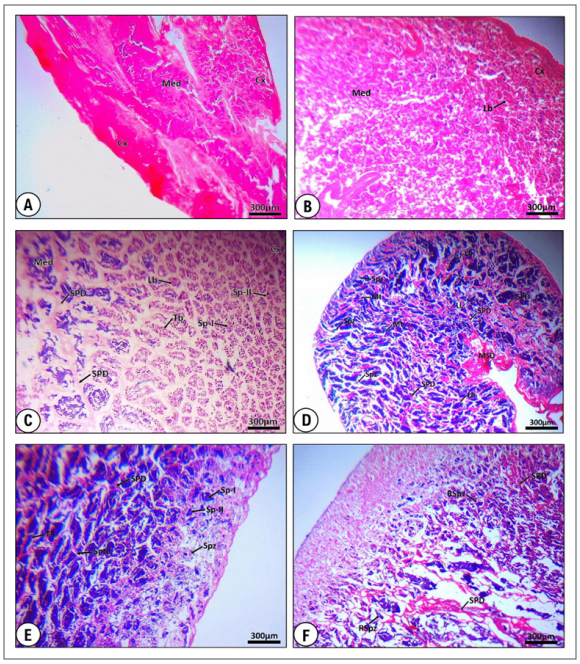

Figure 6. Histoarchitecture of testis of male Eastern Little Tuna (*E. affinis*). (A) Stage 1 (Immature): Med = medulla, Cx = cortex; (B) Stage 2 (Onset Maturation): Lb =lobules; Tb = tubules; (C) Stage 3 (Onset Maturation): SPD = sperm duct, Sp-I = primary spermatocyte, Sp-II = secondary spermatocyte, Lb = lobule; (D) Stage 4 (Spawning): LL = lobular lamina, MSD = main sperm duct, Spz = spermatozoa, Mt = milt; Sptd = spermatid (E) Stage 5 (Spent): Sptd = spermatid, Spz = spermatozoa; (F) Stage 6 (Recovering/Resting): RSpz = residual spermatozoa. (M × 400). (H&E). Bar = 300µm. Photo by Entia JCD and Nabre NMB.

stages, landmark characteristic(s) of each stage, testis length, and corresponding fork lengths for these can be found in Figure 7 and Tables 1 and 2.

4. DISCUSSION

For the majority of teleosts, gonadal development is cyclical and seasonal. Gonadal changes that distinguish reproductive stages from one another are caused by the renewal, differentiation, growth, and release of sperm and oocytes throughout each breeding cycle. Changes in the morphology of gonadal fish during reproduction indicate significant ecological and behavioral adaptations (Coward et al. 2002; Fishelson and Gon 2008; Martins et al. 2012). Fish gonad maturation phases must also be understood for commercially relevant concerns, such as identifying mature stocks and determining their size or age at first maturity (Bagenal 1978), determining the reproductive potential of fish populations, keeping track of changes in the biological characteristics of exploited fish stocks (Williams 2007), and determining the reproduction and gonadal maturation period are all important data needed to ensure that fisheries management plans are implemented correctly (Gonçalves et al. 2006). In this study, the cyclical nature of the maturation of *E. affinis*, where the gonad regenerates after releasing its contents, revealed that its development strategy is asynchronous.

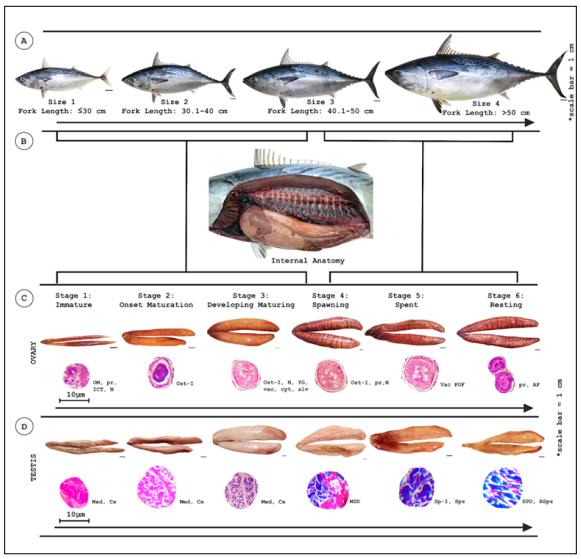

Fig. 7. Comprehensive relationship concerning fish sizes to macrostructure and histoarchitecture of male and female gonads. (A) Fish sizes: Size 1 (≤ 30 cm), Size 2 (30.1–40.0 cm), Size 3 (40.1–50.0 cm), Size 4 (> 50.0 cm), (B) Fish Internal anatomy, (C) Ovarian development through gonad macrostructure and its histoarchitecture: 1 (Immature), 2 (Onset Maturation), 3 (Developing/Maturing), 4 (Spawning), 5 (Spent), 6 (Recovering/Resting) and (D) Testicular development gonad macrostructure and its histoarchitecture: 1 (Immature), 2 (Onset Maturation), 3 (Developing/Maturing), 4 (Spawning), 5 (Spent), 6 (Recovering/Resting). Bar = 2.0 cm (fish); 1.0 cm (gonad); 10 µm (photomicrographs). Photo by Entia JCD, Nabre NMB, and Dela Cruz NA.

Table 1. Female reproductive maturity stages based on macro (gonad appearance) and micro (histological characteristics) in relation to fork length (FL) of *E. affinis*. Size classes based on FL were as follows: Size 1 (≤ 30.0 cm), Size 2 (30.1–40.0 cm), Size 3 (40.1–50.0), and Size 4 (> 50.0 cm). Nomenclature for both macro and micro aspects are the same: Stage 1 (Immature), Stage 2 (Onset Maturation), Stage 3 (Developing/Maturing), Stage 4 (Spawning), Stage 5 (Spent), Stage 6 (Recovering/Resting). Highlighted row shows that at Size 3 (40.1–50.0 cm), female *E. affinis* is spawning/participating in reproduction, characterized by 38.50–41.70 cm in FL based on (a) microstructures secondary vitellogenic oocyte (Ost-II), vacuoles (vac), follicular epithelium (FE), yolk granules, and mature follicles (FO); of its macrostructure, ovary length is between 11.55 –19.80 cm, fullness of the gonads advanced and the contents freely escaping under light pressure; blood vessels conspicuous on the surface. FL based on microstructure is 41.50–56.70 cm, while FL based on (b) macrostructure is 41.50–49.70 cm. Size classes in relation to FL overlap due to the asynchronous reproductive strategy of tuna species.

FISH BODY SIZE	STAGE	PHASE	GROSS MORPHOLOGY LANDMARK (MACRO)	FL OF FISH BASED ON MACRO (cm)	HISTOLOGY LANDMARK (MICRO)	FL OF FISH BASED ON MICRO (cm)	SIZE CLASS
Size 1 ≤ 30 cm	1	Immature	dark pink in color, with no visible eggs (granules) visible to the naked eye, length at 3.00 - 16.00 cm (min – max)	14.0 - 44.80	previtellogenic oocytes (pr)	20.40 - 36.0	Sizes 1, 2, 3
Size 2 30.1 – 40.0 cm	2	Onset Maturation	ovary is lighten, where the color is changing to orange (pink/yellow) from red, venation was visible at this phase, length at 9.30-14.35 cm	25.30 - 47.00	primary vitellogenic oocyte (Ost – I)	21.70 - 27.40	Sizes 1, 2, 3
Size 3 40.1 – 50.0 cm	3	Developing/ Maturing	eggs visible to the naked eye although visible at this point, the eggs did not freely escape under light pressure, ovary length at 10.65 - 15.85 cm	38.50 - 48.30	secondary vitellogenic oocyte (Ost-II)	38.50 - 41.70	Sizes 2, 3

Continuation Table 1. Female reproductive maturity stages based on macro (gonad appearance).....

FISH BODY SIZE	STAGE	PHASE	GROSS MORPHOLOGY LANDMARK (MACRO)	FL OF FISH BASED ON MACRO (cm)	HISTOLOGY LANDMARK (MICRO)	FL OF FISH BASED ON MICRO (cm)	SIZE CLASS
Size 3 40.1 – 50.0 cm	4	Spawning	fullness of the gonad is more advanced that the contents freely escaped under light pressure and the blood vessels are much more conspicuous on the surface, ovary length at 11.55 - 19.80 cm	41.50 - 49.70	secondary vitellogenic oocyte (Ost-II), vacuoles (vac), follicular epithelium (FE), mature follicle (FO), yolk granules (YG)	41.50 -56.70	Sizes 3, 4
	5	Spent	decreased in size, evidently due to the release of the contents, color is dark red, with the walls flaccid, containing remnants of disintegrating opaque and translucent eggs, length at 14.15 -14.20 cm	45.10 - 49.70	empty follicle (EF)	49.0 - 58.60	Sizes 3, 4
	6	Recovering/ Resting	the ovaric walls, visibly flaccid, had regained comparable gain in mass, venation also beginning to reappear, ovaries also lightened from red to pink, length at 14.35 - 47.00 cm	47.00	follicle in resorption state (FR)	52.0 - 64.70	Size 4

Table 2. Male reproductive maturity stages based on macro (gonad appearance) and micro (histological characteristics) in relation to fork length (FL) of *E. affinis*. Size classes based on FL were as follows: Size 1 (≤ 30.0 cm), Size 2 (30.1–40.0 cm), Size 3 (40.1–50.0), and Size 4 (> 50.0 cm). Nomenclature for both macro and micro aspects are the same: Stage 1 (Immature), Stage 2 (Onset Maturation), Stage 3 (Developing/Maturing), Stage 4 (Spawning), Stage 5 (Spent), Stage 6 (Recovering/Resting). Highlighted row shows that at Size 3 (40.1–50.0 cm), male *E. affinis* is spawning/participating in reproduction, characterized by 40.1 – 50.0 cm in FL based on (a) microstructures spermatid (Sptd), spermatozoa (Spz); of its macrostructure, testis length is between 14.50–26.05 cm, fullness of the gonads advanced and the contents freely escaping under light pressure; blood vessels conspicuous on the surface. FL based on microstructure is 26.40–28.30 cm, while FL based on (b) macrostructure is 42.50–64.70 cm. Size classes in relation to FL overlap due to the asynchronous reproductive strategy of tuna species.

FISH BODY SIZE	STAGE	PHASE	GROSS MORPHOLOGY LANDMARK (MACRO)	FL OF FISH BASED ON MACRO (cm)	HISTOLOGY LANDMARK (MICRO)	FL OF FISH BASED ON MICRO (cm)	SIZE CLASS
Size 1 ≤ 30 cm	1	Immature	thin and pale in color, with red-to-brown pigmentation on the surface, comparably more opaque, legth at 3.0 - 16.25 cm (min – max)	20.50 - 44.60	medulla (Med), cortex (Cx)	13.70 - 16.40	Sizes 1, 2, 3
Size 2 30.1 – 40.0 cm	2	Onset Maturation	lobes by this point had developed to become elongated and symmetrical, occupying almost half of the body cavity, measuring 7.80 -13.95	24.10 - 40.0	tubules (Tb), lobule (Lb)	24.50 - 26.0	Sizes 1, 2
Size 3 40.1 – 50.0 cm	3	Developing/ Maturing	fully opaque and white, with limited red-orange pigmentation at the end of the lobes, occupies almost two-thirds of the body cavity as the lobes gain more mass and thickness in size, length at 13.00 - 17.75 cm	38.0 - 44.80	primary spermatocyte (Sp-I), secondary spermatocyte (Sp-II), spermatid (Sptd)	23.20 - 27.0	Sizes 1, 2, 3
	4	Spawning	fully white in color, and wrinkled in texture, with the length spanning from two-thirds to the entire body, contents escaping freely in more advanced conditions, length at 14.50 - 26.05 cm	42.50 - 64.70	spermatid (Sptd), spermatozoa (Spz)	26.40 - 28.30	Sizes 1, 2, 3, 4

Continuation Table 2. Male reproductive maturity stages based on macro (gonad appearance).....

FISH BODY SIZE	STAGE	PHASE	GROSS MORPHOLOGY LANDMARK (MACRO)	FL OF FISH BASED ON MACRO (cm)	HISTOLOGY LANDMARK (MICRO)	FL OF FISH BASED ON MICRO (cm)	SIZE CLASS
Size 3 40.1 – 50.0 cm	4	Spawning	fully white in color, and wrinkled in texture, with the length spanning from two-thirds to the entire body, contents escaping freely in more advanced conditions, length at 14.50 - 26.05 cm	42.50 - 64.70	spermatid (Sptd), spermatozoa (Spz)	26.40 - 28.30	Sizes 1, 2, 3, 4
Size 4 >50.0 cm	5	Spent	loss in mass was also the most noticeable attribute, regained their red-orange pigmentation, smoothness in appearance. 14.05 - 15. 05	44.70	less spermatozoa (Spz)	31.80 - 33.0	Sizes 1, 2, 3
	6	Recovering/ Resting	comparably lighter than in the previous stage–orange, regeneration of sperm occurs, observed to be increasing in size, length at 16.00 -17.00 cm	55.70	residual spermatozoa (RSpz)	28.0 - 33.0 cm	Sizes 1, 2, 3, 4

4.1. Morphology and histoarchitecture of gonads: Female

In a study by Beytullah and Yusuf (2019), the ovarian phase, gonad growth, and gamete development in *M. barbatus* were observed to possess the formation of its perinucleolus (immature) stage oocytes with multiple nuclei and it observed that the ovaries were yet to be active and functional. In this present study on *E. affinis*, meanwhile, the immature stage was also found to possess the same features; at Stage 1, the tissue space is mostly occupied by interstitial connective tissue (ICT), cytoplasm (Cyt), oocyte membrane (OM), and previtellogenic cells (pr)—the primitive form of what would later become actual oocytes. At this stage, the previtellogenic cells are on the periphery of the gonad, and instances of the nucleus being differentiated are rare and are found at the center. Similar findings were also found in this investigation according to studies by Valdés et al. (2004), Mackie and Mackie (2009), and Kokokiris et al. (2014) on snapper (*Pagrus auratus*), red mullet (*Mullus barbatus*), and common pandora *Pagellus erythrinus (L.)*, where the oogonia in the chromatin-nucleolus stage have big nuclei and light-stained cytoplasm. The huge, spherical nucleus is present. The majority of the cell is made up of a nucleolus that is encased in fibrous connective tissue.

In this study, at stage 2 (Onset Maturation), while previtellogenic oocytes (pr) are still present, primary vitellogenic oocytes (Ost-I) begin to dominate the space, with the much more enlarged, prominent nucleus (N) being the landmark. In the reports of Marino et al. (2001), McMillan (2007), and Mahmoud (2009), small previtellogenic oocytes are visible in the peri nucleolus stage, where the outer layer of follicular epithelial cells surrounding the oocyte thickens but is not differentiated. Towards the end of this stage, the oocyte is characterized by the appearance of one or several small vacuoles in the cytoplasmic mass. These vacuoles begin to arrange themselves around the nucleus later in the stage. Similar to the results of other studies, the oocytes contain a homogeneous cytoplasm, a large nucleus, and a large number of small elongated-looking peripheral nucleoli, which are more numerous and peripheral in the distribution in this stage than in the other stages.

In comparison to our study, upon actual maturation (Stage 3; Developing or Maturing), the secondary vitellogenic oocyte (Ost-II) begins to show as the necessary successor to the primary vitellogenic oocyte (Ost-I), with the prominent presence of vacuoles (vac), zona radiata (ZR) becoming its apparent boundary and the theca (TQ) providing the follicle the nutrients it needs as it further matures. The alveoli (alv), being blood vessels, also serve this function. Yolk granules (YG) begin to appear in this stage as well.

Common between Stage 3 and Stage 4 (Spawning) are the yolk granules (YG), which contain the necessary proteins for the embryo that will develop once the oocyte is fertilized. It should be noted that Ost-II cells dominate most of the space in Stage 4. The yolk granules (YG) are mixed up and scattered throughout the cytoplasm (Cyt). Moreover, the follicular epithelium (FE), mature follicle (FO), and cortical alveoli are discernible during this developmental stage, as highlighted by McMillan (2007), ÇakıcıÖ and Üçüncü (2007), and Çelik (2005),

along with findings from Kokokiris et al. (2014) and Mackie and Mackie (2009). These sources collectively affirm the presence of cortical alveoli proximal to the outer surface of the oocyte's glycoprotein structure. Based on Koç et al. (2008), the ooplasm's granular structures grow, and the cortical alveolar structures expand in size as the oocyte and follicle also grow. Also, at this stage, the region around the oocyte nucleus turns opaque and the nucleus develops. The zona radiata is pushed across the nucleus as the nucleolus develops, thickening the follicle. There is a frequent increase in the nucleus membrane's invaginations, and the nucleus structure appears shapeless.

According to Mahmoud (2009), who investigated the gonadal histology of Epinephelus areolatus (areolate grouper) and Lethrinus nebulosus (spangled emperor), atresia is a risk for fish that do not lay eggs because a substantial portion of total consumed ovaries contains a lot of underdeveloped follicles. Incidentally, these occurrences were also found in this study, in which atretic follicles (AF) were found in the ovaries in Stage 5 (Spent). The structures left behind post-spawning—the invaginated follicular epithelium (invaFE), empty follicles (EF), and post-ovulatory follicle (POF)—all histoarchitectural indications that the fish has exerted its reproductive potential.

In the study of *M. barbatus* by Junqueira in 1992, atretic follicles were observed in the post-ovulation stage—the nucleus starts to disintegrate following the chromatin deformation, and space is formed in the vitelline membrane (Koç et al. 2008; Mahmoud 2009), supporting the findings in this report that atretic follicles, although potentially harmful for fishes (Mahmoud 2009), is an inevitable occurrence in the oogonial matrix at the conclusion of the release of eggs. Furthermore, in Stage 6 (Recovering or Resting) of this study, while atretic follicles (AF) are also still present in this stage, the reconstitution of the interstitial connective tissue (recICT) and the follicles in resorption state (recICT) and most importantly, the reappearance of the previtellogenic oocyte (pr) signal the regeneration of the tissues in preparation for the next breeding opportunity.

4.2. Morphology and histoarchitecture of gonads: Male

In the histological analysis of the reproductive properties of frigate tuna *(Auxis thazard*) conducted by Bahou et al. (2017), the testis in Stage 1 (Immature) lacks definable structures—only the medulla and cortex when viewed at low magnification. Similarly, only the medulla and cortex are the definable features in this study. As noted by Valdes et al. (2004) in their study on the reproductive maturation stages in common pandora (*Pagellus erythrinus L.)*, testicular development this early in development exhibits only a modest number of primary spermatocytes.

Lobules (Lb) and tubules (Tb) begin to be apparent in Stage 2 (Onset Maturation), as sites where germ cells condense, as evidenced by the increase in the concentration of hematoxylin, which binds to chromatin. The transition between the medulla and the cortex becomes more defined, with a higher occurrence of Tb and Lb towards the center. In the investigation conducted by Beytullah and Yusuf in 2019 on the seasonal gonad maturation of red mullet (*Mullus barbatus* L. 1758), both spermatozoa and a large number of spermatids were found in the testis and deferens ducts. The stages of spermatogonium, primary and secondary spermatocytes, spermatid, and finally, spermatozoa were finished in the lobules, which were divided into compartments by thick connective tissue.

At Stage 3, within the lobules and tubules, we observed primary spermatocytes (Sp-I), secondary spermatocytes (Sp-II), and spermatids (Sptd) in increasing density. The sperm duct (SPD) was observed at this stage, which serves as the conduit of the spermatocytes from its place in the testis to the urogenital orifice. In the investigation of the cyclic variations of gonad development in air-breathing fish (Chama striata), Al Mahmud et al. (2016) observed that the presence of spermatozoa, the smallest cells of the spermatogenic lineage, appeared as dark (almost black) spots under the microscope. These dark-colored spots were also observed at this stage in this study.

Histological analysis in this study also showed that in Stage 4 (Spawning), the arrangement of testicular lobular and tubular tissues was observed with spermatozoa (Spz) appearing in small, dark spots with a high concentration of hematoxylin, along with the lobular lamina (LL), main sperm duct (MSD), sperm duct (SPD), primary spermatocytes (Sp-I), secondary spermatocytes (Sp-II), and spermatids.

Sp-I, Sp-II, Sptd, and Spz occur less in Stage 5 (Spent) due to their release in the event of Spawning. Mohapatra et al. (2020) reported a similar pattern of testicular development in the post-spawning stage of the reproductive cycle and gonadal development in the climbing perch, *Anabas testudineusgon*, where the stage was characterized by loosely packed spermatozoa or empty lumens of the constricted seminiferous tubules.

Residual spermatozoa (RSpz), along with the widespread presence of the sperm duct (SPD), occur in Stage 6 (Recovering or Resting). Germ cells are largely absent from the lobules and tubules. In the testicular tissue of male fish in this particular stage, the connective tissue predominates while spermatozoa are not produced, as mentioned by Kokokiris et al. (1999), Valdes et al. (2004), and Macki et al. (2009). Additionally, they observed that the sperm sinuses shrink during the resting stage and that there is little residual sperm. The existence of regressed testicular tissue in this report after the spawning season is consistent with the other findings of earlier investigations.

5. CONCLUSION AND RECOMMENDATIONS

To date, this study is the first report on the reproductive status in relation to the fish length of Eastern Little Tuna *(Euthynnus affinis)* in the Philippines. This study reveals that male and female *E. affinis* are sexually mature at Size 3, between 40.1–50.0 cm, and spawning thereof follows. While asynchronous strategies and variations in size ranges may introduce overlapping elements, our focus pertains to the examination of Stage 4 gonadal development and the subsequent investigation of its histoarchitecture These endeavors have revealed that *E. affinis* exhibit spawning behavior during this developmental stage. However, this study considers only the months of collection in the Southern Philippines, the high proportion of immature individuals, opportunistic sampling, and the natural loss of the fish species in the area. Therefore, further studies investigating the waters not covered in this report are suggested. We also advocate for additional experiments on scanning electron microscopy (SEM) and delve deeper into the molecular aspects of reproduction within this species. Thus, we propose that future tuna management plans and policies involving the collection areas be geared towards protecting those under 50 cm in length so the young can mature fully and thus ensure their reproductive success and potential.

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CONFLICTS OF INTEREST

We claim to have no knowledge of conflicting personal or financial interests that could have been found to impact the research described in this study.

AUTHOR CONTRIBUTIONS

Entia JCD: Writing - Original Draft, Methodology, Data curation, Formal analysis, Investigation and Visualization. N**abre NMB**: Data curation, Formal Analysis, Investigation, Visualization, and Project Administration. **Castrence GA**: Supervision. **Arellano BJG**: Writing - Review & Editing. **Amoncio RADA**: Data Curation. **Wetzel JT**: Supervision. **Guevarra EP**: Conceptualization, Supervision.

ETHICS STATEMENT

Authorization and collaboration were granted by the Department of Environment and Natural Resources - Protected Area Management Board (DENR- PAMB) Region XII, BFAR-NSAP Region XI and XII, Philippine National Police Maritime Group (PNP-MG) Regional XII, Philippine Coast Guard (PCG) Region XII, Local Government Units (LGUs) of General Santos City and, 6 Municipalities of Sarangani Province (Alabel, Malapatan, Glan, Maasim, Kiamba, and Maitum). Our research adhered to significant institutional and national guidelines and used independent and dependent sampling techniques.

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