Gametogenesis, spawning and larval development of *Isostichopus* sp. aff *badionotus*

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Abstract

The sea cucumber family Stichopodidae is distributed throughout the Caribbean Sea. *Isostichopus* species are widespread in the entire Caribbean region, but little is known about the biology of this native species, especially on the Colombian coast. This study presents a description of the gametogenesis, natural spawning, and embryonic and larval development of *Isostichopus* sp. aff *badionotus*. Observed gonadosomatic index values were highest between August and November. Recovery, growth, mature, partially spawned and postspawning were the stages recorded by female gonad histology while in males, the observed stages were recovery, growth, mature and postspawning. The growth stages occurred in almost all of the sampling periods. During two consecutive years, natural spawnings were observed in the laboratory in the second half of each year (from July to November). Larval rearing reached the doliolaria stage. The animals were fed with a mix of *Thalassiosira* sp. (commercial food) and microalgae (*Nannochloropsis* sp). Larval mortalities were associated with infestation by copepods in the culture.

Introduction

Sea cucumbers are benthic marine invertebrates that are known worldwide for their ecological importance and economic value. During recent years, Caribbean sea cucumbers became the target in the search of sea cucumber species of a similar quality to those of Asian origin. But, sea cucumbers of the Colombian Caribbean Sea have not been well studied (Caycedo 1978; Borrero-Pérez et al. 2003; Toral-Granda 2008; Toral-Granda et al. 2008; Rodríguez et al. 2013; Agudelo and Rodríguez 2015; Vergara and Rodríguez 2016). There are many gaps in basic knowledge, including the reproductive biology of native Caribbean sea cucumbers. This study describes the gonadal morphology and gonadal development of wild *Isostichopus* sp. aff *badionotus*, including the spawning period, sex ratio, and evaluation of synchronisation stages of maturity between females and males. It also describes the larval development until the doliolaria stage. *Isostichopus* sp aff *badionotus* is roughly similar to I. *badionotus* but differs from it on some morphological and molecular (DNA) levels that will be discussed in another paper. The work provides records on I. sp aff *badionotus* reproduction, early development, and economic value, and constitutes a significant contribution to the biological knowledge of this of native sea cucumber species of the Colombian Caribbean. Our goal was to have a benchmark for *I.* sp aff *badionotus* reproductive characteristics in an aquaculture environment, for its sustainable management in the wild.

Material and methods

Collection of animals

Between February 2013 and January 2014 adult broodstock of Caribbean sea cucumbers were purchased from local artisanal fishers in Rodadero Bay (11°13′ 22,73″N and 74°13′32, 59″ W) and Airport Bay (11°07′10″N and 74°13′50″W) in Santa Marta, Magdalena, Colombia (Fig. 1). Sea cucumbers were quickly taken alive in 20-L plastic tanks filled with seawater to the Aquaculture Laboratory of the Universidad del Magdalena. On arrival, they were conditioned in 550-L tanks (temperature: 26 ± 0.6°C; salinity: 36 ± 0.8 ppt; oxygen: 5.36 ± 0.74 mg L^{-1}), where they were kept until the time of dissection. The next day, individuals were sacrificed by hypothermia and photographed. At that time, they were weighed (± 0.1 g) (using a Navigator-OHAUS scale, USA), and total weight (TW), drained weight (DW) and gonad weight (GW) were recorded.

Gametogenesis study

The gonadosomatic index (GI) was approximated in accordance with Abdel-Razek et al. (2005):

$$GI = \frac{gw}{dw} \times 100$$

In total, 120 individuals were examined microscopically during the sampling period. In the months during which the gonad weight was low (≤ 1 g), a complete analysis of gonadal material was carried

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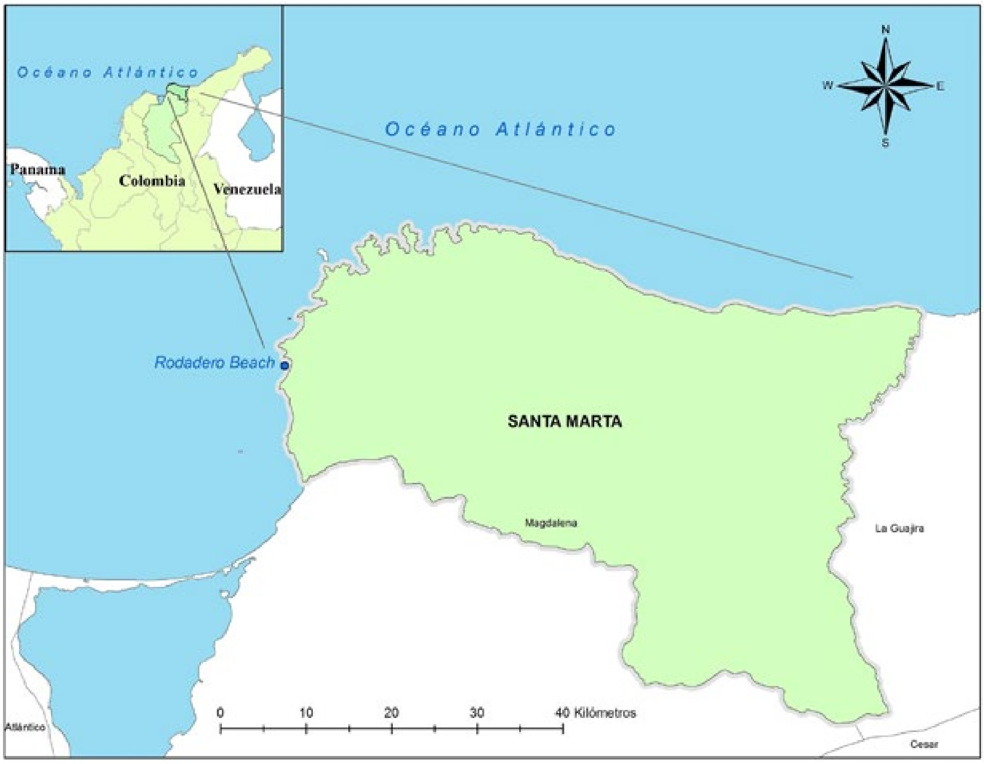

Figure 1. Marine and coastal areas of the Colombian Caribbean Sea (by Jose Viillacob). Blue point indicates the study area.

out, but in the months during which gonad weight was higher (> 1 g), one specific portion was selected for histological analysis (Fig. 2). The gonads were fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.2), for at least 24 h, then dehydrated in graded ethanol solutions and embedded in paraffin; they were subsequently sectioned in 5 microns lengths with a microtome (Sakura ® Accu-cut SRM), and the sections were stained with hematoxylin-eosin (H-E) (Drury and Walington 1980). A Zeiss microscope, equipped with camera AxioVision 4.8.2 software, was used to evaluate gametogenesis development and to measure the diameter of gonadal tubules and oocytes.

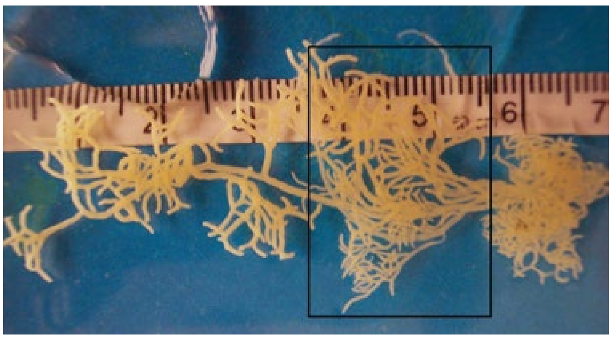

Figure 2. A fragment of gonadal tissue of *Isostichopus* sp. aff *badionotus* used for histological processing.

Sex determination, maturity stage and occurrence of reproductive events were assigned using the classification made by Ramofafia et al. (2000) and Fajardo-León et al. (2008).

Spawning and larval development

During the second half of 2014 and 2015, 100 adult sea cucumbers each year (232.09 ± 73.25g) were placed in ten 550-L tanks filled with sterilised seawater (temperature 26 ± 2.3°C; pH 7.8 ± 0.04; O2: 5.7 ± 0.22 mg L-1; salinity 36.5 ± 0.41 ppt), equipped with a biological filter, and aerated by air stones. The sea cucumbers were exposed to a 12-h light–dark photoperiod using overhead fluorescent lights. Faeces were siphoned out every day and water salinity was adjusted when needed. For broodstock maintenance, adults were fed with a mixture of marine sediment (previously washed and dried) and Spirulina powder (Artemia-International®) at a rate of 0.5 g per 100 g of sediment. Broodstock reproductive behaviour was monitored daily during the new moon phase. Two days before spawning, food was removed to avoid poor sea water quality (Agudelo and Rodríguez 2015). Natural spawning took place during the night or early in the morning. Successful fertilisation was recognised by the appearance of a fertilisation membrane. Fertilised eggs were siphoned and washed with filtered and sterilised sea water. To estimate the total number of eggs, samples were withdrawn using a 1-mL aliquot. After that, they were incu¬bated in 250-L tanks (1 larva mL^{-1}) at room temperature (26°C). Descriptions of the early development of *Isostichopus* sp. aff *badionotus* were made with the aid of light microscopy observations (Carl Zeiss, Modelo Primo Star), and photographic records were made with a digital camera (Axiocam ERC 5S). During 2013, the larvae were supplied with live algae (*Chlorella* sp.) at a concentration of 5,000 cells mL^{-1} but larvae did not grow (Agudelo and Rodriguez 2015). In 2014 and 2015, larvae were fed with a mix of live microalgae *Nannochloropsis* sp. and TW 1200 (Instant Algae® Products): early auricularia = 20,000 cells mL^{-1}; mid auricularia =40,000 cells mL^{-1} and late auricularia = 50,000 cells mL^{-1}. During 2015, we recorded the larval development until the doliolaria stage and described some abnormal eggs obtained.

Data analysis

Data from the GI were analysed by a simple analysis of variance test using Statgraphics program, where months were a factor and the variable response was the index value obtained in each of these months. To determine significant differences in the index during the months, nonparametric analysis of variance of Kruskal-Wallis (H) was applied, after verifying that the data did not meet the assumptions of normality (Kolmogorov-Smirnov test, $p < 0.05$) and homogeneity variance.

Results

Gametogenesis

Sea cucumbers had an average weight with standard deviation of 271.25 ± 84.93 g; while their average DW was 233.67 ± 78.10 g, and no significant differences were found between weights by gender: females had an average weight of 236.91 ± 82.40 g, while males had an average weight of 237.65 ± 74.87 g.

Isostichopus sp. aff *badionotus* is a gonochoric species that lacks external sexual dimorphism; the gonad has a bifurcated attachment to the dorsal mesentery, located on either side at the front of the body (Fig. 3). Gonads are organized by numerous branched tubules that group into a single duct that opens by the gonopore located in the anterior dorsal portion of the body.

Gonad sizes showed a gradual increase and the cell stages distribution was linked to the degree of development (Figs. 4a and b). During the reproductive cycle, some differences in colour and size of gonadal tubules for both sexes were found. Female gonads always presented a beige-yellow colouration and tubules were larger, reaching a diameter of 405.07 ± 248.19 μm (Figs. 5a and b), while males exhibited a whitish colour and thinner tubules with a diameter of around of 272.18 ± 56.37 μm (Figs. 5c and d). In most months, these characteristics were permanent in almost all samples, although sometimes two colours were apparent, allowing identification of gonadal development status, which also was corroborated by histological analysis.

From the 120 individuals examined 51.3% were male, 43.3% were females and 5.4% could not be differentiated because they had no gonads. The male:female ratio varied during the sampling

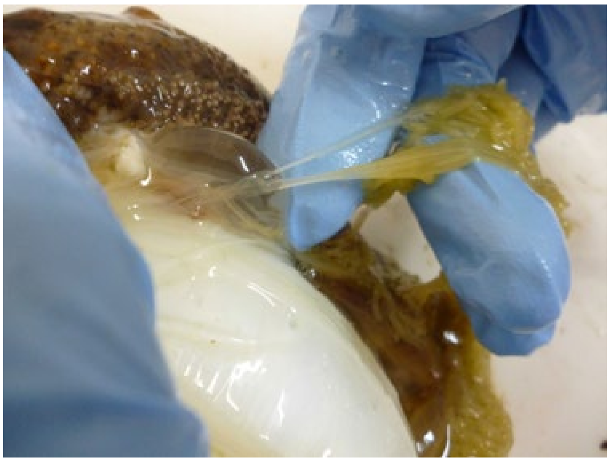

Figure 3. Bifurcated gonad on the *Isostichopus* sp. aff *badionotus* mesentery.

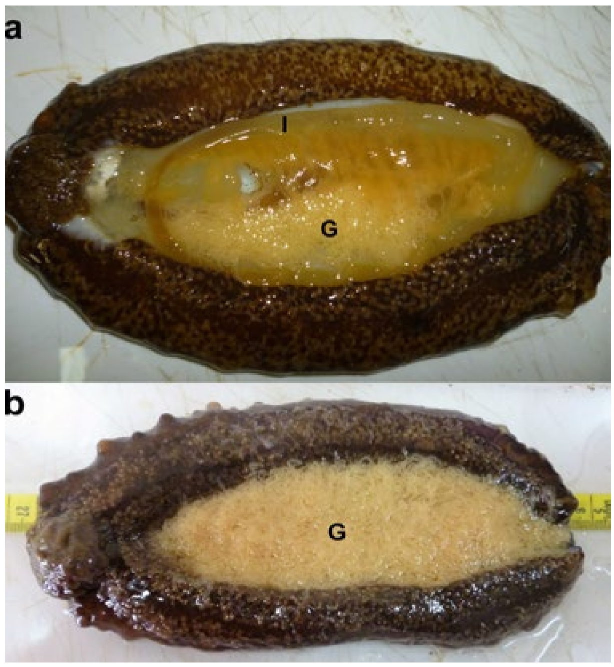

Figure 4. Gradual increase in female gonad size: a) gonad in growth (G) and differentiated intestine (I); and b) fully mature gonad.

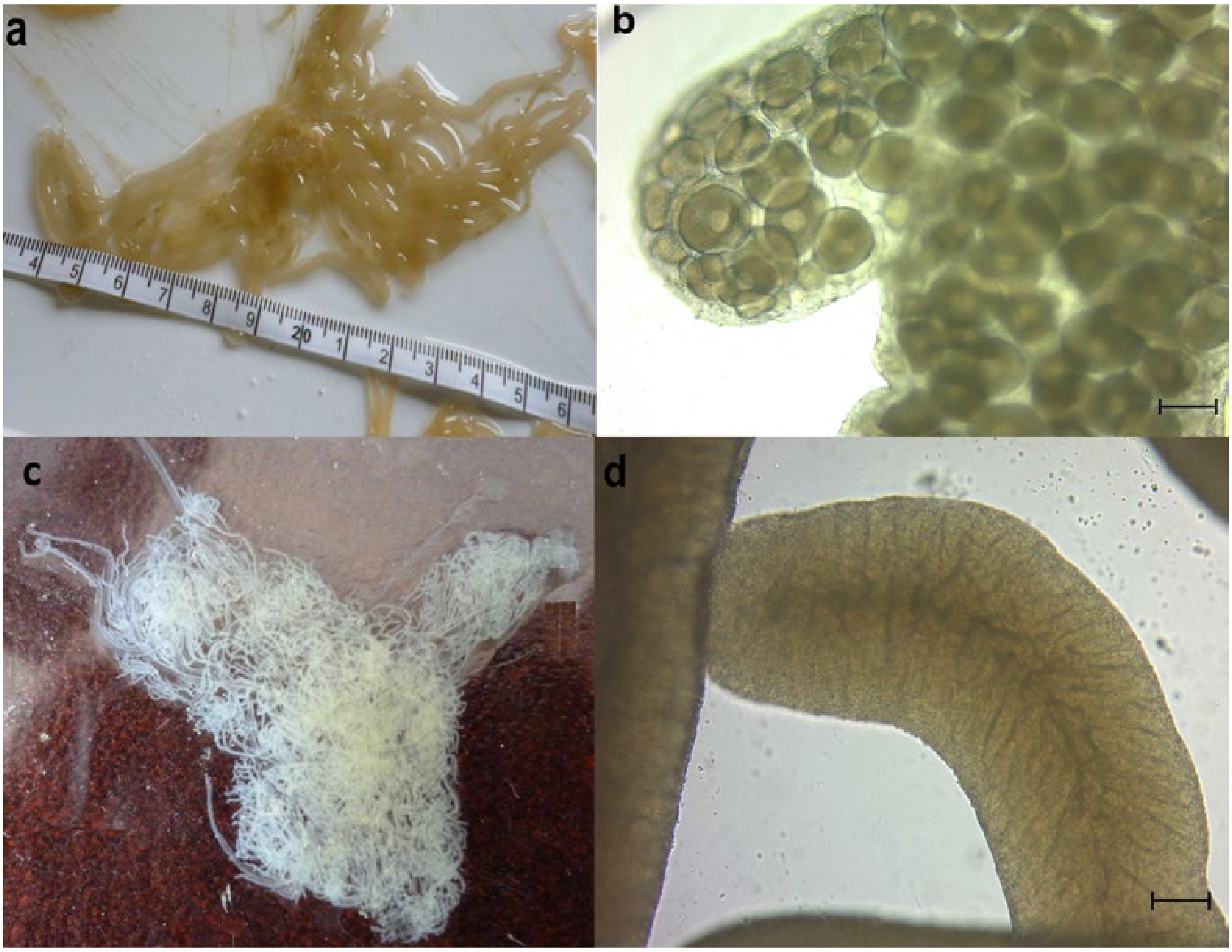

Figure 5. *Isostichopus* sp. aff *badionotus*. Gonads – a) macroscopic view of female gonad that shows tubules with phagocytes (P); b) Light microscopy showing "fresh" oocytes in the process of growth, GV: germinal vesicle; c) macroscopic view of whitish coloured male gonad; d) Light microscopy of "fresh gonadal male" that shows seminiferous channels directed towards the lumen.

Figure 6. Sex ratio of *Isostichopus* sp. aff *badionotus*. during one year of sampling February 2013 to January 2014. n = 120. (X2 = 0.314, p < 0.05).

period: in February, August and October the male:female ratio was 1:2.33; during March, May, June and November the ratio remained 1:1; while in April, July, September and December, a greater proportion of males (Fig. 6) was recorded. Total sexual ratio obtained during the sampling was not significantly different from the expected distribution ($x^2 = 0.314$, df = 44, $p < 0.05$); therefore, no difference in sex ratio (1:1) was found.

Description of the reproductive cycle

Gonadosomatic index

The maximum values of GI were observed when the gonad was ripe; while in the resting phase and post spawning phase, lower values were observed due to the reduction in size. Higher values of GI were observed from July to November. The reproductive peak was recorded in October with an average value of 8.72 ± 4.4. Statistical analysis for months showed significant differences during the study ($p < 0.05$), and a relationship between August and November was evident (Fig. 7).

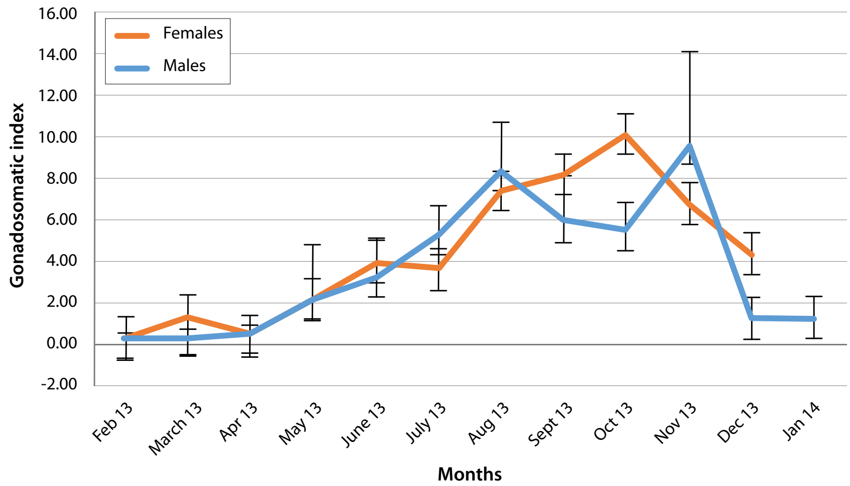

Figure 7. Monthly gonadosomatic index of *Isostichopus* sp. aff *badionotus*, with standard deviation (SD) from February 2013 to January 2014. n = 120. (X2 = 0.314, P < 0.05).

In the analysis by sex, GI showed a similar trend. Males showed GI values of 0.08–16.17, while females showed GI values in the range of 0.06–18.48. When analysing the values based on indicators by gender, no significant differences ($p < 0.05$) between males and females were found, indicating synchronous gonadal development.

The reproductive cycle began in February (2013), when GI values were the lowest. In July, a significant GI increase signalled the onset of ripening. This trend continued for females until October when GI began to decrease rapidly, while males showed a decrease during September and October but GI increased again in November and then declined dramatically from December to January (Fig. 7).

Gonadal histology

Histological analysis of the gonads and gonadosomatic index showed an annual pattern of gonadal development for both sexes corresponding to a complete cycle. Based on the morphological and histological characteristics, the gonadal development for females and males were identified and described as follows:

Oogenesis

In females, five stages of gonadal development were observed: recovery, growth, mature, partially spawned and postspawning (Fig. 8).

Recovery. Gonadal tubule wall is thick and lined by an outer or coelomic epithelium. In this study this structure was found in most stages of gonadal development. Immature oocytes at the periphery of the gonad were observed, delineated by a germ layer that also presented a basophil cytoplasm and nucleus with peripheral nucleoli. The average size of these oocytes was 30.14 ± 10.9 µm. This state was found during February.

Growth. The thickness of the connective tissue that runs along the gonadal tubules becomes thinner. In the germinal epithelium, few immature oocytes were observed, and in the centre of the gonadal tubule, abundant basophilic oocytes at the vitellogenic and previtellogenic stages can be seen. In the cell tissue, we observed a large basophilic cytoplasm and eosinophil nucleus, in which was located a peripheral nucleolus. The average size of these oocytes was 70.5 ± 27.92 µm. This stage was present from March to July.

Mature. Wide tubules were observed. Gonadal tissue showed a reduced wall thickness, making the cœlomic epithelium almost imperceptible. The tubules were completely filled with mature oocytes, formed by an abundant eosinophilic cytoplasm, a large nucleus and nucleolus located in the central or peripheral position, supported on a thin layer of connective tissue. On the periphery of the gonad (germinal epithelium), few immature oocytes were apparent. The average size of mature oocytes was 125.24 ± 13.11 µm. This stage of development was found from June to November.

Partially spawned. Gonadal tubules were observed with abundant mature oocytes, interspersed with empty areas that show the expulsion of the oocyte during spawning. This stage was established before the appearance of phagocytes, which feed waste oocytes. This condition is most common during the months of spawning (August to December).

Post-spawning. The tubular wall was thin and composed of connective tissue, surrounded by squamous cell and a clear coelomic epithelium. Inside the structure, few basophils residual oocytes were

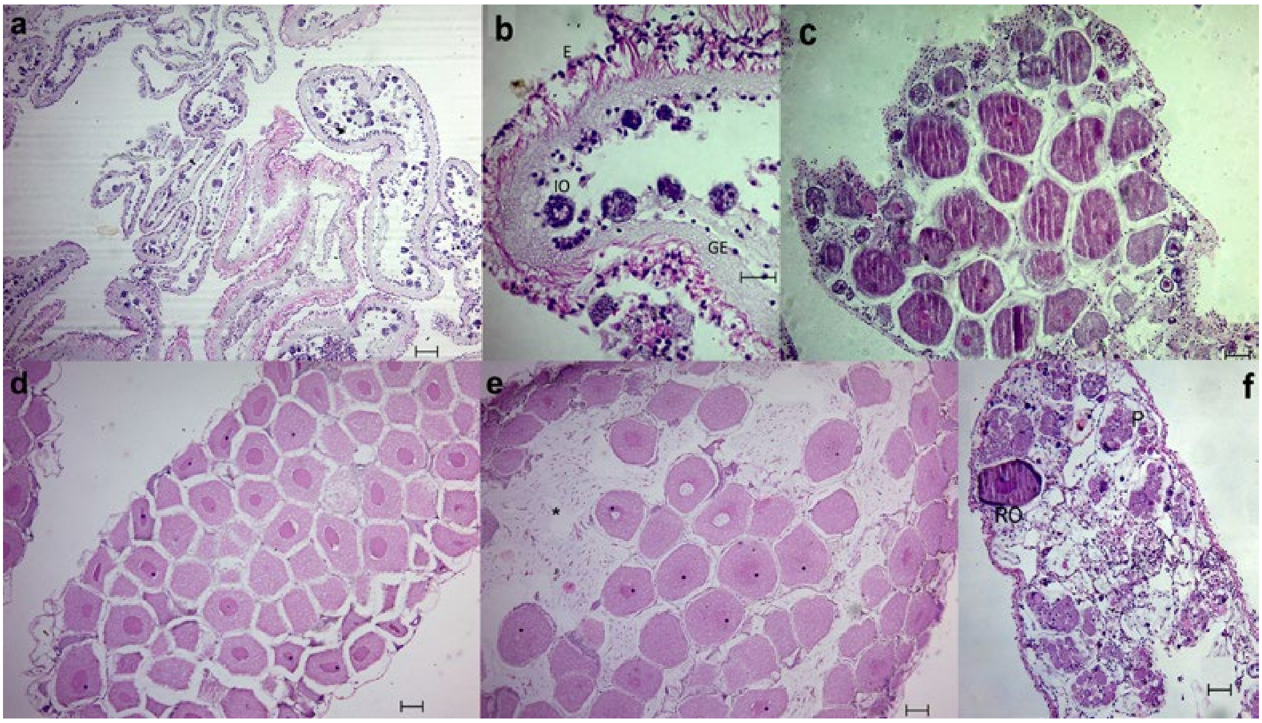

Figure 8. Photomicrography of female gonad development of *Isostichopus* sp. aff *badionotus*. a) Recovery 4x; b) Recovery: outside epithelium (E); Germinal epithelium; immature basophils oocytes. 40x; c) Growth: abundant immature oocytes and other states of previtellogenic development and vitellogenic ovocites, markedly eosinophils. 10x; d) Mature: abundant previtellogenic oocytes and vitellogenic oocytes, containing a large germinal vesicle are seen 10x. e). Partly spawned. Displays empty follicles (*) and vitellogenic oocytes with germinal vesicle and a peripheral nucleoli; f) Postspawning: A damaged tissue with residual basophils oocytes and the presence of abundant phagocytes can be seen (February to December 2013). The scale bar equals 50 µm.

observed, which have different degrees of deterioration; at this stage, phagocytes appear within the area bounded by the remains of the vitelline membrane surrounding the oocyte. This stage was found in February, August, September and November.

Spermatogenesis

Male gonads were characterised by four stages of gonadal development: recovery, growth, mature and post spawning (Fig. 9).

Recovery. The tubular wall was thick, comprising a thin layer of connective tissue with abundant germinal tissue layers on which primary sperm cells are generated. These cells were basophilic, circular in shape and uniform in size. The tubular lumen was empty and no sperm was apparent.

Growth. A thin layer of connective tissue that surrounds the spermatic tubules was observed. Abundant sperm cells (spermatids, spermatocytes) were apparent, organised in a centripetal direction (i.e. from the periphery toward the lumen and then, in like a labyrinth (Fig. 9b).

Mature. Small cells were located in the centripetal position in the tubular lumen, which were identified as mature sperm. The tubular wall was smooth, and in its periphery were numerous spermatocytes.

Post-spawning. The lumen of the tubule was virtually empty and free of sperm. The wall of the tubule showed some invaginations, typical of the germinal epithelium, and the route of sperm was evidenced by the lumen outward.

Gonadal development showed an asynchrony between sexes.

Most females during February were in "post-spawning", while a slight portion (33.4%) was in "recovery". In March, gonads in "growth" were observed and this stage was dominant in all individuals sampled over the next two months. During June, July and August, females with "growth" ovaries were observed, indicating the beginning of the reproductive period. In June, there was a high proportion of females in the "mature" stage (60%) accompanied by females in the "growth" stage. A similar pattern was observed during July with a 1:1 ratio between "mature" and "growth". In August, "partially spawned" (50%), "post-spawning" (16.7%) and "mature" (33.3%) females were seen. "Post-spawning" and "partially spawned" sea cucumbers were found only in September. In October, once again,

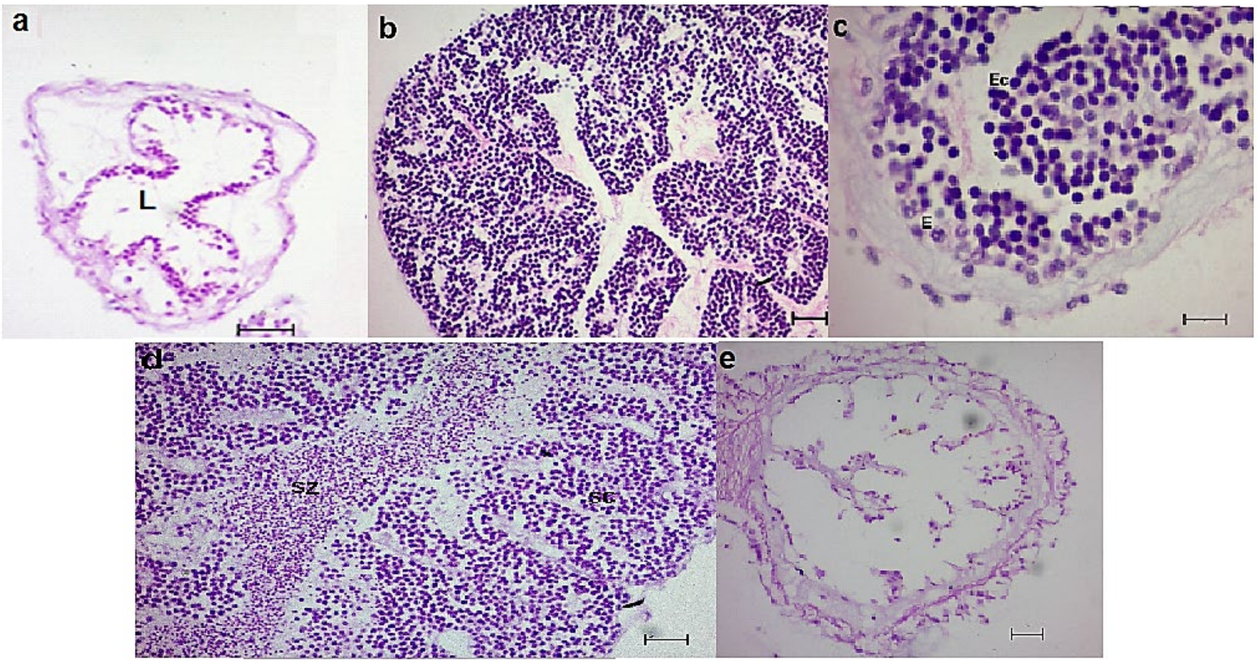

Figure 9. Photomicrography of histology of gonadal development of male *Isostichopus* sp. aff *badionotus*. a) Recovery. A thick layer of connective tissue and basophilic germinal tissue layers is observed. Lumen is empty. 40x; b) Growth. Thin layer of connective tissue surrounding the spermatic tubules (40x), with abundant spermatogonia and spermatocytes, more apparent in photo c). 100x. d) Mature. Sperm in the tubular lumen ready for release. Abundant follicles in the seminal spermatocytes are observed. 10x. e) Postspawning. Seminal follicle is empty. Germinal epithelium tubular wall. 40x. (February 2013 to January 2014). The scale bar equals 20 µm.

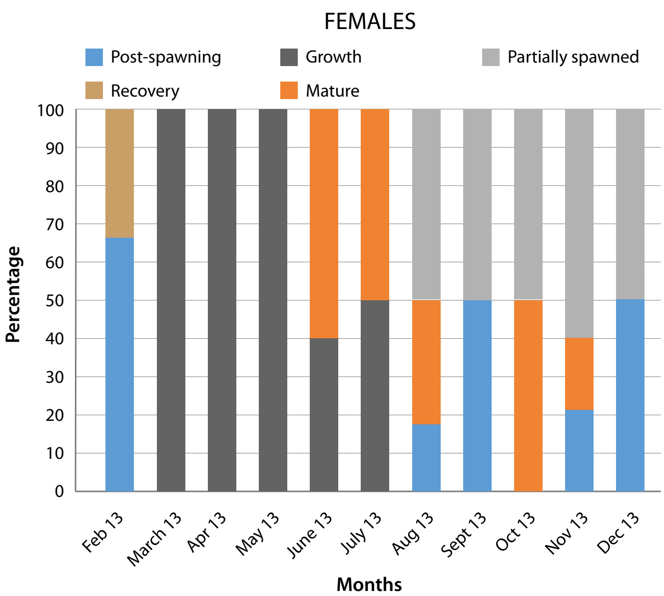

Figure 10. Frequency of gonadal development of female *Isostichopus* sp. aff *badionotus* from February 2013 to December 2013. n = 120.

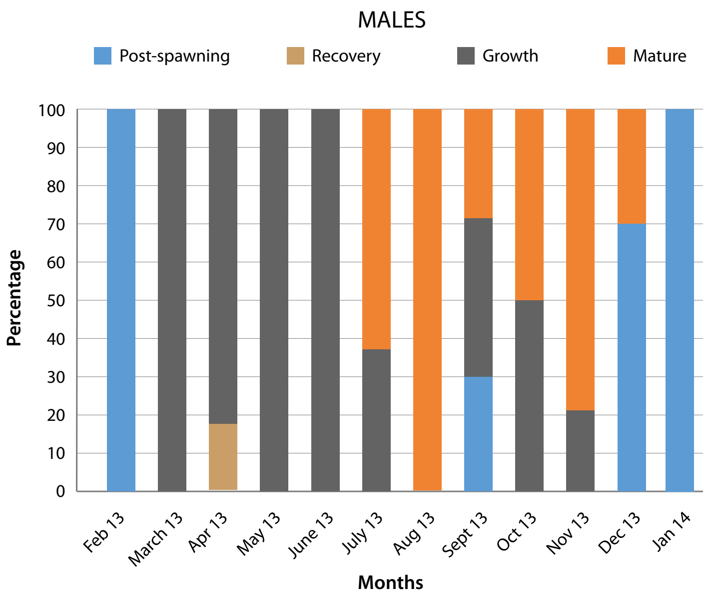

Figure 11. Frequency of gonadal development of male *Isostichopus* sp. aff *badionotus* during February 2013 to January 2014. n = 120.

"mature" and "partially spawned" individuals were present in equal proportion (50%). In November, "partially spawned" (60%), "mature" (20%) and "post-spawning" (20%) stages were identified. In December, the same conditions as in September were observed (Fig. 10).

Males showed two reproductive periods as evidenced by the presence of mature individuals from March to May and July to November. August was the reproductive peak month, with 100% of mature individuals. Males were found to be 100% "post-spawning" only in February 2013 and January 2014. "Growth" and "mature" males were observed almost every month and were present in 100% of the individuals sampled in June. "Recovery" was only found in April (16.7%) (Fig. 11).

Spawning and larval development

Several spawnings were obtained with 4.9 x 10^6 and 6.4 x 10^6 of fertilised eggs produced each month from August to November, at night and dawn. Most of these spawnings took place during the new moon phase (80%). Males begin by releasing sperm followed by females spawning. That behaviour was consistent every year during spawning season.

Fertilisation characteristics were noticeable by the formation of the membrane around the fertilised oocytes. Figure 12 shows different phases of *Isostichopus* sp. aff *badionotus* with embryonic development, from fertilised eggs to gastrula, and then from early auricularia until the doliolaria stage. The development lasted for 28–30 days up to the doliolaria stage at 26°C. In 2014, larval development reached the late auricularia stage, while in 2015 it reached the doliolaria stage (Table 1).

Table 1. Development of *Isostichopus* sp. aff *badionotus* from fertilisation to the doliolaria stage at 26°C. n =40 samples in each stage.

	Size (µm)	
Larval stage	**2014**	**2015**
Fertilised egg	138.02–175.05	146.45–187.75
Blastula	262.72–302.79	286.45–354.67
Gastrula	278.40–356.30	298.34–376.56
Early auricularia	425.30–476.50	496.43–563.93
Mid auricularia	609.28–788.83	576.58–663.07
Late auricularia	787.59–1087.66	976.01–1045.01
Doliolaria		726.98–765.08

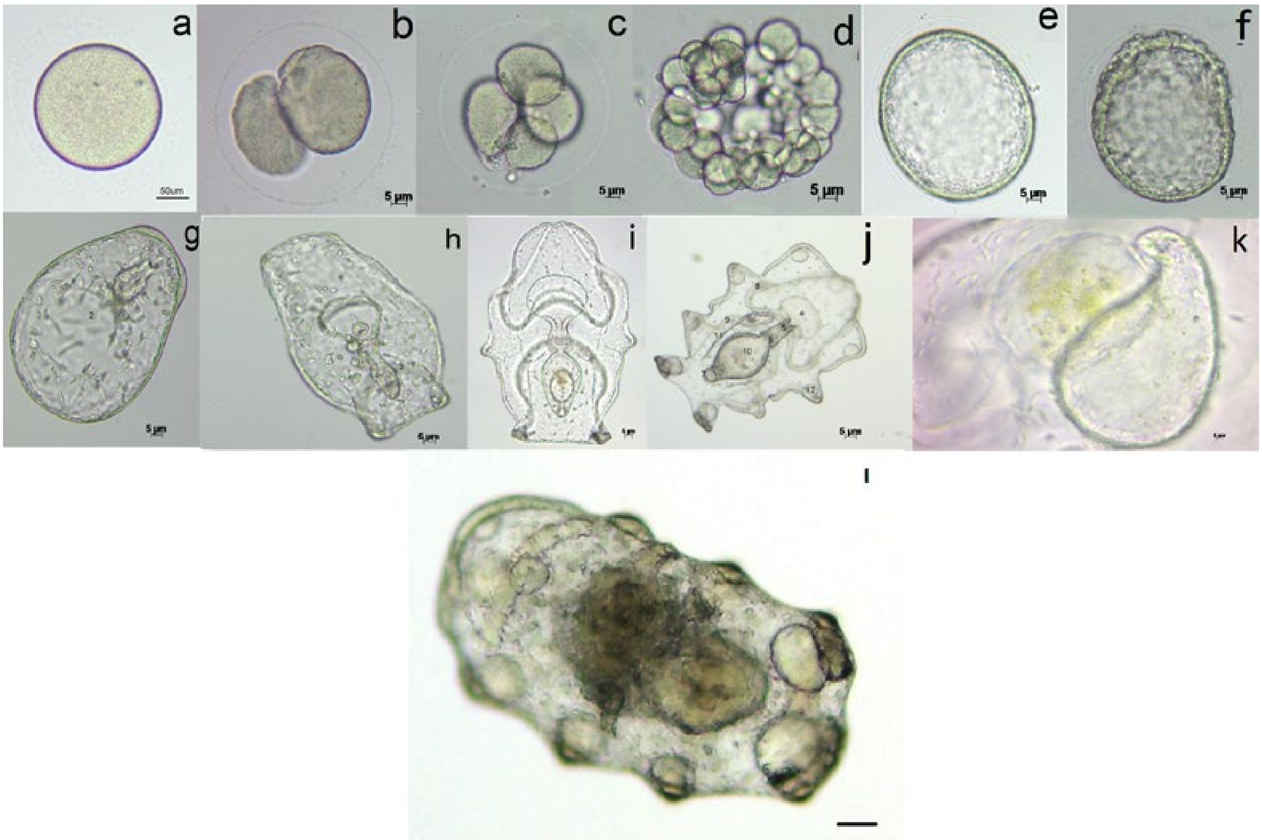

Figure 12. Embryonic and larval development of *Isostichopus* sp. aff *badionotus*.
a) Fertilised egg; b–d) Cleavage stages; e) Early blastula; f) Late blastula; g) Gastrula – (1) archenteron, (2) mesenchyme cells; h) Early auricularia – (3) dorsal pore; i–j) Auricularia – (4) buccal ciliated cavity, (5) oesophagos, (6) stomach, (7) cloaca, (8) ventral pre-oral band, (9) ventral post-oral band, (10) Intestine, (11) Anxohydrocoel (axo), (12) Hyaline sphere; k) Digestive tract with food; l) Doliolaria.

Discussion

Gametogenesis

The sex ratio estimated for *Isostichopus* sp. *nov.* did not differ significantly from 1:1, coinciding with what has been reported for *I. badionotus* and *Holothuria mexicana* (Guzmán et al. 2003), and *H. spinifera* (Asha and Muthiah 2008). Several studies report that this ratio is characteristic of holothurid species with sexual reproduction (Conand 1993; Herrero-Perezrul et al. 1999). The tubules were present in most individuals sampled throughout the year and were not absorbed after spawning; the absence of tubules were evidenced in only five individuals representing 4.16% of the total sampled population. Studies carried out by Herrero-Pérezrul et al. (1999) argue that the absence of gonads can be associated with the evisceration processes, but in our case, sea cucumbers did not show any evidence of evisceration as all organs were present (intestine and respiratory tree), except gonads. During the histological monitoring of gonadal development, the stage of resorption was not recorded. The same situation has been reported for other species of sea cucumbers such as *Holothuria leucospilota* (Ong Che 1990), and *H. fuscogilva* and *Actinopyga mauritiana* (Ramofafia et al. 2000). Some authors, such as Hamel et al. (1993), suggest that the presence of phagocytes inhibits tubular reabsorption of gonads; for this reason, the tubule recruitment model proposed by Smiley (1988) may not apply to *Isostichopus* sp. aff *badionotus*.

For female *Isostichopus* sp. aff *badionotus*, five stages were described; whereas in males only four stages were described. The fluctuation of GI during the sampling period revealed a significant increase from July to November, with a peak in October, indicating that *Isostichopus* sp. *nov.* is a species with an annual reproductive cycle, which presents a single spawning event during the warmer months of the year (Agudelo-Martinez and Rodríguez-Forero 2015). Smiley et al. (1991) considered that this reproductive preference for the warmer months is related to sea temperature and food availability for planktonic larvae development. The same situation was reported for *I. badionotus* (Herrero-Pérezrul et al. 1999; Foglietta et al. 2004; Zacarías-Soto et al. 2013). This situation is also common to most aspidochirotides of temperate and tropical zones (Hyman 1955; Smiley et al. 1991; Zacarías-Soto et al. 2013; Agudelo and Rodríguez 2015).

Spawning and larval development

Spawning season was linked to the increase in GI from July until November. During December to June natural spawning was not viable. Our results are in agreement with those obtained for *Isostichopus badionotus* in the tropical western Atlantic (Guzmán et al. 2003; Foglietta et al. 2004; Zacarias-Soto et al. 2013). It is well known that moon phases play an important role in the onset of spawning in holothuroids (Babcock et al. 1992; Kubota and Tomari 1998; Morgan 2000; Battaglene et al. 2002; Guzmán et al. 2003; Hu et al. 2010); this study showed that is also the case for *Isostichopus* sp. aff *badionotus*.

Embryonic and larval development of *Isostichopus* sp. aff *badionotus* was similar to other tropical sea cucumbers, showing the same phases: blastula, gastrula, auricularia and doliolaria.

Commercial value

Isostichopus badionotus is one of the sea cucumber species collected by a large artisanal and semi-industrial sea cucumber fisheries established in Colombia for several years. It is possible that most of the sea cucumber catches reported as *I. badionotus* were in fact *Isostichopus* sp. aff *badionotus*. Export statistics to Asian countries would, therefore, be wrong. In 2013, sea cucumbers were sold by artisanal fishers at low prices (USD 0.5 unit^{-1}, or USD 1–3 tkg^{-1}) (Rodríguez et al. 2013). Currently, commercial sea cucumber fisheries are illegal in Colombia. Considering that *Isostichopus* sp. aff *badionotus* has a healthy chemical composition similar to that of sea cucumbers internationally traded, it could be a species with a competitive commercial future (Vergara and Rodríguez 2016), and recent progress with its aquaculture (Agudelo and Rodríguez 2015) could make it a suitable candidate for the international market.

Acknowledgements

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