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Gonad development and gonad fatty acid composition of *Holothuria* (Halodeima) *grisea* (Echinodermata:Holothuroidea) in Southern Brazil: new perspectives

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Abstract: Holothurians fulfill crucial ecological roles in marine ecosystems and are widely studied for their biological and commercial properties. Their reproduction is strongly influenced by seasonal, environmental and metabolic factors, with fatty acids being essential elements in energy supply and gamete viability. This study analyzed reproductive aspects (Gonadal Index: GI and macro and microscopic diagnosis) and the lipid composition of the gonads of *Holothuria (Halodeima) grisea* in southern Brazil, considering seasonal variations (winter and summer). The results showed greater reproductive activity in summer, with females investing more energy in development of tubules, gamete production, reflected in the greater amount of total lipids. The fatty acids composition of gonads varied between sexes and seasons, with an increase in polyunsaturates in males during summer, associated with gametogenesis. Gonads aspects (tubules colors and diameter) showed seasonal variability, although they were not sufficient to diagnose reproductive stages. This reinforces the importance of detailed histological and biochemical analyses to understand the reproductive cycle of sea cucumbers as *H. (H.) grisea*, contributing to knowledge of reproductive biology, establishing strategies for sustainable management, aquaculture and conservation of the species.

Key words: gametogenesis; energy allocation; gonadal morphology; biochemical profiling.

Desenvolvimento gonadal e composição de ácidos graxos em gônadas de *Holothuria (Halodeima) grisea* (Echinodermata:Holothuroidea) no sul do Brasil: Novas Perspectivas.

Resumo: As holotúrias desempenham papéis ecológicos cruciais em ecossistemas marinhos e são amplamente estudadas por suas propriedades biológicas e comerciais. Sua reprodução é fortemente influenciada por fatores sazonais, ambientais e metabólicos, sendo os ácidos graxos elementos essenciais no fornecimento de energia e na viabilidade dos gametas. Este estudo analisou aspectos reprodutivos, incluindo análises histológicas, e a composição lipídica das gônadas de *Holothuria (Halodeima) grisea* no sul do Brasil, considerando variações sazonais (inverno e verão). Os resultados mostraram maior atividade reprodutiva no verão, com fêmeas investindo mais energia na produção de gametas, refletido na maior quantidade de lipídeos totais. A composição de ácidos graxos variou entre sexos e estações, com aumento de poliinsaturados nos machos no verão, associado à gametogênese. Embora as cores das gônadas apresentem variabilidade sazonal, não foram suficientes para diagnosticar estágios reprodutivos. Assim, reforça-se a importância de análises histológicas e bioquímicas detalhadas para compreender o ciclo reprodutivo de H. (H.) grisea, contribuindo para o conhecimento da biologia reprodutiva, estabelecer estratégias de manejo sustentável, aquicultura e conservação da espécie.

Palavras-chave: Gametogênese; Alocação de Energia; Morfologia Gonadal; Perfil Bioquímico.

Introduction

Holothuroid echinoderms (sea cucumbers) are presently one of the most exploited groups of marine invertebrates (Rupp & Marenzi, 2021). In addition, there is a high number of scientific evidence supporting the importance of these marine invertebrates in pharmacological activities, including anticoagulant, anti- inflammatory, antimicrobial, antioxidant, antitumor and wound healing (Santos *et al.* 2015, Miranda & Tavares 2024). In the order Aspidochirotida, the families Stichopodidae and Holothuriidae include the sea cucumber species most frequently exploited for commercial purposes (Junus, Kwong & Khoo 2018). On the Brazilian coast, among the few dozens of known species of holothuroids, *Holothuria (Haloidema) grisea* stands out, its populations are abundant from Piauí to Santa Catarina, inhabiting diverse environments such as sandy and rocky substrates, soft sandstone banks, coral reefs and marine prairies; from the intertidal zone to a depth of 25 m (Bueno *et al.* 2018, Rupp *et al.* 2021, Rupp *et al.* 2024).

H. (H.) grisea, like others holothurians, have lost pentamerism in their reproductive system and have a single gonad composed of one or two tufts of tubules that tend to fill most of the interior cavity of the body at their peak maturity stage. Their gametes are released into the water through a gonopore located at the dorsal mesentery (Navarro, Garcia-Sanz & Tuya 2012, Tahri *et al*, 2019). Studies on the reproduction of marine invertebrates would allow a better understanding of the population ecology can offer a baseline for the potential sustainable exploitation not only for proposing management plans and closed seasons for commercial catches but also to analyze the potential use of this species in an aquaculture production (Marquet *et al.* 2017, Rupp *et al*., 2021; Tavares *et al*., 2025).

Considerations about energy allocation have been largely discussed for echinoderms and the ways resources are allocated to structures and functions are important to understand life-history characteristics (McClintock 1989, Hill & Lawrence 1999, Tavares *et al.* 2004). Lipids perform essential structural and energetic functions in animals and can be stored in the form of triacylglycerol (TAG), diacylglycerol ether (DAGE) and sterols stored in organelles called lipid droplet (LD) which are regulated during lipogenesis and lipolysis (Murphy 2012, Yu & Li 2017).

In the gonads of sea cucumber, lipids are the main molecules usually stored as an energy source and are the most abundant biomolecules, with their composition directly influencing the reproductive success of these organisms (Hoegh-Guldberg & Emlet 1997, Byrne & Cerra 2000, Villinski *et al*, 2002, Prowse *et al*, 2009). These constituents-fatty acids, TAG, DAGE and carotenoids- can vary in chemical composition and tissue proportion depending on the temperature, diet and sex (Gao, Xu & Yang 2011, Zhang e*t al.* 2023). Additionally, carotenoids are responsible for giving color to the sea cucumber's gonad.

These organisms are dioecious and have a reproductive system composed of a single gonad, branched into several tubules of varying thickness and length and with different colors (Gao & Yang, 2015). Gonads color is often used as a macroscopic parameter to determine sex and reproductive stage, but it can vary between species and gametogenic stages and should not be used as the only attribute for diagnosing sex due its high variability (Table I) (Marquet *et al.* 2017, Setiawati *et al.* 2021).

The aim of this study was to analyze seasonal reproductive aspects and the composition of fatty acids in the gonads of a population of *Holothuria (Halodeima) grisea* in southern Brazil.

Materials and methods

Sample collection and processing: Adult specimens of *Holothuria (Halodeima) grisea* (the largest individuals) were collected manually and randomly in the winter (July/2022) and summer (January/2023) from rocky shores at Guaratuba beach (Paraná, southern Brazil) (25°53’23”S/48°33’41”W). The sea cucumbers were weighed while still in situ and then individualized in zip-lock plastic bags and anaesthetized with ice and menthol. After that, all animals were then transported to the laboratory.

Gonadal index (GI), gonadal macro- and microscopic diagnoses and sexual ratio: All specimens were cleaned and eviscerated to remove the gonads and obtain the gonadal wet weight. The contribution of the gonads to the total weight of the animal was determined by calculating the gonadal index (GI):

Table I. Synthesis of the studies focusing on gonadal macroscopic diagnoses (color, sex and and gonadal development stage – GDS) in holothuroids.

Species	**Authors**	**Color**	**Sex/GDS**
Holothuria arguinensis	Marquet *et al*. (2017)	Pale orange	♀ Mature
		Pale pink	♀ Growing
		Cream	♂ Mature
		Brown	♀ ♂ Spawning
Holothuria mammata		Orange	♀ Growing
		White	♂ Growing
		Dark pink	♀ Mature
		Beige	♂ Mature
		Brown	♀ ♂ Spawning
Holothuria scabra	Rasolofonirina *et al*. (2005)	Translucent	♀ ♂ Spent/Recovery
		White	♂ Growing
		Yellow	♀ Growing
		Yellow cream	♂ Mature
		Pale Yellow	♂ Spawning
		Orange	♀ Mature/Spawning
Holothuria tubulosa	Pasquini *et al*. (2022)	Pale and translucent	♀ ♂ Recovery
		Matt pale yellow	♂ Growing/Mature/Partly spawned
		Translucent red–coral red	♀ Growing/Mature
		Matt red-coral red	♀ Partly spawned
		Different colors and rust blotches	♀ ♂ Spent
Psolus fabricii	Hamel, Himmelman & Dufresne (1993)	Cream	♂ Immature
		Pink	♂ Mature
		Reddish brown	♀ Mature
Stichopus herrmanni	Setiawati *et al*. (2021)	Orange	♂ Mature
Bohadschia vitiensis	Omar *et al*. (2013)	Red	♀ ♂ Mature
		Yellow white	♀ ♂ Immature

(wet weight of gonads)/[(total weight – dry gut content) x 100] (Santos *et al.* 2015).

The gonadal macroscopic diagnosis was done as follows: the gonadal tubules of each individual were placed in a Petri dish with a reference scale (cm) and photographed. To measure the thickness of the tubules, a four-quadrant template with randomly dispersed points was inserted into each photograph of the gonads. Ten points overlapping the tubules in each quadrant were selected and measured using ImageJ software version 1.53t (Rueden *et al.* 2017). Gonadal colors were determined using a reference scale – the Munsell system Soil Survey Division Staff (2017) - by visual comparison. For this the following parameters were considered: Hue (dominant color), Value (hue/lightness) and Chroma (weak/strong).

The majority of the gonadal tissue of each specimen was frozen and freeze-dried. The sexing and determination of gametogenic stages were carried out under optical microscopy. For this, a fraction of the gonads (previously preserved in a 10% saline formalin solution) was processed using standard histotechniques. The histological preparations were obtained from 7 µm sections of tissue using a manual microtome (Minot type) stained in Hematoxylin and Eosin (Santos *et al.* 2021).

Subsequently, each histological preparation was analyzed under an optical microscope (Olympus, model CX43) at 4X and 10X magnification and photomicrographs were taken with an EPView 50©

5.2.35 camera integrated with EPview 1.3 software. The stages were defined according to Bueno *et al.* (2015) and Tavares et al (2023). A theoretical 1:1 sex ratio (female:male) was tested using Chi-square test (χ^2) considering both month (season) samples.

Measurements of non-vitellogenic and vitellogenic oocytes were performed along the major axis and only those whose nucleus was visible. Feret's diameter measurement (the longest distance between any 2 points along the selection perimeter) of all oocytes/females was recorded with Image J software (Benítez-Villalobos et al., 2013; Tavares et al. 2025). Size classes intervals of oocyte diameter were determined by the Sturges' rule (Sturges 1926).

Total lipids extraction: The freeze-dried gonads were ground to powder and the total lipid was extracted with $CHCl_3$/MeOH (3:1, v/v) in a ratio of 1:30 (w/v), stirring constantly for 2 hours at room temperature (24 ± 2 ºC). The extracts were filtered through quantitative filter paper (Whatman, N1, 90 mm), evaporated with a flow of N_2 and quantified by gravimetry on an analytical balance and stored at - 18 ºC (Bligh & Dyer, 1959).

Determination of the fatty acid profile by derivatization in fatty acid methyl esters (FAME): Samples of the total lipids extracted (3-5 mg) were dissolved in 600 µL of 1 N MeOH-HCl and kept at 100 ºC for 2 hours. Then, they were partitioned with 1 mL hexane and 1 mL distilled water and the apolar portion was collected, evaporated under a N_2 flow, dissolved in acetone and analyzed using GC-MS (Ichihara & Fukubayashi 2010, Chiu & Kuo 2020). GC-MS analyses were carried out using a gas chromatograph (GC) coupled to a SHIMADZU QP2020NX electronic ionization (EI) mass spectrometer, with a scan between 30 and 1000 m/z, electron generation energy of 70 eV and equipped with a DB-5 ms (SHIMADZU) fused silica capillary column (30 m x 0.25 mm i.d.) (Santana Filho, 2013). Helium gas was used as carrier gas at a pressure of 119 kPa and a flow rate of 1 mL/min. The injector temperature was 250 ºC. The temperature programming was 100 ºC for 2 min, followed by an increase of 5 ºC/min up to 280 ºC and maintained for 7 min. The total programming time was 45 min. The ion source temperature was 300 ºC and the split ratio was 1:10.

Data Analysis: The sex ratio was statistically analyzed using the χ^2 test (CI=95%). The temporal variation of the mean oocyte diameter (Dm) was analyzed using size frequency histograms constructed by determining class intervals using Sturges' Rule. The Bhattacharya method (FISAT II software - FAO-ICLARM Fish Stock Assessment Tools, 1.2.2) (Sparre & Venema, 1997) was used to analyze the modal progression and determine the representative cohorts of Dm over time and subsequently delimit the representative modes and intervals characteristic of the oogenesis stages (Benítez-Villalobos, Avila-Poveda & Gutiérrez-Méndez 2013). Results were presented using seasonal relative frequencies histograms of the repletion degree and Dm.

All data were first tested for normality. The parametric Student's t-test (unpaired) was applied to normally distributed data, while non-normally distributed data were analyzed using the non-parametric Mann-Whitney test. The means are presented with the standard deviation (±). To check the seasonal variation in the percentages of fatty acids (saturated, monounsaturated and polyunsaturated) between the sexes, a factorial ANOVA was carried out and homogeneous groups were determined using the Tukey HDS test. The graphs were displayed considering the weighted averages and the standard error. Statistica© 10 and OriginPro® 8.5 software were used for statistical analysis and/or graph construction.

Results

Reproductive aspects: A total of 32 specimens of *H. (H.) grisea* were colleted (12 in winter and 20 in summer). The sex ratio did not differ significantly from the 1:1 ratio, with 17 males (53.1%) and 15 females (46.4%). The average weight of the males was 156.5 ± 37.8 g (62.0–209.0 g), while the average weight of the females was 171.6 ± 55.3 g (103.0–306.0 g), respectively. Moreover, the average values of the Gonadal Index (GI) in summer were significantly higher than in winter for females (4.02 ± 2.4%, p = 0.0421) and males (3.29 ± 2.0%, p = 0.003) (Fig.1).

Gonadal tubules average thickness (Dtg) showed significant variations ($p< 0.001$) between seasons and sexes (Fig. 2a and Fig 2b). The data for females was higher than for males and ranged from 0.099±0.059 mm (summer) to 0.110±0.041 mm (winter). In males, Dtg ranged from 0.065±0.021 mm (winter) to 0.071±0.028 mm (summer).

Female gonads in winter exhibited the greatest color variability, including N8, 10YR 8/6, 5RP 7/8, 5RP 7/4, 10YR 8/6, 7.5RP 7/10 and 5B 6/6 (Fig. 3). The males collected in winter predominantly showed neutral colors, such as N7, N8 and N9, as well as 10YR 8/6. In summer, the colors observed in the males' gonads were N7 and 10YR 8/6, while the females showed the colors 5RP 7/8 and 5B 6/6. There was a reduction in the variety of colors in the summer, with

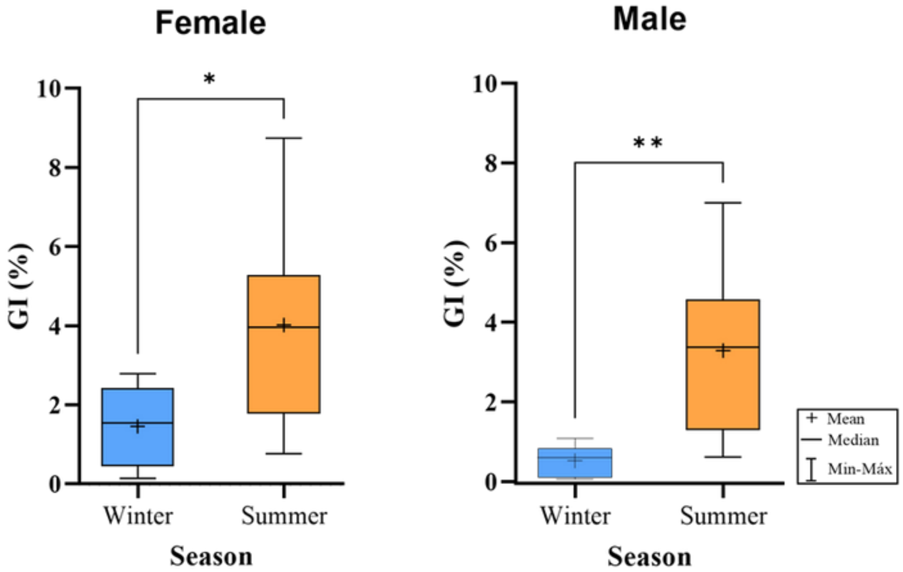

Figure 1. Gonadal Index of *Holothuria (Halodeima) grisea* females and males from Guaratuba beach (Paraná, Brasil) in winter and summer 2022.

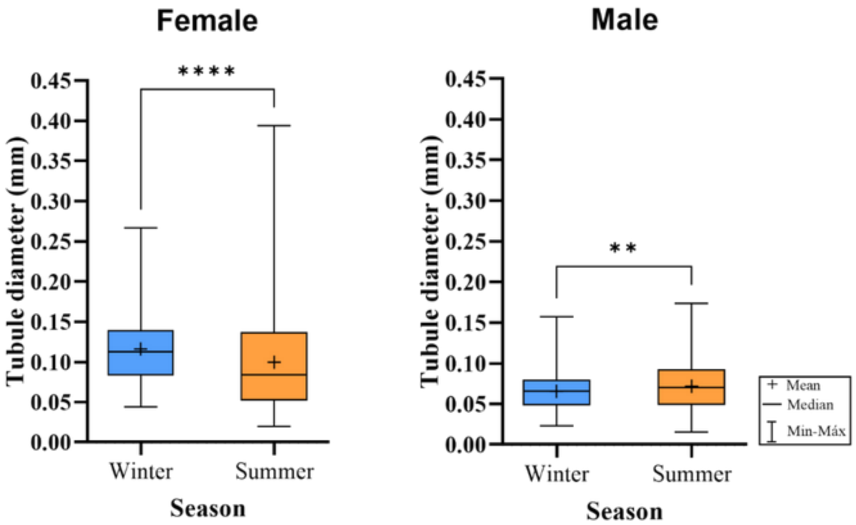

Figure 2. Seasonal variation of tubule diameter of *Holothuria (Halodeima) grisea* females and males from Guaratuba beach (Paraná, Brasil) between winter and summer 2022.

90% of the females' gonads showing the color 5RP 7/8 and 10% the color 5B 6/6. In the male s, 70% showed the color 10YR 8/ 6 and 30% the color N7.

The following images illustrate the fresh gonads of *H. (H.) grisea* (Fig. 4). Color differences in the gonads of the females are evident, with pink and lilac tones and in the males the predominance of white and yellow colors.

Microscopic diagnosis Gametogenic stages: The gonadal tubules could be diagnosed in at least one of the five stages: growing, premature, mature, spawning or partly spawning and post-spawning (depleted or spent) according Bueno et al. (2015) and Tavares et al. (2025) (Fig. 5). In winter, growing, premature and mature stages were observed in females; and spawning and post-spawning stages in males. In summer, post- spawning tubules predominate in females, followed by images of mature, spawning or partly spawning tubules. Male gonadal tubules were mature in most individuals, although spawning or partly spawning tubules have also been observed.

Biometric oocyte analysis: A total of 1,492 oocytes were measured (winter: 1.094; summer: 398) with diameter average (Da) ranging from 4,7 to 135.4 µm The Sturges' method distributed the population into 23 size classes of 6 µm. Bhattacharya's method (Sparre & Venema, 1997) identified 2 main cohorts (20.1 and 78.5 µm) and then established three cytometric intervals for oogenesis: (1) growing: Da<20.1

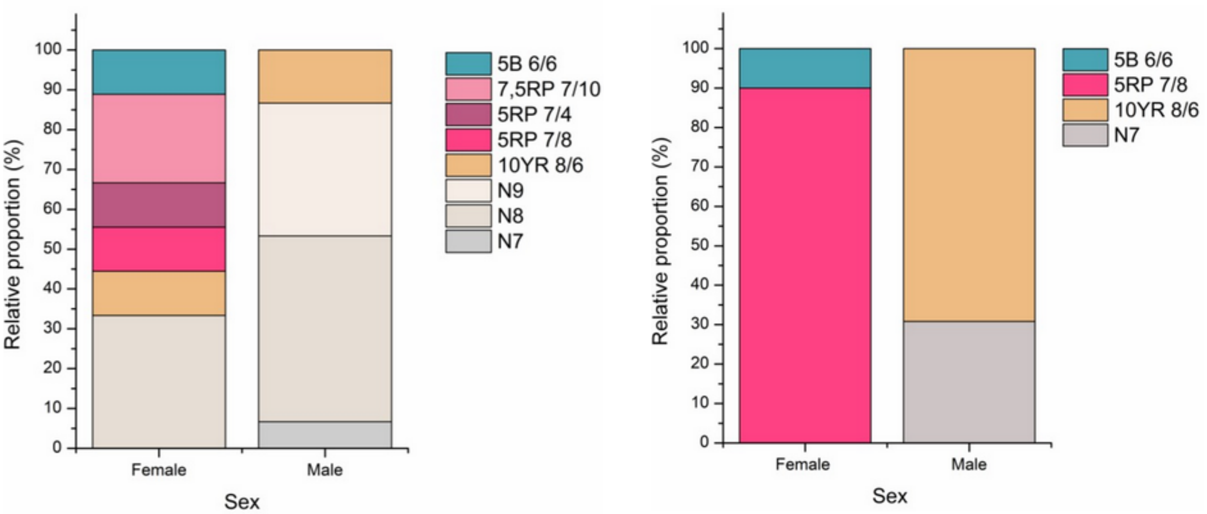

Figure 3. Relative percentage of colors present in the gonads of females and males of *Holothuria (Halodeima) grisea* in winter (left) and summer (right). The notations N7, N8, N9, 10YR 8/6, 5RP 7/8, 5RP 7/4, 10YR 8/6, 7.5RP 7/10 and 5B 6/6 correspond to the original notations in Munsell color identification system. Colors in the figure are just approximate to the original notation.

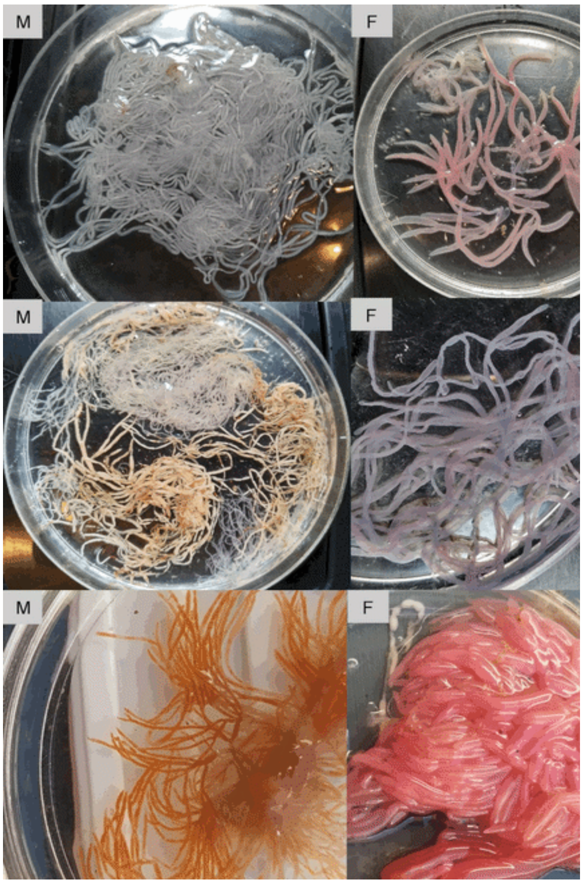

Figure 4. Photographs of fresh gonads observed in males (M) and females (F) of *Holothuria (Halodeima) grisea* from Guratuba beach (without a standardized scale).

μm, (2) premature: 20.1 <Da<78.5 μm and (3) mature: Da>78.5 μm. Vitellogenic oocytes (completely mature) were observed mainly in summer with average diameters from 82,7 to 106,7 μm.

In winter, oocyte population was distributed with parsimony between growing (34.6%), premature (40.2%) and completely mature (25.2%) (Fig. 6a). In summer, the majority of cells measured were mature (66,8%) followed by premature intervals (32.7%). An inexpressive amount of primary oocytes was observed (0,5%) (Fig. 6b).

Total lipid: Total lipids average varied statistically (p= 0.0376) between the sexes (males: 55.75±54.8 mg and females 122.3±108.4 mg) but not between the seasons (winter: 70.4±69 mg and summer 102.7±100.2 mg, p= 0.3442). It should also be noted that females in winter showed a significant difference compared to males (p 0.0344, ΔM 84.48 ± 33.9 mg).

Total lipids per gonad development stage: Females with tubules in the stages of maturation and spawning with proliferation and males pre-mature and spawning showed the highest amounts of lipids in winter (Table II). In summer, lipid amounts in females were higher in pre-mature, maturation and spawning with proliferation stages while in males, in the spawning with proliferation stage. In some cases, the gametogenic stages were diagnosed in just one individual, which prevented any statistical analysis to be done. Thus, these results are presented as averages or individually according to the situation.

Relative Fatty Acid Composition: The fatty acids with the highest proportions (≥ %5) in both sexes were C16:0, C16:1, C18:0, C18:1, C19:0, C20:4n-3, C20:4n-6, C20:0 and C20:1 (Table III). The saturated

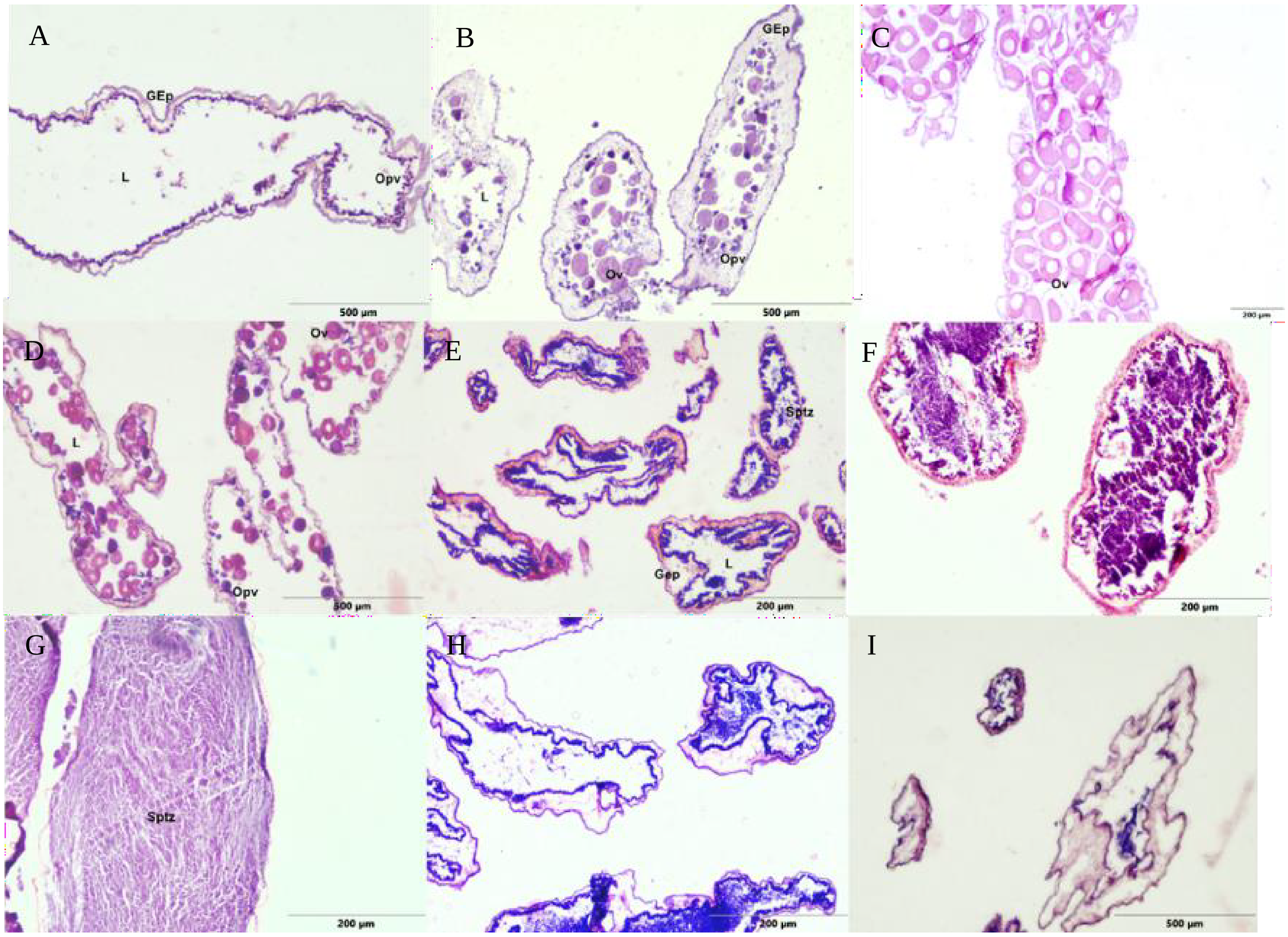

Figure 5. Photomicrographs of the gonadal tubules of *Holothuria (Halodeima) grisea* from Guaratuba beach in the different stages. Female. A: growing (winter); B: premature (winter); C: mature (summer); D: partly spawning (winter). Male. E: growing (winter); F: premature (winter); G: mature (summer); H: partly spawning (summer); I: post-spawning (winter). Abbreviations: Ov, vitellogenic oocyte; Opv, pre- vitellogenic oocyte, Gep, germinal epithelium; L, lumen; Sptz, sperm mass.

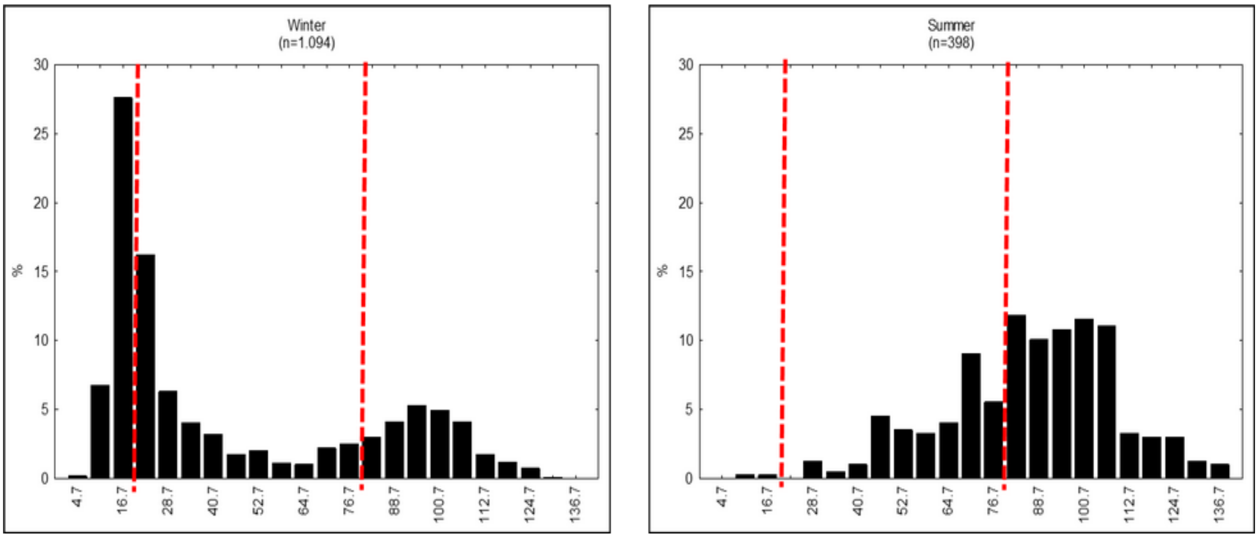

Figure 6. Distribution frequencies of oocytes average diameter of *Holothuria (Halodeima) grisea* from Guaratuba beach in winter (left) and summer (right). The dotted lines represent the cohorts based on Bhattacharya's method.

Table II. Lipid values (mg) of *Holothuria (Halodeima) grisea* from Guaratuba beach at different reproductive stages for females and males in winter and summer. Mean values are indicated by "a"; n: number of specimens sampled; Un: Unmeasurable; I: Growing, II: Premature, III: Mature, IV: Spawning, V: Partly spawning and VI: post-spawning or spent

Stage	**Lipid (mg)**			
	Winter		**Summer**	
	Female	**Male**	**Female**	**Male**
I	5,3 (n=1)	-	-	-
II	79,8 (n=1)	76,7 (n=1)	221[a] (n=2)	-
III	156,6 (n=1)	-	180,5[a] (n=3)	-
IV	-	55,8 (n=1)	74,5 (n=1)	35,4[a] (n=2)
V	170,4[a] (n=2)	14,9[a] (n=4)	106,5 (n=1)	102,3[a] (n=6)
VI	-	Un (n=1)	29[a] (n=3)	-

fatty acids (SFA) that stood out in both sexes were palmitic acid (C16:0), stearic acid (C18:0) and nonadecanoic acid (C19:0). A total of 12 monounsaturated fatty acids (MUFA) were identified in the gonads of both sexes: C16:1, C17:1, C18:1, C19:1, C20:1, C21:1, C22:1, C23:1 and C24:1. The highest MUFA were C20:1, with values ranging from 2.2 to 10.6%, while the lowest MUFA was C17:1, which did not reach 2%. Polyunsaturated fatty acids (PUFA) varied between seasons and sex, with the highest values being observed in males in summer (C20:3n-3, 19.2%; C20:4n-6, 16.8%) and females in winter (C20:3n-3, 10.1%; C20:4n-6, 8.3%). For males, there was a reduction in SFA and MUFA between winter and summer and an increase in PUFA. In females, SFA and PUFA decreased and MUFA increased.

ANOVA: The fatty acid composition between males and females was similar during winter, with saturated fatty acids (SFA) being predominant in both sexes. In females, SFA accounted for approximately 50%, while monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) represented 35% and 15%, respectively. In males, SFA were slightly lower (45%), MUFA remained at 35%, and PUFA reached 20%. During summer, significant seasonal variation was observed, particularly in females. MUFA increased from 35% (winter) to approximately 45% (summer), while PUFA decreased significantly from 15% to 10% ($p = 0.0000$). SFA decreased slightly from 50% to around 45%, although this change was not statistically significant. In males, the proportions of SFA and MUFA remained relatively stable between seasons (45% and 35%, respectively), while PUFA increased from 20% in winter to 25% in summer, representing the only seasonal variation observed in this group.

Discussion

Gonadal index (IG) and gonad tubules stages: Physiological and biochemical demands on females and males of *H. (H.) grisea* differed between seasonal extremes. Winter was characterized by a lower percentage of gonad participation (expressed by GI) and thin tubules with few or no gametes. Thus, in this season the individuals were found in a total exhaustion of reproductive activity that Bueno *et al.* (2015) diagnosed as the post-spawning or depleted stage. On the other hand, in summer both sexes had heavier gonads (higher GI) and in most of the individuals analyzed the tubules had greater average thickness, characterizing the sexually mature animals, especially the females. The positive relationship between tubule diameter and gonad maturity has also been observed by Bueno *et al.* (2015) and Leite-Castro *et al.* (2016) in *H. (H.) grisea*, as well as in others species (Benítez-Villalobos, Avila-Poveda & Gutiérrez-Méndez 2013, Kazanidis, Lolas & Vafidis 2014).

In addition, the increase in water temperature in the warmer months is closely associated with the reproductive periods of the populations already studied in the country, regardless of latitude (Bueno *et al.* 2015, Pereira 2017, Leite-Castro *et al.* 2016). This pattern was also observed in *Holothuria polii* (Tehranifard *et al.* 2006), *Holothuria fuscogilva* (Muthiga, Kawaka & Ndirangu, 2009), *Stichopus herrmani* (Tolon & Engin, 2019), *Holothuria fuscocinerea* (Benítez-Villalobos, Avila-Poveda & Gutiérrez-Méndez, 2013), *Holothuria arguinensis* (Marquet *et al.* 2017) and *Holothuria sanctori* (Navarro, Garcia-Sanz & Tuya 2012). Females of *H. (H.) grisea* may have more robust gonads than males due to the oocytes size (> 100 µm diameter), lipid content (Setiawati *et al.* 2021, Venâncio *et al.* 2022) and may have tubules at different reproductive stages in the same individual, which indicates an asynchronous reproductive cycle (Foglietta *et al.* 2004). In addition, intra- and interspecific variations are possible. In mature individuals of *H. scabra* from different populations, gonads with maximum values of 15 g and 315 g were observed (Ramofafia, Byrne & Battaglene 2003, Rasolofonirina *et al.* 2005,

Table III. Relative percentage of fatty acid (% of total FA, mean values) in gonads of *Holothuria (Halodeima) grisea* collected in winter and summer per sex.

Fatty acid	Male (n=6)	Female (n=5)	Male (n=7)	Female (n=10)
C 14:0	1.8	3.9	0.6	3.8
C 15:0	1	3.2	0.3	3.3
C 15:0*iso*	0.4	2.5	0.0	1.2
C 15:0*anteiso*	0.9	2.3	0.1	1.7
C 16:1	2.7	5.8	2.0	5.9
C 16:2	0.4	0.5	0.0	0.9
C 16:0	9	15.8	4.4	12.3
C 17:1	0.3	0.4	0.4	1.7
C 17:0	1.5	2.5	0.4	0.0
C 17:0*iso*	1.7	2.1	0.9	2.0
C 18:1	10	11.8	9.5	8.5
C 18:0	12.2	12.2	17.2	5.7
C 19:0	3	2.3	3.5	9.0
C 20:4n-6	11.1	8.3	16.8	2.6
C 20:3n-3	11.2	10.1	19.2	7.6
C 20:1	9.7	7.8	10.2	17
C 20:0	5.2	4.9	4.3	1.7
C 21:1	2.2	2.4	0.7	2.8
C 21:0	2	2.9	1.7	1.2
C 22:1	2.7	3.7	1.7	1.7
C 22:0	2.9	4.5	2.1	1.9
C 23:1	2.1	2.4	1.4	4.3
C 23:0	1	1.5	0.8	0.3
C 24:1	4.6	5.3	1.8	2.6
C 24:0	0.5	1.1	0.0	0.2
Saturated	43.1	51.6	36.3	44.4
Monounsat.	34.2	33	27.6	44.6
Polyunsat.	22.7	15.4	36.1	11

Rahantoknam 2017) while for *Athyonidium chilensis* it only reached 35 g (Peters-Didier *et al.* 2018).

Winter was generally characterized by the presence of individuals with less reproductive activity. The description of more gametogenic stages, even in just two seasons, indicates the greater variability of the tubules in the organisms in winter, which was not described by Bueno *et al.* (2015) in the same location. Although a low sampling frequencies could compromise the interpretation of the gametogenic cycle throughout the year, the present study highlight that spawning phenomena could be associated with and without proliferative events, not pointed out by Bueno *et al.* (2015) or Leite-Castro *et al.* (2016) for the northern population (Ceará).

In both sexes, the presence of different stages in the gonadal tubules of the same individual agrees with the tubule recruitment model (Hamel, Himmelman & Dufresne 1993, Hamel & Mercier 1996, Ramofafia & Byrne 2001; Ramofafia, Byrne & Battaglene 2000, 2003). We also believe that a major point to be discussed is whether the absence of synchronization in tubule development within each gonad could lead to high variability within the same individual (Hamel *et al*., 1993; Mercier & Hamel, 1996; Marquet *et al*., 2017; Rasolofonirina *et al*., 2005; Tavares *et al*., 2025).

The use of other quantitative methods to determine the stages of gonadal development has already been proposed, specifically the thickness, length or branching of the tubules (Rasolofonirina *et al.* 2005, Gaudron *et al.* 2008, Muthiga *et al.* 2009, Dissanayake & Stefansson 2010, Navarro, Garcia-Sanz & Tuya 2012, Santos *et al.* 2015, Rogers *et al.* 2018, Venâncio *et al.* 2022). For *H. (H.) grisea* the description of the diameter of the tubules was proposed for the population of the northeast (Leite-Castro *et al.* 2016) and present discrepant values from those recorded in the present study. However, it is agreed that the diameter is substantially greater in the period of greatest maturity in both sexes.

Female gametic investment expressed by oocyte average diameter was another parameter used as a quantitative descriptor. Cytometric measurements obtained with image analysis in marine invertebrates have been shown to be an efficient method for characterizing the process of gonadal maturation, helping to reduce the subjectivity of histological observations (Corte *et al.* 2014, Stakowian *et al.* 2020). The temporal evolution of oogenesis in *H. (H.) grisea* was distinct between the extreme seasons: in winter there is a large gamete production with an expressive gonial burst to the detriment of summer, where the number of gametes drops drastically (about ⅓ of that recorded in winter), but the egg size increases, these

Table IV. Macroscopic diagnosis for female (♀) and male (♂) gonads of *Holothuria (Haloidema) grisea* described in Brazil. The colours correspond to the description of the Munsell code presented earlier.

Author	**Stage**	**Tubule description**	
		Color	**Aspect**
Bueno *et al.* (2015)	Growth	yellow white♀♂	shorter, thinner and branched
	Mature	red♀	long, thick, branched maximum volume tubules
		cream♂	
	Post spawning or depleted	whitish and translucent♀♂	thinner and branched, less consistent texture
Leite-Castro *et al.* (2016)	Early growth	transparent or bluish♀♂	thick and coiled
	Growth	transparent pink♀	thin
		transparent cream♂	thinning and with slight convolution
	Mature	dark pink♀ cream♂	highly dilated
	Post-spawning or depleted	brownish♀♂	extremely convoluted
Present study	Growing	grayish white♀♂ bluish♀	average thickness
	Premature	grayish white♀♂ pale rose♀	very dilated and elongated
	Mature	yellow, light raspberry or dark pink♀ yellow♂	
	Spawning	pale rose ♀ grayish white, white or yellow♂	thinner and less consistency
	Partly spawning	pale rose♀ yellow♂	
	Post-spawning or spent	pale rose ♀♂ grayish white or white♂	thin, convoluted and highly compressed

these dimensions represent mature cells (2⁄3) and confirm greater expression of the gonadal index in this season.

The allocation of reproductive energy to a larger or smaller number of offspring, based on egg size, is an important life history strategy for any species. In comparison with other studies, the percentages of the size range from which oocytes are considered vitellogenic are similar to those recorded by Bueno *et al.* (2015) in Paraná and smaller than the values indicated in the Ceará population (Leite-Castro *et al.* 2016) which may point to different investment strategies that should be investigated.

Color pattern: The gonads of *H. (H.) grisea* show different coloration patterns between sexes and seasons. Intense and opaque shades were related to the maturity of the tubules, while the presence of less intense or even neutral colors, were related to the lower quantities of gametes and the consequent emptying of the gonadal tubules (Navarro, Garcia-Sanz & Tuya 2012). During the maximum reproductive period when the tubules are full of gametes, both the female and male gonads show greater uniformity in color which has also been observed in other Brazilian populations (Bueno *et al.* 2015; Leite-Castro *et al.* 2016; Pereira, 2017). The description of the colors used in two of these studies also lacks any standardization, which increases the subjectivity of this qualitative aspect of the gonads. Table IV summarizes the macroscopic (qualitative) information on the species' gonads in Brazil.

Many studies have indicated that the colors in the gonads of holothuroids are the result of the incorporation of metabolites produced by algae, protists, bacteria and/or fungi consumed by sea cucumbers, the main molecular class being carotenoids that exhibit yellow, orange, red, blue or purple colors (Rocha- Santos & Duarte 2014, Chasanah *et al.* 2016, Galasso, Corinaldesi & Sansone 2017, Maoka 2020). Commonly found in the digestive tract are the pigments chlorophyll c, fucoxanthin (brown macroalgae) and neoxanthin (green macroalgae) (David *et al.* 2020). These carotenoids have antioxidant activity and are a protective mechanism for gametes against the effects of photo-oxidation and reactive oxygen species (Matsuno & Tsushima 1995, Galasso, Corinaldesi & Sansone 2017).

In *Apostichopus japonicus* there is endogenous production of Astacin (Zhao, Chen & Yang 2015). The compounds astaxanthin, β-equinenone, canthaxanthin and cucumariaxanthin have been identified in the gonads of *Holothuria atra, Holothuria leucospilota, Stichopus japonicus, Holothuria moebi* and *Holothuria pervicax*, and they are responsible for color variation (Matsuno *et al.* 1969, Matsuno & Tsushima 1995, Bandaranayake & Rocher, 1999). Similarities and differences in color may indicate a specific profile in biochemical composition and we can also deduce that color variability is not related to the amount of total lipids. However, studies focused on identifying these compounds are further necessary to confirm this.

Thus, it is not recommended to use the color parameter as the sole/dominant characteristic for determining sex or gametogenic stage in holothurians. Colors vary between stages and can be influenced by diet, lipid composition and habitat, as they depend on the availability and metabolization of pigments (Pasquini *et al.* 2022).

Total fat content: There is clearly a difference between males and females in terms of the total amount of lipids and the fatty acids composition, even between seasons. The greater amount of lipids in the female gonads in summer compared to winter reiterates greater investment in gamete production during the reproduction period (David *et al.* 2023).

Maternal investment is aimed at the quality and viability of the larva, while in males the energy reserves of the sperm are directed towards motility and survival during fertilization (Prowse, Sewell & Byrne 2008, David *et al.* 2020). The metabolic pattern between total lipids and gametogenic stage is characterized by an increase in lipids in stages of greater maturity or intense food capture and lipid storage, associated with gametogenesis and a reduction in the amount of lipids after gamete emission (Hughes *et al.* 2006, Carboni *et al.* 2013). However, in the present study, we would point out that the small sample size made it difficult to establish a precise correlation between stages and total lipids, although the data obtained indicate a convergence between stages of greater maturity and greater amounts of lipids.

An important event for the gametogenesis in echinoderms is energy translocation. This phenomenon is characterized by the transfer of molecules (carbohydrates and lipids) between different tissues and/or between substructures of the same tissue (Martin 1969, Hamel & Mercier 1996, Byrne e*t al.* 1998, Jiang e*t al.* 2009, Hansen, Flatt & Aguilaniu 2013). Hence, we suggest that the increase in summer lipids is the result of the translocation of lipids from other tissues (body wall and digestive tract) to the gonads and/or from the germinal epithelium to the gametes, as presented by Miranda & Tavares (2024) for the same species. This phenomenon has also been reported in *H. scabra* (Krishnan 1968), A*mperima rósea, Bathyplotes natans* and *Laetmogone violacea* (Hudson *et al.* 2004).

Fatty acids: The gonads fatty acid composition is strongly influenced by diet and intestinal microbiota. Bacteria, microalgae and heterotrophic protists are the central organisms that modify saturated fatty acids by re- synthesizing polyunsaturated and odd fatty acids, C20:3n-3, C20:6n-3, C 19:0 and C 21:0 (Dalsgaard e*t al.* 2003, Monroig, Tocher & Navarro 2013, Zhang *et al.* 2023). Some fatty acids are characteristic biomarkers of diatoms (C14:0 and C16:0) and others (*iso*/*anteiso*) are indicators of microbiological activity in the organic matter present in the marine sediment, being recognized by C15:0*iso*, C15:0*anteiso* and C17:0*iso* (Cooper & Blumer 1968, Opute 1974, Volkman *et al.* 1989).

The dominance of some fatty acids in the reproductive period (warms), such as C16:0, C19:0 and C20:1 for females and C18:0, C20:4n-6 and C20:3n-3 for males, indicates metabolic differences related to the processes of oogenesis and spermatogenesis (Martínez-Pita, García & Pita 2010b, Zárate *et al.* 2016). The reduction in the proportion of C14:0, C16:0 and C18:0 in summer may also indicate that they are being used as energy for gametogenesis, since they are the main saturated fatty acids used in β-oxidation (Houten & Wanders 2010, Martínez-Pita, García & Pita, 2010a). Differences in the fatty acid profile may also be related to two factors: a) activation of enzymes (desaturases and elongases) of lipid metabolism; b) stages of gonadal development (Hughes *et al.* 2006, Carboni *et al.* 2012). In this study, differences were observed in the level of unsaturated fatty acids between sexes and seasons, which may indicate that these enzymes were recruited differently in the organisms. The fatty acid profile in summer also expresses the composition of mature gametes and gonads, since the gametes have not been released (Díaz de Vivar *et al.* 2019).

In the population studied, polyunsaturates in males were more abundant than in females, suggesting greater metabolic importance, as observed in holothurians such as *Holothuria forskali* (David *et al.* 2023), and echinoids such as *Psammechinus miliaris*, *Paracentrotrus lividus*, *Arbacia lixula* and *Arbacia dufresnii* (Hughes *et al.* 2006, Martínez-Pita, García & Pita, 2010b, Zárate *et al.* 2016). Alternatively, in *Stichopus japonicus* there is a slight difference in the composition of fatty acids between the sexes (Kasai 2003) or a greater amount of polyunsaturates in the female gonads depending on the habitat (Xu *et al.* 2016). The importance of unsaturated fatty acids for gametes is their antioxidant and energetic activity, which is influenced by diet, since it directly affects the production, quality and viability of gametes, increasing the amount of TAG and the proportion of polyunsaturated fatty acids (Gianasi *et al.* 2016).

The absence of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in gonadal tissues — both crucial fatty acids for reproduction — may be related to the limited availability of their metabolic precursors (Blair et al., 2003; George, Fox & Wakeham, 2008) or the period in which the organisms were collected from the environment. In *Apostichopus japonicus*, increased concentrations of these fatty acids were observed in the cell-free coelomic fluid prior to spawning (Jiang et al., 2021), suggesting a seasonal or phase-specific mobilization of these compounds. On the other hand, the presence of 20:4n-6 (arachidonic acid, ARA) and 20:3n-3 is particularly relevant in the context of male reproductive physiology. ARA is the precursor of eicosanoids involved in sperm motility, capacitation, and cell signaling during reproduction (Chimsung, 2014). The detection of these fatty acids may indicate selective lipid allocation to support sperm function and male fertility. Thus, even in the absence of EPA and DHA, the presence of ARA and 20:3n-3 may reflect a compensatory mechanism to ensure reproductive performance.

Therefore, understanding how and which fatty acids are utilized by the gonads guides holothurian cultivation strategies. Diet biochemical composition must contain the essential nutrients for both energy and gametogenic metabolism, which is only possible by deepening the knowledge on basic biochemistry of each species and understanding how diets and environmental factors influence the variation in biochemical composition. It is also imperative to standardize techniques in both macro and microscopic determination in order to better interpret the reproductive cycle of both individuals and populations.

Conclusion

The gametogenic cycle of *Holothuria (H.) grisea* shows differences between extreme seasons (winter and summer) with little reproductive activity and lower expression of the gonadal index in the former. Macroscopic observations of the gonadal tubules indicated a great heterogeneity in the thickness, branching, consistency and colors, which confirms the existence of the tubule recruitment model in this species, especially when individuals were not in the mature stage. In this way, these aspects have been shown to be ineffective tools for reproductive diagnosis, requiring the use of microscopic (histological) analysis and biochemical techniques. In females the cytometric analysis of oocyte size was an accurate diagnosis of gametes maturation and cell production. Furthermore, it is crucial that the histological analysis covers a larger area of the gonad, reducing uncertainties in the evaluation of gametogenic events in the whole gonad. The composition of fatty acids in the gonads is shaped by diet and intestinal microbiota, but may also be related to the distinct metabolic demand between males and females during gametogenesis and suggest a differentiated metabolic importance.

Ethical statement

The present investigation did not involve regulated animals and did not require approval by an Ethical Committee.

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