# Reproductive biological characteristics and fatty acid profile of *Holothuria mammata* (Grube, 1840)

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## Abstract

In southern Europe, the interest in sea cucumbers is relatively recent, but several fisheries have now been developed, as well as aquaculture projects. *Holothuria mammata* is one of the holothurian species targeted. In this study, some reproductive biological characteristics of *H. mammata* were studied, such as sex ratio, weight, gonadosomatic index, gonadal tubules morphology, and oocytes diameter in different stages of maturation. The lipid profile was also analysed in order to highlight the fatty acid requirements in a diet for broodstock conditioning. The spring period, from March to April, corresponds to maturation and subsequent spawning. Total fat content was 1.00% ± 2.45, and highest values of fatty acids were obtained for stearic acid (C18:0) (8.91% ± 1.44), arachidonic acid (C20:4 ω6) (19.97% ± 1.30), and eicosapentaenoic acid (C20:5 ω3) (10.85% ± 0.37). The findings lead us to consider higher requirements of long-chain polyunsaturated fatty acids (or LC–PUFAs) in broodstock diets, and their potential uses as functional foods and nutraceuticals. It is crucial to develop studies to increase the biological knowledge of *Holothuria mammata*, and create conditions to domesticate broodstock of this species and improve the rearing of sea cucumbers in Europe.


## Introduction

Interest in sea cucumber species from the Mediterranean Sea is fairly recent, and focues particularly on *Holothuria arguinensis, H. mammata, H. polii, H. sanctori, H. tubulosa* and *Stichopus regalis* (Aydin 2008; Ramón et al. 2010; Navarro et al. 2012). These species are currently being fished in Turkey, Spain and France and traded in Asian markets (Chakly et al. 2004; Vannuccini 2004; Aydin 2008).

Sea cucumbers are also consumed for their beneficial effects on human health. Several studies in the last two decades have demonstrated that sea cucumber extracts possess biological attributes that promote wound healing and exhibit antimicrobial, antioxidant, and anticancer properties (Beauregard et al. 2001; Jawahar et al. 2002; Roginsky et al. 2004; Ogushi et al. 2015; Tian et al. 2005; Hing et al. 2007; Zhong et al. 2007; Li et al. 2008 a, b; Lu and Wang 2009; Janakiram et al. 2010; Bordbar et al. 2011; Santos et al. 2015 a,b). Furthermore, sea cucumbers are healthy food. They are rich in protein and low in fat, and are particularly rich in the essential fatty acids EPA (eicosapentaenoic acid), DHA (docosahexanoic acid), and ARA (arachidonic acid) (Fredalina et al. 1999; Chen 2004; Santos et al. 2015 a, b).

The present study aimed to characterise the reproductive biology of *Holothuria mammata*,by monthly examination of the gonadosomatic index (GI) and histological analyses of the gonadal tubules in order to improve the knowledge of this species for its aquaculture production. Total fat content and the fatty acid profiles were analysed.

## Materials and methods

### *Sampling and gonadosomatic index*

In total, 73 *Holothuria mammata* individuals were collected from the Peniche coast in Portugal (39°21′14.5″N and 9°23′43.4″W). The sampling procedure was carried out at low tide and sea cucumber individuals were transported alive to the Aquaculture Laboratory of MARE-IPleiria in Portugal. A longitudinal incision was made along the dorsal surface and the coelomic fluid and gonads were removed. Drained body weight (dwt) and gonad weight (gwt) were measured, and gonads were fixed in 10% buffered formalin for 24 hours. The gonadosomatic index (GI) was calculated using the following equation (Ramofafia et al. 2000):

$$GI = \frac{gwt}{dwt} \times 100$$


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### *Macroscopic examination of the gonadal tubules, and histological analyses*

A maturity scale, based on macroscopic examination of the gonadal tubules, was followed and included: stage I – indeterminate, stage II – growing, stage III – mature, stage IV – partly spawned and stage V – spent (Ramofafia et al. 2000).

Ten tubules of each gonad were removed, and their length and diameter measured. For histology, the gonadal tubules were dehydrated and embedded in paraffin. Sections (6 $\mu$m thick) were stained with haemotoxilin and eosin, and five gametogenic stages were defined, following previous works, as recovery, growing, mature, partly spawned and spent (Ramofafia et al. 2000, 2003; Shiell and Uthicke 2006; Navarro et al. 2012). The diameter of oocytes was measured with LAS Leica imaging software on a Leica DM microscope (Leica, Bensheim, Germany).

### *Quantification of total fat content and fatty acid profile*

#### *Lipid extraction and fatty acid profile*

The total lipid extraction method was adapted from Bligh and Dyer (1959) following a dry weight basis. Fatty acid methyl esters were prepared according to the methods of Lepage and Roy (1986) and Masood et al. (2005): 0.015 mg of crude fat was dissolved in 5 mL acetyl chloride:methanol (1:19 v/v) and heated in a water bath at 80 ºC for 1 h. Then, 1 mL ultrapure water and 2 mL n–heptane were added and the solution was vortex-stirred for 1 min followed by centrifugation at 1,500 g for 5 min. The organic upper phase was recovered and analysed by gas chromatography (GC).

A Finnigan Ultra Trace gas chromatograph equipped with a Thermo TR–FAME capillary column, an auto sampler AS 3000 from Thermo Electron Corp. (Boston, Mass., U.S.A.), and a flame ionization detector were used to quantify fatty acid methyl esters. Fatty acid methyl esters were identified in comparison to an external standard, fatty acid methyl ester mix (PUFA No 3 from Menhaden oil) was purchased from Supelco (Bellefonte, PA, USA).

### *Statistical analysis*

A chi-square test ($\chi 2$) was done to evaluate the differences in relation to the unit. Also, two-way analysis of variance ANOVA, followed by a *Bonferroni* multiple comparation test, was performed on the GI and weight (gutted).

For all statistical tests, the significance level was set at $p \leq 0.05$. All statistical tests were performed with IBM SPSS Statistics 23.

## Results

### *Sex ratio and weight*

The sex ratio obtained, 3:4, did not differ significantly from the unity ($\chi 2(1) = 0.961$; $p > 0.05$). The gutted body weights of individuals varied from 60–140 g for males, 85–135 g for females, and 50–140 g for individuals lacking gonads.

### *Gonadosomatic index*

The monthly gonad maturation pattern showed a high peak in April for both sexes. From January until April there was an increase in the GI values for both sexes, followed by a decrease in May and a steady increase in June. It was also noted that the GI values did not vary greatly between individuals, suggesting that the pattern of spawning may be synchronous among sexes. The maximum GI values obtained for April were 13.87% for females and 14.11% for males. No significant differences were detected between the GI values of both sexes ($F(1, 45) = 0.009$; $p > 0.05$) and months ($F(2, 45) = 2.238$; $p > 0.05$), nor was there a significant correlation between sexes and months ($F(2, 45) = 0.363$; $p > 0.05$).

The gutted body weight did not fluctuate significantly over the months except for the individuals lacking gonads. No significant differences were detected among the months ($F(2, 45) = 0.138$; $p > 0.05$) and in the interaction between sexes and months ($F(2, 45) = 2.655$; $p > 0.05$).

### *Gonad morphology*

The gonads of both sexes consisted of a saddle-shaped gonad base from which extended numerous branching tubules of varying sizes. The gonoduct opened externally at the gonopore, on the dorsal side above the mouth. In total, five tubule size classes were defined based on tubule size and appearance (Table 1). Seasonal changes were observed. Overall, gonad growth involved the formation of new tubules arising from the gonad base, with a subsequent increase in tubule length and diameter. This was never, however, observed at stage I (indeterminate). At stage II (growing), both females and males could be identified by the presence of developing oocytes and spermatocytes. As the gonads approached maturity, tubule colouration also changed, depending on the stage of maturity. In mature females the tubules appeared orange. Mature gonads were bright orange in colour and tubules had transparent thin walls through which oocytes were visible. In mature males the gonads were whitish. Tubule length was a good indicator of the reproductive maturity stage, with females generally having a longer tubule length than males.

**Table 1.** Five maturity stages of *Holothuria mammata*, based on gonad tubule morphology (n=51).

| Maturity stage and sex | Gonad wt (g) | Tubule | | | | |
|---|---|---|---|---|---|---|
| | | Length (mm) | Diameter (mm) | Branching | Condition | Colour |
| I indeterminate | Not identified | | | | | |
| II Females | 6.4–12.8 | 29.8–43.6 | 1.8–2.1 | 1–3 | Growing oocytes (32.3–50.6 µm) | Orange |
| Males | 5.8–15.1 | 34–40.4 | 0.3–1.7 | 1–3 | Developing sperm | White |
| III Females | 7.8–22.1 | 36.4–50.6 | 2.1–2.2 | 1–4 | Oocyte visible through tubule wall (108–122 µm). | Bright orange |
| Males | 8.9–25.6 | 35.2–41.7 | 2.3–3.5 | 1–4 | Tubules packed with sperm | Creamy white |
| IV Females | 4.8–7 | 38.6–43.9 | 1.7–2.1 | 1–4 | Reduced tubules, relict oocytes present, empty lumen visible | Bright orange |
| Males | 3.2–6.9 | 29–41.6 | 2.1–2.3 | 1–4 | Unspawned tubules with residual spermatozoa | White |
| V Females | 2.1–5.3 | 16.2–18 | 2.3–2.5 | 1–4 | Tubules shrunken and wrinkled in size. Relict oocytes. | Orange (transparent) |
| Males | 3.3–5.4 | 16.4–22 | 2.4–3.3 | 1–4 | Relict sperm presented | Transparent |

Throughout the spawning season, the simultaneous presence of both spawned and unspawned tubules indicated that partial spawning was occurring (stage IV), which could be a characteristic of this species. Spent gonad tubules (stage V) were wrinkled and greatly reduced in size.

Females showed translucent and light pinkish colours during the growing phase (stage II), while orange or even reddish dominated during maturity (stage III).

## *Histology*

The histological analysis revealed that the four gonad maturity stages (indeterminate stage was never observed) correlated well with the four stages of gametogenic development. A description of the histological features of each gametogenic stage is detailed below.

### *Females*

Stage II: *growing*

The growing stage (Fig. 1A) was characterised by active vitellogenesis. Early and mid-vitellogenic oocytes were present. These oocytes had a distinct germinal vesicle. Vitellogenic oocytes were surrounded by follicle cells throughout development.

Stage III: *mature*

Mature ovaries were densely packed (Fig. 1B). The oocytes remained within their follicle and the germinal vesicle. An increase in oocyte diameter occurs and it is possible to visualize a well–defined nucleus.

Stage IV: *partly spawned*

It was observed that not all ovarian tubules released gametes during spawning. Partly spawned ovaries contained both spawned and unspawned tubules (Fig. 1C). Partly spawned tubules had a reduced diameter and a wrinkled appearance.

Stage V: *spent*

Spent ovaries were wrinkled and shrunken, with the presence of relict oocytes in the lumen (Fig. 1D), the gonad wall was thick.

### *Males*

Stage II: *growing*

A striking feature of growing testis was the presence of numerous infolds of the germinal epithelium, which extent into the lumen (Fig. 2A). These infolds were lined by a dense layer of spermatocytes organised in short columns. It was observed in late growing-stage testis due to the growing number of spermatozoa abundance in the lumen.

Stage III: *mature*

The infolds of the germinal epithelium were reduced or absent and the lumen was packed with spermatozoa (Fig. 2B). A few spermatocytes were present along the germinal epithelium. The gonad wall was at its minimal thickness.

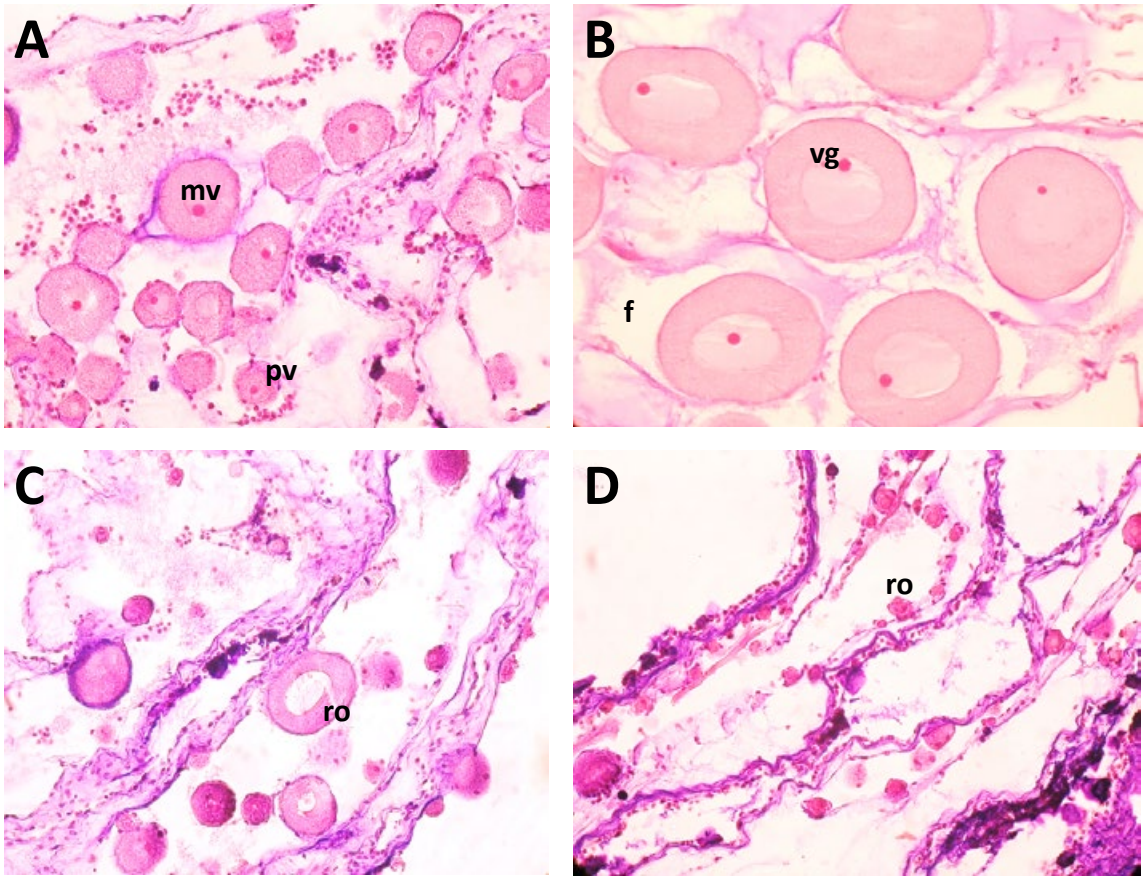


**Figure 1.** Oogenesis. **A**: growing ovaries, with oocytes in pre–vitellogenic (pv) and mid–vitellogenic (mv) stage. **B**: mature ovaries with oocytes enclosed within their follicle (f) and germinal vesicle (gv). **C**: partly spawned ovaries, with the presence of mature unspawned oocytes, relict oocytes (ro). **D**: spent ovaries with intensive shrinkage of the tubules occurred and a few relict oocytes persisted (ro). (Scale bars = 100 $\mu$m).

Stage IV: *partly spawned*

It was observed that not all gametes were released during spawning, and dense aggregations of spermatozoa and phagocytes were present in the lumen (Fig. 2C).

Stage V: *spent*

Spent tubules were shrunken and generally had an empty lumen, except for a few relict spermatozoa (Fig. 2D).

### *Quantification of total fat content and fatty acid profile*

The total fat content for *H. mammata* was 1.00 ± 2.45%. Table 2 shows the fatty acid profile, with a high abundance of araquidonic acid (C20:4 ω6) (19.97 ± 1.30%), eicosapentaenoic acid (C20:5 ω3) (10.85 ± 0.37%), and stearic acid (C18:0) (8.91 ± 1.44%). The ω3:ω6 ratio obtained was 0.48.

**Table 2.** Fatty acid profile of *Holothuria mammata* by mean of fatty acid ± standard deviation (n = 3).

| Fatty acid | *Holothuria mammata* |
|---|---|
| ∑ SFA[a] | 14.18 ± 1.68 |
| C 12:0 | 0.65 ± 0.03 |
| C 14:0 | 0.88 ± 0.02 |
| C 16:0 | 3.74 ± 0.18 |
| C 18:0 | 8.91 ± 1.44 |
| ∑ MUFA[b] | 4.48 ± 0.26 |
| C 18:1 ω7 | 2.89 ± 0.15 |
| C 18:1 ω9 | 1.59 ± 0.11 |
| ∑ PUFA[c] | 46.30 ± 2.18 |
| C 16:3 | 7.27 ± 0.13 |
| C 18:3 ω3 | 1.07 ± 0.19 |
| C 20:2 ω6 | 6.35 ± 0.17 |
| C 20:4 ω6 | 19.97 ± 1.30 |
| C 20:5 ω3 | 10.85 ± 0.37 |
| C 22:6 ω3 | 0.79 ± 0.02 |
| ω3/ω6 | 0.48 |

a saturated, b monounsaturated, c polyunsaturated

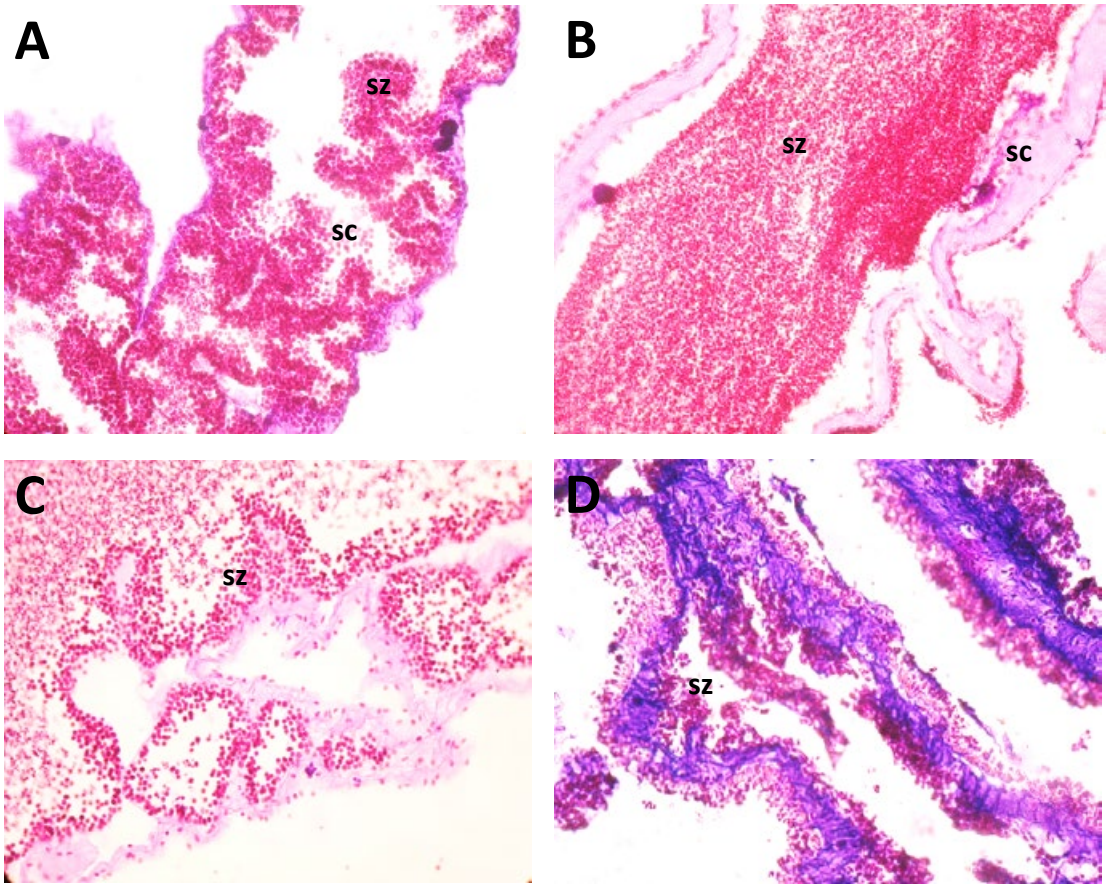


**Figure 2.** Spermatogenesis. **A**: growing testis with developing spermatocytes (sc) and spermatozoa (sz) beginning to fill lumen as part of the growth progress. **B**: mature testis with spermatocytes (sc) persisting along gonad wall and full spermatozoa (sz) accumulation. **C**: partly spawned testis, with spermatozoa (sz) in the lumen. **D**: spent testis with residual spermatozoa (sz) or empty lumen. (Scale bars = 100 $\mu$m).

## Discussion

Although *Holothuria mammata* has a wide geographic distribution, data concerning its reproductive biology and biotechnological potential are not reported in the literature. There is only one study from Aydin et al. (2011) concerning the food potential of *H. mammata*. Therefore, our data were compared with those previously reported for similar species of the genus *Holothuria* or other species from the order Aspidochirotida of different geographic regions.

### *Sex ratio*

The sex ratio obtained for *H. mammata* did not differ significantly from 1:1, although it has presented an unbalanced ratio of 3:4. In most holothurians from the order Aspidochirotida, the sex ratio often coincides with a balanced 1:1 ratio (Ramofafia et al. 2001; Rasolafonirina et al. 2005; Asha and Muthiah 2008). However, some species demonstrated an unbalanced ratio of 1:2 or 2:3, due to fishing pressure, which affects the population of a given area, or due to species that undergo asexual reproduction (Harriott 1982; Uthicke and Klumpp 1998; Uthicke and Karez 1999; Shiell and Uthicke 2006; Muthiga et al. 2009). In this study, the site where samples were collected is not considered to be a locality with high fishing activity. However, this site is seasonally affected by strong waves, which could be considered a factor affecting the balanced sex ratio.

### *GI and gutted body weight*

Sea cucumbers often show one annual reproductive cycle (Cameron and Fankboner 1986; Smiley et al. 1991; Tuwo and Conand 1992; Conand 1993a,b; Chao et al. 1995), although semi–annual cycles (Harriott 1985; Conand 1993b) or even continuous reproduction activity throughout the year (Harriott 1985) are also frequent, particularly in tropical species. On the Peniche coast, *H. mammata* followed one single annual cycle, as would any typical temperate sea cucumber species (Sewell and Bergquist 1990; Tuwo and Conand 1992; Sewell et al. 1997). The maximum reproductive activity was observed in spring. For echinoderms, the annual reproductive cycle is clearly related to sea watr temperature as gametogenesis only occurs when sea water temperature rises. Generally, cold waters are associated with high nutrient availability that triggers phytoplankton blooms and favours larval development

(Boidron–Metairon 1995). Indeed, a synchronisation of echinoderms spawning according to phytoplankton availability is considered to be an advantageous adaptation (Starr et al. 1990), and our study revealed that the peak of gonad maturation was synchronised with phytoplankton availability, which in southern Europe is at a maximum in spring. The only observation of spawning individuals belonging to *H. mammata* was recorded at Gran Canaria (Spain) in August during a study by Navarro et al. (2012) for *H. sanctori*. This could suggest that *H. mammata*, like *H. sanctori,* reproduces in the months leading up to and including the summer. These observations may indicate that the reproductive cycles of *H. sanctori* and *H. mammata* may be synchronised, as reported for other echinoderms (Ramofafia et al. 2003; Shiell and Uthicke 2006; Muthiga et al. 2009).

The gutted body weights of both sexes showed slight differences between males and females. In general, females were heavier than males due to higher fecundity rates, requiring greater nutrient storage. The weight of individuals lacking gonads was generally slightly lighter than sexed individuals. This fact demonstrates the importance of gonads in establishing the weight of an individual, which is in agreement with observations made of other species (Morgan 2000; Ramofafia et al. 2000, 2003; Asha and Muthiah 2008; Navarro et al. 2012).

### *Macroscopic examination of gonadal tubules and histological analysis*

The monthly assessment of gonadal tubule length showed some discrepancies between the sexes. It is a common feature of sea cucumbers for females to have a greater number of tubules (given its highest reproductive output) than males (Shiell and Uthicke 2006; Toral-Granda and Martinez 2007). However, it is described for a large number of species of the order Aspidochirotida, such as *Holothuria fuscogilva*, *H. nobilis, H. scabra,* and *H. atra*, that male gonadal tubules are slightly longer in length than female gonadal tubules (Conand 1993 b). Nevertheless, *H. mammata* counteracted this trend by having a distinct gonadal morphology that may be characteristic of the species or may possibly be related to the collection of young males with less weight and smaller gonads.

With regard to oocyte diameter, the range of values in stage III of oogenesis (mature) was 108–122 $\mu$m. These values are lower than the common values in tropical species, which range from 150 to 210 $\mu$m (Conand 1993b). However, the results are consistent with those observed by Tuwo and Conand (1992) for *Holothuria forskali*, where in stage III, the values were 90–120 $\mu$m. In the partly spawned stage, residual mature oocytes were present, and in stage V, trace oocytes were observed. These findings could indicate that spawning is not complete and different stages of oocyte development could be found throughout the year, as observed for other tropical sea cucumbers species (Ramofafia et al. 2000; 2003; Navarro et al. 2012).

### *Quantification of total fat content and fatty acid profile*

A recent study by Aydin et al. (2011) on sea cucumber species in Turkey, including *H. mammata*, showed a substantially lower total fat percentage (0.09% ± 0.08) than the values obtained in our study (1% ± 2.45). This may be explained by seasonal changes in feeding behaviours and geographical variations (Chang-Lee et al. 1989) and, according to Ozer et al. (2004), the handling procedures are also likely to affect the chemical composition of sea cucumbers. Chang-Lee et al. (1989) have defined a range in total fat percentage for holothurians of 0.1–0.9%, but Santos et al. (2015a, b) report that the total fat content for *H. forskali* was 4.83% ± 2.33% and 3.63% ± 0.11% for *Stichopus regalis*.

Regarding fatty acid profile, in our study, *H. mammata* presented lower total percentages of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, than was reported by Aydin et al. (2011). On the other hand, *H. mammata* presented higher values of EPA, DHA and ARA fatty acids as well as of stearic acid (C 18:0). Several studies have shown that high values of the ω3:ω6 ratio have resulted in increased protection against degenerative and cardiovascular diseases (Russo 2009; Smith et al. 2009). According to FAO (2004), ω3/ω6 ratio should range between 1:8 and 2:5; the ratio observed for *H. mammata* in this study (± 1:2) is therefore consistent with those reported for other species.

## Acknowledgements

This study was supported by the Fundação para a Ciência e Tecnologia (FCT), through the strategic project UID/MAR/04292/2013 granted to MARE project "Sea cucumber: economic valorization of fisheries rejects" Project nº 31–04–01–FEP–188 GacOeste, PROMAR, Operational Programme for Fisheries, Coordinated by MARE–IPLeiria, Portugal.

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