

Reproductive cycle of the traditionally exploited sea cucumber *Holothuria tubulosa* (Holothuroidea: Aspidochirotida) in Pagasitikos Gulf, western Aegean Sea, Greece

Georgios KAZANIDIS^{1,2,*}, Alexios LOLAS¹, Dimitris VAFIDIS¹

¹Department of Ichthyology and Aquatic Environment, School of Agricultural Sciences, University of Thessaly, Nea Ionia, Magnesia, Greece
Current address: Oceanlab, University of Aberdeen, Newburgh, Aberdeenshire, UK

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Abstract: The reproductive cycle of the traditionally exploited sea cucumber *Holothuria tubulosa* was investigated in the Pagasitikos Gulf (39°18'457"N, 23°05'869"E) from June 2007 to July 2008. The study examined the microscopic characteristics of the gonads and was based on maturity index (MI) and oocytes' size-frequency distribution. The reproductive cycle was found to be synchronous between sexes, following an annual pattern. The minimum MI values were recorded in December and January, marking the onset of the reproductive cycle. During the spring, MI increased due to gamete development and proliferation. Mature specimens were prominent in summer while spawning occurred between July and September. The maximum MI values were recorded in mid-autumn, when most specimens were at the postspawning stage. The oocytes' size-frequency distribution showed analogous seasonal variability. The present findings are crucial for establishing an efficient management strategy for this commercial and ecosystem-engineering species, since its exploitation in Greek waters has not been under official control for more than a century. The authors propose a seasonal ban on the exploitation of the species from July to September and a minimum individual drained weight limit of 250 g.

Key words: Histology, maturity index, gametogenesis, spawning, eastern Mediterranean

1. Introduction

Holothuria tubulosa Gmelin, 1788 is a species of sea cucumber with a Mediterranean distribution (Borrero-Pérez et al., 2009), recorded in various habitats but usually found in the seagrass meadows of *Posidonia oceanica* (Gustato et al., 1982; Bulteel et al., 1992). During the last 2 decades, specimens harvested in Turkish regions have been exported as a frozen or dried product to Far East countries (Aydin, 2008; Aydin et al., 2011; Sicuro and Levine, 2011), where they are regarded as a gastronomic delicacy (Sicuro et al., 2012) and a source of pharmaceutical compounds (Bordbar et al., 2011). In Greece, *H. tubulosa* is harvested by hand or by using hooks and has been utilized as bait in longline fisheries for more than a century (Antoniadou and Vafidis, 2011). In Greek regions, this kind of exploitation is not under official control, and preliminary studies have suggested its contribution to low stocks of natural populations (Antoniadou and Vafidis, 2011). This is of high concern, as *H. tubulosa* is considered an ecosystem-engineering species, mainly because of its contribution to the bioturbation of the sediments by its feeding behavior (Meysman et al., 2006; MacTavish et al., 2012); it stabilizes the bacterial community in the sediment (Amon and

Herndl, 1991; Coulon and Jangoux, 1993) and converts detritus in nitrogenous compounds, thus enhancing primary productivity (Işgören-Emiroğlu and Günay, 2007).

The most comprehensive study on *H. tubulosa* reproductive biology was carried out in the Adriatic Sea, focusing on the temporal distribution of maturity stages (Despalatović et al., 2004), contrary to a number of other regions across the Mediterranean, where information has been provided through the use of lower-precision techniques like gonad index (Bulteel et al., 1992; Kazanidis et al., 2010). However, life-history information acquired using state-of-the-art techniques is a prerequisite for the efficient management of natural resources.

The aim of the present study was to elucidate the reproductive cycle of *H. tubulosa* in the Pagasitikos Gulf through the histological examination of its gonads, focusing on the temporal allocation of the spawning period, as well as to provide an indication regarding the environmental conditions involved in the release of the gametes. Up to now, parameters such as temperature (e.g., Conand, 1993; Chao et al., 1995), photoperiod (e.g., Ramofafia et al., 2000; Muthiga, 2006), phytoplankton blooms (e.g., Chao

* Correspondence: r02gk11@abdn.ac.uk

et al., 1995), or lunar cycle (e.g., Purwati and Luong-van, 2003) have been suggested as potential spawning cues for temperate and tropical holothurian species. Eventually, it could prove a difficult task to determine which of these environmental factors is the key driver to this behavior (Olive, 1995).

Since no official regulation exists regarding the exploitation of the species in Greece, information on the reproductive cycle of *H. tubulosa* will be crucial in the development of a potential conservation strategy within the regions of the Greek Exclusive Economic Zone.

2. Materials and methods

The present survey took place in the Pagasitikos Gulf, which is located in the central-western region of the Aegean Sea (Figure 1). It is a semienclosed shallow water basin (mean depth is 69 m) in which the water masses are cold and homogeneous in winter (12.6 °C) and highly stratified in the summer (27.5 °C) (Petihakis et al., 2005). In the sampling area (Kato Gatzea: 39°18'457"N, 23°05'869"E), the bottom consists mainly of a soft substratum with a sparse meadow of the seagrass *Zostera marina* Linnaeus,

1753 and patches of rocks, dominated by communities of photophilic algae (Lolas and Vafidis, 2013). The soft sediment consists mainly of sand fractions and silt, with large amounts of detritus that provide a favorable environment for the establishment and development of the sea cucumbers (Mercier et al., 1999, 2000).

Samples were collected between June 2007 and July 2008 on a monthly or semimonthly basis via scuba diving, reaching a total of 15 samplings. In each sampling event, 20–25 specimens were collected from the soft sediment, in depths of up to 10 m, while the seawater temperature was recorded with a Seabird SBE 19Plus CTD instrument (measurement range: -5 to $+35 \pm 0.0074$ °C). The body size at first maturity was calculated in terms of the drained weight of the individuals (DW_{50}), according to Conand (1981). After dissection, the gonads from each individual were fixed in 10% seawater formalin and preserved afterwards in 70% ethanol pending further analysis.

The histological examination was carried out using the hematoxylin-eosin regressive staining method, accompanied by the use of a light microscope and a digital camera. From each female, the diameter of 60 oocytes

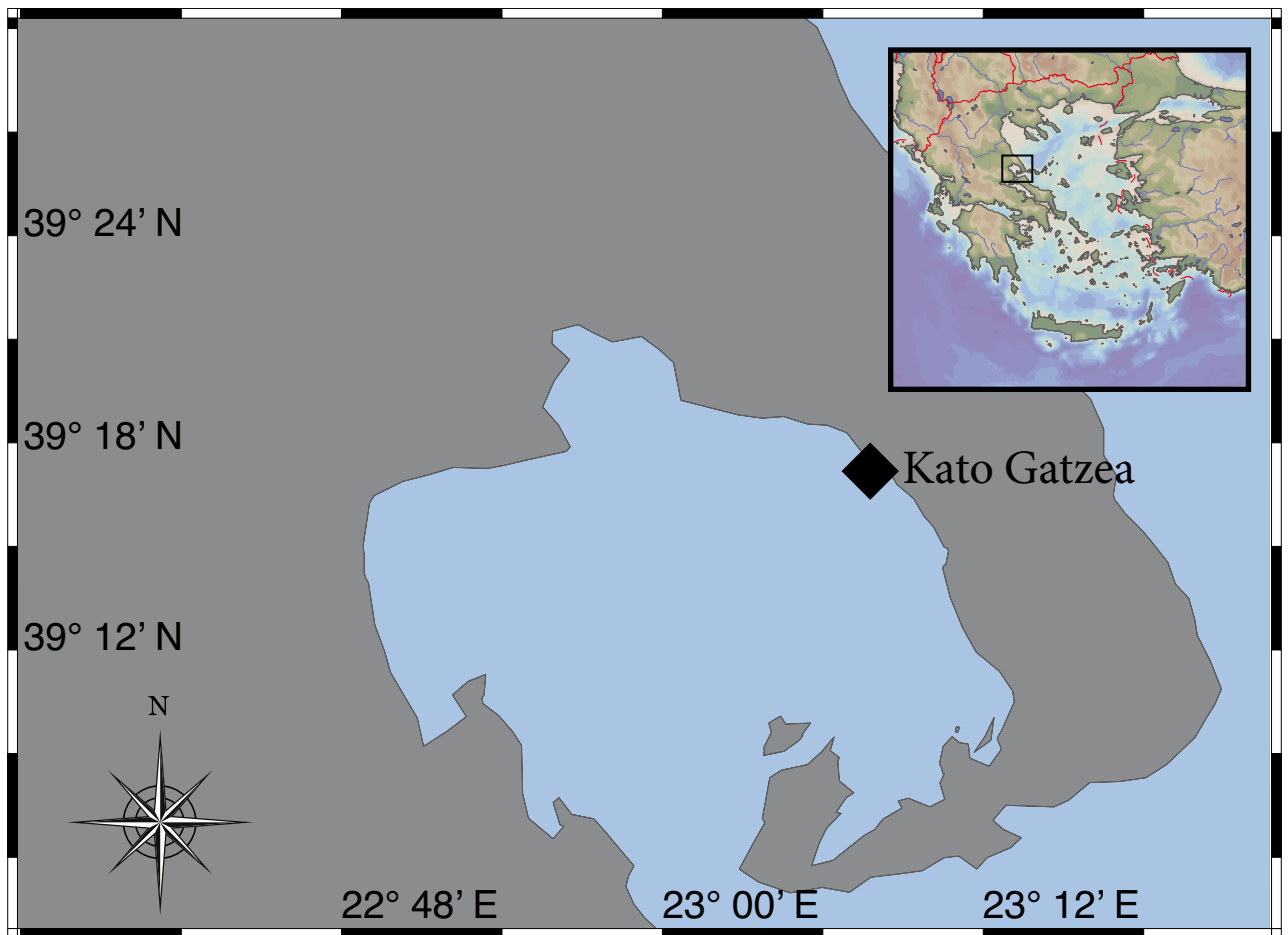


Figure 1. Map of the study area with the sampling location.

whose nuclei could be observed was recorded (Doyle et al., 2012) and the values were grouped in 20- μm size classes (Despalatović et al., 2004). The null hypothesis of no significant differences in oocyte diameter between the samplings and between the seasons was tested using one-way ANOVA, while individual group differences were assessed by Fisher's LSD procedure. The homogeneity of variances was tested (Levene test) and, whenever necessary, the log transformation $\log(x + 1)$ was used (Zar, 1996).

Microscopic features (i.e. type of gametes present in the lumen, presence of connective tissue) were used to assign the individuals to 1 of the 5 developmental stages described for *H. tubulosa* by Despalatović et al. (2004): recovery stage (I), growing stage (II), mature stage (III), spawning stage (IV), and postspawning stage (V). The relative percentage of the developmental stages at each sampling was used in the estimation of the maturity index (MI) for males and females using the following equation:

$$MI = \frac{\sum_{i=1}^5 i \cdot n_i}{N},$$

where i = developmental stage, n_i = number of individuals in developmental stage i , and N = total number of individuals in the sample (Yoshida, 1952).

3. Results

From the microscopic examination of the gonads from a total of 314 individuals, 133 were identified as males, 126 were identified as females, and 55 were classified as immature. The sex ratio did not reveal a deviation from the 1:1 equilibrium ($\chi^2 = 0.189$, 1 df, $P = 0.664$). The DW_{50} was estimated to be approximately 220 g (Figure 2).

The temporal allocation of the developmental stages (Figure 3) and the pattern of the MI (Figure 4) revealed synchronous development between sexes, characterized by clear seasonal variability. The recovery stage (I) occurred over the winter months (December–January), corresponding with the lowest MI values and marking the

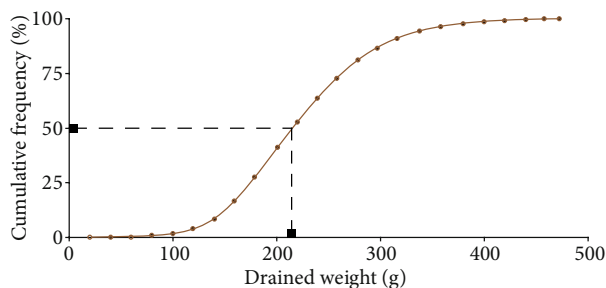


Figure 2. Estimation of the drained weight at first sexual maturity (DW_{50}) in *H. tubulosa* individuals from Pagasitikos Gulf.

onset of the reproductive cycle. The growing stage (II) was observed between March and May, with slight differences between sexes, thus contributing to the increase in MI values over the spring months. The presence of individuals in the mature stage (III) was prevalent over the summer period (June–August) for both sexes. Individuals in the spawning stage (IV) were present from July to September, with slight differences in frequency between the sexes. The spawning period was followed by a short postspawning period (V) in October and November, which in turn led to the highest values of the MI during the autumn months, designating the end of the reproductive cycle.

The variation in the values of oocyte diameters was significantly different between the samplings ($F_{14,7545} = 1718.06$, $P < 0.0001$), as well as between the seasons ($F_{3,7556} = 3856.96$, $P < 0.0001$), while the post hoc analysis by Fisher's LSD procedure classified the seasons in 4 different groups in the increasing order of winter < spring < autumn < summer (Figure 5)

The oocytes' size frequency distribution showed a well-marked seasonal pattern (Figure 6; Table 1). During December and January, small previtellogenic oocytes with a diameter of up to 40 μm were abundant. As oogenesis progressed during the spring months, a wide spectrum of sizes ranging from 40 to 140 μm was recorded. During the summer months, the allocation of the size spectrum showed a clear shift towards the upper size classes (up to 200 μm). In spawning and postspawning stages, the presence of oocytes in the upper size classes was reduced and the vast majority of relict oocytes were found in the size classes of 120 and 140 μm .

4. Discussion

From a brief review of the known literature, it seems that the dynamics of reproduction in sea cucumbers are known to be different between geographically separated populations (Table 2). The spawning period in the dendrochirotid *Aslia lefevrei* (Barrois, 1882) at Galway Bay (west coast of Ireland, ca. 53°N) showed a 2-month lag compared to that from Ría de Arosa (northwest Spain, ca. 43°N), probably as a response to lower temperature values in the northern region. In both populations, the release of gametes coincided with the first substantial increase in temperature after the winter minima (approximately from 6 °C to 10 °C in Galway Bay and from 11 °C to 14 °C in Ría de Arosa) (Costelloe, 1988). A similar time lag in the temporal allocation of mature and spawning individuals of *Parastichopus parvimensis* (Clark, 1913) has been reported from the northern coasts of Mexico compared to southern ones, indicating a latitudinal gradient. Although their contribution was not evaluated, it was suggested that geographical differences in the increase of daylight length/intensity and phytoplankton biomass had a role (Fajardo-León et al., 2008).

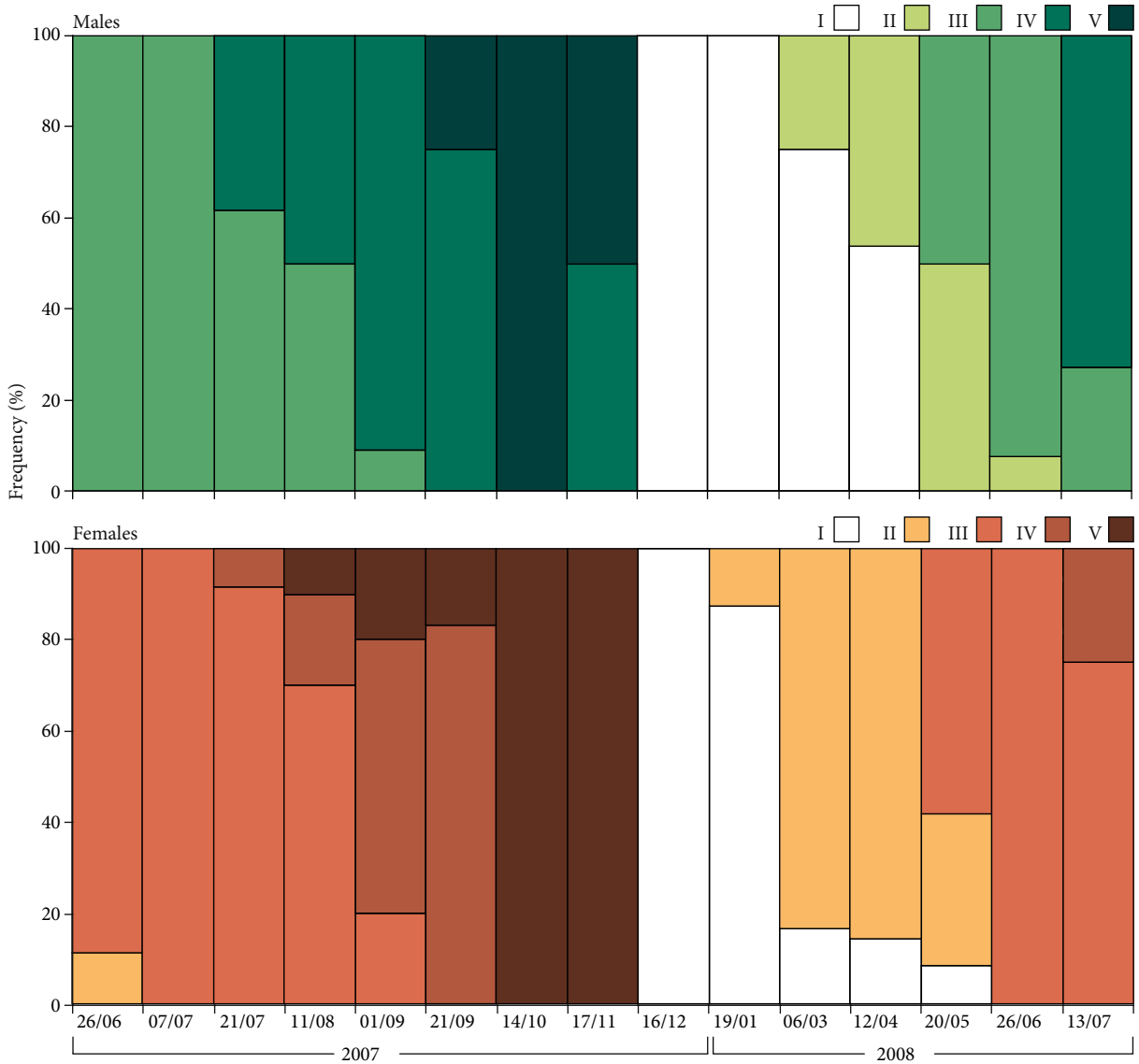


Figure 3. Temporal allocation of the developmental stages in males and females of *H. tubulosa* at Pagasitikos Gulf. I, recovery stage; II, growing; III, mature; IV, spawning; V, postspawning.

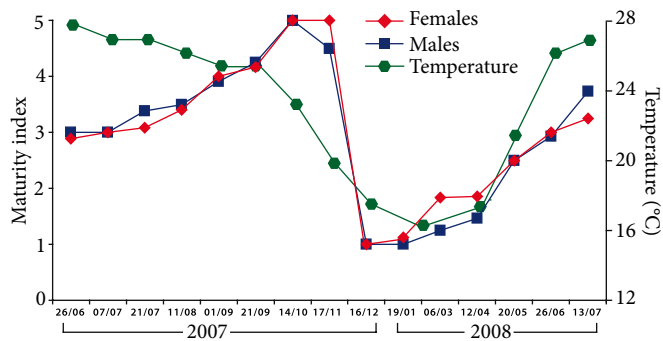


Figure 4. Temporal pattern of seawater temperature and the maturity index of males and females of *H. tubulosa* from the Pagasitikos Gulf.

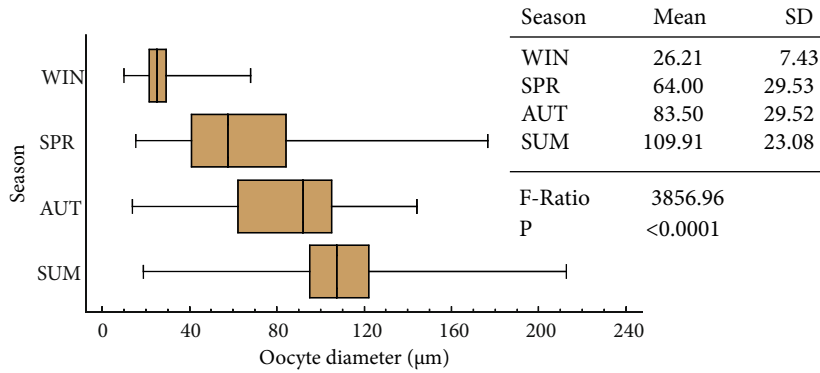


Figure 5. Results from one-way ANOVA of the variation of oocyte diameter among seasons. The order of appearance on the graph is the result of Fisher's LSD procedure. WIN: Winter (December–January), SPR: spring (March–May), SUM: summer (June–August), AUT: autumn (September–November).

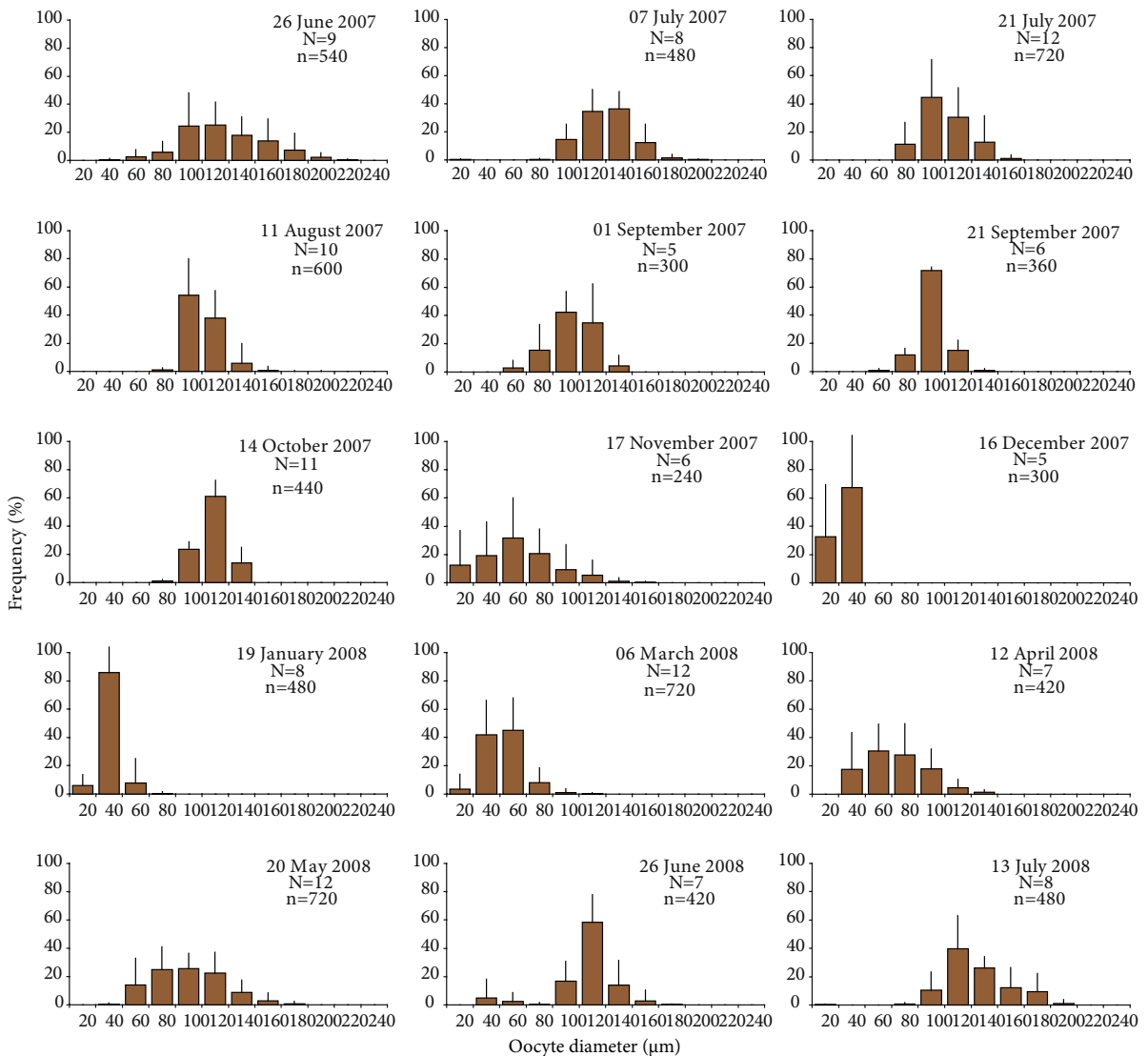


Figure 6. Oocyte size-frequency distribution of *H. tubulosa* females from each sampling. N: number of females; n: number of oocytes; error bars: standard deviation.

Table 1. Mean values \pm standard deviations of the diameter of oocytes from female individuals of *H. tubulosa* at the Pagasitikos Gulf. N: number of females; n: number of oocytes.

Date	Mean \pm SD, μm	N	n
26/06/2007	116.5 \pm 30.3	9	540
07/07/2007	119.9 \pm 19.5	8	480
21/07/2007	99.3 \pm 17.1	12	720
11/08/2007	100.2 \pm 12.90	10	600
01/09/2007	97.6 \pm 14.6	5	300
21/09/2007	90.2 \pm 10.9	6	360
14/10/2007	107.0 \pm 12.5	11	440
17/11/2007	54.1 \pm 27.3	6	240
16/12/2007	22.4 \pm 5.2	5	300
19/01/2008	28.6 \pm 7.6	8	480
06/03/2008	41.8 \pm 13.8	12	720
12/04/2008	63.3 \pm 22.6	7	420
20/05/2008	89.0 \pm 26.3	12	720
26/06/2008	105.7 \pm 23.1	7	420
13/07/2008	123.6 \pm 23.5	8	480

Table 2. Intraspecific comparison of reproductive characteristics in *H. tubulosa* and 4 species from temperate regions. Methods used: 1: histological examination; 2: GI; 3: field spawning observations; 4: presence of eggs and embryos in plankton; 5: macro- and microscopic analysis of the physical characteristics of tubules.

Species	Latitude	Method	GI max	Reproductive pattern	Spawning period	Reference
<i>H. tubulosa</i>	43°N	1	-	Annual	Jul-Sep	Despalatović et al., 2004
	42°N	2	-	-	Jul	Valls, 2004
	40°N	3	Aug	Annual	Aug-Sep	Bulteel et al., 1992
<i>H. forskali</i>	47°N	1, 2	Feb-Mar	Annual	Apr-Jun	Tuwo and Conand, 1992
	43°N	1	-	-	Jun-Aug & Dec	Despalatović et al., 2003
<i>H. sanctori</i>	28°N	2, 5	Jun-Jul	Annual	Jun-Aug	Navarro et al., 2012
<i>Aslia lefevrei</i>	53°N	1, 2	Nov-Mar	Annual	Mar-May	Costelloe, 1988
	43°N	2, 4	Oct-Jan	Annual	Jan-Mar	Costelloe, 1988
<i>Parastichopus parvimensis</i>	37°N	3	-	-	May-Jun	McEuen, 1988
	27°N	1, 2	Feb-Apr	Annual	Jan-Jul	Fajardo-León et al., 2008

The reproductive pattern observed in our study, an annual cycle with spawning over summer months, follows that described in the Adriatic Sea by Despalatović

et al. (2004). Although geographically separated, these populations are found within a relatively narrow latitudinal range (Table 2), in which abiotic factors, such as seawater

temperature, have well-defined similarities in their seasonal patterns. Considering that these parameters may have a significant role in the initiation of spawning (Smiley et al., 1991; Mercier and Hamel, 2009), the congruent patterns in the release timing of the gametes in *H. tubulosa* could be attributed to the similar profiles of temperature in the study regions (Bosc et al., 2004). Furthermore, the results from the studies analyzing the microscopic characteristics of the gonads (Despalatović et al., 2004; present study) are in agreement with findings based on different methods carried out in the Mediterranean Sea/eastern Atlantic Ocean over different years (Table 2). The obvious convergence around the temporal allocation of spawning over summer months is an indication that high seawater temperatures (22–27 °C) may have an influence on the initiation of spawning in *H. tubulosa* individuals within the boundaries of the Greek Exclusive Economic Zone.

Findings on the reproduction of the Atlanto-Mediterranean *Holothuria forskali* seem to constitute an intriguing exception to expectations around similar intraspecific characteristics within a narrow latitudinal range (Conand et al., 2002; Shiell and Uthicke, 2006; Mercier and Hamel, 2009). In the Glenan Archipelago (Brittany, ca. 47°N), the species followed an annual cycle with spawning being allocated in early spring when sea temperature was increasing (Tuwo and Conand, 1992), while in the Adriatic Sea, mature and spawning individuals were found during summer and winter months (Despalatović et al., 2003). The reasons for this divergence are unclear and further investigation is required to determine the extent of this phenomenon and underlying mechanisms.

The temporal allocation of spawning recorded in *H. tubulosa* follows findings from several tropical *Holothuria* species releasing their gametes during the warm period of the year (Table 3). Reproductive patterns of tropical

Table 3. Characteristics of reproduction in tropical *Holothuria* species. Methods used: 1: histological examination; 2: GI; 3: spawning-induction trials.

Species	Latitude	Location	Method	GI max	Reproductive pattern	Spawning period	References
<i>H. arenacava</i>	04°S	Kenya	2	Mar	Annual	Mar–May	Muthiga, 2006
<i>H. atra</i>	21°N	Taiwan	1	-	Annual	Jun–Sep	Chao et al., 1994
	08°N	Sri Lanka	1, 2	Sep & Mar	Biannual	Oct & Apr	Dissanayake and Stefansson, 2010
	11°N–9°S	Various	1	-	Continuous	All year	Pearse, 1968
	23°S	Great Barrier Reef	1, 2	May & Dec	Biannual	Jun & Jan	Harriott, 1982
<i>H. cinerascens</i>	21°N	Taiwan	1, 2	Jun	Annual	Apr–Jun	Chao et al., 1995
<i>H. difficilis</i>	21°N	Taiwan	1, 2	Aug	Annual	Sep	Chao et al., 1995
<i>H. fuscogilva</i>	01°N	Maldives	1, 3	-	Continuous	Dec–Mar	Reichenbach, 1999
	09°S	Solomon Islands	1, 2, 3	Aug–Oct	Annual	Aug–Oct	Ramofafia et al., 2000
	22°S	New Caledonia	2	Nov–Dec	Annual	Nov–Jan	Conand, 1993
	22°S	New Caledonia	2	Nov–Dec	Annual	Dec–Feb	Conand, 1993
<i>H. fuscopunctata</i>	23°S	Great Barrier Reef	1, 2	Dec	Annual	Jan–Feb	Harriott, 1985
<i>H. leucospilota</i>	21°N	Taiwan	1, 2	Jun–Jul	Annual	Jun–Sep	Chao et al., 1995
	12°N	Vietnam	1, 2	Mar & Jul	Biannual	Feb–Mar & Jun–Aug	Nguyen and Britaev, 1992
	12°S	Darwin	1	-	Annual	Late Apr	Purwati and Luong-van, 2003
	21°S	Réunion	1, 2	Jan & Apr	Annual	Feb & May	Gaudron et al., 2008
<i>H. lubrica</i>	24°N	Bahía de La Paz	1, 2	Aug	Annual	Jul–Aug	Skarbnik López et al., 2010
<i>H. mexicana</i>	09°N	Panama	1, 2	Jul	Continuous	Jan, Aug, Sep	Guzmán et al., 2003
<i>H. nobilis</i>	22°S	New Caledonia	2	Jun–Jul	Annual	Jun–Aug	Conand, 1993
<i>H. scabra</i>	13°N	Philippines	1, 2	-	Continuous	Jan, Jul	Ong Che and Gomez, 1985
	05°S	Indonesia	1, 2	Apr & Oct	Biannual	May–Jun & Dec–Jan	Tuwo, 1999
	9°S	Solomon Islands	1, 2	Aug & Oct	Continuous	Sep–Dec	Ramofafia et al., 2003
	22°S	New Caledonia	2	Aug–Dec	Biannual	Aug–Sep & Dec–Jan	Conand, 1993
	27°S	Australia	1, 2	Nov	Annual	Nov–Dec	Morgan, 2000
<i>H. scabra versicolor</i>	22°S	New Caledonia	2	Oct	Annual	Nov–Feb	Conand, 1993
<i>H. spinifera</i>	8°N	India	1, 2	Sep–Oct	Annual	Nov–Mar	Asha and Muthiah, 2008
<i>H. whitmaei</i>	23°S	Australia	1, 2	Apr–Jun	Annual	Jun–Oct	Shiell and Uthicke, 2006

representatives have shown intraspecific variability between different latitudes, especially in number, timing, and duration of spawning event(s), but this variability does not seem to follow a consistent geographic pattern (i.e. longer spawning periods in regions closer to the equator compared to regions of higher latitude), with the only possible exception being the species *H. atra* Jaeger, 1833.

Information on the life-history parameters is a prerequisite for management measures on holothurian fisheries (Guzmán et al., 2003; Muthiga et al., 2009; Purcell et al., 2013). Unfortunately, relevant data on *H. tubulosa* biology are absent (e.g., recruitment, growth, mortality, longevity) or rather limited (e.g., population density, biometrics, and reproduction) (Bulteel et al., 1992; Despalatović et al., 2004; Kazanidis et al., 2010). This situation constitutes a fundamental barrier for the establishment of an efficient management strategy for the natural resources of this ecosystem-engineering species.

The present study provides important information on the reproduction of *H. tubulosa* in the eastern Mediterranean and especially in the Greek regions of the Aegean Sea through the use of histological examination. In this region, *H. tubulosa* has been under uncontrolled exploitation for more than a hundred years. Our study revealed an annual and synchronogamic pattern characterized by a short spawning period over summer months. High values of seawater temperature seem to

have a role in the release of the gametes (Despalatović et al., 2004; Kazanidis et al., 2010; present study).

Although the role of this species in ecosystem functioning could justify even its total exclusion from lists of harvested species (Amon and Herndl, 1991; Purcell et al., 2013), here we suggest the establishment of a seasonal ban from July to September, i.e. over its spawning period (Guzmán et al., 2003; Muthiga, 2006; Toral-Granda and Martínez, 2007; Gaudron et al., 2008). Furthermore, a limit of 250 g should be placed as the minimum drained weight of harvested individuals, in order to make sure that the majority of local stocks reach maturity before being collected. Further research in fundamental aspects such as recruitment dynamics and postsettlement survivorship are essential steps to further understand the population ecology of this commercially and ecologically important species.

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