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Reproductive biology of the holothurian *Holothuria tubulosa* (Echinodermata) in the Adriatic Sea

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The reproductive cycle of a local population the aspidochirote *Holothuria tubulosa* in Kaštela Bay (Adriatic Sea) was analysed from July 1994 to August 1995 by histological examination and observations of macroscopic features of the gonads. Five gonadal stages were described: recovery stage, growing stage, mature stage, spawning stage, and post-spawning stage. The distinguishing features used to describe gonad stages in both sexes included the main features of reproductive cells and gonad wall. The reproductive cycle showed a clear annual pattern and was synchronous in both sexes. Spawning occurred during the warm season, from July to September, when the surface water temperature ranged from about 22°C to 26°C. From October to January individuals were in resting phase and had no gonads. In both sexes, variations in maturity indices were related to the seasonal changes in temperature.

INTRODUCTION

The aspidochirote *Holothuria tubulosa* Gmelin, 1788 is distributed in the Mediterranean Sea and in the Eastern Atlantic Ocean from Gibraltar to the Bay of Biscay (Tortonese, 1965). It is very common in the Adriatic Sea to depths of approximately 100 m and on various bottom types (Šimunović et al., 2000). *Holothuria tubulosa* is a continuous deposit-feeder and significantly contributes to the overall energy budget of the ecosystem (Coulon et al., 1992).

Although the majority of the studies on aspidochirote holothurians focus on reproductive biology, little information is available on the reproductive cycle of *H. tubulosa*, despite its abundance in benthic communities (Bulteel et al., 1992). Most studies have investigated tropical and commercially important species (Chao et al., 1995; Conand, 1981, 1993; Hopper et al., 1998; Smiley, 1988).

The objective of this paper is to present the main features of the reproductive biology of the *H. tubulosa* population in Kaštela Bay (Adriatic Sea). The description is based on observation and histological examination of the gonads of both sexes during a one-year period, as well as comparison of maturity indices to temperature data and oocyte diameter measurements in females.

MATERIALS AND METHODS

From July 1994 to August 1995, specimens were collected on 12 occasions from a local population of *Holothuria tubulosa* in Kaštela Bay (Adriatic Sea; 43°30′N 16°22′E) at depths of 0 to 10 m (Figure 1). At each sampling 20 individuals were collected.

Surface seawater temperatures were recorded at the study site during each sampling.

Specimens were measured underwater from mouth to anus with ±0.5 cm precision, with care taken to avoid body contraction. They were put into individual plastic bags and taken to a boat. In the laboratory, all specimens were dissected and their gutted weight was recorded following removal of gonads, alimentary canal and respiratory trees (Conand, 1981). The null hypothesis that average length of individuals and average gutted weights were the same between sexes was tested with analysis of variance (ANOVA).

To test the hypothesis that the sex ratio was not significantly different from 1:1, a χ^2-test was carried out.

Gonads were removed and preserved in 10% seawater-formalin. Gonad samples were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at 7 μm and stained with haematoxylin and eosin. Gonad examination and photography were

Figure 1. Sampling station in Kaštela Bay (43°30′N 16°22′E; asterisk), Adriatic Sea (open arrow) for investigation of reproductive cycle of *Holothuria tubulosa*.

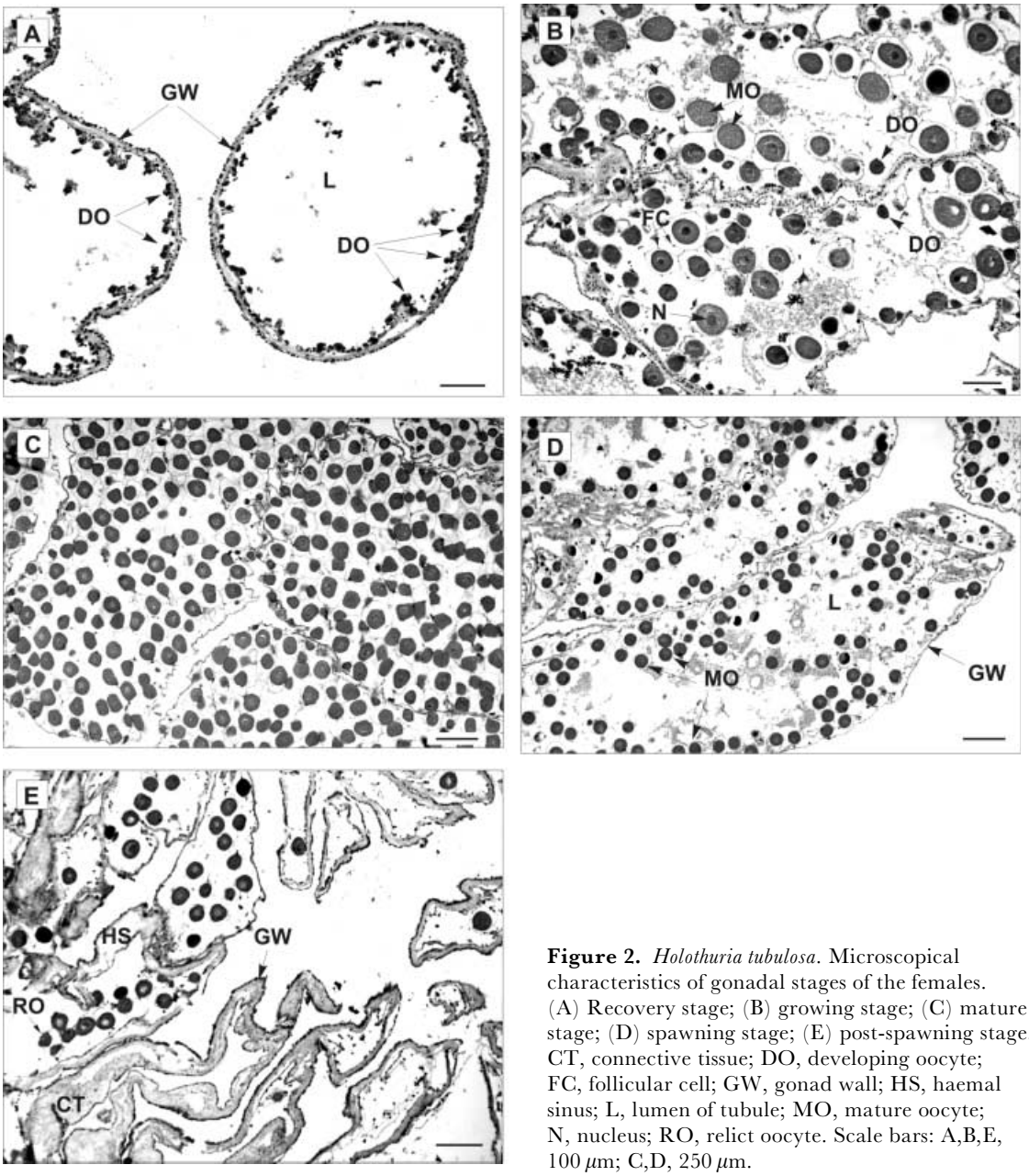

Figure 2. *Holothuria tubulosa*. Microscopical characteristics of gonadal stages of the females. (A) Recovery stage; (B) growing stage; (C) mature stage; (D) spawning stage; (E) post-spawning stage. CT, connective tissue; DO, developing oocyte; FC, follicular cell; GW, gonad wall; HS, haemal sinus; L, lumen of tubule; MO, mature oocyte; N, nucleus; RO, relict oocyte. Scale bars: A,B,E, 100 μm; C,D, 250 μm.

carried out with a light microscope. The reproductive status was assessed by using macroscopic features of the gonads (form, colour and consistency), histological examination and changes in the maturity index.

Gonadal development was classified into five stages: recovery stage (I), growing stage (II), mature stage (III), spawning stage (IV) and post-spawning stage (V), that were adapted from earlier studies of holothurians (Conand, 1981; Costelloe, 1985; Tuwo & Conand, 1992). Each gonad was examined and assigned to one of these stages. The distinguishing features used to describe these stages of the ovaries included the number, size and position of the oocytes and the width of the ovary wall. In males, these stages were assigned by assessing the spermatogenic condition of the germinal layer, the amount of spermatozoa in the lumen, and status of the development of the haemal layer in the gonad wall.

The maturity index (MI) is based on that of Yoshida (1952) and is calculated according the following formula:

$$MI = \frac{\sum_{i=1}^{5} i \cdot n_i}{N} \quad (1)$$

where i=Stage; n_i=number of individuals in Stage i; N=total number of individuals in the sample. The maturity indices were calculated separately for the females and the males in each sample.

Measurements of 60 or more oocytes in different stages of vitellogenesis were taken from each female. The results were grouped into 20 μm size-classes. Following Costelloe (1985) oocyte measurements were taken across the greatest axis and only from those cut across the nucleus.

RESULTS

The single gonad of *Holothuria tubulosa* is located in the anterior of the coelom and consists of numerous elongated

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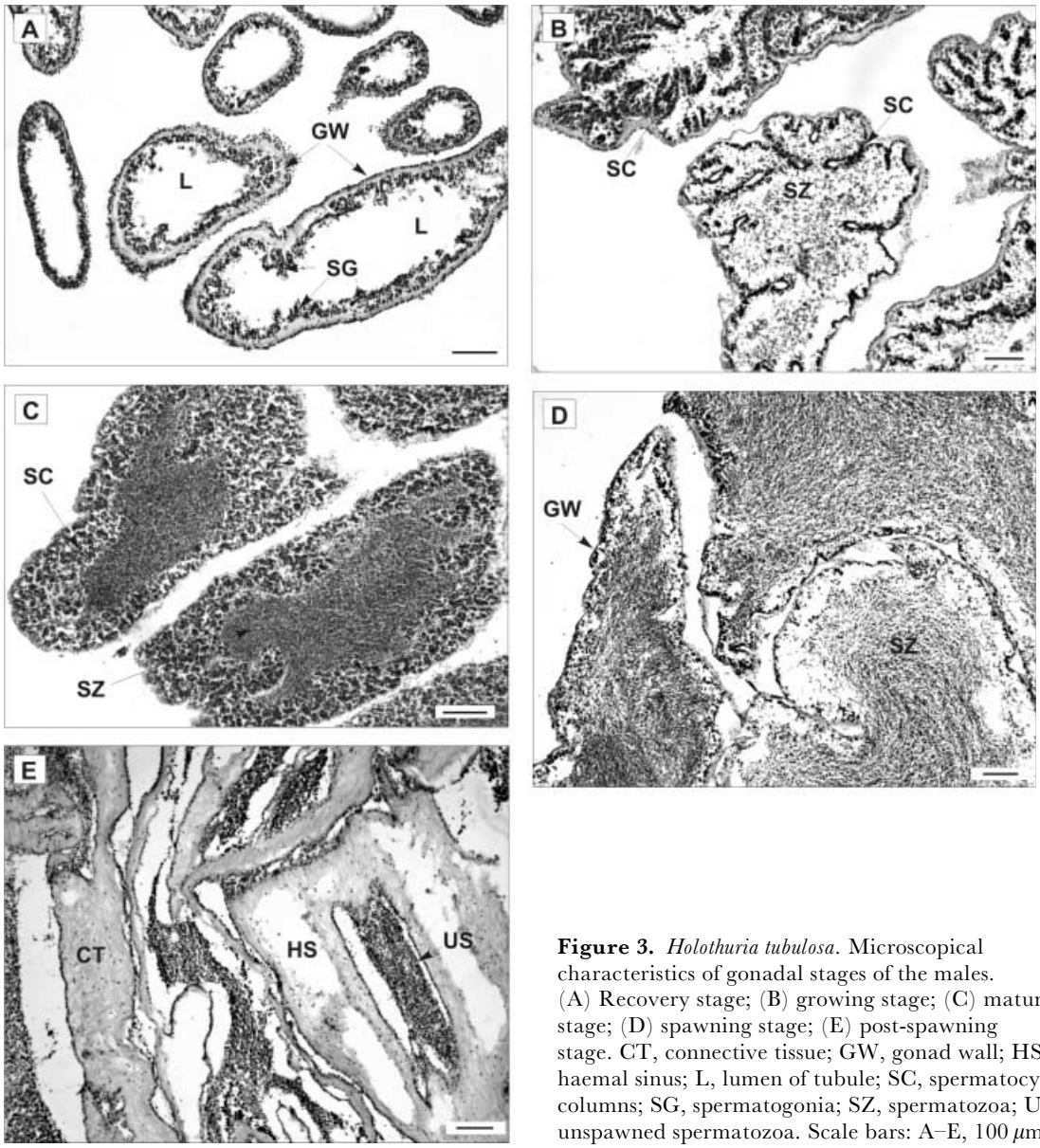

Figure 3. *Holothuria tubulosa*. Microscopical characteristics of gonadal stages of the males. (A) Recovery stage; (B) growing stage; (C) mature stage; (D) spawning stage; (E) post-spawning stage. CT, connective tissue; GW, gonad wall; HS, haemal sinus; L, lumen of tubule; SC, spermatocyte columns; SG, spermatogonia; SZ, spermatozoa; US, unspawned spermatozoa. Scale bars: A–E, 100 μm.

and branched tubules. The gonads are white in male and pink in female individuals.

Of the 238 specimens of *H. tubulosa* 26% were males, 29% females and 45% undetermined sex. Chi squared test results indicated that the sex ratio was not significantly different from 1:1 ($\chi^2=0.618$; df= l; $0.25<P<0.5$). The highest number of individuals of undetermined sex was collected during October and November when gonads were completely reduced, making sex determination impossible. Hermaphroditic individuals were not found.

The average length ±SD of the males was 26.30 ±5.52 cm and of females 28.33 ±7.88 cm. An ANOVA showed that these differences were not significant ($P>0.05$). The average gutted wet weights ±SD were 75.81 ±34.77 g for males and 87.14 ±38.77 g for females. An ANOVA also found these differences to be not significant ($P>0.1$). The smallest individual was 11 cm long and had a gutted wet weight of 28 g. Its sex was undetermined.

On the basis of morphological and histological observations, five gonadal stages were described for *H. tubulosa* for each sex.

Developmental stages of the female

Stage I: recovery stage (Figure 2A)

In recovering ovaries, the oogonia are embedded in the germinal layer, making them difficult to identify. Small developing oocytes are arranged in a single layer. The gonad wall is thick and three basic layers are easily distinguished.

Stage II: growing stage (Figure 2B)

The germinal layer is lined with oocytes at various stages of development. As vitellogenesis intensifies, fully-grown oocytes with large nuclei begin to take up a central position in the lumen of the tubules. Small follicular cells surround the oocytes. The gonad wall is still thick but in the advanced stages of growth it becomes attenuated.

Stage III: mature stage (Figure 2C)

The gonad wall is thin. Fully-grown mature oocytes that have reached their maximum size are densely packed in the lumen of tubules. In the germinal layer previtellogenic oocytes could be present and continuing their development.

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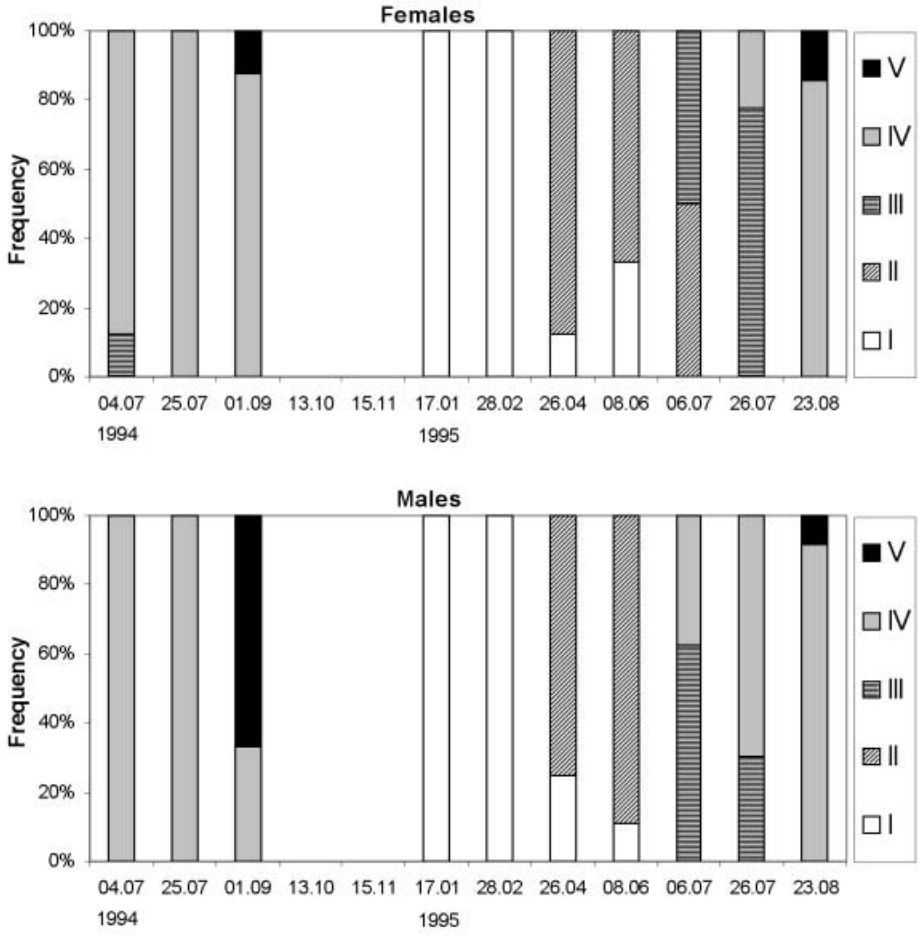

Figure 4. *Holothuria tubulosa*. Relative frequencies of the maturity stages for females and males during the sampling period (July, 1994 to August, 1995). I, recovery stage; II, growing stage; III, mature stage; IV, spawning stage; V, post-spawning stage.

Stage IV: spawning stage (Figure 2D)

In spawning ovaries, the gonad wall is still thin. There is a noticeable decrease in the abundance of oocytes inside the tubules. Empty spaces are observed in the lumen due to gamete release. The number of oocytes and the size of empty spaces in the lumen depend on the stage of spawning. Few developing oocytes may remain in the germinal epithelium.

Stage V: post-spawning stage (Figure 2E)

In post-spawning ovaries, the gonad wall is thick again and large amounts of connective tissue are present. Genital haemal sinus is present too. Unspawned relict oocytes are present in the lumen and start to show signs of degradation due to phagocytic activity.

Developmental stages of the male

Stage I: recovery stage (Figure 3A)

The recovery stage is characterized by a thickening of the outer and inner epithelium of the gonad wall. Spermatogonia appear on the germinal layer, which may have numerous folds that increase the surface area for spermatogenesis.

Stage II: growing stage (Figure 3B)

The growing stage is characterized by very active spermatogenesis. Columns of spermatocytes extend towards the lumen and spermatozoa start to fill the lumen. As the growing proceeds, the folds of the germinal epithelium straighten out. The gonad wall is thinner and the follicles increase their size.

Stage III: mature stage (Figure 3C)

In the mature stage, densely packed spermatozoa in the lumen of the tubules characterize mature testes. Spermatocyte columns are present in the germinal layer and spermatogenesis proceeds. The gonad wall is thin.

Stage IV: spawning stage (Figure 3D)

In the spawning stage, the spermatozoa are less densely packed. Empty spaces are present in the lumen due to gamete release. In the early period of the spawning, a spermatogenic layer is present. There is an empty space between the spermatogenic layer and the mass of gametes in the central part of the lumen. The gonad wall is still very thin.

Stage V: post-spawning stage (Figure 3E)

In the post-spawning stage, the gonad wall is very thick and characterized by the presence of large amounts of connective tissue. The genital haemal sinus starts to expand. Unspawned spermatozoa are present in the lumen. Phagocytes are observed.

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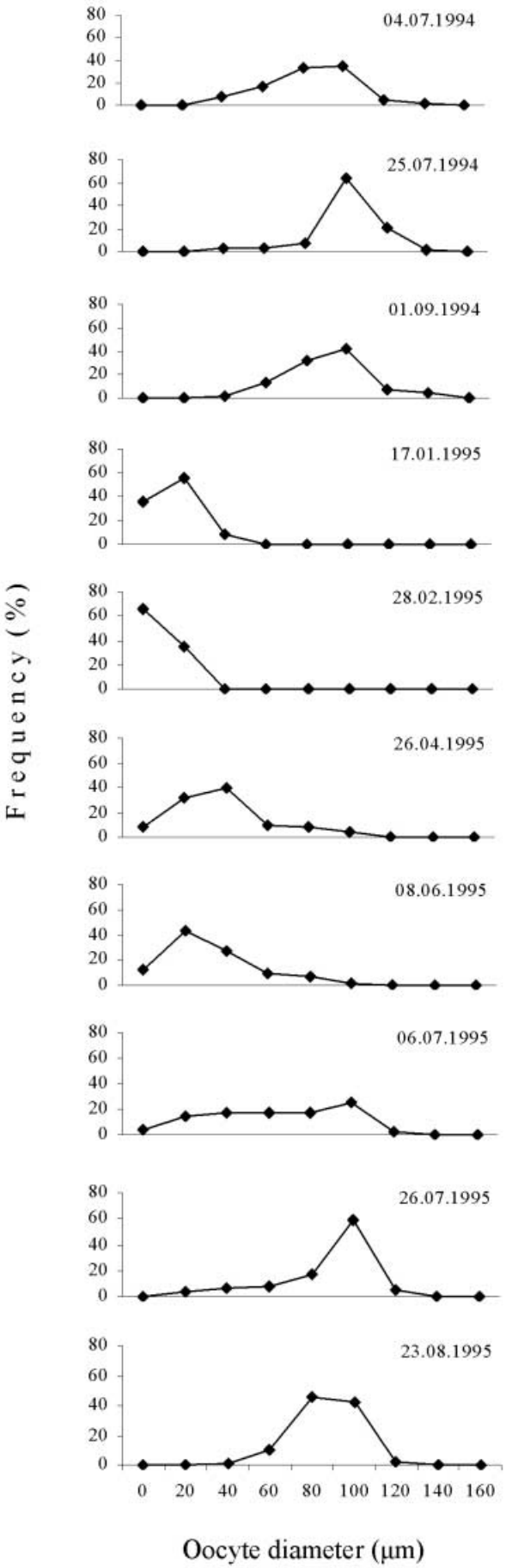

Figure 5. *Holothuria tubulosa*. Oocyte diameter frequencies grouped in 20 μm size-classes during the sampling period (July, 1994 to August, 1995).

Reproductive cycle

The reproductive cycle of *H. tubulosa*, monitored over a one-year period from July 1994 to August 1995, showed a clear annual pattern and was synchronous in both sexes.

Relative frequencies of the maturity stages for both sexes are illustrated in Figure 4. Spawning occurred from July to September. From October to January individuals were in resting phase and had no detectable gonads. Recovering ovaries and testes were prevalent in January and February, but remained present until June. Growing gonads were prevalent from April to June in both sexes. Growing ovaries were still present in some individuals in the beginning of July, when the mature gonads were observed in both sexes. Spermatogenesis and oogenesis continued to some extent through the breeding season in parallel with spawning. Spawning started in the beginning of July in both sexes. The post-spawning stage was short, extending from the end of August to September.

Monthly oocyte diameter gave a more detailed picture of the seasonal gonadal development in female individuals (Figure 5). In January and February oocyte diameter ranged from 14.5 to 65.25 μm. During the growing stage from April to July oocytes showed a gradual increase in average diameter. In spawning season from July to September average oocyte diameter ranged from 91.77 to 109.96 μm. Maximum measured diameter of mature oocytes was 174 μm. Analysis of variance test results show statistically significant differences ($P<0.01$) in oocyte diameter among each sample.

Changes in maturity indices in both sexes were related to the seasonal variations in temperature (Figure 6). The lowest surface sea temperatures (11–12°C) and maturity indices were in January and February 1995, at which time the recovery of the gonads started. Maturation and higher maturity indices were observed to begin during water warming in spring. Spawning occurred during the warm season when the surface water temperature ranged from about 22°C to 26°C. In these months, maturity indices were the highest, especially at the end of August and at the beginning of September when post-spawning individuals were observed.

DISCUSSION

During investigation of the reproductive cycle of *Holothuria tubulosa* various size parameters were measured. The length and the total mass in this species are highly variable. Therefore, only the total length of the relaxed specimen on the sea-floor and the body wall wet weight (gutted weight), as Conand (1981) suggested as a 'mass parameter', were chosen.

The results showed no statistically significant difference in length or mass between the sexes. Juvenile individuals were not found during our sampling. Prior studies of holothurian species had similar findings with few or no juveniles in the samples (Conand, 1981; Tuwo & Conand, 1992).

Although *H. tubulosa* is a dioecious species, there is no external sexual dimorphism. Sex is only determined by the appearance and colour of the gonads. Variation in gonad colour is also known for some other species in the family Holothuriidae (Tuwo & Conand, 1992; Chao et al., 1995).

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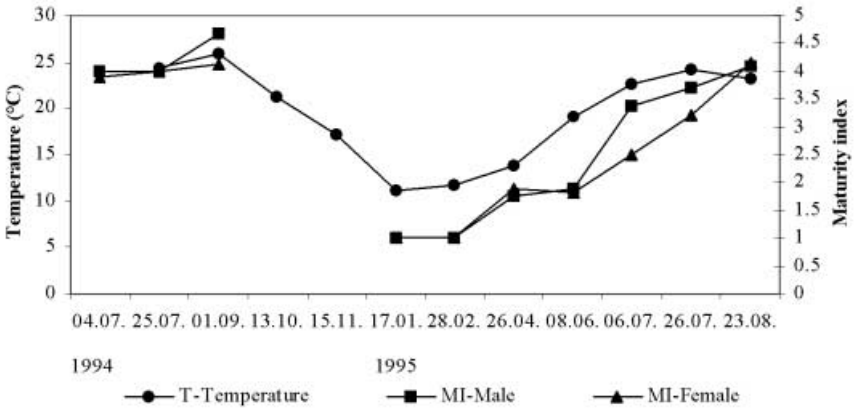

Figure 6. *Holothuria tubulosa*. Changes of maturity indices values in both sexes in relation to seasonal changes of temperature during the sampling period (July, 1994 to August, 1995).

The population sex ratio for *H. tubulosa* was approximately 1:1, as is the case for most aspidochirotes (Conand, 1981; Hopper et al., 1998; Tuwo & Conand, 1992).

According to our observations the tubule recruitment model described in Smiley (1988) for gonad development in holothurians is not applicable for the *H. tubulosa* population in the investigated area. Complete reabsorption of the gonad after the spawning period was found, with no gonad material evident during the winter months. Also, according to Sewell et al. (1997), the model is not applicable where immature previtellogenic and mature vitellogenic oocytes are found in the same ovarian tubule as is the case in our investigation.

In Kaštela Bay in the Adriatic Sea *H. tubulosa* showed a clear annual reproductive pattern and spawning occurred from July to September when the sea temperature was highest. The species appears to spawn during the summer period in the Mediterranean Sea (Pladellorens & Subirana, 1975; Bulteel et al., 1992). Previous data suggested that the spawning in the Adriatic Sea occurs year-round, with the maximum intensity from June to September (Tortonese, 1965; Zavodnik & Šimunović, 1997). Temperature is probably one of the most important factors in determining the period of reproduction. The connection between increasing seawater temperature in warm periods of the year and the spawning period is already known for some Holothurioidea species (Conand, 1981; Costelloe, 1985; Hopper et al., 1998; Tuwo & Conand, 1992). The majority of investigated tropical species have an annual reproductive cycle and spawning occurs during the summer months (Conand, 1981; Chao et al., 1995; Hopper et al., 1998). Until now, the only member of the family Holothuriidae in temperate seas whose reproductive cycle has been investigated is *Holothuria forskali*, and its spawning occurs in spring (Tuwo & Conand, 1992).

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