Size at first maturity, oocyte envelopes and external morphology of sperm in three species of lucinidae (Mollusca: Bivalvia) from Florida Keys, U.S.A.

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SIZE AT FIRST MATURITY, OOCYTE ENVELOPES AND EXTERNAL MORPHOLOGY OF SPERM IN THREE SPECIES OF LUCINIDAE (MOLLUSCA: BIVALVIA) FROM FLORIDA KEYS, U.S.A.

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ABSTRACT

Gonads in the tropical lucinid bivalves *Ctena orbiculata*, *Codakia orbicularis*, and *Lucina pensylvanica* are located in the cephalopodial mass above the foot and behind the gills. Age estimates for *Lucina pensylvanica* suggest that individuals less than one year old are immature, as also is *C. orbicularis*, and that both males and females mature at two years. For *Ctena orbiculata*, we have no age data, but we consider that this species may also have early development. In July 2002 when water temperatures were high, mature individuals of all three species were spawning or in resorption, and oocyte envelopes were present. Parasites were found in the digestive diverticula of *C. orbiculata* and *L. pensylvanica*. External ultrastructure of the spermatozoon shows differences between the three species. *Codakia orbicularis* and *L. pensylvanica* have a long-headed spermatozoa, whereas *Ctena orbiculata* has a middle-size head. *Codakia orbicularis* possesses a flagellum with a lateral undulating membrane, absent from the two other species.

Key words: Lucinidae, reproduction, oocytes, sperm.

INTRODUCTION

The Lucinidae is the most diverse and geographically widespread of the bivalve families possessing chemoautotrophic sulphide-oxidising bacteria (Distel, 1998). Species live in a wide variety of marine habitats ranging from intertidal mangrove muds to hydrothermal vents, and the family has a long and rich fossil history (Taylor & Glover, 2000). Many tropical lucinids live in close association with shallow water seagrass beds and are particularly diverse in the Florida Keys area, where 21 species have been recorded from all depths and habitats (Mikkelsen & Bieler, 2000). Of these, ten species were found living in the shallow water habitats of the Middle Keys sampled during the International Marine Bivalve Workshop (IMBW) in 2002 (Mikkelsen & Bieler, 2004). Three species, *Ctena orbiculata* (Montagu, 1803), *Codakia orbicularis* (Linnaeus, 1758), and *Lucina pensylvanica* (Linnaeus, 1758) were sufficiently abundant and present in a range of size classes to attempt an investigation of some aspects of their reproductive biology. The objectives of this study were to determine the size of maturity, the presence of oocyte envelopes and to describe the external morphology of their sperm. Additionally, the maturation sizes of *L. pensylvanica* were related to the age of the individual as estimated from acetate peel replicas of sections through the shell.

Previous research on Lucinidae has primarily focused on aspects of functional anatomy, chemosymbiosis and evolution, and there are few studies of their reproductive biology. The exception is *Codakia orbicularis*, one of the most studied species. General aspects of the reproductive biology of this large, edible clam have previously been studied by Alatalo et al. (1984), Berg & Alatalo (1984), and Prieto et al. (1999), larval development by Gros et al. (1997), and sperm morphology by Mouëza & Frenkiel (1995). The potential role of chemosynthesis in molluscan mariculture has also been investigated for this species (Berg & Alatalo, 1984).

Many invertebrates possess oocyte envelopes with different layers. An inner protein-polysaccharide layer (vitelline envelope) can be formed by the Golgi apparatus of the oo-

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cyte itself and deposited at the outer surface by exocytosis (Jong-Brink et al., 1983; Huebner & Anderson, 1976). When present, a thick jelly coat covers the outer surface of the vitelline envelope, and appears as a loose association of striated fibrous material (Hodgson & Eckelbarger, 2000). Follicle cells are also believed to produce secondary compounds or cellular egg envelopes around oocytes (Eckelbarger, 1994). In *Codakia orbicularis*, the jelly coat that covers the oocyte is formed by an inner layer (the vitelline envelope) and an outer layer (Gros et al., 1997). In another lucinid, *Phacoides pectinata* (Gmelin, 1791), this jelly coat is made up of glycoproteins and proteoglycans (Frenkiel et al., 1997), synthesized by the oocyte during vitellogenesis (Frenkiel, unpubl.). No information exists concerning the composition and formation of oocyte envelopes in *Ctena orbiculata* and *L. pensylvanica*, and the reproductive biology of these species has received no attention probably due to their smaller size and lack of commercial importance.

MATERIAL AND METHODS

The lucinid bivalves *Codakia orbicularis, Ctena orbiculata* and *Lucina pensylvanica* were collected between July 2002, at different localities in Florida Keys, USA, during the International Marine Bivalve Workshop 2002. Sampled habitats included sandy bottoms at 6 m depth (sampled by scuba diving) and shallow subtidal sandy substrata colonized by the seagrasses *Thalassia, Halodule, Syringodium* and *Halophila*. Most of the seagrass-covered sediments were anoxic with hydrogen sulphide concentrations detectable by smell. Bivalves were collected by digging and sieving sediment through 2 mm mesh screens. Sampling localities included a number of oceanside intertidal and shallow water sites in the Middle Keys (IMBW-FK-622, 628, 635, 638, 642, 647, 649; Mikkelsen & Bieler, 2004: fig. 1 for map). *Ctena orbiculata* was most abundant at inshore bayside sites in sparsely vegetated sand patches; *Codakia orbicularis* at several oceanside sites with thick *Thalassia* growths, and *Lucina pensylvanica* was found commonly only at oceanside Station IMBW-FK-642 on Lower Matecumbe Key in shallow sand on rock.

Twenty individuals of each species were sexed macroscopically observing the texture of gonads (females had granulose and males homogeneous texture) and their shell parameters measured. A preliminary scale of gonad maturation was compiled from light microscope observations of fresh tissue, and this was used for comparison with thin sections of gonads prepared later. External gonad morphology was described from fresh animals. Samples of gonad tissue were fixed in Bouin's solution for 48 h and stored in 70% alcohol. In order to determine the first maturation stage and the presence of oocyte envelopes, sections of the gonads were cut at 6 μm with a Leitz microtome and stained with hematoxilin and eosin. We use the term oocyte or egg envelope for the inner layer and jelly coat for the outer layer of the oocyte. For scanning electron microscopy (SEM) of spermatozoon ultrastructure, pieces of male gonad were cold fixed in 2.5% glutaraldehyde solution in Sorenson's phosphate buffer. Slices of gonad were then cut with a razor blade, dehydrated through an ascending acetone series, critical point dried, sputter coated with gold, and examined by scanning electron microscopy (SEM) with a Philips XL30 field emission SEM operated at 5kV.

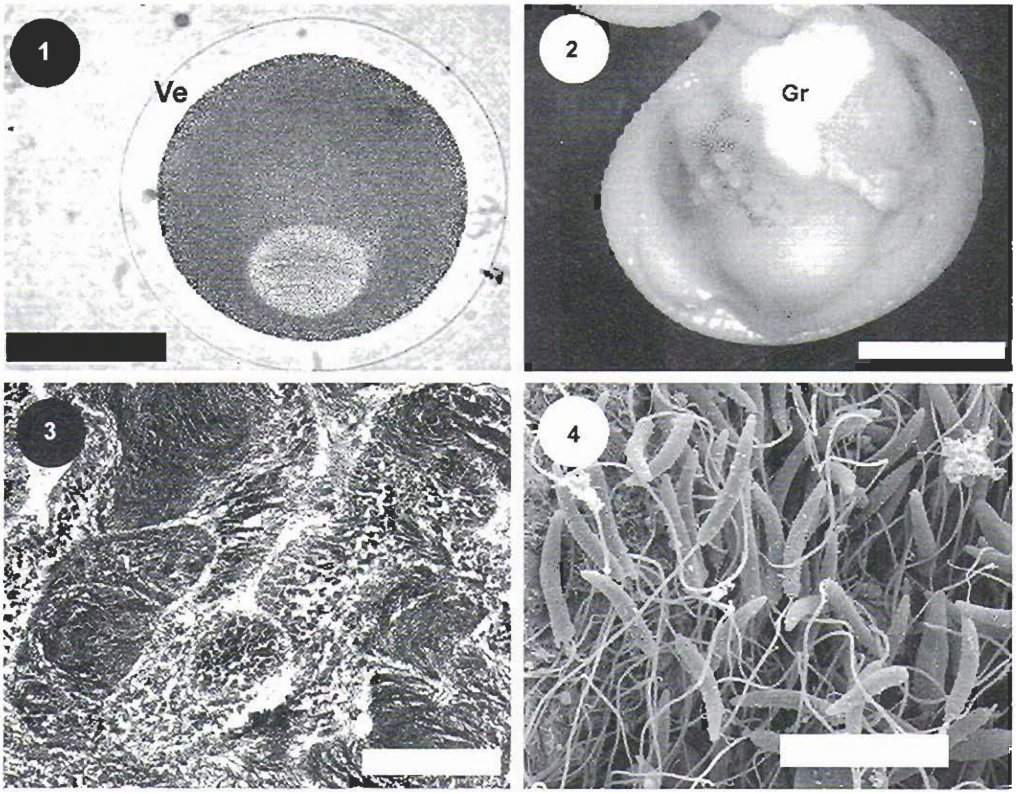
For age determination, 20 dry shell valves of *Lucina pensylvanica* were embedded in MET20 resin (Struers Ltd), sectioned from the umbo to the ventral edge, ground, polished, and etched for 20 min in 0.01M HCl and acetate peel replicas prepared (Richardson, 2001). The age of each shell was estimated by three observers using the major growth lines present in acetate peels of the umbonal region and the outer prismatic shell layer (Richardson, 2001). These major growth lines were treated as annual lines by comparison with the results of a study of *Codakia orbicularis* from the Bahamas by Berg & Alatalo (1984). Data were fitted to the von Bertalanffy growth function $L_t = L_\infty(1-e^{-k(t-t0)})$ using the Fisheries Programme "Fisat".

Voucher specimens of the species studied are deposited in the Mollusca collections of the Department of Zoology, The Natural History Museum, London.

RESULTS

Ctena orbiculata

Specimens collected measured between 5.5 mm and 13.8 mm in length. No gonad development was observed in individuals smaller than 5.6 mm. All males larger than 5.6 mm

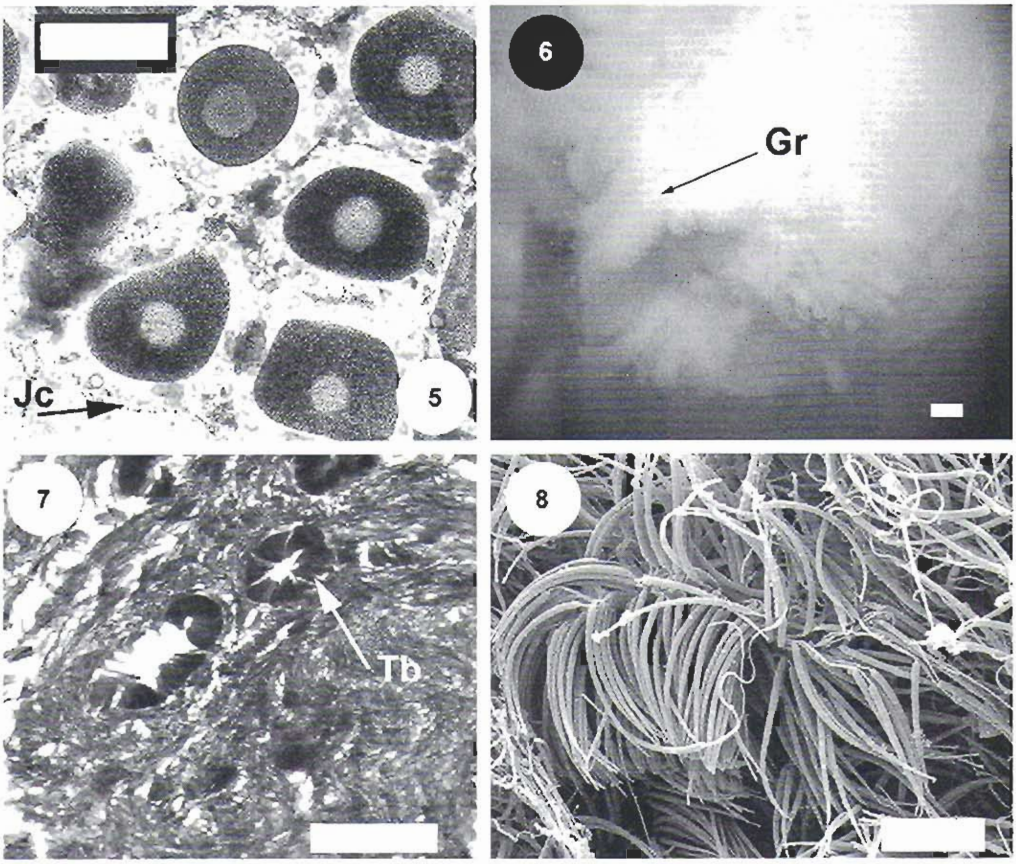


FIGS. 1–4. *Ctena orbiculata*. FIG. 1: Living mature oocytes covered by the vitelline envelope. Scale bar = 50 µm; FIG. 2: Female gamete release. Scale bar = 3 mm; FIG. 3: Sperm in follicles. Scale bar = 100 µm; FIG. 4: SEM image of sperm. Scale bar =10 µm. Gr, gamete release; Ve, vitelline envelope.

were mature. The spermatozoa were contained in follicles and orientated in relation to the lumen of the follicle (Fig. 3). Females less than 7.1 mm in length were immature; oocytes of individuals of 7.1 mm had a mean diameter of 20 mm (SD = 1.8) and no envelope was present. Developing oocytes were present in specimens between 7.2 mm and 7.8 mm length with maturity reached at 7.9 mm. Some individuals were spawning at this size, but no resorption was observed. The maximum oocyte diameter measured was 137.5 mm, with an oocyte envelope width of 10 mm, with no jelly coat covering it. When the gonad is ripe, an egg mass full of gametes forms a thin layer, that covers the ctenidia and sometimes all of the pallial cavity (Fig. 2). The egg mass has no defined organization. At this stage, the oocytes are sticky and covered by a jelly coat and have a total mean diameter of 360 µm (Fig. 1). This corresponds to the time of gamete release and possibly the oocytes are retained by mucus in the pallial cavity until fertilization. After release, the oocytes are covered by the jelly coating and this probably provides protection to the egg until fertilization occurs. No evidence of either protandry or simultaneous hermaphroditism was found in this species.

Male gametes are released as sperm strings comprising hundreds of sperm attached to each other at the head. SEM images (Fig. 4) show that sperm cells are relatively short-headed, with a long, cylindrical flagellum. The heads are cylindrical and tapering, slightly curved with a visible, short acrosome. Heads have a mean length of 7.5 µm and a width of 1–1.2 µm, with the acrosome about 0.6 µm long and the mid piece 0.9 µm. The tail measures around 28 µm, giving a total length for the sperm cell of around 36 µm.

Parasites were observed in the digestive diverticula lying adjacent to the gonad follicles

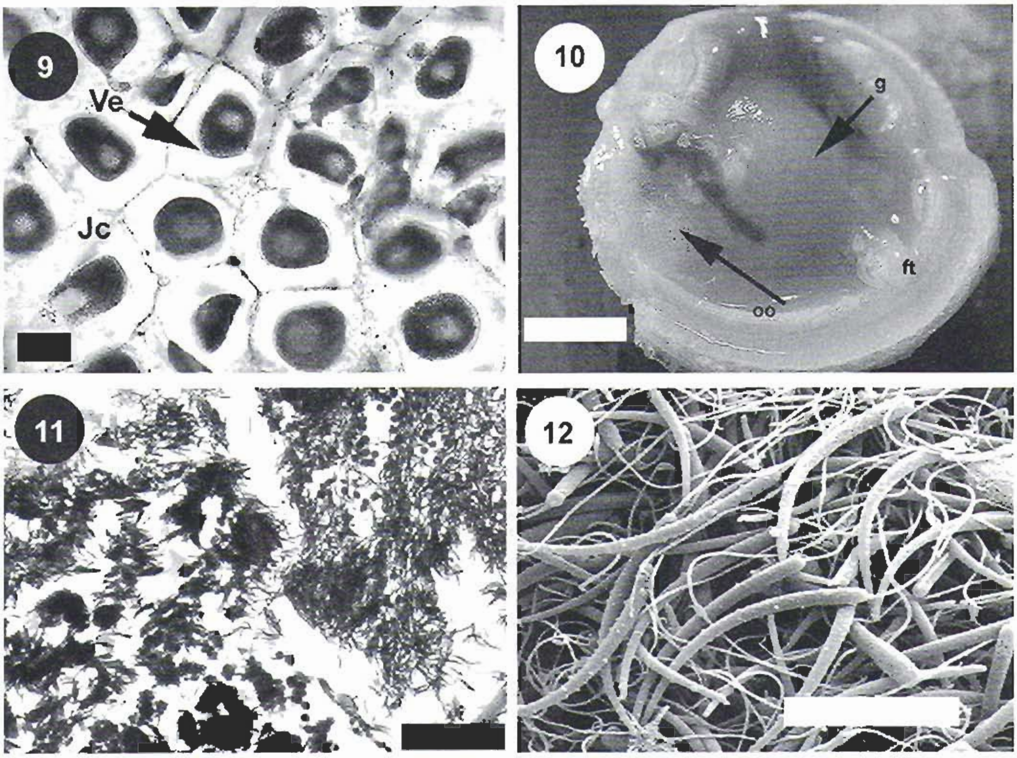


FIGS. 5–8. *Codakia orbicularis*. FIG. 5: Mature oocyte with jelly coat. Scale bar =100 µm; FIG. 6: Female gamete release. Scale bar =500 µm (arrow shows the finger-shaped mass); FIG. 7: Sperm ready to spawn. Scale bar = 50 µm; FIG. 8: SEM image of sperm. Scale bar = 10 µm. Gr, gamete release; Jc, jelly coat; Tb, tubules.

(Fig. 13). They are probably cercaria of the digenean family Monorchiidae (D. Gibson, personal communication). We also found yellow granules in the digestive diverticula, fluxing to the gonads.

Codakia orbicularis

The bivalves sampled measured between 11.3 mm and 64.8 mm shell length. Individuals less than 12.5 mm were immature, while one individual of 12.6 mm had started to form follicles. Males larger than 13.2 mm had begun to mature with some sperm visible. All males larger than 25.9 mm were mature. In individuals larger than 46 mm, sperm were observed in tubules ready to spawn (Fig. 7). All females sampled had already spawned with visible oocyte resorption. Females larger than 46.8 mm had oocytes of 100 µm average diameter (SD = 5.8), covered by an oocyte envelope of about 7.5 µm thick. The largest male sampled was 47.1 mm in length. Unfortunately, no larger-sized *Codakia* were available to determine if protandry occurs in this species. No hermaphrodites were found. Release of female gametes occurs in a mucous mass held within the mantle cavity similar to that of *Ctena orbiculata*, but in *C. orbicularis* it is finger shaped (Fig. 6). At this stage, the oocytes are sticky and slightly negatively buoyant. Upon release from the gonads, oocytes are spherical and covered by a 50 µm thick jelly coat that, together with the oocyte envelope, forms the external envelope (Fig. 5). Further expansion of the envelopes results in an egg with a total diameter of around 200 µm.



FIGS. 9–12. *Lucina pensylvanica*. FIG. 9: Mature oocyte with irregular jelly coat. Scale bar = 100 µm; FIG. 10: Ova release. Scale bar = 5 mm; FIG. 11: Mature sperm in gonads. Scale bar = 50 µm; FIG. 12: SEM image of sperm cells. Scale bar = 10 µm. ft, foot; g, gonad; Jc, jelly coat; Oo, oocyte; Ve, vitelline envelope.

The sperm have long, slender, tapering, curved heads (Fig. 8) with a long flagellum that possesses a narrow, lateral, undulating membrane to either side (Fig. 14). The head measures around 14–15 µm in length with a width of 0.8 µm at the posterior end. The lengths of the flagella were difficult to measure in our preparations, but were at least 25 µm long, with the undulating membrane having a width of 0.42 µm.

Lucina pensylvanica

Individuals sampled measured between 12.8–42.6 mm shell length. No gonad development was observed in individuals of less than 13 mm. Sexual differentiation with incipient follicular formation begins at a size of 13.8 mm (specimens of one to two years old). Individuals larger than 15.9 mm had well-developed gonads; males were all mature and females exhibited different maturity stages. Male gametes are released as sperm strings comprising hundreds of sperm attached at the head as in *Ctena orbiculata* (Fig. 11). Females begin maturation at a shell length of 17.7 mm and reach maturity at around 26.6 mm. Individuals having oocytes with a mean diameter of 130 µm (SD = 6.7) with an oocyte envelope of approximately 10 µm were classified as mature females. Release from the gonads occurs as a finger-shaped mucous mass containing the ova, similar to that of *Codakia orbicularis* (Fig. 10). After release from the gonad, oocytes are spherical with an irregular jelly coat (Fig. 9). This jelly coat is approximately 47 µm thick, covering the ova, which has an external maximum diameter of 245 µm.

Sperm have long, curved, tapering heads (Fig. 12), with a long flagellum without a lateral membrane. The mean length of the head

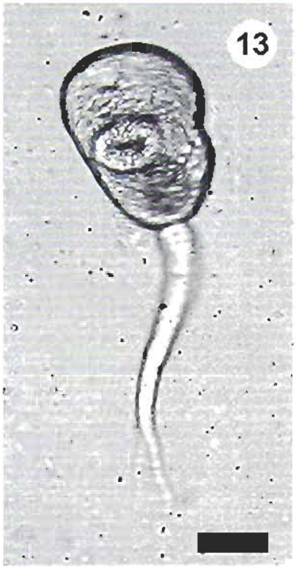


FIG. 13. Parasite found in the digestive diverticula of *C. orbiculata* and *L. pensylvanica*. Scale bar = 100 µm.

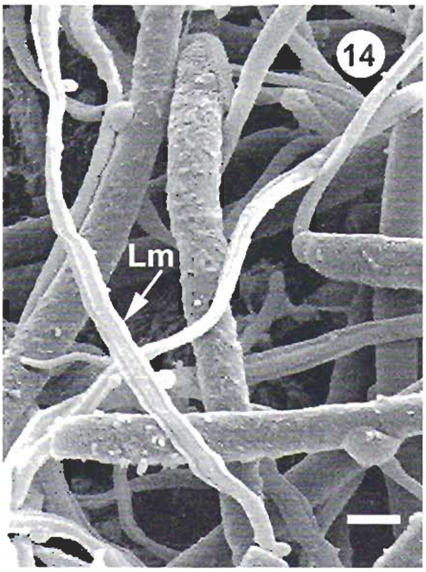


FIG. 14. SEM image showing the lateral undulating membrane (Lm) of the flagellum of *C. orbicularis*. Scale bar = 1 µm.

is 15.5 µm and the width at the posterior end is 1.1 µm. Flagella measured around 33 µm in length. Cercaria similar to those of *Ctena orbiculata* were found as parasites in the digestive diverticula adjacent to the gonad follicles.

From the analysis of major growth halts observed in shell sections, the oldest individual studied had attained an age of 5 years. Analysed shells showed individual variations in growth (Taylor et al., 2004). The asymptotic shell length was 38.37 ± 9.36 mm (Fig. 15).

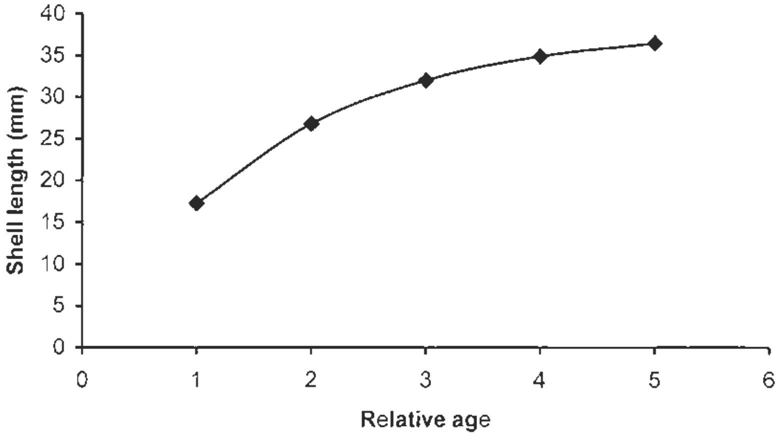


FIG. 15. Growth curve for *Lucina pensylvanica* fitted using the Von Bertalanffy growth equation: $L=38.37\ (1-e^{-0.60t+0.37})$.

DISCUSSION

It should be emphasised that this was a very short-term study conducted over a period of only two weeks. Nevertheless, the results show that interesting similarities and differences exist in the reproductive biology among the three coexisting lucinid species.

For mature individuals of the three lucinid species studied, the gonads were ripe, spawning or in resorption in July 2002. This spawning period corresponds to the hottest time of the year, and surface water temperatures measured by the National Data Buoy Center (LONF1, Long Key, Florida) ranged between 29–30ºC for the period 20–30 July. At the sampling sites, temperatures reached around 35°C in water depths of between 0.5–1 meter.

According to Berg & Alatalo (1984), the annual size classes of *Codakia orbicularis* at Grand Bahama Island are 12, 29, 42, 52 and 63 mm for years 1 to 5, respectively. By comparison with these results, our data suggest that *C. orbicularis* from the Florida Keys are immature at less than one year old, with males maturing between 1 and 2 years and females spawning at 3 years. In the Bahamian *Codakia*, shell growth was not continuous throughout the year, as evidenced from sharp growth rings on the external surface of the shell, and the number of these external growth rings coincided with annual year classes predicted from the von Bertalanffy growth equation (Berg & Alatalo, 1984). Spawning and recruitment of *C. orbicularis* occur over a long period, with variation in individual growth rates, and at Grand Bahama Island growth rates decreased during winter months. Follicular development in *C. orbicularis* was first observed at a shell length of 19.8 mm, corresponding to an age of 1.5 years, and the smallest animal with fully developed gametes measured 25.4 mm (Berg & Alatalo, 1984). In our study, we were unable to record the size of first maturity for females, but one individual of 12.6 mm length was observed with incipient follicular formation. According to Berg & Alatalo (1984), this corresponds to an individual of about one year old. They also recorded three hermaphrodites from a total sample of 224 bivalves analysed. We recorded no hermaphrodites in our Florida Keys sample, but all individuals examined less than 46.6 mm long were males, with the larger sizes all females. This result suggests protandry for this population, but more data are needed for confirmation. The observation from the Bahamas that most animals greater than 30 mm were ripe during the summer months indicates the same maturity pattern for this species as at Florida Keys, for we found maturity at sizes above 25.9 mm length. Animals within the population at Gold Rock Creek, Bahamas, did not spawn completely, nor simultaneously, but appeared to continue spawning over a period of at least a month (Berg & Alatalo, 1984). The spawning season for *C. orbicularis* in Bahamas (Alatalo et al., 1984) and in Venezuela, Sucre State (Prieto et al., 1999), agrees with our results for July 2002 in Florida Keys. In our case, we found spawned females towards the end of July, with oocyte resorption visible, but only in individuals greater than 46.8 mm. Observations on animals of both smaller and larger sizes are necessary to complete this study.

Parasites observed in the digestive diverticula of *Ctena orbiculata* and *Codakia orbicularis* resembling the general body form of *Cercaria caribbea* LXIV of Cable (1963) were previously recorded from the lucinid bivalve host *Ctena pectinella*. Cable suggests that the adult is a species of *Proctotrema* that occurs in the porkfish *Anisotremus virginicus*. The cercaria were observed only *in vivo*, and further studies are needed to confirm this. The yellow granules found in the digestive diverticula of *Ctena orbiculata* could be sulphur granules, related to the chemoautotrophic habits of this species.

Our age estimates for *Lucina pensylvanica* suggest that individuals less than one year old are immature, as in *C. orbicularis*, and that both males and females mature at two years. For the smallest species, *Ctena orbiculata*, we have no age data, but from the size range we consider that this species may also have early development. Selection for rapid gonad development could assure the recruitment of new spat when environmental conditions are favourable. Unfortunately, no further data is available concerning reproductive biology of *Ctena orbiculata* and *L. pensylvanica*.

Another lucinid from the western Atlantic, the large *Phacoides pectinata*, a protandric species inhabiting mangrove swamps and reducing mud, has permanently mature gonads (Frenkiel et al., 1997). This reproductive strategy, which, in addition to the sulphur oxidising bacterial endosymbionts and high bacteriocyte haemoglobin concentration, is considered an adaptation to a high-stress environment. Resorption of oocytes and recovery of metabolites through the follicular cell lysosomal function appears to be the most efficient means to minimize the metabolic cost of maintaining the state of maturity. In the cases of *Ctena orbiculata* and *L. pensylvanica*, resorption of oocytes was found, suggesting the same reproductive strategy in these species also living in similar hypoxic habitats. In the case of *C. orbiculata*, we observed only females spawning, but larger sizes were not found. Probably the same strategy occurs in this species, as a possible adaptation to reducing substrata.

We consider the oocyte envelope as a vitelline envelope. The composition and location of its synthesis is still unknown for *Ctena orbiculata* and *Lucina pensylvanica*. For *Codakia orbicularis*, Gros et al. (1997) considered that the jelly coat is made up of glycoproteins and proteoglycans synthesised by the oocyte itself during vitellogenesis. After spawning, it is swollen by hydration of the proteoglycan components. In *Phacoides pectinata*, the same jelly coat is observed and the glycoproteins are likely to support recognition receptors for sperm (Frenkiel et al., 1997). In bivalves from Antarctica, such as *Laternula elliptica* (King & Broderip, 1832), a similar oocyte envelope is observed, allowing storage of mature oocytes for the whole year until environmental conditions are favourable (Bigatti et al., 2001). This vitelline envelope and the jelly coating protect the embryos of *Codakia orbicularis* in the veliger stage (Alatalo et al., 1984) and are digested by enzymes from the larva (Gros et al., 1997). This is another possible adaptive response to a high-stress environment. For *Ctena orbiculata* and *Lucina pensylvanica*, studies concerning oogenesis and the origin and composition of oocytes envelopes are needed, but as they are members of the same family and live in similar environments, a similar pattern could be expected.

For the Florida lucinids, our observations of the release of gametes adhering within the pallial cavity suggests that these species retain oocytes until fertilization. However, this observation does not suggest the existence of a brood chamber, such as occurs in *Ostrea* spp. (Morriconi & Calvo, 1979), because no embryos or divided cells were identified. Probably the oocytes were recently spawned when we observed them. This could be another adaptation that enhances reproductive success after the eggs are released to the exterior environment.

Electron microscopy of molluscan sperm has provided an important set of characters for phylogenetic studies (Healy, 1995, 1996). Most detail is provided by TEM studies, but scanning microscopy of external features reveals a set of morphological characters useful for generic and specific differentiation. Our observations show that *Lucina pensylvanica* and *Codakia orbicularis* have similarly proportioned large sperm with long, curved, tapering heads, but the latter differs in having an undulating membrane to the flagellum. By contrast, *Ctena orbiculata* sperm have much shorter and less tapering heads.

The length of the spermatozoa of *Lucina pensylvanica* and *Codakia orbicularis* is comparable with that of *Scrobicularia plana* (da Costa, 1778) (Souza et al., 1989), which is the longest described spermatozoon in bivalves. TEM studies show that *Codakia orbicularis* sperm have a short acrosome (Mouëza & Frenkiel, 1995), as does *Codakia punctata* (Linnaeus, 1758) (Healy, 1995) and *Loripes lucinalis* (Lamarck, 1818) (Johnson et al., 1996). It has been suggested by Mouëza & Frenkiel (1995) that occurrence of this feature, along with long tapering heads, in species having large oocytes with a gelatinous coat might be adaptations to facilitate penetration of the spermatozoa through this coating and the vitelline envelope.

The undulating lateral membrane of the flagellum in *C. orbicularis* (Fig. 14), previously described by Mouëza & Frenkiel (1995), is an unusual feature of bivalve sperm, although well-developed undulating membranes appear characteristic of corbiculid sperm (Komaru & Konishi, 1996; Konishi et al., 1998). Mouëza & Frenkiel (1995) suggest that the structure is somehow related with sperm locomotion prior to fertilization. The sperm of too few lucinid species has been studied to evaluate the possible systematic significance of this feature.

Finally, longer-term studies of the reproductive biology of *Lucina pensylvancia* and *Ctena orbiculata* are necessary to confirm and expand our preliminary findings based on the two-week survey. Both of these species are abundant, but poorly studied, chemosymbiotic bivalves inhabiting the shallow waters of the Florida Keys and the populations are highly vulnerable to environmental disturbance of these fragile habitats.

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