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Ultrastructure of Mature Sperm and Eggs of the Brooding Hermaphroditic Chiton, *Lepidochitona fernaldi* Eernisse 1986, With Special Reference to the Mechanism of Fertilization

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ABSTRACT Most chitons are gonochoric and free spawning with fertilization occurring in seawater. Brooding hermaphroditic chitons retain their embryos in the pallial groove but whether the eggs are fertilized internally in the female genital tract or externally in the pallial groove was not previously known. Furthermore, the transition to this mode of reproduction could be expected to involve other changes in the functional morphology of eggs and sperm. We describe for the first time observations on live spawning, as well as the ultrastructure of sperm and eggs, in the brooding hermaphrodite, *Lepidochitona fernaldi*.

Sperm and eggs are spawned through common gonoducts and the eggs are self-fertilized in the pallial groove. Sperm structure is unmodified from that typical of other Lepidochitonidae, which suggests that the medium filling the pallial groove is seawater and not some more viscous fluid. The egg hull is reduced in this species to a series of flattened hexagonal plates that block access to sperm. Sperm must, therefore, penetrate the egg in between hull plates. In this region are located numerous micropores that provide more direct access to the vitelline layer beneath the hull. This region also overlies an area of the egg membrane that is rich in microvilli. The biological significance of these results is discussed with reference to the evolution of self-fertilization.

The large majority of chitons free spawn gametes and are gonochoric (Pearse, '79). Exceptions to free spawning include about 30 species that brood embryos. Brooders typically retain embryos or recently hatched trochophore larvae in the pallial groove (see reviews by Pearse, '79 and Eernisse, '88). Exceptions to gonochorism include only two known cases of hermaphroditism (Eernisse, '88), *Lepidochitona fernaldi* Eernisse 1986 and *L. caverna* Eernisse 1986; both hermaphroditic species are also brooders. Moreover, there is good evidence that both normally self-fertilize (Eernisse, '88), but the site and mechanism of fertilization are unknown. Fertilization in chitons has been studied in detail in only one species (Buckland-Nicks et al., '88a,b), the free-spawner *Tonicella lineata* (Wood, 1815). *T. lineata* is characterized by having eggs with elaborate extracellular hull cupules that are opened by the retraction of follicle cells at maturity (Buckland-Nicks et al., '88b). Sperm swim inside the cupules to fertilize the eggs. Certain other members of the same family, Lepidochitonidae, such as *Lepidochitona dentiens* (Gould, 1846), have cupules that remain permanently closed (Eernisse, '88), suggesting that the mechanism of fertilization is probably different in some respects (Buckland-Nicks, '92).

Eernisse ('88) maintained separate populations of *L. fernaldi* and *L. caverna* on transparent substrates in the laboratory and on several occasions observed, for both species, synchronous early cleavage stages in neighboring brooders. These observations were interpreted as evidence that at least occasional instances of cross-fertilization might occur. Any cost to the behavior of synchronous brooding in close proximity could not otherwise be explained by increased fertilization or outbreeding success. Despite this pattern of brooding synchrony, other reproductive and population genetic studies revealed no evidence for even rare cross-fertilization. *L. fernaldi* and *L. caverna* both successfully brood fertilized embryos in isolation (Eernisse, '88) with no indication of sperm storage. Allozyme studies noted a highly significant absence of heterozygous *L. fernaldi* individuals at electrophoretically inferred loci known to be polymorphic within single popu-

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lations (Eernisse, '84). Moreover, paired paternity analyses of *L. fernaldi* brooders known to be homozygous for different enzyme alleles produced only homozygous progeny (Eernisse, unpub.).

As self-fertilization (or some unusual form of self-sperm-activated amictic or apomictic parthenogenesis) appears to be the normal mode of reproduction in *L. fernaldi* and *L. caverna,* in contrast to the cross-fertilization noted for most chitons (Pearse, '79), it would be interesting to know if sperm ultrastructure and the process of fertilization itself have become modified as well. Franzén ('55) has documented that sperm structure usually becomes modified when the biology of fertilization shifts from external to internal. Structural modifications generally include an increase in the size and number of mitochondria, increased structural support in the flagellum, and a more filiform shape. Embryos brooded in the pallial cavity are not brooded internally, but fertilization could conceivably occur internally before eggs are spawned. This paper examines for the first time the ultrastructure of sperm and eggs from the brooding hermaphroditic chiton, *L. fernaldi,* and interprets the sperm-egg interaction based upon direct observations of fertilization in this species.

MATERIALS AND METHODS

Specimens of *L. fernaldi* were obtained in May '90 from tide pools in association with the barnacle, *Semibalanus cariosus* (Pallas, 1788), and the anemone, *Anthopleura elegantissima* (Brandt, 1835), at Deadman Bay on the west coast of San Juan Island, Washington [48°30'N123°9'W]. Animals were kept on a running seawater table at 10°C (±2°C) in disposable 35 mm plastic petri dishes or in thin-walled polyethylene jars. When a chiton was adhering to the surface of the petri dish, the contents of the pallial groove could be seen through the plastic. On May 24, '90, chitons without brooded embryos were observed at frequent intervals until an ongoing spawning event was discovered. About 20 eggs were removed with a toothpick and a few were observed immediately with an Olympus BH2 light microscope using DIC optics. Four or five eggs were held on the seawater table to confirm cleavage. The remaining eggs were fixed for electron microscopy (E.M.) in cold 2.5% glutaraldehyde in millipore-filtered seawater at pH 8.0 for 2–3 hr or overnight. The ovotestis was exposed by slicing lengthwise down the pallial groove between the shell plates and the foot. Pieces of ovotestis were fixed immediately as above for S.E.M. and T.E.M. The pieces of ovotestis were then diced smaller and these pieces and the fertilized eggs were postfixed in separate vials with cold 2% osmium tetroxide in 1.25% sodium bicarbonate buffer (final concentrations) at pH 7.2 for 1 hr. Tissues were then washed in distilled water and dehydrated in an acetone series, with three changes in 100% acetone.

For T.E.M., the acetone was gradually exchanged with Spurr's resin (Spurr, '69) and allowed to infiltrate overnight in pure resin. The following day samples were oriented in embedding molds and baked overnight in a 70°C oven. One micrometer sections were cut with glass knives on a Sorvall MT2C ultramicrotome and stained with Richardson's stain (Richardson et al., '60). Thin sections were cut with a diamond knife (Diatome), picked up on naked 150 μm mesh copper grids, and stained sequentially with saturated aqueous uranyl acetate (10 min) followed by aqueous lead citrate (6 min) prior to examination in a Philips 300 E.M.

For S.E.M., eggs were pipetted into microporous specimen capsules (SPI Supplies, West Chester, PA, USA) and critical point dried from 100% acetone. The vial was inverted over an aluminum stub coated with a double-sided sticky tab and tapped so that the eggs fell onto the tab. The specimens were sputter-coated with gold and examined in a Cambridge S150 or S250 stereoscan scanning electron microscope.

RESULTS

The eggs of species that brood, such as *L. fernaldi,* are released into the pallial groove and retained. One can routinely find brooded embryos in various stages of development, but it is very rare to spot the eggs at the moment of their release. We were fortunate on one occasion to observe spawning and spawned eggs were immediately transferred to a drop of filtered seawater using a toothpick. We assume that we witnessed the moment of spawning since the eggs were uncleaved (Fig. 1) and a few sperm could be seen swimming around them. Furthermore, cleavage did occur subsequently, confirming successful fertilization. On two occasions sperm were observed penetrating an egg between hull plates, but this event was not captured on film. Subsequently, during sectioning of plastic embedded eggs a sperm was encountered in the act of fertilizing the egg at the same site (Fig. 2). The number of sperm present was very low, which is consistent with observations of the ovotestis of *L. fernaldi* (Eernisse, '88) and of other self-fertilizing hermaphrodites (Strathmann et al., '84). These are the first reported observations of fertilization in a brooding hermaphroditic chiton.

Fine structure of the sperm

The *L. fernaldi* sperm nucleus is bullet-shaped, extending into a long needle-like anterior filament (Fig. 4) which is capped, at the end, by a tiny acrosomal vesicle (Fig. 3). The nucleus comprises highly condensed chromatin with occasional lacunae (Fig. 4). Earlier in spermatogenesis the chromatin forms into a series of strands approximately 20 nm thick. Three mitochondria are associated with the main body of the nucleus, one more anterior and two posterior (Figs. 6–9). A fibrous body is situated posterior to the centrioles and below the level of the annulus attached to axonemal doublets 3, 4, and 5 (following the system of Afzelius, '59) (Fig. 9). Proximal and distal centrioles are embedded in dense granular secretions forming a basal body which is housed in a posterior indentation of the nucleus (Fig. 6). The proximal centriole, which lies perpendicular to the distal centriole, is connected to the base of the nucleus by dense strands. The distal centriole is parallel to the axis of the sperm and produces the flagellum posteriorly (Fig. 6). Extending from the peripheral doublet microtubules of the distal centriole are nine satellite projections. These connect with an annulus which is attached to an infolding of the plasma membrane (Figs. 6–9). Glycogen granules have been seen in the vicinity of the centrioles. The elongate flagellum emanates from the distal centriole with a typical 9 + 2 configuration of microtubules, but terminates in an end-piece containing only the two central microtubules.

Egg structure

The structure of the egg is in essence the same as that reported for other *Lepidochitona* (Richter, '86), with the exception that the hull elaborations are reduced to a series of plates which are usually hexagonal (Fig. 10). Follicle cells are closely associated with the developing oocyte, helping form the characteristic sculpturing pattern. The follicle cells are shed as the oocyte reaches maturity and is released into the ovarian lumen. In the egg cytoplasm numerous yolk granules are visible as well as lighter stained vesicles, which may be cortical granules (Fig. 5). Some of the latter have been observed undergoing release by exocytosis.

Examination of three eggs with S.E.M. did not reveal any sperm in the act of penetration, but between the hull plates we found numerous micropores (Figs. 10, 11). In thin sections one sees microvilli embedded in the underside of the hull primarily between hull plates (Fig. 5). Micropores located only in the area between hull plates (Fig. 5) may extend right through to the egg surface. These micropores are perpendicular to the egg surface but they follow an irregular path, so that it is rare to find a section right through one, even in the thinnest part of the hull (Fig. 5). No micropores or obvious sperm entry points were observed on the hull plates themselves. However, in an S.E.M. examination of fertilization in a related species, *Lepidochitona dentiens,* sperm were found penetrating the hull in the region of micropores between hull cupules (Fig. 12) but were not found inside hull cupules.

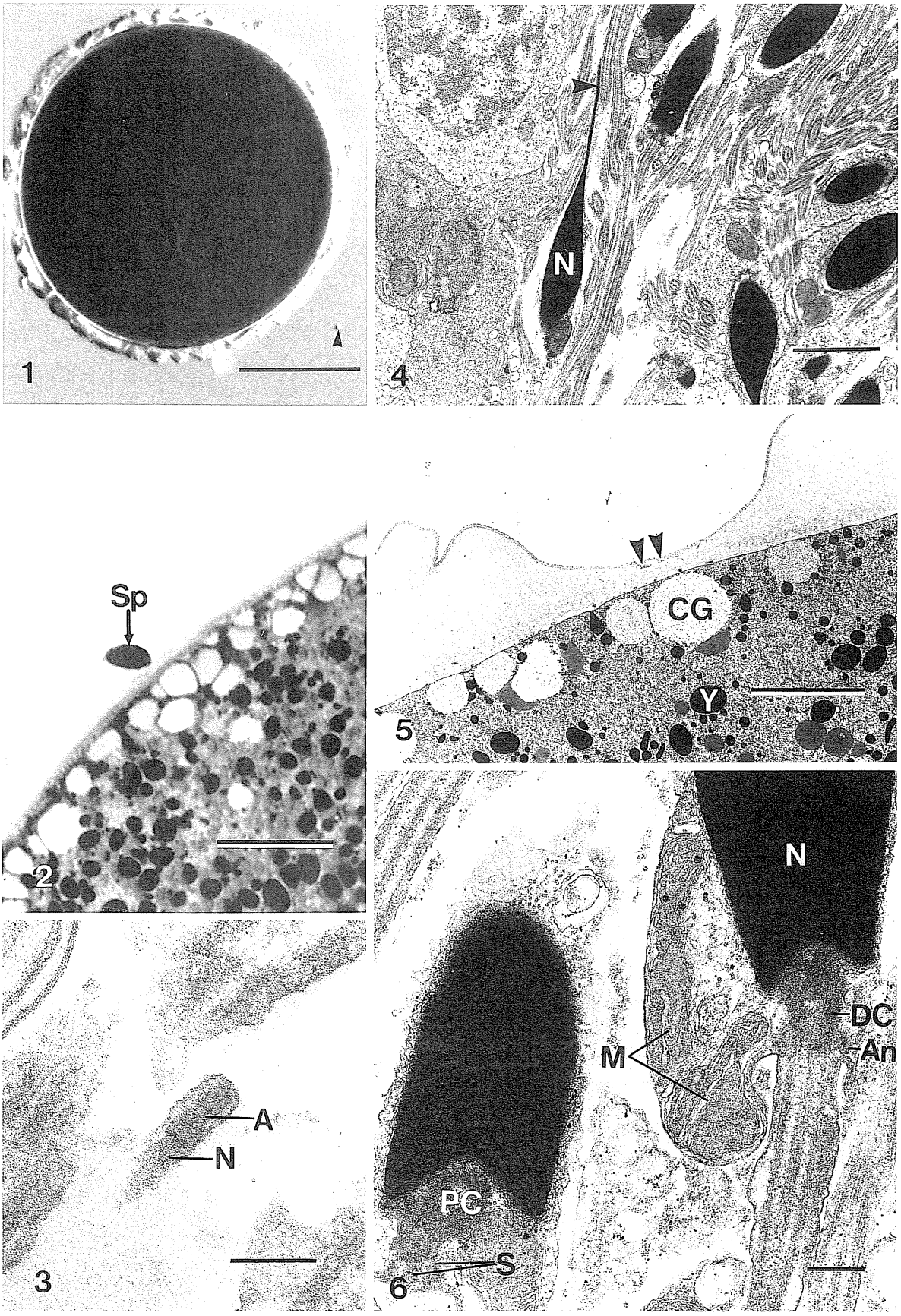
DISCUSSION

On fertilization

An important question that is unresolved for the brooding hermaphroditic chitons is whether fertilization occurs externally in the pallial groove or internally in the female genital tract. Franzén ('55) has shown that sperm structure usually becomes modified when the biology of fertilization shifts from external to internal. Structural modifications generally include an increase in the size and number of mitochondria, increased structural support in the flagellum, and a more filiform shape. We found that the structure and shape of the sperm of *L. fernaldi* is virtually identical to that of free-spawning members of recent chiton groups such as the Mopalidae, Acanthochitonidae, and other Lepidochitonidae. This leads us to suspect that the basic mechanism of fertilization in *L. fernaldi* is similar.

Our direct observations of fertilization in *L. fernaldi* support the hypothesis that the pallial groove essentially provides an external environment in which the sperm swim to the eggs, perhaps assisted by ciliary currents produced by the brooder's multiple gills. Sperm and eggs are probably released through the shared gonopores, simultaneously or nearly so, as this species self-fertilizes (Eernisse, '88) and sperm were still active when the uncleaved eggs were removed for observation.

Like other western North American members of *Lepidochitona,* the hull cupules remain permanently closed. Sperm must therefore penetrate the area between cupules (Buckland-Nicks, '92). In this location one finds numerous micropores in the surface of the hull. Located beneath these micropores and concentrated in the areas between the cupules are elongate microvilli which occasionally protrude above the surface. In *Tonicella lineata,* sperm fuse with individual microvilli (Buckland-Nicks et al., '88b). It is likely that *L. fernaldi* sperm fuse with egg microvilli located beneath the micropores in the region between the hull plates, since there appears to be no access through the hull plates themselves. The hull plates represent the thickest part of the



Figures 1–6.

hull and in the area between the plates, where the hull is thinnest, the micropores extend down to the vitelline layer. Two observations of living sperm at the moment of sperm penetration as well as one of fixed sperm indicate that indeed sperm first penetrate the egg between hull plates. This is supported by direct S.E.M. observations of fertilization in the related species *L. dentiens*.

The acrosomal vesicle in this and some other species, including *Chaetopleura apiculata* (Buckland-Nicks et al., '90), may be composed of a single granule. It has not been possible to determine if more than one granule is involved in this species, as is the case in *T. lineata* (Buckland-Nicks et al., '90). If there is only one granule, this would suggest a modification in the fertilization process. Possibly the anterior filament of the sperm enters a micropore without the aid of the acrosome, which then fires only on contact with the vitelline layer. The resolution of this point will have to await more detailed examination of closely related species such as *L. dentiens* that can, in our experience, be induced to undergo polyspermy.

The possibility remains that the acrosome reaction could occur on contact with a protruding egg microvillus, not requiring digestion of a pathway through egg hull and vitelline layer. If this were true in *L. fernaldi* or other species then, following fusion, retraction of the microvilli could create the series of pores that we observe on the surface. This situation is known to occur among polychaetes (Eckelbarger and Chia, '78; Sato and Osanai, '83). It will be difficult to confirm or eliminate this possibility.

Fig. 1. Uncleaved zygote of *L. fernaldi* removed from pallial groove. Note single sperm (arrowhead). The hull cupules are reduced to flattened plates in this species. Scale bar = 100 μm.

Fig. 2. Sperm (Sp) fertilizing egg in the area between hull plates. Scale bar = 40 μm.

Fig. 3. Apex of sperm showing acrosome (A) atop nuclear filament (N). Scale bar = 0.2 μm.

Fig. 4. Section of testis illustrating various spermatogenic stages. Note late spermatid with bullet-shaped nucleus (N) and its filamentous extension (arrowhead). Scale bar = 2 μm.

Fig. 5. Section of fertilized egg showing region of micropores (arrowheads) between hull plates. Note example of cortical granules (CG) and yolk platelets (Y). Scale bar = 10 μm.

Fig. 6. Sections of late spermatids showing nucleus (N), two posterior mitochondria (M) proximal centriole (PC) embedded in dense secretions, distal centriole (DC), annulus (An), and satellite fibers (S) emanating from distal centriole. Scale bar = 0.25 μm.

Biological significance

Eernisse ('84,'86,'88, unpubl.) concluded that, based on phylogenetic analysis of allozymes and morphology, the hermaphroditic condition of *L. fernaldi* is almost certainly derived, as the species most closely related to *L. fernaldi* all cross-fertilize, including its likely sister taxon, *L. thomasi* (Pilsbry, 1898). This more southern species is a gonochoric brooder with nearly equal sex ratios and pronounced intrapopulational brooding synchrony (Eernisse, '84,'88). *L. thomasi* eggs must also become fertilized while they reside within the female's pallial grooves during an early stage of brooding. The *L. fernaldi* situation differs in that sperm and eggs both are spawned through shared bilaterally-paired gonopores from the same gonad. Thus spawning could involve practically simultaneous, self-fertilization of eggs and sperm.

Perhaps seawater or some other extrinsic factor may be required to activate spawned sperm, as occurs in other molluscs (Buckland-Nicks, '81) as well as in other invertebrates (Bibring et al., '84; Lee et al., '88), so that internal fertilization is at best infrequent. Our results here suggest that fertilization is nearly simultaneous with spawning. Self-fertilization apparently involves a relatively simple hermaphroditic development of small amounts of testicular tissue within an ovary, although one could imagine that some mechanism to coordinate spawning of gametic types, or for preventing internal fertilization, might also be involved. Hermaphroditism and selfing do not appear to have led to any observable modifications of egg or sperm morphology or differences in sperm-egg interactions, based on superficially identical eggs in the gonochoric *L. thomasi* (Eernisse, '88) and the highly similar description of gametes and gametic interactions in the cross-fertilizing free spawner *L. dentiens* (Buckland-Nicks et al., '90; Buckland-Nicks, '92).

The relatively simple transformation to self-fertilization inferred in *L. fernaldi* raises the question of why there are not more selfing chiton species. Selfing lineages could at least theoretically spread much more rapidly than cross-fertilizing lineages, since all of a selfing brooder's progeny could themselves potentially brood. The answer must lie either in the rarity of mutations leading to hermaphroditic individuals or in the presumed selection against such individuals, should they happen to arise.

Strathmann et al. ('84) and Eernisse ('84,'88) argued that inbreeding depression could be invoked as the mechanism normally selecting against selfing individuals, overcoming assumed advantages of selfing. Furthermore, selfing should only be

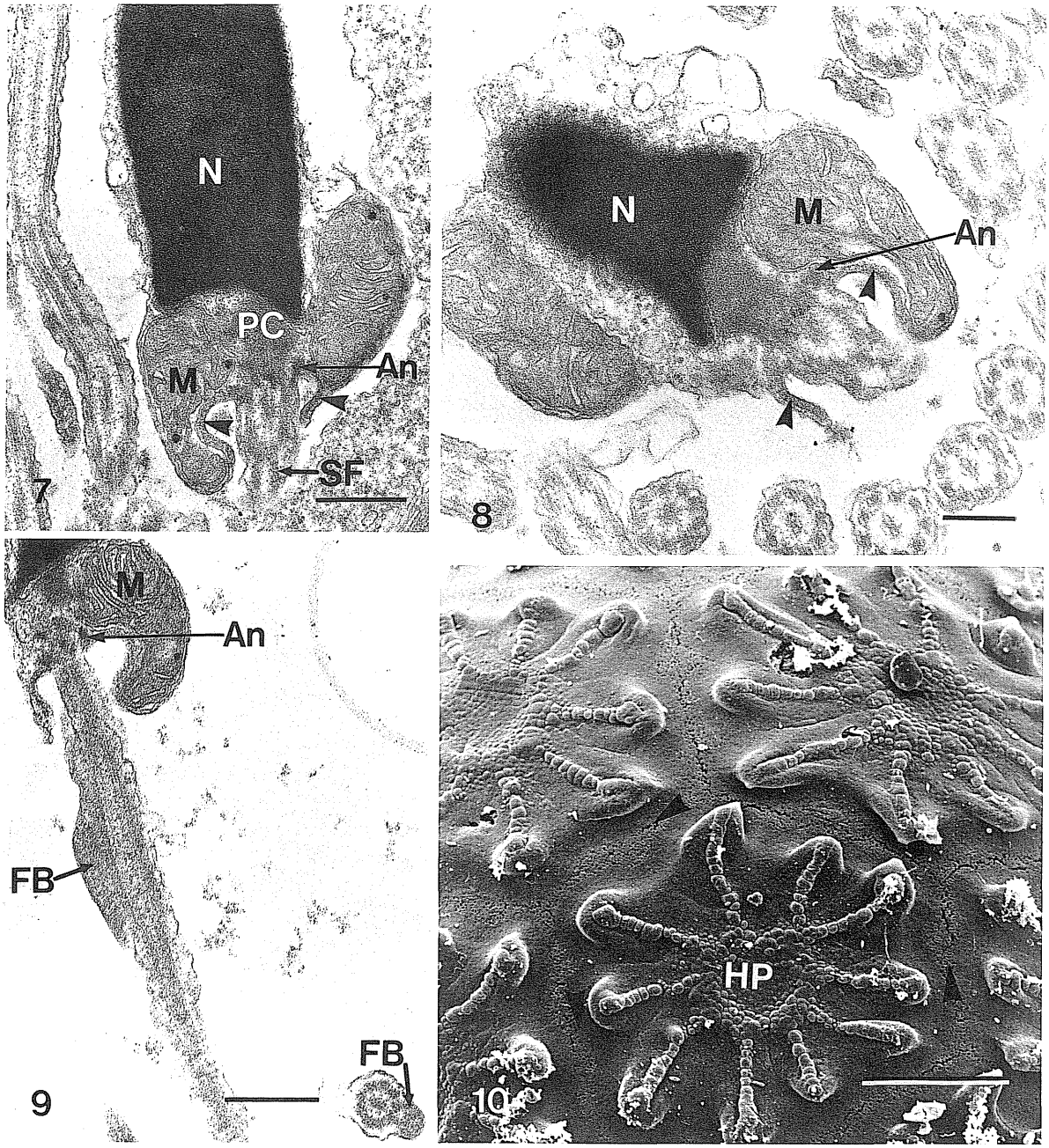


Fig. 7. Section of late spermatid showing nucleus (N), posterior mitochondria (M), proximal centriole (PC), annulus (An), fibrous thickening of plasma membrane (arrowheads), and satellite fibers (SF). Scale bar = 0.4 μm.

Fig. 8. Section of late spermatid showing mitochondrion (M), annulus (An), and fibrous thickening of plasma membrane (arrowheads). Scale bar = 0.2 μm.

Fig. 9. Section of late spermatid showing relationship between posterior mitochondrion (M), annulus (An), and fibrous body (FB). Note also fibrous body in cross section of flagellum below. Scale bar = 0.4 μm.

Fig. 10. S.E.M. of hexagonal hull plates (HP) and regions of micropores (arrowheads) between. Scale bar = 25 μm.

observed in lineages already highly inbred and so not prone to substantial additional inbreeding depression due to selfing. These authors suggest that brooding, with its potential for nondispersal of offspring, creates situations in which hermaphroditism can arise in an already highly inbred population. The inference that the cross-fertilizing *L. thomasi* is sister taxon to *L. fernaldi* and also is a brooder helps support their hypothesis. Allozyme studies of members of this genus revealed that brood-

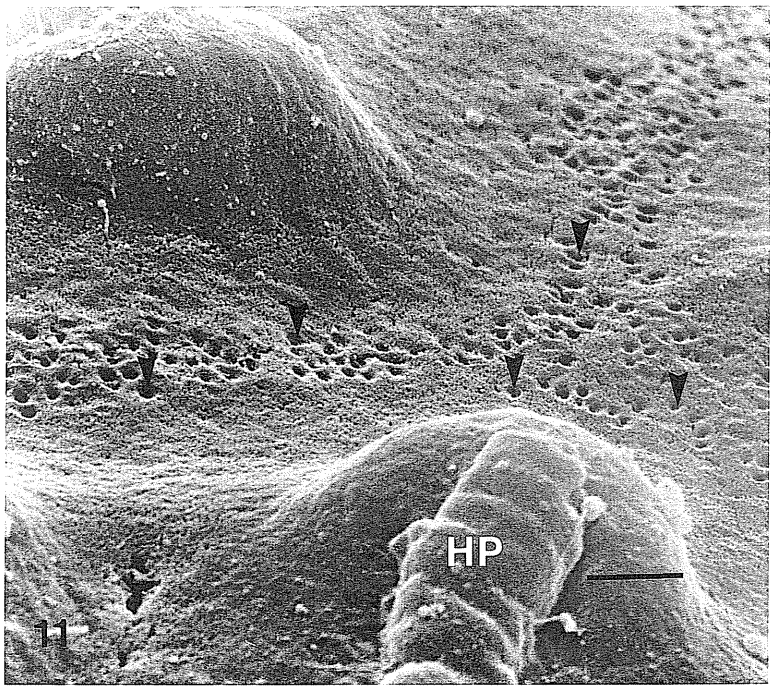


Fig. 11. Micropores (arrowheads) in region between hull plates (HP). Scale bar = 2 μm.

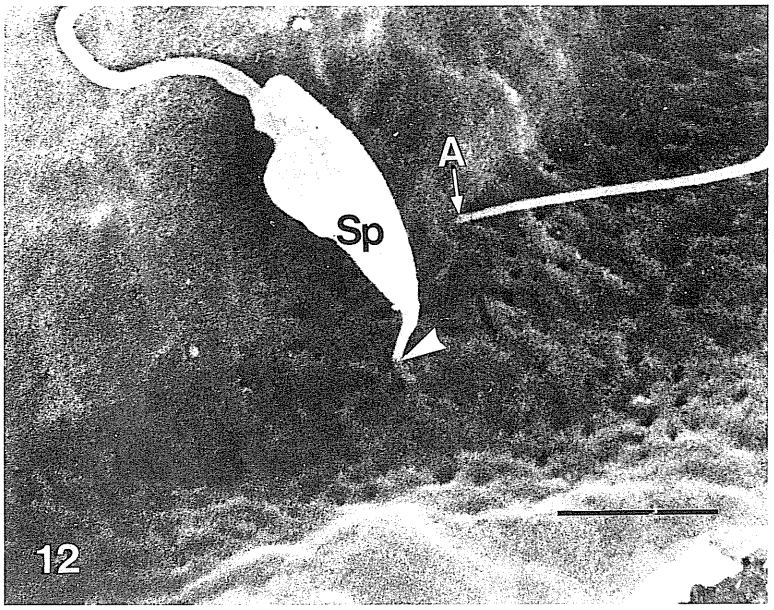


Fig. 12. Sperm (Sp) of *L. dentiens* penetrating egg (arrowhead) in region of micropores between hull cupules. Note acrosome (A) at tip of unreacted sperm. Scale bar = 2 μm.

ers are much less heterozygous than free spawners, whether or not they self-fertilize (Eernisse, '84), further suggesting that brooders are more highly inbred. The potential reproductive advantage to be gained by selfing could explain its occurrence in *L. fernaldi*. Nevertheless, any realized advantage of selfing does not appear to have led to selfing lineages of marine animals of high taxonomic rank. Such lineages must therefore be short-lived on a geologic time scale.

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