Gamete Research 24:219–228 (1989)

Role of Spermatozeugmata in the Spawning Ecology of the Brooding Oyster *Ostrea edulis*

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Ostrea edulis spawns spermatozeugmata, each composed of radially arrayed sperm cells attached by an extracellular matrix (ECM) to a core of acellular vesicles. The acellular vesicles are formed from excess nuclear and plasma membranes produced during spermatid condensation, and the ECM is topologically restricted to the interstices between acellular vesicles and sperm heads, being absent from the flagellar surface. When released into seawater, spermatozeugmata retain their structural integrity for varying periods (up to 24 hours) and become demersally distributed in still water. The flagella activate, initially beating in a non-synchronized, languid manner; however, both the tempo and amplitude of the flagellar action gradually increase to resemble that of typical "primitive" sperm once the cells are released from the spermatozeugma. This increase in flagellar activity and subsequent gamete release coincide with an erosion of the ECM and suggest that the ECM may modulate sperm motility in addition to adhering the cells to the spermatozeugma. Sperm are capable of fertilizing eggs only after dissociating from the spermatozeugma. The net effect of spermatozeugma formation in *Ostrea edulis* may be the retention of viable sperm in high concentrations at the benthic/water column interface for prolonged periods after spawning, at least in low current regimes. Unfertilized *O. edulis* eggs are also concentrated at this location, in the inhalent chambers of female oysters. Spermatozeugmata are entrained by the inhalent current of females and carried into the brood chamber where fertilization occurs. The *Ostrea edulis* spermatozeugma likely functions as an efficient sperm transfer mechanism if two conditions are met: 1) eggs are retained as broods within benthic females, 2) egg masses are at intermediate distances from spawning males.

Key words: bivalvia, spermatozeugma, ultrastructure

INTRODUCTION

The spawning ecology of broadcast spawning aquatic invertebrates differs in important details from that of species engaging in parental care. Gamete interaction in broadcasting species occurs while both gamete types are dispersing into the ambient water body. High rates of fertilization are achieved by releasing large numbers of free swimming sperm in close proximity to spawning females, thereby preempting prohibitive gamete dilution [Pennington, 1985; Run et al., 1988]. The simplified, "primitive" sperm morphology of broadcast spawners is highly conservative [Franzén, 1956; Baccetti and

Received January 3, 1989; accepted April 28, 1989.

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Afzelius, 1976] and broadcast sperm typically exhibit high motility over a relatively short life span [Pennington, 1985].

Aquatic invertebrates that engage in parental care (brooders) retain newly spawned eggs in high concentrations at specific fertilization sites upon or within the female. Efficient fertilization in cross-fertilizing brooders is achieved by the bulk transfer of aggregated sperm to these sites, an event that may be temporally separated from egg spawning in species with sperm storage capability [Daly and Golding, 1977; Ó Foighil, 1985b]. Brooders that lack copulatory organs employ either dwarf males [Morton, 1981; Turner and Yakovlev, 1983; Ó Foighil, 1985a], or one of a variety of indirect sperm transfer methods where sperm are spawned as aggregates of varying structural complexity and individual cells initially exhibit little or no motility. In the simplest cases of indirect sperm transfer, such as the externally brooding holothuroid *Cucumaria lubrica,* the aggregations are composed of quiescent, adherent sperm and lack auxillary structures [Atwood and Chia, 1974; Engstrom, 1982; McEuen, 1988]. Spermatozeugmata are produced in a wide variety of taxa [Fretter, 1953; Austin, 1965; Werner, 1973; Buckland-Nicks and Chia, 1977; Lynn, 1987] and consist of a central modified cell or mass of acellular membranes to which the sperm are attached. Spermatophores differ from spermatozeugmata in that the sperm mass is enclosed by a sheath or capsule of varying complexity in the former [Zimmer, 1967; Flügel, 1977; Rice, 1980; Hadfield and Hopper, 1980; Kress, 1985; Ó Foighil, 1985b].

Spermatozeugma production in brooding oysters has been well documented [Coe, 1931; Hori, 1933; Menzel, 1955; Morriconi and Calvo, 1979]. The most detailed investigation to date is that of Coe [1931] on spermatogenesis in *Ostrea lurida.* Sperm are spawned as ellipsoid clusters, each containing 250–2,000 cells descended from a single spermatogonium. The structural integrity of the spermatozeugma is maintained by a central gelatinous core which envelops the sperm heads, but not the radially projecting flagella. When spawned, the flagella activate and after varying intervals, individual sperm swim off leaving the gelatinous core behind as an amorphous mass.

This present study aims to characterize ultrastructurally the spermatzeugma of *Ostrea edulis,* paying particular attention to the acellular core, the method of sperm attachment, and the structural changes associated with spermatozeugma breakdown. It also describes the role of the spermatozeugma in the spawning ecology of this species.

MATERIALS AND METHODS

Mature specimens of *Ostrea edulis* were obtained from Wescott Bay Gourmet Shellfish Company, San Juan Island, WA, in spring and summer 1987. Spermatozeugmata were dissected from the gonad and their developmental stage assessed by light microscopy. Mature spermatozeugmata were rinsed twice in artificial seawater (AFSW) and were either fixed immediately for electron microscopy, or were first incubated in AFSW at room temperature (20°C) for 6 hours to promote their dissociation. The fixation recipes and tissue processing techniques used for both scanning and transmission electron microscopy (SEM, TEM) are detailed in Ó Foighil [1985c]. Specimens were viewed with a JEOL JSM-35 scanning electron microscope and a Philips EM-300 transmission electron microscope. Spermatozeugmata and unfertilized eggs were also obtained from spontaneously spawning, laboratory-held individuals; and sperm-egg interaction was studied by light microscopy and SEM. Low-density spermatozeugmata

monolayers were incubated in AFSW at ambient seawater temperatures (14°C) in Falcon tissue culture wells to determine dissociation rates.

RESULTS

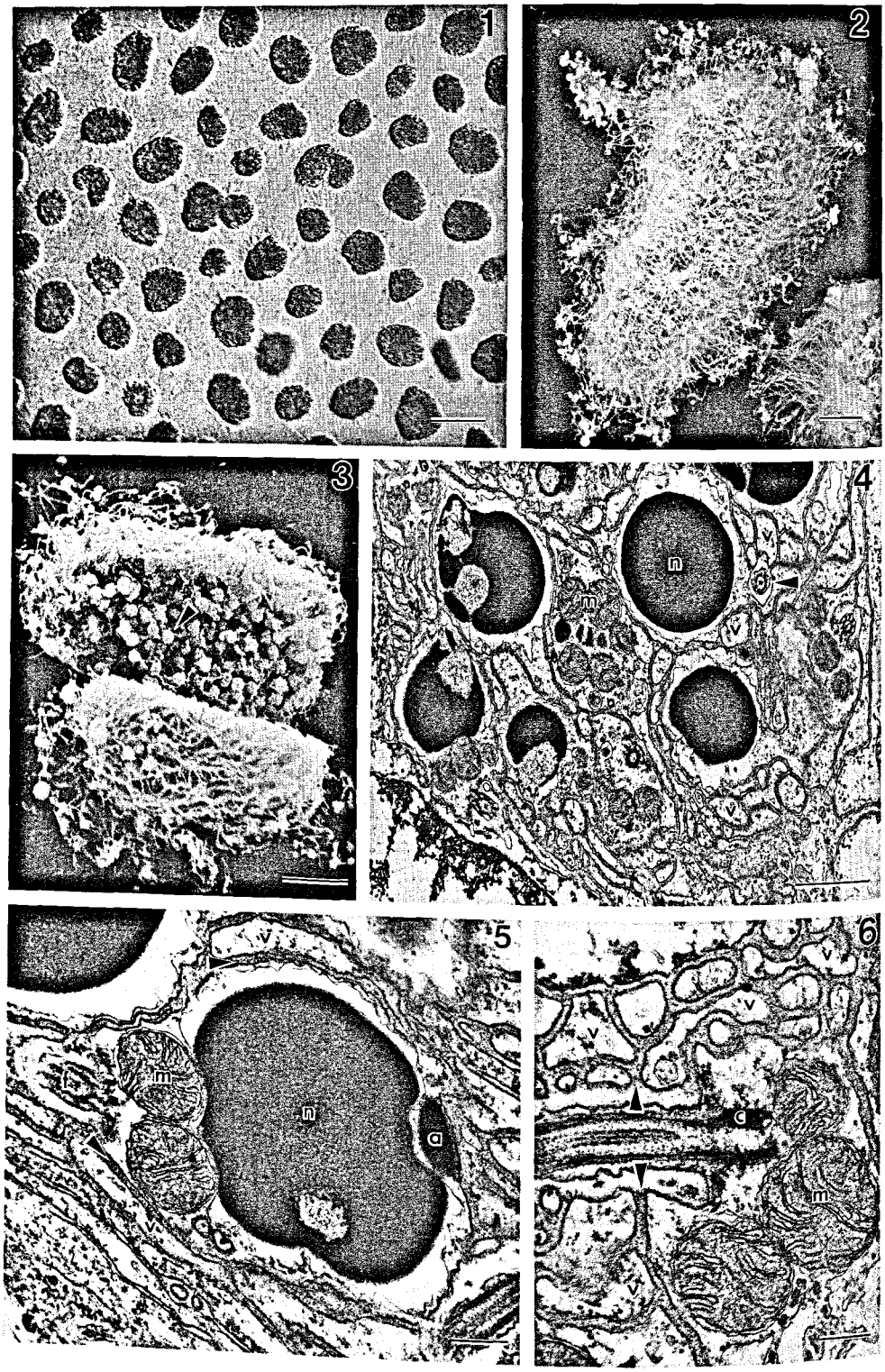
Ostrea edulis spermatozeugmata are very similar in their overall structure to those of *O. lurida* [Coe, 1931]. They are, however, variable in their individual gross morphology and range from 25 to 80 μm in diameter (Fig. 1). While part of an intact spermatozeugma, each sperm cell transverses a well-defined micro-environmental boundary. The flagella radiate freely into the ambient environment (Fig. 2), whereas the sperm heads are embedded in the central core of the spermatozeugma (Fig. 3). *Ostrea edulis* sperm conform to the "primitive" sperm architecture typical of externally fertilizing aquatic organisms [Franzén, 1956; Bacetti and Afzelius, 1976]. This is apparent from the apical acrosomal vesicle; small, spherical nucleus; a simple mid-piece, and a long flagellum (Figs.4–6).

In addition to sperm heads, the spermatozeugma core is composed of a large number of acellular vesicles which envelop the sperm heads (Figs. 4–6). These vesicles are formed during the mid-late spermatid stage of development from the excess membrane discarded by the gametes during nuclear condensation. Although the vesicles lack organelles, they may share some cytoplasmic constituents with the sperm cells in the form of flocculent material visible by TEM (Figs. 4–6). The vesicles are bound to each other and to the sperm heads by a distinct, fibrillar, extracellular matrix (ECM) which occupies the interstices separating the vesicles from each other and from the sperm heads (Figs. 4–6). Ontogenetically, the ECM first appears at the mid-late spermatid stage of spermatogenesis. Its distribution on the sperm surface appears to be restricted to the sperm head and it is lacking from flagella (Figs. 5, 6), including those that originate from sperm heads located deep within the spermatozeugma core (Fig. 4).

When spermatozeugmata are released into seawater, either by spawning or by dissection from the gonad, the flagella activate, gently beating in a non-synchronized, languid manner. Spermatozeugmata are denser than seawater and sink in still water. The sinking rates of individual spermatozeugmata, however, vary considerably, being positively related with their size.

Spermatozeugmata retain their structural integrity and the attached sperm maintain their flagellar action for varying periods in seawater, in some cases (including spontaneous spawnings) for up to 24 hours when held at ambient seawater temperatures (14°C). The longevity of the attached sperm was not a laboratory artifact due to crowding [Pennington, 1985] because the spermatozeugmata were held in low-density monolayers as depicted in Figure 1. Observations of laboratory-held, spawning females revealed that near-bottom suspended spermatozeugmata were entrained by the inhalent current of spawning females and carried into the brood chamber where fertilization occurred.

Dissociation of spermatozeugma is preceded by a gradual increase in the rate and amplitude of flagellar beating. Individual cells break free and swim off, leaving behind the vesicular core (Figs. 7–9). An interesting structural change occurs in the vesicular core that coincides with sperm release. The ECM, which completely occupies the interstices between sperm heads and acellular vesicles before dissociation (Figs. 4, 5), becomes eroded and persists only in isolated patches around relict sperm (Figs. 10, 11). Once liberated from the spermatozeugma, *Ostrea edulis* sperm are active swimmers and



Figs. 1–6.

bind to the surface of conspecific eggs by undergoing a prominent acrosomal reaction (Fig. 12). Incubation of unfertilized eggs with intact spermatozeugmata did not result in fertilization events prior to sperm release.

DISCUSSION

Ostrea edulis sperm cells have a typically "primitive" morphology and differ from those of broadcast spawning oysters only in minor ultrastructural details [e.g., lack of a well-developed axial rod and one additional mitochondrion in the middle piece; Daniels et al., 1969, 1971; Kyozuka and Osanai, 1985]. Once released from the spermatozeugmata, *O. edulis* sperm exhibit typical "primitive" behaviour when the free swimming cells experience a brief period of high motility and are capable of undergoing an acrosomal reaction and fertilizing eggs. Spermatozeugmata breakdown, however, may not occur until up to 24 hours after spawning. Prior to this, *O. edulis* sperm remain aggregated, display little motility, become demersally distributed (at least in low current regimes), and are incapable of fertilizing eggs.

The differences in postspawning behaviour of *Ostrea edulis* sperm from those of broadcasting spawners originate in modifications of the spermatogenic process in this species. Unlike broadcast spawning bivalve molluscs, spermatogenic stages in brooding oysters remain aggregated throughout their development Coe [1931]. Results from the present study indicate that two distinct ontogenic modifications, production of acellular vesicles and of an ECM, occurring during the spermatid stage of development in *O. edulis*, are responsible for maintaining the structural integrity of the spermatozeugma for considerable periods after spawning.

Spermatids undergo a rapid reduction in nuclear and total cell volumes during their transition to mature sperm cells [Longo and Dornfeld, 1967]. A by-product of this shrinkage is the formation of excess membrane, both nuclear and plasmalemma. In broadcast spawners, this surplus membrane is discarded by cytoplasmic sloughing [Longo and Dornfeld, 1967] or by the formation and release of myelin figures on the cell surface [Tilney, 1976; Buckland-Nicks et al., 1984]. In *Ostrea edulis*, the excess membrane form vesicles (see Fig. 10) which are retained as the acellular core of the developing spermatozeugma.

Fig. 1. Differential interference contrast (DIC) light micrograph of intact *Ostrea edulis* spermatozeugmata. Note variation in shape and size. Scale = 50 μm.

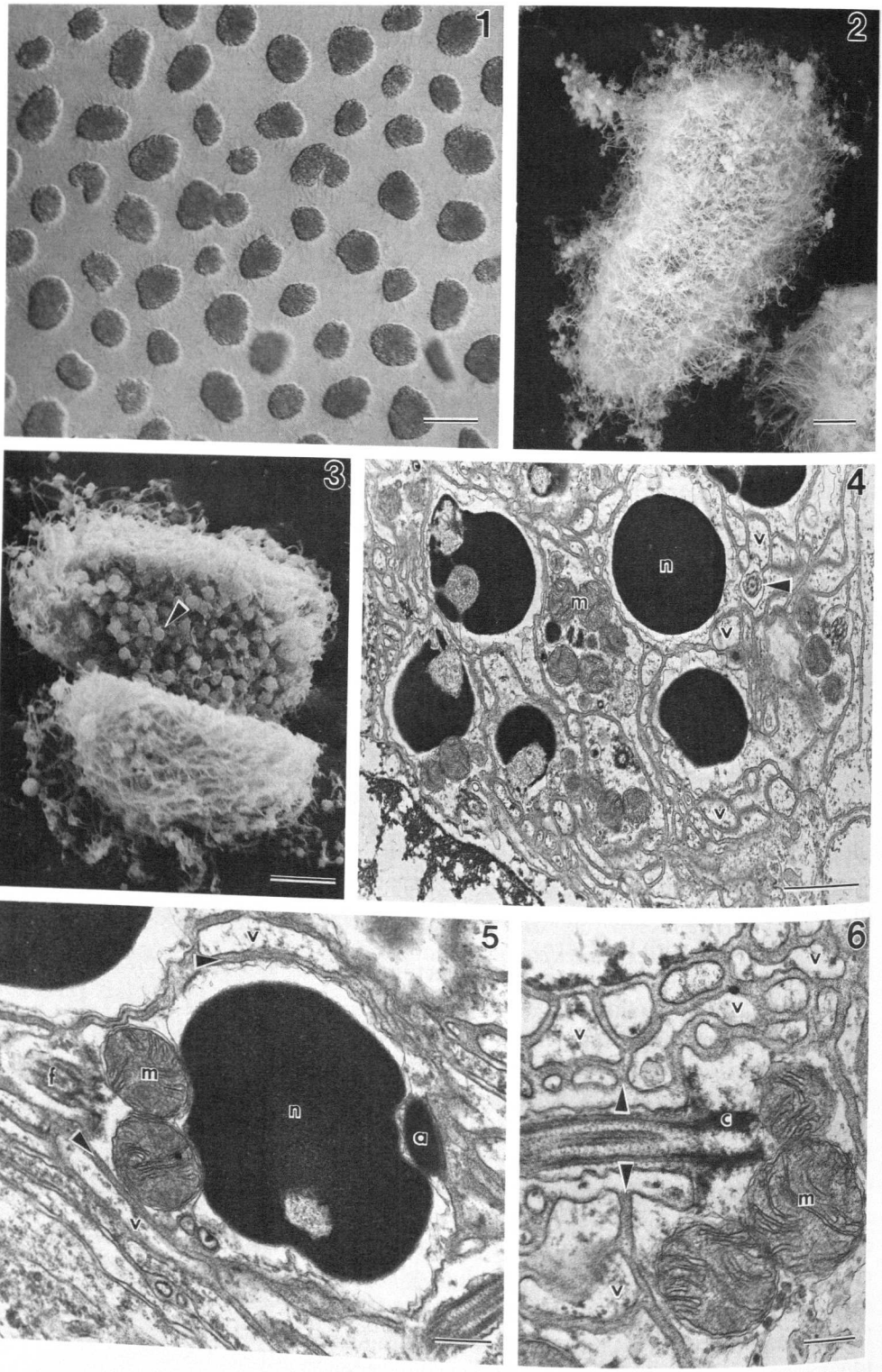
Fig. 2. Scanning electron micrograph (SEM) of an intact *O. edulis* spermatozeugma showing radiating flagellae. Scale = 10 μm.

Fig. 3. SEM of a sectioned, undissociated *O. edulis* spermatozeugma. Arrow points to a central sperm head. Scale = 8 μm.

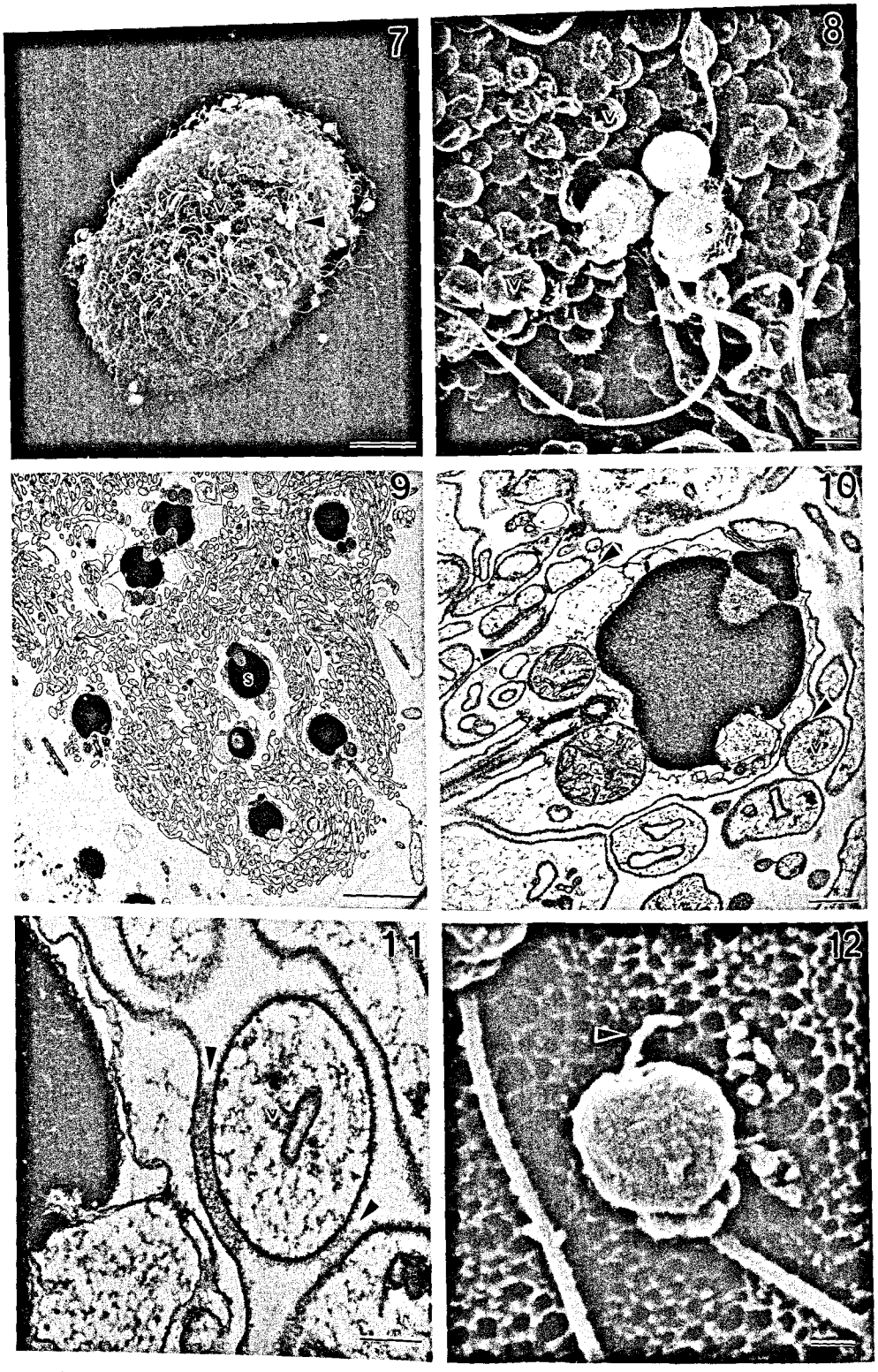
Fig. 4. Transmission electron micrograph (TEM) of a section through an intact *O. edulis* spermatozeugma. m, mitochondria in mid-piece; n, nucleus; v, acellular vesicles. Arrow indicates a cross section of a flagellum originating from a central sperm head. Scale = 1 μm.

Fig. 5. TEM of a longitudinal section through an *O. edulis* sperm head in an intact spermatozeugma. a, acrosomal vesicle; f, flagellum; m, mitochondrion; n, nucleus; v, acellular vesicle. Arrow points to the extracellular matrix (ECM) on periphery of the sperm head. Scale = 0.3 μm.

Fig. 6. TEM of longitudinal section through mid-piece of an *O. edulis* sperm in an intact spermatozeugma. c, distal centriole; m, mitochondrion; v, acellular vesicles. Arrows indicate limitation of ECM to the interstices between acellular vesicles and the sperm heads. Note that the ECM is not present on the flagellar surface. Scale = 0.2 μm.



Figs. 1–6.



Figs. 7–12.

Extracellular matrices play important roles in cell adhesion, migration, and differentiation [Hay, 1984]. The *Ostrea edulis* ECM is interesting for a number of reasons. It is formed during the mid-late spermatid stage of development and may represent a rare example of a "haploid effect," i.e., postmeiotic expression of the sperm genome [Sivinski, 1984]. Topologically, the ECM is restricted in its distribution to the sperm heads and to the acellular vesicles. Two lines of evidence indicate that the ECM is directly responsible for sperm adhesion to the acellular vesicular core of the spermatozeugma. The ECM is absent from the flagella, which are the only part of the cells unattached to the vesicular core, including the entire length of the flagella projecting from centrally located sperm heads. Release of individual sperm cells from the spermatozeugma coincides with the erosion of the surrounding ECM.

Sperm of externally fertilizing marine organisms are quiescent in seminal fluid, but motility is activated when these gametes are diluted in seawater [Morisawa and Suzuki, 1980; Bibring et al., 1984; Morisawa, 1985]. This onset of motility is triggered by the change in ambient ion concentrations experienced by the cells when spawned into seawater [Morisawa, 1985]. *Ostrea edulis* sperm are also activated when released into seawater, but they show a markedly different pattern of motility from that of broadcast spawning marine invertebrates. Instead of undergoing a brief burst of high motility, *O. edulis* sperm exhibit a prolonged period of reduced flagellar beating while attached to the spermatozeugma. The tempo and amplitude of the flagellar action gradually increase as the ECM is eroded and resembles that of typical "primitive" sperm once the cells are released from the spermatozeugmata. These observations suggest that the ECM may modulate sperm motility.

The mechanism of ECM dissolution is of interest because the ECM apparently plays a pivotal role in maintaining the structural integrity of the *O. edulis* spermatozeugma, and sperm are only capable of fertilizing eggs when they are released from these aggregations. Available data suggest two possibilities: ECM structural integrity is dependent on an ambient ionic milieu found in gonadal fluid but not in seawater; an ECM digesting enzyme is activated upon spawning. While there is insufficient data to determine which, if any, of these two potential mechanisms operate, the sharply defined boundaries and patchy distribution of intact ECM around relict sperm in disassociating spermatozeugmata (Figs. 10, 11) may favour the former hypothesis.

Competition for fertilization is thought to be the primary influence on the evolution of sperm diversity [Sivinski, 1984]. Does the formation of spermatozeugmata in *Ostrea edulis* enhance fertilization efficiency or could higher rates of fertilization be obtained if

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Fig. 7. SEM of dissociating *O. edulis* spermatozeugma. Arrow indicates some of the residual sperm attached to the acellular vesicular core (v). Scale = 10 μm.

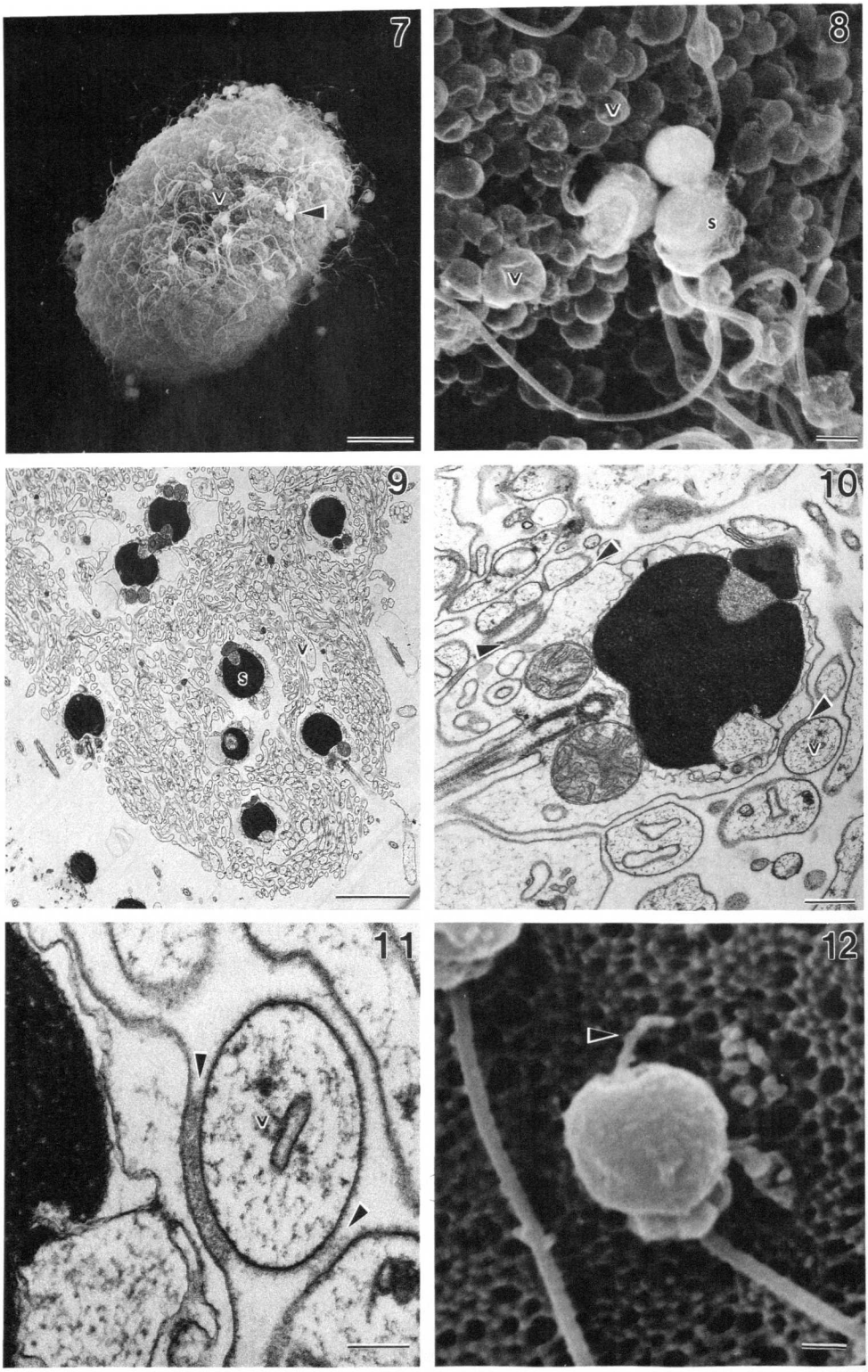
Fig. 8. SEM showing detail of *O. edulis* spermatozeugma in Figure 13. Note acellular vesicles (v) and residual; sperm cells (s). Scale = 1 μm.

Fig. 9. TEM of section through a dissociating *O. edulis* spermatozeugma. s, sperm heads; v, acellular vesicles. Scale = 2 μm.

Fig. 10. TEM of a longitudinal section through a relict sperm cell in a dissociating *O. edulis* spermatozeugma. Arrows point to residual patches of ECM. v, acellular vesicles. Note presence of intracellular vesicles and excess plasmalemma in this sperm cell, indicating it is not yet fully mature. Scale = 0.3 μm.

Fig. 11. TEM showing detail of Figure 16. Arrows indicate residual ECM patches. v, acellular vesicles. Scale = 0.1 μm.

Fig. 12. SEM of dissociated *O. edulis* sperm cell attached to the vitelline coat of a conspecific egg. Arrow points to the acrosomal filament. Scale = 0.5 μm.



Figs. 7–12.

short-lived, free-swimming sperm cells were released? Pennington [1985] used concentrations of 1,600 echinoid eggs per ml of sperm-containing ambient seawater in his field spawning experiments and found that percentages of fertilization dropped rapidly at distances >10 cm from spawning males. Differences in the spawning ecology of *O. edulis* from that of broadcast spawners suggest that the spatial constraint experienced by *O. edulis* would be considerably greater than that of echinoids, if it released its sperm as single cells. Female *O. edulis* retain their eggs in very high concentrations in their mantle cavities, approximately 90,000/ml (Ó Foighil, unpubl.) and would therefore require a correspondingly concentrated uptake of individual sperm cells to achieve high rates of fertilization. In addition, free-swimming sperm cells are relatively short-lived [Pennington, 1985]; however, spawning in female *O. edulis* is a prolonged affair; eggs have to be forced through the gill ostia into the inhalent chamber of the mantle cavity by rhythmic valve contractions [Andrews, 1979].

The net effect of spermatozeugma formation in *Ostrea edulis* may be the retention of viable sperm in high concentrations at the benthic/water column interface for prolonged periods after spawning, at least in areas lacking strong currents. Unfertilized *O. edulis* eggs are also concentrated at this location—in the inhalent chambers of female oysters. The demersal sperm densities achieved in natural spawning events are determined by environmental features, e.g., density of male spawners and current flow rates. Sinking rates of the spermatozeugmata are directly related to their size and the wide range of sizes produced by single individuals imply that at least the smaller spermatozeugmata will remain in suspension under low current regimes. Adult *Ostrea edulis* pump an average of 6 litres of seawater per hour in laboratory conditions [Wilson, 1983], although this rate is likely to be reduced in spawning or brooding specimens. Spermatozeugmata entrained in the inhalent currents of spawning females pass into the mantle cavities and gain access to the unfertilized eggs.

Aggregated transport of sperm cells, however, also bears a price for the participating gametes by resulting in the closer proximity of competitors [Sivinski, 1984]. When spawning males are typically positioned very close to females, sperm dilution is less of a problem, and the competitive costs of sperm aggregation begin to outweigh the benefits. This phenomenon can be seen in the brooding oyster *O. puelchana,* where males occur in two distinct forms, large solitary individuals and small dwarf males that attach to the valves of adult females at the entrance to the brood chamber [Calvo and Morriconi, 1978; Castro and Lucas, 1987]. Solitary *O. puelchana* males form spermatozeugmata that appear identical to those of *O. edulis* [Morriconi and Calvo, 1979], whereas spermatogenesis in the dwarf males results predominently in unassociated sperm cells [Calvo and Morriconi, 1978]. The dwarf *O. puelchana* males, unlike the larger, solitary conspecifics and male *O. edulis,* release their gametes directly into the brood chamber of the females and experience minimal sperm dilution and no indeterminate period of transfer between mantle cavities. Fertilization success in this case is presumably greater for the unassociated sperm that are immediately capable of undergoing an acrosomal reaction and fertilizing the eggs.

The *Ostrea edulis* spermatozeugma likely function as an efficient sperm transfer mechanism if two conditions are met: 1) eggs are retained as broods within benthic females, 2) egg masses are at intermediate distances (perhaps 10 cm to 1–2 m) from spawning males. If spawning females are more than a few metres from spawning males,

sperm dilution, although less than in species releasing unaggregated sperm, is likely to be severe and fertilization efficiency low.

ACKNOWLEDGMENTS

My thanks to Wescott Bay Gourmet Shellfish Company for supplying specimens of *Ostrea edulis*. Damhnait McHugh and the comments of two anonymous reviewers helped improve early drafts. Financial support was provided by a Friday Harbor Laboratories Post-Doctoral Fellowship, and I thank Dennis Willows, FHL Director, for the use of facilities.

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