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Spermatozeugmata structure and dissociation of the Australian flat oyster *Ostera angasi*: Implications for reproductive strategy

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ABSTRACT

Variation in reproductive strategy is one of the key factors contributing to recruitment success of molluscs in different habitats. Spermcasting is a unique mode in mollusc reproduction where males produce spermatozeugmata, a radially arrayed sperm cluster wrapped by gelatinous membrane. In this study, spermatozeugmata structure and their dissociation in the Australian flat oyster *Ostrea angasi* were investigated to elucidate the reproductive strategy in spermcasting molluscs. The histological observation indicated that spermatogonia gradually aggregated in the gonad follicle at the early gonad development stages and developed into spermatozeugmata and became tightly packed at the advanced stages. Even though mature male and female gametes could be found in a hermaphroditic individual, the animal may prevent self-fertilization by shedding different sex gametes at different time. The *O. angasi* sperm are similar in size and shape to broadcasting oysters, but have one additional mitochondrion. Variations in maintaining spermatozeugmata integrity and sperm motility between individuals depended on the level of masculinity or femineity. The durations of spermatozeugmata dissociation and sperm viability were longer in males than in hermaphrodites. The unique structure and capability for spermatozeugmata to maintain the functional integrity after spawning have adaptive significance for fertilization and gamete dispersal in this species.

1. Introduction

The dynamics of reproductive pattern are vitally important in understanding the life history and adaptation of marine invertebrates. The general mode of reproduction in marine invertebrates is broadcast spawning in which the male and female release gametes in water where fertilization and embryonic development occur. Spermcasting is another type of reproduction where males broadcast sperm in water and females inhale sperm to fertilize eggs in the body cavity (Bishop and Pemberton, 1997; Pemberton et al., 2003). In most of the spermcasting species, fertilized eggs are incubated inside the female body and develop to a free swimming larva before being released into the water (Jackson, 1985). In this paper, the term 'spermcasting' is adopted from Falese et al. (2011) though its synonyms are also used, such as spermcast mating (Bishop and Pemberton, 2006), egg brooding (Phillippi et al., 2004), egg brooding free-spawner (Johnson and Yund, 2004) and larviparity (Buroker, 1985).

Spermcasting differs from broadcasting in the structure of male gametes. Rather than releasing individual sperm, the spermcasting species spawn spermatozeugmata which can be carried by water movement to the female mantle cavity for fertilization. In a spermatozeugma, the sperm heads are clustered by a gelatinous membrane with tails extending outside (Foighil, 1989). Spermatozeugma is also termed as 'sperm-balls' (Coe, 1931), 'sperm spheres' (Ishibashi et al., 2000) and 'sperm morule' (Jespersen et al., 2001). Spawning of spermatozeugmata is found in some aquatic invertebrates including polychaetes (Drozdov and Galkin, 2012), worms (Maiorova and Adrianov, 2005; Bohn and Heb, 2014), and bivalves (Jespersen et al., 2001; Geraghty et al., 2008), and some vertebrates such as fishes (Meisner et al., 2000; Fishelson et al., 2007).

Past research on sperm structure is mainly focused on broadcasting oysters such as the Pacific oyster *Crassostrea gigas* (Bozzo et al., 1993; Komaru et al., 1994; Dong et al., 2005; Drozdov et al., 2009; Yurchenko, 2012), eastern oyster *Crassostrea virginica* (Daniels et al., 1971; Eckelbarger and Davis, 1996), Portuguese oyster *Crassostrea angulata* (Sousa and Oliveira, 1994), Iwagaki

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oyster *Crassostrea nippona* (Yurchenko, 2012), Jinjiang oyster *Crassostrea rivularis* (Yurchenko, 2012), Sydney rock oyster *Saccostrea commercialis* (Healy and Lester, 1991) and small rock oyster *Saccostrea mordax* (Yurchenko, 2012). These studies have revealed the dimensional differences in sperm components to support species specificity of sperm morphology. As such, the spermatozeugmata dimensions are also different among taxonomic groups (Ferraguti et al., 1989). So far, the oysters that have showed spermcasting behaviour all belong to the genus *Ostrea*; including the European flat oyster *Ostrea edulis* (Foighil, 1989), Chilean oyster *Ostrea chilensis* (Chaparro et al., 1993), Puelche oyster *Ostrea puelchana* (Castanos et al., 2005) and Australian flat oyster *Ostrea angasi* (O'Sullivan, 1980). Among the spermcasting oysters, the spermatozeugma structure is studied only in the European flat oyster while the information on other species is lacking. Furthermore, the implications of spermatozeugmata structures have not been applied to explain the adaptive strategy in reproduction success of spermcasting oysters.

The structural integrity of a spermatozeugma is maintained by gelatinous membrane that envelops sperm heads. However, once the spermatozeugma is released in seawater, sperm become activated and gradually swim-off by dissociating the membrane (Foighil, 1989). A bulk of aggregated sperm in a spermatozeugma improves the fertilization efficiency but only the dissociated sperm can successfully fertilize eggs (Foighil, 1985). In spermcasting oysters, spermatozeugmata could be released by both hermaphroditic and male individuals. In *O. edulis*, spermatozeugma dissociation took place within 24 h after release but the relationship between dissociation rate and the level of masculinity (i.e., testis dominance) or femineity (i.e., ovary dominance) has not been defined. The understanding of the dissociation of spermatozeugmata would provide an insight into the unique reproductive biology of these species.

The Australian flat oyster *O. angasi* is a spermcasting species that release spermatozeugmata to fertilize eggs. Our understanding on the basic biology of *O. angasi* is limited to growth, survival (Dix, 1980; Mitchell et al., 2000), and fertility (O'Sullivan, 1980). After settlement, oysters have no movement capacity to find potential mates. Therefore, the properties of their gametes are intrinsically related to the evolution of reproductive biology in these species. In this study, we aimed to understand the structure and dissociation of spermatozeugmata in an attempt to elucidate the reproductive strategies of the spermcasting mollusc *O. angasi*.

2. Materials and methods

2.1. Oyster source and maintenance

The flat oysters were collected monthly from April to November, 2014 from the Pristine Oyster Farm in Coffin Bay, South Australia and shipped to South Australian Research and Development Institute (SARDI) in a chilled Styrofoam box within 24 h. Our monthly sampling revealed that the oysters with brooded larvae occurred from May to December in South Australia. After arrival, the oysters were cleaned with a brush and kept in a rectangular tank supplied with flow through seawater and aeration. The temperature was maintained at $20 \pm 0.5\,^{\circ}C$ and the oysters were fed with mixed microalgae of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*. The oysters were two years old having shell length 75.6 ± 5.5 mm and live weight 69.1 ± 13.3 g.

2.2. Gonad tissue histology

The gonad of a flat oyster located around the digestive gland and the gonad and non-reproductive tissues are not anatomically separate. The middle portion (3 mm thick) of the gonad-visceral tissues was cross sectioned and immediately placed in Davidson's fixative. The gonad-visceral tissue sections were prepared by an existing histological procedure (Kim et al., 2006). Briefly, the tissues were submerged through the graded alcohol solution and xylene before being embedded in paraffin wax. A 5 μm cut section was mounted on a microscope slide and stained with haematoxylin and counter stained with eosin. The specimen slides were scanned and the photos were taken on an inverted microscope (Nikon Eclipse TS100-F). The gonad developmental stages in this study were based on the criteria used for the European flat oyster *O. edulis* (da Silva et al., 2009). Briefly, gonad development was categorized into five stages; (i) inactive or resting gonad, (ii) early gametogenesis, (iii) advanced gametogenesis, (iv) mature gonad and (v) spawned gonad.

2.3. Sperm collection

After the oyster shells were opened, spermatozeugmata/sperm were collected by stripping from the gonad using a 3.5 ml pipette and placed in 1.5 ml Eppendorf tubes. As there were a large percentage of simultaneous hermaphrodites in the flat oyster population, the presence of male gametes in the gonad was confirmed on a light microscope at 200× magnification. The sperm collected from 3 to 5 individuals were pooled for each observation at different masculine levels such as male, predominant male hermaphrodite and predominant female hermaphrodite.

2.4. Specimen preparation for electron microscopy

For scanning electron microscope (SEM) observation, the suspended spermatozeugmata in filtered seawater were collected on 0.2 μm polycarbonate membrane filters and placed in a fixative (2.25% glutaraldehyde in phosphate buffer solution + 4% sucrose, pH 7.2) for 30 min. After fixation, the specimens were placed in a buffer (4% sucrose in phosphate buffer solution) for two consecutive washing of 5 min each. After washing, the samples were post-fixed with 2% osmium tetroxide (OsO_4) for 30 min. The specimens were dehydrated twice in 70%, 90% and 100% ethanol for 10 min each. Then the samples were critical-point dried twice with (i) 1:1 hexamethyldisilazane (HMDS) and 100% ethanol, and (ii) 100% HMDS for 10 min each. The samples were placed in the fume hood at room temperature to vapourize extra moisture. The dried filters were coated with platinum at a thickness of less than 100 Å and six filters were observed with an SEM (Philips XL 30).

2.5. Dissociation of spermatozeugmata

Sperm surrounded by a gelatinous membrane were considered non-dissociated whereas sperm swimming freely were considered dissociated. The dissociation of spermatozeugmata was observed by placing an aliquot of 20 μl suspension on a glass slide under a light microscope at 200× magnification. The time required for the dissociation of spermatozeugmata collected from the male, predominant male hermaphrodite and predominant female hermaphrodite was compared.

2.6. Sperm motility

Although the sperm attached to a spermatozeugma had flagella beating but this effect was not considered while studying sperm motility because the number of sperm with an active flagellum in a spermatozeugma cannot be quantified. The typical individual sperm with active forward movement was counted motile while those without such a movement were considered non-motile. The sperm suspension was produced by passing the sample through