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FERTILIZATION AND EMBRYONIC DEVELOPMENT IN THE BASKET COCKLE, *CLINOCARDIUM NUTTALLII*

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ABSTRACT Sperm motility, fertilization, and embryonic development were investigated in the hermaphroditic basket cockle, *Clinocardium nuttallii*. Motility of the sperm was more prolonged over a 2-h storage period at 4°C than at 19°C. The sperm-to-egg ratio resulting in the highest fertilization rate was 10,000:1, or an equivalent final sperm concentration of 6×10^5 cells mL^{-1}, using a density of 60 eggs mL^{-1}. However, at least some degree of self-fertilization appeared to be unavoidable during the release of the eggs from the broodstock. The biological zero point, below which embryonic development ceased, was estimated to be 2.8°C to 2.9°C for *C. nuttallii*.

KEY WORDS: biological zero point, *Clinocardium nuttallii*, cockle, embryonic development, fertilization, temperature

INTRODUCTION

The basket cockle, *Clinocardium nuttallii*, is a common bivalve normally found in the low intertidal and shallow subtidal zones of the coastal waters of British Columbia (BC), Canada. Basket cockles are a traditional food for regional First Nations, but they are not harvested commercially within BC. Recent interest in developing commercial aquaculture of *C. nuttallii*, especially in the colder waters of the north coast of BC, has led to research into the development of hatchery techniques for seed production of this species (present study, Liu et al. 2008).

Gamete fertilization is an important step that needs to be controlled carefully in bivalve hatchery operations to achieve high fertilization rates. High sperm-to-egg ratios for fertilization may result in polyspermy, dissolution of egg membranes, and/or abnormal development of zygotes (Stephano & Gould 1988, Clotteau & Dubé 1993, Encena et al. 1998). On the other hand, there may be a lack of fertilization caused by a decrease in the viability of sperm before fertilization (O'Connor & Heasman 1995), a low sperm-to-egg ratio used during manipulation, or a delay or failure in male spawning (Narvarte & Pascual 2003). However, factors affecting fertilization success have not been examined in the hermaphroditic *C. nuttallii*.

Knowledge of the influence of environment on the early ontogeny of a target species is necessary for its hatchery management. Water temperature is generally regarded as the dominate factor controlling the development of poikilotherms. Within a suitable temperature range, the duration of any developmental period increases at lower temperatures or decreases at higher temperatures (Weltzien et al. 1999). Several studies have quantified the relationship of timing and the temperature-dependent developmental stage with abalone larvae and shown that larval development is theoretically arrested below a threshold temperature or biological zero point (BZP), which is the key value in calculation of the effective accumulative temperature (EAT) for prediction of the duration of any developmental stage at any particular temperature (see Grubert & Ritar 2004 for a review). Despite their economic importance, relevant published information on BZP for bivalves has been scarce.

The aim of the present work was to examine the effect of low-temperature storage on sperm motility, the influence of sperm-to-egg ratio on fertilization rate, and to determine the BZP for embryonic development of *C. nuttallii*.

MATERIALS AND METHODS

Broodstock Maintenance and Collection of Gametes

Ripe adult *C. nuttallii* with a mean (±SD) shell length of 61.5 ± 5.1 mm ($n = 100$) were collected in June 2006 from a shellfish farm site (48°58′20″N, 123°41′27″W) in the Strait of Georgia (BC Fishery Management Area 14), transported to the Center for Shellfish Research (CSR) at Malaspina University-College, and placed in a round, 430-L flat-bottom tank supplied with a continuous flow of recirculated seawater at a temperature of 15°C–16°C and a salinity of 32.0‰ to 33.0‰. The cockles spawned spontaneously after overnight maintenance without further intervention. The spawning animals were removed from the holding tank and transferred to individual containers with 2 L of seawater for easier collection of gametes for experimentation.

Sperm Motility

The motility score of sperm over time was investigated at two temperatures, 4°C and 19°C (room temperature). Sperm from three cockles spawned at 16°C were combined to give a suspension with a concentration of 3×10^6 cells mL^{-1}, as determined with a hemocytometer, and 40-mL aliquots were added to each of two 10-mL glass test tubes. One test tube was then held at 4°C and the other at 19°C for a 2-h period. At 10, 20, 30, 60, 90, and 120 min after collection of the sperm, 200-μL aliquots were transferred from each tube onto a Sedgwick

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Rafter counting cell for immediate recording of sperm motility, using a digital video camera attached to a light microscope. Preliminary trials showed that drops of sperm suspensions put on such counting cells did not spread, but maintained similar forms of well-shaped beads; this allowed the videos to be taken at the same depth of view among samples as quickly as possible. Each video clip was played repetitively on QuickTime for Windows for three 1/5-sec time intervals, and numbers of actively swimming *versus* weakly swimming and inactive sperm were scored and averaged for each sample (time point).

Fertilization

Sperm pooled from five cockles was diluted to a concentration of 6×10^6 cells mL^{-1}, stored at 4°C, and checked for motility before use. Eggs were collected from three other cockles, to avoid self-fertilization, and adjusted to a density of 60 eggs mL^{-1}, as determined with a Sedgwick Rafter counting cell. Fifty-mL egg suspensions (containing 3000 eggs) were subsequently dispersed into each of thirty 125-mL glass flasks. Aliquots of 5, 10, 15, 20, 25, 50, 250, 500, and 5,000 µL of sperm suspensions were then added to different flasks to fertilize the eggs at sperm-to-egg ratios of 10, 20, 30, 40, 50, 100, 500, 1,000, and 10,000:1. These corresponded to final sperm concentrations of 600, 1,200, 1,800, 2,400, 3,000, 6,000, 30,000, 60,000, and 600,000 spermatozoa mL^{-1}, respectively. A blank control, without addition of sperm, was used to monitor any uncontrolled fertilization. Each treatment had three replicate flasks. After a 2-h period of incubation postfertilization at 19°C, the fertilized eggs had developed into two or four cells. Fertilization rates were then determined as the percentage of dividing cells in a random sample of 100 eggs for each flask. Because of an uncontrolled fertilization occurring in the blank control, further experiments were carried out to examine if this resulted from self-fertilization or contamination. Consequently, on a different spawning occasion, three cockles that were found to be releasing clumps of eggs were removed from the spawning tank. They were well rinsed (to remove residual sperm which may have been adhering to the cockle shells or gills) in seawater, which had been disinfected with sodium hydrochloride (200 ppm) and subsequently neutralized by sodium thiosulphate. Each cockle was rinsed for three separate times of five minutes. The cockles were then placed separately inside aquaria containing 10 L of similarly disinfected seawater, and the eggs being shed from the opening of exhaling siphons were collected over time with Pasteur pipettes and fixed in seawater containing 4% formalin for later examination of rates of fertilization.

Embryonic Development and BZP

Eggs were collected from two cockles and pooled in a 1-L beaker where they were fertilized with sperm pooled from four other cockles. The fertilized eggs were then held at 16°C to 17°C for a further 30 min to allow formation of polar bodies before the start of the experiment with different temperature treatments. This short period approximated that required for female and male pronuclei to fuse and form diploid nuclei as observed in the zygotes of a scallop (Desrosiers et al. 1995). After this procedure, 10 mL of the fertilized egg suspensions (containing 12,000 eggs) were transferred to 1-L glass beakers that had been prefilled with 600 mL seawater (to produce a concentration of 20 fertilized eggs mL^{-1}) and preset to each of six test temperatures of 2.6, 4.2, 6.2, 8.2, 15.7, and 19.2°C (±SD = 0.3°C for each value, $n = 25$) in water baths, for further development. There were three replicate beakers for each temperature treatment. Samples were taken from the replicate beakers at 10-min intervals for the first 1 h after the commencement of temperature treatment (ACTT), and then at 1-h intervals until 12 h ACTT when the experiment was terminated. On each sampling occasion, 10-mL subsamples were removed from each beaker and preserved in seawater containing 4% formalin for later examination of embryonic stages of 100 randomly chosen zygotes, using a compound microscope. Curves of percentage of the development stages *versus* time were plotted for each temperature with best-fit regressions, and times ACTT taken for 50% occurrences of the first and second cell divisions were determined. The reciprocal of time was then plotted against temperature for each development stage using linear regression analysis, and the *x*-axis intercept of each regression line was extrapolated to yield the BZP (Grubert & Ritar 2004, Moss 2004).

Statistical Analysis

The effect of sperm-egg ratio on fertilization rate was examined with one-way ANOVA, followed by a Student-Newman-Keuls multiple range test to detect which means differed significantly ($P < 0.05$). Normality of data was confirmed by Kolmogorov-Smirnov's test and homogeneity of variances by Levene's test. All statistics were performed using the statistical software NCSS (Kaysville, UT).

RESULTS

Sperm Motility

The sperm of *C. nuttallii* remained highly (>90%) motile over the 2-h storage period at a temperature of 4°C (Fig. 1). In contrast, motility of the sperm held at 19°C decreased progressively from 96% to 66% in the first hour, and further decreased to 27% in the next 0.5-h period of time after collection. At the end of the 2-h storage period, only 24% of the sperm stored at 19°C were observed to be weakly motile (oscillating). This represented a decrease in sperm motility of 74% compared with the treatment at 4°C at the same time.

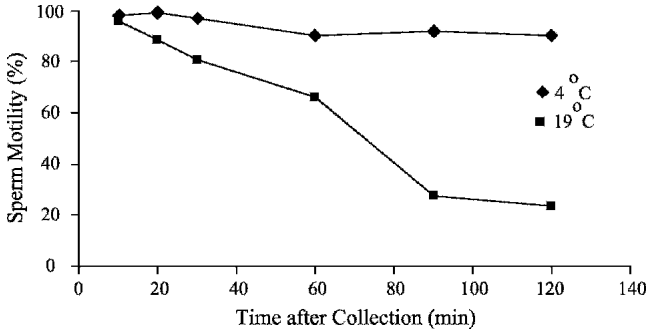

Figure 1. Motility over time of *Clinocardium nuttallii* sperm stored at two temperatures.

Fertilization

The effect of different sperm-to-egg ratios on the fertilization rates of *C. nuttallii* eggs is shown in Figure 2. There were no significant differences ($P > 0.05$) in the fertilization rates (40.7% to 50.7%) obtained when the sperm-to-egg ratios used ranged between 10:1 and 100:1 (Fig. 2). Further increases in the sperm-to-egg ratios increased the fertilization rates substantially, the highest rate being 93.7%—significantly ($P < 0.05$) higher than all other sperm-to-egg ratios—achieved at the highest ratio of 10,000:1. In addition, there was a fertilization rate of 25.7% in the blank control, which was significantly lower ($P < 0.05$) than all the rates attained in the various sperm-to-egg ratio treatments (Fig. 2). In the follow-up experiment to determine the possible source of this uncontrolled fertilization, it was found that a proportion of the eggs (varying between 30% and 95%) collected directly from the openings of exhalent siphons had already been fertilized. This was evinced by the elevated fertilization membrane and the numerous spermatozoa surrounding the eggs (Fig. 3H). Thus, this uncontrolled fertilization was attributable to self-fertilization. The variation in self-fertilization rate was attributed to the amount of eggs being liberated at the time of collection. During the moments of peak egg liberation the percentage of self-fertilization obtained was lower compared with that obtained when eggs were being "dribbled" out. In contrast, no eggs were found in the sperm suspensions collected during the several spawning occasions.

Embryonic Development and BZP

Within the test temperature range of 2.6°C to 19.2°C, the development rate of *C. nuttallii* embryos increased with rising temperature (Fig. 3A–G). At the highest temperature of 19.2°C, the fertilized eggs rapidly reached the 2- and 4-cell stages at 10 and 44 min ACTT, respectively, became motile blastulae at 10 h ACTT, and developed further into early gastrulae (Fig. 3G) at 12 h ACTT when the experiment was terminated. In contrast, development of the zygotes held at the lowest temperature of 2.6°C was marginal. No signs of cell division were evident until 10 h ACTT and by 12 h ACTT onset of the first cleavage was observed in only a small proportion (7%) of the eggs and limited to the occurrence of the first cleavage furrow (Fig. 3A). The eggs incubated at other temperatures had reached 2–4-cell, 8-cell, multicell, and the blastula stages (Fig. 3B–F) at 4.2, 6.2, 8.2, and 15.7°C, respectively, at the end of the experiment. These results show that a temperature of 2.6°C is close to the BZP for embryonic development of *C. nuttallii* despite the fact that a complete arrest of embryonic development was not observed during the experiment. When the relationships between timing and temperature were examined, the BZP was estimated to be 2.8°C (95% confidence interval of 0.6°C–5.2°C) from the first cell division and 2.9°C (95% confidence interval of 1.7°C–4.2°C) from the second cell division (Fig. 4). Note that in calculating the BZP, the lowest temperature treatment of 2.6°C was omitted because by the termination of the trial at 12 h ACTT, few zygotes had reached the 2-cell stage (Fig. 3A).

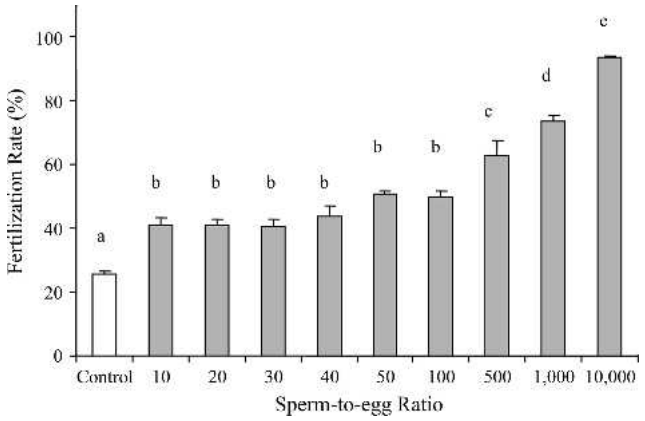

Figure 2. Fertilization rate of *Clinocardium nuttallii* eggs at different sperm-to-egg ratios. Means with different superscripts differ significantly (Student-Newman-Keuls multiple range test, $P < 0.05$). Error bars are standard errors ($n = 3$).

DISCUSSION

Sperm motility has been used as an indicator of the quality or the fertilization ability of the sperm for bivalves (O'Connor & Heasman 1995, Narvarte & Pascual 2003). Results obtained in the present study suggest that the sperm of *C. nuttallii* should be used within 1 h of release when kept at a room temperature of 19°C. However, a cold storage temperature of 4°C greatly prolonged the motility and hence the viability of the sperm of *C. nuttallii*. Similar effects of storage temperature on sperm motility have been reported for other molluscs (O'Connor & Heasman 1995, Naud & Havenhand 2006), and also for many species of fish (Alavi & Cosson 2005).

The reported values of optimal sperm-to-egg ratios for fertilization in bivalves vary widely (O'Connor & Heasman 1995). Whereas lower ratios of 16–30:1 were found to be optimal for fertilization in the scallop *Placopecten magellanicus* (Desrosiers et al. 1995) and of 50–100:1 in the clam *Spisula solidissima* (Clotteau & Dubé 1993), the use of a comparable ratio of 50 sperm per egg did not lead to the best fertilization rates in the scallop *Aequipecten tehuelchus* (Narvarte & Pascual 2003). Conversely, higher sperm-to-eggs ratios of 500–5,000:1 were shown to be suitable for fertilization in the oyster *Crassostrea rhizophorae* (Santos & Nascimento 1985) and the scallop *Chlamys asperrima* (O'Connor & Heasman 1995). In the present study, significant increases in fertilization rates with *C. nuttallii* were not found until the sperm-to-egg ratio reached 500:1 and higher, and the highest fertilization rate was attained at a ratio of 10,000:1 (the highest ratio tested). Unfortunately, the sperm-to-egg ratio in many studies is not an independent variable of the egg density and therefore the final sperm concentration adopted (and *vice versa*). With the use of a same sperm-to-egg ratio of 1,000:1 across different densities of eggs, O'Connor and Heasman (1995) found that the fertilization rate of *C. asperrima* increased significantly at higher densities of eggs (and therefore with higher final sperm concentrations), indicating that fewer sperm are required to elicit maximum fertilization of the same number of eggs with increasing egg densities (and *vice versa*). In light of this, the lower range of optimal sperm-to-egg ratios of 16–100:1, obtained in the studies of Desrosiers et al. (1995) and Clotteau and Dubé (1993), are likely to be associated with the use of higher densities of eggs in the range of 500–5,000 mL^{-1}. This contrasts with the use of a much diluted density of 1–60 eggs mL^{-1} in those other fertilization studies mentioned above and in the present one, in which higher

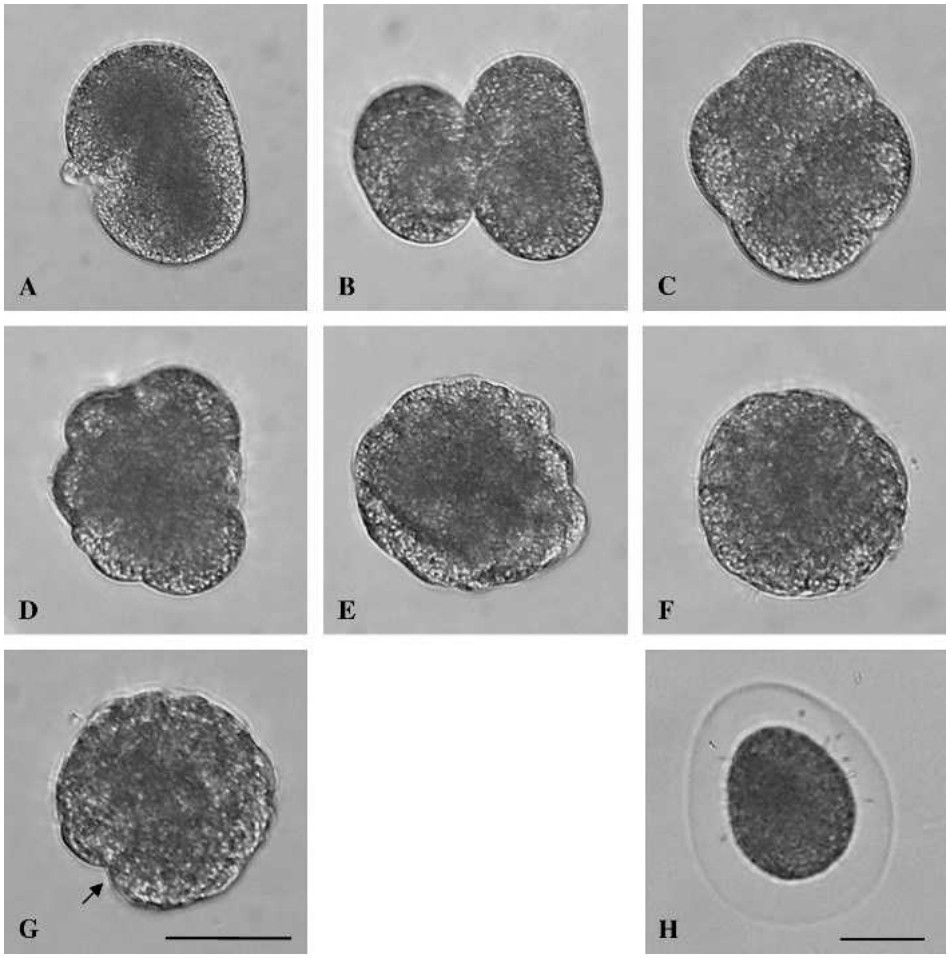

Figure 3. Embryonic development of *Clinocardium nuttallii* at different temperatures after a 12-h period (A–G) and fertilization membrane (H). (A) onset of first cleavage at 2.6°C. (B) 2-cell stage at 4.2°C. (C) 4-cell stage at 4.2°C. (D) 8-cell stage at 6.2°C. (E) multicell stage at 8.2°C. (F) motile blastula at 15.7°C. (G) early gastrula at 19.2°C (arrow indicating gastrulation). (H) eggs collected directly from the opening of exhalent siphon, showing fertilization membrane and numerous spermatozoa surrounding the eggs. Bars = 50 μm.

sperm-to-egg ratios were required to ensure high fertilization rates. Nonetheless, the final sperm concentration itself has to be maintained above certain critical levels during fertilization so that it does not become a limiting factor and cause profound decreases in fertilization success (Levitan et al. 1991, Dexon & Benzie 1994). This also has a tendency to increase the sperm-to-egg ratios with the use of low densities of eggs, to achieve maximum fertilization. Alternatively, the number of sperm visible at the periphery of the eggs after addition of sperm, that is, a number of 1–10 sperm per egg as verified microscopically (Bourne et al. 1989, Cropp 1993, O'Connor & Heasman 1995), would probably be a better criterion for optimal fertilization in bivalves for practical consideration, unless strict synchronization in fertilization is a priority.

The BZP for larval (embryonic) development has been determined for several species of temperate abalone to be in the range of 5°C to 9°C (Grubert & Ritar 2004), on the basis of analyses of the relationship between timing and temperature-dependent developmental stage, as performed in the present study. However, relevant information published for bivalves has been scarce and equivocal. Beaumont and Budd (1982) demonstrated that larvae of the mussel *Mytilus edulis* still had limited growth when held at a temperature of 5°C. Using the developmental rates described by Sprung (1984), we recalculated the BZP for larval *M. edulis* to be 0.8°C. In the present study, the BZP for embryonic development of *C. nuttallii* was predicted to be between 2.8 and 2.9°C. Further experimental evidence, by direct observation, suggests that the actual BZP for embryonic development in *C. nuttallii* could be below the estimated value, because the zygotes kept at 2.6°C were still dividing, though at a rather delayed rate. We also showed that a temperature of 2.5°C was still not fully inhibitory to the gonadal development of *C. nuttallii* (Liu et al. 2008). However, the present experimental design did not permit us to detect such small differences in temperature or to examine the viability of larvae surviving such critical conditions, as studied by Beaumont and Budd (1982) and Hayhurst (2001).

Partial self-fertilization is a common event in the laboratory spawning of hermaphroditic pectinids (Beaumont & Budd 1983, Ibarra et al. 1995, Narvarte & Pascual 2003, Winkler & Estévez 2003). Evidence of self-fertilization was also present in the histologically-prepared gonad samples of the hermaphroditic freshwater clams *Corbicula fluminea* and *C. leana* (Park & Chung 2004). In the present study, a self-fertilization rate of

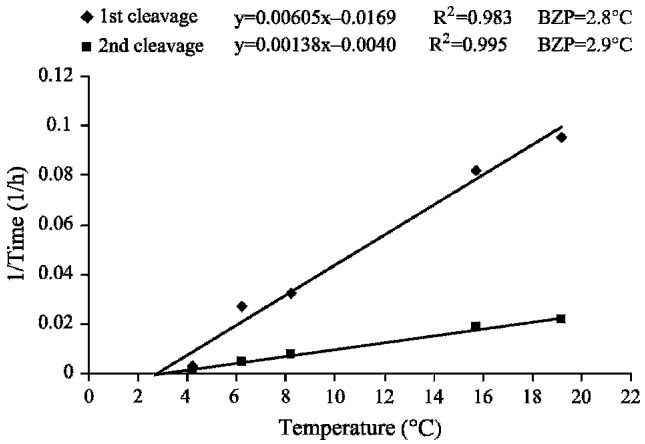

Figure 4. Relationships between reciprocal of development time and temperature for the first and the second cell cleavages of the zygotes of *Clinocardium nuttallii*. The biological zero point (BZP) is the *x*-axis intercepts of the regression lines.

25.7% was observed in the eggs of *C. nuttallii* when examined at 2–4-cell stages, and the rate was even able to reach as high as 95% with the newly released eggs. Ibarra et al. (1995) pointed out that in hermaphroditic pectinids, self-fertilization is probably a rule rather than an exception. Based on both field observations and laboratory evidence regarding the hermaphroditic barnacle *Balanus improvisus*, Furman and Yule (1990) argued that the ability to self-fertilize is especially advantageous for individuals of a species which often has sparse and isolated populations. This is probably applicable to *C. nuttallii* (Gallucci & Gallucci 1982). The effect of self-fertilization on the growth and survivorship of *C. nuttallii* at different life stages—as has been studied in various pectinid species (Beaumont & Budd 1983, Ibarra et al. 1995, Winkler & Estévez 2003)—needs to be addressed further.

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