Running Head: Geoduck embryogenesis and fertilization

Practical fertilization procedure and embryonic development of the New Zealand geoduck clam (*Panopea zelandica*)

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Abstract

Despite the fact that a successful aquaculture industry for the geoduck Panopea zelandica *(Quoy & Gaimard, 1835) must rely on hatchery spat production, little is known about the embryonic development of the species, which appears to be critical for larval rearing success. The present study investigated the development of* P. zelandica *embryos at 15°C and 35 ppt and the optimal sperm:egg ratios for fertilization under hatchery conditions.* P. zelandica *broodstock were induced to spawn by serotonin injection. Sperm and eggs were collected within 30 min post-release, and then stored at 4°C for up to 4 h and 1.5 h, respectively. Fertilization was conducted at sperm:egg ratios of: 50:1, 100:1, 500:1, 1000:1, and 10,000:1 with a sperm-egg contact time of 40 min. The optimal sperm:egg ratio was determined to be < 500:1 and the normal embryo yield at 3 and 18 h post-fertilization (hpf) ranged from 83-96%. Since there was only one female spawned, the sperm:egg ratio results cannot be generalized to the population, but can be considered to be useful information for future studies.* P. zelandica *eggs (~ 80 µm diameter) developed the first and second polar bodies within 15 - 20 and 50 - 55 min post-fertilization, respectively. The blastula appeared at ~ 8 hpf, including the X^R and X^L cells and the presumptive shell field depression. Gastrulation occurred at 12 - 18 hpf with organic material shell apparent at the shell field depression. The mid-stage trochophore, which appeared at around 35 hpf had an apical plate with an apical tuft. The shell field spread to form the periostracum, which expanded and folded into right and left segments covering the late trochophore. The early D-stage veliger appeared at 45 hpf with the soft body being enclosed by two valves and the appearance of the velum. These observations will serve as the basis for future*

analyses of P. zelandica *embryogenesis and for optimization of commercial production of D-veliger larvae.*

Keywords: *Panopea zelandica*, New Zealand geoduck, embryogenesis, blastula, gastrula, trochophore, fertilization, sperm:egg ratio.

INTRODUCTION

The New Zealand aquaculture sector has set a target to achieve annual sales of $1 billion NZD by 2025 (Carter, 2012), more than doubling current revenues. Alongside adding value to existing aquaculture species (e.g. salmon, Pacific oysters, Greenshell™ mussels), another strategic priority to accomplish this goal is to identify new shellfish species with commercial potential and develop techniques for their production (Carter, 2012). Geoducks are a high value species, currently selling for up to $200 - $300 USD/kg in Asian restaurants (Shamshak & King, 2015). The endemic geoduck clam *Panopea zelandica* (Quoy & Gaimard, 1835) has been chosen as an emerging species for aquaculture within this strategy (King, 2010). *P. zelandica* populations have been found in both North and South islands of New Zealand (Breen *et al.*, 1991; Gribben *et al.*, 2004). However, the wild fishery is unlikely to fulfil potential market demands sustainably (see review in Gribben & Heasman, 2015). Thus, geoducks have become an object of significant aquaculture research and development.

The success of any shellfish aquaculture depends on the availability of seed/spat to stock farms. For many bivalves, such as mussels and oysters, intensive recruitment of wild juveniles onto spat-catching ropes or frames can result in a relatively efficient way to obtain wild seed to supply the farms (Buestel *et al.*, 2009; Alfaro *et al.*, 2010). However, geoduck spat do not attach or cement to substrates but bury in sand. This attribute makes it practically impossible to collect wild geoduck spat; hence, the geoduck aquaculture industry must rely on hatchery-based spat production.

Successful embryo development is critical for reliable spat production. The yield of embryos can be substantially affected by the ratio of sperm:egg during fertilization

(Dong *et al.*, 2012). For example, low sperm:egg ratios can reduce the probability of gamete contact, while high ratios can increase the risk of polyspermy (Gribben *et al.*, 2014). Polyspermy can then cause dissolution of egg membranes and abnormal embryo development (Stephano & Gould, 1988; Clotteau & Dubé, 1993; Encena *et al.*, 1998). Abnormal embryos either terminate prior to the shell development or result in deformed D-larvae, which cannot survive to the pediveliger stage. Hence, it is important to determine the optimal sperm:egg ratio so that polyspermy can be avoided without compromising fertilization ratios. This optimal ratio varies among different bivalve species. For example, a sperm:egg ratio of 10,000:1 is optimal for the cockle *Clinocardiuim nuttallii* (Liu *et al.*, 2008), whereas 1000:1 is optimal for the oysters *Crassostrea virginica* and *Crassostrea gigas* (Alliegro & Wright, 1985; Stephano & Gould, 1988), and a ratio of ≤ 200:1 is ideal for the blood clam *Tegillarca granosa* (Dong *et al.*, 2012).

Although the hatchery production of Pacific geoduck (*Panopea generosa*) spat is commercially well-established in the USA and Canada, limited information on optimal sperm:egg ratios has been released. In a study to investigate the production of triploid *P. generosa*, Vadopalas & Davis (2004) successfully used a sperm:egg ratio of 40:1. More recently, in New Zealand, Gribben *et al.* (2014) conducted a comprehensive study to investigate the fertilization kinetics of *P. zelandica*, and recommended a broad sperm:egg ratio of 5,000–50,000:1 for hatchery production with fresh gametes (< 30 min old), a starting egg density of 20 eggs mL^{-1}, and a sperm-egg contact time of 5 – 10 min. Under these conditions, greater sperm densities resulted in high percentages of polyspermy and poor fertilization success. While the fertilization kinetics model

provided highly valuable information, the suggested gamete age and sperm-egg contact time by Gribben *et al.* (2014) may not be feasible for commercial hatchery operations. It is well-established that gamete age and sperm-egg contact time considerably affects fertilization success and the optimal sperm:egg ratio (Levitan, 2006; Stephano & Gould, 1988). A more practical commercial scenario would be to cold-store gametes for up to 2 h, enabling a sufficient number of eggs to be used (Adams *et al.*, 2004), and then to provide sperm-egg contact times of > 30 min in order to evaluate fertilization success as is routine with other bivalve species (Helm *et al.*, 2004). Thus, there is a need to determine the optimal *P. zelandica* sperm:egg ratio for commercial fertilization purposes.

Fundamental biological knowledge of embryonic and larval development can be an important source for phylogenetic hypothesis generation and for the hatchery culture of bivalves. Bivalve embryogenesis has two notable features that relate to organ development and shell formation of early larvae (Kin *et al.*, 2009). The cleavage pattern feature determines the normal development of embryos, and consequently the normal development of organs, such as the velum, mouth, apical tuft and stomach in D-larvae (Hashimoto *et al.*, 2014). The normal shape and integrity of larval shells is dependent on the successful cleavage and development of the shell-founding cell during the zygote and morula stages, the invagination, evagination, and expansion of the shell field during the gastrula stage, and the secretion of shell matrices and calcification during the trochophore stage (Kin *et al.*, 2009). Surprisingly, there are very few studies on embryonic development for any geoduck species. Most studies on geoduck embryos have only focused on the effects of temperature and salinity on the success of

119 embryogenesis (i.e., D-larval yield), but not on the embryonic development itself. A
120 detailed description of the embryogenesis of this unique genus of soft-sediment bivalve
121 will contribute to our understanding of its evolution. However, comparative embryology
122 might need a more thorough examination across several species. In the meantime, *P.*
123 *zelandica* embryogenesis, particularly the timing of developmental stages and
124 characterization of key phenotypes, would be extremely valuable for future
125 advancements and optimization of hatchery technologies. Specifically, without
126 information on optimal sperm:egg ratios and embryonic development, the deformities
127 we have observed in geoduck larvae may not be well understood and the yield of larvae
128 and spat may not be reliably optimized. Thus, the aims of the current study are to
129 describe the normal embryonic development and determine the optimal sperm:egg ratio
130 under hatchery conditions in *P. zelandica*. This information will not only assist in the
131 development of hatchery protocols for this species, but also other related bivalve
132 species.

133

134 MATERIALS AND METHODS

135 **Broodstock conditioning and gamete collection**

136 *P. zelandica* broodstock (105 – 130 mm shell length, 500 - 800 g live weight) were
137 collected from Golden Bay (South Island, New Zealand) and conditioned in flow-through
138 1 µm-filtered seawater at 15°C with microalgae (*Tisochrysis lutea* and *Chaetoceros*
139 *muelleri*, 1:1 cell counts) for 3 months (after Le *et al*., 2014). Geoduck broodstock were
140 induced to spawn by injecting 1 – 2 mL of 2 mM serotonin solution into their mantle.
141 After their sex was revealed by initial gamete release, males and females were

separated into different containers. Gametes were collected within 30 min of release, then rinsed through a 100 µm sieve to remove particulate matter. Eggs were caught on a 40 µm mesh screen and re-suspended in 500 mL seawater. Sperm and egg solutions were then stored at 4°C for up to 2 h and 1 h, respectively. Before fertilization, gametes were examined for quality and quantity. All gametes were in good quality according to the characterization of egg shape and sperm motility as in Baker & Tyler (2001). Sperm and egg concentrations were determined from three replicate counts of 20-µL and 200-µL aliquots, respectively. Sperm aliquots were diluted 1000x, transferred to a haemocytometer, and cells were counted under a light microscope (BX41, Olympus America Inc., New York, USA) at 400x magnification. Egg densities were counted under 200 × magnification under an inverted light microscope (Olympus CKX41).

Embryonic development

About 1 million eggs were fertilized in a 10 L bucket with a sperm:egg ratio of 500:1, screened (22 µm) and washed with fresh 1 µm-filtered seawater. Approximately 500,000 embryos were transferred to a beaker containing 5 L of 1 µm-filtered seawater and 4 µmol EDTA. The temperature of the incubation seawater was maintained at 15°C in a thermostat-controlled incubator. Triplicate 1 mL samples of suspended embryos were pipetted from the 5 L beaker every 10 min for the first 2 h, then every 30 min for the next 4 h, and every 2 h thereafter until the D-veliger larval stage. Samples were fixed in Davidson's solution and stored at 4°C until visual assessment. Embryos were observed using a light microscope and a scanning electron microscope (SEM, Hitachi SU-70 Skottky). The cleavage pattern was described following the standard terms in Hashimoto *et al.* (2015).

Scanning electron microscopy

Preserved embryos were washed with phosphate buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH = 7.4) for 5 min, then rinsed for 1 min with deionized water. Embryos were then dehydrated through an ascending series of analytical grade ethanol 50, 60, 70, 80, 90, and 100% for 15 min intervals each (Turner & Boyle, 1974). After dehydration, samples were soaked in 98% chloroform for 30 sec, and then dried for 12 h in a desiccator. To dry samples in a vaporous condition, a chloroform-soaked filter paper was also placed in the desiccator as suggested by Wassnig & Southgate (2012). Dried samples were placed on adhesive carbon discs and mounted on aluminium stubs. Samples were sputter coated with carbon for 40 s using an ion sputter coater (Hitachi E-1045), then imaged via SEM at 5.0 kV.

Sperm:egg ratio optimization trial

Approximately 3,000 eggs from one female were fertilized and incubated at 15°C in each of fifteen 50 mL Falcon™ tubes containing 30 mL of 1 µm filtered seawater and 4 µmol EDTA. Sperm aliquots from two males were pipetted into the Falcon tubes to provide sperm:egg ratios of 50:1, 100:1, 500:1, 1000:1, and 10,000:1 (3 replicates for each ratio). After a 40 min contact time, embryos and any unfertilized eggs were filtered and washed on a 22 µm mesh screen to remove excess sperm. Samples were incubated in 50 mL Falcon™ tubes containing fresh 1 µm filtered seawater with 4 µmol EDTA. After 3 and 18 h post-fertilization (hpf), embryos were carefully resuspended and 1 mL of each 30 mL embryo suspension was fixed in Davidson's solution and stored at 4°C for subsequent visual assessment. A total sample of 2230 and 3890 embryos were assessed in the 3 and 18 hpf groups, respectively. The embryonic

development was assessed visually at 400 × magnification using the inverted light microscope. Embryos that showed signs of irregular cleavage, incomplete blastula development and discoloration were recorded as 'abnormal' (Lewis & Galloway, 2009). Unfertilized eggs were also categorized as 'abnormal' for the calculations. The proportion of normally developed embryos was determined by expressing the number of normal embryos as a percentage of the number of eggs initially present.

Statistical analysis

The ratios of normal embryos were arcsine-transformed (Sokal & Rohlf, 1995) to achieve homogeneity of variance and normality. The effect of sperm:egg ratios on the normal embryo yield were analyzed by one-way analysis of variance (ANOVA), followed by Tukey pairwise comparison at the significance level $\alpha = 0.05$ using the statistical software Minitab v. 17. All data are expressed as mean ± SD.

RESULTS

Embryonic development

Newly released eggs were pear-shaped and then became more spherical (with a diameter of 75 - 80 µm) immediately post-spawning. The first polar body became evident after 15 – 20 min post-fertilization (Figure 1a, 2a). The second polar body was typically observed about 35 min later (50 – 55 min post-fertilization; Figure 1b). The first cleavage started with polar lobe formation occurring at 1.5 hpf from the vegetal region, resulting in two unequal cells (small cell: AB, and large cell: CD; Figure 1c, 2b). The polar body was located in the plane of cleavage. The second cleavage appeared at 2.5

hpf. Polar lobe formation occurred again, producing three smaller cells of similar size, referred to as the A, B, and C blastomeres and one larger blastomere (D; Figure 1d, 2d). The third cleavage occurred at 4 hpf. The third cleavage was uneven, creating the first quartet of smaller apical micromeres (1a – 1d; Figure 1e, 2e). The fourth cleavage occurred at 5 hpf, producing 16-cell embryos with the second micromere quartet ($1a^2$ – $1d^2$; Figure 2f). The fifth cleavage appeared at 6 hpf, producing 32 - cell embryos, or morulae, with the third micromere quartet (Figure 1f, 2g - h). The blastula appeared at ~ 8 hpf and showed a symmetric division pattern. The bilaterally-symmetric cell division yielded X^L and X^R regions and a presumptive shell field (Figure 1h - i, 2i - l). Occasional cilia were apparent surrounding the anterior circular margin, forming the early prototroch. Two cellular depressions started at the late blastula within the vegetal side. The shell field depression in the dorsal region was recognizable as a crescent-shaped orifice in the blastomere X region. The other depression within the ventral region represented the blastopore. The early gastrula appeared at 12 hpf. The shell field and blastopore depressions at this stage were deeper than at the blastula stage (Figure 2m - n). The prototrochal pad developed and correlated well with the general timing at which embryos began rotating, following circular trajectories within the water column. Gastrulation appeared at 18 hpf, by which time overall shape was no longer spherical. The dorsal region was distinguishable by an open orifice, which expanded under and posterior to the developing prototrochal pad (Figure 2o). The new shell material (pellicle) appeared as a wrinkle and accumulated at either side of the orifice. A mid-stage trochophore appeared at around 35 hpf. The trochophores were ovoid with a broad animal region and narrower vegetal region (Figure 2p). The well-developed

prototroch was characterized as a crown of motile cilia (Figure 1j) and divided the trochophore into two regions (Figure 2p). The posterior region contained the blastopore on the ventral side and the shell field on the dorsal side. The anterior region contained the apical plate on which the cilia elongated and thickened to form an apical tuft that acts as a sensory organ (Figure 2p - r). The cilia developed on the posterior area of embryos and formed the presumptive telotroch (Figure 2s). Late-stage trochophores appeared at 39 hpf. The shell field spread out to form a flat and smooth periostracum on the posterior-dorsal region (Figure 2t). The periostracum then expanded and folded into right and left segments covering the trochophore (Figure 2u). Early D-stage veligers appeared at 45 hpf with the soft body enclosed by two valves and the appearance of the velum (Figure 2v - x). Mineralization began along the hinge, and then continued along the shell edge while the center of the valve remained uncalcified. A summary of the timing of development stages is given in Table 1.

Sperm:egg ratio

The original aim of this study was to determine the optimal sperm:egg ratio; unfortunately, only one female geoduck spawned. Hence, the result in this study cannot be generalized to the entire geoduck population. The sperm:egg ratio significantly affected the percentage of normally-developed embryos at 3 hpf (One way ANOVA, df = 14, F = 6.62, $P = 0.007$) and at 18 hpf (One way ANOVA, df = 14, F = 27.99, $P < 0.001$; Figure 3). The highest normal embryo percentage was achieved at a sperm:egg ratio of 50:1 as confirmed by both the 3 hpf and 18 hpf sampling events. The percent of normal embryos after 3 hpf with a sperm:egg ratio of 50:1 was significantly higher than those obtained from 500:1 and 10,000:1 ratios. However, there was no significant difference

256 in the quantity of normal embryos obtained after 3 hpf between sperm:egg ratios of 50:1
257 and 100:1. Moreover, at 18 hpf, significantly higher numbers of normal embryos were
258 obtained at a sperm:egg ratio of 50:1 compared with those obtained at ratios of 1000:1
259 and 10,000:1 (Figure 3). However, there was no significant difference in normal embryo
260 development after 18 hpf among sperm:egg ratios from 50:1 to 500:1. Although the
261 power of the statistical analysis might not be strong, overall, there was a decreasing
262 trend in the quantities of normally-developed embryos as the sperm:egg ratio increased.

263

264 DISCUSSION

265 **Embryonic development**

266 The developmental time of *P. zelandica* embryos to D-veliger larvae was < 65 h at 15°C
267 and < 48 h at 17°C in our commercial batches (unpublished data). These
268 developmental periods were similar to those determined for *P. japonica* by Lee & Rho
269 (1997), who incubated embryos at 14 and 17°C (Table 1). However, the incubation
270 period for *P. japonica* embryos could be shortened to 27 h at 19°C (Nam *et al.*, 2014).
271 While it may be beneficial for geoduck hatcheries to maximize the developmental rate,
272 the thermal threshold for normal development should not be exceeded (Santo &
273 Nascimento, 1985). Thus, the development of *P. zelandica* embryos at higher
274 temperatures may be examined in future research, to improve hatchery efficiency and
275 understanding impacts of climate change.

276 In the current study, the formation times for the first and second polar bodies at 15°C
277 and 35 ppt were 20 - 25 and 50 - 55 min, respectively. The appearance times of the

second polar body of *P. zelandica* observed in this study were similar to those of the geoduck *P. generosa* at 15°C (Vadopalas & Davis, 2004). This information is important for the triploidy induction in bivalves, when using chemicals to block the second polar body formation (Barber *et al.*, 1992; Gerard *et al.*, 1994; Vadopalas & Davis, 2004). The present study provides the first record of early shell formation in geoducks. The presumptive shell field depression appeared at the blastomere X of *P. zelandica* blastula and started to depress at late blastula stage. The shell field depression occurring when the X^R and X^L were still present may confirm that the differentiation of the shell gland in *P. zelandica* occurs at the late blastula stage, while there are only a small number of cilia associated with the prototroch, and the embryos are spherical. The commencement of shell field depression in *P. zelandica* embryos was earlier than in other clams, e.g. *Ruditapes decussatus* (gastrula stage, Aranda-Burgos *et al.*, 2014) and *Spisula solidissima* (early trochophore stage, Eyster & Morse, 1984). The process of shell field depression at the gastrula stage for *P. zelandica* was similar to that of other clams (e.g. *Chione cancellata*, *Venerupis pullastra*, and *Ruditapes decussatus*) in which the shell field did not undergo invagination (Mouëza *et al.*, 2006; Aranda-Burgos *et al.*, 2014). However, the shell invagination needed to close either completely or partially before the shell could be formed in other bivalves (e.g. the mussel *Mytilus galloprovincialis* (Kniprath, 1980), the scallop *Pecten maximus* (Casse *et al.*, 1998), the clam *Spisula solidissima* (Eyster & Morse, 1984), and the oysters *Saccostrea kegaki* (Kin *et al.*, 2009) and *C. gigas* (Zhang *et al.*, 2012). This study also revealed that the shell mineralization only commenced once the periostracum covered the whole embryo, and began along the hinge, then continued along the shell margin, but did not initially

include the center of the valves. This shell mineralization process was similar to *M. galloprovincialis* (Kniprath, 1980) and *Tridacna squamosa* (LaBarbera, 1974). Furthermore, we observed that the shell valves preceded the ligament formation in *P. zelandica*. The same observation has been reported in *C. cancellata* (Mouëza *et al*., 2006).

Sperm:egg ratio

The reported values of optimal sperm:egg ratios for fertilization and successful development vary greatly for different bivalve species. In agreement with the present study, the lower range of sperm:egg ratio (≤ 100:1) was also optimal for fertilization in the scallop *Placopecten magellanicus* (Desrosiers *et al*., 1996) and the clams *Spisula solidissima* (Clotteau and Dubé, 1993), and *Tegillarca granosa* (Dong *et al*., 2012). The medium range of sperm:egg ratio (100-1000:1) has been found to optimize D-veliger larval yields in *C. gigas* (Song *et al*., 2009) and normal embryo yields in *C. gigas* (Stephano & Gould, 1988) and *C. virginica* (Alliegro & Wright, 1985). In addition, a high range of sperm:egg ratio (1000-5000:1) has been found to be optimal for normal D-larvae yield in the oyster *Crassostrea rhizophorae* (Santos & Nascimento, 1985) and the scallop *Chlamys asperrima* (O'Connor & Heasman, 1995). An even higher range of sperm:egg ratio (≥ 10,000:1) has been found to be optimal for fertilization in the cockle *Clinocardium nuttallii* (Liu *et al*., 2008).

Since the sperm:egg ratio and the fertilzation ratio are usually confounded by other factors (i.e. sperm motililtiy, egg density, oocyte maturation or/and contact time) we discuss here some potential reasons underlining the discrepancy between results on *P. zelandica*. The fertilization ratio for *P. zelandica* (81 – 91% 3 hpf and 88 – 96% 18

hpf) in the present study is higher than that (max. 70% 9 hpf) reported by Gribben *et al.* (2014). The procedures common to both the present study and Gribben *et al.* (2014) are spawning method and sperm motility evaluation before fertilization. The low range of sperm:egg ratio (≤ 100:1), which was found to be optimal for normal embryo yields for *P. zelandica* in the present study was also used for the Pacific geoduck *P. generosa* by Vadopalas & Davis (2004). In contrast, Gribben *et al.* (2014) found the ultra-high range (≥ 10,000:1) of sperm:egg ratio to be optimal for fertilization of the *P. zelandica*. The egg density was fixed at 100 eggs mL^{-1} in the present study, while 20 eggs mL^{-1} were used by Gribben *et al.* (2014), and Vadopalas & Davis (2004) did not report the egg density. The egg density may affect the numbers of sperm reaching the egg in marine invertebrates (Gould & Stephano, 2003). O'Connor & Heasman (1995) observed that the higher the egg density was the less sperm were required to elicit maximum fertilization. Besides, the egg density affected the fertilization ratio in the clam *S. solidissima* (Clotteau & Dubé, 1993). Similarly, the percentage fertilization in the scallop *C. asperrima* significantly increased from about 87% to 97% while the egg density increased from 1 to 500 eggs mL^{-1} (O'Connor & Heasman, 1995). In contrast, Levitan *et al.* (1991) did not find an effect of egg concentration on fertilization for the sea urchin *Strongylocentrotus franciscanus*. However, their lowest egg concentration was over 600 eggs mL^{-1}. Over 500 eggs mL^{-1} did not increase the fertilization ratio in *C. asperrima* (O'Connor & Heasman, 1995). Hence, the difference in optimal sperm:egg ratios and fertilization ratio results between this study and Gribben et al., (2004) might be due to the differences in the egg density. Further investigation of the effect of egg density on the fertilization ratio for *P. zelandica* should be conducted.

347 The sperm-egg contact time in the present study (40 min) was also longer than that (5 -
348 10 min) used by Gribben *et al.* (2014). Interestingly, Gribben *et al.* (2014) also
349 observed fertilization at low sperm concentrations if the contact time was increased.
350 Another potential factor influencing the higher normal embryo yield or lower polyspermy
351 in the present study may be the age of eggs prior to fertilization (1.5 h), which was a
352 longer storage period than that (< 30 min) used by Gribben *et al.* (2014). It must be
353 noted that *P. zelandica* eggs obtained in the present study and Gribben *et al.* (2014)
354 were the result of serotonin-induced spawning. Serotonin-spawned eggs have been
355 suggested to be more vulnerable to polyspermy (Misamore *et al.,* 1996). However, the
356 polyspermic susceptibility of serotonin-spawned eggs can be reduced if incubated for
357 over 1 h (O'Connor & Heasman, 1995). Similarly, the incidence of polyspermy of *C.*
358 *gigas* artificially stripped eggs was significantly reduced from 98 to 4% if eggs were
359 incubated 1 - 1.5 h prior to fertilization (Stephano & Gould, 1988). This might be due to
360 the maturation of oocytes after incubation in seawater. As oocytes become mature,
361 they develop an effective membrane potential barrier to polyspermy (Schlichter &
362 Elinson, 1981). For instance, it took the clam *Tivela stultorum* oocytes, which were
363 treated with serotonin, up to 40 min to become mature (Alvarado-Alvarez *et al*., 1996).
364 Moreover, a decreased conductance, which strengthens the polyspermy block,
365 developed slowly in serotonin treated oocytes (Alvarado-Alvarez *et al*., 1996).

366 In addition to those potential factors mentioned above (i.e. egg density, contact
367 time and egg age), the temperature for storing gametes is a critical factor influencing
368 fertilization practices and success. Gribben *et al.* (2014) found that *P. zelandica*
369 gametes stored at 15°C for over 30 min had reduced viability. This reduction in viability

has also been observed in other bivalves (e.g. *Clinocardium nuttallii*, Liu *et al.*, 2008) at their spawning temperatures. However, when gametes are stored at lower temperatures the gamete viability can be maintained for up to 1.5 - 4 h (O'Connor & Heasman, 1995; Liu *et al.*, 2008; Adams *et al.*, 2004, 2009). Similarly, in the present study no negative effects of storing *P. zelandica* gametes at 4°C were found. Thus, it seems that reducing the temperature may be a factor in resolving inconsistencies between the age of eggs and their susceptibility to polyspermy.

Inevitably, in a commercial operation, eggs need to be pooled until sufficient quantities have been collected to stock an incubation tank, which may take several hours. Thus, cold storage adds flexibility to spawning and fertilization times and prolongs the viability of both sperm and egg. Further research may usefully be focused on the mechanisms underlying the viability of geoduck gametes at low temperatures.

In conclusion, embryo cleavage follows a spiral and unequal pattern while the shell field depresses and expands to create the periostracum. However, the ligament is not formed until the shell field covers the entire embryo. Sperm:egg ratios of 50-500:1 with a 40 min sperm-egg contact time gave the highest normal embryo yield under the experimental conditions. Eggs and sperm can be stored at 4°C to extend their viability up to 1.5 h, making the fertilization practical since geoducks typically continue to spawn for 4 h. In addition, incubating eggs at 4°C for over 1 h may make the eggs less susceptible to polyspermy. An experiment with more female spawned would confirm the finding of sperm:egg ratio in this study. Further research is needed to determine the extent to which cold storage can prolong gamete viability, and whether incubation times

exceeding 1 h can reduce the polyspermic susceptibility of eggs, as well as confirming the shell field pattern for *P. zelandica*.

ACKNOWLEDGEMENT

This project was funded by the Cawthron Cultured Shellfish Programme (NZ Ministry of Business, Innovation and Employment contracts CAWS0802, CAW1315). Logistical and technical support was provided by the School of Applied Sciences, Auckland University of Technology (AUT). We are grateful to the Aquaculture Biotechnology Group at AUT for fruitful discussions that improved this research. We would like to thank Patrick Conor and Marcel Schaefer from School of Engineering, AUT for SEM guidance and Clara & Richard for editing photos. This project is part of a Ph.D. thesis, which was supported by a New Zealand Aid scholarship awarded to D. V. Le under the supervision of A. C. Alfaro.

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542 **Table 1.** The approximate post-fertilization developmental time sequence for geoduck
543 embryos. *P. zelandica* data are derived from the current study and compared to *P.*
544 *japonica* raised at different temperatures by Lee and Rho (1997).

	***P. zelandica* at**	***P. japonica* at**		
Stage	**15°C**	**11 °C**	**14 °C**	**17 °C**
1st polar body	15 - 20 min			
2nd polar body	50 - 55 min			
2 cells	1.5 h	2 h		
4 cells	2.5 h	4 h		
8 cells	4 h	9 h	5.4 h	4.3 h
16 cells	5 h	15 h		
32 cells	6 h			
Morula	6 h			
Blastula	8 h	23 h	18.7 h	12.3 h
Early gastrula	12 h			
Gastrula	18 h			
Early trochophore	28 h			
Trochophore	35 h	2 d	33.8 h	23.6 h
Late trochophore	39 h			
Early veliger	45 h			
D-Veliger	62 h	3 d	62.4 h	42.7 h

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548 **Table 2.** List of abbreviations used in Figure 1 and 2.

ap	apical plate
at	apical tuft
b	blastopore
Ci	cilia
dp	depression
h	hinge
pb	polar body
pel	pellicle
ps	periostracum
psb	pseudo-blastopore
pSF	presumptive shell field
pt	prototroch
s	shell
SF	shell field
sp	sperm
tt	telotroch
Ve	velum

549

550

Figure 1.

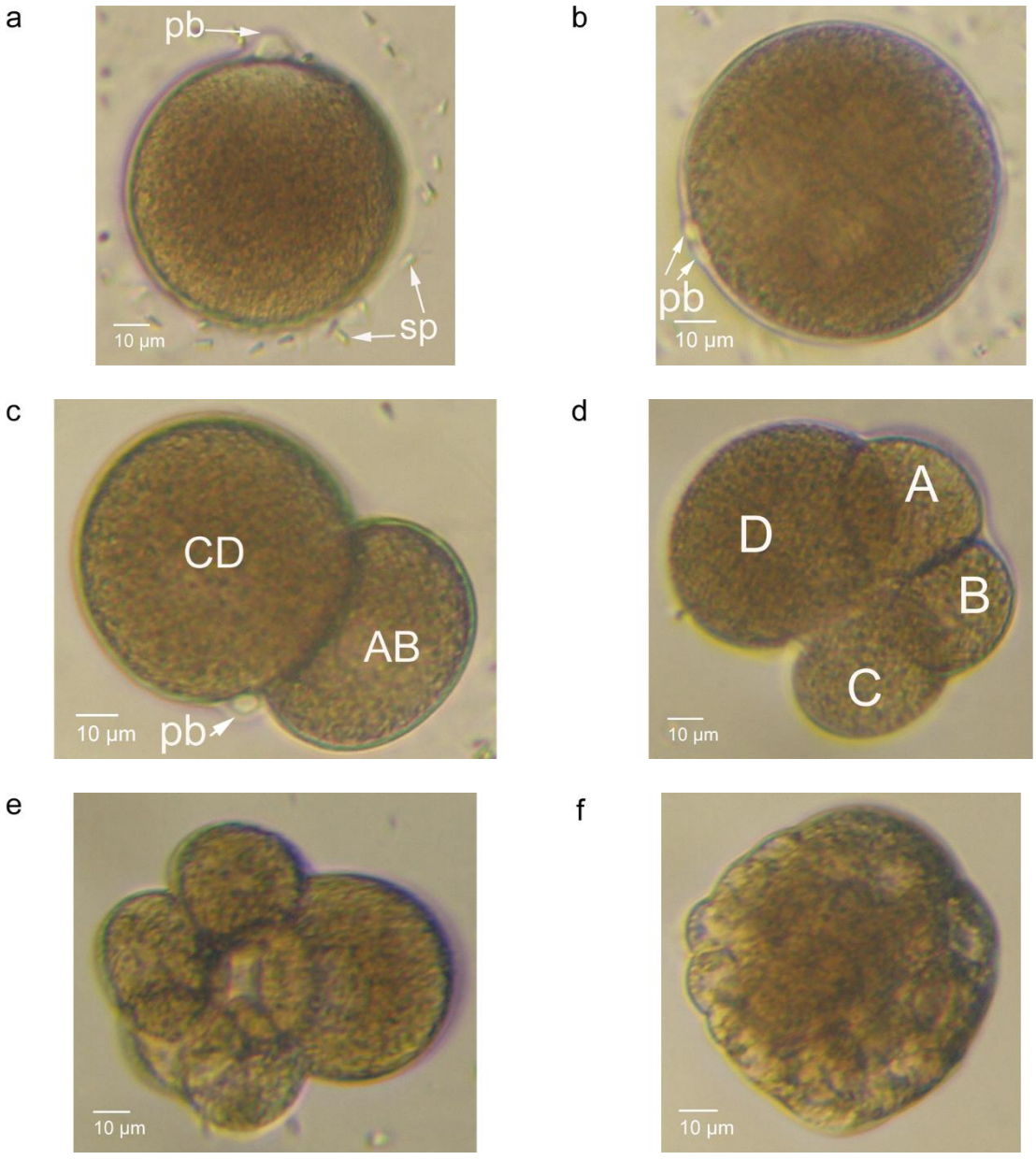


Figure 1 cont.

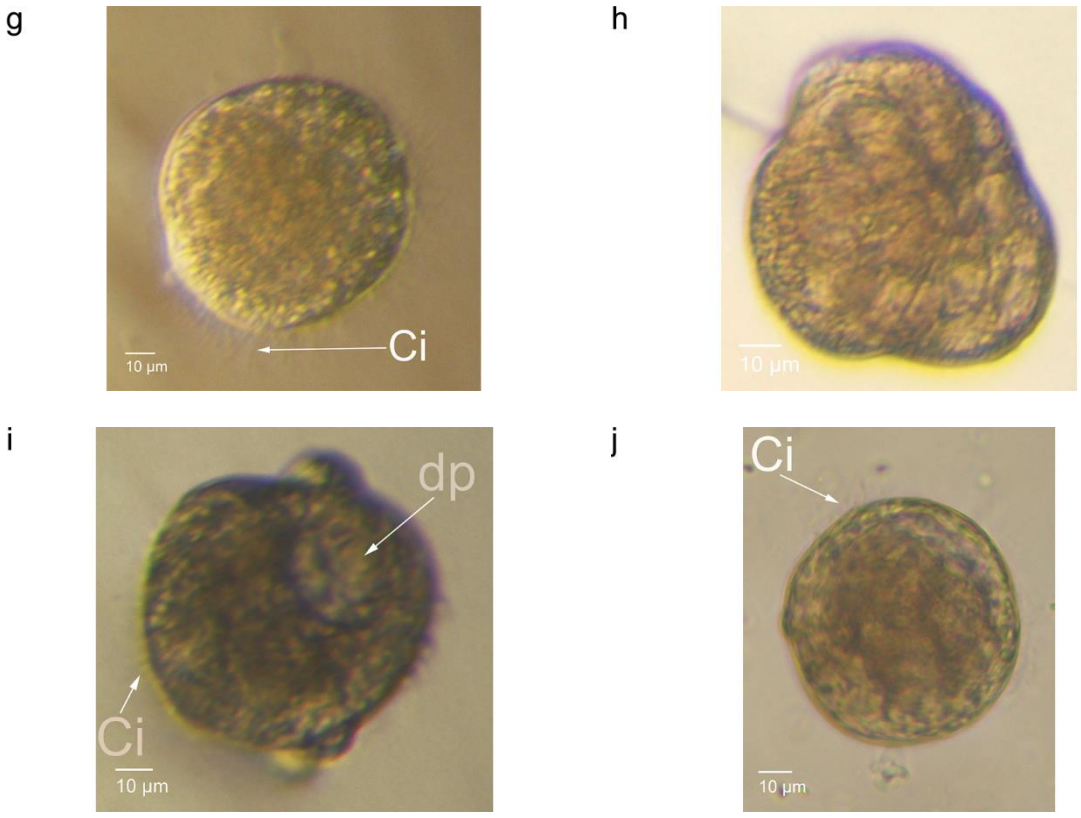
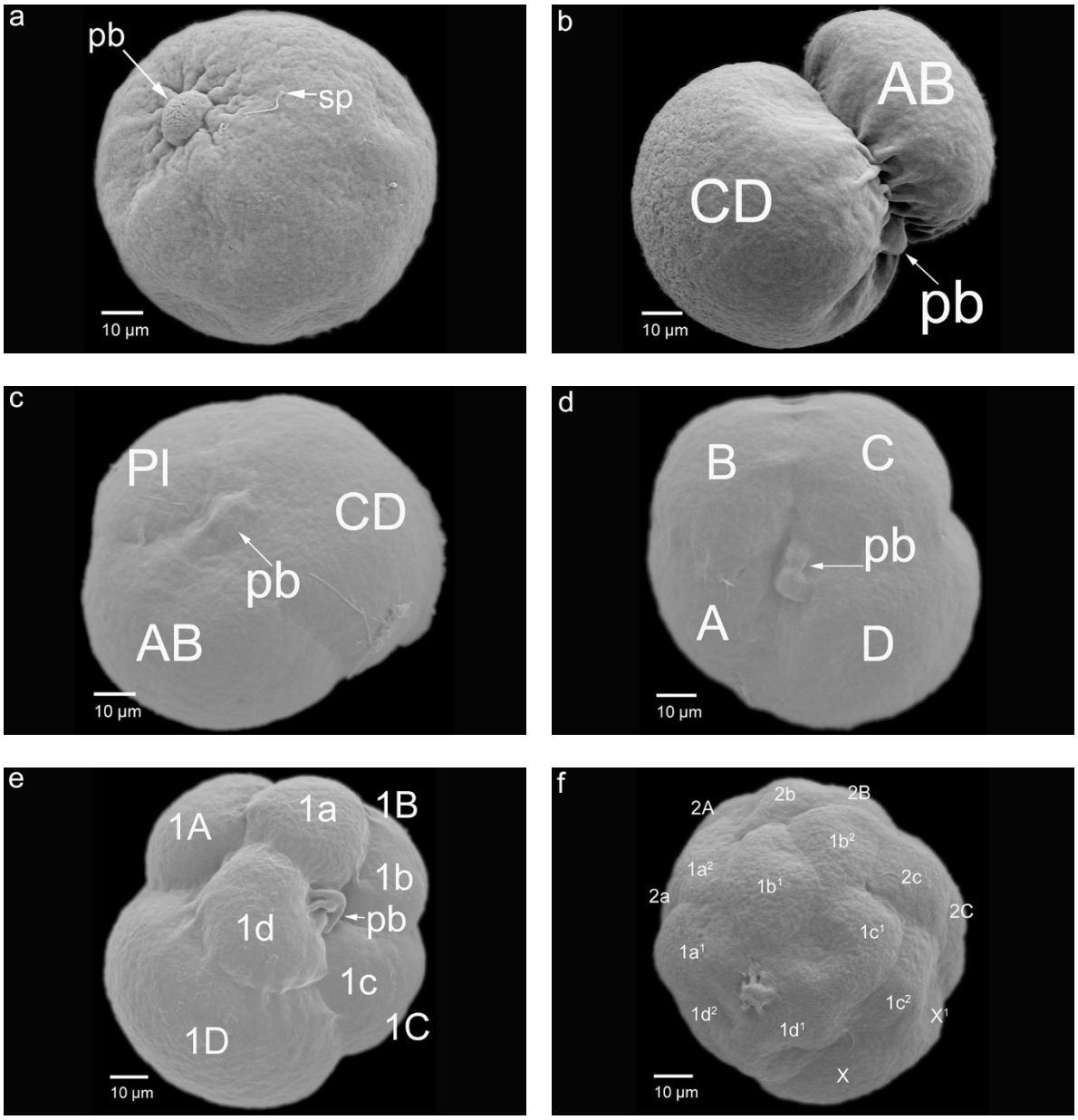


Fig. 1. Light microscopy images of *P. zelandica* embryonic development. a) - e) initial cell divisions; f) morula; g) blastula; h) - i) gastrula and j) trochophore. Abbreviations are summarized in Table 2.

Figure 2.


a
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sp
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1c
1D
1C
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f
2A
2b
2B
1b²
1a²
1b¹
2c
2a
2C
1c¹
1a¹
1d²
1c²
X¹
1d¹
X
10 µm

Figure 2 cont.

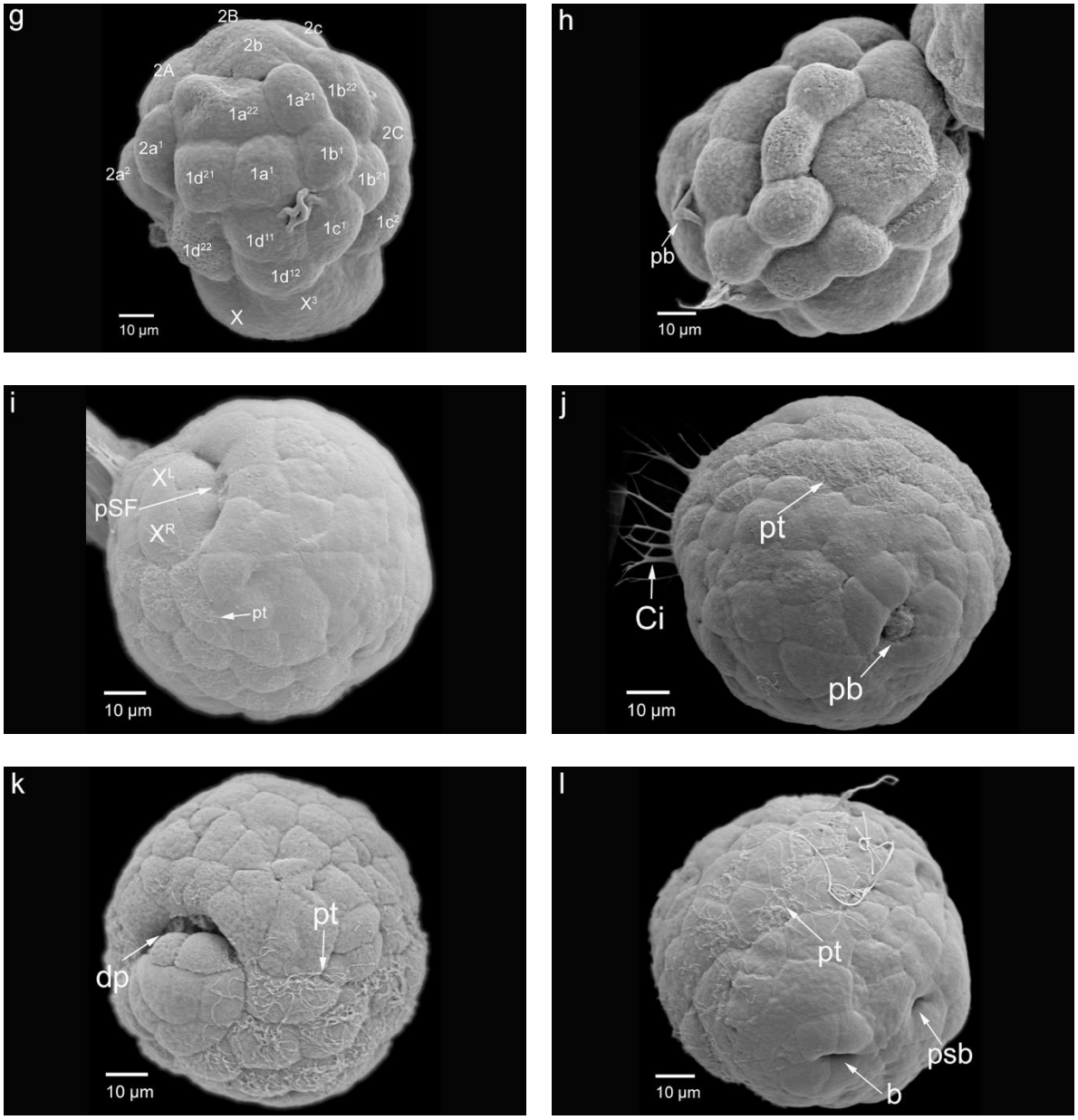

Figure 2 cont.

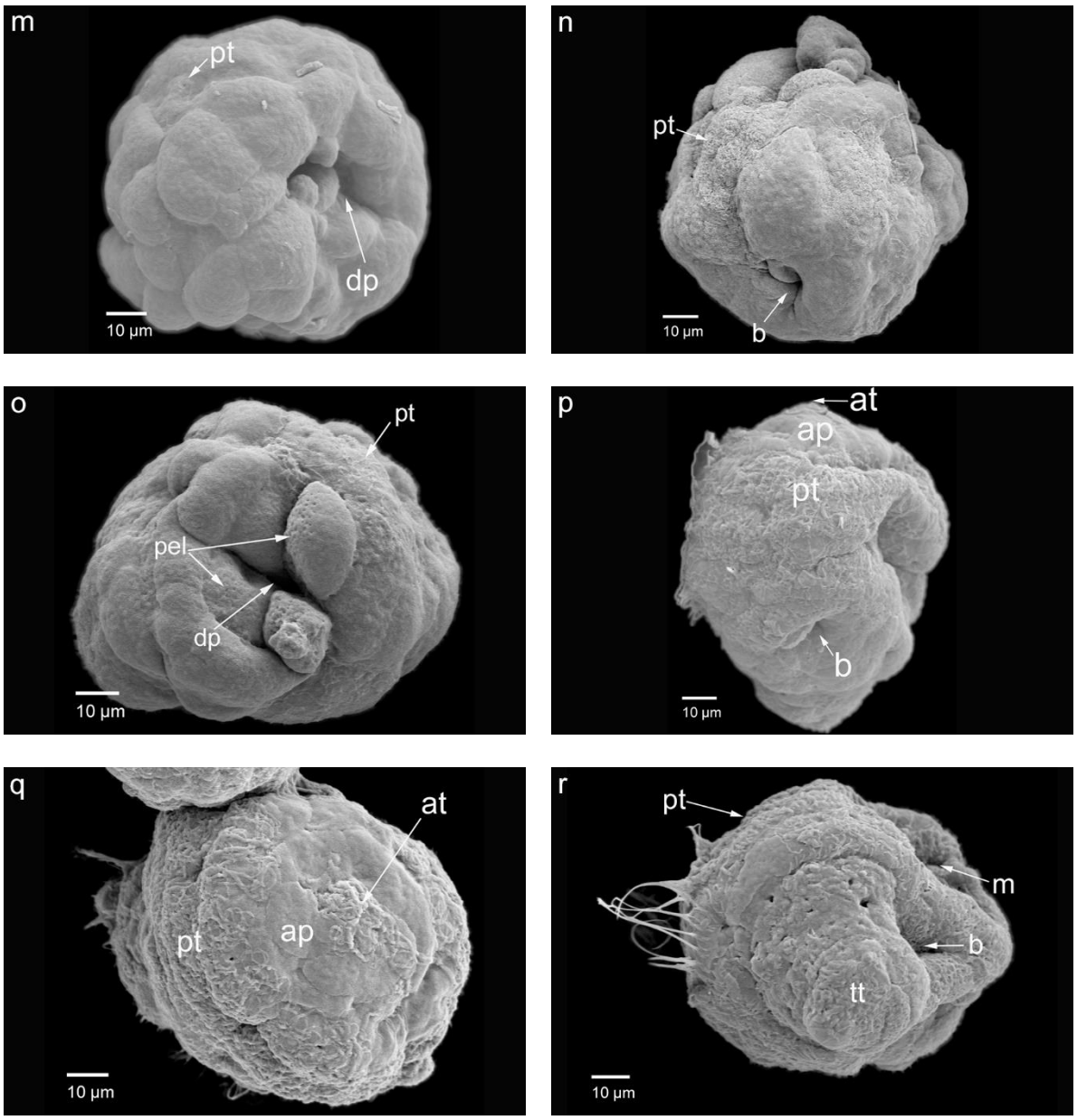

Figure 2 cont.

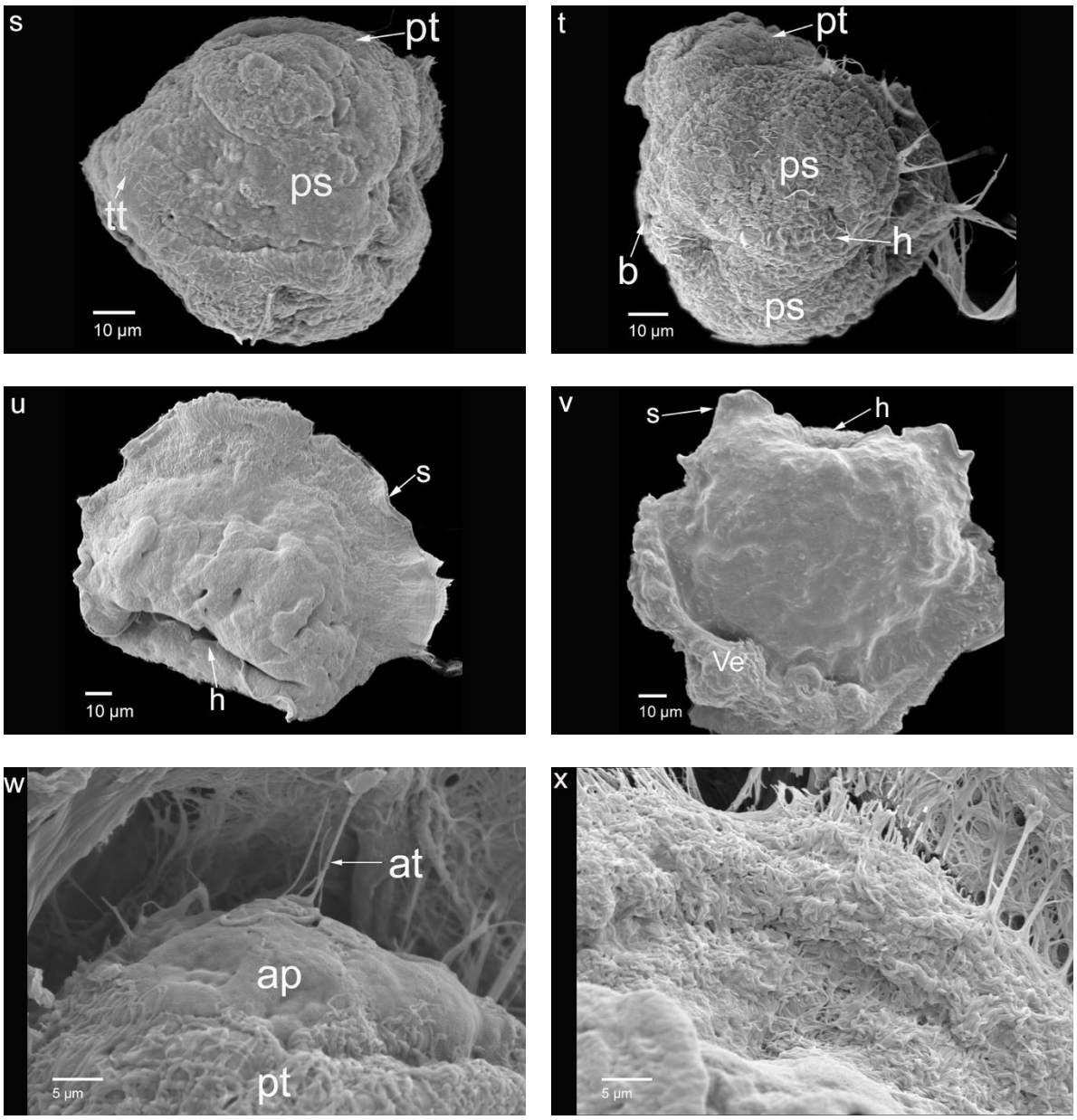

Fig. 2. SEM images of *P. zelandica* embryonic development. a) fertilized egg; b) 2 cell stage; c) 3 cell; d) 4 cell; e) 8 cell; f) 16 cell; g) 32 cell; h) morula; i) - j) early blastula; k) - l) late blastula; m) - n) early gastrula; o) gastrula; p) - s) trochophore; t) - v) late trochophore and w) - x) early D-veliger. Abbreviations summarized in Table 2 and the results text.

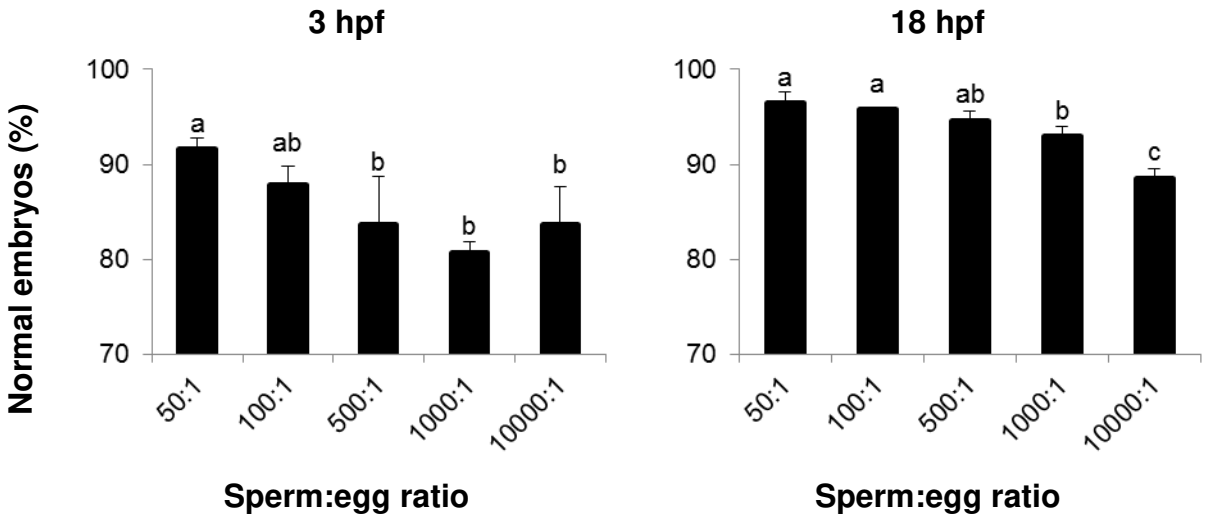

Fig. 3. Proportion of apparently normal embryos, expressed as a percentage of initial egg numbers, 3 and 18 hpf using different sperm:egg ratio treatments. Bars represent mean ± SD, n = 3; significant differences are identified by distinct letters ($P < 0.05$).