Invertebrate Reproduction and Development, 44:2–3 (2003) 131–137
Balaban, Philadelphia/Rehovot
0168-8170/03/$05.00 © 2003 Balaban

Effects of cytochalasin B on fertilization and ploidy in the Pacific oyster *Crassostrea gigas*

CHRISTOPHE LEDU* and HELEN MCCOMBIE**
Laboratoire de Génétique et Pathologie, Institut Français pour la Recherche et Exploitation de la Mer (IFREMER), 17390 La Tremblade, France
Tel. +33 (5) 46 36 98 36; Fax +33 (5) 46 36 37 51; email: hmccombi@ifremer.fr

Received 23 June 2003; Accepted 22 September 2003

Summary

The effects of cytochalasin B on fertilization and ploidy in *Crassostrea gigas* were examined in a controlled experiment. This enabled us to better understand why variable ploidy results have been found with the retention of polar bodies in previous studies on bivalve mollusks, and what the interaction with fertilization may be. By looking at the early development of *Crassostrea gigas* embryos with sequential samples stained using Hoechst 33258, a fluorescent DNA-specific dye, we compared normal evolution of fertilized eggs with that obtained with cytochalasin B treatment. In treated eggs, embryos with both three and four pronuclei were found after the second meiotic division. When embryos had three pronuclei, these were identified as the female pronucleus plus two chromatin groups arising from the retention of the two polar bodies. The fourth pronucleus present in some embryos was the male pronucleus. The absence of the male pronucleus in the embryos with only three pronuclei was interpreted as gynogenetic development due to the inhibitory effects of cytochalasin B on penetration of the sperm into the eggs. This result explains the findings of several previous studies where different ploidies resulted from double polar body inhibition in bivalve species. Implications of such treatments for post-embryogenic development are discussed.

Key words: Polyploidy, polar body, cytochalasin B, fertilization, *Crassostrea gigas*

Introduction

Cytochalasin B (CB) treatments have been used to produce triploids in the Pacific cupped oyster *Crassostrea gigas* and other commercial shellfish species where they offer advantages for aquaculture in terms of sterility and enhanced growth over their diploid counterparts (reviews in Beaumont and Fairbrother, 1991; Nell, 2002). The principle of this treatment is the inhibition of the first or second polar body (PB1 or PB2) during the early stages of embryonic development (Longo, 1972). Either of these methods can be used to produce triploids in *C. gigas* (e.g., Downing and Allen, 1987; Yamamoto et al., 1988; Gérard et al., 1999). However, blocking the expulsion of the first

*Current address: Laboratoire d'Aquaculture tropicale, Centre Océanographique de la Pacifique (COP), Institut Français pour la Recherche et Exploitation de la Mer (IFREMER), BP 7004, 98719 Taravao, Tahiti, French Polynesia.
**Corresponding author.

polar body in different bivalves has been seen to produce not only triploids (3n) but also tetraploids (4n) and in some cases pentaploids (5n) or numerous aneuploids of varying chromosome number (Yamamoto et al., 1988; Guo et al., 1992a; Gérard et al., 1999; Yang et al., 2000a). Diverse ploidies issued from a single treated batch are presently explained by disordered chromosome segregation (Komura et al., 1990; Guo et al.; 1992b; Longo et al., 1993; Yang et al., 2000b), or accidental retention of both polar bodies (Yamamoto et al., 1988; Komura et al., 1990), but it has also been suggested that a treatment to suppress PB1 might affect ploidy through effects on fertilization and first mitosis (Diter and Dufy, 1990; Gendreau and Grizel, 1990). It is known that cytochalasin B inhibits actin polymerization (Maclean-Fletcher and Pollard, 1980) and that this could lead to the non-penetration of spermatozoids (Longo, 1978) in addition to the targeted retention of polar bodies in polyploidy induction experiments. The principle objectives of our present study were to examine the effects of the chemical on fertilization, to see whether non-penetrating sperm could nevertheless activate eggs and to observe subsequent inhibition of polar bodies for the potential effects on ploidy.

In previous studies, the retention of both polar bodies produced mostly tetraploid and pentaploid larvae in mussels *Mytilus galloprovincialis* (Scarpa et al., 1993). In the dwarf surf clam *Mulinia lateralis* (Peruzzi and Guo, 2002), triploids, tetraploids and pentaploids were found. Embryos from similar treatments on *C. gigas* (Cooper and Guo, 1989) and the Japanese pearl oyster *Pinctada fucata martensii* (Komura et al., 1990) were pentaploid.

Tetraploids are valued in aquaculture for the production of triploids via 2n×4n crosses, which in contrast to chemical methods give 100% triploids (Chourrout et al., 1986; Guo et al., 1996) and are now used in hatchery production of triploid oysters (Nell, 2002).

The present experiment was also designed to explain why high numbers of tetraploids can sometimes be seen when both polar bodies are blocked, rather than uniquely the expected pentaploids given by the addition of four genomes from the female (female pronucleus plus the content of two non-expulsed polar bodies, the second twice the size of the first) and one from the male. Our study of CB-treated embryos showed evidence for the expected inhibitory effect of CB on fertilization. Following this, our examination of the meioses and the first metaphase allowed us to examine in detail the effects of the treatment on development and how it affected subsequent ploidy of the embryos.

Materials and Methods

Preparation of gametes

Oysters collected from the Marennes-Oléron basin (Charente-Maritime, France) were matured under controlled conditions in the IFREMER hatchery at La Tremblade (Charente-Maritime, France) for 2 months. The gametes were removed from the ripe animals (10 females, 6 males) by stripping the gonads. The oocytes were then filtered on 100 µm screen and the spermatozoids on 25 µm and the male and female gametes pooled separately in 1 µm filtered seawater. A water bath was prepared at 25°C for the fertilization and treatments.

CB treatments and embryo sampling

All treatments and samplings were made at precise points in time after the sperm and oocytes were mixed ($t = 0$ min). The experiment comprised a control with no CB (Batch 1) and two separate CB treatments (Batches 2 and 3, started at $t = 2$ and $t = 3$ min, respectively). The three batches each had 15 million oocytes with spermatozoids (oocyte:spermatozoid ratio=1:200) in 1 L filtered seawater at 25°C. CB (Sigma C-6762), dissolved in dimethylsulfoxide (DMSO, Lab-Osi D-770) was added to each of the treatment beakers to produce final concentrations of 0.5 mg/l. The CB treatments lasted 25 min in order to block the expulsion of both polar bodies. The CB was then removed by filtration on a 10 µm screen and the embryos rinsed in DMSO (1 ml/L) in seawater for 15 min. They were subsequently re-filtered and put in clean filtered seawater.

Samples for embryo study were taken at fertilization and every following 5 min until 80 min post-fertilization. One ml was taken at each interval and was immediately fixed in formaldehyde in a glucosamine-acetate buffer (Dubé et al., 1985).

Embryo coloration and observation

The sampled embryos were colored with Hoechst 33258 (Sigma B-2883), a fluorochrome, and examined by epifluorescence using an Olympus BH-2 UV microscope. One hundred embryos were studied per sample for their state of development: expulsion of polar bodies and cell division. Polar body counting (Beaumont, 1986) allows the inhibitory action of the CB to be assessed in a manner corresponding to the ploidy level.

Results

The control provides an example of normal diploid development (Figs. 1a–g). Expulsion of the first PB started between 10 and 15 min after fertilization (Fig. 1b), followed by the second PB during meiosis II (Fig. 1c and d). Expulsed polar bodies can be readily observed because they remain stuck to the outside of the embryo from expulsion until after the first cleavage. The spermatozoid decondenses gradually (Fig. 1d) and fuses with the female pronucleus for karyogamy (Fig. 1e). The first mitosis then takes place (Fig. 1f), followed by division (Fig. 1g), which began between 45 and 50 minutes in our control. These events took place more slowly in the presence of cytochalasin B; meiosis II appeared 5–10 min later than in the control. Mitosis and cell division, where observed, were about 20 min later than the control.

In both the CB treatments, the retention of the two polar bodies within the embryos could be observed resulting in two extra nuclei. The number of nuclei should be four (male pronucleus, female pronucleus, PB 1 and PB2) (Fig. 2a). At 50 min, 21% of batch 2 and 16 % of batch 3 showed this type of development. However, there were more cases in both batches where there were only three nuclei and no expulsed polar bodies (26% and 25% in batches 2 and 3 respectively; Fig. 2b). The missing nucleus was identified as the male pronucleus, given that the male and female pronuclei can be easily distinguished because the female pronucleus is found under the polar bodies at the edge of the cell (Fig. 2a and b).

Karyogamy took place with either the three or four pronuclei, after the removal of the CB. Finally, whilst in the control, first cell division had started by 50 min,

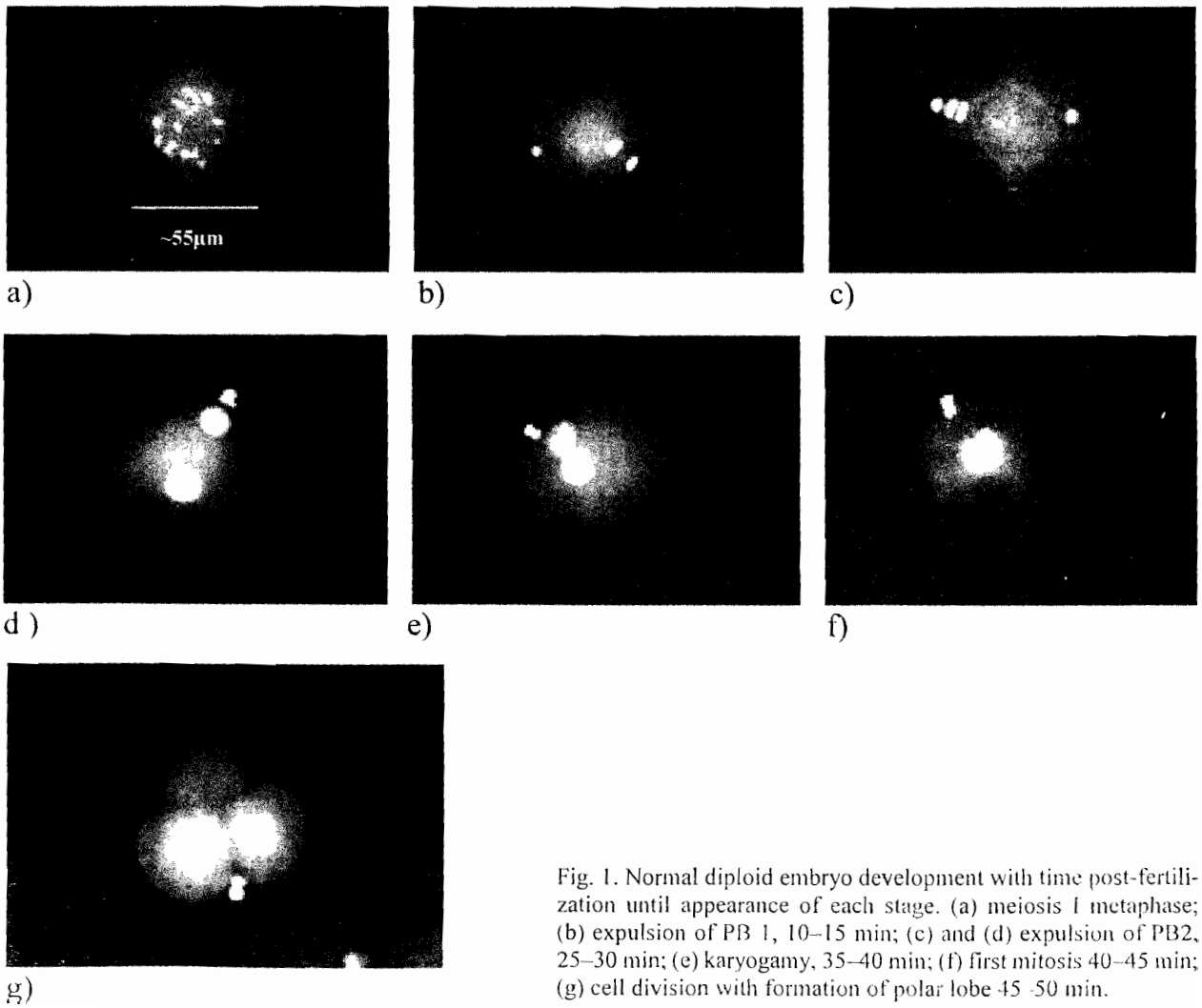

Fig. 1. Normal diploid embryo development with time post-fertilization until appearance of each stage. (a) meiosis I metaphase; (b) expulsion of PB 1, 10–15 min; (c) and (d) expulsion of PB2, 25–30 min; (e) karyogamy, 35–40 min; (f) first mitosis 40–45 min; (g) cell division with formation of polar lobe 45–50 min.

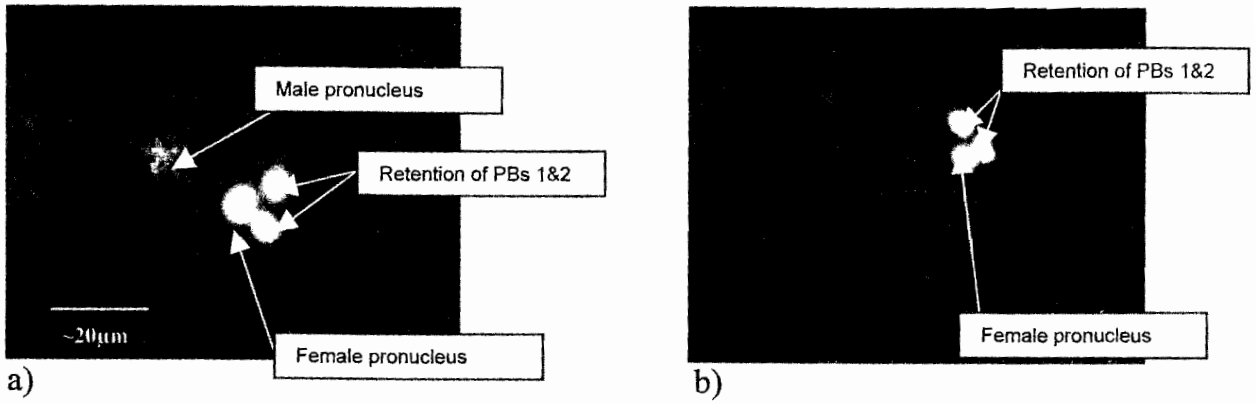

Fig. 2. Embryos resulting from the retention of both Polar Bodies (PBs) (a) with male pronucleus; (b) without male pronucleus.

in the treatments, the first divided cells were only seen at 70 min implying that the treatment could have had an effect on capacity for, or speed of, subsequent mitosis and division. Delayed mitosis in the treated batches could be partly explained by the delay in meiosis II mentioned above, although the difference with the control had become greater by mitosis. Based on the amount of genetic material, specimens with four pronuclei and no released polar bodies (Fig. 2a) would develop as pentaploids and those with three (Fig. 2b) as tetraploids.

The two CB treatments were not markedly different in timing of development or numbers of pronuclei. Very few expulsed PBs were observed, maximum 8% in batch 2 and 5% in batch 3. These started to be seen just after the removal of the CB, and though they were probably delayed PB1s might also have been PB2s judging by the control time scale where some eggs were still releasing either PB1 or PB2 at this time.

Although the two types of development shown in Fig. 2 were the most common at 50 min, others were also seen. The rest of the samples showed a variety of formations present at low frequencies (≤5%), some of which were difficult to interpret. There were examples with five or more pronuclei, which were observed both in the treatments and control. The most likely reason for this is polyspermy (multiple fertilization) (Togo and Morisawa, 1999). Tripolar segregation at meiosis II and the formation of two nuclei were also occasionally observed. Tripolar segregation can lead to expulsion of a PB2 containing variable amounts of genetic material (Guo et al., 1992b). The most extreme formation that we observed was the expulsion of all chromatin groups in a single polar body. There were some also unfertilized eggs in all samples which remained at metaphase of meiosis I (Fig. 1a).

Discussion

Our results show that early fertilization, embryo development and ploidy outcome are markedly different between treated and untreated *C. gigas* eggs. Under normal fertilization conditions in marine invertebrates, the membranes of the spermatozoid and oocyte fuse. The sperm then achieves its entry into the oocyte via a cytoplasmic bridge which is created by actin polymerization (Colwin and Colwin, 1963; Longo and Scarpa, 1991). Cytochalasin B inhibits actin polymerization (Maclean-Fletcher and Pollard, 1980), and therefore, if applied close to fertilization, could prevent sperm from penetrating the egg as has already been demonstated in molluscs and echinoderms (Longo, 1978; Schatten and Schatten, 1981). In the present experiment, the CB treatments were made at 2 or 3 min after mixing sperm and oocytes, meaning that effects on the fertilization mechanism are likely. In previous studies aiming to retain PB1 (or both PBs) in bivalves (e.g., Yamamoto et al., 1988; Cooper and Guo, 1989; Scarpa et al., 1993; Guo et al., 1992a; Gérard et al., 1999; Yang et al., 2000a), the added sperm inhibition effect may have been partial or absent because the treatment was later relative to sperm penetration and embryogenic development. This would explain the absence of tetraploid *C. gigas* embryos in the double retention experiment of Cooper and Guo (1989). In addition, the development of *Crassostrea* sp. eggs is asynchronous (Longwell and Stiles, 1968; Desrosiers et al., 1993), meaning that in our study some eggs would have been ready to be fertilized before the CB treatment was applied but that for others the penetration of the spermatozoids would have been inhibited by the CB. In the latter case, the eggs would be activated but unfertilized, leading to gynogenetic development. This effect would have been enhanced by our use of stripped oocytes from several

females which could have increased variability in states of development.

In a previous study with early CB treatment to retain PB1 for triploid induction, Yamamoto et al. (1988) suggested that the percentage of tetraploid and pentaploid *C. gigas* embryos that they subsequently observed had been generated by the double retention of PB1 and PB2. Diter and Dufy (1990), working on *Ruditapes philippinarum* and Gendreau and Grizel (1990), working on *Ostrea edulis*, suggested that tetraploidy arose from inhibition of the first mitosis due to absence of the spermatozoid inhibited by the CB. None of these studies examined embryogenesis in detail. In our experiment, with double polar body inhibition, the resulting embryos would be pentaploids if fertilized and tetraploids if gynogenetic. This is in accordance with Guo et al.'s (1993) observations of 4n *C. gigas* embryos produced by double PB retention and fertilization with UV-inactivated sperm, which gave 4n gynogens, and with the 5n embryos observed in the same species by Cooper and Guo (1989) where viable sperm was used. In 2002, Peruzzi and Guo suggested that the 4n/5n embryo mixture they obtained with double PB inhibition on *Mulinia lateralis* could have been due to chromosome loss from some 5n embryos or accidental release of PB2 associated with abnormal segregation, although embryogenic events were not recorded. Very few PBs were released in our treated samples, and the identification of the male pronucleus or its absence using epifluoresence provides a clear explanation for a mixture of 4n/5n ploidies. Our results do not preclude a subsequent effect on first mitosis due to inhibition of sperm penetration. In fact, we observed, as in Scarpa et al. (1993), that the first division was later (70 min) in the treated embryos than in the control (50 min), which may also be a consequence of spermatic inhibition. Scarpa et al. (1992) suggested that the absence of sperm in gynogenetic induction could prevent first mitosis, cleavage and subsequent development because of the absence of a paternal centrosome and Diter and Dufy (1990) that the first mitotic division and cleavage would be delayed by this means but that development could resume later. However, Heilbrunn (1925) suggested for *Cummingia* (Mollusca) and Washitani-Nemoto et al. (1994) for *Asterina pectinifera* (Echinodermata) that the inhibition of polar body expulsion in unfertilized eggs could compensate for this lack of centrosome due to the presence of meiotic centrosomes which would allow normal mitosis and first cleavage to take place. This would explain why we observed continued embryo development, although further study will be necessary to establish if the early cell divisions we observed occurred in embryos where fertilization had been inhibited. Detailed study of the centrosomes themselves, using more precise techniques like fluorescent immunocytochemical marking (Stephano and Gould 2000), could be used to establish whether or not a maternal meiotic centrosome could play this important role in mitosis.

Some previous authors have also noted five or variable numbers of pronuclei in studies on other species where both polar bodies were retained (Longo, 1972; Komura et al., 1990; Scarpa et al., 1993). Other types of development we observed may result from abnormal segregation following the inhibition of PB1 as observed by Guo et al. (1992b) or from polyspermy.

A further study of the subsequent stages of development will help to understand the importance of the events we have observed for future development and survival of induced polyploid *C. gigas*. At 4 h post-fertilization, we did observe some tetraploid and pentaploid mitotic metaphases on microscope preparations made from the embryos of the CB treatments, which underline that mitotic divisions continue beyond the early embryonic stages. However, we know of no reports of viable surviving pentaploid progenies in bivalves. This could be due to physical problems of cell division associated with large nucleus to cell volume ratio (Guo and Allen, 1994). Tetraploids induced directly from diploid bivalves have been seen to survive to post-metamorphic stages in several bivalve species (Scarpa et al., 1993; Allen et al., 1994; Peruzzi and Guo, 2002). For aquaculture, this method avoids selecting fertile triploid females otherwise required as maternal parents in a cross to make tetraploids (Guo and Allen, 1994), which might select for undesired fertility in future triploid progeny. Exploitation of fertilization inhibition combined with polar body retention could also provide a means of producing tetraploid and diploid gynogens for selection programs and genetic studies.

Acknowledgements

We are indebted to André Gerard for initiating this project. We would also like to thank the technical staff at Ifremer La Tremblade for the care and preparation of the animals used in this study and Pascal Phelipot for practical assistance during the induction experiment. Finally we thank Pierre Boudry, Carol McCombie and Alexandra Leitão who gave us useful advice on the preparation of this manuscript.

References

Allen, S.K. Jr., Shpigel, M., Utting, S. and Spencer, B., Incidental production of tetraploid Manila clams, *Tapes philippinarum* (Adams and Reeve). Aquaculture, 128 (1994) 13–19.

Beaumont, A.R., Genetic aspects of hatchery rearing of the scallop *Pectin Maximus* (L.). Aquaculture, 57 (1986) 99–110.

Beaumont, A.R. and Fairbrother, J.E., Ploidy manipulation in shellfish: a review. J. Shellfish Res., 10 (1991) 1–18.

Chourrout, D., Chevassus, B., Kreig, F., Happe, A. Burger, G. and Renard, P., Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females. Potential of tetraploid fish. Theor. App. Gen., 72 (1986) 193–286.

Colwin, A.L. and Colwin, L.H., Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneustra). I. The acrosome reaction and its changes in the early stages of fertilisation. Cell, 19 (1963) 477–500.

Cooper, K. and Guo, X., Polyploid Pacific oysters produced by inhibiting polar body I and II with cytochalasin B. J. Shellfish Res., 8 (1989) 412 (abstract).

Desrosiers, R.R. Gérard, A., Peignon, J-M., Naciri, Y. Dufrense, L. Morasse, J., Ledu, C., Phelipot, P. Guerrier, P. and Dubé, F., A novel method to produce triploids in bivalve molluscs by the use of 6-dimethylaminopurine. J. Mar. Biol. Ecol., 170 (1993) 29–43.

Diter, A. and Dufy, C., Polyploidy in the Manila clam, *Ruditapes philippinarum*. II. Chemical induction of tetraploid embryos. Aquat. Living Resour., 3 (1990) 107–112.

Downing, S.L. and Allen, S.K., Jr., Induced triploidy in the Pacific oyster *Crassostrea gigas*: optimal treatments with cytochalasin B depend on temperature. Aquaculture, 61 (1987) 1–15.

Dubé, F., Schmidt, T. Johnson, C.H. and Epel, D., The hierarchy of requirements for an elevated intracellular pH during early development of sea urchin embryos. Cell, 40 (1985) 657–666.

Gendreau, S. and Grizel, H., Induced triploidy and tetraploidy in the European flat oyster, *Ostrea edulis* L. Aquaculture, 90 (1990) 229–238.

Gérard, A., Ledu, C., Phélipot, P. and Naciri-Graven, Y., The induction of MI and MII triploids in the Pacific oyster *Crassostrea gigas* with 6-DMAP or CB. Aquaculture, 174 (1999) 229–242.

Guo, X., Hershberger, W.K., Cooper, K. and Chew, K., Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*. I Ploidy of resultant embryos. Biol. Bull., 183 (1992a) 381–386.

Guo, X., Hershberger, W.K., Cooper, K. and Chew, K., Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*. II Segregation of chromosomes. Biol. Bull., 183 (1992b) 387–393.

Guo, X., Hershberger, W.K., Cooper, K. and Chew, K., Artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster, Crassostrea gigas. I. Induction and survival. Aquaculture, 113 (1993) 201–214.

Guo, X. and Allen, S.K. Jr., Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thunberg) produced by inhibiting polar body 1 in eggs from triploids. Mol. Mar. Biol. Biotech., 3 (1994) 42–50.

Guo, X., Debrosse, G.A. and Allen, S.K. Jr., All triploid oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture, 142 (1996) 149–161.

Heilbrunn, L.V., Studies in artificial parthenogenesis. IV. Heat Parthenogenesis, J. Exp. Zool., 41 (1925) 243–260.

Komura, A., Matsuda, H., Yamakawa, T. and Wada, K.T., Chromosome behaviour of mitosis inhibited eggs in Japanese pearl oyster *Pinctada martensii*. Nippon Suisan Gakkaishi, 569 (1990) 1419–1422.

Longo, F.J., The effects of cytochalasin B on the events of fertilization in the surf clam, *Spisula solidissima*: I. Polar body formation. J. Exp. Zool., 182 (1972) 321–344.

Longo, F.J., Effects of cytochalasin B on sperm-egg interactions. Dev. Biol., 67 (1978) 157–173.

Longo, F.J. and Scarpa, J., Expansion of the sperm nucleus and association of the maternal and paternal genomes in fertilized *Mulinia lateris* eggs. Biol. Bull., 180 (1991) 56–64.

Longo, F.L., Mathews, L. and Hedgecock, D., Morphogenesis of maternal and paternal genomes in fertilized oyster eggs (*Crassostrea gigas*): effects of cytochalasin B at different periods during meiotic maturation. Biol. Bull., 185 (1993) 197–214.

Longwell, A.C. and Stiles, S.S., Fertilization and completion of meiosis in spawned eggs of the American oyster *Crassostrea virginica*. Caryologia, 21 (1968) 65–73.

Maclean-Fletcher, S. and Pollard, T., Mechanism of action of cytochalasin B on actin. Cell, 20 (1980) 329–341.

Nell, J.A., Farming triploid oysters. Aquaculture, 210 (2002) 69–88.

Peruzzi, S. and Guo, X., Tetraploid induction by meiosis inhibition with cytochalasin B in the dwarf surfclam, *Mulinia lateris* Say: Effects of temperature. J. Shellfish Res., 21 (2002) 677–684.

Scarpa, J., Wada, W. and Allen, S.K., Jr., Pathenogenetic development of dwarf surf clam, *Mulina lateralis*, oocytes treated with polar body suppressing agents. Invert. Reprod. Develop., 22 (1992) 47–56.

Scarpa, J., Wada, K.T. and Komura, A., Induction of tetraploidy in mussels by suppression of polar body formation. Nippon Suisan Gakkaishi, 59 (1993) 2017–2023.

Schatten, G. and Schatten, H., Effects of motility inhibitors during sea urchin fertilization. Exp. Cell Res., 135 (1981) 311–338.

Stephano, J.L. and Gould, M.C., MAP Kinase, a universal suppressor of sperm centrosomes during meiosis? Dev. Biol., 222 (2000) 420–428.

Togo, T. and Morisawa, M. Mechanisms for blocking polyspermy in oocytes of the oyster *Crassostrea gigas*. J. Exp. Zool., 283 (1999) 307–314.

Washitani-Nemoto, S., Saitoh, C. and Nemoto, S., Artificial parthenogenesis in starfish eggs: Behaviour of nuclei and chromosomes resulting in tetraploidy of pathenogenotes produced by the suppression of polar body extrusion. Dev. Biol., 163 (1994) 293–301.

Yamamoto, S., Sugawara, Y., Nomura, T. and Oshino, A., Induced triploidy in Pacific oyster *Crassostrea gigas*, and performance of triploid larvae. Tohoku J. Agric. Res., 39 (1988) 47–59.

Yang, H., Zhang, F. and Guo, X., Triploid and tetraploid zhikong scallop *Chlamys farreri* Jones & Preston, produced by inhibiting polar body 1. Mar. Biotech., 2 (2000a) 466–475.

Yang, H., Que, H., He, Y. and Zhang, F., Chromosome segregation in fertilized eggs from zhikong scallop *Chlamys farreri* (Jones & Preston) following polar body 1 inhibition with cytochalasin B. J. Shellfish Res., 19 (2000b) 101–105.