Asian Fisheries Science **10** (1997):131-137
Asian Fisheries Society, Manila, Philippines
https://doi.org/10.33997/j.afs.1997.10.2.005

Triploidy and Tetraploidy Induction in the Chinese Mitten-Handed Crab (*Eriocheir sinensis*) by Cytochalasin B

L.Q. CHEN, Y.L. ZHAO, Y.F. WANG, W. CHEN, L.Y. TANG, N.S. DU AND W. LAI

Department of Biology
East China Normal University
Shanghai 200062, China

Abstract

Induction of triploidy and tetraploidy in the Chinese mitten-handed crab, *Eriocheir sinensis*, was successfully carried out for the first time by administration of cytochalasin B (CB) treatment to zygotes in January-April 1992 and January-May 1993, in Shanghai, China. Results of studies showed that CB treatment in chromosome manipulation was feasible for this species. In the treatment with 0.5-2.0 $mg \cdot l^{-1}$ CB, begun 10-20 minutes after insemination and lasting 10-20 minutes, the proportion of triploid embryos as judged from chromosome counts was 14.29-58.18%. Following the results of triploidy induction, tetraploids were also produced by 18-minute treatments with 1.5 $mg \cdot l^{-1}$ CB prior to first cleavage. The optimum CB treatment for inducing tetraploidy appeared to be 9.2 and 9.5 h post-fertilization at 20°C, which yielded 56.94 and 57.89% tetraploids, respectively. The ef-fects of CB treatment on the extent of embryo development are also discussed. Sterile trip-loids may continue to moult and grow after reaching maturity as diploid crabs. The suc-cessful induction of chromosome polyploidy in the crab has great scientific significance on conservation and modern breeding programs for this species.

Introduction

The Chinese mitten-handed crab *Eriocheir sinensis* is widely distributed in fresh and brackishwater ecosystems and has great potential for aquaculture in China. However, overfishing, severe pollution and irrigation activities threaten its survival. The abundance of this stock has been decreasing day by day. Artificial stock enhancement of the crab has been initiated to rebuild its natural population. Unfortunately, stocking juveniles in lakes is difficult to manage, and its effect on survival and yield is uncertain.

Crab culture in small waterbodies faces two major difficulties. One is the prematurity of 1- year old crabs, and the other is that 2-year old mature crabs are small compared to the wild ones. These lead not only to a decline in

its economic value, but also to the genetic adaptation of original gene pools being disrupted due to interbreeding between wild and cultured stocks. Chromosome manipulation can be used to solve these problems. So far, polyploidy has been successfully induced in over 20 species of fish and about 10 species of mollusc (Refstie et al. 1977; Stanley et al. 1981; Purdom 1983; Lou 1984; Bidwell et al. 1985; Fujino et al. 1987; Allen and Bushek 1992; Thorgaard 1992; Mair 1993). Some of these techniques have been used with great economic benefits. However, artificial induction of polyploidy has been reported in only two decapod species, i.e., *Penaeus chinensis* (Xiang et al. 1992) and *Eriocheir japonicus* (Lu et al. 1993). No study has yet been reported on chromosome manipulation in *E. sinensis*, the most economically and nutritionally important decapod species in China. This paper presents the induction of triploidy and tetraploidy in this species using cytochalasin B (CB).

Materials and Methods

Healthy and sexually mature crabs were purchased from a market in Shanghai from October to November of 1992 and 1993. Males and females were reared separately in aquaria with freshwater for 2-3 months before introducing single couples to separate aquaria containing aerated and filtered seawater with 1.0-1.2% salinity. Water temperature for mating and spawning was controlled at 20±1°C.

Cytochalasin B (Sigma Ltd.) was dissolved in a solution of dimethysulphoxide (DMSO) and made to a concentration of 10 $mg \cdot l^{-1}$ with ddH_2O. This stock solution was kept at 4°C prior to the experiments. Fresh seawater was then added to form 0.5, 1.0, 1.5 and 2.0 $mg \cdot l^{-1}$ concentrations of CB with 0.01% DMSO.

The spawning behavior of the females 10 - 20 h after copulation was observed closely. They usually spawned late in the evening or at dawn. Before beginning to spawn, the female cleans the non-plumose setae of her endopodites with her first thoracopods once or twice.

For triploidy induction, the procedure was as follows: 1) the carapace was opened when the females were about to or just beginnning to spawn; 2) unfertilized ripe eggs were collected from the ovary and mixed homogeneously with sperm from the spermatheca; 3) after 2-3 minutes, the inseminated eggs were washed twice with fresh seawater, and incubated in pylen mesh containers in a circulating seawater system at 20°C. For tetraploidy induction, the eggs were spawned naturally, and the beginning of the spawning period was considered as the fertilizing time.

Triploidy induction: 10-25 minutes after insemination, the batches of eggs were submitted to the CB concentrations of 0.5-2.0 $mg \cdot l^{-1}$ for 10-20 minutes, then returned to the hatchery aquaria. Control groups were maintained in a solution of 0.01% DMSO for the same length of time as the treatment groups. Each treatment was repeated at least thrice. In each test and control group, 2,000-3,000 inseminated eggs were used.

Tetraploidy induction: in various phases (8.5-9.8 h) after fertilization (a.f.), the females' abdomens with fertilized eggs attached were transferred to the CB solution of 1.5 $mg \cdot l^{-1}$ for 18 minutes, with the exposed eggs treated equally. The females were then returned to the hatchery aquaria. Several spawned crabs were treated in 0.01% DMSO as control.

The survival rates of embryos at the blastula and gastrula stages were estimated by examining 100-200 embryos randomly removed from each treatment (Du et al. 1992). Chromosome preparation was done according to the method of Hong (1987) with the following modifications: 1) the embryos were exposed to a 500 $ug \cdot ml^{-1}$ colchicine solution for 1-2 h at 14-16°C; 2) this solution was replaced with 0.1 M KCL hypotonic solution at 37°C; 3) after 40-50 minutes, the hypotonic solution was removed, and the embryos mixed with methanol-acetic acid (3:1); 4) then the solution was replaced with methanol-acetic acid (1:1); 5) several embryos were put on a clean slide, one or two drops of 50% glacial acetic acid was added for 3-5 minutes, then the embryos were minced gently with fine forceps; 6) after air-drying thoroughly, the chromosome preparations were stained with 15% Giemsa, then counted and photographed under the oil objective of an Olympus universal microscope.

Results

Triploidy Induction

In experiments 1 and 2, treatments using 1.5 $mg \cdot l^{-1}$ CB solution for 10-20 minutes, 10-25 minutes a.f., induced triploidy successfully (14.29-51.61%). Batches subjected to 1.0 and 1.5 $mg \cdot l^{-1}$ CB for 18 minutes, starting 15 minutes a.f., resulted in 53.83 and 58.18% triploidy, respectively, in experiment 3 (Table 1) . The chromosome number of triploid embryos in the experimental groups was about 219 (Fig. 1A), while in control embryos only diploids were found with about 146 chromosomes (Fig. 1B). The blastula survival rate decreased with increased concentrations of CB solution and duration of treatment. However, it was not directly linked to the commencement time of the CB treatment, although the survival rate of embryos in all experiment groups (80.85-85.23%) was lower than in control groups (91.95%).

Treatment with 0.5 $mg \cdot l^{-1}$ CB had almost no effect on the cleavage of developing eggs; but abnormal cleavage formation was observed when the CB concentration was increased (Fig. 1C). These abnormal eggs died at an early stage of development. No abnormal egg development was found in control groups. There was no difference between the experimental and control groups in the developmental speed of embryos at 20.

Tetraploidy Induction

After the success of triploidy induction, further work was carried out with tetraploidy induction using a concentration of 1.5 $mg \cdot l^{-1}$ CB. In the subsequent

Table 1. Relationship of cytochalasin B treatment with the rate of triploidy and the survival rate of the embryos at the blastula stage.

Experiment no.	Time after fertilization (minutes)	Duration (minutes)	Cytochalasin B concentration ($mg \cdot l^{-1}$)	S_1 (%) (C_1)	C_2	Percent triploid (%)
1	10	18	1.5	82.24(107)	22	31.82
	15	18	1.5	80.85(92)	31	51.61
	20	18	1.5	85.23(88)	49	40.82
	25	18	1.5	83.54(79)	40	20.00
Control	--	--	--	91.95(87)	42	--
2	15	10	1.5	87.25(102)	21	14.29
	15	15	1.5	85.84(113)	46	43.48
	15	18	1.5	84.69(98)	57	42.11
	15	20	1.5	75.76(99)	39	38.46
Control	--	--	--	93.55(93)	37	--
3	15	18	0.5	87.84(74)	47	14.89
	15	18	1.0	84.47(103)	39	53.85
	15	18	1.5	80.90(89)	55	58.18
	15	18	2.0	73.12(93)	62	37.10
Control	--	--	--	90.43(94)	42	--

S_1 : Survival rate = Survival of blastulae/inseminated eggs
C_1 : Blastulae counted
C_2 : Cells counted

series of experiments, the time from fertilization to beginning of treatment varied from 8.5 to 9.8 h. The percentage of tetraploids and the survival rates in the gastrula stage are shown in Table 2. The initial time of treatment obviously affected the percentage of tetraploids in treatments with 1.5 $mg \cdot l^{-1}$ CB for 18 minutes. The percentage of tetraploids was 56.94 and 57.89% for treatments starting after 9.2 and 9.5 h, respectively, following fertilization (Fig. 1D). Control embryos were found to have 146 chromosomes. The survival rate of embryos at the gastrula stage in these treated groups is not explicitly different from those in the control groups. Fertilized eggs in the experimental groups, particularly those treated with higher concentrations of CB, developed slower than those in control groups.

Discussion

This is probably the first report on successful induction of triploidy and tetraploidy in *E. sinensis* by CB. Because it provides a potential way to prevent prematurity in 1-year old crabs and miniaturization in 2-year old adults in artificial culture, it has great scientific significance in genetic conservation and modern polyploidy breeding of this species.

Unlike fish, mollusc and *Penaeus chinensis*, the fertilized eggs of crabs must attach themselves to the pleopods of the female for embryonic development. Artificial insemination was used in spawning (physiologically mature)

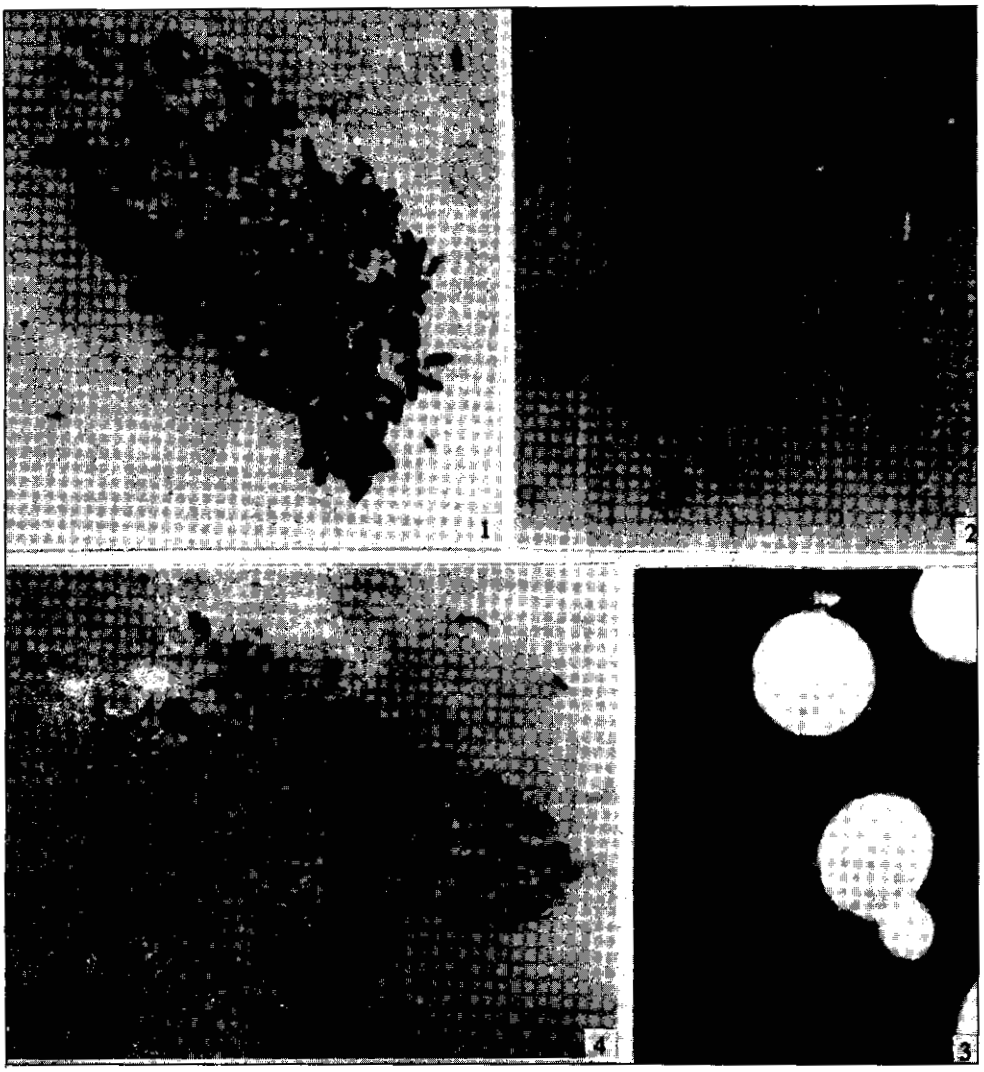

Fig. 1. A) Chromosomes of a triploid crab embryo treated with 1.0 mg·l^{-1} cytochalasin B 15 minutes after insemination for 18 minutes. B) The diploid of the control crab embryo with about 146 chromosomes. C) Abnormal cleavage of the crab embryo treated with 2.0 mg·l^{-1} cytochalasin B. D) The tetraploid of the crab embryo treated with 1.5 mg·l^{-1} cytochalasin B, 9.2 h after fertilization.

crabs to circumvent the difficulty of incubating the fertilized eggs *in vitro*. The suitable CB concentration and duration of treatment might be found by selective tests on triploidy induction. The efficiency of tetraploidy induction would then be improved. When the eggs attach firmly to the pleopods of females (8 h after oviposition), they do not come off easily after treatment with CB. This provides considerable savings in materials and test time.

Compared to thermal shock, the weaker stimulation from CB does not make the fertilized eggs attached to pleopods come off easily. Downing and Allen (1987) reported that CB treatment and thermal and pressure shock induce polyploidy in Pacific oyster by means of different mechanisms. CB treatment depresses formation of the microfilament net in the cell membrane, thus

Table 2. Relationship of cytochalasin B treatment with the rate of occurence of tetraploidy and survival rate of the embryos at gastrula stage.

Group	Time after fertilization (h)	Duration (minutes)	Cytochalasin B concentration ($mg \cdot l^{-1}$)	S_2 (%) (C_1)	C_2	Percent tetraploid (%)
Experiment	8.5	18	1.5	93.55(93)	112	29.46
	8.8	18	1.5	92.55(94)	94	43.62
	9.2	18	1.5	93.52(108)	72	56.94
	9.5	18	1.5	95.32(107)	76	57.89
	9.8	18	1.5	91.84(98)	51	35.29
Control	--	--	--	95.87(121)	72	--

S_2 : Survival rate = Survival of blastulae/inseminated eggs
C_1 : Gastrullae counted
C_2 : Cells counted

inhibiting cell division, but it has no effect on nucleus division. Thermal and pressure shock, on the other hand, inhibit both microfilaments and microtubles, and consequently stop not only the movement of chromosomes, but also cell division. It may be said that thermal and pressure shock completely block cell development. Thus, CB treatment is more effective in inducing polyploidy than thermal and pressure shock. In the present experiment, the highest percentage of triploids and tetraploids induced by CB was 58.1 and 57.89% respectively, a bit higher than induction by heat shock (50.00% for triploids and 52.69% for tetraploids) (Chen Liqiao et al. 1993, unpubl. paper). Further experiments are needed to determine the best combination of treatment parameters to optimize polyploid production.

Identification of starting time, CB concentration and duration of treatment are critical to polyploidy production and vary from species to species. The sensitive period for inducing first polar body retention is short and at the metaphase and anaphase of meiotic division I. Before this period, CB treatment would disrupt embryo development in fertilized eggs. After this sensitive period, the tendency of the first meiotic division is so strong that application of CB would fail to control and retain the first polar body. Unsuitable treatments damage fertilized eggs and fail to induce polyploidy. This experiment shows that administering CB to eggs 25 minutes a.f. led to a sharp decrease in the rate of triploids, only 20%. When fertilized eggs were subjected to 1.5 $mg \cdot l^{-1}$ CB solution for 18 minutes, earlier than 8.8 h or later than 9.8 h a.f., the incidence of tetraploidy induction was much lower than in treatment shocks 9.2-9.5 h a.f. Zhao et al. (1993) reported that cleavage speed of eggs is slower when hatching temperature is lower, so the long interval between fertilization and first mitotic karyokinesis of eggs might be more effective in inducing tetraploidy in this species.

When fertilized eggs were treated with 2.0 or 1.5 $mg \cdot l^{-1}$ CB for over 20 minutes, the number of deformed embryos increased and survival rate declined remarkably, the same results as in the polyploidy induction of mollusc and *Penaeus chinensis* (Stanley et al., 1981; Allen et al. 1982; Downing and Allen 1987; Bao et al. 1993). If a fertile tetraploidy strain can be built up, it can be crossed with normal diploid crabs to obtain sterile triploids. The induction of

tetraploidy may be more effective and feasible than triploidy in this species because of the characteristics of its propagational physiology.

Acknowledgments

The authors thank Dr. Yimin Ye, Fishery Resources Group at Shanghai Fisheries University, for constructive comments on the manuscript. We gratefully acknowledge the support of K.C. Wong Education Foundation, Hong Kong; and the Shanghai Young Stars of Science and Technology.

References

Allen, S.K., Jr. et al. 1982. Induced triploidy in the soft-shell clam: cytogenetic and allozymic confirmation. Journal of Heredity 73: 421-428.

Allen, S.K. and D. Bushek. 1992. Large-scale production of triploid oysters *Crassostrea virginica* (Gmelin) using "stripped" gametes. Aquaculture 103: 241-251.

Bidwell, C.A., C.L. Chrisman and G.S. Libey. 1985. Polyploidy induced by heat shock in channel catfish. Aquaculture 51(1): 25-32.

Bao, Z.M., Q.Q. Zhang, H. Wang, J. Gao and J.X. Dai. 1993. Studies on the triploid induction in *Penaeus orientalis* II. Cytochalasin B treatment. Acta Oceanologica Sinica 15(3): 101-106. (In Chinese with English abstract.)

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

Du, N.S., Y.L. Zhao and W. Lai. 1992. A study on the embryonic development of the Chinese mitten-handed crab, *Eriocheir sinensis* (Crustacia, Decapoda). Transactions of the Chinese Crustacean Society 3: 128-135. (In Chinese with English abstract.)

Fujino, K. et al. 1987. Temperature tolerance difference among normal diploid and triploid Pacific abalone. Bulletin of the Japanese Society of Scientific Fisheries 53: 15-21.

Hong, Y. 1987. A rapid sample preparation for one embryonic chromosome of fish. Freshwater Fisheries 140: 35-36 (In Chinese.)

Lou, Y. D. 1984. Studies on fish polyploid breeding abroad. Journal of Fisheries of China 8(4): 344-356. (In Chinese.)

Lu, R.H., J. Zhang, X. Liu and H.J. Yu. 1993. An experiment on induction of triploidy in *Eriocheir japonicus* hepuensis Dai by heat treatment. Chinese Journal of Oceanology and Limnology 110(1): 21-24.

Mair, G.C. 1993. Chromosome-set manipulation in tilapia - techniques, problems and prospects. Aquaculture 111: 227-244.

Purdom, C.E. 1983. Genetic engineering by the manipulation of chromosomes. Aquaculture 33(4): 287-300.

Refstie. T., V. Vassvik and T. Gjedrem. 1977. Induction of polyploidy in salmonids by cytochalasin B. Aquaculture 10: 65-74.

Stanley, J.G., S.K. Allen, Jr. and H. Hidu. 1981. Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin B. Aquaculture 23: 1-10.

Thorgaard, G.H. 1992. Application of genetic technologies to rainbow trout. Aquaculture 100: 85-97.

Zhao, Y.L., N.S. Du and W. Lai. 1993. Effects of different gradient temperature on embryonic development of the Chinese mitten-handed crab, *Eriocheir sinensis* (Crustacea, Decapod). Zoological Research 14(1): 49-53. (In Chinese with English abstract.)

Manuscript received 20 June 1996; accepted 2 May 1997.