Introduction

Giant clams are an important fishery resource specific to the tropical Indo West Pacific area (Heslinga et al. 1984, Shokita et al. 1991, Mingoa-Licuanan & Gomez 2007). They are utilized as ornamentation, food, tourism resource and companion animal (Knop 1996). To enhance the stock of giant clams depleted owing to overfishing and environmental destruction (e.g. Heslinga et al. 1984, Iwai et al. 2006), seed production has been developed in many countries (Shokita et al. 1991).

In a primitive method of seed production, multiple adults are allowed to release gametes in the same tank (e.g. Heslinga et al. 1984). Many of the adults release both sperm and eggs, since giant clams are simultaneous hermaphrodites (Wada 1952; Fig. 1A). This appears to have led to self-fertilization, considering previous reports: Benzie & Williams (1996) found allozyme patterns specific to self-fertilization in the seeds of the giant clam *Tridacna gigas* (Linnaeus) that had been produced with the primitive method. Murakoshi & Hirata (1993) showed that the mixture of the sperm and eggs released from the same individual of *Tridacna crocea* Lamark developed into D-shaped larvae. Because self-fertilization can cause deleterious effects (e.g. low survival, growth, and fecundity) in some organisms (e.g. Jarne et al. 1991), the primitive seed-production method for giant clams, probably causing self-fertilization, has been considered harmful (Ellis 1998).

To avoid self-fertilization, therefore, more sophisticated methods have recently been recommended (Crawford et al. 1986, Ellis 1998, Mingoa-Licuanan & Gomez 2007). Briefly, the methods are based on an apparent characteristic of giant clams that each adult individual ejaculates first and, after completely stopping ejaculation, release eggs. In the seed production method aimed at preventing self-fertilization, each adult clam is induced to ejaculate in a tank and then release eggs in another tank. Giant clams, however, have recently been suggested to continue ejaculation for a period after the beginning of egg release. The overlap between ejaculation and egg release might lead to self-fertilization in the tanks used for egg release, especially for the eggs released just at the beginning of spawning. We examined the possibility of such self-fertilization for the giant clam *Tridacna crocea* and obtained three results. (1) In observations with the naked eye in a laboratory, 2 of 38 *T. crocea* simultaneously ejaculated and released eggs. (2) In a laboratory experiment, 1.5 to 80.0% of eggs released from each adult clam developed into D-shaped larvae without artificial cross-fertilization. Such development occurred more frequently for the eggs released earlier from each adult clam than for the eggs released later from the clam. (3) In observations at a hatchery, 2 to 94% of the eggs released from 4 of 5 adults were found to develop into D-shaped larvae without artificial cross-fertilization. The three results suggest that at least some *T. crocea* adults continue ejaculation for a period after starting spawning eggs, which causes self-fertilization.

Key words: aquaculture, selfing, tridacnid

Abstract: Giant clams are simultaneous hermaphrodites and are assumed to ejaculate first and, after completely stopping ejaculation, release eggs. In the seed production method aimed at preventing self-fertilization, each adult clam is induced to ejaculate in a tank and then release eggs in another tank. Giant clams, however, have recently been suggested to continue ejaculation for a period after the beginning of egg release. The overlap between ejaculation and egg release might lead to self-fertilization in the tank used for egg release, especially for the eggs released just at the beginning of spawning. We examined the possibility of such self-fertilization for the giant clam *Tridacna crocea* and obtained three results. (1) In observations with the naked eye in a laboratory, 2 of 38 *T. crocea* simultaneously ejaculated and released eggs. (2) In a laboratory experiment, 1.5 to 80.0% of eggs released from each adult clam developed into D-shaped larvae without artificial cross-fertilization. Such development occurred more frequently for the eggs released earlier from each adult clam than for the eggs released later from the clam. (3) In observations at a hatchery, 2 to 94% of the eggs released from 4 of 5 adults were found to develop into D-shaped larvae without artificial cross-fertilization. The three results suggest that at least some *T. crocea* adults continue ejaculation for a period after starting spawning eggs, which causes self-fertilization.

Key words: aquaculture, selfing, tridacnid
of stimulants (e.g. serotonin, macerated gonads of giant clams) into the tank. (2) Some of the clams begin ejaculation, and each is temporarily transferred to a different “sperm tank” from which released sperm is used later for artificial cross-fertilization. (3) Some of these ejaculating adults on starting to release eggs are moved to one of the egg tanks prepared for several adults. The eggs released therein are artificially cross-fertilized with sperm of other adults gathered in sperm tanks. In the hatchery observations, however, we collected eggs without artificial cross-fertilization. This method has been believed to prevent self-fertilization in giant clams (Ellis 1998).

It has, however, not been examined whether this widely-used method (Fig. 1B) actually prevents self-fertilization in giant clams. The method might still allow self-fertilization. This is inferred from Murakoshi & Hirata (1993): giant clams might continue to ejaculate for a while after starting to spawn eggs (Fig. 1A). If so, an adult clam possibly ejaculates when its eggs are collected, which leads to self-fertilization. The possibility is especially high, when its eggs start to be collected just after the egg release begins and ejaculation might continue.

To examine whether or not the widely-used method for seed production (Fig. 1B) completely prevents self-fertilization in T. crocea, we carried out the following. (1) Spawning-behavior observations: these examined whether T. crocea can continue to ejaculate for a while even after starting to spawn eggs. (2) Individual-stimulation experiment: this tested whether the eggs of T. crocea in the laboratory develop into D-shaped larvae without artificial cross-fertilization. The experiment also tested whether the eggs released earlier from a clam develop into D-shaped larvae more frequently than the eggs released later from the same clam (Fig. 1C). (3) Hatchery observations: these checked whether the eggs of T. crocea produced in a hatchery also develop into D-shaped larvae without artificial cross-fertilization. Based on the experiment and observations, we discuss the possibility of self-fertilization occurring in hatcheries.
Materials and Methods

Tridacna crocea

Adult *Tridacna crocea* were collected in Sekisei Shoko Lagoon, Okinawa, Japan (24.4°N, 124.0°E). In Okinawa, *T. crocea*>55 mm in shell length matures as bisexual (Shokita et al. 1991). In hatcheries, *T. crocea* typically begins ejaculation within 1 d after stimulation, continuing ejaculation for several min to 1 h. Several min to 1 d after apparent stopping of the ejaculation, the clam begins spawning eggs and continues spawning for several min to 1 h. The eggs develop into trochophores and D-shaped larvae (“D-larvae”, hereafter) about 15 h and 20 h, respectively, after artificial cross-fertilization (Jameson 1976, Shokita et al. 1991).

Spawning-behavior observations

Spawning-behavior observations were made 15 times in total between May 2007 and October 2008. For each time, 4 to 19 *T. crocea* with shell length 61.2 to 127.9 mm (155 individuals in total) were put into a “stimulation tank” (volume: 100 to 2001, Fig. 1B) including FSW (i.e. sea water filtered through sand and anthracite). The clams then were stimulated through drainage of FSW from the tank and through addition of frozen sperm and eggs that had been taken from other *T. crocea*. Each specimen that began releasing eggs was moved to a different “egg tank” (Fig. 1B) and whether the clam still continued ejaculation was checked with the naked eye.

Individual-stimulation experiment

The individual-stimulation experiment was conducted at Ishigaki Tropical Station, Seikai National Fisheries Research Institute. We used four different clams (Adults 1 to 4) in total, two on 20 June 2008 and another two on 13 July 2008 (shell length: 77.74 to 100.58 mm). For each adult, we prepared a different stimulation tank to avoid the incorporation of other adults’ sperm and egg tanks 1 to 3 to collect eggs at the intervals after initiation of egg release (Fig. 1C). The clams were stimulated through exposure to air (30.7 to 31.8°C) for about 1 h. Each of the clams was then put separately into a different stimulation tank (Fig. 1C) including FSW (30.4 to 32.1°C). These clams then were further stimulated with frozen sperm and eggs. The sperm and eggs were dead, because several months before the experiment they had been released from clams and had been left in FSW for 1 day to become inactive prior to being frozen. In the individual-stimulation experiment, clams given these stimulants began ejaculation 1 to 4 h later and, further, egg release after the ejaculation. Each clam on beginning to release eggs was taken out from the stimulation tank and was washed with flowing FSW for about 5 s to clear sperm off the surface of the clam. The clam was then moved to egg tanks in the order of egg tank 1, 2 and 3 and was left to release eggs for 2 to 30 min in each egg tank. During the egg release, whether the clams still continued ejaculation was checked with the naked eye. From each egg tank, eggs were collected and kept at a density of about 3 eggs ml⁻¹ in five 18-ml bottles (28.6 to 29.1°C). Twenty hours later, the offspring were fixed with 1% formalin and were categorized into eggs, trochophores and D-larvae under a microscope.

Hatchery observations

The hatchery observations were conducted on 23 to 25 August 2007 during the actual process of seed production (Iwai et al. 2006) at Ishigaki Branch, Okinawa Prefectural Fisheries and Ocean Research Center. *T. crocea* (N=19, shell lengths=93.7 to 115.3 mm) were stimulated generally in the above-mentioned manner. But the clams were put all together in one stimulation tank, as is typical for seed production (Ellis 1998, Fig. 1B). Of these clams, five specimens (shell lengths=93.7 to 115.3 mm) ejaculated and began to release eggs after stopping ejaculation visible to the naked eye. Each of the clams beginning to release eggs was transferred separately to a different egg tank after the body was washed with flowing FSW for about 5 s. Some of the eggs in egg tanks were sampled before being artificially cross-fertilized for seed production. The eggs sampled were kept in a 30-ml bottle (26.7 to 31.9°C) for each adult clam at a density of about 170 ml⁻¹. These eggs were fixed with 1% formalin 20 h later. For each bottle 33 to 335 offspring were randomly chosen and were categorized into eggs, trochophores and D-larvae under a microscope.

Statistical analyses

In the individual-stimulation experiment and hatchery observation, the count data for each developmental stage were used to calculate the D-larvae development rate (the number of D-larva/the number of offspring at all the stages). Mean D-larvae development rate was defined as the number of D-larvae/the number of offspring at all the developmental stages. In the individual-stimulation experiment, a mixed-effects logistic regression (Crawley 2002) was performed for each adult clam to test whether the D-larvae development rate gradually decreases after the initiation of egg release:

\[
p_{x_i} = \frac{1}{1 + \exp \left( -\mu - \beta t - \epsilon_i \right)}
\]

where \( p_{x_i} \): expected D-larvae development rate for egg-release timing \( t \) and replication \( i \), \( \mu \): intercept, \( \beta \): coefficient for egg-release timing, \( \epsilon_i \): random effect varying across replication. The count data were fitted with the “glmmML” package (Broström 2008).
Results

Spawning-behavior observations

Of 155 *Tridacna crocea* stimulated, 38 clams spawned eggs. Of the 38 clams, 2 specimens were considered to continue ejaculation even after beginning egg release, since the two were found to release white, suspended material after starting to spawn eggs.

Individual-stimulation experiment

Some of the eggs spawned from each adult clam developed into D-larvae (Fig. 2). Mean D-larvae development rate varied between 1.5% and 80.0% among clams. Mean D-larvae development rate was higher for the eggs spawned earlier from each adult clam than for the eggs spawned later from the adult. Such a decrease according to egg-release timing was significant (Table 1; mixed-effects logistic regression, $\beta = -0.10$, $P < 0.001$), except for Adult 1 ($P = 0.779$) which spawned eggs in only two egg tanks. Visual observations with the naked eye revealed that Adult 2 ejaculated while releasing eggs.

Hatchery observations

D-larvae developed from the eggs spawned from 4 of 5 adult clams (Table 2). For these eggs, the D-larvae development rate varied from 1.8 to 94.0% among adult clams.

Discussion

Eggs of the giant clam *Tridacna crocea* developed into D-larvae with no artificial cross-fertilization in the individual-stimulation experiment. The causes of such development possibly include (1) self-fertilization, (2) cross-fertilization, and (3) parthenogenesis. Of these, self-fertilization is the most likely cause of the development of D-larvae for the following reasons.

(1) Self-fertilization: In the individual-stimulation experiment the D-larvae development rate was higher for the eggs spawned earlier from each adult clam than for the eggs spawned later from the adult. This finding conforms to the result of the spawning-behavior observations that at least some adult giant clams continue to ejaculate for a period even after beginning to release eggs (Fig. 1A). Such overlap between ejaculation and egg release is also suggested by Murakoshi & Hirata (1993). The sperm and eggs released from the same adult clam in the laboratory and hatcheries will cause self-fertilization, as demonstrated for giant clams including *T. crocea* (Murakoshi & Hirata 1993, Benzie & Williams 1996).

(2) Cross-fertilization: during the individual-stimulation experiment, each adult clam had no chance of obtaining and storing sperm from other adults because the clam was kept in a stimulation tank with no other adults (Fig. 1C). Therefore, to cross-fertilize its eggs, each adult should have received sperm from other adults and stored the sperm until spawning eggs in the experiment. This is unlikely since giant clams are not reported to have a sperm reservoir that stocks sperm from different adults (Harrison & Kohn 1997).

(3) Parthenogenesis: although parthenogenesis is found for a few bivalves (*Lasaea* spp.; Thiriart-Quivvreux et al. 1988, Taylor & Ó Foighil 2000), there seems to be no bivalve species reported to carry out both parthenogenesis and cross-fertilization. Therefore, *T. crocea*, which cross-fertilizes, is rather unlikely to carry out parthenogenesis. Further, it is unlikely that the development of D-larvae due to parthenogenesis occurs more frequently for the eggs...
clams and D-larvae. (2) The second study needed is to determine whether the self-fertilization causes deleterious effects (e.g. low survival, growth, and fecundity). The effect is unclear at present. Although Ellis (1998) and Mingoa-Licuanan & Gomez (2007) have suggested low survival rate of larvae, they have given no data. (3) The third study needed is to determine how to avoid self-fertilization, if it is harmful. A promising method is to collect eggs in a later phase of egg-release behavior during which the self-fertilization rate appears to be lower (Fig. 2). Another promising method is to introduce sperm of an adult into an egg tank before, not after, another adult is induced to spawn eggs in the tank. This should increase the chance that the introduced sperm reaches the eggs faster than the sperm ejaculated from the egg-releasing adult.

### Acknowledgements

We thank Kenzo Yoseda and Taku Sato for their comments on this paper; Kenji Iwai, Hirofumi Kubo, and Nobuhiro Ooshiro for their information on aquaculture of giant clams; and the staff of Seikai National Fisheries Research Institute and Okinawa Prefectural Fisheries and Ocean Research Center for their logistic support and advice on this study.

### References

Benzie JAH, Williams ST (1996) Limitations in the genetic variation of hatchery produced batches of the giant clam, *Tridacna gigas*. Aquaculture 139: 225–241.

Boudry P, Collet B, Cornette F, Hirvouet V, Bonhomme F (2002) High variance in reproductive success of the Pacific oyster (*Crassostrea gigas*, Thunberg) revealed by microsatellite-based adult age analysis of multifactorial crosses. Aquaculture 204: 283–296.

Broström G (2008) glmmML: Generalized Linear Models with
Clustering. http://cran.r-project.org/web/packages/glmmML/index.html (accessed on 1 July 2008)

Crawford CM, Nash WJ, Lucas JS (1986) Spawning induction, and larval and juvenile rearing of the giant clam, *Tridacna gigas*. Aquaculture 58: 281–295.

Crawley MJ (2002) Statistical Computing. An Introduction to Data Analysis Using S-Plus. John Wiley & Sons, West Sussex, 761 pp.

Ellis S (1998) Spawning and Early Larval Rearing of Giant Clams (Bivalvia: Tridacnidae). Center for Tropical and Subtropical Aquaculture. http://www.ctsa.org/upload/publication/CTSA_130631672860873095404.pdf (accessed on 22 July 2008)

Harrison FW, Kohn AJ (1997) Mollusca II. Microscopic Anatomy of Invertebrates. Vol. 6A, Wiley-Liss, New York, 414 pp.

Heslinga GA, Perron FE, Orak O (1984) Mass culture of giant clams (f. Tridacnidae) in Palau. Aquaculture 39: 197–215.

Iwai K, Kiso K, Kubo H (2006) Biology and status of aquaculture for giant clams (Tridacnidae) in the Ryukyu Islands, southern Japan. In: Proceedings of the Regional Technical Consultation on Stock Enhancement for Threatened Species of International Concern (eds Primavera JH, Quintio ET, Eguia MRR), 13–15 July 2005, Iloilo City, Philippines. Southeast Asian Fisheries Development Center, pp. 27–38.

Jameson SC (1976) Early life history of the giant clams, *Tridacna crocea* Lamark, *Tridacna maxima* (Röding) and *Hippopus hippopus* (Linnaeus). Pac Sci 30: 219–233.

Järne P, Finot L, Delay B, Thaler L (1991) Self-fertilization versus cross-fertilization in the hermaphroditic freshwater snail *Bulinus globosus*. Evolution 45: 1136–1146.

Knop D (1996) A Comprehensive Guide to the Identification and Care of *Tridacnid* Clams. Dühne Verlag, Ettlingen, 255 pp.

Mingoa-Licuanan SS, Gomez ED (2007) Giant Clam, Hatchery, Ocean Nursery and Stock Enhancement: Aquaculture Extension Manual No. 37. Southeast Asia Fisheries Development Center, Iloilo, 109 pp.

Murakoshi M, Hirata H (1993) Self-fertilization in four species of giant clam. Nippon Suisan Gakkaishi 59: 581–587.

R Development Core Team (2006) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. http://www.R-project.org. (accessed on 3 July 2009)

Shokita S, Kakazu K, Tomori A, Toma T, Yamaguchi M (1991) Aquaculture in Tropical Areas. Midori Shobo, Tokyo, 360 pp.

Taylor DJ, Ó Foighil D (2000) Transglobal comparisons of nuclear and mitochondrial genetic structure in a marine polyploid clam (*Lasaea*, *Lasaeidae*). Heredity 84: 321–330.

Thiriot-Quivvreux C, Soyer J, De Bovee F, Albert P (1988) Unusual chromosome complement in the brooding bivalve, *Lasaea consanginea*. Genetica 76: 143–151.

Wada SK (1952) Protandric functional hermaphroditism in the tridacnid clams. Oceanogr Mag 4: 23–30.