# Factors Affecting the Fertilization Success in Laboratory Hybridization between *Haliotis discus hannai* and *Haliotis gigantea*

Author(s): Xuan Luo, Caihuan Ke, Weiwei You and Dexiang Wang
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# FACTORS AFFECTING THE FERTILIZATION SUCCESS IN LABORATORY HYBRIDIZATION BETWEEN *HALIOTIS DISCUS HANNAI* AND *HALIOTIS GIGANTEA*

**XUAN LUO,[1,2] CAIHUAN KE,[1,2,*] WEIWEI YOU[1,2] AND DEXIANG WANG[1,2]**

[1]*State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, PR China;*
[2]*Department of Oceanography, Xiamen University, Xiamen, 361005, PR China*

***ABSTRACT*** In this study, effects of sperm concentration and gamete age on fertilization success of *Haliotis discus hannai* (D) × *H. gigantea* (G) were investigated. Results showed that the fertilization rates of heterologous crosses *H. discus hannai*♀ × *H. gigantea*♂ (DG) and *H. gigantea*♀ × *H. discus hannai*♂ (GD) were consistently lower than those of homospecific groups *H. discus hannai*♀ × *H. discus hannai*♂ (DD) and *H. gigantea*♀ × *H. gigantea*♂ (GG). In sperm concentration experiments, the sperm concentrations that yielded maximum fertilization rates with the least abnormality in subsequent development were $4.66 \times 10^7$ sperm/mL for the DG cross and $2.6 \times 10^7$ sperm/mL for the reciprocal cross GD. In gamete age experiments, the optimal fertilization rates were achieved in heterologous crosses when freshly spawned ova were fertilized with sperm that were released within 0.5 h. Furthermore, in heterospecific crosses, when ova were fertilized 10 min after being spawned, fertilization rates declined significantly with increasing ova age. It is suggested to use fresh gametes and higher sperm concentrations for hybridization between *H. discus hannai* and *H. gigantea*.

***KEY WORDS:*** abalone, *Haliotis discus hannai*, *Haliotis gigantea*, hybridization, artificial fertilization, sperm concentration, gamete age

## INTRODUCTION

Aquaculture of Pacific abalone (*Haliotis discus hannai*) has become increasingly popular in Northeast Asia, especially in China, because of its consistent high demand and market value (Zhang et al. 2004). To meet the increasing commercial demand, abalone farming of *H. discus hannai* has grown rapidly in China since the 1980s (Guo et al. 1999). Several problems emerged in the Pacific abalone industry associated with long-term cultivation, such as seed quality degeneration, slow growth rates, and decline of disease resistance. These problems may be the result of inbreeding depression, environmental deterioration, and improper culture methods in the abalone aquaculture industry. Genetic improvement such as selection and hybridization should be applied to overcome these obstacles.

*H. gigantea*, which is called Xishi abalone in China, is a valued commercial species along the coast of Japan. This species was introduced from Japan to China for mariculture in 2003 because of its excellent disease resistance and taste (Gao et al. 2000, Luo et al. 2006). These traits make it a potential species for abalone mariculture in China.

Hybridization can increase productivity through hybrid vigor, produce animals that are sterile, or combine desirable characteristics found in one species with those of another (Chevassus 1983, Hedgecock 1987, Benzie et al. 1995, Rahman et al. 2005). Interspecific hybrids among various abalone species have the potential to gain positive heterosis in terms of growth, survival, and adaptation to particular environmental conditions (Owen et al. 1971, Leighton & Lewis 1982, Koike et al.1988, Hoshikawa et al. 1998). Laboratory crosses of *H. discus discus* and *H. gigantea* have demonstrated that $F_1$ hybrids can be produced with low fertilization rates (Ahmed et al. 2008). *H. discus hannai* and *H. discus discus* are two closely related subspecies, but can be distinguished by means of 18S rDNA sequences (Naganuma et al. 1998). Therefore, it is possible that hybridization between *H. discus hannai* and *H. gigantea* would occur when these 2 abalone species are cultured in the same environment. For hybridization to occur, either prezygotic or postzygotic, barriers to gene exchange must be overcome. Some effective studies on sea urchins have provided insight that might help to identify those critical data needed to begin to estimate gamete interactions between closely related species (Levitan et al. 1992, Levitan 1998, Rahman & Uehara 2004). However, available information concerning factors influencing fertilization success in hybridization among abalone species is also limited. In the current study, we investigate how sperm concentration and gamete age might affect fertilization success in both intra- and interspecific crosses between *H. discus hannai* and *H. gigantea*.

*Corresponding author. E-mail: chke@xmu.edu.cn

## MATERIALS AND METHODS

### *Broodstock*

The broodstock of *H. gigantea* (G) was collected from Nagasaki, Japan, and the broodstock of the Pacific abalone *H. discus hannai* (D) was collected from Dalian City, Liaoning Province, China. Each sample was transported to the Jiefeng Abalone Farm, Fuzhou City, China. The 2 species were placed separately in 2 20-$m^3$ concrete tanks. Abalone was fed to satiation with *Laminaria japonica* and water was changed daily.

A random sample of 40 mature males and 60 mature females from each of the 2 species was selected for spawning. Individual abalone were placed in separate containers to prevent inadvertent fertilization. The mature abalone were then induced to spawn with the procedures described by Zhang et al. (2004).

### *Experimental Design*

A series of experiments was conducted to develop a better understanding of factors affecting the fertilization success for both conspecific and heterospecific crosses. Cross-fertilization between *H. discus hannai* and *H. gigantea* was conducted using a 2 × 2 factorial mating from each species. Two reciprocal crosses (DG, *H. discus hannai*♀ × *H. gigantea*♂ and GD, *H. gigantea*♀ × *H.*

*discus hannai*♂) and 2 purebred controls (DD, *H. discus hannai*♀ × *H. discus hannai*♂ and GG, *H. gigantea*♀ × *H. gigantea*♂) were produced. All experiments were replicated 3 times with different parents in each replicate to reduce the chance that results were dependent on the quality of gametes from any 1 abalone.

As spawning occurred, gametes were pooled within each sex and species. Ova from individual females were siphoned through a 200-mm mesh into 500-mL glass beakers and examined under a microscope. Only batches of ova that were well rounded (approximately spherical) and undamaged were retained. The ova were gently rinsed 3 times in filtered, sterilized seawater to remove any extraneous gonadal material. Concentration of ova was determined volumetrically using a stereomicroscope. A total of 500 ova from each species was then placed in 500-mL glass beakers with 100 mL 5-μm filtered, ultraviolet-irradiated seawater.

Sperm were poured into a 200-ml beaker and observed for motility using a microscope. The density of sperm suspensions was quantified 3 replicates with a hemocytometer.

### *Sperm Concentration Experiments*

In the experiment on sperm concentration, ova from each species were fertilized within 10 min postspawning using freshly spawned sperm. The appropriate volume of stock sperm suspension was added to make final concentrations in different crosses. In heterospecific crosses (DG and GD), the final sperm concentrations were $8.67 \times 10^5$ sperm/mL, $2.15 \times 10^6$ sperm/mL, $3.85 \times 10^6$ sperm/mL, $6.77 \times 10^6$ sperm/mL, $8.66 \times 10^6$ sperm/mL, $2.6 \times 10^7$ sperm/mL, $4.66 \times 10^7$ sperm/mL, $6.75 \times 10^7$ sperm/mL, $8.77 \times 10^7$ sperm/mL, and $2.07 \times 10^8$ sperm/mL. In conspecific crosses (DD and GG), the final sperm concentrations were $7.5 \times 10^2$ sperm/mL, $2.2 \times 10^3$ sperm/mL, $6.0 \times 10^3$ sperm/mL, $2.0 \times 10^4$ sperm/mL, $4.0 \times 10^4$ sperm/mL, $7.33 \times 10^4$ sperm/mL, $3.0 \times 10^5$ sperm/mL, $6.77 \times 10^5$ sperm/mL, $2.15 \times 10^6$ sperm/mL, and $4.77 \times 10^6$ sperm/mL[1]. Each treatment had 6 replicates, and the temperature for all crosses was 18.5 ± 0.5°C.

### *Gamete Age Experiments*

To compare the effects of ova age on fertilization rate for intra- and interspecific crosses, fresh conspecific or heterospecific sperm at an optimal concentration (obtained from the previous experiment) were added separately to ova of each species. In DG and GD crosses, ova from each species were artificially fertilized 10, 20, 30, 40, 50, 60, 70, 80, and 90 min postspawning using freshly spawned heterospecific sperm in each container. Moreover, in conspecific crosses (DD and GG), ova were fertilized at 0.5, 1, 2, 3, 4, 5, and 6 h postspawning using freshly spawned conspecific sperm at a final optimal sperm concentration in each container.

For each species, sperm of different ages (0.5, 1, 2, 3, 4, 5, and 6 h postspawning) were used to fertilize conspecific or heterospecific ova within 10 min of ova being spawned. Each treatment had 6 replicates and was conducted at 19 ± 0.5°C.

### *Sampling*

Fertilization rate and hatching rate were assessed by counting 3 random samples from each cross, with each sample consisting of about 50 eggs. After 1–1.5 h postfertilization, the eggs of all crosses were collected and examined microscopically in each experiment. The eggs were either classified as fertilized eggs if they had at least reached the 4-cell stage, or classified "unfertilized" if development had not occurred. The hatching rate was evaluated as soon as the larvae hatched (about 15 h postfertilization, 19°C), and only normal trochophore larvae were contribute to the hatching rate.

### *Statistical Analysis*

For all experiments, fertilization rate and hatching rate were arcsine transformed for statistical analyses, and then means and errors back-transformed for presentation. Differences among the mean fertilization rate and hatching rate in each of the 4 crosses were compared using a 1-factor analysis of variance, followed by a multiple comparison test (Tukey method).

## RESULTS

### *Effects of Sperm Concentration*

Figures 1 and 2 show effects of different sperm concentrations on fertilization rate and hatching rate of conspecific and heterospecific crosses of *H. discus hannai* and *H. gigantea*. In conspecific crosses, there was a rapid increase in the proportion of fertilized eggs when sperm concentrations reached $10^4$ sperm/mL (Fig. 1). At a sperm concentration of $4.0 \times 10^4$ sperm/mL in the DD crosses, fertilization rate generally exceeded 85%. Similar results were observed for other intraspecific mating with *H. gigantea* sperm and eggs, where fertilization rate exceeded 80% for almost all trials when sperm concentrations reached $7.33 \times 10^4$ sperm/mL. However, there were sharp declines of fertilization rate in conspecific crosses when sperm concentrations were higher than $3.0 \times 10^5$ sperm/mL in the DD crosses and $6.77 \times 10^5$ sperm/mL in the GG crosses. At sperm concentrations more than $4.0 \times 10^4$ sperm/mL in DD cross, hatching rate declined. The same trend was also found in the GG crosses' hatching rate reduced when sperm concentration exceeded $3.0 \times 10^5$ sperm/mL.

In heterologous crosses, there was a rapid increase in fertilization rate when sperm concentrations reached $10^6$ sperm/mL, but fertilization rates decreased sharply at higher concentrations, with a range of $6.75 \times 10^7$–$2.07 \times\times 10^8$ sperm/mL (Fig. 1). Low fertilization rates were obtained in the DG crosses using sperm concentrations from $8.67 \times 10^5$–$6.77 \times 10^6$ sperm/mL. The eggs from *H. discus hannai* were unable to be

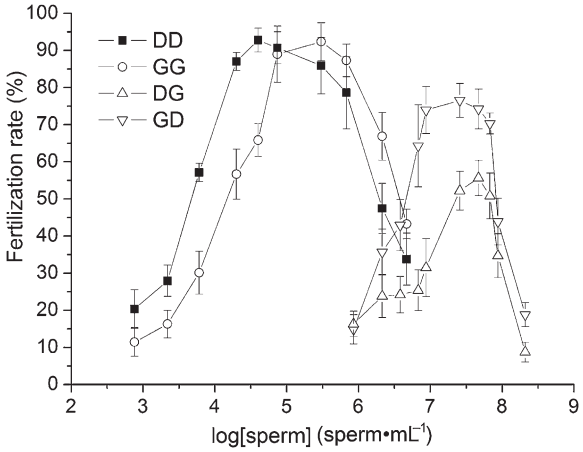


**Figure 1. Fertilization rates (mean ± SD) as a function of log-transformed sperm concentration for conspecific and heterospecific crosses between *H. discus hannai* and *H. gigantea*.**

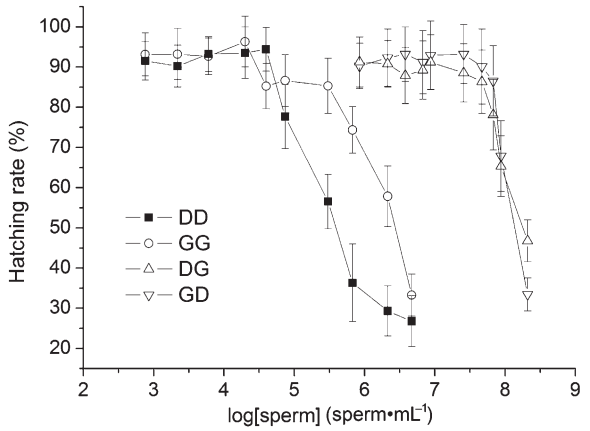


**Figure 2. Hatching rates (mean ± SD) as a function of log-transformed sperm concentration for conspecific and heterospecific crosses between *H. discus hannai* and *H. gigantea*.**

fertilized using sperm from *H. gigantea* when the sperm concentration was less than $4.0 \times 10^5$ sperm/mL. Fertilization rate of the DG crosses peaked at 55.7 ± 4.79% when sperm concentration was $4.66 \times 10^7$ sperm/mL (Fig. 1), and hatching rates of the DG crosses were not significantly different ($P > 0.05$) for sperm concentrations ranging from $5.25 \times 10^5$–$4.66 \times 10^7$ sperm/mL (Fig. 2). In the GD cross, sperm concentrations between $8.66 \times 10^6$ sperm/mL and $6.75 \times 10^7$ sperm/mL gave consistent fertilization rates (more than 75%) with no significant differences ($P > 0.05$). Average hatch rates in the GD crosses exceeded 85% for sperm concentration between $8.67 \times 10^5$ sperm/mL and $6.75 \times 10^7$sperm/mL, but declined steeply at higher sperm concentrations (Fig. 2).

### *Effects of Gamete Age*

Figures 3 and 4 show fertilization rate and hatching rate of ova fertilized at different time postspawning using sperm within 20 min of its release. Fertilization rate in conspecific crosses exceeded 90% for ova less than 2 h old, but declined with further ova aging.

In heterospecific crosses, fertilization rate and hatching rate decreased sharply if ova were fertilized after 20–40 min postspawning. In DG crosses, the highest fertilization rate was

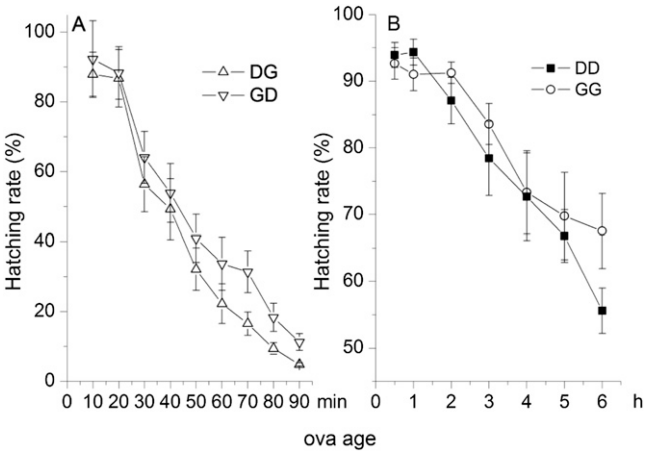


**Figure 4. (A, B) Hatching rates (mean ± SD) of conspecific (A) and heterospecific (B) crosses between *H. discus hannai* and *H. gigantea* using different levels of ova age at optimal sperm concentration.**

57.5 ± 5.96% when eggs were cross-fertilized with fresh *H. gigantea* sperm 10 min postspawning. Subsequently, fertilization rate in DG crosses fell to less than 30% when ova were fertilized after 40–50 min postspawning (Fig. 3), and hatching rate also reduced with ova aging (Fig. 4). Similar to DG crosses, fertilization rate and hatching rate of GD crosses varied greatly at different ova ages. The fertilization rate and hatching rate reached 75.1 ± 10.99% and 92.2 ± 9.56%, respectively, for ova that were fertilized 10 min after they were spawned. Subsequently, fertilization rate of GD crosses decreased to 5.4 ± 5.97% when fresh sperm were added to ova 70 min after they were spawned.

The effect of sperm age on fertilization and hatch rates is shown in Figures 5 and 6. Fertilization rates of conspecific GG crosses and both heterospecific crosses (DG and GD) noticeably declined when ova were fertilized with sperm ≥2 h old (Fig. 5), and hatching rates of heterospecific crosses (DG and GD) decreased sharply with sperm aging (Fig. 6). *H. discus hannai* sperm lost their potency after 4 h in DD crosses, whereas in GG crosses, *H. gigantea* sperm usually lost their potency after 3 h. The conspecific crosses gave higher fertilization and hatching rates than the heterospecific crosses, and the GD crosses appeared more successful than the DG crosses (Figs. 5 and 6).

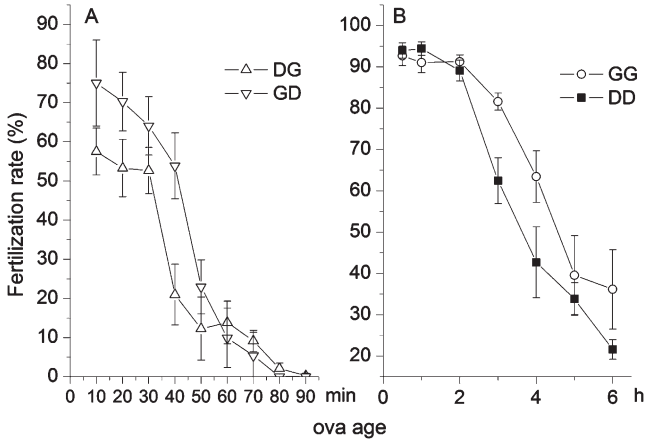


**Figure 3. (A, B) Fertilization rates (mean ± SD) of heterospecific (A) and conspecific (B) crosses between *H. discus hannai* and *H. gigantea* using different levels of ova age at optimal sperm concentration.**

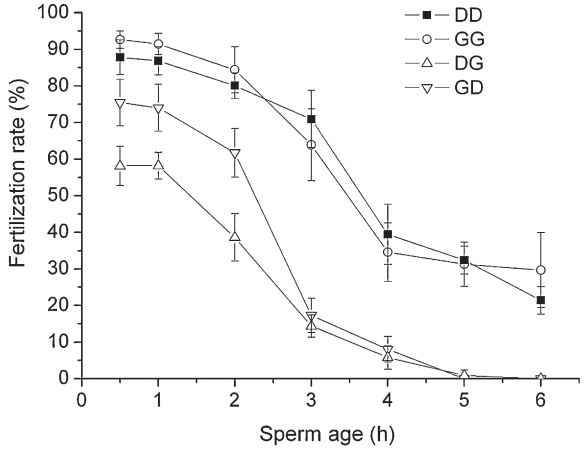


**Figure 5. Fertilization rates (mean ± SD) in *H. discus hannai* (DD), *H. gigantea* (GG), and their reciprocal hybrids (DG and GD) using different levels of sperm age.**

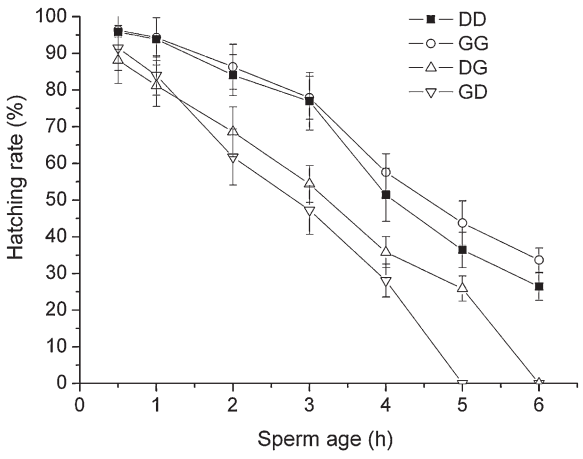


**Figure 6. Hatching rates (mean ± SD) of *H. discus hannai* (DD), *H. gigantea* (GG), and their reciprocal hybrids (DG and GD) using different levels of sperm age.**

## DISCUSSION

Gamete fertilization is a critical step in hybridization. The affinity between heterologous gametes is determined by a number of factors, including sperm concentration and gamete longevity. The results of this study supported previous findings that abalone sperm could more easily attach to conspecific ova than to heterospecific ones (Leighton & Lewis 1982). Similar results have been obtained in other invertebrate groups such as sea urchins (Rahman et al. 2000) and oysters (Lyu & Allen 1999). It has been suggested that this is caused by the binding of the acrosome or the glycoprotein of the eggs, which could be different in 2 distinct species. Previous investigations of gamete recognition have also suggested that the membrane proteins may play a role in mediating sperm–egg interactions. Surface-bound proteins can promote species-specific fertilization, and variation in such factors may be sufficient to drive reproductive isolation (Palumbi 1994, Biermann 1998). Reproductive isolation can be generated through both pre- and postzygotic processes. Prezygotic mechanisms include ecological separation, behavioral separation, and gametic incompatibility. Postzygotic processes generally involve lowered viability of hybrids or hybrid sterility (Arnold 1997). It is well known that abalone show broadcast spawning behavior (Murayama 1935, Shepherd 1986) and it is, therefore, not surprising that hybrids may form among sympatric species (Palumbi 1994). The natural occurrence of hybrids among California abalones has been studied since the 1970s (Owen et al. 1971). Leighton and Lewis (1982) demonstrated that hybrid fertilization among California abalone required prompt addition of sperm to newly spawned eggs (Leighton & Lewis 1982). In our study, we also found the sperm concentration used to fertilize the heterospecific eggs was higher than that used in conspecific crosses. Compared with conspecific controls, the hybrid crosses had lower fertilization rates for a given sperm concentration. This may be the result of the low gamete affinity for *H. discus hannai* and *H. gigantea*. The results of our gamete age experiment under laboratory conditions demonstrated that sperm lost their potency within 1–2 h in heterospecific crosses, and increasing egg age also decreased fertilization success even though the eggs were cross-fertilized using freshly released sperm at an optimal sperm concentration. It was suggest that the rapid decrease in the potency of gametes in heterospecific crosses compared with that in conspecific crosses might decrease the likelihood of cross-fertilization in the field. As a free-spawning marine invertebrate, asynchronous spawning and the combined effect of lower concentrations and aging of gametes might contribute to the reproductive isolation of *H. discus hannai* and *H. gigantea* where they co-occur.

We also noticed that a high percentage of *H. gigantea* ova were fertilized by *H. discus hannai* sperm, but, in the reciprocal cross, a relative lower fertilization success was produced even when *H. discus hannai* ova were fertilized with freshly released *H. gigantea* sperm. Similar asymmetrical gametic incompatibility between 2 species of *Haliotis* had been found in experimental hybridization of California abalone (Leighton & Lewis 1982). Asymmetrical blockage to fertilization was also shown in hybrid oyster crosses. Banks et al. (1994) reported 1-way genetic incompatibility; *Crassostrea sikamea* eggs × *C. gigas* sperm formed viable hybrids. However, *C. sikamea* sperm could not fertilize *C. gigas* eggs. Lyu and Allen (1999) also found a significant difference in gamete binding between *C. gigas* and *C. virginica*, and suggested that the reciprocal difference in fertilization level between *C. gigas* and *C. virginica* might be explained by the reciprocal differences in acrosome reaction. In the current study, we found that abnormal development of zygotes occurred much at higher sperm concentration (>$2.6 \times 10^7$ sperm/mL) in heterospecific crosses than in conspecific controls (>$4.0 \times 10^4$ sperm/mL). Polyspermy resulting from high sperm density could be the cause of the abnormal development.

In conclusion, the results of this study indicate that sperm concentration of $4.66 \times 10^7$ sperm/mL and $2.6 \times 10^7$ sperm/mL are required for maximal fertilization and normal trochophore development in DG and GD crosses, respectively. For practical purposes, it is recommended to use fresh sperm with an optimal sperm concentration to fertilize ova within 10 min of their release.

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