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Hybridization between *Haliotis rufescens* and *Haliotis discus hannai*: evaluation of fertilization, larval development, growth and thermal tolerance

Fabiola Lafarga-De la Cruz, Gustavo Núñez-Acuña & Cristian Gallardo-Escárate

Laboratorio de Biotecnología y Genómica Acuícola, Departamento de Oceanografía, Centro de Biotecnología, Universidad de Concepción, Concepción, Chile

Correspondence: C Gallardo-Escárate, Departamento de Oceanografía, Centro de Biotecnología, Universidad de Concepción, Casilla 160-C, Concepción, Chile. E-mail: crisgallardo@udec.cl

Abstract

Two introduced abalone species are currently produced in Chile, red abalone *Haliotis rufescens* and Japanese abalone *Haliotis discus hannai*. However, red abalone accounts for 99% of total production, while the Japanese abalone has not adapted well to Chilean coastal waters. This study reports the hatching, growth and thermal tolerance performance in interspecific hybrids produced between red (R) and Japanese (J) abalone. Our results show that egg age and sperm concentration were critical factors to produce hybrids. The cross R♀ × J♂ showed a fertilization rate of 55.3 ± 3.5% using 20-min-old eggs and sperm concentrations of 14×10^6 cells mL^{-1}, while the reciprocal cross (J♀ × R♂) was not successful. Further, larval development stages were similar in RR, JJ and RJ hybrid abalones. Among the experimental trials, settlement rate varied from 12.3% to 18.6% and final survival from 20.1% to 31.7%, being the RJ hybrid rates intermediate between parental species. The final shell lengths were similar between RR and RJ hybrids, but significantly higher in JJ abalones. In addition, thermal tolerance was ascertained due its pivotal role for the abalone physiology. Thus, RJ hybrids showed the highest *HSP70* gene expression and offers new possibilities to expand Chilean abalone production in warm waters zones.

Keywords: *Haliotis rufescens*, *Haliotis discus hannai*, hybridization, hatchery performance, *HSP70*

Introduction

The Chilean abalone industry is supported by two species: the red abalone *Haliotis rufescens* from California and the Japanese abalone *Haliotis discus hannai* (Flores-Aguilar, Gutierrez, Ellwanger & Searcy-Bernal 2007). By 2010, total production had reached 843 tons, with an estimated value of US$ 27.1 millions, establishing Chile as the fifth-largest producer of abalone worldwide (FAO 2011). The red abalone accounts for practically 99% of total production due to its good adaptation to intensive culture conditions in northern and southern Chile (Enríquez & Villagrán 2008). In contrast, Japanese abalone production has not reached important levels due to the poor performance and lower resistance of the species to intensive production conditions, with only two farms out of the 25 now in operation. However, the Japanese abalone is produced intensively in others parts of the world as Korea (An, Lee, Kim & Myeong 2011). Improvements to abalone aquaculture worldwide have been achieved mainly through genetics and selective breeding (Elliott 2000; Kijima, Li & Park 2002; Zhang & Liu 2006). There have been advances recently through selection, crossbreeding, hybridization, polyploidy, gynogenesis and gene manipulation (You, Ke, Cai, Wang & Wang 2005; Cai, Ke, You, Wang, Wang & Wang 2007; Dunstan, Elliott, Appleyard, Holmes, Conod, Grubert & Cozens 2007; Kube, Appleyard & Elliott 2007). Interspecific hybridization has been used as a conventional breeding method in many fish and

shellfish, such as carp, catfish, salmonid, oyster, and crayfish, to improve production traits (i.e. growth and survival rates and disease resistance) and to manipulate sex ratios (Refstie 1983; Harrell 1997; Lawrence & Morrissy 2000; Hulata 2001). In abalones, interspecific hybridization has shown the potential to increase production through adaptation to particular environmental culture conditions and desired traits for the industry (Robinson, Li & Hayes 2010). Experimental abalone hybridization has been carried out for more than 50 years with multiple purposes such as basic research, genetic and ecological studies, phylogeny studies and finally for aquaculture production. Since then, natural and artificial hybrids among *Haliotis* species account for more than 50 crosses reported worldwide (Lafarga-De la Cruz & Gallardo-Escárate 2011).

Several abalone hybrids have shown positive heterosis (*hybrid vigour*), such as faster growth, higher survival rates and/or wider temperature tolerance ranges than at least one of the parental species (Lafarga-De la Cruz & Gallardo-Escárate 2011). On the other hand, even when some interspecific hybrids do not exhibit positive heterosis for any trait, they may still be of interest for aquaculture purposes because of a good combination of other beneficial traits from the parental species (Elliott 2000; Leighton 2000; Hulata 2001). Additionally, intra- and interspecific hybridization could help to reduce some inbreeding effects, like high larval deformity rates, low metamorphosis success and low juvenile and adult survival rates (Deng, Liu, Zhang & Guo 2005; Kobayashi & Kijima 2009).

The target species of our study, *H. rufescens* (California) and *H. discus hannai* (Japan) are allopatric species that are closely related and are grouped into a North Pacific abalone species clade (see Streit, Geiger & Lieb 2006). The two species have been used before to produce interspecific hybrids among several species in California and Japan (Leighton & Lewis 1982; Inoue, Kito, Uki & Kikuchi 1986; Hoshikawa, Sakai & Kijima 1998; Wang & Fan 1999). Furthermore, Wang and Fan (1999) published the first brief report describing the artificial hybridization between *H. rufescens* and *H. discus hannai*, establishing the feasibility of producing reciprocal crosses, but with very low fertilization rates (1.8–2.1%) and higher growth rates than the parental specie *H. discus hannai*. The goal of this research was to evaluate rates of fertilization, hatching, settlement, growth, survival and yield recovery of an abalone hybrid species, as well as morphometrics and gene expression of *HSP70* (response to thermal stress).

Materials and methods

Broodstock conditioning

Three hundred red abalones and 800 Japanese abalones composing our broodstock were obtained from commercial abalone farms located in northern and southern Chile (Spinetech Chile S. A., 26° 55′S–70°48′W; Chiloé Island farm 42°53′S–73° 28′W and Cultivos Marinos San Cristóbal S. A., 26°57′S–70°48′W). Abalones were measured, weighed, tagged and maintained in 100 L tanks (1 kg 50 L^{-1}) by species and sex, in a flow-through seawater system. The average shell length (SL) of Japanese broodstock was 70 ± 7.6 mm and the average weight (W) was 51.5 ± 17.7 g. Average SL and W were 96 ± 6.0 mm and 175.1 ± 41.1 g, respectively, for red abalone broodstock. The feeding regime started 2 days after reception, supplying 5% of wet body weight once daily for a week, after which it was increased to 15% of wet body weight. A mixed diet was provided containing 90% brown algae *Macrocystis integrifolia* and 10% red algae *Gracilaria chilensis*. Mean conditioning temperatures for red and Japanese abalone were 16 ± 1°C and 18 ± 1°C, respectively, while salinity was 32–34 ‰ and dissolved oxygen ranged from 7.2 to 8.5 mg L^{-1}. Maturation was assessed every 2 weeks, using the Visual Gonad Index (VGI) which is a semi-quantitative expression of macroscopically observable gonad growth (size and shape) with four categories (0–3) according to the following criteria: 0 – sex indistinguishable; 1 – sex distinguishable, thin gonad with pointed tip; 2 – gonad partially enlarged with pointed tip; 3 – gonad swollen with rounded tip (Kikuchi & Uki 1974; Ebert & Houk 1984).

Spawning induction

Gravid abalones with a grade 3 VGI were selected from the broodstock tanks and placed separately on trays covered for 1-h desiccation under room temperature (about 20°C). Then, abalones were distributed into 8 L containers with 1-μm-filtered UV-irradiated seawater (FSW), where spawning was artificially induced by the TRIS-H_2O_2 protocol as described by Morse, Duncan, Hooker and Morse

(1977). Males were usually induced 30 min before females to ensure sperm availability in the quantities required for hybrid fecundation of an order of magnitude of 10^7 spermatozoids mL^{-1} (Leighton 2000; Luo, Ke, You & Wang 2010). After 2.5 h, the animals were washed gently with clean water and containers were filled with clean FSW.

Egg age and sperm concentration effects on fertilization and hatching success

Previous experiments showed that JR offspring were not reproducible, with high degree of variation among the trials and more than 5% of success (data not published). Consequently, we focused our experiments on the RJ abalone hybrids. The effect of egg age on RJ hybrid fertilization and hatching success was tested by using fresh Japanese abalone sperm to fertilize red abalone eggs of 10, 20, 30 and 60 min postspawning (four treatments). Crosses of both parental species were used as controls [*H. rufescens* ♀ × *H. rufescens* ♂ (RR) and *H. discus hannai* ♀ × *H. discus hannai* ♂ (JJ)] and as experimental cross *H. rufescens* ♀ × *H. discus hannai* ♂ (RJ hybrid). Nine red abalones (6♀; 3♂) and 12 Japanese abalones (8♀; 3♂) were induced to spawn with the procedure described previously. After 5 min postspawning, eggs were collected and pooled by species. Immediately after, they were cleaned in a series of sieves of different mesh sizes (200/150/100 μm) and eggs retained at 150 μm were recovered and distributed at average egg densities of 146.5 ± 23 eggs mL^{-1} into 18 L fertilization tanks (by triplicate per egg age treatment). Fertilization was carried out at different times (10, 20, 30 and 60 min egg age), using sperm concentrations of $2.5 \pm 0.2 \times 10^6$ cells mL^{-1} for crosses RR and JJ and $12.5 \pm 0.8 \times 10^6$ cells mL^{-1} for the RJ hybrid cross in a room with controlled temperature at 17°C. The fertilization contact time was 2 min, after which fertilized eggs were carefully washed with fresh FSW several times, and fertilization tanks were filled and kept water-static until larval hatching. Fertilization and hatching rates were recorded at 2- and 16-h postfertilization respectively. The calculation formulae were as follows: Fertilization rate (%) = [total number of fertilized eggs in the first cleavage (2 cells)/total number of eggs] × 100; Hatching rate (%) = [total number of hatching larvae (trocophora)/total number of fertilized eggs in first cleavage (2 cells)] × 100.

The effect of sperm concentration on RJ hybrid fertilization and hatching success was tested by fertilizing red abalones eggs of 20 min postspawning (egg age) with five Japanese abalone sperm concentrations: 1.4 ± 0.2, 2.8 ± 0.4, 5.6 ± 0.7, 11.2 ± 0.5 and $14.1 \pm 1.4 \times 10^6$ cells mL^{-1}. As control, to assess egg quality and viability, a pure cross of red abalone *H. rufescens* ♀ × *H. rufescens* ♂ (RR) was performed using $1.4 \pm 0.15 \times 10^6$ cells mL^{-1}. Eight red abalones (5♀; 3♂) and three Japanese abalones (3♂) were induced to spawn. At 10-min postspawning, red and Japanese abalone spermatozoids were collected separately, then directly counted with Neubauer chamber and distributed at required concentrations. Subsequently, after 5-min postspawning, red abalone eggs were collected, washed, classified (150 μm) and dosed at densities of 100 ± 10 eggs mL^{-1} in 2 L fertilization containers (in triplicate per treatment). Fertilization was carried out simultaneously for each spermatic concentration treatment when eggs were 20 min of age at 17°C. Fertilization contact time was 2 min, after which fertilized eggs were carefully washed with fresh FSW several times, and containers were filled and kept water-static until larval hatching. Fertilization and hatching rates were record and analysed as described above.

Embryonic and larval development of hybrid abalones

Embryonic and larval development of RJ hybrid abalones and RR abalones (control) were record during 7 days (168 h). Five red abalones (3♀; 2♂) and three Japanese abalones (3♂) were induced to spawn, and fertilization was carried out using spermatozoids concentrations of 1.0 ± 0.1 and $10.5 \pm 0.7 \times 10^6$ cells mL^{-1} of red and Japanese abalone, respectively; 20-min postspawning eggs and 2 min of fertilization time at 17°C, under conditions described above. After hatching, trocophore larvae were transferred to 80 L tanks with constant low water-flow at 17 ± 1°C, where they were daily washed and treated with Ditral 12 antibacterial hydro solution (0.2 mL L^{-1} of seawater; Veterquimica®; Biovac, Santiago, Chile). Observations of cleavage, embryo and larval development were recorded using a microscope Nikon Eclipse 80i (10X, 40X; Nikon Instruments Inc., Melville, NY, USA) and images were randomly captured using a digital monochromatic camera Nikon DS-Qi1(Nikon

Instruments Inc.). According to Courtois De Viçose, Viera, Bilbao and Izquierdo (2007), time required to reach different development stages were recorded for each cross. Observations started immediately after fertilization, cleavage development was record continuously within the first 5 h, and after that embryonic development was recorded each 1 h until larvae age of 30 h. However, from day 2 (48 h) to day 7 (168 h), larvae development was registered on a daily base. Size measurements of embryos and larvae were record using NIKON IMAGING SOFTWARE NIS180 ELEMENTS (version 3.1). The daily growth rate (DGR) of larva, for the period of 7 days, were calculated with DGR (μm day^{-1}) = $[(S_i - S_f)/t]$, where S_f, S_i are the final larva size and initial embryo size, respectively, and t is the time between measures in days (Hopkins 1992).

Performance of hybrid abalones under commercial culture conditions

The RJ hybrid abalone growth performance was evaluated for 461 days (January 2009–April 2010) in Caldera, III Region (26°55′S–70°48′W). Twenty-two red abalones (15♀; 7♂) and 21 Japanese abalones (15♀; 6♂) were selected from the farm's broodstock to produce RR, JJ and RJ hybrid abalones. Spawning and fertilization procedures were carried out using 20-min-old eggs, 2 min of fertilization time, $1.5 \pm 0.2 \times 10^6$ cells mL^{-1} for RR and JJ crosses, and $11.2 \pm 0.3 \times 10^6$ cells mL^{-1} to produce RJ hybrids, at 17°C water temperature. Newly hatched trocophore larvae were reared in 80 L upwelling tanks with constant low water-flow at 16.5 ± 0.5°C. On the sixth day of larval culture, when the third tubule on cephalic tentacle was observed (Hanh 1989), veliger larvae were induced to settle on polycarbonate plates, preconditioned with benthic diatoms, in 5 m^3 settlement tanks (36 holders/720 plates at a seed density of 352 ± 50 larvae per plate) with no water-flow and moderate air-flow. After 48 h, water-flow was restored and the settlement rate was evaluated by counting 0.5% of randomly selected plates in the settlement tank, and calculated using the following equation: settlement rate (%) = [total number of spat/total number of veliger larvae] × 100. The commercial conditions involve procedures for the equalization or calibration or abalone's densities per tanks to avoid differences in growth due to density. Herein, after settlement stage each experiment trial was subdivided in at least five tanks with densities of 800.000 spats $tank^{-1}$ and feeding regime of benthic diatoms and brown macroalgae (*M. integrifolia* at 1% of wet body weight daily). At day 150, juvenile abalones were transferred to 5 m^3 grow-out tanks with shelters (25 000 abalones $tank^{-1}$) and fed with a fresh mixed diet of 90% brown algae *M. integrifolia* and 10% red algae *G. chilensis* at 10% of wet body weight daily.

These measures correspond to the cultivation under commercial conditions. Shell length measurements and abalone densities were recorded at 461 days. Shell length was measured with a vernier calliper to the nearest 0.02 mm of randomly selected abalones (n = 80) from the tanks, and abalone densities were determined sampling and counting 0.5% of plates or shelters randomly selected (20% was added considering abalones attached to the wall and floor surfaces of the tanks). Growth was reported as the specific growth rate (SGR) and the daily increase in shell length (DISL), with SGR equations (% day^{-1}) = $[(\text{Ln } SL_f - \text{Ln } SL_i)/t] \times 100$; and DISL (μm day^{-1}) = $(SL_f - SL_i)/t$, where Ln is the natural log, SL_f, SL_i are final and initial mean shell length, respectively, and t is the time in days (Hopkins 1992; Mai, Zhu & Wu 2007). The yield recovery rate (%) was calculated as the density of abalones in t time divided by the total number of fertilized eggs (day 0) × 100 The survival rate (%) was calculated as the density of abalones in t time divided by total larval settlement (spat; day 8) × 100.

Additionally, morphometric shell measurements were taken from RR, JJ and RJ hybrid abalones (n = 50 for each group). Six measurements were recorded: SL, shell breath (SB), shell height (SH), distance between apex and first respiratory pore (DAFRP), distance between apex and third respiratory pore (DATRP) and distance between first and third respiratory pore (DFTRP) (Park, Hur, Im, Seol, Hur, Park, An, Kim & Han 2008). The number of open respiratory pores (ORP) and shell weight (SW) were also recorded. Data were standardized as calculated ratios (%) among the measured traits: SW:SL, SB:SL, SH:SB, DAFRP:SB, DFTRP:SB and DATRP:SB.

Thermal tolerance response and genetic certification

To evaluate thermal tolerance, *Heat Shock Protein70 (HSP70)* gene expression was performed

according to Cheng, Liu, Zhang and Deng (2006). Juveniles of red, Japanese and RJ hybrid abalones ($n = 18$) were randomly selected from grow-out tanks and placed separately in 18-L tanks. Three abalones for each group were exposed to 1-h heat shock at different temperatures: 20, 22, 24, 26, 28 and 30°C. Control temperature was at 18°C. Then, individual epipodium samples were collected and stored at −80°C for the RNA isolation procedure. In addition, hybrid abalones were genetically certified using molecular DNA markers *Hco97*, *Awb036* and *Hruf300* according to Lafarga-De la Cruz, Amar-Basulto and Gallardo-Escárate (2010).

Statistical analysis

All percentage data (fertilization, hatching, survival) were square-root arcsine transformed prior to statistical analysis (Zar 1999). To compare growth and morphometric trait ratios, data expressed in millimetres were used directly. Data normality was tested using the Shapiro–Wilk test and homogeneity of variances with the Levene test. After that, a one-way ANOVA was performed to test differences in the variables measured among the three-abalone groups (RR, JJ and RJ hybrid). Finally, *a posteriori* a Tukey test was used to compare mean values when the one-way ANOVA showed statistically significant differences at a 95% confidence level ($\alpha = 0.05$; Dixon & Massey 1983). Statistical analyses were carried out using STATISTICA 6.1 (StatSoft).

Results

Effects of egg age and sperm concentration

Fertilization rates for control groups varied from 79% to 90%, and no significant differences were observed with respect to egg age until 30-min postspawning. With eggs beyond this age, the fertilization rate decreased to 33% in JJ crosses (Fig. 1a). Whereas the RJ hybrid cross had presented fertilization rates significantly different from those of the RR and JJ crosses at all tested egg ages. The RJ hybrid fertilization rate decreased from 64% to 15%, with an increase in egg age from 10 to 60 min (Fig. 1a), but no statistical differences were observed using eggs of 10, 20 and 30 min. For all crosses, hatching rates did not present a tendency according to egg age (Fig. 1b). Instead, they varied from 91–93% (10–30 min) to 85% (60 min) for the RR cross and from 95–97% (20, 30 min) to 77–85% (10, 60 min) for the JJ cross. For the RJ hybrid, hatching rates ranged from 11% (60 min) up to 72% (20 min), being significantly different ($P < 0.05$) from the other crosses.

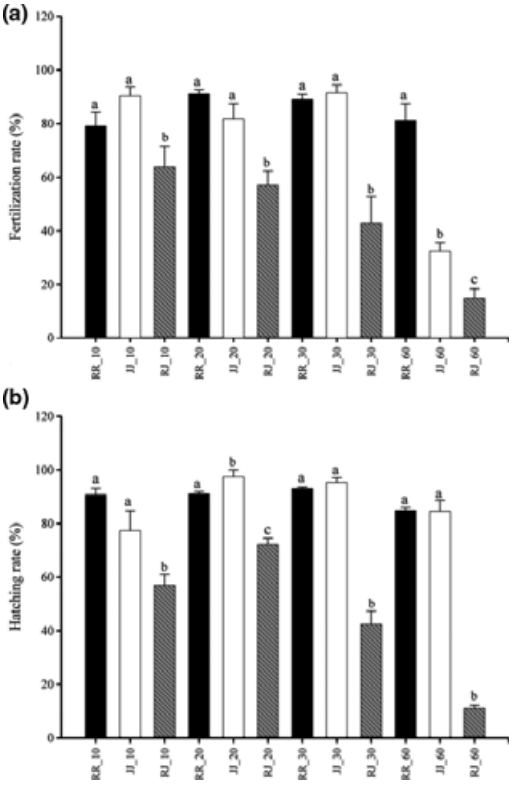

Figure 1 Fertilization (a) and hatching (b) rates for red abalone *Haliotis rufescens* (RR), Japanese abalone *Haliotis discus hannai* (JJ) and *H. rufescens* ♀ × *H. discus hannai* ♂ (RJ hybrid). The *x*-axis represents crosses fertilized at different egg ages (10, 20, 30 and 60 min). Standard error and differences among treatments are shown ($P \leq 0.05$).

To test sperm concentration on fertilization and the hatching rate, we used the RR cross as control group fertilized at a normal sperm concentration (10^6 cells mL^{-1}). Fertilization and hatching rates for the RR cross were 75.9 ± 4.5% and 98.1 ± 0.6% respectively. The RJ hybrid fertilization rate showed a tendency to increase from 13% to 55%, as sperm concentration increased from 1.4 to 14 × 10^6 cells mL^{-1} (Fig. 2a). Between sperm concentrations of 1.4 and 11.2 × 10^6 cells mL^{-1}, no significant differences were observed in the RJ hybrid fertilization rate ($P > 0.05$), being on average 18.3 ± 1.5%. On the other hand, the RJ

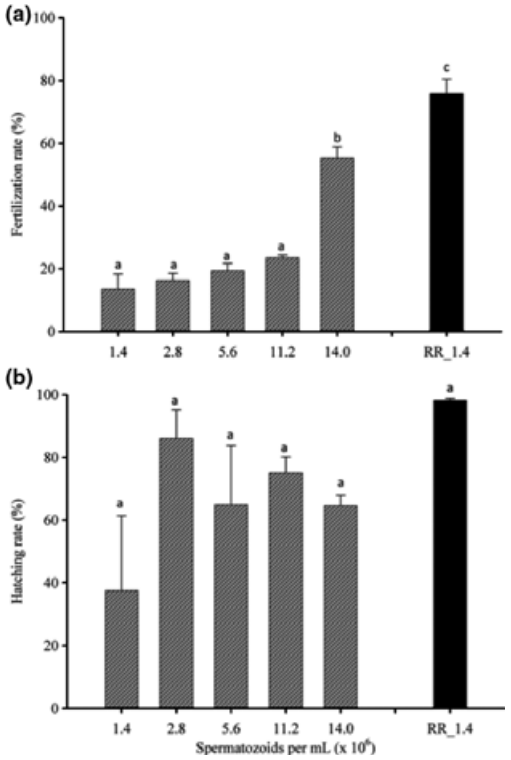

Figure 2 Fertilization (a) and hatching (b) rates for *Haliotis rufescens* ♀ × *Haliotis discus hannai* ♂ (RJ hybrid) at increasing concentrations of Japanese abalone spermatozoids ($\times 10^6$ mL^{-1}). Standard error and differences among treatments are shown ($P \leq 0.05$).

hybrid hatching rates did not show any trend related to sperm concentrations, but were significantly lower in the minimal concentration used with the highest standard error deviation (37.5 ± 23.9%; Fig. 2b). No significant differences were observed among RJ hybrid treatments and the RR control ($P = 0.0581$) because of the high level of data dispersion (Fig. 2b). In the treatment with the maximum fertilization rate (14.0×10^6 cells mL^{-1}), the RJ hybrid hatching rate was 64.6 ± 3.2%.

Embryonic and larval development of RJ hybrids

Cleavage and embryonic and larval development of RR, JJ and RJ hybrid abalones were recorded in terms of daily size and the time required to reach the different development stages (Table 1). The fertilization rate was significantly higher for the RR cross, which reached 85 ± 5%, while for RJ hybrid it was 54 ± 3%. The hatching rate was 64.3 ± 3%, with no significant differences among treatments ($P > 0.05$). Recently fertilized red eggs had a mean size of 218.6 ± 0.55 μm, with no differences among treatments. During 7 days of culture, statistical differences on larvae size among groups were observed on days 1, 3 and 5 ($P = 0.0463$; 0.00007; 0.0168). However, the final larvae size was similar, reaching a mean size of 261.8 ± 2.6 μm and a mean DGR of 6.2 μm day^{-1}. During the first 30 h, the required time to reach different cleavage and embryonic development stages, no relevant differences were presented (less than 15 min) among RR, JJ and RJ hybrid abalones, considering that around 80% of the population had reached each evaluated stage. Likewise, daily larval development stages were similar among treatments. However, for RJ hybrid abalones, a 10–15% of fertilized eggs stopped development after the first meiotic division and 15–20% of the hatching larvae showed an abnormal development and a characteristic erratic swimming behaviour.

Performance of hybrid abalones under commercial culture conditions

Fertilization rates for the parental RR and JJ control crosses were 84.3 ± 3.4% and 82.2 ± 3.3%, respectively, with no significant differences between them ($P = 0.9513$). The fertilization rate of the RJ hybrid was significantly lower, at 42.5 ± 3.1% ($P = 0.0001$). Hatching rates varied from 63.3% to 74.8% among treatments with no significant differences ($P = 0.2019$). Settlement rates were 18.6% for the JJ cross, 16.7% for the RJ hybrid cross, and 12.3% for the RR (see the summary in Table 2).

These results correspond to the cultivation under commercial conditions. The yield recovery rate, calculated in relation to total fertilized eggs, decreased drastically during larval development (day 0–6) and the settlement period (day 6–8) to 64.3 ± 1.3% and 10.1 ± 1.0% respectively (Fig. 3a). Final yield recovery rate values (at day 461) were 3.0%, 2.6% and 2.4% for the RJ hybrid, RR and JJ abalones respectively. The survival rate, calculated in relation to settled larvae (spat, day 8), tended to decrease to 55.7 and 40% during the first 90 days of culture for RR and RJ hybrid abalones, while the JJ abalones had a 94.7% survival rate for that period (Fig. 3b). At day 150, when juvenile abalones were transferred to grow-out tanks, survival rates were 31.7%, 50.6% and 77.2% for RJ hybrid, RR and JJ abalones respectively. Final sur-

Table 1 Embryonic and larval development rate of RR, JJ and RJ abalones at 17 ± 1°C

		Time (h:min)		
Seq*	Larval development stage	RR	JJ	RJ
1	Fertilization (*in vitro*)	0:00	0:00	0:00
2	Discharge of first polar body	0:15	0:17	0:14
3	Discharge of second polar body	0:24	0:26	0:20
4	First cleavage (2 cells)	0:53	0:55	0:53
5	Second cleavage (4 cells)	1:42	1:50	1:57
6	Third cleavage (8 cells)	2:21	2:30	2:29
7	Fourth cleavage (16 cells)	2:48	2:51	2:54
8	Fifth cleavage (32 cells)	3:18	3:22	3:43
9	Morula	6:18	6:26	6:24
10	Blastula	7:47	7:55	7:54
11	Gastrula	8:26	8:30	8:38
12	Appearance of cilia forming the prototochal girdle	9:27	9:36	9:35
13	Stomodeum	11:36	11:51	11:50
14	Complete formation of prototochal girdle and cilia	12:15	12:20	12:28
15	Trocophore larvae in the egg membrane ready to hatch out	13:38	13:42	13:50
16	Larval shell formation	17:00	17:05	17:12
17	Veliger larvae exhibiting flat apical region and completely developed velum with cilia	22:44	22:48	22:57
18	Appearance of larval retractor muscle	30:00	30:23	30:15
19	Appearance of integumental attachment to larval shell	–	–	–
20	Development of foot mass	–	–	–
21	Appearance of eye spot	–	–	–
22	Completion of larval shell	–	–	–
23	90-degree torsion of the cephalo-pedal mass	–	–	–
24	180-degree torsion of the cephalo-pedal mass	–	–	–
25	Spines at the end of metapodium and formation of operculum	–	–	–
26	Operculum	46:14	46:20	47:12
27	Appearance of cilia on the foot sole	–	–	–
28	Vertical groove formation in velum	–	–	–
29	Appearance of propodium	–	–	–
30	Appearance of cilia on propodium	73:40	73:52	74:00
31	Appearance of cephalic tentacles	94:14	94:25	95:34
32	Appearance of cilia in mantle cavity	–	–	–
33	Appearance of apophysis on propodium	–	–	–
34	Formation of epipodal tentacles	–	–	–
35	Appearance of otolith	–	–	–
36	Appearance of spines on cephalic tentacles	–	–	–
37	Protrusion of snout underneath the velum	–	–	–
38	Appearance of two tubules on cephalic tentacles	117:35	117:39	117:45
39	Third tubule appearance on cephalic tentacles	146:00	146:05	146:10

–, Time not measured.
*Sequence according to Courtois De Viçose *et al.* (2007).

vival rates at day 461 were 31.7% for RR abalones, followed by 28.6% for RJ hybrid and 20.1% for JJ abalones (Table 2 and Fig. 3b).

For 461 days of commercial culture, RR, JJ and RJ hybrid abalone growth curves were recorded in respect to SL (mm) as shown in Figure 3c. With respect to fertilized egg and larval size (day 0–6), no significant differences were observed among treatments ($P = 0.9823$). Subsequently, the growth curves among treatments showed significant differences ($P < 0.05$), the SL measurements being lower than those of RJ hybrid abalones (Fig. 3c). However, on day 461, RR and RJ hybrid abalones showed similar final SLs of 33.5 ± 0.6 and 34.1 ± 0.8 mm, respectively, whereas the final SL of JJ abalones was significantly higher with 38.1 ± 0.8 mm. In addition, DISL and SGR values were similar among RR, JJ and RJ

Table 2 Hatchery parameters of RR, JJ and RJ abalones under commercial culture conditions during 461 days

Parameters	RR	JJ	RJ hybrid
Fertilization % (2 h)	84.3 ± 3.4 (6)[a]	82.2 ± 3.3 (9)[a]	42.5 ± 3.1 (5)[b]
Hatching % (18 h)	74.8 ± 5.9 (7)[a]	69.1 ± 4.1 (8)[a]	63.3 ± 2 (5)[a]
Settlement % (8 days)	12.3	18.6	16.7
Final survival % (461 days)	31.7	20.1	28.6
Final yield recovery %*	2.6	2.4	3.0
Final shell length (mm)	33.5 ± 0.6 (80)[a]	38.1 ± 0.8 (80)[b]	34.1 ± 0.8 (80)[a]
DISL (μm day^{-1})	75.9 ± 6.3 (7)[a]	65.1 ± 12.6 (7)[a]	66.1 ± 19.2 (7)[a]
SGR (% day^{-1})	1.07 ± 0.4 (7)[a]	0.93 ± 0.5 (7)[a]	1.11 ± 0.3 (7)[a]

Data presented are mean values ± standard error, and sample size *n* is indicated in parenthesis. Values with different superscript letter in a row are statistically significant ($P \leq 0.05$).

DISL, Daily Increment in shell length; SGR, Specific Growth Rate.

*Percentage of abalones recovered on day 461 according to the total fertilized eggs (2 h), average.

hybrid abalones (Table 2; $P = 0.6959$; 0.2259, respectively); with an average DISL and SGR among treatments of 69.0 ± 7.6 μm day^{-1} and 1.03 ± 0.2% day^{-1}.

Morphometric ratios, reported as percentage, of RR, JJ and RJ hybrid abalones are summarized in Table 3. The RJ hybrid shared one morphometric ratio, SW:SL, with its maternal species (red abalone), which is related to shell thickness. This means that the RJ hybrid has a shell as thick and strong as the red abalone. While for JJ abalones, shell thickness had a significantly lower score ($P = 0.0000$), resulting in a much thinner shell than that of RR and RJ hybrid abalones. Two others RJ hybrid morphometric ratios were shared with its paternal species, the Japanese abalone, these being DAFRP:SB and DFTRP:SB, which are related to shell elongation in relation to respect to the position and separation of respiratory pores. These scores were significantly higher than those of RR abalones ($P = 0.0000$; 0.0030). The SB:SL score of the RJ hybrid abalones was significantly different from those of the both parental species, with an intermediate score between theirs (Table 3). Other scores as SH:SB and DATRP:SB were similar among the three kinds of abalones ($P = 0.0616$, 0.0539). The number of ORP varied 3–5 for RR abalones, 4–5 for JJ abalones and 3–4 for RJ hybrid abalones.

Response to thermal stress (*HSP70* gene expression)

For RR abalones, the temperature of maximal *HSP70* expression (Tpeak) was 24°C, showing an expression gradient from 18°C to Tpeak. Once the Tpeak was exceeded, *HSP70* gene expression was down regulated at 26°C, and additionally, individuals showed a loss of the ability to adhere to the substrate, preventing performance under heat shock at higher temperatures. Similarly, JJ abalones showed the up/down-regulation pattern, but with a Tpeak at 26°C and a down-regulation at 30°C. While for RJ hybrid abalones, the Tpeak was recorded at 28°C, showing a gene expression gradient from 18°C to the Tpeak. Herein, the RJ hybrid abalones had showed a higher up-regulation of *HSP70* compared with the response of RR abalones (Fig. 4).

Genetic certification of RJ hybrid abalones

Microsatellite locus *Hco97* showed a species-specific allelic range for parental species that varied from 76 to 90 bp for the JJ abalone and 200–258 bp for the RR abalone (Fig. 5). For most of the RJ hybrid offspring (85%), locus *Hco97* was heterozygous, with the presence of both parental bands (Fig. 5). For RJ hybrids, that were homozygotes for *Hco97* loci (15%), positive parental control microsatellite loci *Awb036* (Japanese control; 175–200 bp) and *Hruf300* (red control; 150–180 bp) confirmed the contribution of genetic material from both parental species were analysed (data not shown).

Discussion

RJ hybrid fertilization and hatching success

As pointed out by Leighton (2000), some Californian species, such as the green abalone *Haliotis fulgens* and the white abalone *Haliotis sorenseni,* do not present natural hybrids because they differ

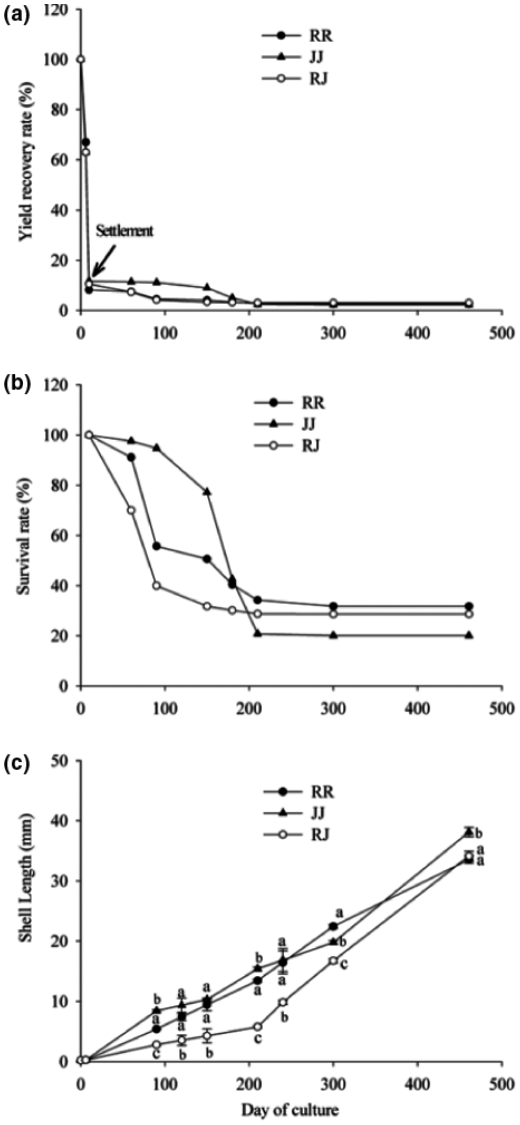

Figure 3 Yield recovery rate (a), survival rate (b) and average shell length (c) for RR, JJ and RJ abalones, culture under commercial conditions during 461 days.

markedly in bathymetrical distribution and reproductive season. However, under laboratory conditions, it is possible to produce 'improbable hybrids' with widely varied fertilization success, depending on the genetic proximity between target species and fertilization conditions, mainly temperature, gametic age and sperm concentrations (Leighton & Lewis 1982; Hanh 1989; Cai, Wang, Wang & Wang 2006; Luo *et al.* 2010). As reported in most abalone hybridization studies, rapid fertilization of eggs after spawning is critical to produce the largest quantity of hybrid embryos (Leighton & Lewis

Table 3 Morphometric traits ratios of RR, JJ and RJ abalones

Ratio (%)	RR	JJ	RJ hybrid
SW:SL	28.5 (1.7)^{a}	12.9 (0.3)^{b}	26.2 (0.6)^{a}
SB:SL	72.1 (0.4)^{a}	65.4 (0.7)^{c}	67.9 (0.7)^{b}
SH:SB	22.7 (0.5)^{a}	23.8 (0.5)^{a}	21.8 (0.6)^{a}
DAFRP:SB	102.5 (0.9)^{a}	114.7 (1.6)^{b}	112.3 (1.8)^{b}
DFTRP:SB	47.0 (2.9)^{a}	57.7 (2.4)^{b}	56.1 (2.4)^{b}
DATRP:SB	82.7 (0.9)^{a}	85.9 (1.0)^{a}	86.5 (1.0)^{a}

Data presented are mean values with standard error ($n = 50$). Values with different superscript letter in a row are statistically significant ($P \leq 0.05$).
SW, shell weight; SL, shell length; SB, shell breadth; SH, shell height; DAFRP, distance between apex and first respiratory pore; DATRP, distance between apex and third respiratory pore; DFTRP, distance between first and third respiratory pore.

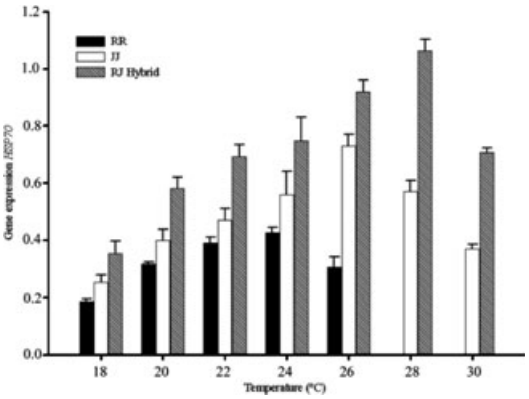

Figure 4 Mean *HSP70* gene expression of RR, JJ and RJ abalones exposed to increasing temperatures (18–30°C). Standard error and differences among treatments are shown ($P \leq 0.05$).

1982; Hanh 1989; Cai *et al.* 2006; Ahmed, Koike, Strüssmann, Yamasaki, Yokota & Watanabe 2008; Luo *et al.* 2010). Our results showed a decline of fertilization rates depending on egg age. The fertilization rate varied from 57% to 15% when we used eggs 10- and 60-min postspawning respectively. Similar findings were reported for the inter-specific cross of *H. rufescens* ♀ × *H. fulgens* ♂, where red eggs fertilized after 8 min postspawning reached a fertilization rate of 23%, while after 45 min, the rate dropped to only 4% (Leighton & Lewis 1982). As described by Leighton (2000), timing is a critical factor to avoid the sperm blocking process.

Regarding con-specific crosses of red abalones (RR), we observed that fertilization rates were similar among the different egg ages evaluated. Yet,

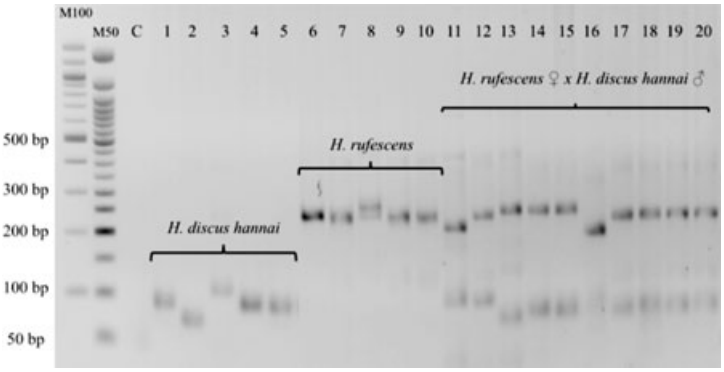

Figure 5 Genetic certification of RR, JJ and RJ abalones using microsatellite locus *Hco97*. M100, DNA ladder 100 bp; M50, DNA ladder 50 bp; C, negative control; 1–5, *Haliotis discus hannai*; 6–10 *Haliotis rufescens*; 11–20, *H. rufescens* ♀ × *H. discus hannai* ♂.

for the con-specific cross of Japanese abalones (JJ), eggs of 60-min postspawning decreased the fertilization rate from 87.7 ± 2.2% (10–30 min) to 33%. Ahmed *et al.* (2008) reported that the Japanese species *Haliotis gigantea* had short egg viability, as con-specific cross fertilization rates declined to 0.3% within 5 min of egg release, providing a clue to the differences in success observed between reciprocal hybrid crosses. As reported by Hoshikawa *et al.* (1998), the hybrid cross of *H. discus hannai* ♀ × *Haliotis kamtschatkana* ♂ had a fertilization rate of 20%, whereas the reciprocal cross *H. kamtschatkana* ♀ × *H. discus hannai* ♂ was never successful under similar fertilization conditions. In our case, preliminary studies to produce the reciprocal cross of *H. discus hannai* ♀ × *H. rufescens* ♂ (JR hybrid) showed fertilization rates of only 4.5% when Japanese eggs were fertilized 10-min postspawning (data not shown), which is significantly lower than the RJ hybrid rate (range 30–60.5%). Nevertheless, our results were significantly higher than those observed by Wang and Fan (1999) for reciprocal hybrid crosses between red and Japanese abalone. These authors reported 2.1% and 1.0% for RJ and JR hybrids, respectively, where fertilization was carried out 30–60 min after egg release and 5000 spermatozoids per egg at 18°C. Another relevant factor that should have an effect on hybrid fertilization success is the state of gonad maturation. As the method used by our study to evaluate the gonad maturation is visual appraisal of gonad size and shape, the quality of the eggs (in terms of lipid and protein content) is unknown (Kikuchi & Uki 1974; Ebert & Houk 1984). For example, gonad histology of *H. discus hannai* ovaries had showed that two egg cohorts matured during a single spawning season, and the second egg cohort being of higher quality than the first cohort but similar size (Fukazawa, Kawamura, Takami & Watanabe 2007). On the other hand, low viability of hybrid progeny could be related to postzygotic barriers like chromosomal or genetic incompatibility (Mayr 1968). Partial unviability for RJ hybrids was observed as a proportion of fertilized eggs stopped development after the first meiotic division and abnormal larvae development. Moreover, some differences at the chromosomal level among RR, JJ and RJ abalones were found, despite their conservative chromosome number of $2n = 36$ (Amar-Basulto, Lafarga-De la Cruz, Iturra-Constant & Gallardo-Escárate 2010).

Other factors affecting fertilization success are sperm concentration, the ratio of sperm and egg and gamete contact time (Leighton & Lewis 1982; Leighton 2000; Cai *et al.* 2006; Luo *et al.* 2010). For con-specific abalone crosses, the optimal spermatic concentration for maximum fertilization varies among species due to differences in egg size, vitelline membrane permeability, lysin efficacy, success of the acrosomal reaction or spermatozoid morphology (Baker & Tyler 2001; Grubert, Mundy & Ritar 2005). For *H. rufescens* and *H. discus hannai,* spermatic concentrations to attain maximum fertilization rates varies from 10^5 to 10^6 spermatozoids mL^{-1} with a sperm:egg ratio more than 100 (Kikuchi & Uki 1974; Leighton 2000). However, for the inter-specific cross of *H. rufescens* ♀ × *H. discus hannai* ♂ sperm concentrations of 10^7 spermatozoids mL^{-1} were required to achieve maximum fertilization success, as observed previously for other inter-specific abalone crosses using *H. rufescens*, *Haliotis corrugata*,

H. fulgens, *H. sorenseni*, *Haliotis diversicolor*, *H. discus discus*, *H. discus hannai* and *H. gigantea* (Leighton & Lewis 1982; Cai *et al.* 2006; Luo *et al.* 2010). In abalones, sperm-egg interaction is mediated by the sperm proteins lysin and 18-kDa, and their, respectively, egg receptor proteins vitelline envelope receptor for lysine (VERL) and vitelline envelope zona pellucida domain protein 14 (VEZP14), located on the vitelline envelopment (VE) which are specie-specific (Vacquier, Swanson, Metz & Stout 1999; Aagaard, Vacquier, MacCoss & Swanson 2010). Lysin binds to VERL, breaking apart the VE and creating a hole for spermatozoid passage, while 18-kDa protein mediates the fusion between gametes plasmatic membranes (Vacquier, Carner & Stout 1990; Swanson & Vacquier 2002). However, it had been proved that specie-specificity of lysin-VERL complex is not complete, and high lysin concentration of one species can dissolve the VE of other species (Vacquier *et al.* 1999; Swanson & Vacquier 2002). Taking this into account, we can suppose that fertilization success of inter-specific hybrid abalone crosses is related to some extent to the efficacy of lysin to dissolve the VE of hetero-specific eggs, as increased spermatic concentrations result in a significant increase in the fertilization rate of RJ hybrid crosses. But the fertilization rate can be also affected by the interaction between 18-kDa and VEZP14 and more complex relationships among other proteins located in the VE in abalone eggs (Aagaard, Yi, MacCoss & Swanson 2006; Aagaard *et al.* 2010). Moreover, homologies between reproductive proteins of red and Japanese abalones are 73.2% for lysin, 88.9% for VERL and 79% for VEZP14 (Lee & Vacquier 1995; Galindo, Vacquier & Swanson 2003; Aagaard *et al.* 2010), which can explain the moderate success to produce RJ hybrids as compare with other inter-specific hybrid abalones crosses. Leighton and Lewis (1982) had reported the highest fertilization rate of 98.7% for *H. sorenseni* ♀ × *H. rufescens* ♂ (SR hybrid), with homologies of 91.6% for lysin, 90.7% for VERL and 86.6% for 18-kDa reproductive proteins (Vacquier, Swanson & Lee 1997; Galindo *et al.* 2003).

Embryonic and larval development of hybrid abalones

Cleavage, embryonic and larval development of several abalones species had been reported previously (Hanh 1989; Leighton 2000; Courtois De Viçose *et al.* 2007). But, there are only two studies related to hybrid abalones embryonic and larval development that showed a significant delayed development with respect to their parental species (Leighton 2000; Lu, Chen, Wu, Zeng & Su 2001; Luo, You, Ke, Yang & Wu 2006). For example, Leighton (2000) found that inter-specific reciprocal hybrids between *H. rufescens* (R) and *H. fulgens* (G) showed retarded hatching rates as compare with both parental species. For con-specific parental cross larvae usually hatch as trocophore after 12–16 h postfertilization, while for RG and GR hybrids, larvae hatch as an early shelled veliger after 22- to 26-h postfertilization. In our study, RJ hybrids did not showed significantly differences in times required for embryonic and larval development with respect to its parental species. In the other hand, differences in larvae sizes observed on days 1, 3 and 5 among RR, JJ and RJ abalones can be due to asynchronic larval development (Hanh 1989). However, by day 7, the RJ hybrid larvae size was similar to that observed in RR and JJ abalones.

Hybrid abalone performance

Abalone hybrid production is difficult and success may reflect genetic affinities between species (Meyer 1967; Brown & Murray 1992). In natural conditions, wild hybrids are found between sympatric species in California, Japan and Australia (Owen, McLean & Meyer 1971; Fujino 1992; Brown 1995). Moreover, induced hybridization has been successful to some extent between allopatric abalone species that are closely related (Hoshikawa *et al.* 1998; Wang & Fan 1999). Our results showed that the fertilization rate of RJ hybrid could be significantly improved, allowing RJ hybrid production to be viable for commercial culture. Wang and Fan (1999) recorded a fertilization rate of 1–2%, a hatching rate of 65–80%, a mean DGR of 62.5 ± 3.5 μm day^{-1} and a mean survival rate of 49.4 ± 5.4% for 10 month-old RJ and JR hybrids. Our experiments showed fertilization rates of 30–60.5%, hatching rates of 30.4–74.5% and settlement rates of 1.6–16.7%. Additionally, 15.3-month-old RJ hybrid abalones cultured under commercial conditions showed an average DGR of 66.1 ± 19 μm day^{-1} and a final survival rate of 28.6%, without differences in final SL between RR and RJ abalones (33.5 ± 0.6 and 34.1 ± 0.8 mm).

Moreover, with respect to phenotypic features, RJ hybrid shell density resembles that of RR abalones, as shells were thicker, stronger and more resistant than JJ abalone shells. Subsequent experiences with 2-year-old RJ hybrids have shown a reproductive ability of the hybrids to produce F_2 hybrid progeny (RJ ♀ × RJ ♂) and to backcross with their maternal parental red abalone (RR ♀ × RJ ♂), with fertilization rates of 6.9% and 55% respectively (Lafarga-De la Cruz & Gallardo-Escárate 2011). Gonad development and reproductive ability of wild and artificially induced hybrid abalones have been observed previously (Owen *et al.* 1971; Leighton & Lewis 1982; Fukagawa & Tachiyama 1997; Leighton 2000; Ahmed *et al.* 2008).

Regarding thermal stress, there was a clear relationship between *HSP70* gene expression under thermal stress conditions and individual tolerance. In the case of mollusks, this relationship has been reported in a number of cases. Laursen, di Liu, Wu and Yoshino (1997) showed that *HSP* expression varies in a mollusk cell line when the cells are under different heat-shock conditions, making it possible to establish the relationship between the relative abundance of *HSPs* in the cell and the thermal stress level that the organism is able to tolerate. In parallel, Roberts, Hofmann and Somero (1997) showed that seasonal climate changes affect the gene expression of different *HSP70* isoforms in *Mytilus californianus*, this being one of the first approximations to show a relationship between seasonal temperature variation and the stress levels generated in natural mytilid populations. In abalones, Farcy, Serpentini, Fievet and Lebel (2007) identified the *HSP70* and *HSP90* coding sequences in *Haliotis tuberculata*. They also performed an expression analysis on a primary hemocyte culture exposed to thermal stress, showing over-expression of both genes when higher temperatures stressed cells. Our results concur with previous studies, showing significant up- and down-regulation according to the thermal stress. On the other hand, it was possible to correlate the *HSP70* levels with the behaviour of the individuals reported by Díaz, del Río-Portilla, Sierra, Aguilar and Re-Araujo (2000) in red abalone, who found that abalones turned their shells 180º in relation to the foot and that there was a loss in their capacity to adhere at Tpeak (24°C). This behaviour was not shown in hybrid abalones. Furthermore, it is hypothesized that the thermal tolerance archived in RJ hybrid abalones represents a new genomic conformation and is an indicator of a heterosis effect. Moreover, we certified the hybrid status of RJ hybrid abalones using three microsatellite loci (*Hco97, Awb036, Hruf300*), with consistent results like those described previously by Lafarga-De la Cruz *et al.* (2010).

The implications of RJ hybrid abalone production for the Chilean industry

In northern Chile, abalone producers have reported that hybridization between red and Japanese abalones is possible during the summer, due to the natural synchrony between female red abalones and male Japanese abalones. During this season, larval production problems arose due to the low availability of mature red abalone males from spontaneous spawning events, which in turn is the result of higher water temperatures in the region (above 20°). However, producers have observed that mature Japanese abalone males could be used to produce larvae during those months. In this sense, the hiatus in continuous red abalone larvae production could be filled during summer months by producing hybrid abalones. Given that the Japanese abalone is the most popular and best priced abalone product in the international market, due to the quality of its meat and traditional consumer preferences (Cook & Gordon 2010), the selection of specific hybrid genotypes could preserve the phenotypic attributes of Japanese abalone meat. Future studies should characterize the quality traits of abalone meat, such as flavour, tenderness and texture, as well as the biotechnological solutions to improve fertilization rates of RJ hybrid abalones, given its potential to diversify the Chilean abalone industry.

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