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E ects of temperature and salinity on survival, growth and DNA methylation of juvenile Pacific abalone, Ino*

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Temperature and salinity are two of the most potent abiotic factors influencing marine mollusks. In this study, we investigated the individual and combined e ects of temperature and salinity on the survival and growth of juvenile Pacific abalone, Ino, and also examined the DNA methylation alteration that may underpin the phenotypic variation of abalone exposed to di erent rearing conditions. The single-factor data showed that the suitable ranges of temperature and salinity were 16–28°C at a constant salinity of 32, and 24–40 at a constant temperature of 20°C, respectively. The two-factor data indicated that both survival and growth were significantly a ected by temperature, salinity and their interaction. The optimal temperature-salinity combination for juveniles was 23–25°C and 30–36. To explore environment-induced DNA methylation alteration, the methylation-sensitive amplified polymorphism (MSAP) technique was used to analyze the genomic methylation profiles of abalone reared in optimal and adverse conditions. Neither temperature nor salinity induced evident changes in the global methylation level, but 67 and 63 di erentially methylated loci were identified in temperature and salinity treatments, respectively. The between-group eigen analysis also showed that both temperature and salinity could induce epigenetic di erentiation in . Ino. The results of our study provide optimal rearing conditions for juvenile ę ъ D ӟ Ξ ' 1 INTRODUCTION

In China, the Pacific abalone, Ino, is a commercially important mollusk, whose production was around 50 000 metric tons in 2010, accounting for approximately 90% of the worldwide yield (FAO, 2012). Beginning in the early 1980s, its commercial-scale mariculture was mainly distributed in its natural habitats, the Liaodong and Shandong Peninsulas (Zhang et al., 2004). Since the 2000s, farming of . Ino has extended substantially from the northern Yellow Sea to the East China Sea (Li et al., 2012), which exposes the abalone to various environmental conditions. Even within the same sea area, the ambient environment can vary considerably according to the season.

In marine mollusks, temperature and salinity are regarded as the most potent environmental factors (Kinne, 1963), which not only limit their natural distribution and farming area (Allan et al., 2006; Cáceres-Puig et al., 2007), but also induce variation in survival, growth and hence overall mariculture production (Laing, 2002; Searle et al., 2006; Cáceres-

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Puig et al., 2007; Xue et al., 2010). Thus, it is necessary to explore the e ects of temperature and salinity on abalone performance to optimize development of the industry. A number of studies have dealt with the individual e ects of temperature and salinity on . Ino (Nie et al., 1984; Gao et al., 1990; Liu et al., 2005; Zhang et al., 2005; Wang, 2012), but their interaction, which would be more physiologically valid, has not been covered. The present study aimed to examine the individual and combined e ects of temperature and salinity on the survival and growth of juvenile Ino and determine the optimal rearing conditions for abalone mariculture.

Much e ort has been devoted to uncovering the mechanism underlying phenotypic variation in response to environmental heterogeneity (Gao et al., 2010). Growing evidence indicates that epigenetic modification, especially DNA methylation, plays an important role in helping individuals cope with di erent environments (Gao et al., 2010; Lira-Medeiros et al., 2010; Tan, 2010; Morán et al., 2013). DNA methylation, predominantly occurring at the 5-position of cytosine, is crucial for epigenetic control of gene expression (Li et al., 2009). It can be altered by environmental stimuli and may contribute to phenotypic variation by regulating transcriptional opportunities of stress-responsive genes (Roberts and Gavery, 2012). Navarro-Martín et al. (2011) observed that high temperature increased the DNA methylation of the gonadal aromatase promoter, which was involved in temperature-dependent sex ratio shifts in European sea bass. Marconi et al. (2013) found that salinity could induce genome-wide changes in DNA methylation, which might result in the di erential expression of stress-related genes. So far, the environment-induced methylation alteration has been well characterized in vertebrates and plants, but little is known in invertebrates, especially in Ino.

Methylation-sensitive amplified polymorphism (MSAP) analysis, a modification of the amplification fragment length polymorphism (AFLP) technique, represents an e cient and cost-e ective approach to profile genomic methylation patterns without requiring any genome information (Lira-Medeiros et al., 2010). MSAP is based on the use of isoschizomers II and I, which recognize the same restriction site 5'-CCGG-3' but display di erential sensitivity to cytosine methylation. Recently, the MSAP technique has been applied extensively in (Jiang et al., 2013), (Sun et al., 2014), (Morán et al., 2013) and (Zhao et al., 2015), and has proved useful to uncover epigenetic variability in marine organisms. In this study, we first analyzed the extent and pattern of DNA methylation in abalone reared at di erent temperatures and salinities using the MSAP technique, expecting to uncover the epigenetic regulatory mechanism underlying the phenotypic variation and adaptive responses to thermal and salt stresses.

The results of our study may provide a physiological basis for the mariculture of juvenile . Ino, and also lay a foundation for epigenetic research in the abalone.

2 MATERIAL AND METHOD

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The abalone used in our study were derived from a selective breeding population known as P-97, which had experienced six generations of selection for rapid growth since 1997. Three-month-old juveniles of uniform size were collected from Xunshan Fishery Company, Shandong Province, and acclimated in a closed circulating water tank (temperature: 20±0.5°C, salinity: 32±0.5) for 2 weeks. Water was totally replaced every other day and vigorous aeration was provided. The animals were fed daily with an artificial diet (250 μm sieved Gold Feed, Weihai, China). Of these, 200 randomly chosen individuals, which were not used in the following culture experiment, were measured on the day of stocking to obtain the initial values of growth-related traits. The abalone were gently dried using blotting paper before being measured. Shell length and width were measured to the nearest 0.01 mm using a vernier caliper, and the wet weight was measured to the nearest 0.01 g using an electronic balance.

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The individual and combined treatments of temperature and salinity were implemented simultaneously. The experiment was carried out in closed circulating water tanks, each with an e ective water volume of 450 L and a turnover rate of 7 200 L/day. The tanks acted as water baths and aeration ensured complete mixing of the water. Each tank was equipped with a thermostat-controlled immersion heater or a cooling titanium pipe to regulate the water temperature. Each tank had space for partially submerged 2-L plastic beakers, which were used to

hold the abalone and ensure they were exposed to continuous aeration. An 11 cm×6 cm black corrugated sheet was placed in each beaker as substrate.

Temperature was controlled using a water bath, and salinity was adjusted by adding a commercial sea salt (Tropical Marine, Cnsic Marine Biotechnology Co., Tianjin, China) or dechlorinated tap water to ambient seawater. Temperature and salinity were adjusted daily to keep the variations within ±0.5°C and ±0.5, respectively, using a mercury thermometer and a portable refractometer. Dissolved oxygen (5.70–7.90 mg/L) and pH (7.90–8.20) were monitored three times a week using a water-quality meter (YSI Model 556MPS, Yellow Springs Instrument, Yellow Springs, OH, USA).

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To study the individual e ect, juveniles were exposed to 16, 18, 20, 22, 24, 26, 28, 30 and 32°C at a constant salinity of 32, and 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44 and 46 at a constant temperature of 20°C. To study the combined e ect, four temperatures (16, 20, 24 and 28°C) and five salinities (24, 28, 32, 36 and 40) were tested in a full factorial experiment.

After the acclimation, 60 abalone in triplicates (=20/replicate) were randomly selected for each treatment. Each day at 17:00 h, the abalone were fed with the artificial diet (as above) to excess. The following morning, each beaker was drained, washed and refilled with appropriate temperature/salinity-adjusted water. Abalone were cultured for 77 days. At harvest, the shell length, shell width and wet weight were individually measured using the above-mentioned method. Specific growth rate (SGR, %/d) was calculated using the formula: SGR=100×(ln $_t$– ln $_0$)/ (the days of the experiment), with $_t$ and $_0$ as final and initial values of shell length, shell width and wet weight. Mortalities were recorded daily and used to calculate the survival rate.

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To study the e ect of temperature and salinity on DNA methylation, we performed MSAP analysis on the abalone from selected temperature (20, 24 and 30°C) and salinity (22, 32 and 42) treatments in the single-factor experiment. Treatments of 24°C and 32, which had the best survival and growth, were regarded as control groups, with the other treatments used as the corresponding stress groups. Sixteen individuals were sampled from each treatment, except for 30°C, which only provided seven abalone for the analysis.

Genomic DNA was extracted from the foot muscle using a modified phenol-chloroform protocol (Li et al., 2006). DNA samples were checked on 1% agarose gels and normalized to 100 ng/μL using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For each individual, 100 ng DNA were independently digested with RI/ II and RI/ I (2 U each) in a restriction bu er totaling 10 μL, and subsequently ligated with adapters. Pre-selective amplification was performed in a 10 μL reaction using 2 μL of 1:10 ligation dilution, 1×PCR bu er, 0.2 mmol/L of dNTPs, 1.5 mmol/L of $MgCl_2$, 2.5 pmol of RI-A and II/ I-T pre-selective primers (Jiang et al., 2013) and 2.5 U of Taq polymerase. PCR conditions for pre-selective amplification were as follows: 72°C for 2 min, 20 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 2 min and a final step of 60°C for 30 min. Selective amplification was performed in a 10 μL reaction using 1.5 μL of 1:20 pre-selective amplification dilution. Ten selective primer combinations were chosen to generate all the MSAP profiles. PCR contained 1×PCR bu er, 0.2 mmol/L of dNTPs, 1.5 mmol/L of $MgCl_2$, 2.5 pmol of each selective primer and 2.5 U of Taq polymerase. Cycling conditions for selective amplification were 94°C for 2 min, 10 cycles of 94°C for 20 s, 66°C (decreasing by 1°C each cycle) for 30 s, and 72°C for 2 min; followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, ending with 60°C for 30 min. PCR products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the fragment analysis was performed using GeneMapper v.4.0 software (Applied Biosystems, Foster City, CA, USA). DNA fragments less than 50 bp in length, longer than 500 bp or less than 200 RFU (Relative Fluorescent Units) were excluded from the analysis because of low levels of reproducibility.

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Analysis of variance (ANOVA) was used to test the individual and combined e ects of temperature and salinity on the survival and growth of juvenile . Ino. Data were assessed for homogeneity of variance using Levene's test. When significant di erences were found (<0.05), Student-Newman-Keuls test was used to compare means of various treatments. Response-surface contour plots were generated to graphically demonstrate the combined e ect using Surfer v.8.0 software (Golden Software,

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Temperature (°C)	Survival (%)	SGR of shell length (%/d)	SGR of shell width (%/d)	SGR of wet weight (%/d)
16	90.00±8.66 a	0.54±0.12 a	0.51±0.11 a	1.59±0.36 a
18	86.67±5.77 a	0.59±0.05 a	0.59±0.05 a	1.74±0.14 a
20	91.67±5.77 a	0.62±0.03 a	0.62±0.03 a	1.80±0.15 a
22	88.33±10.41 a	0.63±0.10 a	0.63±0.10 a	1.87±0.29 a
24	90.00±5.00 a	0.83±0.05 b	0.79±0.04 b	2.47±0.14 b
26	85.00±10.00 a	0.66±0.09 a	0.63±0.09 a	1.96±0.22 a
28	83.33±7.64 a	0.57±0.05 a	0.55±0.05 a	1.71±0.14 a
30	21.67±7.64 b	0.19±0.04 c	0.15±0.05 c	0.50±0.07 c
32	0.00±0.00 c	-	-	-

Data are means±standard errors of three replicates. Within columns, di erent superscripts indicate significant di erences (<0.05).

Golden, CO, USA).

In the MSAP analysis, three types of bands were identified by presence (1) or absence (0) of RI/ II and RI/ I digests, respectively: (a) present in both (1-1), non-methylation; (b) present in only RI/ II (1-0, hemi-methylation) or RI/ I (0-1, full methylation), methylation; (c) absent in both (0-0), uninformative because of the absence of a fragment or hyper-methylation. The MSAP locus, at which the observed proportion of methylated scores exceeded a 5% threshold, was classified as a "methylation-susceptible locus" (MSL); an MSL was classified as a ''methylation-susceptible polymorphism locus" (MSP) if the non-methylated or missing scores exceeded a 5% threshold. The MSP binary matrix was scored as 1 (1-0 or 0-1), 0 (1-1) and missing (0-0) (Liu et al., 2012).

Methylation level (%) in di erent treatments was calculated and compared using the Kruskal-Wallis test. There might be a situation where the treated abalone could di er in methylation at many loci, but the direction might not be consistently toward hypo- or hyper-methylation. Therefore, we tested the di erence in methylation frequency at each MSP between the control and stress treatments via a two-tailed Fisher's exact test using a 5% criterion (Blouin et al., 2010). Multivariate analysis was also used to investigate individual profiles because it is a band-based approach that does not assume Hardy-Weinberg equilibrium. Principal component analysis (PCA) on inter-profile covariance matrix followed by between-group eigen analysis (BPCA) was computed on MSP

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Salinity	Survival (%)	SGR of shell length (%/d)	SGR of shell width (%/d)	SGR of wet weight (%/d)
16	0.00±0.00 a	-	-	-
18	11.67±2.89 ab	0.17±0.10 a	0.15±0.09 a	0.37±0.21 a
20	20.00±5.00 b	0.37±0.12 b	0.32±0.10 b	1.03±0.44 b
22	56.67±5.77 c	0.41±0.02 b	0.38±0.03 b	1.17±0.08 b
24	78.33±5.77 de	0.56±0.02 c	0.54±0.03 c	1.59±0.08 c
26	76.67±7.64 de	0.61±0.05 c	0.54±0.03 c	1.62±0.07 c
28	83.33±10.41 de	0.68±0.07 c	0.66±0.07 c	1.92±0.23 c
30	86.67±5.77 e	0.64±0.02 c	0.63±0.02 c	1.89±0.11 c
32	91.67±5.77 e	0.62±0.03 c	0.62±0.03 c	1.80±0.15 c
34	91.67±2.89 e	0.64±0.05 c	0.62±0.04 c	1.88±0.12 c
36	86.67±7.64 e	0.58±0.03 c	0.53±0.10 c	1.77±0.10 c
38	78.33±11.55 de	0.63±0.04 c	0.63±0.04 c	1.90±0.13 c
40	70.00±5.00 d	0.54±0.08 c	0.56±0.06 c	1.64±0.23 c
42	56.67±7.64 c	0.36±0.03 b	0.38±0.03 b	1.11±0.12 b
44	8.33±2.89 ab	0.37±0.05 b	0.39±0.07 b	1.07±0.16 b
46	0.00±0.00 a	-	-	-

Data are means±standard errors of three replicates. Within columns, di erent superscripts indicate significant di erences (<0.05).

binary profiles using ADE-4 software. BPCA (i.e. PCA among groups based on the PCA among individuals) divides the variance into within- and between-group components and is regarded as analogous to -statistics (called β_{ST} here) given that it is a Euclidean approach. Statistical significance was assessed by the Romesburg randomization test (10^4 permutations).

3 RESULT

Tables 1 and 2 show the summarized data for temperature and salinity treatments, respectively. One-way ANOVA indicated that both temperature (<0.001) and salinity (<0.001) had significant e ects on the survival and growth of juvenile . Ino.

All juveniles reared at 32°C died within 4 days. Survival at temperatures from 16 to 28°C, ranging from 83.33% to 91.67%, was significantly higher than that at 30°C. With temperature rising from 16 to 30°C, the specific growth rates first increased and then decreased. Maximum specific growth rates of shell

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Temperature (°C)	Salinity	Survival (%)	SGR of shell length (%/d)	SGR of shell width (%/d)	SGR of wet weight (%/d)
16	24	75.00±8.66 abc	0.49±0.05 abc	0.44±0.05 ab	1.38±0.15 ab
	28	83.33±7.64 bc	0.60±0.02 cde	0.56±0.02 bcd	1.71±0.12 bcd
	32	90.00±8.66 bc	0.54±0.12 bcde	0.51±0.11 bcd	1.59±0.36 abcd
	36	88.33±2.89 bc	0.49±0.01 abc	0.47±0.01 abc	1.49±0.04 abcd
	40	81.67±2.89 bc	0.45±0.05 ab	0.44±0.05 ab	1.42±0.19 abc
20	24	78.33±5.77 abc	0.56±0.02 bcde	0.54±0.03 bcd	1.59±0.08 abcd
	28	83.33±10.41 bc	0.68±0.07 ef	0.66±0.07 de	1.92±0.23 de
	32	91.67±5.77 c	0.62±0.03 cde	0.62±0.03 de	1.80±0.15 bcd
	36	86.67±7.64 bc	0.58±0.03 bcde	0.53±0.10 bcd	1.77±0.10 bcd
	40	70.00±5.00 ab	0.54±0.08 bcde	0.56±0.06 bcd	1.64±0.23 abcd
24	24	75.00±8.66 abc	0.66±0.05 def	0.60±0.06 cde	1.93±0.13 de
	28	78.33±5.77 abc	0.74±0.05 fg	0.71±0.04 ef	2.25±0.16 ef
	32	90.00±5.00 bc	0.83±0.05 g	0.79±0.04 f	2.47±0.14 f
	36	86.67±5.77 bc	0.81±0.02 g	0.79±0.04 f	2.37±0.08 f
	40	61.67±10.41 a	0.62±0.04 cde	0.65±0.05 de	1.88±0.13 cde
28	24	70.00±5.00 ab	0.40±0.06 a	0.37±0.04 ag	1.21±0.17 a
	28	90.00±5.00 bc	0.45±0.03 ab	0.42±0.03 ab	1.41±0.05 abc
	32	83.33±7.64 bc	0.57±0.05 bcde	0.55±0.05 bcd	1.71±0.14 bcd
	36	73.33±7.64 abc	0.53±0.01 bcd	0.51±0.03 bcd	1.63±0.05 abcd
	40	20.00±5.00 d	0.29±0.05 h	0.30±0.08 g	0.89±0.29 g

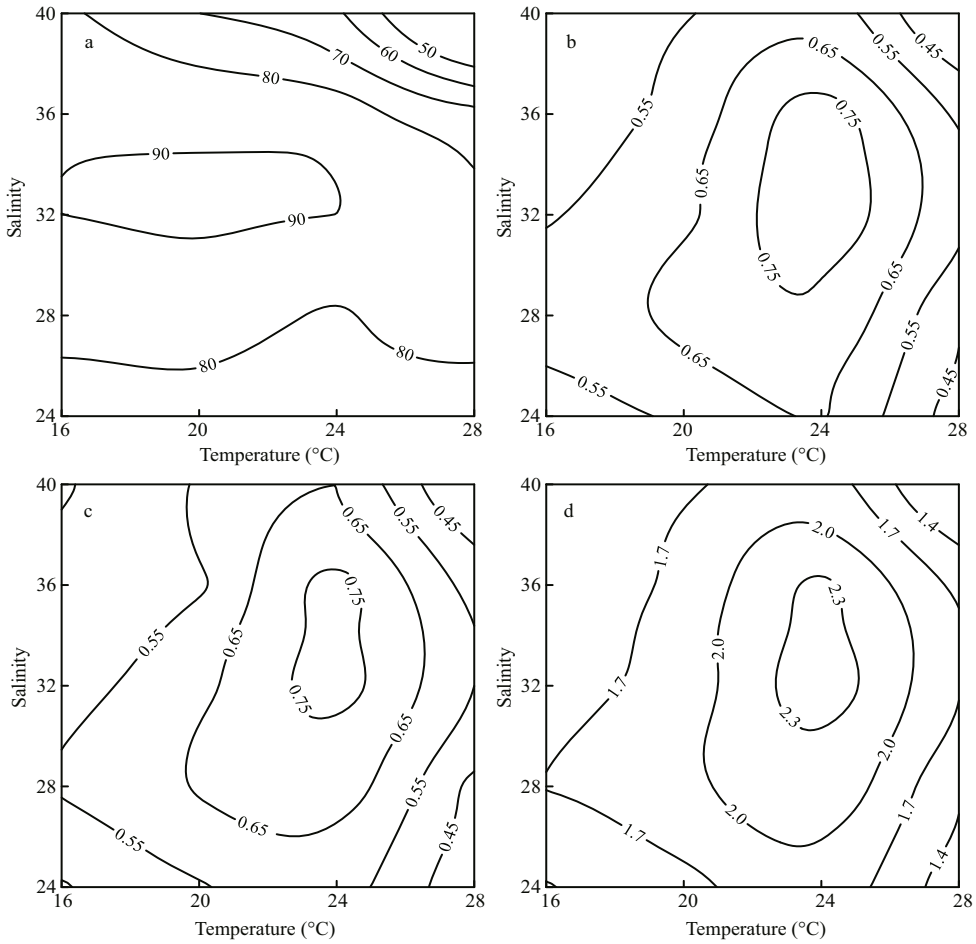
Data are means ± standard errors of three replicates. Within columns, di erent superscripts indicate significant di erences (<0.05).

length, shell width and wet weight were obtained at 24°C, which were significantly higher than those in the other treatments, with the values being 0.83%/d, 0.79%/d and 2.47%/d, respectively. Survival at salinities from 24 to 40 was not less than 70%, whereas no juvenile survived at 16 or 46. Specific growth rates at salinities from 24 to 40 were similar and significantly higher than those in the other treatments. Based on the above data, the suitable ranges of temperature and salinity for juvenile . Ino were determined as 16–28°C at a constant salinity of 32 and 24–40 at a constant temperature of 20°C, respectively.

Table 3 shows the summarized data for combined treatments of temperature and salinity. Two-way ANOVA indicated that temperature (<0.001), salinity (<0.001) and their interaction (<0.05) all significantly a ected the survival and growth of juveniles (Table 4). The mean survival rates were 83.67%, 82.00%, 78.33% and 67.33% at 16, 20, 24 and 28°C, respectively. Regardless of temperature, survival was higher at 28, 32, 36 (83.75%–88.75%) than at 24 (74.58%) and 40 (58.34%). Juveniles reared at the highest salinity of 40 had a relatively low survival at 24°C and 28°C. At all salinities, the specific growth rates increased as temperature ascended from 16 to 24°C, and decreased at 28°C. Specific growth rates at salinities from 28 to 36 were greater than those in the other treatments regardless of temperature. The poorest growth was observed at the combination of 28°C and 40. Response-surface contour plots in Fig.1 show that the optimal conditions for survival and growth coincided best at 23–25°C and 30–36.

The 10 primer combinations assayed with the MSAP analysis provided a total of 1 494 loci in the temperature treatments, with 612 and 498 of these loci being classified as MSL and MSP, respectively. In the salinity treatments, 675 loci were classified as MSL among 1 476 screened loci, with 542 of them

	Source of variance	df	Sum of squares	Mean square	F	Sig.
Survival	Error	40	0.190	0.005		
	Temperature	3	0.243	0.081	17.041	0.000
	Salinity	4	0.696	0.174	36.632	0.000
	Temperature×salinity	12	0.493	0.041	8.649	0.000
Shell length	Error	40	0.107	0.003		
	Temperature	3	0.695	0.232	86.424	0.000
	Salinity	4	0.228	0.057	21.219	0.000
	Temperature×salinity	12	0.094	0.008	2.923	0.005
Shell width	Error	40	0.125	0.003		
	Temperature	3	0.666	0.222	71.092	0.000
	Salinity	4	0.177	0.044	14.153	0.000
	Temperature×salinity	12	0.107	0.009	2.859	0.006
Wet weight	Error	40	1.164	0.029		
	Temperature	3	5.616	1.872	64.309	0.000
	Salinity	4	1.847	0.462	15.860	0.000
	Temperature×salinity	12	0.732	0.061	2.097	0.040



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The isobars show percentage survival in (a) and specific growth rates in (b) (%/d), (c) (%/d) and (d) (%/d)

/ *Eco* *Hpa*) *Msp*

Primer set	RI selective primer	II/ I selective primer	Temperature			Salinity		
			Total loci	MSL	MSP	Total loci	MSL	MSP
Set 1	ACT	TGG	111	32	25	100	30	20
Set 2	ACC	TGG	118	61	46	98	46	39
Set 3	ATC	TTG	183	71	58	170	85	74
Set 4	AAC	TAC	157	60	45	119	58	53
Set 5	ATC	TTC	239	106	89	257	129	94
Set 6	ATC	TAA	159	82	66	143	72	58
Set 7	AAC	TCT	144	42	36	151	50	35
Set 8	ACC	TTC	167	70	58	180	77	59
Set 9	ACC	TAA	121	57	49	149	80	69
Set 10	ACC	TTG	95	31	26	109	48	41
Total			1 494	612 (41.0%)	498 (33.3%)	1476	675 (45.7%)	542 (36.7%)

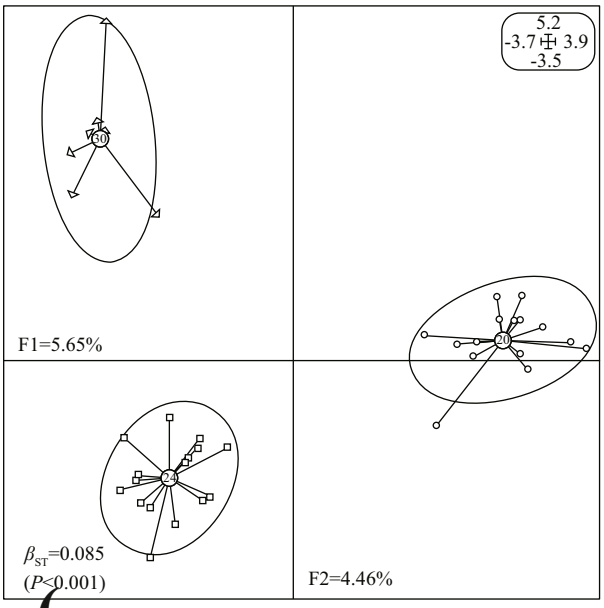
Methylation state	Temperature (°C)			Salinity			Average
	20	24	30	22	32	42	
Non-methylation (1-1)	68.81	67.65	66.04	66.51	66.17	65.66	66.81
Hemi-methylation (1-0)	9.55	9.53	10.44	11.08	12.46	12.62	10.95
Full methylation (0-1)	21.64	22.82	23.51	22.42	21.37	21.72	22.25
Methylation (1-0 + 0-1)	31.19	32.35	33.96	33.49	33.83	34.34	33.19

showing methylation-susceptible polymorphism (Table 5).

Table 6 shows the level of di erent DNA methylation states observed in selected treatments. The average level of full methylation among all treatments was greater (22.25%) than hemi-methylation (10.95%), indicating that fully methylated in internal cytosine was the dominant methylation pattern in the . Ino genome. In temperature treatments, the total methylation (hemi- and full methylation) level ranged from 31.19% to 33.96%, with a mean of 32.50%. No significant di erence was detected in the level of hemi-methylation (Kruskal-Wallis χ^2=0.328, df=2, =0.849), full methylation (Kruskal-Wallis χ^2=0.692, df=2, =0.708) or total methylation (Kruskal-Wallis χ^2=0.661, df=2, =0.719). Total methylation levels were also quite similar among salinity treatments, with values of 33.49%, 33.83% and 34.34% for 22, 32 and 42, respectively. There was no significant di erence in the level of hemi-methylation (Kruskal-Wallis χ^2=1.208, df=2, =0.574), full methylation (Kruskal-Wallis χ^2=0.472, df=2, =0.790) or total methylation (Kruskal-Wallis χ^2=0.196, df=2, =0.907).

However, in Fisher's exact tests, 67 di erentially methylated loci were identified among 498 MSP in temperature treatments with a percentage of 13.5%, which was higher than expected by chance (at 5% criterion). Among these loci, 48 loci were detected in 20°C treatment and 20 loci in 30°C treatment when compared with the control treatment of 24°C. Similarly, 63 out of 542 (11.6%) loci showed significant di erences in methylation frequency attributable to salinity, with 40 and 33 loci detected in 22 treatment and 42 treatment, respectively (Table 7).

Figure 2 shows the epigenetic structure di erentiation attributable to temperature. The PCA on the covariance matrix based on the MSP binary profile summarized 10.11% of total inertia in the first two axes. The three treatments showed pronounced separation, with 30°C treatment exhibiting greater variation than the others. The multivariate BPCA based on the PCA scores resulted in a significant β_{ST} of 0.085 (<0.001), suggesting that epigenetic variance could be divided into distinct between- (8.5%) and within- (91.5%) group components.



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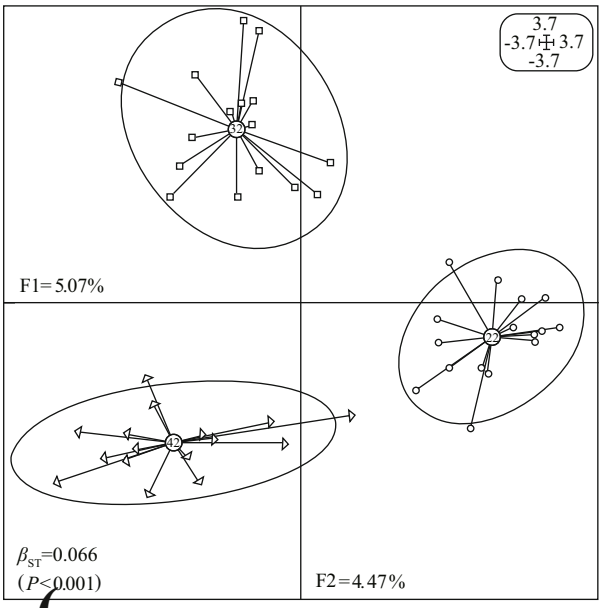
F1 and F2 values show the contribution of the two principal components summarizing the total variance of each data set. β_{ST} was calculated by BPCA for epigenetic profiles and tested with 10^4 Romesburg randomization permutations. Circles, squares and triangles represent individuals from 20, 24 and 30°C treatments, respectively. The group label shows the centroid for the points cloud in each group. Ellipses represent the dispersion of those points around their center.



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F1 and F2 values show the contribution of the two principal components summarizing the total variance of each data set. β_{ST} was calculated by BPCA for epigenetic profiles and tested with 10^4 Romesburg randomization permutations. Circles, squares and triangles represent individuals from 22, 32 and 42 treatments, respectively. The group label shows the centroid for the points cloud in each group. Ellipses represent the dispersion of those points around their center.

Primer set	No. significant		Total/No. tested	No. significant		Total/No. tested
	20°C[a]	30°C[b]		22[c]	42[d]	
Set 1	2	2	4/25	2	0	2/20
Set 2	6	3	8/46	1	2	3/39
Set 3	5	4	9/58	4	4	7/74
Set 4	4	3	7/45	4	9	12/53
Set 5	8	2	10/89	14	7	18/94
Set 6	7	2	9/66	5	0	5/58
Set 7	1	0	1/36	0	1	1/35
Set 8	7	2	9/58	3	2	3/59
Set 9	5	1	6/49	5	3	7/69
Set 10	3	1	4/26	2	5	5/41
Total	48	20	67/498 (13.5%)	40	33	63/542 (11.6%)

[a], [b], [c] and [d] are the number of loci showing a significant di erence in methylation frequency between treatments of 20°C and 24°C; 30°C and 24°C; 22 and 32; and 42 and 32, respectively. Total/No. tested is the total number of di erentially methylated loci over the total number tested.

In the salinity treatments, three groups also showed obvious clustering in four quadrants by the first two axes (summarizing 9.54% of the total inertia), and the multivariate BPCA output a significant β_{ST} of 0.066 (<0.001), which indicated that the proportion of between-group epigenetic variance caused by salt stress was 6.6% (Fig.3).

4 DISCUSSION

Traditionally, . Ino is regarded as a stenothermal and stenohaline species, preferring low-temperature and high-salinity waters. However, the single-factor data in our study confirmed a relatively wide tolerance to temperature (16–28°C) and salinity (24–40), which may have resulted from the selective breeding and long-term adaptation of the species to environmental heterogeneity in mass production. Compared with previous studies (Gao et al., 1990; Zhang et al., 2005; Cho and Kim, 2012), the abalone used in our study exhibited much greater tolerance to high temperature, with survival exceeding 80% at 28°C and the best growth obtained at 24°C. Di erent

temperature tolerances and optima may result from variations in size and genetic background. Searle et al. (2006) suggested that the thermal tolerance and temperature optima of . Gmelin were size-specific and decreased as abalone size increased. Liu et al. (2005) indicated that the di erences in genetic background mainly contributed to the variation in temperature optima of . Ino. It is also worth noting that the abalone in our study had experienced six generations of selection for rapid growth. There is a possibility that the fast-growing abalone have stronger environmental tolerances, expanding the span of growth time when they are exposed to adverse conditions. Hence, during the selection for rapid growth, the thermal tolerance of Ino was also improved.

It is the first time that the combined e ect of temperature and salinity on . Ino has been investigated. Two-way ANOVA indicated that the survival and growth of juvenile . Ino were significantly influenced by temperature, salinity and their interaction. Calabrese (1969) stated that the interactive e ect of temperature and salinity was significant when the tolerance of either of these was approached. Consistent with this statement, the two factors in our study showed strong interaction at extreme combinations such as 28°C and 40, at which both the survival and growth were the poorest. At salinity of 40, the survival rates at 16–20°C were higher than those at 24–28°C, suggesting that the juveniles had greater tolerance to extreme salinities at lower temperatures than at higher temperatures. This observation is of value to abalone mariculture, recommending the culturists lower the seawater temperature appropriately to ensure good survival when the ambient salinity fluctuates considerably. Two-way ANOVA showed that temperature accounted for greater variance in specific growth rates than salinity, while it was the reverse in survival. Ruscoe et al. (2004) obtained similar results to ours, in finding that the growth of was influenced more by temperature than by salinity, although both had significant e ects.

Pronounced variation in the survival and growth of . Ino attributable to temperature and salinity has been observed in this and previous studies (Nie et al., 1984; Gao et al., 1990; Liu et al., 2005; Zhang et al., 2005; Wang, 2012), but the underlying epigenetic mechanism remains uncharacterized. In the present study, we first compared the extent and pattern of DNA methylation in . Ino reared in optimal and adverse conditions using the MSAP technique. The results showed that the methylation level of . Ino was 33.19%, which was higher than that of . (26.4%) (Jiang et al., 2013) and . (20.9%–21.7%) (Sun et al., 2014). The abalone used in our study were derived from the same selective breeding population, indicating that there was high homogeneity in their genetic backgrounds. Hence, it is reasonable to hypothesize that the extensive phenotypic variation among di erent temperature and salinity treatments was largely a ected by the epigenetic events.

Kruskal-Wallis tests showed that neither temperature nor salinity induced evident changes in the global methylation level, in contrast to the results in (Zhu et al., 2013) and var. (Marconi et al., 2013). It is possible that the methylation alteration occurred in a number of key genes, without inducing a large change in the methylation level. Therefore, the di erence in methylation frequency at each MSP was tested. As we speculated, 67 and 63 loci exhibiting di erential methylation frequencies were identified in temperature and salinity treatments, respectively. These loci may be involved in the stress acclimation of . Ino, and thus constitute a core set of epimarker resources that would facilitate further epigenetic studies in this species (Sun et al., 2014). It is worth noting that much more di erential loci were found in the low-temperature treatment of 20°C (48 loci) than in the high-temperature treatment of 30°C (20 loci), and this might be related to the phenotypic di erences in the two treatments. The result indicated that more stress-related genes were involved in the cold acclimation, ultimately resulting in better survival and growth of . Ino in the low-temperature treatment.

The BPCA plots based on methylation profiles showed that the abalone from di erent treatments were obviously separated, suggesting that both temperature and salinity could induce epigenetic di erentiation in . Ino. Significant β_{ST} values also indicated that 8.5% and 6.6% of epigenetic variation was structured into the between-group component when stressed by temperature and salinity, respectively. The results provided evidence of the correlation between epigenetic reprogramming and phenotypic variation in . Ino, and verified the important role of DNA methylation in enabling abalone acclimation to environmental stresses.

5 CONCLUSION

Our study established the optimal rearing conditions of juvenile . Ino in terms of temperature and salinity, which may provide important guide to farming practice and industry development. We also analyzed the DNA methylation alteration in abalone reared in di erent conditions, presenting the first examination of the epigenetic regulatory mechanism of abalone in response to thermal and salt stresses.

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