



## MEAN PROLOCULUS SIZE AS A SALINITY INDEX IN BENTHIC FORAMINIFERA *AMMONIA AOMORIENSIS*: BASED ON CULTURE AND SEASONAL STUDIES

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### ABSTRACT

Limited studies on proloculus size of foraminifera inhibits its reliable application in palaeoceanographic reconstruction. This study shows that salinity has a significant effect on the proloculus size of shallow water benthic foraminifera *Ammonia aomoriensis*. Four culture experiments and 13-month long seasonal study from May 2014 to May 2015 in an intertidal flat, Qingdao Bay was conducted on the proloculus size of living *A. aomoriensis*. The mean proloculus size (MPS) of *A. aomoriensis* in both the offspring reproduced by culture experiments and the Rose Bengal stained specimens from the field gives a negative correlation with salinity: MPS ( $\mu\text{m}$ ) =  $65.2737 (\pm 4.3334) - 0.3357 (\pm 0.1468)$  Salinity (%) (Eq. (1),  $p = 0.0297$ ,  $r = -0.3908$ , 24–33%) in cultures; MPS ( $\mu\text{m}$ ) =  $214.7030 (\pm 20.7783) - 5.4215 (\pm 0.6902)$  Salinity (%) (Eq. (2),  $p < 0.0001$ ,  $r = -0.6979$ , 29–31%) in the field. Regression in the case of field study displayed a sharper slope as compared to the laboratory cultures, probably owing to the appearance of both microspheric agamont and megalospheric schizont in *A. aomoriensis* induced by the fluctuating environment in nature. As a result, salinity could be one of the prime factors responsible for the dimorphism behavior of *A. aomoriensis* and we suggest that Eq. (1) is more appropriate for optimum condition where megalospheric gamont is exclusively dominant, while Eq. (2) is more appropriate for unstable environment where trimorphism is observed. Thus, the MPS of *A. aomoriensis* can be used in palaeoenvironmental reconstruction.

**Keywords:** *Ammonia aomoriensis*, mean proloculus size, salinity, culture experiment, field study

### INTRODUCTION

In recent years a number of studies are done dealing with the application of foraminifera in past climate reconstruction. For example, the bottom water temperature reconstruction by Mg/Ca ratio (Tripati and Elderfield, 2005; Quillmann *et al.*, 2012; Rosenthal *et al.*, 2013; Morley *et al.*, 2014; Elmore *et al.*, 2015) and the ice volume rebuilding by paired stable oxygen isotope (Lear *et al.*, 2010; Bohaty *et al.*, 2012; Fukuda *et al.*, 2012). There are also studies concerning with biostratigraphic correlations and corresponding climate shift in the geologic past based on the coiling direction of foraminifera (Galeotti and Coccioni, 2002; Angue Minto'o *et al.*, 2015). The proloculus size of foraminifera is considered as an ideal index of salinity to infer the monsoon variation (Nigam and Khare, 1995; Khare *et al.*, 2008; Panchang and Nigam, 2012; Yu *et al.*, 2014).

The size of foraminiferal proloculus is a function of dimorphism which is a phenomenon related to reproductive behavior of foraminifera. The dimorphic forms are known as megalospheric (asexual reproduction) and microspheric (sexual reproduction) specimens (Nigam, 1986; Nigam and Rao, 1987). Megalospheric generation has smaller test but larger proloculi, while microspheric generation has larger test but smaller proloculi. This is observed in *Ammonia beccarii* (Brooks, 1967; Goldstein and Moodley, 1993), *A. parkinsoniana* (Colburn and Baskin, 1998), *Rotalidium annectens* (Nigam and Khare, 1992) and several species of *Bolivina* (Smith, 1963; Qvale and Nigam, 1985; Staines-Urías and Douglas, 2009). The dimorphism is somewhat indistinguishable, thus, mean proloculus size

(MPS) is used to understand the proportion of foraminiferal dimorphism (Nigam and Rao, 1987). The controlling factor of dimorphism phenomenon is less understood. Nigam (1986) first suggested temperature dependence of the dimorphic forms of *Bolivina skagerrakensis*. Then the research of *R. annectens* showed MPS was inversely proportional to both temperature and salinity (Nigam and Rao, 1987). On the contrary, living *A. parkinsoniana* exhibited a larger proloculi during a period of higher salinity (50‰) as compared to lower salinity (14‰) (Colburn and Baskin, 1998). Most laboratory culture studies are focused on the Mg/Ca ratio of foraminiferal test (de Nooijer *et al.*, 2014; Mewes *et al.*, 2014; Evans *et al.*, 2015; Mewes *et al.*, 2015). Moreover, no foraminifera laboratory culture has demonstrated the specific control on proloculus size so far.

*Ammonia aomoriensis* is an epifaunal species which lives in the upmost centimeter of the sediment, field study indicates that living *A. aomoriensis* is distributed in tidal flats, marshes and brackish lake of North Sea, Northeast China and Japanese coasts (Hayward *et al.*, 2004; Toyofuku *et al.*, 2005). This species was found occurring frequently in muddy sediments of Baltic Sea (Haynert *et al.*, 2012). It is widely distributed in the Yellow Sea of China and is also one of the most abundant species at the intertidal area of Qingdao Bay (Lei and Li, 2015). Lei *et al.* (2016) studied morphological variations of *A. aomoriensis* and its molecular identification and a significant positive correlation was detected between the ratio of microspheric/megalospheric forms and salinity. This paper discusses potential salinity control on the proloculus size of live *A. aomoriensis* from both field and culture studies.

## MATERIALS AND METHODS

### Field study

Living individuals of *A. aomoriensis* were collected monthly from May 2014 to May 2015 in the intertidal flat of Qingdao Bay (Fig. 1 a, b, c). The top 1 cm sediment was collected and fixed with 2% Rose Bengal–90% alcohol for 48 hours to impregnate the protoplasm of living *A. aomoriensis*. Fixed sample was washed over a 300 mesh sieve and was oven-dried under 50 °C. Temperature of sediment ( $T_s$ ) was measured by spirit thermometer in situ. Salinity was measured after samples were transported to the laboratory by handheld salinometer.

### Culture experiment

Living individuals of *A. aomoriensis* were collected from the intertidal flat of the Qingdao Bay, Yellow Sea. Organisms were kept intact and taken back to the laboratory. The coarser residual was reserved in a stock culture dish under room temperature for about one week after the sample was wet sieved to remove residual <300-mesh fraction with seawater. Living *A. aomoriensis* were picked under a stereo microscope (LEICA S8AP0). Individuals with yellow protoplasm and strong pseudopodial activity were isolated, transferred to culture vessels.

Glass beakers with a bottom area of about 28 cm<sup>2</sup> and a height of 4 cm were used for stock cultures, and the overlying silver papers were used to avoid evaporation. These culture vessels have the advantage of holding a sufficiently large volume of seawater and they are easy to examine under the microscope. These vessels containing ambient seawater (salinity 30‰) were kept in six thermostatic incubators (6 °C, 10 °C, 12 °C, 18 °C, 20 °C, 24 °C). Salinities of 24‰, 27‰ and 33‰ were adjusted

by either diluting with deionized water or evaporating ambient seawater, and kept in 18 °C only. Three repetitions were set up in each treatment, placed in a water bath to avoid salinity change. To distinguish newly born offspring under controlled conditions precisely (only the proloculus size of the offspring was meaningful and measured in this study), fluorescent calcite stain calcein (~7mg/L) was added to each culture medium. Calcein-method has been successfully used in foraminiferal culturing (Bernhard *et al.*, 2004; Allison *et al.*, 2010; Dissard *et al.*, 2010a; Dissard *et al.*, 2010b; Raitzsch *et al.*, 2010; Dueñas-Bohórquez *et al.*, 2011; Diz *et al.*, 2012). When examining specimens under a fluorescence microscope (OLYMPUS BX53, blue excitation, 470–495 nm), the calcite precipitate during culture experiment would be bright green, which was easily separated from the calcite precipitated before the culture experiment (Fig. 1f).

### MPS measurement and analysis

Only living (stained) individuals collected from the field were picked out for MPS measurement (Fig. 1e), The MPS size was measured in different size fractions, viz., 200–250 µm, 250–300 µm, 300–350 µm, 350–400 µm, 400–450 µm, 450–500 µm, 500–550 µm. All the proloculus size of the offspring of *A. aomoriensis* in culture were measured (Fig. 1g). Test and proloculus size of *A. aomoriensis* were measured along the longest axis, under an objective micrometer using software cell Sens Standard. The offspring of *A. aomoriensis* from four cultivations were pooled together to refine the accuracy of the MPS calculation and increase the repetitions for statistical analysis. Linear regression was performed on MPS using Sigma-Plot 12.5. Analysis of variance (ANOVA) was used in order to determine the quality of a regression model. Normality test (Shapiro-Wilk) and constant variance test were passed for every regression.

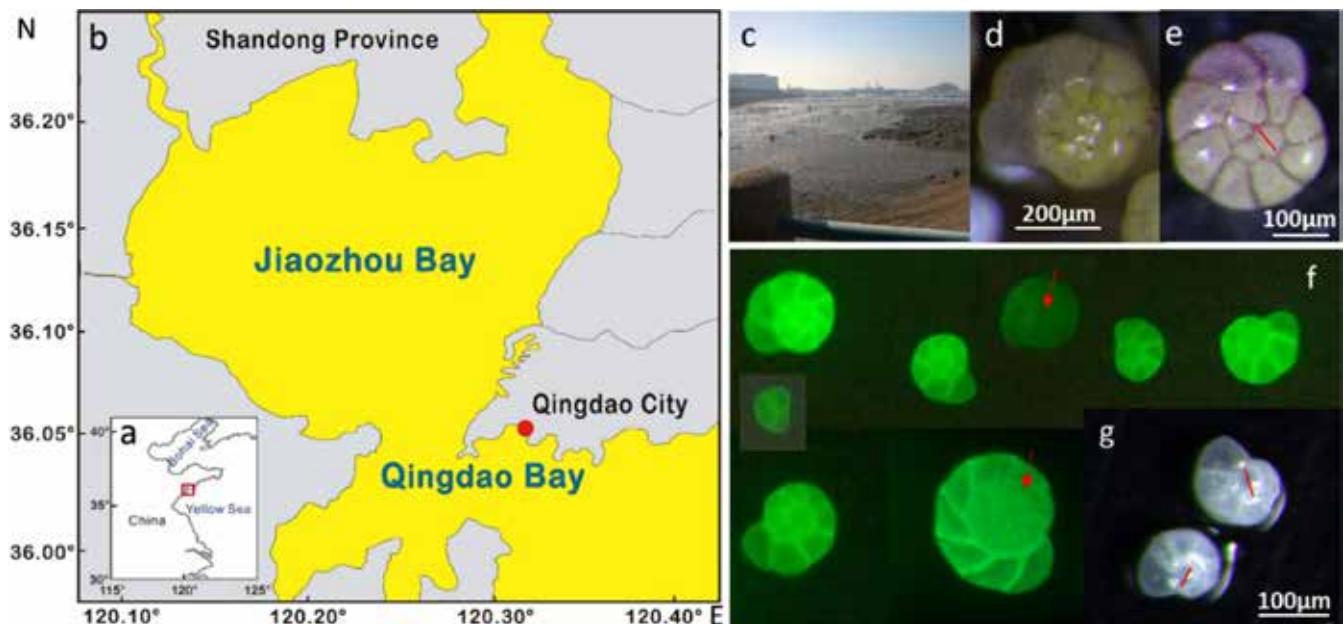


Fig. 1. (a, b) Sampling site of living *Ammonia aomoriensis*, Qingdao intertidal flat, Shandong Province. (c) Spot picture of intertidal flat in Qingdao Bay. (d) Living *A. aomoriensis* with yellow protoplasm. (e) Rose Bengal stained individuals of *A. aomoriensis*, red lines show the proloculus size. (f) Calcein stained specimens observed under fluorescence microscope: the upper arrow indicates the dim specimen which does not precipitate any calcite during the culture experiments; the under arrow indicates the adult specimen which precipitates the last five tests (bright suture) during the culture experiments; the rest specimens are the offspring of *A. aomoriensis* used in MPS measurement, and all the tests are bright green. (g) Proloculus size (red line) measurement of the offspring of incubated *A. aomoriensis*.

## RESULTS

### Temperature experiment in culture

Six temperature gradients ( $6^{\circ}\text{C}$ ,  $10^{\circ}\text{C}$ ,  $12^{\circ}\text{C}$ ,  $18^{\circ}\text{C}$ ,  $20^{\circ}\text{C}$ ,  $24^{\circ}\text{C}$ ) with controlled salinity (30‰) are set to explore the temperature effect on the proloculus size of *A. aomoriensis*. 1864 offsprings of *A. aomoriensis* are employed in the temperature experiment, gathered from different reproductive events in different treatments. The proloculus sizes range from 27.82–111.60 µm. The mean proloculus size measurement in each treatment is based on 28–176 individuals, which is listed in Table 1. Three repeats of offspring are obtained in  $6^{\circ}\text{C}$  incubation. The maximum and minimum MPS of *A. aomoriensis* are 57.38 µm and 52.42 µm, respectively. The mean MPS in  $6^{\circ}\text{C}$  is 55.19 µm with a standard error of 0.55 µm. The MPS values of *A. aomoriensis* in  $10^{\circ}\text{C}$  vary from 54.53 µm to 58.15 µm, with a mean value of  $56.37 \pm 0.47$  µm. The maximum MPS value among four repeats in  $12^{\circ}\text{C}$  treatment is 55.88 µm, and the minimum is 53.74 µm. The average proloculus size of *A. aomoriensis* in  $12^{\circ}\text{C}$  is 54.59 µm, and its standard error is 0.54 µm. The mean proloculus size of *A. aomoriensis* in temperature  $18^{\circ}\text{C}$  is  $53.11 \pm 0.65$  µm, from the two repeats (52.92 µm and 53.29 µm). The highest and lowest values of the MPS from three repeats in temperature  $20^{\circ}\text{C}$  are 57.92 µm and 52.20 µm, and the calculated average is 54.40 µm, with a standard error of 0.56 µm. Four repeats (MPS values range from 54.82 µm to 57.63 µm) are available in incubation  $24^{\circ}\text{C}$ , the average MPS in this condition is  $56.19 \pm 0.56$  µm.

Statistical analysis by Sigma-Plot is run based on those MPS values from 20 treatments. As shown in Fig. 2, the MPS data is dispersedly distributed and there is no statistical significance between the MPS value of incubated born *A. aomoriensis*

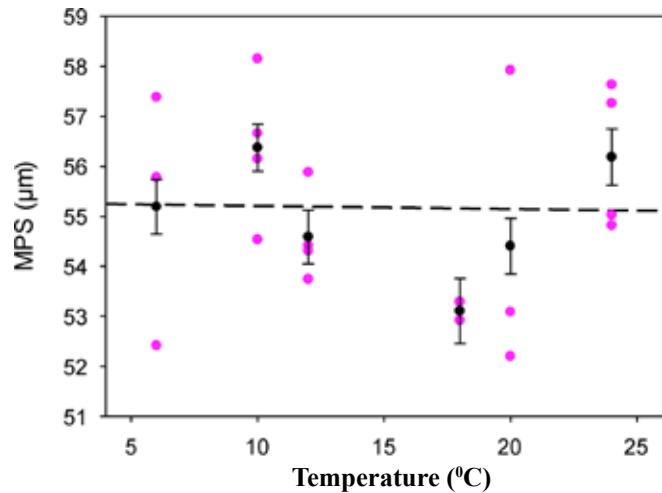


Fig. 2. Linear regression (dash line,  $p = 0.9285$ ) between MPS of the offspring of *A. aomoriensis* in culture experiments and temperature. The violet dots show the MPS of every treatment, black dots and error bars are the average MPS of each temperature and its standard error.

and controlled temperature ( $p = 0.9285$ ) in the six different temperature gradients with ambient salinity.

### Salinity experiment in culture

Salinities of 24‰, 27‰ and 33‰ are adjusted in incubation  $18^{\circ}\text{C}$ , extra 1110 sizes of the first chamber in *A. aomoriensis* are measured, ranging from 29.44–98.74 µm with 41–126 specimens of offspring from each treatment (Table 1). Total four salinity gradients (including controlled salinity 30‰) are designed to

Table 1: Experimental mean proloculus size and its standard error of the offspring of incubated *A. aomoriensis*. Temperature experiment is at controlled salinity 30‰, and salinity experiment is maintained at  $18^{\circ}\text{C}$ .

Condition	Number	MPS (µm)	Mean (µm)	Std. error (µm)	Condition	Number	MPS (µm)	Mean (µm)	Std. error (µm)
<b>Temperature experiment</b>									
6 °C	91	57.38	55.19	0.55	18 °C	102	52.92	53.11	0.65
	96	52.42				86	53.29		
	97	55.78			20 °C	75	52.20	54.40	0.56
10 °C	63	56.66	56.37	0.47		80	53.09		
	176	54.53				99	57.92		
	123	56.15			24 °C	106	57.26	56.19	0.56
	137	58.15				82	57.63		
12 °C	28	53.74	54.59	0.54		50	54.82		
	107	55.88				92	55.03		
	81	54.42							
	93	54.31			total	1864		55.18	0.23
<b>Salinity experiment</b>									
24‰	95	54.28	56.90	0.59	33‰	119	57.15	54.14	0.42
	87	58.64				104	51.20		
	41	57.78				111	53.31		
27‰	113	54.78	56.63	0.46		126	54.89		
	115	58.34							
	98	56.72							
	101	56.67						-	-

investigate the salinity effects on MPS of *A. aomoriensis*. The maximum MPS among the three repeats in salinity 24‰ is 58.64 µm, the minimum value is 54.28 µm, and the average MPS of *A. aomoriensis* is  $56.90 \pm 0.59$  µm. MPS of the four repeats of *A. aomoriensis* reproduced in 27‰ vary from 54.78 µm to 58.34 µm, and the average is 56.63 µm with a standard error of 0.46 µm. The average proloculus size  $54.14 \pm 0.42$  µm is obtained from four groups of the offspring of *A. aomoriensis* (51.20–57.15 µm) grown in salinity 33‰. As to the controlled medium (salinity 30‰), all the data in the temperature experiment are employed. There is no significant influence of temperature on proloculus size of *A. aomoriensis*. The average MPS referred to salinity 30‰ is 55.18 µm, with a standard error of 0.23 µm.

Thus, 31 repeats were analyzed by Sigma-Plot 12.5 to explore a precise expression, the result shows a statistically significant negative correlation between the proloculus size of *A. aomoriensis* and salinity (Fig. 3), which could be described as:

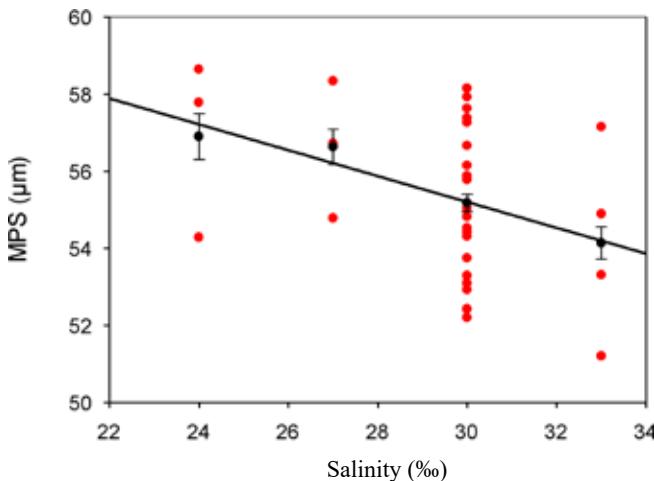


Fig. 3. Linear regression (black line) between MPS value of *A. aomoriensis* and salinity. The red dots show the MPS from every treatment, black dots and error bars are the average MPS of each temperature and its standard error.

$$\text{MPS} (\mu\text{m}) = 65.2737 (\pm 4.3334) - 0.3357 (\pm 0.1468) \text{ Salinity} (\%) \quad (1)$$

The correlation coefficient  $r = -0.3908$ , ANOVA test  $p = 0.0297$ , and the standard error of estimate is 1.91 µm, within salinity range 24–33‰.

#### Salinity experiment in field

The proloculus sizes of 3148 stained *A. aomoriensis* are measured in total, ranging from 9.39 µm to 102.86 µm with 87–568 specimens studied every month. The MPS measurement is grouped by different test-size (200–550 µm, 50 µm in interval). From May 2014 to August 2014, the monthly average MPS values of living *A. aomoriensis* are  $52.20 \pm 0.66$  µm,  $54.80 \pm 0.44$  µm,  $55.60 \pm 0.81$  µm and  $59.00 \pm 1.12$  µm, respectively. The MPS increases progressively with the decreasing salinity (from 30‰ to 29‰), reaching the maximum in summer (Table 2). From September 2014 to November 2014, the average proloculus sizes are  $54.24 \pm 0.81$  µm,  $51.53 \pm 0.70$  µm,  $54.88 \pm 0.66$  µm, respectively, for the same spot measured salinity 30‰. From December 2014 to March 2015, MPS of *A. aomoriensis* decreases to reach the lowest value, with the monthly averages  $50.13 \pm 0.70$  µm,  $48.68 \pm 0.67$  µm,  $43.93 \pm 0.73$  µm and  $43.60 \pm 0.73$  µm. The measured field water salinity is 31‰, restricted by the accuracy of the handheld salinometer. In spring, the average MPS of living *A. aomoriensis* increases again by  $46.92 \pm 0.81$  µm in April and  $47.86 \pm 0.59$  µm in May 2015 when the measured water salinity is 30‰ in both months.

The MPS data marked by # is not grouped in different sizes.

67 MPS of *A. aomoriensis* yielded by test size grouping in Table 2 are used for statistical analysis against salinity by Sigma-Plot 12.5. A wonderful inverse relationship between MPS of stained *A. aomoriensis* in field and water salinity is observed, with correlation coefficient  $r = -0.6979$ , ANOVA test  $p < 0.0001$ , the standard error of this estimate is 4.09 µm (Fig. 4), over a much narrower salinity range (29–31‰) compared to the laboratory experiment. The formula is:

$$\text{MPS} (\mu\text{m}) = 214.7030 (\pm 20.7783) - 5.4215 (\pm 0.6902) \text{ Salinity} (\%) \quad (2)$$

Table 2: Measured MPS values of stained *A. aomoriensis*, individual number, salinity as well as temperature of sediment ( $T_s$ ) of sampling-site seawater, in the intertidal every month from May 2014 to May 2015 and their average values in every salinity interval.

Date	MPS (µm) in different test size							Mean MPS (µm)	Std. error (µm)	Total number	Salinity (%)	$T_s$ (°C)
	200–250 (µm)	250–300 (µm)	300–350 (µm)	350–400 (µm)	400–450 (µm)	450–500 (µm)	500–550 (µm)					
27.05.2014	50.38	51.50	52.66	52.73	53.75			52.20	0.66	265	30	22.8
15.06.2014	52.48	51.70	56.19	55.57	57.47	57.06	53.15	54.80	0.44	568	29.5	23.5
15.07.2014	51.96	53.62	56.57	59.78	59.93	51.71		55.60	0.81	215	29	23
16.08.2014	51.70	55.96	60.51	59.30	63.47	63.09		59.00	1.12	87	29	30
15.09.2014	48.44	52.25	56.40	58.06	56.04			54.24	0.81	195	30	23
14.10.2014	48.28	50.57	52.14	55.83	50.83			51.53	0.70	256	30	21
14.11.2014	51.73	52.25	54.53	52.51	55.70	62.55		54.88	0.68	241	30	14
14.12.2014	44.81	48.89	50.83	52.82	53.31			50.13	0.70	214	31	8
13.01.2015	46.91	47.66	48.51	47.33	49.15	52.51		48.68	0.67	279	31	6.2
11.02.2015	42.06	44.32	39.83	48.52	44.94			43.93	0.73	222	31	3.8
19.03.2015	54.57	46.74	37.11	40.09	39.07	43.99		43.60	0.73	228	31	9
17.04.2015				46.92 <sup>#</sup>				46.92	0.81	177	30	11.5
17.05.2015	47.44	48.38	46.43	49.17				47.86	0.59	201	30	16.9
Total								57.30	0.67	302	29	
								54.80	0.44	568	29.5	
								51.27	0.30	1335	30	
								46.59	0.37	943	31	

From Table 2 and Figure 4, we can see the average MPS value of *A. aomoriensis* in field salinity 29‰ is  $57.30 \pm 0.67 \mu\text{m}$ , and that in salinity 29.5‰ is a little lower,  $54.80 \mu\text{m}$ , with a standard error of  $0.44 \mu\text{m}$ . Furthermore, the proloculus size of *A. aomoriensis* decreases to  $51.27 \pm 0.30 \mu\text{m}$  when water salinity increases to 30‰, which finally reaches to the minimum value  $46.59 \pm 0.37 \mu\text{m}$  at salinity 31‰.

Table 3 and Fig. 5 display the summary of the linear regressions between the proloculus size of benthic foraminifera and water salinity from both culture and field studies. Equation (3) comes from another field study of proloculus variation in *R. annectens* from core-top samples around India, which shows an even stronger dependence of MPS on the salinity variations:  $\text{MPS} = 559.0762 - 14.1027 \text{ Salinity}$ ,  $r = 0.7483$  (Nigam and Rao, 1987). The results show the slope (salinity sensitivity) of linear regression from field survey is steeper (Eq. (2),  $-5.2804$ ) than that from incubations (Eq. (1),  $-0.3357$ ), for the MPS in *A. aomoriensis*. In addition, the salinity sensitivity of *R. annectens* is much higher (Eq. (3),  $-14.1027$ ) than our species *A. aomoriensis*.

## DISCUSSION

### Physiology of *A. aomoriensis*

Studies about *A. aomoriensis* are limited as this species is often referred to as *A. tepida* (Mewes *et al.*, 2014) or *A. beccarii* (Hayward *et al.*, 2004; Haynert and Schönfeld, 2014; Lei and Li, 2015). *A. aomoriensis* is well known for its robustness under laboratory conditions, high survival and asexual reproduction rates, characterized by its insensitivity to alteration in natural conditions (Mewes *et al.*, 2014).

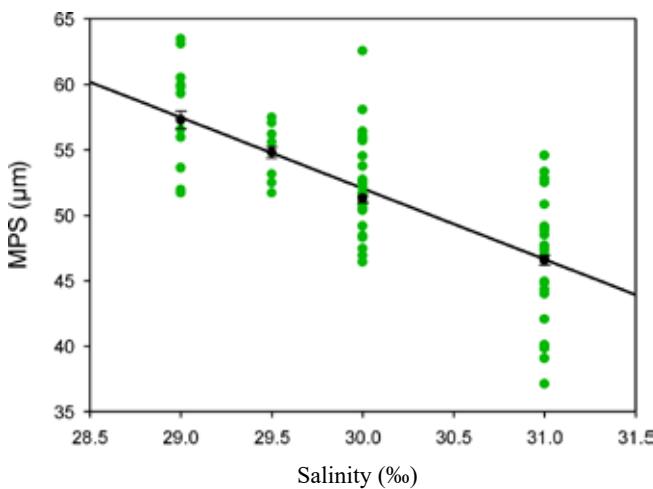


Fig.4. Linear regression (black line) between MPS value of *A. aomoriensis* in field and salinity. The green dots show the MPS in different test size, black dots and error bars are the average MPS of every salinity and its standard error.

Table 3: Summary of relationship between the proloculus size of different foraminiferal species and water salinity from both culture and field studies.

MPS (µm) = b + a * salinity (‰)									
Species	Experiment	S (‰)	T(°C)	a	b	r	p	Equation	Source
<i>A. aomoriensis</i>	culture	24–33	6–24	-0.3357	65.2737	-0.3908	0.0297	(1)	this study
<i>A. aomoriensis</i>	field	29–31	3.8–30	-5.4215	214.7030	-0.6979	< 0.0001	(2)	this study
<i>R. annectens</i>	field	33.7–36	27.5–29.25	-14.1027	559.0762	-0.71	No data	(3)	Nigam and Rao, 1987

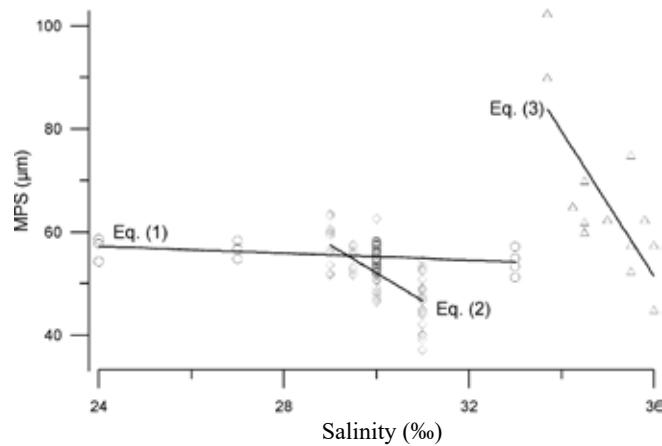


Fig.5. Comparison of MPS in *A. aomoriensis* between culture experiment (Eq. (1)) and field study in Qingdao Bay this study (Eq. (2)), as well as the field MPS values of *R. annectens* from core-top samples around India (Eq. (3)) (Nigam and Rao, 1987).

The maximum growth of *A. aomoriensis* is observed at 18 °C (warm end among 8 °C, 13 °C and 18 °C) which is slightly above the measured bottom water maximum temperature (15.3 °C) in summer in the southwestern Baltic Sea (Haynert and Schönfeld, 2014). However, specimens of *A. aomoriensis* in this experiment grow better at lower temperatures (6 °C, 10 °C and 12 °C) which coincides with the high density of *A. aomoriensis* population found in the winter season of intertidal flat of the Qingdao Bay, also seen in the study of Lei and Li (2015). The significant response of optimum growth to the environment is likely attributed to their different long-term accustomed habitats. *A. aomoriensis* inhabits 13 m water depth with 16.8–26.3‰ and -0.8–15.3 °C throughout the year in Flensburg Fjord, which is a euryhaline species (Haynert *et al.*, 2012; Haynert and Schönfeld, 2014). Whereas the distribution of *A. aomoriensis* in the Yellow Sea and East China Sea is 0–80 m deep, 3–24.5 °C and 31–38‰ (Lei and Li, 2015), and *A. aomoriensis* living in our sample site is within a narrower salinity range (29–31‰, no river input) and a temperature range of 3.8–30 °C, revealing a higher salinity and temperature habitat than Baltic Sea.

Fluorescent calcein was added to the culture medium to mark the newborn offspring of *A. aomoriensis*. Foraminifers incubated in solution of calcein and seawater, precipitated normal-looking chambers during the three-week incubation and the survival rate was similar to the control specimens, the reproduction occurred during or after calcein exposure (Bernhard *et al.*, 2004). The comparable growth, abnormality, mortality and reproduction in both the control and experimental set-ups suggest that short-term exposure (3–4 weeks) to calcein does not adversely affect benthic foraminifera (Kurtarkar *et al.*, 2015). In this study, no abnormality is observed under the exposure of calcein, and we postulate it does not affect the size of the initial chamber in *A. aomoriensis*. During the four culture experiments, reproduction is observed in all temperatures (6–24 °C) and salinities (24–33‰),

expressing a wide reproduction range. Living *A. aomoriensis* is stimulated to asexual reproduction at 20 °C and 32‰ (Mewes *et al.*, 2014). Reproduction of *A. tepida* takes place at temperatures between 20 °C to 30 °C and salinities between 13‰ and 40‰ (Bradshaw, 1957). Megalospheric *A. tepida* undergoes asexual reproduction at salinity 30‰ and temperature 25 °C (de Nooijer *et al.*, 2014). Adults of *A. beccarii* reproduce asexually at 28 °C and 20‰ (Toyofuku *et al.*, 2011).

#### Salinity control in culture and field study on proloculus

The first correlation between foraminiferal proloculus size and ecological parameter was conducted in one-year study on the living *A. beccarii* from a station in the mouth of the West Passage of Narragansett Bay, the proloculus size was roughly grouped but still presented larger values from July to October (Brooks, 1967). While a survey based on proloculus diameter of three morphotypes of *A. beccarii* in natural salinity (7.4–92‰) and temperature (12–28 °C) gradients somewhat beyond the optimum ranges in salinity displayed no clear relationship with the environmental variables (Malmgren, 1984). Then Nigam (1986) analyzed the ratio of dimorphic forms of *B. skagerrakensis* from a 10.5-meter core, which fitting well with the foraminiferal abundance and calcite  $\delta^{18}\text{O}$ , demonstrated a temperature dependence. Moreover, measurement for MPS values of *R. annectens* from 14 surface sediment samples along the west coast of India furnished two regressions: Temperature = 30.1219 – 26.8357 MPS ( $r = -0.78$ ) and Salinity = 37.5224 – 37.5634 MPS ( $r = -0.71$ ), describing its inverse proportion to both temperature and salinity variations (Nigam and Rao, 1987). Furthermore, MPS variation of *R. annectens* in a sediment core near the Kali River coincided with the 100-year rainfall record from the catchment area, confirming that MPS was inversely related to salinity and encouraging its potential use in inferring the past climate (Nigam and Khare, 1995). Then, a 2500-year monsoon precipitation variation was recovered from a core located near the mouth of Kali River, by compilation of the grain size, foraminiferal abundance and the MPS of *R. annectens* (Khare *et al.*, 2008). In addition, the MPS of *R. annectens* from Pearl River Delta fitted well with the ITCZ migration, indicating the summer monsoon variation during the Late Holocene (Yu *et al.*, 2014). It can be seen that the salinity influence on foraminiferal initial chamber is recognized gradually but disagreement still exists. Living *A. parkinsoniana* individuals collected during a period of higher salinity (50‰) exhibited a larger proloculi as compared to those collected during lower salinity (14‰), which was inconsistent with early recognitions (Colburn and Baskin, 1998). As a result, culture experiments where temperature and salinity could be manipulated independently are required to confirm a reliable relationship and develop a method for paleoenvironmental interpretation (Nigam, 1986; Nigam and Rao, 1989).

In this study, two linear regressions were established for the proloculus size of benthic foraminifera *A. aomoriensis* with respect to water salinity: MPS = 65.2737 ( $\pm 4.3334$ ) – 0.3357 ( $\pm 0.1468$ ) Salinity (Eq. (1),  $p=0.0297$ ,  $r=-0.3908$ , 24–33‰) in cultures, and MPS = 214.7030 ( $\pm 20.7783$ ) – 5.4215 ( $\pm 0.6902$ ) Salinity (Eq. (2),  $p<0.0001$ ,  $r=-0.6979$ , 29–31‰) in field. As Table 3 and Figure 5 shown, the slope of equation (2) is much steeper than equation (1): –5.4215 versus –0.3357, and the fluctuant extent of the MPS in *A. aomoriensis* collected from natural circumstance (37.11–63.47 µm for 2 salinity-unit intervals) is much stronger than in the physicochemical

controlled medium (51.20–58.64 µm for 9 salinity-unit intervals). The discrepancy mainly occurs in salinities 30‰ and 31‰. The MPS of living *A. aomoriensis* at salinity 30‰ from incubation is  $55.18\pm0.23$  µm, larger than  $51.27\pm0.30$  µm measured from stained specimens in nature. Additionally, the calculated MPS by equation (1) at salinity 31‰ is  $54.87\pm0.22$  µm, alike larger than  $46.59\pm0.37$  µm measured from the field individuals.

#### Comparison of culture and field studies

The discrepancy of proloculus-salinity relationship in *A. aomoriensis* between culture and field studies may come from two aspects.

On one hand, dimorphism phenomenon exists in field, although the number of microspheric specimens is considerably less than the number of megalospheric form in normal circumstance (Nigam, 1986; Douglas and Staines-Urias, 2007; Staines-Urias and Douglas, 2009). For example, the percentage of microspheric *Elphidium crispum* individuals is about 3% during most of the year, and it increases to 67% in April (Myers, 1942). The microspheric form of investigated *A. beccarii* is less than 7.5% (Brooks, 1967). No conclusion has been reached about the boundary of the dimorphism. Smith(1963) documented that the microspheric generation of *Bolivina* develops from gametes that are typically 1–4 µm. A proloculus diameter less than 14 µm is considered as microspheric form for *A. beccarii* (Brooks, 1967), and 10–20 µm viewed for *B. skagerrakensis* (Qvale and Nigam, 1985). In this study, we take the specimen whose proloculus size less than 21 µm as microspheric, according to observation. In our incubations, all the reproduced juveniles are megalospheric forms, same as observed in earlier culture studies for *A. tepida* (Bradshaw, 1957; Schnitker, 1974; de Nooijer *et al.*, 2014), *A. beccarii* (Toyofuku *et al.*, 2011) and *Amphistegina* spp. (Hallock and Larsen, 1979). Hundreds of gamonts are reproduced by division of the parental cell and the minimum proloculus size value of *A. aomoriensis* in all salinity intervals is 27.82 µm. While in the upmost sediment layer collected from Qingdao Bay, although the megalospheric forms are dominated, microspheric individuals (1.14% throughout the year) of *A. aomoriensis* are co-existing, its proportion reaches the highest to 5.70% in March. The average abundance of microspheric specimens of *A. aomoriensis* is 0.53% in salinity 30‰ and 2.76% in salinity 31%, see Table 4, this may explain the strongest deviation which occurs in winter.

The option of different reproduction mode depending upon the prevailing environmental conditions, would ensure higher survival rate of individuals of the species (Goldstein and Moodley, 1993). Many authors have tried to explain the mechanism which controls dimorphism. Lipps (1976) suggests that in seasonally fertile, upwelling regions in the open ocean, abundant food encourages the population booming by asexual reproduction, yet in areas or at times of decreased fertility, a population can grow more leisurely and slowly and is engaged in more elaborate sexual reproductive strategies. Asexual reproduction occurs in comfortable circumstances because it consumes more cellular material and energy (Zhu, 1993; Colburn and Baskin, 1998; Saraswat *et al.*, 2011), and megalospheric proloculi can survive more easily than microspheric one (Alve and Goldstein, 2003). But microspheric forms are comparatively more numerous under unfavorable harsh conditions such as winter, or conditions when temperature exceed 20 °C (Nigam, 1986), or under variable or fluctuating environmental conditions, because it permits an increase in genetic variability within a population

(Colburn and Baskin, 1998). Megalospheric tests of *Bolivina subadvena* and *B. interjunctablicostata* predominate in higher oxygen environments or the organic-rich benthic boundary above the sediment-water interface, which is consistent with the flux of food to the seafloor (Douglas and Staines-Urias, 2007). In addition, the maximum abundance of megalospheric *B. argentea* and *B. subadvena* are observed between 0 mm and 2 mm in the surface, blooming during eutrophic periods, being opportunistic and short live forms which grow fast. Whereas individuals of the microspheric generation are most abundant deeper in the sediment (1 mm and 6 mm), tolerance to low oxygen concentration and grow slowly (Staines-Urias and Douglas, 2009).

**Table 4: The component of the proloculus size of *A. aomoriensis* included in this study, divided by 21 µm and 33 µm.**

<i>Ammonia aomoriensis</i>	Salinity (%)	< 21 µm (%)	21–33µm (%)	< 33µm (%)
Culture experiment	24	0	0	100
	27	0	0	100
	30	0	0.27	99.73
	33	0	0.65	99.35
Field study	29	0.33	0.99	98.68
	29.2	0.35	1.76	97.89
	30	0.53	4.79	94.68
	31	2.76	10.92	86.32

In addition to classical life cycle, a third form known as megalospheric schizont has been reported in 16 species of foraminifera, such as *A. beccarii*, *E. crispum*, *Quinqueloculina circularis*, *Planorbolina mediterranensis*, *Heterostegina depressa* and so on, which is biologically known as trimorphic life cycle (Röttger *et al.*, 1986; Dettmering *et al.*, 1998; Lehmann *et al.*, 2006). The three size classes of proloculi correlate with: microspheric agamont, smaller megalospheric schizont and larger megalospheric gamont (Lehmann *et al.*, 2006). Megalospheric schizonts are reproduced by autogamy (self-fertilization) (Staines-Urias and Douglas, 2009). For megalospheric schizonts, their growth rate is lower, final test size and proloculus are smaller than normal megalospheric forms (Röttger *et al.*, 1990). Gamonts of larger foraminifera *H. depressa* have a mean proloculus diameter of 135 µm compared to 84 µm in schizonts (Röttger *et al.*, 1986). The agamonts of *Trochammina inflata* range from 16.1–22 µm, those of the supposed schizonts from 28.6–34.2 µm and those of the supposed gamonts from 36.5–41.7 µm (Lehmann *et al.*, 2006). Trimorphism is common in nature, high schizont densities of *H. depressa* were observed in natural populations (Biekart *et al.*, 1985; Lehmann *et al.*, 2006), and all megalospheric individuals collected from the natural habitat were schizonts (Röttger *et al.*, 1986). However, very little could be obtained in laboratory cultures. It was reported only 0.07% megalospheric offspring of agamonts have been shown to be schizonts in *E. crispum*, which is irregular (Röttger *et al.*, 1990). Three of 1369 megalospheric daughter individuals produced from an agamont of *H. depressa* could be convinced as schizonts (Röttger *et al.*, 1990) and all agamonts collected from Hawaii produce only gamonts in the laboratory (Röttger *et al.*, 1986). There is no research about the trimorphism phenomenon in *A. aomoriensis*, but some young individuals with a proloculus of approximately 33 µm are observed in culture studies of *A. tepida*, among most of the forms with a prolocular diameter of approximately 57

µm (Bradshaw, 1957). If we suppose 33 µm as the boundary between the schizont and gamont of *A. aomoriensis*, from Table 4, we see that the proportions of schizont occupied in incubated salinity 30‰ and 33‰ are 0.27% and 0.65% respectively. However, the sediments collected from intertidal region contain more schizonts, about 4.79% and 10.92% in salinity 30‰ and 31‰, respectively, which might be the second factor creating the difference between equation (1) and (2).

The proportions of reproductive products differ strongly in habitats subject to strong fluctuations in water content, temperature and salinity (Lehmann *et al.*, 2006). The alternation of generations may be obligatory or facultative, including one sexual and two or more asexual generations that may or not present trimorphism (Goldstein, 1999).

The earlier study about proloculus size is limited. The first report that discussed the salinity influence on proloculus size was given by Nigam and Rao, 1987 (Fig. 5, Eq. (3)). 14 surface sediment samples were analysed to measure MPS of *R. annectens* from the near-shore region along the west coast of India during pre-monsoon when temperature varies from 27.5 °C to 29.25 °C and salinity from 33.7‰ to 36‰. The results show that MPS is inversely proportional to salinity ( $r = -0.71$ ) variations. Regression (3) exhibits a much larger slope ( $a = -14.1027$ ) than regressions of *A. aomoriensis* which might be attributed to more unstable water regime caused by various sampling locations and river runoff along the west coast of India. Moreover, in foraminifera, life cycles may differ from one species to another with respect to gamete morphology, mode of fertilization, test dimorphism, and the pattern of sexual and asexual generations reflecting innate physiological differences (Goldstein, 1999).

#### Temperature effect on the proloculus size of *A. aomoriensis*

The temperature impact on foraminiferal MPS is raised earlier than salinity (Nigam, 1986), by the comparison between the ratio of dimorphic forms and  $\delta^{18}\text{O}$  in *B. skagerrakensis*. However,  $\delta^{18}\text{O}$  in foraminiferal calcite records not only the information of temperature, but also seawater  $\delta^{18}\text{O}$  (salinity) (Shackleton, 1974; Waelbroeck *et al.*, 2002; Elderfield *et al.*, 2012). In addition, MPS of *A. aomoriensis* was regressed against water temperature from the 14 surface sediments (Nigam and Rao, 1987). The temperature and salinity in nature interrelate in a complex way which further complicates the results (Malmgren, 1984; Colburn and Baskin, 1998). In this study, water temperature probably plays a more important role on the density of *A. aomoriensis* is revealed in both culture and field experiments. Lower temperature is advantageous to the growth of *A. aomoriensis*, but has no significant effect on the proloculus size of *A. aomoriensis*. Morphological study including proloculus size of *A. parkinsoniana* collected in field revealed that temperature probably is of little importance in causing morphological differences, while salinity is much more important (Colburn and Baskin, 1998).

#### CONCLUSIONS

Our results which are based on culture experiments and field study show that the proloculus size of benthic foraminifera *Ammonia aomoriensis* could be a reliable method to be used in palaeoceanographic reconstruction. Culture experiments of the offspring of *A. aomoriensis* display a negative correlation to water salinity: MPS (µm) = 65.2737 ( $\pm 4.3334$ ) – 0.3357

( $\pm 0.1468$ ) Salinity (‰) (Eq. (1),  $p = 0.0297$ ,  $r = -0.3908$ , salinity range 24–33‰), and this equation would be more suitable for application in favourable stable environment, where sexual reproduction does not happen. The field study of stained *A. aomoriensis* also presents a negative correlation to water salinity, but with a much steeper slope: MPS ( $\mu\text{m}$ ) = 214.7030 ( $\pm 20.7783$ ) – 5.4215 ( $\pm 0.6902$ ) Salinity (‰) (Eq. (2),  $p < 0.0001$ ,  $r = -0.6979$ , salinity range 29–31‰). The sharper slope may be attributed to the relatively high densities of the microspheric agamonts and megalospheric schizonts of living *A. aomoriensis* at higher salinity in field, which decrease the mean proloculus size. As a result, equation (2) would be more appropriate to recover the fluctuated environment, where trimorphism occurs. In addition, temperature does not directly control the changes observed in MPS values.

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