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Research Article

Adaptive mechanisms of invasion of *Chthamalus challengeri* (Hoek, 1883) in the trans-oceanic zone of coastal China

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Abstract

Chthamalus challengeri Hoek, 1883 (Crustacea, Cirripedia) is typically found in the Bohai Sea and Yellow Sea along the coast of China. However, until 2009, it was never seen in the East China Sea. In 2010, *C. challengeri* was discovered at Yangshan Port in Zhoushan, East China Sea, and it has since been found to invade several islands in the Zhoushan Islands area successfully. Although the population that invaded Yangshan Port has disappeared in recent years, the population that successfully invaded the other islands in Zhoushan has been increasing in density. To study the ecological adaptability of *C. challengeri* larvae from the Zhoushan Sea Area, we conducted an experiment observing the larvae's response to different temperatures and salinity gradients. The results indicate that the *C. challengeri* larvae are highly adaptable to different temperatures and salinities, and under temperatures ranging from 10–25 °C and salinities of 25–35, nauplius can complete all six stages of development and reach a settlement. We found that the survival and settlement rates during larval development were highest at 20 °C and salinity 30, which could be considered the optimum conditions for *C. challengeri* larvae. At these conditions, it took approximately 11.5 days for the larvae to undergo development from nauplius I to complete settlement. However, lower temperatures slowed down the development rate and settlement of *C. challengeri* larvae to some extent, while high temperatures can directly lead to the death of *C. challengeri*. According to the results of this study, the settlement period of *C. challengeri* in a new habitat can last as long as 7 months (April to November) compared to its original environment. This extended settlement period could provide favorable conditions for the long-distance dispersal of *C. challengeri* and enhance its invasive ability in new habitats.

Key words: stages, development, settlement, Zhoushan Sea, China Sea

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Introduction

The spread and invasion of non-native Marine species are posing a significant challenge to the fields of marine biology and ecology (Rivera et al. 2022; Massé et al. 2023). There are two main invasion routes for exotic marine organisms: unintentional introduction and intentional introduction (Zhao et al. 2005). The number of non-native species being introduced is rising worldwide because of the growing globalization of trade and transportation (Bailey et al. 2020; Seebens et al. 2020). It has been discovered that the barnacle known as *Elminius modestus* Darwins (*Austrominius modestus*) was the initial documented instance of barnacle invasion from Australia to Europe (Bishop 1947; Crisp and Chipperfield 1948; Stubbings 1950; Crisp 1958b). There are many cases of transoceanic spread, such as the *Chthamalus proteus* invasion of the Hawaiian coast and the *Balanus glandula* invasion of the west coast of North America, and the *Megabalanus coccopoma* (Darwin, 1854) invasion of Australia (Southward et al. 1998; Kado 2003; Geller et al. 2008; Yamaguchi et al. 2009). Mainly, they were introduced through shipping from locations like Japan and the western Atlantic. Previous studies have shown that unintentional introduction occurs more frequently in areas with weaker geographic isolation (Zolotarev 1996; Gaston et al. 2003; Protopopova et al. 2006; Chapple et al. 2012). Cirripedia can be transported through fouling on the hulls of vessels or as larvae in ballast water (Torres et al. 2012). Studies have demonstrated that the success of non-native barnacle species in terms of colonization, breeding community, and larval survival rate depends on environmental suitability following their introduction to new habitats (Strathmann 1974; McQuaid and Phillips 2000; Jenkins and Hawkins 2003). The ability of an exotic species to spread in a new environment will be influenced by how well its larvae can adapt to the ecological conditions. (Crisp 1958a). The successful settlement of non-indigenous barnacle species relies heavily on two crucial environmental factors – temperature and salinity (Reed 1969; Wing et al. 1995; Anil and Kurian 1996; Gritti et al. 2006; Tie et al. 2010; Cao et al. 2013). These factors have a direct and significant impact on their growth and survival.

Chthamalus challengeri Hoek, 1883 is a type of cold-water species that is typically found in the Bohai and Yellow Seas along the Chinese coast (Liu and Ren 2007). However, in 2009, Zhu et al. discovered *C. challengeri* while surveying the intertidal *macrobenthic* communities on Dongji Island, Zhejiang Province, China. Interestingly, the Latin name for this species in that report was *Pollicipes mitella*, which was observed to exist in the low tidal zone (Zhu 2010). In 2010, *C. challengeri* was first collected from Yangshan Port, Zhoushan City, Zhejiang Province, East China Sea (Xue et al. 2011). It has been discovered that this species has successfully invaded several islands in the Zhoushan Archipelago and has been officially labeled as invasive, particularly in the middle and high tropics (Liu et al. 2014, 2015). Yangshan Port is located on an island in the sea, and its water ecology is distinct from that of coastal ports on the mainland with low salinity levels. The salinity levels of Yangshan Port are comparatively higher, with a peak of 29.56 during the summer season (Wang et al. 2011). Because Yangshan Port and the Zhoushan Islands are predominantly coastal ports, they offer favorable conditions for the proliferation and reproduction of biologically contaminated organisms on the hulls of ocean-going vessels (Liu et al. 2014, 2015). This method facilitates the migration of *C. challengeri*, a prevalent species in the Zhoushan Sea Area. It can be found and collected from nearly every island in the Zhoushan Area (Liu et al. 2015). In certain areas of the islands, it has become the most prevalent species in regions with high tides.

To study the critical factors for the success of *C. challengeri* invasion, namely the adaptation of the larvae to their new habitat, which will directly affect the colonization, breeding population and spread of the species, we collected *C. challengeri* from new habitats, incubated and obtained the larvae to observe the growth cycle and living conditions at different stages under different temperature and salinity conditions. The metamorphosis settlement rate of the cypris larva was observed and recorded, which provided a basic theoretical foundation for the adaptation and spread of *C. challengeri* in a new habitat. Field surveys were conducted in the Chinese sea area to gain a deeper understanding of the dispersal of *C. challengeri* through adaptive mechanisms.

Materials and methods

Specimen collection

1. Experimental research

Experimental samples (rocks with adult *C. challengeri*) were collected in 2013 and experimental studies were carried out on the effects of temperature and salinity on the adhesion and survival of *C. challengeri*. Rocks with adult *C. challengeri* were collected from Gouqi Island (30°42'N, 122°46'E) in the Zhoushan sea area, Zhejiang Province, China, and brought back to the laboratory (Fig. 1).

Rocks with *C. challengeri* adults were cultured in a rectangular aquarium (200 mm × 300 mm × 300 mm) with salinity of 31 °C and temperature of 22 °C after 12 hours away from light. The rectangular aquarium was covered with black plastic bags and oxygenated with an air pump. The stage I larvae of *C. challengeri* were collected by hobophototaxis. The seawater was renewed every 24 hours to cultivate the larvae.

2. Field investigation

In addition, as field surveys were not carried out and data were not perfected at the end of the culture experiment in 2013, an acclimatization survey of *C. challengeri* was carried out in Zhoushan sea area, China, based on experimental

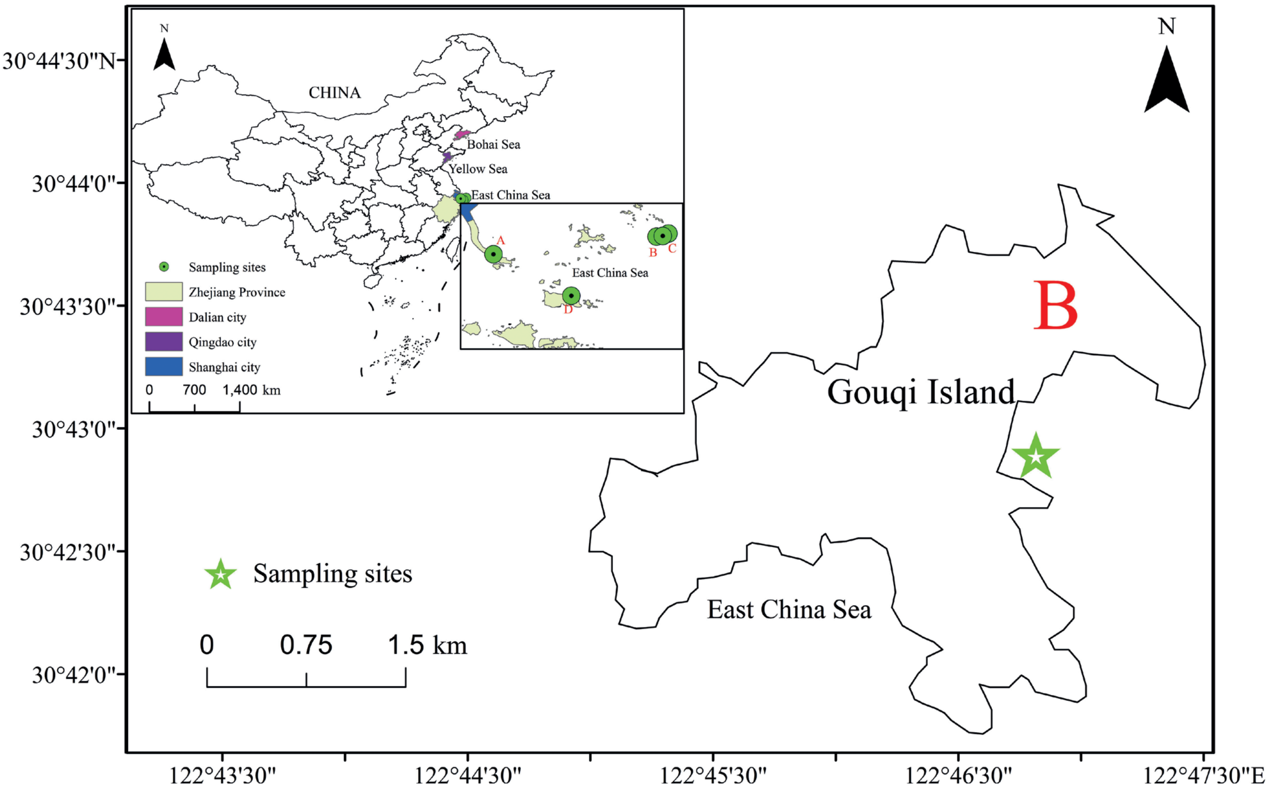

Figure 1. Collection site of adult *Chthamalus challengeri*.

data. *C. challengeri* was surveyed and collected from 4 sites in Zhoushan sea area: Yangshan Port (A), Gouqi Island (B), Shengshan (C) and Qushan Island (D) (Fig. 2). All samples were collected between 2020 and 2023.

Between August 2020 and March 2023, several *C. challengeri* survey collections were carried out on the quay at the passenger terminal of Yangshan Port Terminal 2 (Fig. 2), sample collections of biological communities were carried out in different tidal areas of the quay. A further survey collection of *C. challengeri* was made in April 2023 at Gouqi Island, Shengshan and Qushan Island (Fig. 2). Three 0.25*0.25M sample boxes were used for sampling, and all organisms within the boxes were collected with a scraper. It was divided into quantitative and qualitative sampling. In quantitative sampling, each site of the survey sampling was divided into high, medium and low tide areas, and 3 parallel samples were collected from each tide area, totaling 9 sampling points. In qualitative sampling, strip sampling is carried out within the contour range according to the quantitative sampling sites, with three samples for each site. The tidal areas were divided according to the Zhoushan sea area's tidal characteristics, and each month's low tide was selected as the sampling time. After sample collection, all samples were selected and all benthic organisms were put into pre-prepared sample storage bottles, fixed with alcohol and brought back to the laboratory for specimen identification and counting for subsequent identification.

Experimental methods

Eight temperature gradients (0–35 °C) were designed in the experiment, and seven salinity gradients (5–35) were designed for each temperature culture condition (Table 1).

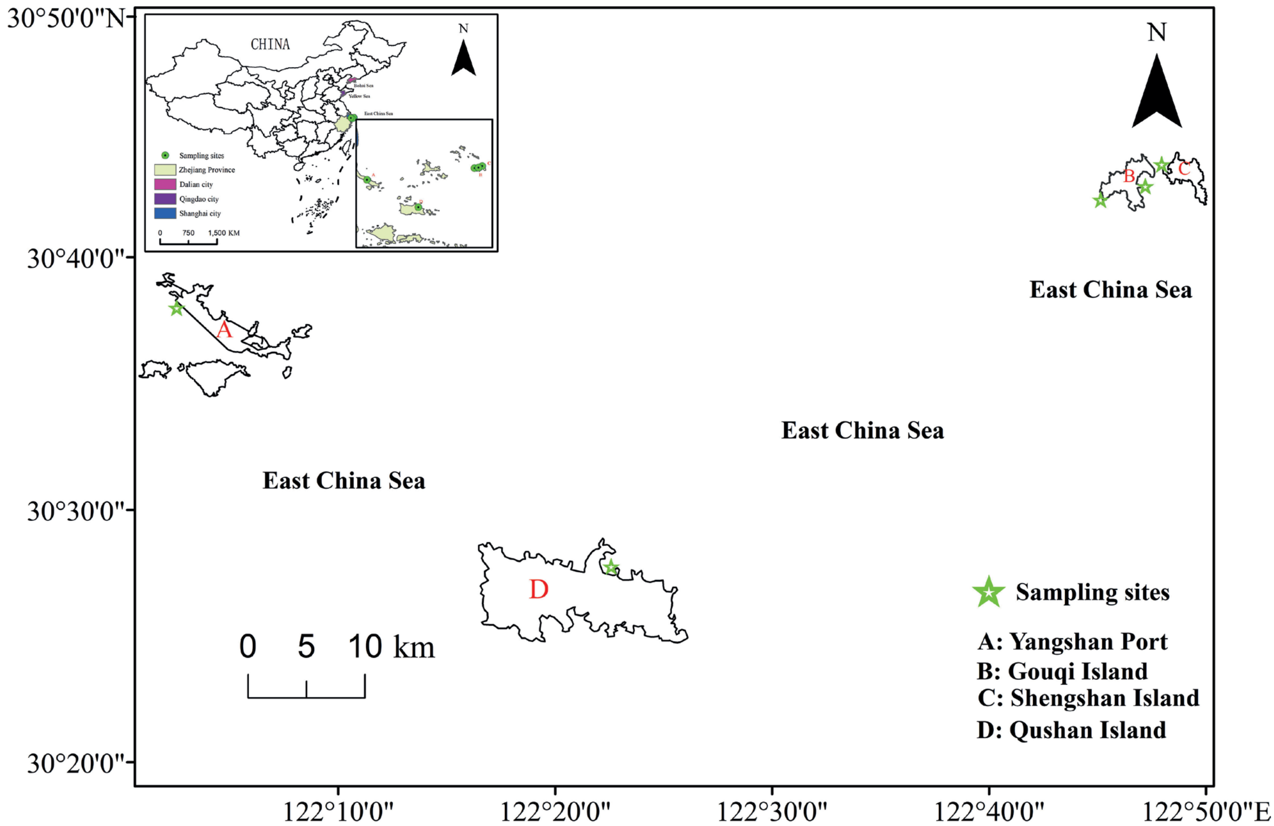

Figure 2. Sampling sites of *Chthamalus challengeri* 's survey in Zhoushan sea area (Yangshan Port, Gouqi island, Shengshan and Qushan island).

Table 1. Experimental conditions.

Salinity Temperature	5	10	15	20	25	30	35
1	0 °C	0 °C	0 °C	0 °C	0 °C	0 °C	0 °C
2	5 °C	5 °C	5 °C	5 °C	5 °C	5 °C	5 °C
3	10 °C	10 °C	10 °C	10 °C	10 °C	10 °C	10 °C
4	15 °C	15 °C	15 °C	15 °C	15 °C	15 °C	15 °C
5	20 °C	20 °C	20 °C	20 °C	20 °C	20 °C	20 °C
6	25 °C	25 °C	25 °C	25 °C	25 °C	25 °C	25 °C
7	30 °C	30 °C	30 °C	30 °C	30 °C	30 °C	30 °C
8	35 °C	35 °C	35 °C	35 °C	35 °C	35 °C	35 °C

The salinity of seawater was approximately 30–32; we used double distilled water to reduce salinity when we required less salinity than seawater. Similarly, we used sea salt to increase salinity when we needed more salinity than seawater. All culture seawater was filtered using a membrane with a pore size of 1.2 μm. Approximately 50 ml of seawater with different salinity gradients was poured into disposable trays and *Isochrysis galbana* cultured in F2 medium was placed in the trays as bait for the larvae.

Around 50–60 nauplius at stage I were placed in various salinity gradients and incubated at different temperatures. The cultures were kept from light, and six experiments were conducted for each temperature and salinity condition. The seawater in the culture tray was renewed every 24 hours, and new bait was added. Each larva's body length, width, and developmental status were also measured and recorded. No larvae were used in the control group. The growth process of *C. challengeri* larvae includes six stages of nauplius, metamorphic cypris larva, and newly settled larva (Fig. 3). The experiment was only terminated once all larvae had completed metamorphosis and settled or died.

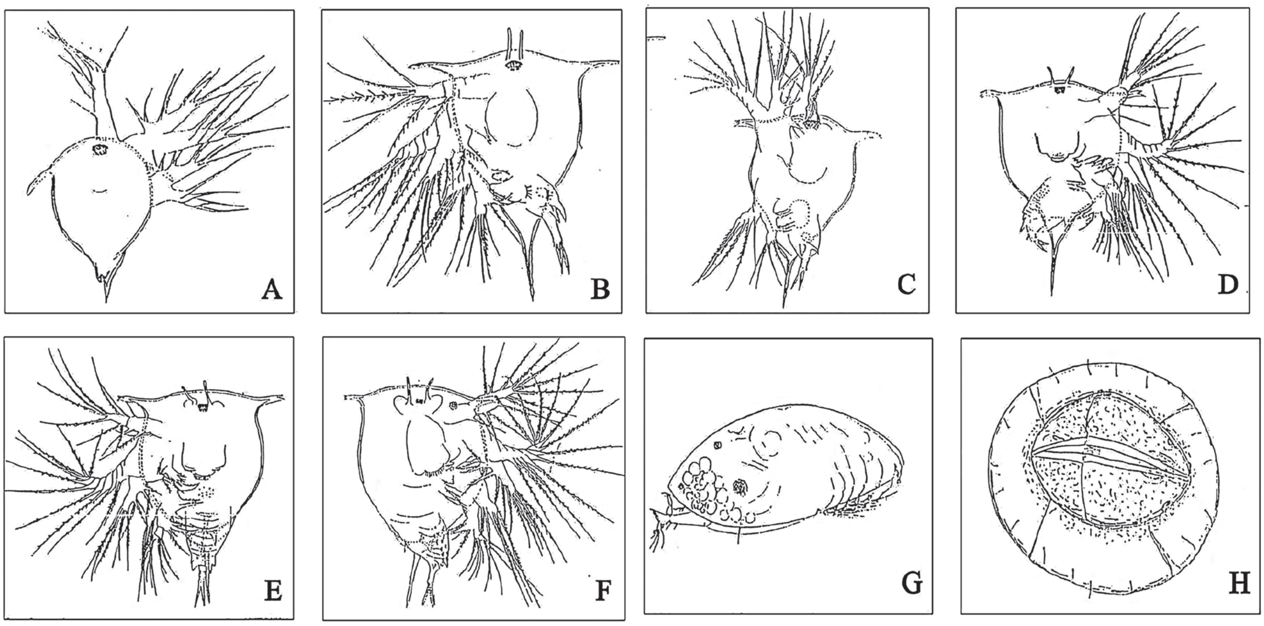

Figure 3. The larval stages of *Chthamalus challengeri*. (Notes: **A.** stage I of nauplius; **B.** stage II of nauplius; **C.** stage III of nauplius; **D.** stage IV of nauplius; **E.** stage V of nauplius; **F.** stage VI of nauplius; **G.** cypris larva; **H.** larva barnacle just settling after metamorphosis).

Data statistics

The survival rate was calculated using the following formula:

$$S_n = \frac{C_n}{C_0} \times 100\% \tag{1}$$

where, S_n (%) = the survival rate for *C. challengeri* in each group; C_n (ind.) = alive *C. challengeri* in each experimental group every day; and C_0 (ind.) = initial living *C. challengeri* in each experimental group.

The daily growth rate was calculated using the following formula:

$$R = \left[\left(\frac{L_t}{L_0}\right)^{\frac{1}{t}} - 1\right] \times 100\% \tag{2}$$

Where, R (%) = daily growth rate; L_t (μm) = average body length of *C. challengeri* in each experimental group at different measurement times; L_0 (μm) = the initial body length of *C. challengeri* in each experimental group; and t (d) = days of the experiment.

Analysis of variance (ANOVA) was performed on the obtained data using SPSS 16.0 software (Norusis 2008).

To better study the relationship between the developmental cycle of *C. challengeri* larvae at various stages and temperature and salinity, analyses were carried out using fitted regression curves. The power function argument formula is as follows:

$$D = bv^{m} \tag{3}$$

where, D = values of the power function ; b = coefficient; v = temperature or salinity; m = index.

The density of *C. challengeri* was calculated according to the following formula:

$$D = \frac{n}{Sa} \tag{4}$$

where, D (individuals/m^2) denotes the density of *C. challengeri*, n is the number of *C. challengeri* in the measurement sample (individuals), and Sa is the area of the measurement sample (m^2) (Cai et al. 1987; Liu et al. 2014).

Results

Morphological changes of *Chthamalus challengeri* in different larval stages

C. challengeri, similar to other crustaceans, has six different nauplius developmental states (Fig. 4), ranging from stage I to stage VI. In stage I, the abdominal terminal and tail were not developed (Fig. 4A). From stage II to stage V, the body shape of the nauplius changed, the body size became more prominent, the number of bristles on the appendages increased, and characterized by a segmented body chest (Fig. 4B–E). After observing the intestinal contents, it can be inferred that *C. challengeri* started feeding from stage II. The abdomen extended further in the stage VI nauplius. At the same time, the compound eyes emerged on both sides of the ocellus (Fig. 4F). The nauplius of stage VI transformed into cypris larva after ecdysis (Fig. 4G). Finally, the cypris larva developed into larva barnacle after swimming, crawling, and settling (Fig. 4H).

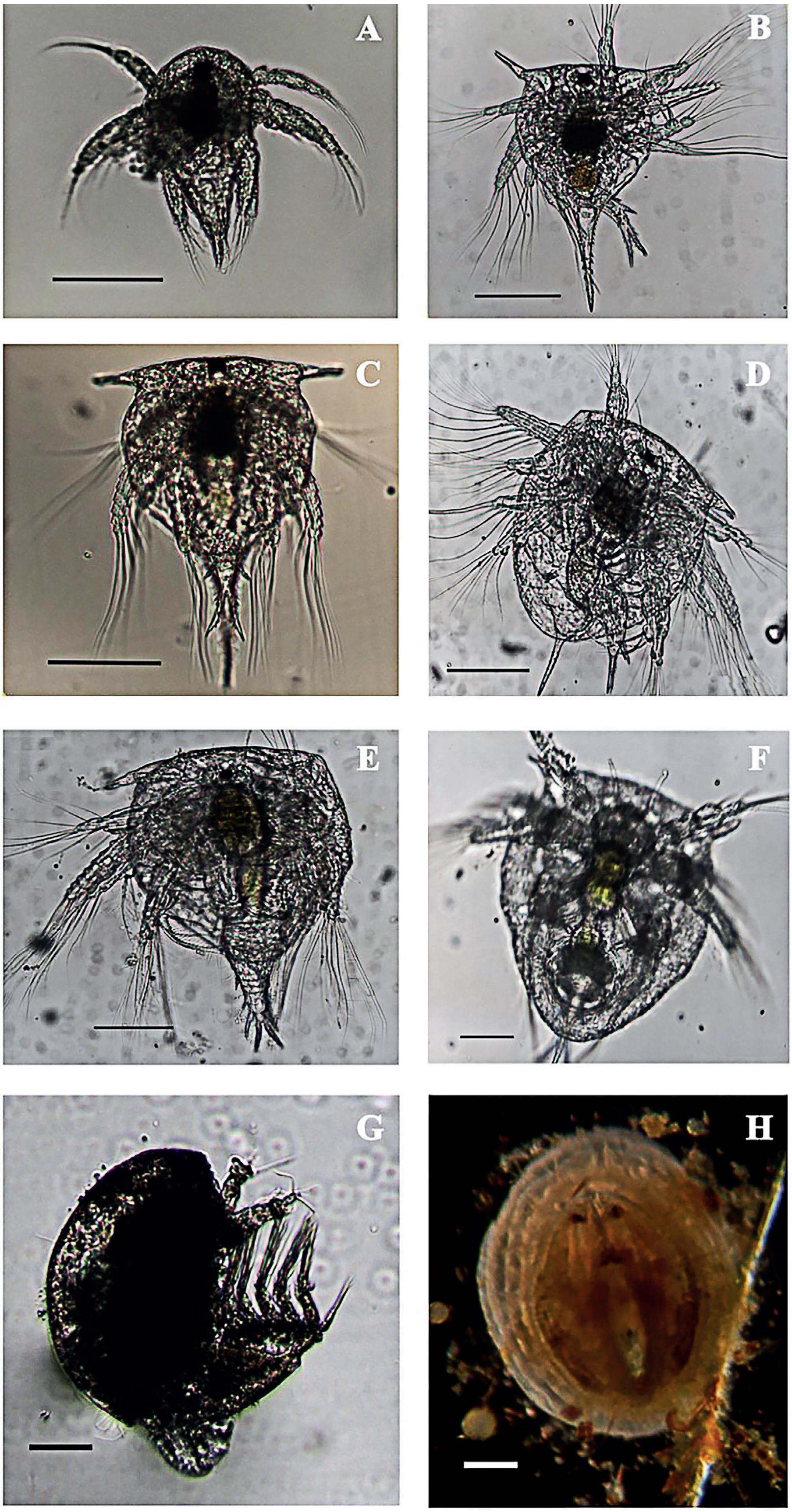

Figure 4. Photographs of the different larval stages of *Chthamalus challengeri* **A.** Stage I of nauplius; **B.** stage II of nauplius; **C.** stage III of nauplius; **D.** stage IV of nauplius; **E.** stage V of nauplius; **F.** stage VI of nauplius; **G.** cypris larva; **H.** larva barnacle just adhering after metamorphosis. Scale bar: 100 μm. Photos by Yan Liu.

Effect of temperature and salinity on the survival of *Chthamalus challengeri* larvae

The survival rate of *C. challengeri* larvae was greatly affected by extreme low and high temperatures. At 35 °C, all larvae died on the first day. At 0 °C and salinity 5–20, the survival rate decreased to 15.03 ~ 21.82% on the first day. At 0 °C and 25–35 salinity, the survival rate of larvae was higher than 50%. The lowest survival rate of 0.65% was observed at 25 °C and salinity 25. The highest survival rate (91.53%) was observed on the first day at 10 °C, salinity 25. When the temperature was 20 °C and the salinity was 30, the survival rate of *C. challengeri* larvae was 40% on average. As can be seen in Fig. 5, this is the highest survival rate under the experimental conditions.

At other salinity levels, survival rates ranged from 65.90% to 90.58%. At a salinity of 5, the survival rate decreased to 0% on days 17–19. At salinities of 10, 35 and 25, survival rates on days 20–24 were 11.06%, 4.98% and 4.39% respectively. At salinities of 30 and 35, *C. challengeri* larvae developed into cypris larvae on the first day, with survival rates of 9.68% and 1.67%, respectively. At a salinity of 25, *C. challengeri* larvae could also develop into cypris larvae, but the survival rate was lower than that of the larvae at high salinity. They successfully developed into cypris larvae after 11.5 days, but the survival rate eventually decreased to 21.82%. The highest survival rate was observed at temperatures of 15 °C and 20 °C and salinities of 25, 30 and 35 (Fig. 6). Survival rates at salinities of 30 and 35 were slightly higher than those at salinity of 25.

Effect of temperature and salinity on the growth and development of *Chthamalus challengeri*

C. challengeri showed different growth and developmental rates under gradients of temperature and salinity. At 25 °C and salinities 30 and 35, the shortest development cycle from stage I to cypris larva was 10.5 days. At 10 °C and salinity 25, the most extended development cycle lasted 24 days.

At 20 °C and 25 °C, stage I larvae developed into stage II larvae only within 0.5 days. At 10 °C and 15 °C, stage I larvae developed into stage II larvae within 1 to 3 days, influenced by salinity. The developmental rate of stage II larvae into stage III larvae increased with increasing temperature. It took approximately 3 days at 10 °C for the longest development time. The development rate for stage II and III larvae was similar, but it took longer for them to develop at 10 °C and 15 °C compared to 20 °C and 25 °C. The growth rate at 25 °C was faster than that at 10 °C. The growth rate of *C. challengeri* larvae varied under different temperature and salinity conditions when they reached stage IV larvae. When the temperature was 10 °C and the salinity was 25, it took 5 days for *C. challengeri* to develop from stage IV to stage V. However, at a salinity of 30, it only took 3 days. At 20 °C and salinities of 30 and 35, stage IV larvae took only 2 days to develop into stage V larvae. The fastest rate of development occurred when *C. challengeri* larvae reached stage V larvae at 25 °C and salinities of 30 and 35, taking an average of only 2 days to develop into stage VI larvae. At 10 °C and salinity 25, the stage V larvae's developmental rate slowed, and it took 5 days for them to develop into stage VI larvae. However, it took approximately 3 to 4 days at other temperatures and salinities. Stage VI larvae took longer to develop from nauplius to cypris larvae than other developmental stages. It took 3 days for stage VI larvae to develop into cypris larvae at salinities of 30 and 35 and temperatures of 15 °C, 20 °C, and 35 °C. However, when the temperature was 10 °C and salinity was 25 or 35, the larvae needed 5 days to develop into cypris

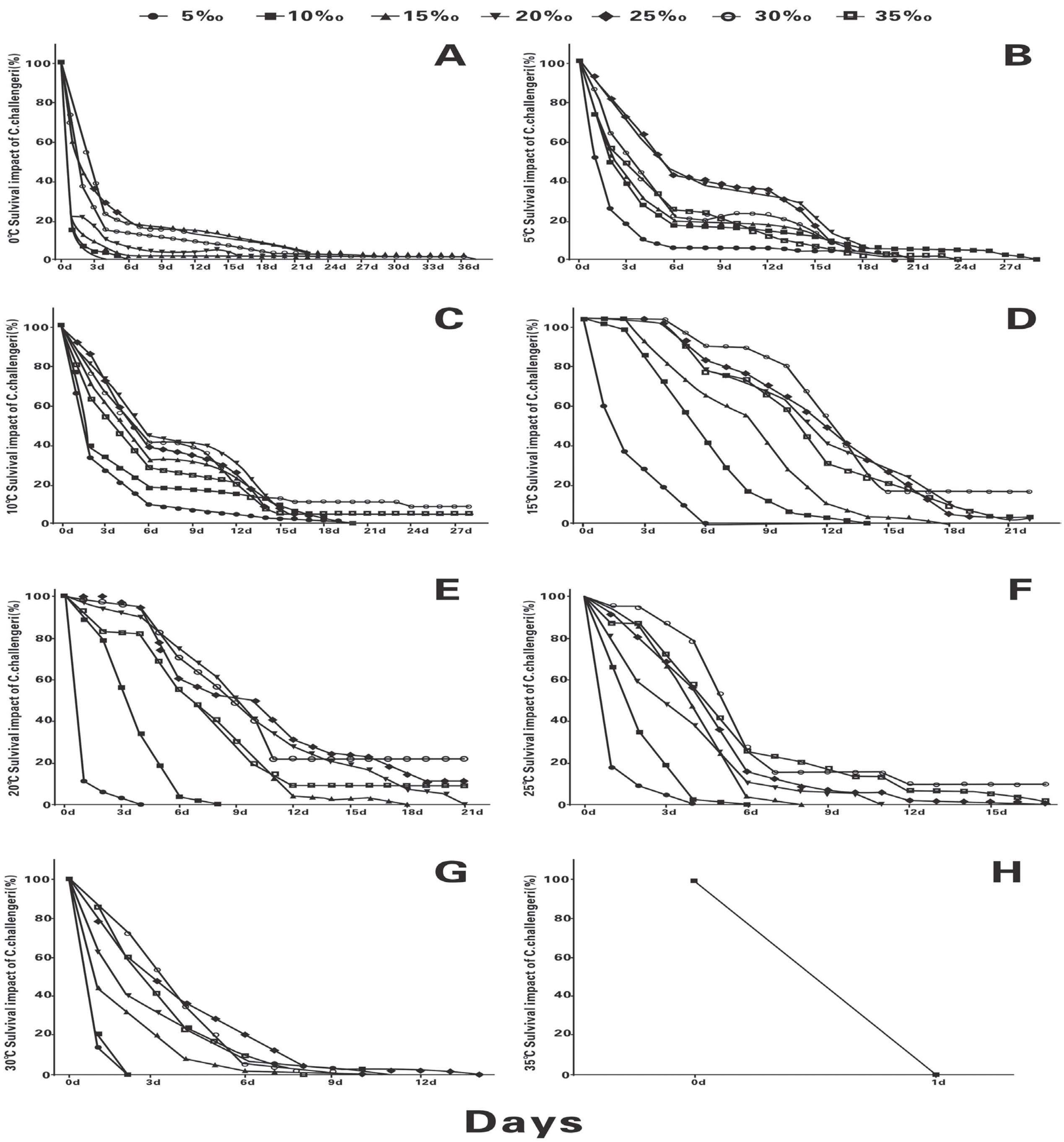

Figure 5. Survival percentage of *Chthamalus challengeri* at different salinity gradient and temperature conditions **A.** 0 °C; **B.** 5 °C; **C.** 10 °C; **D.** 15 °C; **E.** 20 °C; **F.** 25 °C; **G.** 30 °C; **H.** 35 °C.

larvae. According to the results of the culture experiment, the optimal conditions for *C. challengeri* larval development were temperatures of 10 °C, 15 °C, 20 °C and 25 °C, and salinities of 25, 30 and 35. Under the 15 °C salinities of 25, 30 and 35, the *C. challengeri* larvae developed into cypris larvae on the 20th, 16th and 17th day, respectively. At 20 °C and salinity 30, 21.82% of the larvae developed into cypris larvae. The larvae of *C. challengeri* developed into cypris larvae within 20–24 days at salinities of 10, 35 and 25. However, the ability of *C. challengeri* larvae to develop into cypris larvae at salinity 25 was lower than that of larvae at high salinity.

To sum up, the growth cycle of *C. challengeri* was affected by temperature and salinity during every stage of development (Table 2).

Table 2. ANOVA Results for the Effects of Temperature, Salinity, and their Interactions on the Duration of the Larval Stages.

Source of variation	Free degree (df)	Stage I		Stage II		Stage III		Stage IV		Stage V		Stage VI	
		Mean square (MS)	F value	Mean square (MS)	F value	Mean square (MS)	F value	Mean square (MS)	F value	Mean square (MS)	F value	Mean square (MS)	F value
Temperature (A)	3	1.3674	31.033**	0.9471	21.800*	0.2313	127.52**	0.4532	10.755*	0.4532	10.755*	0.2043	9.893*
Salinity (B)	2	0.1266	2.874	0.127	2.924	0.0018	1.012	0.331	7.857	0.331	7.857	0.0848	4.107
A × B	6	0.0441	12.441**	0.0434	1.732	0.0018	0.054	0.0421	1.724	0.0421	1.724	0.0207	0.974
Error	24	0.0035		0.0251		0.0336		0.0244		0.0244		0.0212	

Data were log-transformed to meet the assumptions of ANOVA, *: $P < 0.05$, **: $P < 0.001$

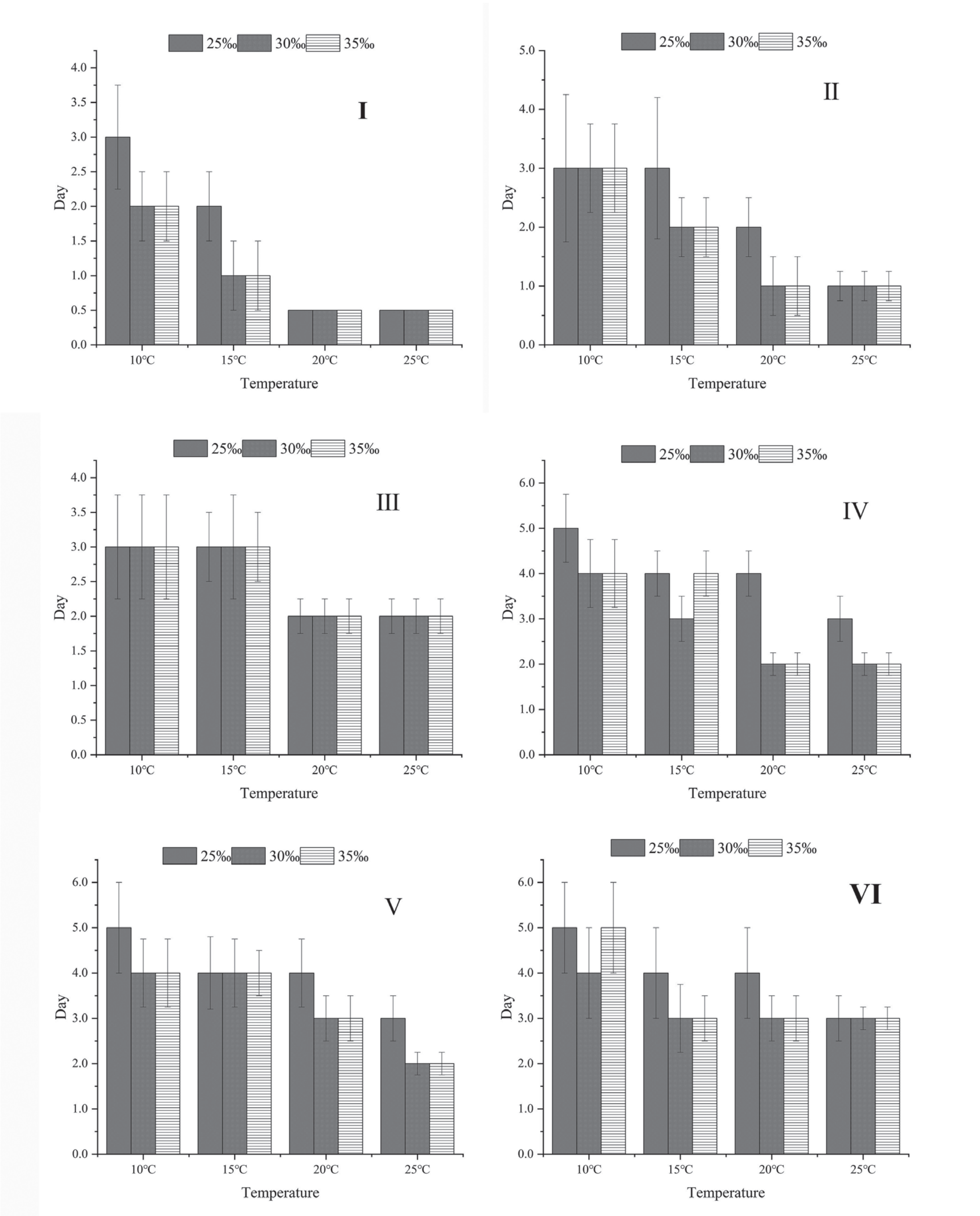

Figure 6. Growth cycle of various larval stages of *Chthamalus challengeri* under different temperature and salinity conditions.

The ANOVA results showed that temperature significantly affected the developmental cycle of *C. challengeri* larvae ($P < 0.05$). In particular, temperature significantly influenced the development of stage I and III larvae ($P < 0.001$). However, salinity did not contribute significantly to the developmental cycle of *C. challengeri* ($P > 0.05$). The results showed that temperature was related to the development cycle of *C. challengeri*. At the same salinity level, the development of *C. challengeri* larvae accelerated with increasing temperature (Table 3).

The coefficient of determination (R^2) values were all higher than 0.4073, indicating that temperature significantly impacted the developmental rate of *C. challengeri* larvae under different salinity conditions. In comparison, the developmental rate of *C. challengeri* larvae was less influenced by salinity. Salinity affected only a few stages of the developmental cycle (Table 4).

As the salinity increased at a temperature of 15 °C, the growth cycle of *C. challengeri* larvae decreased. R^2 values also showed that temperature had a greater effect on the developmental rate of *C. challengeri* larvae than salinity (Tables 3, 4).

Table 3. Values of the Power Function ($D = bv^m$) for the Effects of Salinity (v) on the Duration (D) of the Nauplius Stages (I–VI) at Fixed Temperature Conditions for *Chthamalus challengeri*.

Temperature (°C)	Coefficient (b)	Index (m)	Coefficient of determination R^2	*P*
Stage I				
10 °C	0.017785	-1.39200	0.6059	**0.0135**
15 °C	0.000166	-2.53870	0.7005	**0.0049**
20 °C	0.004309	-1.24180	0.2432	0.1774
25 °C	0.000162	-2.15700	0.8590	**0.0003**
Stage II				
10 °C	0.060465	-1.08540	0.7506	**0.0025**
15 °C	0.017715	-1.38270	0.5604	**0.0203**
20 °C	0.000166	-2.53870	0.5004	**0.0330**
25 °C	0.034610	-0.96649	0.2098	0.2151
Stage III				
10 °C	0.174245	-0.81286	0.4746	**0.0401**
15 °C	9.602500	0.35889	0.0995	0.4084
20 °C	0.051117	-1.00690	0.2302	0.1912
25 °C	0.334706	-0.49155	0.1713	0.2681
Stage IV				
10 °C	0.337120	-0.72485	0.3271	0.1076
15 °C	0.065632	-1.1089	0.7177	**0.0039**
20 °C	0.000332	-2.5387	0.6672	**0.0072**
25 °C	0.017714	-1.3827	0.6725	**0.0068**
Stage V				
10 °C	0.337120	-0.72485	0.3271	0.1076
15 °C	0.410069	-0.63871	0.4413	0.0510
20 °C	0.044619	-1.21810	0.8897	**0.0001**
25 °C	0.004613	-1.75570	0.7655	**0.0020**
Stage VI				
10 °C	0.565204	-0.58559	0.2391	0.1817
15 °C	0.105000	-1.00000	0.5142	**0.0297**
20 °C	0.022444	-1.40640	0.7914	**0.0013**
25 °C	0.154507	-0.82559	0.4857	**0.0370**

Table 4. Values of the Power Function ($D = bv^m$) for the Effects of Temperature (v) on the Duration (D) of the Nauplius Stages (I–VI) at Fixed Salinity Levels for *Chthamalus challengeri*.

Salinity	Coefficient (b)	Index(m)	Coefficient of determination R^2	*P*
Stage I				
25	261.8256	-1.9039	0.8788	**0.0001**
30	230.1488	-2.0696	0.9662	**0.0001**
35	537.7475	-2.3894	0.9519	**0.0001**
Stage II				
25	25.3030	-0.85464	0.7266	**0.0004**
30	36.8994	-1.13020	0.7782	**0.0001**
35	24.8713	-1.00730	0.6899	**0.0008**
Stage III				
25	16.7696	-0.68240	0.7091	**0.0006**
30	9.2676	-0.49031	0.5559	**0.0054**
35	12.1715	-0.62262	0.4073	**0.0255**
Stage IV				
25	14.8156	-0.47123	0.8158	**0.0001**
30	27.6779	-0.83785	0.6767	**0.0010**
35	28.2635	-0.85148	0.7308	**0.0004**
Stage V				
25	15.0233	-0.46870	0.7519	**0.0003**
30	14.2399	-0.52736	0.5498	**0.0058**
35	23.9246	-0.75607	0.7224	**0.0005**
Stage VI				
25	13.7215	-0.43512	0.5683	**0.0046**
30	9.6971	-0.37033	0.5605	**0.0051**
35	19.9657	-0.68670	0.7353	**0.0004**

Effect of temperature and salinity on *Chthamalus challengeri* settlement

In the experiment, the settlement of larvae were observed at different temperatures (10 °C, 15 °C, 20 °C, and 25 °C) and salinities (25, 30, and 35) (Fig. 7). The culture experiment ended when either all the cypris larvae settled or the unsettled ones died. Only a few larvae settled on the first day at 20 °C and 25 °C. At a temperature of 20 °C and a salinity of 30, the average settlement rate was around 20%, with all larvae fully settled by day 2 at an average settlement rate of 60%. Alternatively, at a salinity of 35 and a temperature of 20 °C, the average settlement rate was slightly less than 50%. The longest settlement stage was observed at a temperature of 10 °C and a salinity of 25, with all larvae settled within 6 days at an average settlement rate of 15%. During the first 2 days at either a temperature of 10 °C or salinity of 35, the cypris larvae moved slowly and floated before beginning to settle on day 3. Settlement rates were at their lowest during this time, with an average of 10%.

The results of ANOVA showed that temperature, salinity and days in culture significantly affected the development and settlement of *C. challengeri* larvae (P < 0.001; Table 5). The two-way ANOVA results showed no significant interaction between salinity and days in culture on the settlement of *C. challengeri* (P >0.05) (Suppl. materials 2, 3). However, there was a significant interaction between temperature and salinity, as well as temperature and number of days in culture, on the settlement of *C. challengeri* (P < 0.001) (Suppl. material 1). A more specific analysis for each of the 3 sources of variation is given as Suppl. materials 1–3.

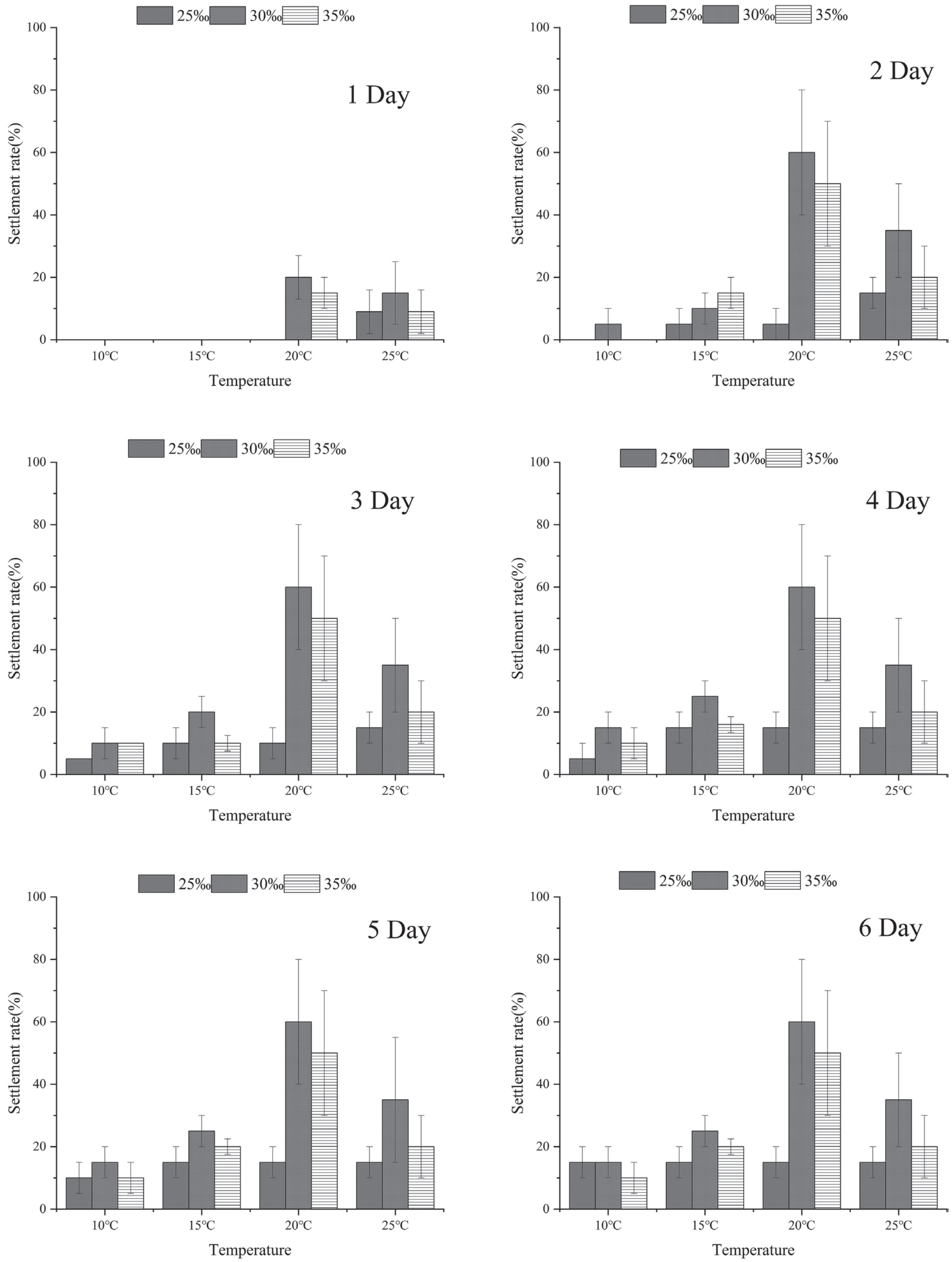

Figure 7. Effects of temperature and salinity on the settlement of *Chthamalus challengeri* cypris.

Table 5. ANOVA parameters for the effects of temperature, salinity, experiment duration, and their Interactions on cypris settlement.

Source of variation	Free degree (df)	Mean square (MS)	*F* value
Temperature(A)	3	8.97	95.3737**
Salinity(B)	2	5.0867	54.0845**
Culture days(C)	5	7.2921	77.5335**
A × B	6	0.9771	10.3891**
A × C	15	0.7352	7.8172**
B × C	10	0.0828	0.88
Error	30	0.0941	

**: $P < 0.001$

Distribution of *Chthamalus challengeri* in Zhoushan Sea

From August 2020 to March 2023, there were no observations of *C. challengeri* in Yangshan Port despite multiple samplings. The survey conducted during this time identified 10 fouling species, including 6 *mollusca* species, in the same area. The main contaminant species found in Yangshan Port were *Balanus albicostatus*, *Littorina scabra* and *Crassostrea ariakensis*. However, in 2013, adult *C. challengeri* was collected from rocks in Gouqi Island located in Zhoushan. A recent survey and sampling conducted in April 2023 revealed the presence of *C. challengeri* in the high tide and medium tide areas of Gouqi Island, Shengshan Island, and Qushan Island in the Zhoushan sea area. The density of *C. challengeri* was the highest in Gouqi Island and Qushan Island, ranging from 1978/m^2 to 16/m^2. *C. challengeri* was predominantly found in the high tide area, indicating that it has become the dominant species in that region of the Zhoushan sea.

Discussion

Effect of temperature and salinity on the survival rate of *Chthamalus challengeri*

Based on relevant studies, it has been observed that no *C. challengeri* organisms were found to be active at temperatures below 9 °C (Tie et al. 2010). In the range of 18 °C to 21 °C, the activity of *C. challengeri* was the highest (Tie et al. 2010). It grows very vigorously. Although the survival rate was lower in the high-temperature segment, their activity rate remained high, indicating that temperature enhanced their metabolism (Tie et al. 2010). In the experiment, *C. challengeri* had a high survival rate at 20 °C with high activity, verifying this conclusion. The effect of temperature on the larva depends mainly on the adult, barnacles in an active state foraged frequently during the light period. The frequency of tentacle opening increased with increasing temperature (Kon and Miki 1994). During this period, the adults released the nauplius occasionally, mainly affected by light intensity and water flow stimulation (Tie et al. 2010). This means that *C. challengeri* can maintain a high survival and reproduction rate during this period. Survival of adult *C. challengeri* declined significantly from spring to summer and stabilized after October (Miku et al. 2020). According to the statistical analysis of China Zhoushan Meteorological Bureau (http://www.tianqihoubao.com/lishi/zhoushan.html), the average annual temperature in Zhoushan is 15–20 °C. This further illustrates the invasion trend of *C. challengeri* into the southern region of China.

Salinity, as one of the critical environmental factors in mariculture, can affect the survival, growth and development as well as reproduction of marine invertebrates, and changes in salinity directly affect the regulation of osmotic pressure and ion concentration in the animals (Li et al. 2002). It has a clear impact on the physiological metabolism of marine organisms. The results of the data in this experiment showed that the survival of *C. challengeri* at different temperatures showed different trends with increasing salinity, however, it generally had higher survival at the salinity of 30. The survival rate of nauplius I- VI was the highest at 15 °C to 20 °C and salinity 30. In the present investigation experiment, the sea water temperature in Zhoushan was around 17 °C and salinity was around 31, which was more in line with the conditions for the survival of *C. challengeri*. This further illustrates the possibility of the trans-oceanic spread of *C. challengeri* in China and the possibility of further spreading invasion of *C. challengeri* in Zhoushan.

Effects of temperature and salinity on the growth and development of *Chthamalus challengeri*

Since the 1980s, many researchers began to pay attention to the effects of temperature and salinity on the growth and development of Marine invertebrates (Pechenik 1987; Holan et al. 2019; Torres et al. 2021). Pechenik found that all types of physical (e.g., temperature, salinity, light, flow velocity, and the roughness and color of fouling objects), chemical (e.g., natural inducers secreted by individuals of the same species and bacteria and artificial inducers such as metal cations and neurotransmitters), and biological (e.g., larva behavior and physiological conditions) factors affected the larval development, metamorphosis, and settlement of invertebrates in marine environments (Pechenik 1987). Among them, temperature and salinity are the most important factors (Pearse et al. 1991). Previous studies demonstrated that invertebrate larvae show a higher rate of metabolism at high temperatures, while low-temperature conditions significantly reduce energy assimilation, which may be directly responsible for the effect of temperature on larval development (Anger and Dawirs 1981; Anil et al. 2001). Chan (2007) compared the reproductive patterns of *C. challengeri* from southern Japan and its congener *C. malayensis* Pilsbry, 1916 from Hong Kong, and reported that the annual reproductive patterns of these barnacles seem to be influenced by seawater temperature (Chan 2007). It was found that individuals that settled in early summer reached their mature size in the following spring and may begin to reproduce and develop. For the newly settled *C. challengeri* in June, the length of the shield and backplate was 0.4–0.6 mm (Miku et al. 2020). In our study, the developmental cycle of *C. challengeri* shortened with increasing temperatures, and the length of *C. challengeri* larva was 0.2–0.5 mm (Fig. 4). This is in agreement with results from *Balanus Amphitrite* (Anil et al. 1995; Qiu and Qian 1999; Anil et al. 2001), *Balanus eburneus* (Scheltema and Williams 1982), *Elminius modestus* (Harms 1984, 1986), and *Semibalanus balanoides* (Harms 1984) larvae.

Previous studies have shown that salinity and temperature may change simultaneously in the wild natural ecology. Therefore, these two parameters usually synergistically affect the development of barnacle larvae in their natural environment (Harms 1986; Anil et al. 1995). The likelihood of *C. challengeri* nauplius developing into cypris larvae was low in this experiment; temperatures of 10 °C, 15 °C, 20 °C and 25 °C and salinities of 25, 30 and 35 had significant effects on stage I nauplius; these temperature and salinity ranges can be found in the natural marine environment of the Zhoushan Sea Area. The developmental cycle of *C. challengeri* was the shortest (the stage I nauplius developmental cycle averaged 10.5–11.5 days) when the temperature ranged from 20 °C to 25 °C and salinity ranged from 30 to 35. These temperature and salinity conditions are consistent with the surface seawater of the new habitat from May to September, which is the breeding and settlement period for *C. challengeri* (Iwaki 1975a). The entire growth and developmental cycle of *C. challengeri* larvae from stage I to stage VI was approximately 12 to 16 days. Experiments have shown that once *C. challengeri* has adapted to its new environment's temperature and salinity conditions, it could complete the whole developmental process, laying the foundation for settlement in Zhoushan sea area. *C. challengeri* was always found in temperate waters (Liu and Ren 2007). In spring (April to May), the water temperature was relatively lower, between 8 °C and 12 °C (Wang Baoqiang et al. 2011), which reduced the rate of development of the *C. challengeri* larvae. By June, the water temperature increased to 15–18 °C (Hu 2013), and most larvae developed to stage VI, and began to prepare for settlement in July. As some of the islands in Zhoushan sea are in the low-latitude subtropical climate zone, the climate is relatively warm and humid (Wang et al. 2011). The water temperature in spring (April–May) can reach 15.2 °C to 19.8 °C (Hou et al. 2013b), with average salinities between 29.1 and 31.8 (Hou 2013a). The conditions are more suitable for the sexual maturation and nauplius development of *C. challengeri*. Therefore, it can be inferred that the breeding and settlement stages of *C. challengeri* in Zhoushan are longer than in other habitats.

Effects of temperature and salinity on the settlement of *Chthamalus challengeri*

Previous studies have found evidence that the nauplius of barnacles can store large amounts of nutrients during their development from stage VI of the nauplius to cypris larvae (Iwaki 1975a). The nauplius stopped feeding after metamorphosing into a cypris larva. Therefore, the survival rate and successful settlement of barnacles depend on the energy stored in their bodies (Lucas et al. 1979; West and Costlow 1988; Satuito et al. 1996; Thiyagarajan et al. 2002). A study in 1989 found that temperature and salinity conditions can promote the metabolic activity of barnacle larvae and regulate osmotic pressure, thus affecting the energy reserves of cypris larvae (Fraser 1989). *C. challengeri* showed different rates of settlement at different temperatures and salinity conditions. The settlement rate increased with increasing temperature at 10 to 20 °C; similarly, at salinity 25–30, the settlement rate increased with increasing salinity. The energy storage stage of an organism is closely related to the environmental temperature, which is mainly associated with its adaptability to the environment (Piola and Johnston 2006; Lee et al. 2012; Blakeslee et al. 2020). It can be inferred that cypris larvae can store different amounts of energy during each developmental stage. Under optimal temperature and salinity conditions, sufficient energy is available to ensure a high success rate in developing barnacle larvae from cypris larvae (Pechenik et al. 2000). This also increases the settlement rate. Under unsuitable conditions, such as low temperature and salinity, the success rate of stage V nauplius developing to cypris larva was lower, thus the settling rate decreases. Previous studies on the growth, settlement, and development of *C. challengeri* have shown that *C. challengeri* began to settle in the wild Marine environment from July to September every year (Iwaki 1975a). At this time, the temperature of the sea varied from 18 °C to 23 °C (Ma et al. 2006; C.L. 2008; Ju and Xiong 2013; Hu 2013), the salinity was about 30 (Xu 2007; Hou et al. 2013b). The water temperature was 17.2–27.7 °C from April to November (Hou et al. 2013b). *C. challengeri*, which lives in Zhoushan, was adapted to the natural marine environment. Suppose adult gonads develop well and nutrition is sufficient. In that case, the settlement period of *C. challengeri* in the wild can theoretically extend from April to around November (about 7 months in total), far exceeding that of the original habitat from July to September (about 3 months in total) (Iwaki 1975a; Hou et al. 2013a, b). The more extended settlement stage of *C. challengeri* may have provided favorable conditions for forming colonies and rapid spread to new habitats. The survival, reproduction, and settlement of the *C. challengeri* in their new habitat are influenced by a variety of environmental factors, such as water current (Gaines et al. 1985), food (Dreanno et al. 2006), and settlement base (Chabot and Bourget 1988). Further field ecological studies are needed to verify the inference of a longer settlement time in new habitats.

Effects of temperature and salinity on the invasion of *Chthamalus challengeri* in Zhoushan, China

C. challengeri is mainly distributed in the coastal high tide areas of the Yellow Sea, Bohai Sea, Korea and Japan Island (Apolincrio 1999; Chan 2007; Liu and Ren 2007; Liu et al. 2015). It is found on islands in northern China, such as Jinzhou in Dalian, Shanhaiguan, Qinhuangdao and Beidaihe in Hebei Province and Laizhou, Yantai, Kongdong Island, Liu Gongdao, Rongcheng Island and Qingdao in Shandong Province (Iwaki 1975b; Tie et al. 2010; Cao et al. 2013). Liu and Ren (2007) specifically mentioned *C. challengeri* in 'Fauna Sinica', which recorded that '*C. challengeri* is dominant intertidal species along Bohai Bay to the north Yellow Sea, but has ever been collected on the Yangtze River Estuary's south coast'. *C. challengeri* was first collected in the south of the Yangtze estuary until 2010, when Xue et al. found three *C. challengeri* settled in the upper part of the high or mid-tide area with a density of 5.0 /m^2 and a biomass of 0.42 /m^2 during an ecological survey in Yangshan Port, Zhoushan sea area, Zhejiang Province (Xue et al. 2011). Subsequently, in 2014 and 2016, both Liu et al. and Wang et al. found *C. challengeri* south of the Yangtze River and showed that *C. challengeri* had a high degree of dominance (Liu et al. 2014; Wang et al. 2016). It was concluded that *C. challengeri* has a robust environmental adaptation to Zhoushan waters and has become an invasive species in Zhoushan sea area. In 2023, a survey of Zhoushan sea area found that *C. challengeri* densities had reached 16/m^2 to 1978/m^2. It has invaded a large area in Zhoushan sea and has become the dominant species. Therefore, the discovery of *C. challengeri* in the southern part of Chinese sea area could indicate that *C. challengeri* used its strong tolerance for complex environments to reach new areas and settle there.

Zhoushan sea are, such as Yangshan Port and its surrounding waters has a complex structure, bordering the Yangtze River mouth, Hangzhou Bay and the East China Sea, forming rich nutrients and other materials that provide suitable growing conditions for the invasion of *C. challengeri* (Liu et al. 2014, 2015). Since the first collection of *C. challengeri* in Yangshan Port in 2010, the follow-up survey found that *C. challengeri* successfully settled and became an invasive species in the high tide area of Zhoushan sea within a few years after its introduction (Liu et al. 2014), and spread widely among the islands in Zhoushan sea area, and *C. challengeri* has become the absolute dominant species in the high tide area of Gouqi Island and Qushan Island. However, according to the survey between 2020 and 2023, *C. challengeri* was not found in Yangshan Port. There may be four reasons why the *C. challengeri* were not collected in Yangshan Port. Firstly, the *C. challengeri* did not adapt to the local environment and gradually died out or migrated. Secondly, Yangshan Port is a cargo port with many ships coming and going, and a certain degree of environmental pollution was found during the survey, which may have caused *C. challengeri* to be unable to settle. Thirdly, Yangshan Port may be affected by the dilution of Yangtze River water, because Yangtze River water is a freshwater resource, which may make the seawater environment in Yangshan Port complex and variable (Huang and Cai 1985). Fourthly, *C. challengeri* may contain parasites in its body, which can affect the reproduction of the host, thus causing *C. challengeri* to be unable to reproduce normally (Miku et al. 2020). There may be two sources of *C. challengeri* collected at Yangshan Port: the spread of northern species; the other is invasion by exotics through ship-bottom settlement, ballast water discharge, etc. In a specific range, the community composition and diversity of the local intertidal zone begin to change, which has a high risk of invasion. Therefore, changes in the nearshore environment and nearshore ocean circulation may also significantly impact the population isolation and diversity of *C. challengeri* in Zhoushan sea area.

The *C. challengeri* can withstand long periods of periodic drying and is a highly adaptable cold water fouling organism (Liu and Ren 2007). The survival rate of *C. challengeri* larvae was seriously influenced by extreme low and high temperatures. According to the results of the culture experiment, the optimum conditions for *C. challengeri* development in Zhoushan were 10 °C, 15 °C, 20 °C and 25 °C, and salinities of 25, 30 and 35. When the temperature was 20 °C and the salinity was 30, the *C. challengeri* larvae in Zhoushan showed a higher survival rate of 40%. It can be inferred that a temperature of 20 °C and salinity of 30 is the suitable breeding environment for *C. challengeri*. According to the investigation (Tie et al. 2010; Cao et al. 2013; Qi et al. 2015; Li et al. 2015), *C. challengeri* in the Dalian and Qingdao sea area of North China, the adaptive temperature is about 16–24 °C, and the salinity is almost 35 and 30, respectively. The comparison between Dalian and Qingdao showed that the colonization season of *C. challengeri* in Zhoushan sea area is similar to the original suitable temperature and salinity in northern China. According to the survey, the Zhoushan sea area has less temperature fluctuation, the average temperature throughout the year is 15–20 °C, and the salinity is around 30. This is suitable for the development and settlement of *C. challengeri*. It can be inferred that *C. challengeri* is easy to adapt to the environment of Zhoushan, and can easily invade and reproduce. With global warming, rising temperature, the temperature difference between north and south in Chinese Seas is gradually decreasing, the adaptation of *C. challengeri* in Zhoushan sea area to its habitat has a tendency to change further, and the change of its invasion risk needs further study.

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Authors’ contribution

Junzeng Xue, Yan Liu, and Ningning Chen conceived and designed the experiments. Yan Liu and Ningning Chen conducted the experiments. Junzeng Xue, Yan Liu and Ningning Chen analyzed the data. Junzeng Xue and Huixian Wu provided experimental reagents, materials and analysis tools. Junzeng Xue, Yan Liu, Huixian Wu and Ningning Chen wrote the paper.

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Supplementary material 1

Multiple comparisons of temperature on settlement rates of *Chthamalus challengeri* cypris

Authors: Ningning Chen, Yan Liu, Lin Yuan, Huixian Wu, Junzeng Xue

Data type: doc

Link: https://doi.org/10.3391/ai.2024.19.1.115111.suppl1

Supplementary material 2

Multiple comparisons of salinity on settlement rates of *Chthamalus challengeri* cypris

Authors: Ningning Chen, Yan Liu, Lin Yuan, Huixian Wu, Junzeng Xue

Data type: doc

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Supplementary material 3

Multiple comparisons of days of culture on settlement rates of *Chthamalus challengeri* cypris

Authors: Ningning Chen, Yan Liu, Lin Yuan, Huixian Wu, Junzeng Xue

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