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Impact of brine acidification on hatchability, survival and reproduction of *Artemia parthenogenetica* and *Artemia franciscana* in salt ponds, Bohai Bay, China*

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Abstract We studied the effect of pH (pH 5, 6, 7 and 8) on the hatching percentage, survival and reproduction of *Artemia* strains in Bohai Bay salt ponds. Strains included parthenogenetic *Artemia* from Bohai Bay (BHB), *Artemia franciscana* from San Francisco Bay, and *A. franciscana* artificially produced in salt ponds in Vietnam. The latter was included as a potential inoculum for biological management of salt ponds. The hatching percentage of cysts after 24 h and the survival rate of the tested *Artemia* strains were significantly reduced when exposed to a culture medium at pH 5 for 18 d (P<0.05). The tolerance of *Artemia* to 48 h acid exposure varied with developmental stage, increasing in the following order: juvenile, nauplii, pre-adult, with maximum tolerance in adults. All strains of *Artemia* tested could not reproduce at pH 5. At pH levels from pH 6–8, a higher pH generally resulted in a shorter brood interval and enhanced ovoviviparity. Hence, we suggest that brine acidification has a negative impact on *Artemia* populations in the Bohai Bay saltworks. Inoculation of *Artemia* with either local parthenogenetic *Artemia* or exotic *A. franciscana* should be feasible at pH 7–8.

Keyword: brine acidification; pH; *Artemia*; hatching percentage; survival; reproductive traits

1 INTRODUCTION

Brine shrimp *Artemia* is a micro-crustacean, inhabiting hypersaline environments such as inland salt lakes and coastal solar saltworks. *Artemia* is a key macroscopic representative in evaporation salt ponds at an intermediate salinity range of 70–200. The shrimps play important roles in balancing the biological system of the salt pond, improving salt production (Tackaert, 1987), and provide an excellent live food source for marine fish and crustacean aquaculture (Dhont and Sorgeloos, 2002). Bohai Bay saltworks are home to the main sea salt production area in China. In recent years, the output of *Artemia* cysts and biomass in the Bay have been threatened by the extensive acidifying discharges (pH 2–3) it receives from a bromine extraction plant at a salinity range of 50–120. Although the neutralization of alkaline soil during evaporation returns the pH of saltpond to a normal level (pH 7–8), the fact that the acidified brine needs to flow over longer distance to get it neutralized over the past years, which indicates that the neutralization capacity of the soil is gradually weakening (information obtained from Chengkou Saltworks, China).

Controlled *Artemia* inoculation is considered part of the biological protocol for proper management of the salt pond ecosystem (Tackaert and Sorgeloos, 1993). As an inoculum for aquaculture application, the selected *Artemia* strain has to be well adapted and capable of easy colonization of the environment.

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Artemia franciscana Kellogg (1906) originating from San Francisco Bay, USA, is in high demand in hatcheries worldwide. It has proved to be a good inoculum (Tackaert and Sorgeloos, 1991; Zhang et al., 1993; Camara, 2001) and a high quality *Artemia* strain with smaller cysts and good hatchability. However, in the United States, the availability of SFB *Artemia* is restricted under the National Wildlife Refuge policy. Thus, an alternative *Artemia* strain is needed as an inoculum, and *A. franciscana* produced artificially in salt ponds in Vietnam, VSFB (Baert et al., 1997; Nguyen et al., 2009; 2010), is considered a potential candidate strain.

Salinity, temperature, food availability, and ionic composition are known as critical factors influencing the occurrence of *Artemia* in nature, with differential responses reported between parthenogenetic and bisexual *Artemia* strains (Browne et al., 1988, 1989; Vanhaecke et al., 1984; Triantaphyllidis et al., 1995; Van Stappen et al., 2002; Sui et al., 2012). However, as *Artemia* live in hypersaline environments, which are slightly alkaline (Van Stappen, 2002), the performance of *Artemia* in acidic environments has been of lesser concern.

In this paper, we examined the effect of pH on the hatchability, survival and reproductive performance of the main *Artemia* stains found in Bohai Bay salt ponds. Strains included the indigenous parthenogenetic *Artemia* (BHB), the exotic species *A. franciscana* (SFB), which has been present in Bohai Bay salt ponds following deliberate inoculation in the 1990s (Tackaert and Sorgeloos, 1991; Zhang et al., 1993, Van Stappen et al., 2007), and the potential inoculum VSFB. This study provides useful information for the sustainable development of Artemia production and bromine extraction from low salinity brine in Bohai Bay saltworks.

2 MATERIAL AND METHOD

2.1 Tested *Artemia* strains

Cysts of the *Artemia* strains, BHB, SFB, and VSFB, were obtained from the *Artemia* Reference Center of Ghent University, Belgium, the Salt Research Institute of China Salt Industry Corporation, China, and Can Tho University, Vietnam, respectively.

2.2 Brine pH adjustment

Acidified brine water (pH 2.4, salinity 50) was taken from the reservoir receiving bromine extraction discharge in Hangu Saltworks, Tianjin, China. The brine pH was adjusted by adding 10% NaOH solution to make final pHs of 5.0, 6.0, 7.0 and 8.0 (Mettler FK20K). To counteract the buffering effect of the brine, the resulting brine water was aerated for 4–5 days and a few drops of 10% NaOH solution then added until the pH had stabilized at the designated level for at least 72 h.

2.3 Hatching of *Artemia* cysts

Artemia cysts were hatched under the conditions described by Van Stappen (1996). Briefly, 0.25 g cysts of each *Artemia* strain were placed into a 250-mL glass cone containing 200 mL brine water (salinity 30) at the designated pH. The cones were incubated at 27±0.5°C, under 2 000 lx illumination and with continuous aeration. Three replicates were used for each treatment. After a 24 h incubation period, four sub-samples (0.25 mL each) were taken from each cone, and the number of nauplii, embryos, and un-hatched cysts of the pooled sample (1 mL) were counted under the binocular microscope (Olympus SZ61). The hatching percentage was calculated as follows:

$$\text{Hatching percentage (\%)} = \text{nauplii}/(\text{nauplii}+\text{embryos}+\text{un-hatched cysts}) \times 100\%$$

2.4 Continuous culture of *Artemia*

For each *Artemia* strain, 1 g of cysts was placed into a 1 000-mL glass cone and incubated in brine water (salinity 30, pH 8.2). After 18–30 h hatching (strain-dependent), 75 instar 1 nauplii were randomly collected, quickly rinsed with test brine and transferred into the culture vessels. The continuous culture experiments were carried out in 250-mL glass cones, each containing 200 mL brine water at the designated pH. *Artemia* were grown at 25±0.5°C for 18 days and fed concentrated *Dunaliella viridis* Teodoresco (1905) throughout the culture period. The feeding ration was verified according to the optimal feeding schedule described by Vanhaecke et al. (1984). To keep the pH of the culture medium constant, the pH of the microalgal concentrate was adjusted according to the treatment by adding a few drops of 10% HCL solution. Survival was counted every 3 days, when 100% of the water was changed. Three replicates were used for each treatment.

2.5 Exposure to pH stress at different *Artemia* developmental stages

Newly hatched nauplii were separated from un-

Table 1 Feeding levels of *Dunaliella viridis* (×10⁶ cells/cone/d)

Culture period	Level
Day 1	2.88
Day 2 to 4	5.76
Day 5, 6	8.64
Day 7, 8	11.52
Day 8, 9	14.40
Day 11, 12	17.28
Day 13 to 18	20.16

hatched cysts and empty shells, and then cultured in brine water (salinity of 50, pH 8.2) at 25±0.5°C and fed ad libitum concentrated *D. viridis*. For each *Artemia* strain, 50 instar 1 nauplii, 30 juveniles, 20 pre-adults and 20 adult *Artemia* were randomly collected from the stock culture, immediately after hatching, and at day 4, day 10 and day 18 after hatching, respectively. After rinsing thoroughly with experimental brine, the animals were transferred into a 250-mL glass cone containing 200 mL brine at the designated pH. The animals were incubated at 25±0.5°C with continuous aeration. No food was given during exposure. Survival rates were counted at 24 h and 48 h. Each treatment contained three replicates.

2.6 Evaluation of reproductive traits

When sex determination was possible, *Artemia* from the stock culture were transferred into a 45-mL Falcon tubes, each containing 40 mL brine water at the designated pH. Each tube contained one female for parthenogenetic *Artemia*, or one female and one male for bisexual strains. 10% of the optimal feeding ration at day 13–18 was given to the parthenogenetic females and 20% given to the bisexual *Artemia* (Table 1). During the 6-week experiment, reproductive traits were monitored daily and the following parameters evaluated: brood interval (days between broods), number of oviparous and ovoviviparous broods per female, number of nauplii and cyst production per brood, and ratio of total nauplii and cyst production per female.

2.7 Statistical analysis

Statistical analysis was conducted on the hatching percentage of the cysts, percentage survival and reproductive parameters of *Artemia* using the software SPSS (version 17.0). For each strain, significant differences among pH treatments were determined using one-way ANOVAs and post-hoc Tukey's tests, at $P<0.05$. Percentage data were arc-sin square root transformed, prior to analysis, to satisfy assumptions of normality and equal variance.

Table 2 Hatching percentage (%) of SFB, VSFB, and BHB *Artemia* cysts at different pH levels

Strains	pH	Hatching percentage (%) at 24 h
SFB	5	51.8±7.0 ^{b}
	6	60.7±4.8 ^{ab}
	7	61.6±6.6 ^{ab}
	8	63.7±1.3 ^{a}
VSFB	5	74.4±3.3 ^{b}
	6	83.1±6.2 ^{ab}
	7	80.9±1.5 ^{ab}
	8	85.6±4.2 ^{a}
BHB	5	17.8±0.6 ^{b}
	6	22.3±3.3 ^{a}
	7	25.4±0.7 ^{a}
	8	25.7±3.3 ^{a}

Values are mean and standard deviation of three replicates. Different superscripts in a column of each strain indicate significant differences ($P<0.05$).

3 RESULT

3.1 Hatching of *Artemia* cysts

The cysts of all tested *Artemia* strains hatched at pH 5–8, and the hatching percentage increased with evaluated pH after 24 h incubation (Table 2). Significantly lower hatching rates were observed in strains cultured at pH 5 ($P<0.05$), although rates were divergent between strains (e.g. 51.8%–63.7% for SFB, 74.4%–85.6% for VSFB, 17.8%–25.7% for BHB). In contrast, when cultured at pH 6–8, hatching percentages did not differ significantly between strains ($P>0.05$). The low hatching percentage of BHB cysts should be due to the long-term stocking. At present, it is almost impossible to obtain pure Bohai Bay parthenogenetic *Artemia* cysts from local salt ponds, and thus the tested BHB cysts were collected in the early 1990s before exotic *Artemia* species were introduced to Bohai Bay saltworks. Although the strains were well preserved in a deep freezer, the hatching rate may have been reduced.

3.2 Survival of *Artemia*

During the 18-day culture period, the pH tolerance of the tested *Artemia* strains declined with decreasing pH, with maximum mortality occurring in the earlier

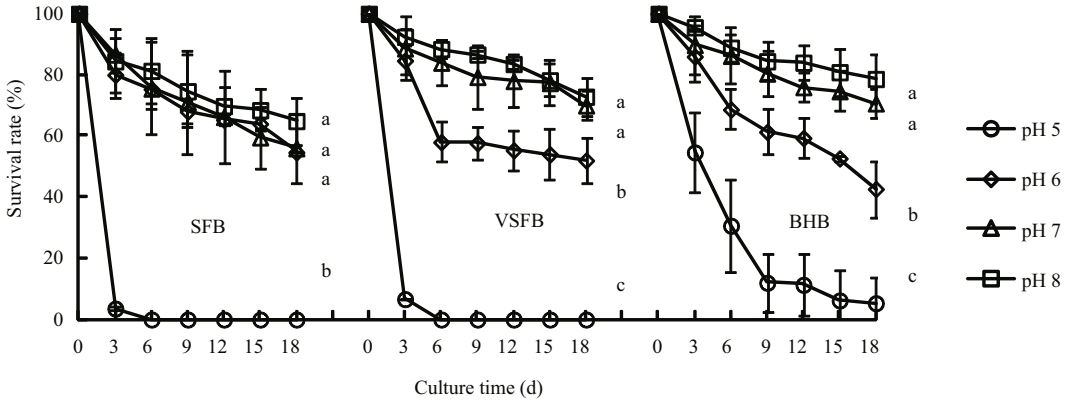

Fig.1 Survival rate (%) of SFB, BHB and VSFB *Artemia* cultured under different pH for 18 days

Bars are the mean value of three replicates. Error bars are the standard deviation of the mean. Different superscripts at day 18 indicate significant difference (P<0.05) for each strain.

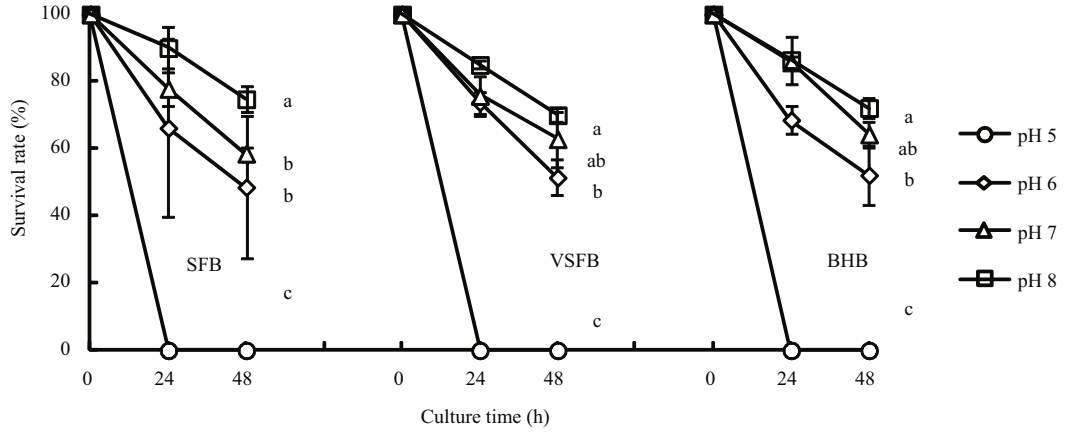

Fig.2 Survival rate (%) of *Artemia* nauplii of SFB, BHB and VSFB strains exposed to different pH levels for 24 h and 48 h

Presented as mean values of three replicates, with standard deviations. Different superscripts at 48 h indicate significant differences between pH levels (P<0.05) for each strain.

culture period (Fig.1). Compared with the BHB strain, SFB and VSFB strains were less tolerant of pH 5, with no *Artemia* surviving after 6 days' culture for both strains. In a pH range of 6–8, the percentage survivals of SFB, VSFB and BHB *Artemia* at day 18 were 54%–65%, 48%–73%, and 42%–79%, respectively. Moreover, after 18 days, significantly higher survival rates were observed for both VSFB and BHB strains exposed to pH 7–8 compared with those exposed to pH 6 (P<0.05).

The pH tolerance of *Artemia* at different developmental stages is shown in Figs.2–5. Overall, juvenile *Artemia* were the least tolerant of pH stress, with tolerance increasing in nauplii and pre-adults, and highest in adults. After 24 h exposure to pH 5–6, only pre-adults and adults survived. Moreover, survival rates improved with increasing pH from 6 to 8, with significant differences observed between pH levels for nauplii and pre-adults (P<0.05), but not for juveniles and adults (P>0.05).

3.3 Reproductive performance

Although a small percentage of *Artemia* survived at pH 5 for a short period (survival data are not presented), none were able to reproduce before dying, and thus reproductive performance was not reported at this pH. Comparing reproductive performance at pH levels from 6 to 8, a lower pH resulted in a prolonged brood interval and *Artemia* tended to reproduce more cysts (oviparity) than nauplii (ovoviviparity) (Table 3). In general, the numbers of ovoviviparous and oviparous broods of bisexual VSFB and SFB strains were higher than those of the parthenogenetic BHB strain. BHB and VSFB *Artemia* produced higher numbers of nauplii, but SFB *Artemia* tended to produce more cysts.

4 DISCUSSION

In the standard hatching protocol, a hatching medium with a pH range of 8–9 is usually proposed to

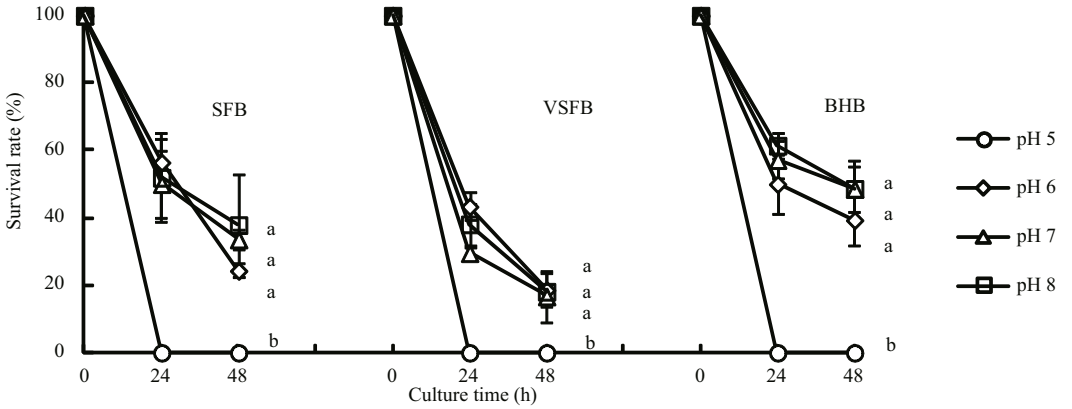

Fig.3 Survival rate (%) of *Artemia* juveniles of SFB, BHB, and VSFB strains exposed to different pH levels for 24 h and 48 h

Presented as mean values of three replicates, with standard deviations. Different superscripts at 48 h indicate significant differences between pH levels (P<0.05) for each strain.

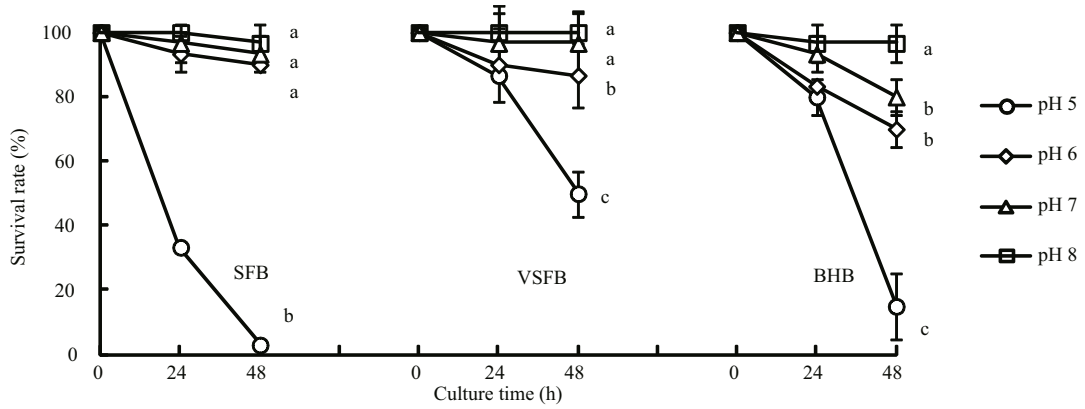

Fig.4 Survival rate (%) of pre-adult *Artemia* of SFB, BHB, and VSFB exposed to different pH for 24 h and 48 h

Presented as mean values of three replicates, with standard deviations. Different superscripts at 48 h indicate significant differences between pH levels (P<0.05) for each strain.

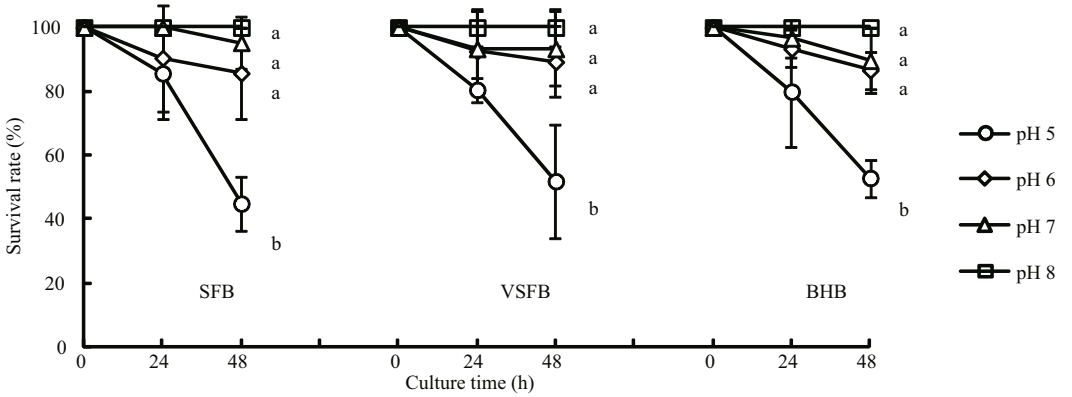

Fig.5 Survival rate (%) of adult *Artemia* of SFB, BHB, and VSFB *Artemia* exposed to different pH for 24 h and 48 h

Presented as mean values of three replicates, with standard deviations. Different superscripts at 48 h indicate significant differences between pH levels (P<0.05) for each strain.

ensure optimal functioning of the hatching enzyme (Van Stappen, 1996). In this study, hatching rates of the tested *Artemia* strains were significantly lower when cysts were exposed to pH 5. This is in agreement with the observations of Doyle and McMahon (1995), who found that the hatching percentage of *A. franciscana* was significantly lower at pH 5–5.5 compared with pH 7.3. Several studies on dormancy of *Artemia* embryos have suggested that decreases in internal pH cause inhibition of metabolic activity and subsequent developmental arrest (Carpenter and Hand, 1986; Conte and Geddes, 1988; Hoffman and

Table 3 Reproductive traits of SFB, VSFB and BHB *Artemia* cultured under different pH levels

Reproductive traits	SFB			VSFB			BHB		
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
Brood interval (days)	4.1±1.2 (14)	4.2±1.4 (14)	3.6±1.4 (12)	3.0±0.4 (13)	3.5±0.8 (14)	3.2±0.6 (14)	5.2±2.2 (4)	4.2±0.8 (8)	4.1±0.6 (9)
Number of ovoviviparous broods/female	1.6±1.9 (8)	1.9±1.1 (12)	3.7±2.1 (10)	4.7±2.7 (14)	3.6±1.9 (14)	4.6±2.4 (12)	2.1±1.8 (11)	1.8±0.9 (14)	2.9±1.6 (12)
Number of oviparous broods/female	5.9±2.7 [a] (13)	3.8±1.5 [b] (13)	3.6±2.0 [b] (13)	3.3±2.9 (14)	2.8±1.4 (14)	3.5±1.7 (12)	2.0±1.0* (4)	6* (1)	2.0±2.0* (4)
Number of nauplii/brood	45.5±10.3 (8)	53.5±17.8 (12)	45.7±13.3 (13)	46.2±15.3 (12)	59.5±24.5 (12)	47.3±14.2 (20)	38.8±9.9 [b] (11)	54±16.5 [a] (13)	51.2±10.1 [a] (12)
Number of cysts/brood	49.8±10.4[ab] (13)	40.2±9.1 [b] (13)	57.6±11.8 [a] (13)	56.6±15.3 [a] (9)	47.4±18.8 [ab] (9)	37.9±9.9 [b] (4)	44.1±0.6* (2)	37* (1)	42.8±13.4* (4)
Ratio of total nauplii and cyst production	0.24	0.56	0.58	1.45	2.15	2.39	4.40	5.56	6.86

* No statistical analysis was performed because of the lack of replicates at pH 7. Values are mean and standard deviation of three replicates. Number between brackets represents the number of observations. Different superscripts in a row of the same strain indicate significant difference (P<0.05).

Hand, 1990). Moreover, it has been indicated that the key enzyme required for hatching (which converts trehalose to glycerol) is inhibited by an acidic pH in vitro (Hand and Carpenter, 1986). Thus, in the present study, the low hatching rates of *Artemia* cysts observed under acidified brine may be caused by the permeability of cyst shells to H^+ and the subsequent acidic internal pH, resulting in irreversible interruption of metabolism or dormancy.

The acute toxicity of pH on crustaceans has been studied for several decapod species. Low pH caused retarded growth of the tiger shrimp *Penaeus monodon* (Wickins, 1984; Allan and Maguire, 1992), disturbed ion regulation in crayfish (Morgan and McMahon, 1982), and reduced survival, growth, molting frequency and feeding of the giant freshwater prawn *Macrobrachium rosenbergii* (Chen and Chen, 2003). Adult crayfish were found to be more tolerant of low pH than juveniles (France, 1984). In the present study, the tolerance of *Artemia* to acid exposure was dependent on the progressing developmental stages of the animals, except for the nauplii that had better survival rates than juveniles at lower pH. Sorgeloos et al. (1978) reported that the 1st instar *Artemia* nauplii are less sensitive to toxicants than 2nd and 3rd instar larvae. This is because the digestive tract of 1st instar nauplii is not yet in contact with the external medium and the larva only consumes its yolk, whereas the digestive tract of animals molted into the 2nd instar stage was well exposed. Nauplii in the present study began as 1st instar nauplii, although they had developed into 2nd to 3rd instar stages within 24 h, and thus adaptation to acidic conditions may have occurred, reducing mortality during the 48 h exposure.

In principle, both oviparity and ovoviviparity are found in all *Artemia* strains, and females can switch between the two reproduction cycles. The switch between the two modes is due to the ability of females to "perceive" forthcoming unstable environmental conditions, either producing dormant cysts when environmental conditions become deleterious or free-swimming nauplii, able to maintain the population, under optimal conditions (Gajardo and Beardmore, 2012). Therefore, the reproductive incapability observed at pH 5, and the prolonged brood interval and shift of reproduction mode from ovoviviparity to oviparity that occurred at pH 6–7, are responses of *Artemia* to acid stress.

Although VCSFB *Artemia* is of San Francisco Bay origin, the characteristic divergence of VCSFB from SFB is attributed to long-term selection in Vietnam (Kappas et al., 2004). This has resulted in different reproductive modes in VCSFB, as shown by higher reproductivity in the current study and our previous study (Sui et al., 2012). We also found that VCSFB produced more nauplii than SFB (and BHB) at all tested pH levels. This indicates that the former strain should be a more effective colonizer than the other strains, as ovoviviparous reproduction facilitates the rapid expansion and hence establishment of the exotic population (Lenz and Browne, 1991).

In conclusion, this study has shown that acidic environments hinder the cyst hatching, survival and reproductive performance of *Artemia*, although the responses were strain-specific. Inoculation of *Artemia* with either local parthenogenetic *Artemia* (BHB) or VSFB *Artemia* from Vietnam should be feasible at pH 6–7.

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