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Effects of light, temperature and salinity on reproductive behaviors of *Artemia sinica* Cai, 1989 (Crustacea: Anostraca)

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ABSTRACT: Reproductive biology of *Artemia* has been extensively studied, yet there are still unresolved issues regarding *Artemia* reproductive behaviors and their relationships with environmental factors. To better understand *Artemia* reproductive behaviors and environmental effects, we observed mating of *Artemia sinica* using visual inspection and video recording, and experimentally examined the influence of light versus dark, temperature (5–37 °C), and salinity (5–240 ppt) on its reproductive behaviors. Mating process in *A. sinica* can be categorized into seven behaviors: detection and orientation, station taking, grasping, pre-copulatory guarding/amplexus, intromission and copulation, post-copulatory guarding/amplexus, and disengagement. Males of *A. sinica* mostly attempt to copulate immediately after grasping a female though often unsuccessful, indicating pre-copulatory guarding is not indispensable. The average copulatory duration of *A. sinica* is 11.5 ± 2.7 min, longer than those recorded for other branchiopods. *Artemia sinica* exhibits no threshold temperature or salinity for initiating amplexus, but prolonged exposure to extreme temperatures or salinities leads to complete suppression of amplexus. Amplexus rates decline under extremely high and low temperatures or salinities, and under darkness. Except at lethal temperatures or salinities, the amplexus duration and amplexus-to-reproduction duration are decreased by the elevation of temperature, and increased by the elevation of salinity. *Artemia* reared to adult at 25 °C can complete reproduction at 7.5 °C, much lower than the lower temperature limit (15 °C) generally considered for completing life cycle. This allows *Artemia* maturing in cooling autumn an opportunity to leave offspring, and may be beneficial to the continuation of populations.

KEY WORDS: amplexus, Anostraca, *Artemia sinica*, environmental factors, reproductive behavior.

INTRODUCTION

Reproductive behaviors in anostracans (Crustacea, Branchiopoda, Anostraca) mainly contain processes of mating, carrying eggs (fertilized eggs developing in ovisac), and spawning. Among them the mating process is often complicated, and follows a basic pattern (Wiman, 1981). It generally contains the following behaviors: (1) detection and orientation (male detects and moves toward a potential mate); (2) station taking (male keeps head around potential mate genital segments and sustains this position more than 1 second); (3) amplexus (also called clasping or riding position; male amplexes potential mate with second antennae); (4) intromission and copulation (male's penis inserts in the female's gonopore near the end of the female's ovisac); (5) disengagement (male and female disengage and return to normal swimming movement) (Wiman, 1981; Belk, 1991; Rogers, 2002; Tsai *et al.*, 2017). However, the mechanics of each behavior/step may vary among taxa (Wiman, 1981). Amplexus, which is common and one of the most notable reproductive behaviors of crustaceans, can be divided into pre-copulatory amplexus and post-copulatory amplexus, with the former being a type of mate guarding that males maximize their chances of reproduction by guarding a potentially receptive female (Benvenuto *et al.*, 2009), and the latter a type of guarding that males secure paternity by clasping the female for a certain period after copulation (Dürbaum, 1995). Previous studies have shown that the length of amplexus differs greatly among anostracans, varying from seconds to days (Rogers, 2002; Tsai *et al.*, 2017).

Brine shrimps (*Artemia* Leach, 1819) (Crustacea, Branchiopoda, Anostraca) are halophilic crustaceans distributed in various permanent and ephemeral hypersaline waters such as salt lakes and salt pans over the world (Van Stappen, 2002). The genus contains up to nine bisexual species and four (diploid, triploid, tetraploid, pentaploid) obligate parthenogenetic lineages (Asem *et al.*, 2024). Both bisexual and parthenogenetic *Artemia* can produce resting eggs through oviparity and produce nauplii through ovoviviparity (Liang and MacRae, 1999; Wang *et al.*, 2017; Yang and Sun, 2023; Asem *et al.*, 2025). The mating system of bisexual *Artemia* is competitive polygyny, with females having the potential to obtain sperm from different males (Belk, 1991). Quite a few studies have documented that *Artemia* performs long (hours to days) amplexus (Wolfe, 1973; Forbes *et al.*, 1992; Correa and Tapia, 1998; Anufriieva and Shadrin, 2014). However, most studies on *Artemia* reproductive behaviors did not describe the entire process of amplexus and copulation in detail. Some studies considered that the amplexus of *Artemia* lasts from before to after copulation (i.e., the male performs both pre- and post-copulatory guarding) (Jensen, 1918; Wolfe, 1973; Huang *et al.*, 2001; Rogers, 2002), but only pre-copulatory amplexus was mentioned in some other studies (Kuenen, 1939; Lent, 1971, 1977; Anufriieva and Shadrin, 2014).

Numerous studies have shown that environmental factors such as temperature, salinity, and photoperiod have significant effects on the maturation and reproduction of *Artemia*. For instance, they can affect the time for sexual maturity (pre-reproductive period) (Wear *et al.*, 1986; Triantaphyllidis *et al.*, 1995; Browne and Wanigasekera, 2000; Abatzopoulos *et al.*, 2003), reproductive mode (Dana and Lenz, 1986; Browne and Wanigasekera, 2000; Abatzopoulos *et al.*, 2003; Agh *et al.*, 2008; Castro-Mejía *et al.*, 2011; Wang *et al.*, 2017), reproductive interval (Wear *et al.*, 1986; Browne *et al.*, 1988; Triantaphyllidis *et al.*, 1995; Abatzopoulos *et al.*, 2003), number of broods (Wear *et al.*, 1986; Aalamifar *et al.*, 2014), and number of offspring (Browne and Wanigasekera, 2000; Abatzopoulos *et al.*, 2003). The effects of environmental factors on reproductive behaviors, however, were much less reported. Anufriieva and Shadrin (2014) demonstrated that the amplexus duration of *Artemia* might be negatively correlated with temperature. Santos *et al.* (2018) found that the non-random amplexus/mating pattern of *Artemia* (males preferentially mating with larger females) could be disrupted by high temperature, and thus impacted sexual selection intensity.

Experimental studies have shown that the lower temperature limit for successful reproduction of *Artemia* is about 15 °C (Browne *et al.*, 1988; Browne and Wanigasekera, 2000). This result, however, reflects the critical temperature for *Artemia* to complete life cycle, rather than the threshold temperature for reproduction itself. Given that adult *Artemia* are more tolerant to low temperatures than *Artemia* of previous stages (Jia *et al.*, 1999), we presume that the lower temperature limit for successful reproduction may be lower than that for completing life cycle.

Therefore, there are still questions regarding the relationships between reproductive behaviors of *Artemia* and environmental factors awaiting to be answered. For instance, is there any critical temperature or salinity for the initiation of *Artemia* mating? What are the effects of temperature, salinity, and light on amplexus? Is the lower temperature limit for successful reproduction different from that for completing life cycle? To answer these questions, the mating behaviors of *Artemia sinica* Cai, 1989 were observed, the effects of light, temperature and salinity on its amplexus, as well as some other reproductive behaviors, were experimentally studied in this work.

MATERIALS AND METHODS

Experimental animals

Resting eggs of *A. sinica* were collected from Yuncheng Salt Lake (34˚59´N, 111˚02´E), Shanxi, China in 2021. They were hatched in seawater (salinity = 30 ppt) under 25 °C and continuous light (~2000 lx), which were controlled by a lighting incubator (GZH-A250, Shanghai Xianxiang Instruments Co., Ltd., China). Nauplii were cultured in media with a salinity of 80 ppt (prepared by adding sea salt to natural seawater) in 2000 ml tanks. The temperature and light conditions were the same as in hatching resting eggs, and were controlled by GZH-A250 lighting incubators aforementioned. The food was a 1:1 mixture of LANSY-Shrimp ZM powder (INVE Asia Services Ltd, Thailand) and *Dunaliella* powder (Bioengineering Branch of Inner Mongolia Lantai Industrial Co., Ltd., China). They were prepared as a working suspension following the method of Yang and Sun (2023).

When *Artemia* developed to L_{14}–L_{15} stage (Cohen *et al.*, 1999), males and females were sorted, and reared separately. Virgin female and male adults were thus obtained for use in the below experiments.

Mating behavior observations

Mating behaviors of *A. sinica* were primarily ascertained by means of visual observations under a temperature of ~25 °C and a salinity of 80 ppt. At the beginning of the experiment, 50 male and 50 female adults were placed in a 500 ml beaker. They were observed for 1–2 h, and a total of 12 batches of *Artemia* were observed by this method. When an amplexus event occurred, the coupled *Artemia* were moved to a well (containing 10 ml experimental medium) of six-well plate. Then behaviors of *Artemia* were either observed visually or recorded using DVOC DanioVison^{TM} Zebrafish Observation Chambers (Noldus, Netherlands). The direct visual observation was primarily employed to determine the copulation duration of *Artemia* that copulated immediately after a male grasped a female. For the DanioVison^{TM} record, videos were captured for 40 *Artemia* pairs (10 pairs, grasping followed by successful immediate intromission; 30 pairs, without successful immediate intromission) at a frame rate of 7.5 frames/s (the duration of video recording was 72–96 h). Using the video tracking software Ethoxision XT 11.5, the interval from amplexus to copulation, the duration of copulation and the interval from copulation to disengagement were determined. Besides, a DP74 digital camera adapted to an SZX16 stereomicroscope (Olympus, Japan) was also used to record the early mating behaviors and copulation.

Light experiment

The experiment contained two treatments, dark and light (2000 lx). The temperature was 25 °C and the salinity was 80 ppt. *Artemia* were acclimated to experimental condition for 12 h prior to experiment. The experiment was conducted in six-well plates, with one male and one female placed in each well (containing 10 ml experimental medium) at the beginning of experiment.

The light treatment contained 60 such *Artemia* pairs. They were checked at 1.5 h, 3 h, 6 h, 12 h, 24 h, and 48 h after the start of the experiment, and the state (in amplexus or not) of each *Artemia* pair was recorded. To avoid the influence of lighting during observation, the dark treatment was divided into six subgroups (each containing 60 pairs of *Artemia*), which were checked at 1.5 h, 3 h, 6 h, 12 h, 24 h, and 48 h, respectively. To determine the cumulative amplexus rate, *Artemia* in the 1.5-h subgroup were also checked at time points posterior to 1.5 h (the lighting time for each check about 2 min).

Temperature experiment

The temperature experiment was conducted at nine treatment temperatures: 5, 7.5, 10, 15, 20, 25, 30, 35, and 37 °C. The salinity was 80 ppt, and the light intensity was about 2000 lx. *Artemia* were acclimated to their treatment temperature for 24 h prior to the experiment. For each treatment, 50 pairs of *Artemia* were cultured in six-well plates as described for the light experiment. Every two days, a 100% water exchange was performed, and 200 µl of food suspension was provided to each *Artemia* pair. The survival, amplexus, disengagement, and reproductive events (laying resting eggs or nauplii) were checked at an interval of 4 h (in initial 72 h) or 12 h (after 72 h). Once the amplexus of an *Artemia* pair terminated, the male was transferred to a different six-well plate, where its survival was observed. When the female died or produced offspring, the experiment of this *Artemia* pair (both female and male) was terminated. For a certain temperature, if there was no reproductive event within seven consecutive days, the experiment of the treatment was terminated.

Salinity experiment

The salinity experiment was carried out at eight treatment salinities: 5, 10, 20, 40, 80, 160, 200, and 240 ppt. The temperature was 25 °C and the light intensity was about 2000 lx. *Artemia* were acclimated to their treatment salinity for 24 h prior to the experiment. Other experimental details were the same as in the temperature experiment.

Data analyses

The following parameters were used:

Time-point amplexus rate: observed number of *Artemia* pairs being engaged in amplexus at a time point / number of *Artemia* pairs tested × 100%. This parameter is only used in the light experiment.

Cumulative amplexus rate: cumulative number of *Artemia* pairs achieved amplexus / number of *Artemia* pairs tested × 100%.

Amplexus duration: Time interval between T_1 and T_2, where T_1 is the time that amplexus was first observed, T_2 is the time that the male and female disengaged.

Amplexus-to-reproduction duration: Time interval between T_1 and T_3, where T_3 is the time that female laid eggs or nauplii.

Significance of differences between percentages (time-point amplexus rate) was examined using Chi-square (X^2) test. For data of amplexus duration and amplexus-to-reproduction duration, a normality test was firstly conducted. If the data followed a normal distribution, One-way ANOVA was performed, and when significant differences ($p < 0.05$) were detected, a post hoc Tukey test was conducted between treatments, and the Pearson simple correlation coefficient between parameters was calculated. If the data did not follow a normal distribution, the non-parametric Kruskal-Wallis test (H test) was performed, and the Spearman simple correlation coefficient between parameters was calculated. All statistical analyses were performed using SPSS 26.

RESULTS

Mating behavior

In general, our observations indicated that the mating behaviors of *A. sinica* were initiated by the male, and comprised up to seven distinct behaviors/stages: detection and orientation, station taking, grasping, pre-copulatory guarding/amplexus, intromission and copulation, post-copulatory guarding/amplexus, and disengagement (for terminology, see Introduction). In some cases, the pre-copulatory amplexus was missing, and some behaviors like grasping and intromission attempt might fail. The pathways to complete the full mating process diverged among individuals or situations (see Fig. 1 and below descriptions).

Across the 12 batches of *Artemia* observed, a total of 152 amplexus events (successful grasping) were recorded. The majority of males (129 out of 152, 84.8%) immediately (within 7 seconds) attempted to copulate (arcing their body and trying to insert their gonopods into the opening of the female's ovisac) (Fig. 2A). However, these immediate copulatory attempts were frequently unsuccessful, occurring in 110 out of 129 cases (85.3%).

When the immediate intromission was successful, the copulation duration was 6.6–18.2 min (12.5 ± 3.2 min / mean ± SD, same below; *N* = 19, including 9 pairs from direct observation and 10 pairs from video records). After copulation, the male and female continued to swim together in amplexus status (Fig. 2B). The post-copulatory guarding duration was 0.3–28.4 h (4.2 ± 8.6 h; *N* = 10). The very large SD came mostly from an extremely large value (28.4 h) recorded in an *Artemia* pair. If it was excluded, the post-copulatory guarding duration would be 0.3–3.9 h (1.5 ± 1.0 h). The whole amplexus duration (copulation duration + post-copulatory guarding duration) was 0.4–28.5 h (4.4 ± 8.5 h; *N* = 10). In addition, one *Artemia* pair was observed to have completed the mating process four times within 4.3 h, with all of their

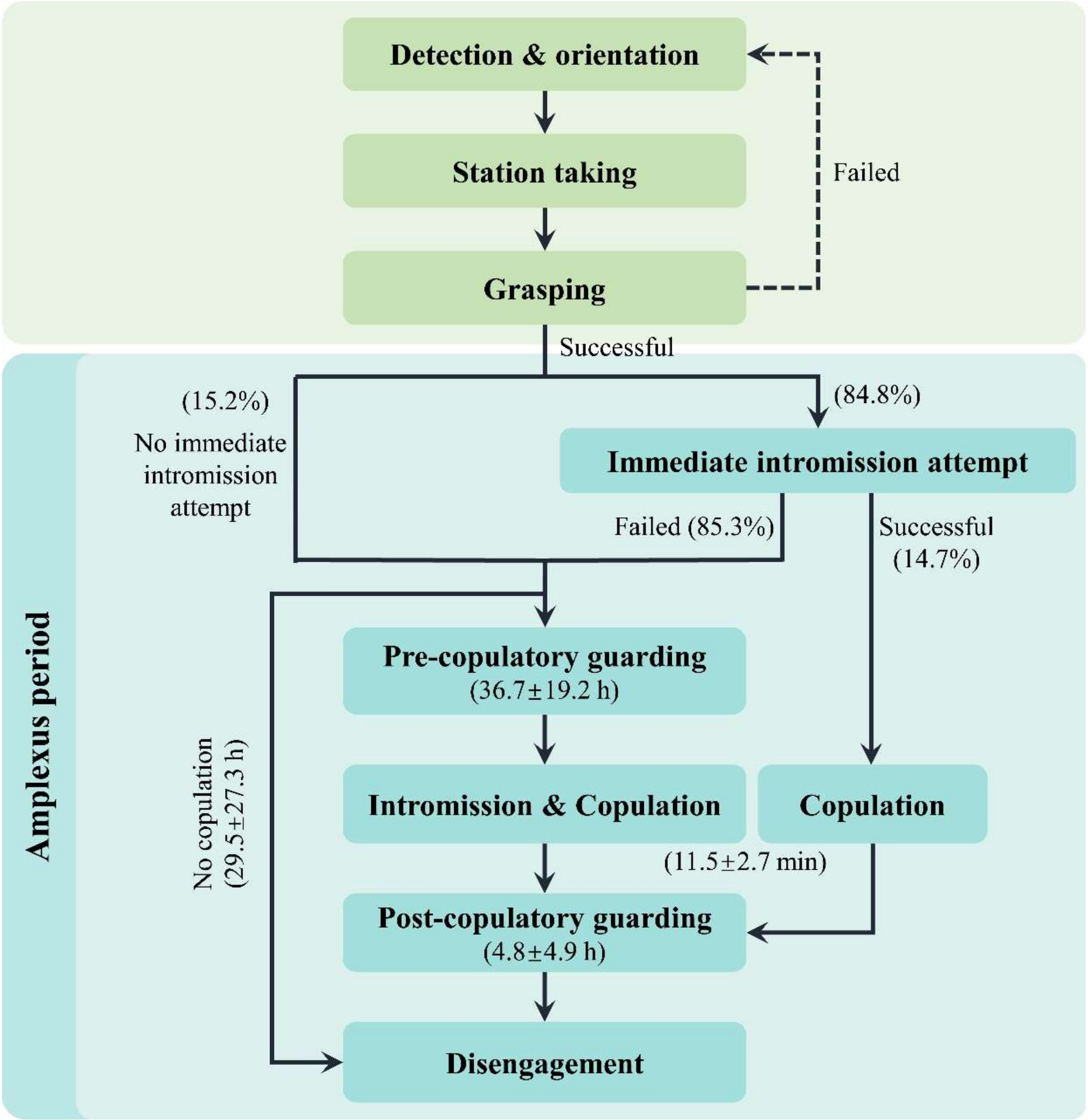

Fig. 1. Mating behavior process of ***Artemia sinica***.

Fig. 2. ***Artemia sinica***. **A.** Copulation and **B.** Amplexus.

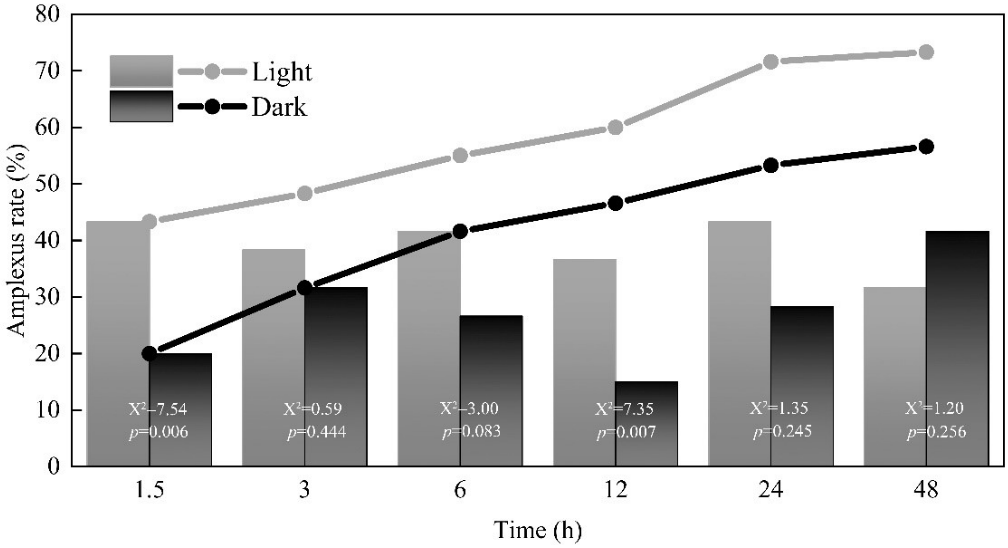

Fig. 3. Amplexus rates of ***Artemia sinica*** under light and dark conditions. Bars show the observed amplexus rates at each time point, with Chi-square test (X^2) test results between treatments shown in bars. Lines show the cumulative amplexus rates of the light group and the 1.5-h dark subgroup.

grasping followed by a successful immediate copulation. The copulation durations were 10.0, 4.0, 8.6, and 8.8 min, respectively, and the post-copulatory guarding durations were 1.2, 0.4, 0.1, and 0.5 h, respectively. The intervals between neighboring mating events were 1, 0.6, and 0.5 h, respectively.

We have recorded the full amplexus process of 30 pairs of *Artemia* that did not have a successful immediate copulation. Among them, 22 pairs engaged in copulation at subsequent time. Their pre-copulatory guarding duration was 9.7–78.7 h (36.7 ± 19.2 h), copulation duration was 7.6–14.2 min (10.7 ± 1.9 min), post-copulatory guarding duration was 2.8–9.7 h (5.1 ± 2.1 h), and whole amplexus duration (pre-copulatory guarding duration + copulation duration + post-copulatory guarding duration) was 17.6–86.1 h (42.0 ± 19.9 h). The ratio of pre-copulatory guarding duration to post-copulatory guarding duration was 1.0–13.7 (7.7 ± 3.0). In the 8 pairs of *Artemia* that did not copulate before disengagement, the amplexus duration was 3.7–77.8 h (29.5 ± 27.3 h).

Counting the two situations (with and without successful immediate copulation) together, the copulation duration was 6.6–18.2 min (11.5 ± 2.7 min; *N* = 41), and the post-copulatory guarding duration was 0.3–28.4 h (4.8 ± 4.9 h; *N* = 32).

Effects of light on amplexus

During the 48 h lighting experiment, *Artemia* exhibited no death. In the initial 24 h, the time-point amplexus rates of *Artemia* under light were higher than those under darkness, though the differences were not statistically significant at some time points (Fig. 3). At 48 h, the time-point amplexus rate of the light treatment was lower than that in the dark treatment (Fig. 3), which should be because more pairs in the light group terminated amplexus (disengaged) before the time point. The cumulative amplexus rates in the light group were always higher than those in the 1.5-h dark subgroup (Fig. 3).

Effects of temperature on reproductive behaviors

In the temperature experiment, the last reproductive event occurred at 46 d (in 7.5 °C treatment), and the experiment was terminated at 53 d. The survival of *Artemia* (both of the paired female and male survived) in the initial 144 h is shown in Fig. 4A. All 50 *Artemia* pairs suffered death (at least one of the paired *Artemia* died) within 96 h and 144 h in the 37 °C and 5 °C treatments, respectively. In the 35 °C treatment 18 *Artemia* pairs survived for 144 h. The survival of *Artemia* at other temperatures were better (33–40 pairs survived for 144 h).

As shown in Fig. 4B, amplexus was observed at all temperatures. The time-course cumulative amplexus rates can be described with the power function $y = ax^b$. At the initial period (e.g., 4 h) of the experiment when the mortalities were low in all treatments, amplexus rates showed great differences among temperatures, ranking as 30 °C > 35 °C > 7.5–25 °C > 37 °C > 5 °C. The time that the cumulative amplexus rates reached the maximum decreased with the elevation of temperature (decreasing from 144 h at 7.5 °C to 24 h at 37 °C) except in the 5 °C treatment (48 h) (Table 1). When the cumulative amplexus rates reached maximum, all surviving *Artemia* pairs in treatments of 7.5–35 °C had achieved amplexus, and the final cumulative amplexus rates showed low difference (86%–98%) (Table 1; Fig. 4B). By contrast, the final cumulative amplexus rates in 37 and 5 °C treatments were markedly lower (34% and 16%, respectively), with a large number of surviving *Artemia*

Table 1. Time and counts (% in parentheses) of ***Artemia*** pairs (categorized by amplexus attainment and survival status) when the cumulative amplexus rate reached its maximum in the temperature experiment

Temperature (°C)	Time (h)	Achieved amplexus	Not achieved amplexus and dead	Not achieved amplexus and alive
5	48	8 (16)	6 (12)	36 (72)
7.5	144	46 (92)	4 (8)	0 (0)
10	120	46 (92)	4 (8)	0 (0)
15	120	45 (90)	5 (10)	0 (0)
20	96	48 (96)	2 (4)	0 (0)
25	96	48 (96)	2 (4)	0 (0)
30	48	49 (98)	1 (2)	0 (0)
35	48	43 (86)	7 (14)	0 (0)
37	24	17 (34)	16 (32)	17 (34)

Note: *Artemia* pair is considered "dead" when either male or female or the both died.

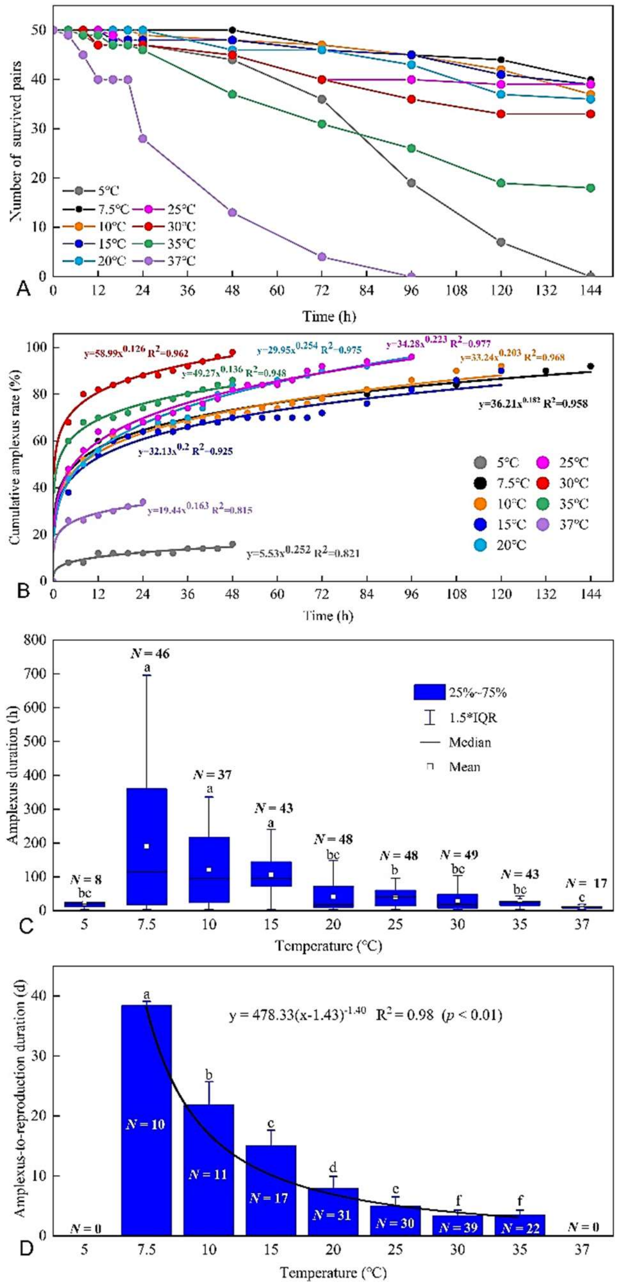

Fig. 4. Effects of temperature on reproductive behaviors of ***Artemia sinica***. **A**. Survival (number of *Artemia* pairs that both female and male survived) during the initial 144 h of experiment. **B**. Cumulative amplexus rates. **C**. Amplexus duration. Values with different characters are significantly different (H test, $p < 0.05$). **D**. Amplexus-to-reproduction duration (mean + S.D.). Values with different characters are significantly different (Tukey test, $p < 0.05$).

pairs (17 and 36 pairs, respectively) failing to achieve amplexus. None of these pairs engaged in amplexus subsequently and all died by 144 h (Table 1; Fig. 4A, B). In summary, extremely high or low temperatures reduced the amplexus rate in *A. sinica*; lower temperatures delayed the initiation of amplexus; prolonged exposure to extreme temperatures (≥48 h at 5 °C or ≥24 h at 37 °C, not including the 24 h accumulation period at their respective temperatures) led to a complete suppression of amplexus in *A. sinica*.

When the temperature dropped from 37 °C to 7.5 °C, the average amplexus duration increased from 11.7 h to 190.5 h, and the durations at 7.5, 10, and 15 °C were significantly higher than at other temperatures. The amplexus duration at 5 °C, however, was only 23.5 h, much lower than at 7.5 °C (Fig. 4C). Spearman Correlation Analysis showed that the amplexus duration was negatively correlated with temperature (R = –0.376, $p < 0.001$; R = –0.416 and $p < 0.001$ if 5 °C not included in analysis).

At 5 °C and 37 °C, all *Artemia* died before reproduction. In treatments of 7.5–35 °C, a small number of "mated" females did not lay any resting eggs or nauplii through the experiment period (excluded from calculating the amplexus-to-reproduction duration). Pearson Correlation Analysis showed that the amplexus-to-reproduction duration was negatively correlated with temperature (R = –0.843, $p < 0.001$). Their relationship conforms to the Bêlehrádek's temperature function ($y = 478.33(x–1.430)^{-1.40}$), with the amplexus-to-reproduction duration at 7.5 °C (38.5 ± 0.6 d) being 11.3 times longer than that at 35 °C (3.4 ± 0.8 d) (Fig. 4D). There were significant differences between different temperatures except between 30 and 35 °C (Fig. 4D).

Table 2. Time and counts (% in parentheses) of ***Artemia*** pairs (categorized by amplexus attainment and survival status) when the cumulative amplexus rate reached its maximum in the salinity experiment

Salinity (ppt)	Time (h)	Achieved amplexus	Not achieved amplexus and dead	Not achieved amplexus and alive
5	48	30 (60)	9 (18)	11 (22)
10	72	35 (70)	10 (20)	5 (10)
20	72	45 (90)	4 (8)	1 (2)
40	96	47 (94)	3 (6)	0 (0)
80	96	48 (96)	2 (4)	0 (0)
160	96	47 (94)	2 (4)	1 (2)
200	120	43 (86)	7 (14)	0 (0)
240	20	18 (36)	5 (10)	27 (54)

Note: *Artemia* pair is considered "dead" when either male or female or the both died.

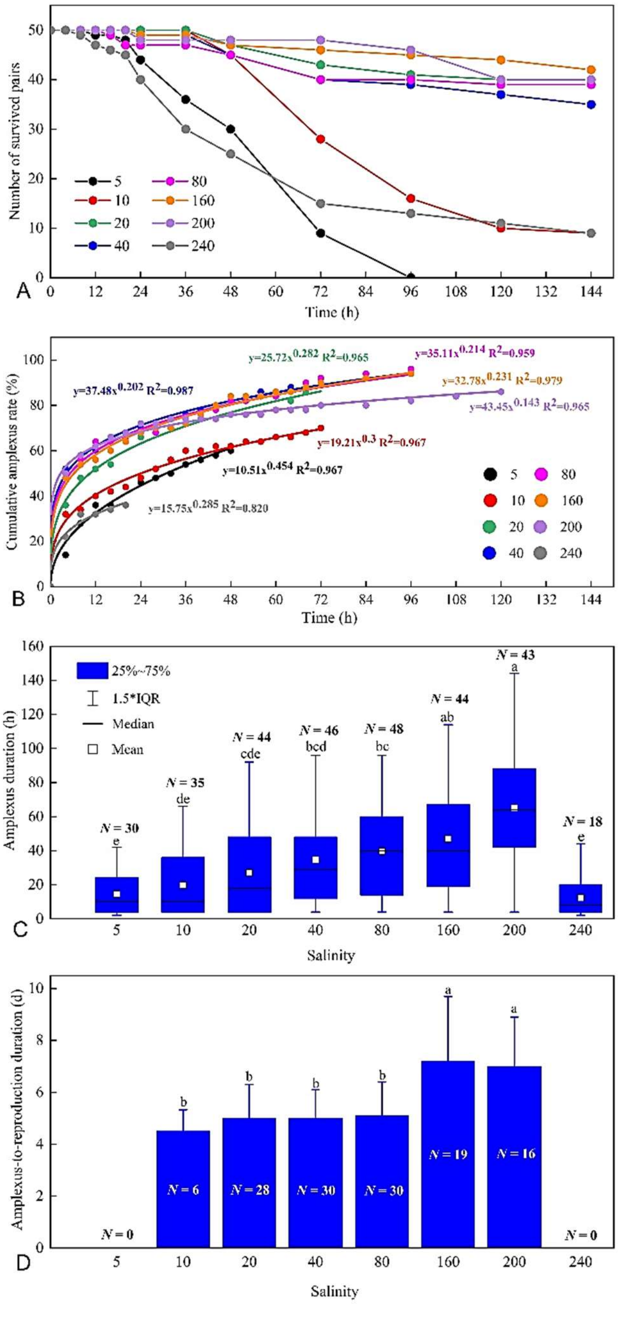

Fig. 5. Effects of salinity on reproductive behaviors of ***Artemia sinica***. **A**. Survival (number of *Artemia* pairs that both female and male survived) during the initial 144 h of experiment. **B**. Cumulative amplexus rates. **C**. Amplexus duration. Values with different characters are significantly different (H test, $p < 0.05$). **D**. Amplexus-to-reproduction duration (mean + S.D.). Values with different characters are significantly different (Tukey test, $p < 0.05$).

Effects of salinity on reproductive behaviors

In the salinity experiment, the last reproductive event occurred at 13 d, and the experiment was terminated at 20 d. All 50 *Artemia* pairs in the 5 ppt treatment died within 96 h, and only 9 pairs in the 10 and 240 ppt treatments survived to 144 h. Treatments of other salinities showed better survival (35–42 pairs survived 144 h) (Fig. 5A).

The time-course cumulative amplexus rates can be described by the power function $y = ax^b$. The amplexus rates began to show obvious among-salinity differences at the initial period (4 h) of the experiment, and the curves of very low (5, 10 ppt) and high (240 ppt) salinity treatments were located below those of the other salinities through the experiment period. The time that the cumulative amplexus rate reached maximum basically extended with increasing salinity (increased from 48 h at 5 ppt to 120 h at 200 ppt), but remarkably decreased in the 240 ppt treatment (20 h) (Table 2; Fig. 5B). The final cumulative amplexus rates showed low difference (86%–96%) at salinities of 20–200 ppt, while obviously decreased under very low and high salinities (60% at 5 ppt, 36% at 240 ppt) (Table 2; Fig. 5B). By the time that the cumulative amplexus rates reached maximum, more surviving *Artemia* pairs in extremely low and high salinity treatments (5, 10 and 240 ppt) had not achieved amplexus (11, 5 and 27 pairs, respectively; all of them suffered death by 144 h) than those in the other treatments (0–1 pair) (Table 2). In summary, extremely high or low salinities reduced the amplexus rate in *A. sinica*; prolonged exposure to extreme salinities (≥48 h at 5 ppt or ≥20 h at 240 ppt, not including the 24 h accumulation period at their respective salinities) led to a complete suppression of amplexus in *A. sinica*.

The average amplexus duration of *A. sinica* increased from 14.5 h to 65.6 h when the salinity increased from 5 to 200 ppt, but significantly decreased to 12.3 h at 240 ppt

(Fig. 5C). Spearman Correlation Analysis showed a positive correlation between amplexus duration and salinity (R = 0.331, $p < 0.001$; R = 0.483 and $p < 0.001$ if 240 ppt not included in analysis).

Under salinities of 5 and 240 ppt, all *Artemia* died before reproduction. Under salinities of 20 to 200 ppt, a small number of "mated" females did not lay any resting eggs or nauplii through the experimental period, which were not considered in calculating the amplexus-to-reproduction duration. The amplexus-to-reproduction duration was significantly prolonged under higher salinities, 4.5–5.1 d at 10 to 80 ppt, and 7.2/7.0 d at 160/200 ppt (Fig. 5D). Pearson Correlation Analysis showed that the amplexus-to-reproduction duration was positively correlated with salinity (R = 0.475, $p < 0.001$).

DISCUSSION

The present study reveals that the mating process of *A. sinica* can be categorized into seven distinct behavioral stages: detection and orientation, station taking, grasping, pre-copulatory guarding/amplexus, intromission and copulation, post-copulatory guarding/amplexus, and disengagement. Overall, this process aligns with the general mating pattern described for anostracans (Wiman, 1981; Belk, 1991; Rogers, 2002; Tsai *et al.*, 2017). However, the present classification differs from previous studies in dividing "amplexus" into three different behaviors/stages (grasping, pre-copulatory guarding/amplexus, and post-copulatory guarding/amplexus). This refinement is based on the observation that in *Artemia*, amplexus may be bisected by the act of copulation, and grasping may be followed directly either by immediate copulation or by a period of pre-copulatory guarding (see below for further details). Additionally, not all behavioral attempts, such as grasping and intromission, lead to immediate success, a phenomenon also reported in other anostracans (Wiman, 1981; Belk, 1991; Tapia *et al.*, 2015). Furthermore, not every grasped *Artemia* pair achieves successful copulation before disengagement.

In some anostracans such as *Branchinecta coloradensis* Packard, 1874 and *Branchinella kugenumaensis* (Ishikawa, 1895), amplexus and copulation occur almost simultaneously, and males do not exhibit copulatory guarding behaviors (Wiman, 1981; Rogers, 2002; Tsai *et al.*, 2017). Other anostracans, like species of genera *Artemiopsis* Sars, 1897, *Chirocephalus* Prevost, 1803 and *Eubranchipus* Verrill, 1870, do engage in copulatory guarding, with their amplexus lasting for hours to days (Rogers, 2002). Although *Artemia* have been known to have mate guarding for over a century (e.g., Jensen, 1918), the time when copulation took place was mentioned in few studies. Early work by Kuenen (1939) noted that "the males of *Artemia* clasp the females some time before the actual copulation takes place", and "this 'riding-position' is kept up for some time after the copulation(s) too". Lent (1971, 1977) reported that the coupled *Artemia* copulates following female molt. Wolfe (1973) found that *Artemia salina* (Linnaeus, 1758) copulates several days after male clasping the female and the post-copulatory guarding persists for approximately 2.5 h (maximum 5.5 h). In *A. sinica*, most males attempted to copulate immediately after grasping a female, though only 14.7% of these initial attempts resulted in successful intromission and copulation, suggesting that pre-copulatory guarding is not indispensable. This finding challenges the long-standing consensus, supported by quite a few studies (e.g., Jensen, 1918; Kuenen, 1939; Lent, 1971, 1977; Wolfe, 1973; Rogers, 2002; Anufriieva and Shadrin, 2014), that copulation is a subsequent event to pre-copulatory amplexus in *Artemia*. Given that the female in amplexus may or may not open her gonopore depending upon her perception of the male's suitability (Rogers, 2002), the low success rate in male's initial copulation attempt may be due to the fact that many females have not accepted the male and opened their gonopores.

When pre-copulatory guarding occurred in *A. sinica*, its duration (36.7 ± 19.2 h) was 7.7 ± 2.9 times longer than the post-copulatory guarding duration (5.1 ± 2.1 h). This pattern of a brief post-copulatory guarding following a potentially prolonged pre-copulatory phase is consistent with that documented for *A. salina* (several days vs. a maximum of 5.5 hours) by Wolfe (1973). Bowen's (1962) experiment demonstrated that mature *Artemia* eggs are fertilized in the oviducts and transferred to the ovisac within 55 minutes, after which they become inaccessible to fertilization by another male. Consequently, the relatively short post-copulatory guarding period in *A. sinica* appears adequate to ensure paternity.

The copulatory duration of *A. sinica* is 11.5 ± 2.7 min, which is longer than that recorded for *A. salina* (~5 min) (Wolfe, 1973), and other branchiopods including the notostracan *Lepidurus apus* (Linnaeus, 1758) (30 s) (Mathias, 1937), the diplostracans *Daphnia pulicaria* Forbes, 1893 (13.8 s) (La *et al.*, 2014), *Cyzicus grubei* (Simon, 1886) (24.6 s) (Pérez-Bote, 2010) and *Polyphemus pediculus* (Linnaeus, 1761) (40 s) (Butorina, 2000), the anostracans *Eubranchipus holmanii* (Ryder, 1879) (16 s) (Moore and Ogren, 1962 as cited in Munuswamy and Subramoniam, 1985), *Eubranchipus serratus* Forbes, 1876 (1.9 min) (Belk, 1984) and *B. kugenumaensis* (2.1 s) (Tsai *et al.*, 2017). In our experiment, one *Artemia* pair was observed to have completed the mating process four times within 4.3 h. This finding of repeated copulation aligns with reports in other anostracans, including *A. franciscana* (Jensen, 1918) and *E. serratus* (Pearse, 1913; Belk, 1984), and supports the earlier view that males are continually ready to mate and will mate repeatedly (Pearse, 1913).

In the fairy shrimps *E. serratus* and *B. kugenumaensis*, males rarely grasp and copulate with females under darkness, suggesting that visual cues play a key role in mate detection and location (Belk, 1991; Tsai *et al.*, 2017). However, other studies indicate that courtship behaviors of anostracans are primarily mediated by female-released semiochemicals. Rogers (2019) found that males of *Branchinecta lindahli* Packard, 1883 entirely isolated from females immediately began searching behaviors when water from female cultures was added. Tapia *et al.* (2016) reported that cuticular compounds (lipids) emitted by con-specifics have an important role in the intra-specific recognition and increase the swimming speed of males in *A. franciscana*. Tapia *et al.* (2015) demonstrated that male *A. franciscana* exhibited station-taking behavior toward other males soaked in female-conditioned water. In *A. sinica*, amplexus occurred under both dark and light conditions, but the amplexus rate declined under darkness (Fig. 3), which suggests that visual cue may have a role in the mating of *A. sinica*, but should not be an indispensable cue. Given that the courtship of *Artemia* depends on semiochemicals (Tapia *et al.*, 2015) and light modulates sex pheromone release in female arthropods (Li *et al.*, 2019), light may affect *Artemia*'s mating behaviors by influencing the release of sex pheromones, in addition to its direct influence on the vision detection (if exists).Furthermore, *Artemia* swim more slowly in the dark than in the light (Huang and Chen, 2000), which may impair males' ability to locate mates.

Artemia sinica engaged in amplexus under diverse thermal (5–37 °C) and osmotic gradients (5–240 ppt), even if the temperature or salinity levels exceeded the normal survival thresholds (Fig. 4B, 5B), indicating the absence of critical conditions for the initiation of mating process. However, the initiation of amplexus could be completely suppressed by prolonged exposure to extreme temperatures or salinities. Similarly, Moore and Ogren (1962, as cited in Belk, 1984) found that *E. holmani* is sexually active only within the temperature range of 13.5 to 19°C. The amplexus rates of *A. sinica* were obviously impacted by temperature and salinity early in the experimental period, and the time that cumulative amplexus rates reaching maximum declined with the elevation of temperature, and increased with the elevation of salinity (e.g., 4 h) (Fig. 4B, 5B). Therefore, temperature and salinity seem to influence *Artemia* amplexus in a pattern that follows both dose-response and time-response relationships. In *A. franciscana*, Santos *et al.* (2018) demonstrated that high temperatures disrupted the non-random amplexus/mating pattern and altered the intensity of sexual selection. When exposed to unfavorable salinities, *Artemia* consume more energy to maintain osmotic balance (Croghan, 1958; Dana and Lenz, 1986; Naegel and Rodriguez, 2002). This energetic trade-off reduces energy allocated to reproduction, which may reduce males' ability to locate and grasp females. Furthermore, studies on insects and spiders have shown that thermal inhibition of mating may result from suppressed production and release of female sex pheromones (Xiang *et al.*, 2009; Leith *et al.*, 2021). Female *Artemia* may likewise attract males via sex pheromones (Tapia *et al.*, 2015). Under extreme temperatures or salinities, the reduction of female sex pheromones and consequent decline of attractiveness to males may be another reason for the decreased rate and delay in amplexus.

Quantitative data on the duration of *Artemia* amplexus are still limited. Under optimal conditions, the amplexus duration of *A. franciscana* is 4–5 d (Jensen, 1918; Correa and Tapia, 1998); that of *Artemia urmiana* Günther, 1890 lasts for 10 and 21 d at 20 and 16 ℃, respectively (Anufriieva and Shadrin, 2014). The average amplexus duration of *A. sinica*, varying from 11.7 h (37 °C) to ~190.5 h (7.5 °C), was negatively correlated with temperature (Fig. 4C), which is consistent with that reported for *A. urmiana* (Anufriieva and Shadrin, 2014), and for crustaceans in general (Ward, 1986). Within the non-lethal salinity range, the amplexus duration of *A. sinica* increased with increasing salinity (Fig. 5C). To ensure the male's paternity, the duration of post-copulatory guarding may be dependent on the duration that eggs are fertilized and transferred to the ovisac (see above). Under low temperature and high salinity conditions, eggs may need longer time to become inaccessible to fertilization (by another male). Hence, the longer amplexus duration may partly attribute to that males prefer a prolonged post-copulatory guarding under low temperatures and high salinities. We do not have a reasonable explanation for prolonged pre-copulatory guarding under low temperatures and high salinities, except that *Artemia's* biological processes, such as hatching, growth, maturation and locomotion, are generally decelerated by low temperature and high salinity (e.g., Sorgeloos, 1980; Dana and Lenz, 1986; Triantaphyllidis *et al.*, 1995; Browne and Wanigasekera, 2000; Abatzopoulos *et al.*, 2003; Anufriieva and Shadrin, 2014; Yang and Sun, 2023).

As the duration from amplexus to the completion of fertilization is relatively short (in case without pre-copulatory guarding, it is less than 1 h (Bowen, 1962); in case with pre-copulatory guarding, it is roughly 36.7 ± 19.2 h the guarding time plus the ~1 h fertilization time), the amplexus-to-reproduction duration (5.0 ± 1.4 d at 25 °C; Fig. 4D) should be mostly composed of the duration of eggs' development in the ovisac. It is not surprising that the relationship between the amplexus-to-reproduction duration and temperature conforms to the Bêlehrádek's temperature function (Fig. 4D), which has long been used to describe the relationship between temperature and development speed of planktons (e.g., McLaren, 1963, 1966; Halsband-Lenk *et al.*, 2002). When salinities were between 10 and 80 ppt, the amplexus-to-reproduction duration of *A. sinica* was about

4.5–5.1 d, while when salinities were 160 and 200 ppt, the time extended to 7.2/7.0 d (Fig. 5D). Similarly, Yang and Sun (2023) documented that some processes related to the reproduction of *A. sinica*, e.g., pre-reproductive period and reproductive interval, were prolonged under higher salinities. The increased energy consumption for osmotic regulation may be a reason for slowing these processes under higher salinities (Dana and Lenz, 1986; Yang and Sun, 2023). It is worth noting that, unlike temperature, the influence of salinity on such reproduction parameters is often variable among *Artemia* species or populations (Yang and Sun, 2023, and citations therein).

It is generally believed that the minimum temperature for *Artemia* to mature and complete life cycle is around 15 °C (Browne *et al.*, 1988; Browne and Wanigasekera, 2000). In the experiment of Browne and Wanigasekera (2000), *A. sinica* failed to reproduce at 15 °C. In the present study, when *A. sinica* reared to adult at 25 °C were transferred to 7.5 °C, they reproduced successfully. Jia *et al.* (1999) demonstrated that nauplii and adults of *Artemia* are more resistant/tolerant to cold. Hence, whether *Artemia* can complete life cycle at low temperatures should mainly depend on their cold resistant/tolerant capacity in stages prior to adult, and the limiting low temperature for reproduction may be lower than that for completing life cycle. This gives *Artemia* maturing in the cooling autumn an opportunity to leave offspring, which is beneficial to the continuation of *Artemia* populations, especially those living in cold areas.

CONCLUSION

This study documents for the first time the mating behaviors, and effects of light, temperature and salinity on reproductive behaviors of *A. sinica.* Its mating process consists of seven behaviors/stages. Males of *A. sinica* generally attempt to copulate immediately after grasping a female, though mostly unsuccessful. *Artemia sinica* possesses both pre-copulatory and post-copulatory mate guarding, but the former is not indispensable. The average copulatory duration of *A. sinica* is longer than those recorded for other branchiopods. There seems to be no threshold temperature and salinity for initiating the mating behaviors of *A. sinica*, but amplexus may be completely suppressed by prolonged exposure to extreme temperatures or salinities. Except at lethal temperatures or salinities, the amplexus duration and the amplexus-to-reproduction duration of *A. sinica* are decreased by the elevation of temperature, and increased by the elevation of salinity. The temperature for *Artemia* to complete reproduction (7.5 °C) is much lower than that for completing life cycle, which gives *Artemia* maturing in cooling autumn an opportunity to leave offspring and is beneficial to the continuation of populations. Future research could focus on the mechanisms through which environmental factors modulate reproductive behaviors, as well as on determining whether specific traits observed in *A. sinica* (such as immediate copulation) also exist in congeneric species.

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