

## Coupled effects of photoperiod, temperature and salinity on diapause induction of the parthenogenetic *Artemia* (Crustacea: Anostraca) from Barkol Lake, China

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**Abstract.** Coupled effects of photoperiod, temperature and salinity on diapause induction were experimentally studied using a diploid clone of the parthenogenetic *Artemia* from Barkol Lake, Xinjiang, China. We conducted three experiments, each containing nine treatments (combinations of two of the above three factors, each with three levels). When photoperiod was set at 12L(light):12D(dark), combinations of salinity 70/140 ppt and temperature 19/23°C led most females to produce diapaused offspring (resting eggs / cysts; percentage of diapaused broods being 81.60±32.51% [mean±SD; same below] to 99.28±3.48%). Under combinations of salinity 70/140 ppt and 27°C most broods were nauplii (percentage of diapaused broods being 28.46±30.68% to 38.43±23.22%). When salinity was set to 70 ppt, treatment conditions of 19/23/27°C + 6L:18D and 19/23°C + 12L:12D induced high percentage of diapaused broods (72.11±36.42% to 100±0.00%). When temperature was set to 25°C, overwhelming cyst production was only observed under 6L:18D + 70/140 ppt (percentage of diapaused broods being 83.33±23.57% to 93.86±14.10%). These results suggest that the modes of offspring production in the studied *Artemia* clone are strongly influenced by photoperiod and temperature and there is significant interaction between the two factors. Lower temperature and shorter daylight tend to induce production of resting eggs, and the effect of photoperiod may dominate over the effect of temperature. Though an interaction between salinity and photoperiod was detected, a sound tendency for the salinity effect could not be determined.

**Key words:** *Artemia*, diapause, oviparity, ovoviviparity, environmental factors.

### Introduction

The brine shrimps from the genus *Artemia*, which occur in saline and hypersaline habitats all over the world (Van Stappen 2002), have long been used as a favorite model system for ecological, physiological and genetic studies (Dhont & Sorgeloos 2002, Baxeveanis et al. 2004, Chu et al. 2014). The embryogenesis of both bisexual and parthenogenetic *Artemia* follows two modes: oviparity with production of encysted embryos (resting eggs/cysts, diapause mode) and ovoviviparity with release of live nauplii (non-diapause mode) (Liang & MacRae 1999). The occurrence of oviparity is maternally controlled by the evolved ability of females to “perceive” forthcoming environmental conditions (Gajardo & Beardmore 1989, Clegg et al. 1996, Gajardo & Beardmore 2012). For a long time, it has been considered that live nauplii were commonly produced when conditions were optimal (low or moderate salinity, high oxygen tension, abundance of food etc.), while resting eggs were often produced when physico-chemical factors were not so favorable (Dutrieu 1960, Clegg

& Trotman 2002, Criel & Macrae 2002). However, some other factors, for which the favorable/unfavorable levels are difficult to identify, may also affect the offspring production mode. For example, females of *Artemia franciscana* Kellogg, 1906 preferred to produce resting eggs when cultured in medium containing chelated iron (Versichele & Sorgeloos 1980); the mode of offspring production could be strongly affected by photoperiod with resting eggs dominating during shorter daylight (Provasoli & Pintner 1980, Nambu et al. 2004).

Considering that the reproduction of bisexual species might be affected by males and wild parthenogenetic populations are sometimes a mixture of strains with different ploidy levels and/or with different genetic structures (Zhang & Lefcort 1991, Stenberg & Saura 2009), it was recommended to use clonal *Artemia* for scoring *Artemia* response to different environmental components (Abatzopoulos et al. 2003). In most studies, the effects of environmental factors on reproductive characteristics were evaluated by the ratio of resting eggs within all offspring (e.g. Triantaphyllidis

et al. 1995, Baxevanis et al. 2004, Agh et al. 2008, Asil et al. 2013, Pinto et al. 2014). Because the reproduction mode of *Artemia* is mutually exclusive for any particular brood (Berthélémy-Okazaki & Hedgecock 1987), and the number of offspring in each brood is variable, the percentage of resting eggs (in all offspring) may be biasedly determined by the broods with larger numbers of offspring. The ratio of diapaused broods in all broods (i.e. number of diapaused broods / number of both diapaused and non-diapaused broods), as used by Nambu et al. (2004), may better reflect the effect of environmental factors on the diapause induction in an experimental *Artemia* population.

Using the production mode of each brood as a major criterion, we experimentally observed the coupled effects of photoperiod, temperature (two main *token stimuli* in arthropod diapause induction [Danks 1987, Huestis & Lehmann 2014]) and salinity (the most important environmental factor affecting the life of hypersaline organisms [Abatzopoulos et al. 2003]) on the reproduction mode of a diploid parthenogenetic *Artemia* clone. The aim is to gain a better understanding for the role of these factors on the induction of embryo-diapause in *Artemia*.

## Materials and methods

### Experimental animals and culture methods

A diploid clone (BRK-C53) was established from the parthenogenetic *Artemia* population of Barkol Lake, Xinjiang, China. Stock cultures of BRK-C53 were maintained at temperature of 25°C, photoperiod of L(light):D(dark) = 18:6, and salinity of 70 ppt.

For all stock and experiment cultures, high-salinity media were prepared by adding sea salt to local seawater (salinity ~31 ppt), temperatures and photoperiods were controlled by lighting incubators (GZX-300, Ningbo Jiangan Instrument Factory, Ningbo, China). *Artemia* were fed with an 1:1 mixture of *Spirulina* sp. powder (Tianjian Biology Technology Co., Ltd, China) and LANSY-Shrimp ZM powder (INVE Asia Services Ltd, Thailand) (previously homogenated with a glass tissue grinder and suspended with culture medium).

### Experiment 1: Coupled effect of salinity and temperature

This experiment was conducted under the combinations of three salinities (35, 70 and 140 ppt) and three temperatures (19, 23, and 27°C), and thus had 9 treatments. The photoperiod was 12L:12D. For each treatment, 50 individuals at the end of post-metanauplius stage (Cohen & Vélez 1999) were selected as test animals, each reared separately in a 50-ml falcon tube containing ~40 ml culture medium.

During the lifespan of the experimental animals, potential reproduction was checked daily. If any individual produced a brood of resting eggs, the brood was recorded as a “diapaused brood”; if it produced nauplii the brood was recorded as a “non-diapaused brood”. In some broods, a small number of “resting eggs” were observed together with a larger number of nauplii. In this case the “resting eggs” were usually irregular in appearance and much smaller than normal cysts, or occasionally were nauplii enclosed in a transparent membrane. Such broods were defined as non-diapaused broods as suggested by Berthélémy-Okazaki & Hedgecock (1987).

### Experiment 2: Coupled effect of salinity and photoperiod

This experiment was conducted under combinations of three different photoperiods (18L:6D, 12L:12D and 6L:18D) and three salinities (35, 70 and 140 ppt). The temperature was 25°C. Other details were the same as in Experiment 1.

### Experiment 3: Coupled effect of temperature and photoperiod

This experiment was done under combinations of three temperatures (19, 23 and 27°C) and three photoperiods (18L:6D, 12L:12D and 6L:18D). The salinity was 70 ppt. Other details were the same as in Experiment 1.

### Data analyses

Following Nambu et al. (2004), data from females that reproduced only once were not taken into analyses. Only data of females that have produced two or more broods were considered as valid and analysed (the maximum number of valid broods observed in present experiments was 8). The effect of environmental factors on inducing diapause was judged by the percentage of diapaused broods in the total valid broods, which was calculated for each individual, and then the mean was determined for the treatment. Data were analyzed by Two-way ANOVAs using SPSS 16. When any significant differences ( $p < 0.05$ ) were found, a post hoc Turkey test was carried out between different treatments.

## Results

### Experiment 1. Coupled effect of temperature and salinity

This experiment lasted for 47 to 93 days (except for the salinity of 35 ppt treatments, for which no valid data were obtained because all individuals died before reproduction or reproduced only once). Two-way ANOVAs revealed that only temperature had significant effect (Table 1). Under salinities of 70 and 140 ppt, the 27°C treatments exhibited lower percentage of diapaused broods than the 19 and 23°C treatments. There were no significant differences between the results of 19°C and 23°C treatments (Table 2).

**Table 1.** *p* values of Two-way ANOVAs for data of three experiments (Temperature × salinity experiment, photoperiod × salinity experiment, photoperiod × temperature experiment).

	Experiment 1 (salinity × temperature)	Experiment 2 (photoperiod × salinity)	Experiment 3 (photoperiod × temperature)
Salinity	0.194	0.889	–
Temperature	0.000	–	0.000
Photoperiod	–	0.000	0.000
Salinity × temperature	0.786	–	–
Photoperiod × salinity	–	0.005	–
Photoperiod × temperature	–	–	0.000

**Table 2.** The number of individuals of *Artemia* that produced two or more broods (N) and the percentage of diapaused broods in total valid broods (P) under combinations of three different salinities and three different temperatures at a constant photoperiod (12L:12D). Data shown as mean ± S.D. Means with different superscripts are significantly different ( $p < 0.05$ ).

Culture regime	19°C		23°C		27°C	
	N	P	N	P	N	P
35 ppt	No valid data	No valid data	No valid data	No valid data	No valid data	No valid data
70 ppt	24	99.28±3.48 <sup>a</sup>	20	85.00±25.39 <sup>a</sup>	16	38.43±23.22 <sup>b</sup>
140 ppt	25	95.1±10.19 <sup>a</sup>	20	81.60±32.51 <sup>a</sup>	23	28.46±30.68 <sup>b</sup>

#### Experiment 2. Coupled effect of salinity and photoperiod

Treatments of this experiment lasted for 38 to 73 days (except for the salinity of 35 ppt treatments, for which no valid data were obtained because all individuals died before reproduction or reproduced only once). Photoperiod had meaningful impacts on the reproductive mode of *Artemia*, but the effect of salinity was not significant. An interactive effect of photoperiod and salinity was also detected (Table 1). Under the salinity of 140 ppt, the percentage of diapaused broods in the 6L:18D treatment was significantly different from those in the other two treatments, while there was not any significant difference between the 18L:6D and 12L:12D treatments. At the salinity of 70 ppt, both 6L:18D and 12L:12D treatments had significantly higher percentage of diapaused broods than the 18L:6D treatment (Table 3).

#### Experiment 3. Coupled effect of temperature and photoperiod

Treatments of this experiment lasted for 44 to 105 days. Statistical analysis showed that temperature,

**Table 3.** The number of individuals of *Artemia* that produced two or more broods (N) and the percentage of diapaused broods in total valid broods (P) under combinations of three different salinities and three different photoperiods at a constant temperature (25°C). Data shown as mean ± S.D. Means with different superscripts are significantly different ( $p < 0.05$ ).

Culture regime	18L: 6D		12L: 12D		6L: 18D	
	N	P	N	P	N	P
35 ppt	No valid data	No valid data	No valid data	No valid data	No valid data	No valid data
70 ppt	18	29.16±22.11 <sup>c</sup>	19	65.74±30.50 <sup>b</sup>	14	83.33±23.57 <sup>ab</sup>
140 ppt	21	44.17±20.80 <sup>bc</sup>	16	42.39±31.80 <sup>bc</sup>	20	93.86±14.10 <sup>a</sup>

**Table 4.** The number of individuals of *Artemia* that produced two or more broods (N) and the percentage of diapaused broods in total valid broods (P) under combinations of three different temperatures and three different photoperiods at a constant salinity (70 ppt). Data shown as mean ± S.D. Means with different superscripts are significantly different ( $p < 0.05$ ).

Culture regime	18L: 6D		12L: 12D		6L: 18D	
	N	P	N	P	N	P
19°C	27	4.94±15.20 <sup>d</sup>	23	99.28±3.48 <sup>a</sup>	25	98.00±9.80 <sup>a</sup>
23°C	30	32.84±30.08 <sup>c</sup>	20	85.00±25.38 <sup>ab</sup>	21	100±0.00 <sup>a</sup>
27°C	14	8.33±21.43 <sup>d</sup>	16	38.43±23.22 <sup>c</sup>	30	72.11±36.42 <sup>b</sup>

photoperiod and the interaction of temperature and photoperiod had impact on the modes of offspring production (Table 1). Shorter daylight as well as lower temperature led to higher proportions of diapaused broods, and almost all broods (98–100%) were resting eggs when mothers were reared under combined conditions of shorter daylight and lower temperatures. Under shorter daylight (6L:18D) plus higher temperature (27°C), the majority of broods were oviparous (72.11±36.42%), but oviparity could not be induced by longer daylight (18L: 6D) plus lower temperature (19,23°C) (Table 4).

## Discussion

Since salinity is one of the most important environmental factors affecting the life of hypersaline organisms, the influence of salinity on reproductive mode/characteristics was studied much more than other factors. Some studies on *Artemia* (e.g., Barigozzi 1939, Berthélémy-Okazaki & Hedgecock 1987) found preponderance of resting egg production at lower salinities, but opposite results were also reported (Ballardin & Metalli 1963, Perez-

Rodriguez as cited in Berthélémy-Okazaki & Hedgecock 1987, Ahmadi as cited in Berthélémy-Okazaki & Hedgecock 1987). Bian & Li (cited as Bian & Li 1988 in Bian 1990; but Bian & Li's (1988) paper was not formally published) compared the reproduction mode of ten parthenogenetic populations under four different salinities, but a sound relationship was not determined between salinity and reproduction mode. Similarly, the present study found no significant difference between treatments of salinities of 70 and 140 ppt, and at the salinity of 35 ppt the studied clone can hardly complete the reproduction process (Tables 2 and 3).

Though Nambu et al. (2004) found that temperature had not significant effect on the reproduction mode of *A. franciscana* (20°C vs 28°C), most other studies have shown that nauplii production was dominant under higher temperatures. For instance, Berthélémy-Okazaki and Hedgecock (1987) reported that an unidentified bisexual species exhibited dominant nauplii production under 24–27°C but resting eggs production under 16°C. Similar higher temperature (26±2°C) effect was also reported for five other populations (Browne 1980). This is well supported by our results, which showed that lower temperatures (19°C, 23°C) usually induced production of resting eggs, while higher temperature (27°C) supported nauplii production (Tables 2 and 4).

Compared to data published on temperature and salinity, fewer studies have documented the photoperiod effect on the reproduction mode of *Artemia*. In *A. franciscana* and a parthenogenetic population from France, diapause was induced by short daylight (Provasoli & Pintner 1980, Nambu et al. 2004). These results were supported by the present study (Tables 2 and 3). Berthélémy-Okazaki and Hedgecock (1987) described that the non-diapause effect of long daylight on the first brood of *A. franciscana* diminished with the increase of salinity. For the BRK-C53 clone, though an interaction between salinity and photoperiod was detected by Two-way ANOVAs, a sound tendency for the salinity effect could not be determined.

Diapause is a natural process related to the metabolic program and developmental pathways, and many invertebrates use this tolerance strategy to adapt to forthcoming adverse conditions (Danks 1987, MacRae 2005, Alekseev et al. 2006, Podrabsky & Hand 2015). In crustaceans like cladocerans, copepods and amphipods, photoperiod

and temperature are effective in inducing diapause, and photoperiod usually plays a leading role in the induction of diapause (Stross 1966; March 1982, Marcus 1986, Hairston & Olds 1987, Alekseev et al. 2006). Studies on insects also showed that photoperiod was the steadiest cue controlling the onset of diapause, other factors mainly serving a compensatory or indirectional role, e.g., food influences the diapause by directly adjusting the developmental or metabolic speed (Danks 1987). Our results showed that temperature had significant interaction with photoperiod. Diapause could be induced by shorter daylight (6L:18D) plus higher temperature (27°C), but could not be induced by longer daylight (18L:6D) plus lower temperature (19 and 23°C) (Table 4), suggesting that decreased daylight might be the prerequisite for inducing diapause of *Artemia*, though lower temperature is also a powerful diapause-inducing signal.

Although environmental factors like temperature and photoperiod have been confirmed to be effective in inducing diapause in *Artemia* by most studies (Berthélémy-Okazaki & Hedgecock 1987, Abatzopoulos et al. 2003, Nambu et al. 2004, present study), some studies have documented that the reproduction mode might be determined also by the genetic traits of species/populations. For instance, Ballardin & Metalli (1963) found there was no relationship between environmental factors and oviparity in an Italian *Artemia* population. While most of the eight studied parthenogenetic populations produced both nauplii and resting eggs, the Hoh Lake population (Qinghai, China) almost constantly reproduced oviparously (Bian & Li cited in Bian 1990, see above). Studies on copepods (e.g., Marcus 1984) and insects (e.g., Beach 1978, McWatters & Sannder 1996, Lai et al. 2008) also demonstrated that *token stimuli* (photoperiod and temperature) had different diapause inducing effects on different populations.

Diapause-inducing signals are perceived during a fixed and specific sensitive period by insects, which is genetically determined and may occur in the parental generation, different stages of embryonal, larval and pupal development, or in adults (Košťál 2006). A recent study on the gene expression of Gahai (China) population of parthenogenetic *Artemia* has suggested that the mode of reproduction might be predestined in the oocyte stage (Dai et al. 2011). However, the exact sensitive period in the diapause-induction of *Artemia* is still a field to be explored.

In conclusion, the present study showed that shorter daylight and lower temperature are effective factors for inducing diapause in *Artemia*, and the effect of either factor can be enhanced by the other one. It is likely that photoperiod plays a predominant role in determining the reproduction mode of *Artemia*.

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