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The critical stage for inducing oviparity and embryonic diapause in parthenogenetic *Artemia* (Crustacea: Anostraca): an experimental study*

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Abstract The brine shrimp *Artemia* exhibits two reproductive modes: 1) oviparity, producing diapause embryos; and 2) ovoviviparity, producing free-swimming nauplii. Previous studies have suggested the existence of a critical stage that determines the reproductive mode. Physicochemical factors, such as photoperiod, temperature, and salinity, have been suggested to irreversibly affect the reproductive mode of oocytes during this critical stage. In this study, experiments were carried out using a photoperiod and temperature-sensitive parthenogenetic *Artemia* clone where maternal *Artemia* were shifted bidirectionally between ovoviviparity (18 h L:6 h D, 27°C) and oviparity (6 h L:18 h D, 19°C) culture conditions. In the main experiment (*Artemia* shifted at six different stages including the post-larva II to adult II), the reproductive mode of first brood was converted when shifting was performed on post-larva II and III but was not converted when females were shifted after post-larva III. A supplementary experiment further revealed that the reproductive mode of first brood could be altered when shifting females at an "early phase of post-larva IV", characterized by a developing ovisac reaching the middle of the third abdominal segment, ventral spines, and some oocytes growing larger than the others. In both experiments, reproductive modes of the second brood were significantly affected when the shifting was performed on post-larva IV. These results suggest that the critical stage for inducing oviparity and embryonic diapause is at the previtellogenic stage of oocytes, or at maternal "early phase of post-larva IV" for the first-brood offspring. During this stage, differential gene expression patterns of the two destined oocytes may be triggered by the token stimuli signals received by the oocytes.

Keyword: *Artemia*; reproduction mode; environmental factors; diapause; critical stage; Anostraca

1 INTRODUCTION

Resistance characterized by actively overcoming environmental stress either at an individual or group level and tolerance characterized by minimizing vital functions or/and forming resistant stages are two main strategies used by organisms to adapt to environmental heterogeneity (Lopes et al., 2004; Alekseev et al., 2006). A wide range of invertebrates exhibits tolerance by undergoing dormancy in some fixed life stages (Alekseev et al., 2007; Koštál and Denlinger, 2011). There are two main types of dormancy, quiescence and diapause (Koštál, 2006). Quiescence refers to a hypo-metabolic state imposed directly by environmental (exogenous) conditions; whereas diapause is a dormancy state where the arrest of development is initiated by internal factors (Lavens and Sorgeloos, 1987; Brendonck, 1996). In insects, diapause-inducing signals (or token stimuli) are always perceived during a fixed and specific sensitive

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period, which is named sensitive stage or critical stage for diapause induction. The stage is genetically determined and ranged from various periods within the parental generation to different stages of embryonal, larval, pupal development and adult individual (Koštál, 2006).

The brine shrimp *Artemia* (Crustacea: Anostraca) is an embryonic diapausing crustacean that has two reproductive modes, oviparity producing diapause embryos / diapause cysts / resting eggs and ovoviviparity producing free-swimming nauplii (Alekseev and Starobogatov, 1996; Nambu et al., 2004). Many studies have shown that physicochemical factors like photoperiod, temperature, and salinity (especially photoperiod) are the token stimuli inducing oviparity in *Artemia* (e.g. Berthélémy-Okazaki and Hedgecock, 1987; Nambu et al., 2004; Dai et al., 2011; Wang et al., 2017).

Previous studies have documented some evidence for the existence of a critical life-stage sensitive to environmental cues (Provasoli and Pintner, 1980; Berthélémy-Okazaki and Hedgecock, 1987), but the stage has not been precisely determined (Dai et al., 2011). The identification of the critical stage receiving the stimulus to switch reproduction mode is an important step to better understand the complex *Artemia* life cycle. To determine the critical stage for inducing oviparity in *Artemia*, shift-culture experiments were carried out in this study. Following the recommendation of Abatzopoulos et al. (2003), a clonal parthenogenetic *Artemia*, sensitive to photoperiod and temperature (Wang et al., 2017), was selected as the experimental animal for two reasons. Firstly, bisexual species might be affected by maternal and paternal heterozygosity (Gajardo and Beardmore, 1989). Secondly, wild parthenogenetic populations have sometimes different genetic structures and/or with different ploidy levels (Zhang and Lefcort, 1991).

2 MATERIAL AND METHOD

2.1 *Artemia* and stock cultures

The previously established diploid parthenogenetic *Artemia* clone (BRK53) obtained from the population at Barkol Lake (43°40′N, 92°47′E), Xinjiang, China, was used in this study. The reproduction mode of this clone is sensitive to photoperiod and temperature. Under longer daylight and higher temperature (18 h L:6 h D and 27°C), more than 90% broods were nauplii and almost 100% females produced nauplii in their first brood; while under shorter daylight and lower temperature (6 h L:18 h D and 19°C), the production of diapaused cysts increased drastically (Wang et al., 2017).

The stock population was cultured in 3-L glass aquaria at the condition of producing nauplii (temperature of 27°C, photoperiod of 18L:6D and salinity of 70). Culture media were prepared by adding natural sea salt (Qingdao Salt Distribution Office, Qingdao, China) to seawater. Temperatures and photoperiods were controlled by illumination incubators (GZX-300, Ningbo Jiangnan Instrument Factory, Ningbo, China). *Artemia* was fed a homogenized mixture of *Dunaliella* sp. powder (Tianjian Biology Technology Co. Ltd., China) and LANSY-Shrimp ZM powder (INVE Asia Services Ltd., Thailand) (1:1) following the protocols of Triantaphyllidis et al. (1995). During the experiment period, the evaporative loss of water was replenished with distilled water. Every time an offspring brood was deposited, the culture medium was exchanged, and the resting eggs or nauplii were removed.

2.2 Shift-culture experiments

Two shift-culture experiments were conducted following the method previously used in insects (Wagner et al., 1999; Kurban et al., 2005). They are mentioned below as “main experiment” and “supplementary experiment”, respectively.

In the main experiment, *Artemia* were shifted by two different ways, from the conditions of producing nauplii (temperature 27°C, photoperiod 18L:6D) to the conditions of producing diapaused cysts (temperature 19°C, photoperiod 6L:18D), and from the conditions of producing diapaused cysts to the conditions of producing nauplii, respectively. Referring to the results of previous studies (Jackson and Clegg, 1996; Dai et al., 2011) and our pilot experiment, post-larva II, post-larva III, post-larva IV, post-larva V (oocytes appearing as opaque dots in ovaries), adult I (oocytes in oviducts) and adult II (oocytes in uterus), were selected as the stages for shifting *Artemia*. Morphological characters, observed and photographed under a Nikon SMZ800 stereomicroscope adapted with a Nikon DS-5M digital camera, were used to distinguish each life stage following Cohen et al. (1999). When transferring from nauplius to cyst production conditions, experimental *Artemia* were selected from the stock culture, and each individual was moved to a 50-mL Falcon tube containing 30 mL culture medium. The

brine shrimp were then cultured under the cyst production conditions. Meanwhile, another set of individuals were prepared by the same method but were cultured under the conditions of the stock cultures (nauplius production conditions). These cultures were designated as control.

In the reverse direction experiments, newly-born nauplii (Nauplius I; see Cohen et al., 1999) in the stock cultures were selected and moved to a different aquarium for the reverse experimental stock cultures. They were reared by the same methods as for the control stock cultures but under the conditions of producing diapaused cysts. When enough (>200) individuals developed to each of the previously mentioned stages, individuals were selected and moved to the Falcon tubes as in the last experiment, but here the control brine shrimps were reared successively under cyst production conditions, while the experimental brine shrimps were shifted to the conditions of producing nauplii.

Each control/experimental consisted of 30 individuals and the experiments were replicated thrice. All individuals were acclimated at 23°C for ~6 h before being transferred from 19°C to 27°C, and vice versa. Other experimental protocols were the same as the stock culture. The stock, control and experimental cultures are summarized in Table 1.

During the experimental period, the reproductive modes of the first two broods were recorded for each brine shrimp. If an individual produced a brood of cysts, an "oviparous brood" was recorded; if the shrimp produced nauplii, an "ovoviviparous brood" was recorded. In some cases, many nauplii and a small number of "irregular" cysts were observed together; these broods were defined as ovoviviparous broods (for more information see Berthélémy-Okazaki and Hedgecock, 1987; Wang et al., 2017). The incidence of oviparous broods (the proportion of oviparous females in total reproducing females) was taken as the criterion for evaluating the oviparity-inducing effect.

After the critical stage (which should be placed between two adjacent stages that could and could not convert the reproductive mode) was roughly determined by the main experiment, a supplementary experiment was conducted to precisely determine the critical stage for oviparity induction. In this experiment, individuals at these two stages (reproductive mode converted, and reproductive mode not converted in the main experiment) and a stage between them (see below) were used as shifting

Table 1 Stock/reverse experimental stock, control, and experimental cultures for the females of BRK53 *Artemia* clone used in the main and supplementary experiments

Culture	Condition	Reproduction mode	Result
Stock	27°C, 18 h L:6 h D	Ovoviviparity	
Control	27°C, 18 h L:6 h D	Ovoviviparity	Tables 2, 4
Experimental	19°C, 6 h L:18 h D	Oviparity	Tables 2, 4
Reverse experimental stock	19°C, 6 h L:18 h D	Oviparity	
Reverse direction control	19°C, 6 h L:18 h D	Oviparity	Tables 3, 5
Reverse direction experimental	27°C, 18 h L:6 h D	Ovoviviparity	Tables 3, 5

for the brine shrimp experiments. As in the main experiment, *Artemia* were also shifted by two different ways. The experimental procedure was the same as that in the main experiment.

The data were statistically analyzed by SPSS 16. Differences among means of the controls or treatments were analyzed by one-way ANOVA coupled with a Tukey's multiple-comparison test ($P<0.05$). The *t*-test was used to determine significant differences ($P<0.05$) between controls and treatments at the same stage.

2.3 Histological studies

During the supplementary experiment, specimens ($n=3$) at each stage were sampled to determine the histological characteristics of the female reproductive organs. They were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffered saline (PBS, pH 7.4) for about 24 h. After dehydration in gradient alcohol series, the tissues were passed through xylene and then embedded in paraffin. After the blocks were cut at 6 μm transversely, the sections were mounted on slides and then stained with hematoxylin and eosin (HE). The ovarian development stages were photographed with a Nikon E600 microscope adapted with an Olympus DP72 camera.

3 RESULT

3.1 Reproduction modes of females shifted at different development stages

Results of the main experiment are shown in Tables 2 and 3. When shifting *Artemia* from nauplius

Table 2 Incidence (%) of females producing diapause cysts among all reproducing females of BRK53 *Artemia* clone in the main experiment

Stage	1st brood			2nd brood		
	Control	Experimental	*P* (*t*-test)	Control	Experimental	*P* (*t*-test)
Post-larva II	1.1±2.0[a]	98.9 ±1.9[b]	<0.000 5	1.3±2.2[a]	96.1±4.2[c]	<0.000 5
Post-larva III	2.2±3.8[a]	96.7 ±3.3[b]	<0.000 5	0.0±0.0[a]	100.0±0.0[c]	<0.000 5
Post-larva IV	1.1±1.9[a]	3.4±3.4[a]	0.363	3.4±3.3[a]	30.7±9.8[b]	0.010
Post-larva V	2.2±1.9[a]	4.5±1.9[a]	0.211	4.5±2.1[a]	3.4±3.3[a]	0.650
Adult I	2.3±2.0[a]	1.2±2.1[a]	0.540	1.2±2.1[a]	3.7±3.7[a]	0.370
Adult II	1.1±1.9[a]	3.4±3.4[a]	0.370	1.1±1.9[a]	1.1±1.9[a]	1.000
F value (ANOVA)	0.202	928.900		1.819	280.164	
P value (ANOVA)	0.955	<0.000 5		0.183	<0.000 5	

Note: For the Experimental, "stage" refer to individuals at their development when transferred from the control conditions (18 h L:6 h D and 27°C; inducing nauplius production) to the experimental conditions (6 h L:18 h D and 19°C; inducing cyst production). In each column, different superscript letters show a significant difference (ANOVA, Tukey, $P<0.05$). In each row, *P* value is the result of *t*-test between the pairwise control and treatment.

Table 3 Incidence (%) of females producing diapause cysts among all reproducing females of BRK53 *Artemia* clone in the main experiment

Stage	1st brood			2nd brood		
	Control	Experimental	*P* (*t*-test)	Control	Experimental	*P* (*t*-test)
Post-larva II	97.7±2.0[a]	1.3±2.2[b]	<0.000 5	100.0±0.0[a]	1.3±2.2[b]	<0.000 5
Post-larva III	98.7±2.3[a]	1.6±2.7[b]	<0.000 5	98.7±2.3[a]	1.6±2.7[b]	<0.000 5
Post-larva IV	97.7±2.0[a]	94.6±2.0[a]	0.134	97.7±2.0[a]	7.1±7.1[b]	<0.000 5
Post-larva V	100.0±0.0[a]	96.3±3.6[a]	0.218	100.0±0.0[a]	96.3±3.6[a]	0.218
Adult I	97.7±2.0[a]	98.3±2.9[a]	0.772	97.7±2.0[a]	98.3±2.9[a]	0.759
Adult II	98.9±1.9[a]	97.3±2.4[a]	0.413	98.9±1.9[a]	97.1±2.6[a]	0.387
F value (ANOVA)	0.755	1 007.650		1.149	523.216	
P value (ANOVA)	0.599	<0.000 5		0.388	<0.000 5	

Note: For the Experimental, "stage" refer to individuals at their development when transferred from the control conditions (6 h L:18 h D and 19°C; inducing cyst production) to the experimental conditions (18 h L:6 h D and 27°C; inducing nauplius production). In each column, different superscript letters show a significant difference (ANOVA, Tukey, $P<0.05$). In each row, *P* value is the result of *t*-test between the pairwise control and treatment.

production condition to cyst production condition (Table 2), low percentages of oviparity (0.0%–4.5%) in both the first and second broods of the controls showed no significant differences among the different stages (post-larva II, post-larva III, post-larva IV, post-larva V, adult I and adult II). For the first brood of treatments, almost all females produced diapause cysts when individuals were shifted at post-larva II stage (98.9%±1.9% of the reproducing females (same below)) and at post-larva III stage (96.7%±3.3%) which both were significantly different ($P<0.01$) from the controls. Females shifted at post-larva IV, post-larva V, adult I and adult II stages produced diapause cysts (1.2%–4.5%) which were not significantly different from the controls. Similar results were obtained in the second brood; however, significantly more ($P=0.01$) females shifted at post-larva IV stage produced diapause cysts (30.7%±9.8%) compared to the controls (3.4%±3.3%). For both broods, percentages of oviparity (96.1%–100.0%) in the females shifted at post-larva II/III stage were significantly higher ($P<0.05$) compared to those shifted at the later stages (1.1%–30.7%). For either brood, no significant difference was detected between treatments of post-larval II and post-larval III stages. Among the other treatments, only the second brood of the post-larval IV treatment had a higher oviparity (30.7%) compared to treatments of the post-larval V, adult I and adult II (1.1%–3.7%).

When shifting females from cyst to nauplius production condition (Table 3), the percentages of oviparous broods in all controls (97.7%–100.0%) showed no significant differences among them. For the first brood of treatment, the oviparity (1.3%–

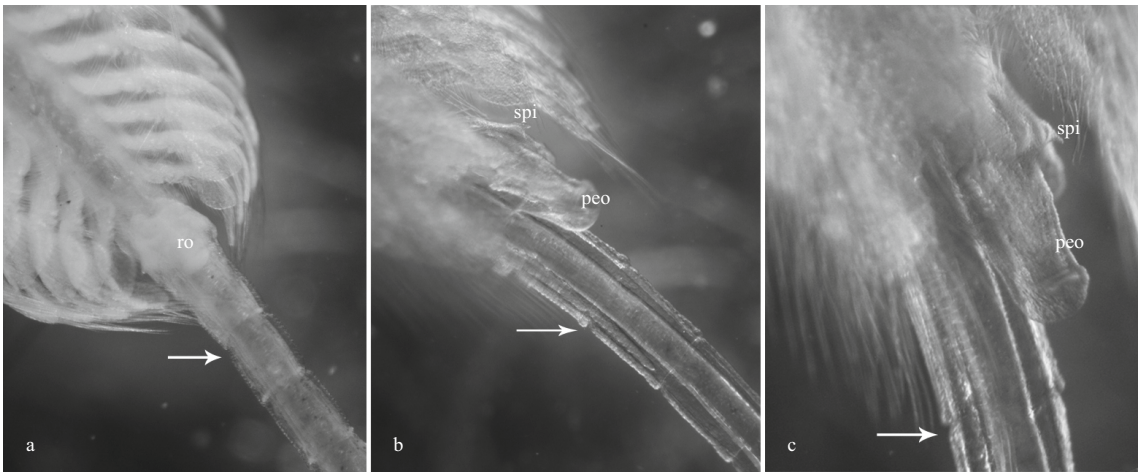

Fig.1 Morphology of the genital segments of the parthenogenetic *Artemia* from Barkol Lake, to show the difference between the "early phase of post-larva IV" stage and post-larva III and post-larva IV stage

a. post-larva III (ventral view); b. "early phase of post-larva IV" stage (ventrolateral view); c. post-larva IV (ventrolateral view). peo: posterior end of ovisac, ro: rudimentary ovisac, spi: spine of the ovisac. Arrows point to the boundary between the 3rd and 4th abdominal segments.

1.6%) of the post-larva II and III stages showed significant differences (P<0.01) compared to the controls (97.7%–98.7%); however, there were no significant differences between the control and the treatment when the females were shifted after the post-larva III stage. For the second brood, oviparity of the treatments post-larva II/III/IV stages (1.3%–7.1%) and the corresponding controls (97.7%–100.0%) showed significant differences (P<0.01); however, no significant differences were found when females were shifted after the post-larva IV stage.

Based on the results of the main experiment, females at post-larva III and post-larva IV stages, and a stage between them were shifted in the supplementary experiment. The morphological definition of the "between post-larva III and IV" stage and its comparison with the two other stages are shown in Fig.1. At post-larva III stage, the ovisac was a rudimentary hump that reached the beginning of the third abdominal segment (Fig.1a). At the stage post-larva IV, the ovisac concealed two-thirds of the third abdominal segment and a pair of sharp ventral spines were visible (Fig.1c). At the "between post-larva III and IV" stage, a developing ovisac was observed and reached the middle of the third abdominal segment, and its blunt ventral spines were visible (Fig.1b). These characters are closer to post-larva IV than post-larva III, and thus the stage is called an "early phase of post-larva IV". In all the three stages, the ovaries were transparent. Opaque dots, which was a character of the post-larva V (Criel, 1989) were not observed microscopically on the ovaries. (Fig.1).

In the supplementary experiment when females were shifted from the nauplius to cyst production conditions, the results were similar to those of the main experiment. Low oviparity (3.6%–5.8%) was observed in females from the control groups. Significantly (P<0.01) more females shifted at post-larva III and "early phase of post-larva IV" stages produced cysts (96.5%±3.4% and 67.7%±4.7%, respectively), compared to the controls (Table 4). In contrast, low oviparity (6.1%±2.2%) was observed in individuals shifted at the post-larva IV stage in the first brood, a non-significant result compared to that of the controls. All three treatments were significantly different from each other (P<0.01). For second brood, females shifted at the post-larva III and "early phase of post-larva IV" stages showed high oviparity (97.7%±4.0% and 96.4%±3.6%, respectively). Additionally, higher oviparity (31.9%±7.6%) was found in females shifted at the post-larva IV stage compared to that of the first brood. Oviparity values of the post-larva III and "early phase of post-larva IV" stages were significantly different (P<0.01) compared to those of the post-larva IV stage. The oviparity of the treatments between the post-larva III and "early phase of post-larva IV" stages showed no significant differences. All oviparity values of the three treatments were significantly different (P<0.01) compared to those of the controls.

In the experiment of shifting females from cyst to nauplius production condition (Table 5), most control females (97.6%–100%) produced diapause cysts in both the first and second broods. In the first brood of treatment groups, low oviparity (1.1%±1.9%) was observed in females shifted at post-larva III stage;

Table 4 Incidence (%) of females producing diapause cysts among all reproducing females of BRK53 *Artemia* clone in the supplementary experiment

Stage	1st brood			2nd brood		
	Control	Experimental	*P* (*t*-test)	Control	Experimental	*P* (*t*-test)
Post-larva III	3.6±3.5[a]	96.5±3.4[c]	<0.000 5	5.4±4.7[a]	97.7±4.0[b]	<0.000 5
Early phase of post-larva IV	5.0±5.4[a]	67.7±4.7[b]	<0.000 5	5.8±2.6[a]	96.4±3.6[b]	<0.000 5
Post-larva IV	5.2±6.2[a]	6.1±2.2[a]	0.830	4.0±4.0[a]	31.9±7.6[a]	0.005
F value (ANOVA)	0.086	501.646		0.175	148.315	
P value (ANOVA)	0.918	<0.000 5		0.844	<0.000 5	

Note: For the Experimental, "stage" refer to individuals at their development when transferred from the control conditions (18 h L:6 h D and 27°C; inducing nauplius production) to the experimental conditions (6 h L:18 h D and 19°C; inducing cyst production). In each column, different superscript letters show a significant difference (ANOVA, Tukey, $P<0.05$). In each row, *P* value is the result of *t*-test between the pairwise control and treatment.

Table 5 Incidence (%) of females producing diapause cysts among all reproducing females of BRK53 *Artemia* clone in the supplementary experiment

Stage	1st brood			2nd brood		
	Control	Experimental	*P* (*t*-test)	Control	Experimental	*P* (*t*-test)
Post-larva III	100.0±0.0[a]	1.1±1.9[a]	<0.000 5	98.9±2.0[a]	0.0±0.0[a]	<0.000 5
Early phase of post-larva IV	98.9±1.9[a]	33.9±9.8[b]	0.006	100.0±0.0[a]	4.9±5.0[a]	<0.000 5
Post-larva IV	97.6±2.1[a]	89.9±3.2[c]	0.026	98.7±2.2[a]	1.8±3.0[a]	<0.000 5
F value (ANOVA)	1.634	165.875		0.503	1.581	
P value (ANOVA)	0.271	<0.000 5		0.628	0.281	

Note: For the Experimental, "stage" refer to individuals at their development when transferred from the control conditions (6 h L:18 h D and 19°C; inducing cyst production) to the experimental conditions (18 h L:6 h D and 27°C; inducing nauplius production). In each column, different superscript letters show a significant difference (ANOVA, Tukey, $P<0.05$). In each row, *P* value is the result of *t*-test between the pairwise control and treatment.

while higher oviparity (33.9%±9.8%) was found in "early phase of post-larva IV" stage; both values were significantly different ($P<0.01$) compared to those of the controls. Although observed oviparity between females shifted at post-larva IV stage (89.9%±3.2%) and the control (97.6%±2.1%) was relatively similar, the difference was significant ($P=0.03$). Oviparity among all the three treatments was significantly different ($P<0.01$). For the second broods, low oviparity (<5%) was observed; oviparity among the females shifted at the three different stages were non-significant.

3.2 Histology

Histological observations showed that the ovary expanded markedly from the stage post-larva III to the stage post-larva IV, with its area (single ovary) in the section increasing from less than 2% to more than 4% of the whole section area (Fig.2a, b, c). Ovaries at post-larva III stage had similar-sized cells (5–7 μm in diameter) (Fig.2a′), while ovaries of *Artemia* at the "early phase of post-larva IV" and post-larva IV stages contained cells of different diameter sizes, with the smaller ones being 5–10 μm, the median ones 12–15 μm (observed in "early phase of post-larva IV" and post-larva IV stages; Fig.2b′, c′), and the larger ones 25–40 μm (seen only at post-larva IV stage; Fig.2c′).

4 DISCUSSION

Abiotic factors exerting on some developmental stages have been proposed to affect changes in the reproductive modes of *Artemia*. Provasoli and Pintner (1980) showed that a change of photoperiod from shorter to longer daylight could shift the reproductive mode of *Artemia* to ovoviviparity, when the shift was applied 10–12 days before the production of first brood. Berthélémy-Okazaki and Hedgecock (1987) suggested that the critical time for the oviparity determination of *Artemia franciscana* Kellogg should happen in the late juvenile stage (6–7 mm long), just before the ovaries become visible microscopically. According to the studies on morphology and expression of p26 gene, Liang and MacRae (1999) considered that the reproductive mode of *A. franciscana* was determined at early oocyte stage

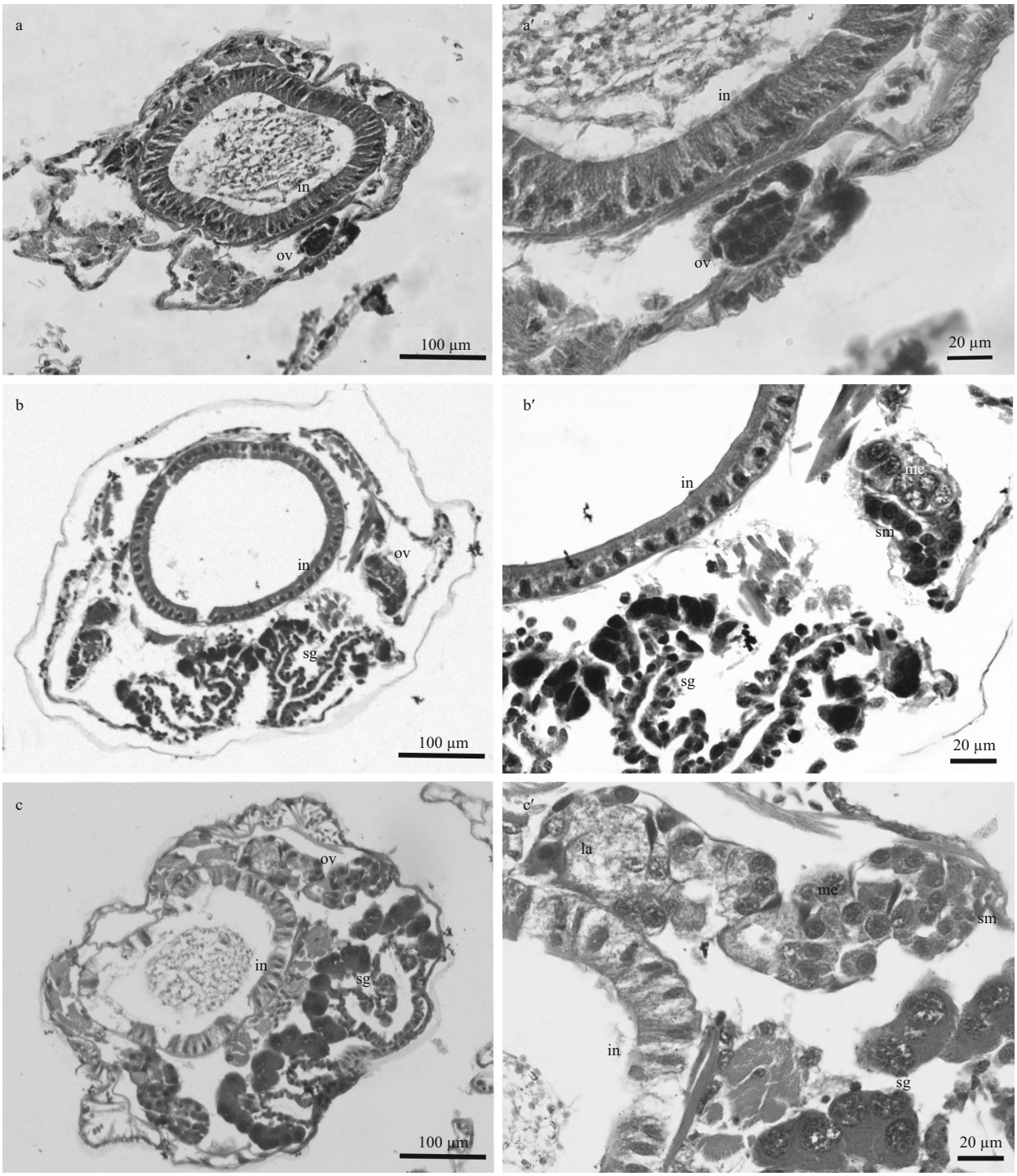

Fig.2 Transverse sections through genital segments of the parthenogenetic *Artemia* at different stages

a & a′: the post-larva III stage; b & b′: "early phase of post-larva IV" stage; c & c′: post-larva IV stage. in: intestine, la: larger cells of the ovary, me: median cells of the ovary, ov: ovary, sg: shell gland, sm: smaller cells of the ovary.

when shell glands were already differentiated. Dai et al. (2011) recently showed that the differential gene expression determined the reproductive mode of *Artemia* at oocyte stage.

In the main experiment of this study, the reproductive mode of the first brood could not be converted when *Artemia* were shifted at post-larva IV stage, but it was effectively converted when females were shifted at the post-larva III stage. This finding confirms the existence of a sensitive stage for oviparity/diapause induction in the life history of *Artemia*.

The results of supplementary experiment further indicated that the exact sensitive stage was placed at the so-called "early phase of post-larva IV" stage, which is characterized by the ovisac reaching posteriorly to the middle of the third abdominal segment, ventral blunt spines visible, and ovary with dimorphic cells. Before and at this stage, either reproductive modes (i.e. oviparity or ovoviviparity) could be switched to another by changing the culture conditions; whereas after this stage the reproductive mode of the first brood would be fixed. However, the token stimuli exerted on this critical stage could not alternate the reproductive mode of the second brood, suggesting that reproductive cells, rather than the ovary or whole animal, could be the final effectors responding to the token stimuli.

Oocytes of *Artemia* are known to mature in batches (Versichele and Sorgeloos, 1980). The larger cells observed in the ovary of *Artemia* at post-larva IV stage, as well as the early phase of post-larva IV might represent oocytes destined to become offspring of first brood. The reproductive mode of these larger oocytes may be first influenced by the token stimuli (at the early phase of post-larva IV), while the reproduction mode of the smaller ones could be determined in the later stages to become the offspring of later broods (e.g. second brood). Since the size and cytoplasmic volume of reproductive cells have begun to increase at the early phase of post-larva IV, the development stage of oocytes should be at the previtellogenesis phase (Criel, 1989). During this stage, numerous free ribosomes, mitochondria and single cisterns of the rough endoplasmic reticulum are in the cytoplasm (Poprawa, 2005), indicating the beginning of active gene expression. Dai et al. (2011) reported when oocytes enter the oviduct, many genes, including several known diapause-specific genes (e.g. *ArHsp22*), are differentially expressed between diapause- and nauplii-destined oocytes. The previtellogenesis is likely the stage that oocytes receive the token stimuli signals, which trigger the differential gene expression of the two destined oocytes.

Regarding the second brood, this study showed that its reproductive mode could be converted when *Artemia* were shifted at post-larva IV or earlier stages (Tables 2–5) but could not be converted when shifting performed on post-larva V or later stages (Tables 2, 3). When shifting at post-larva IV, the conversion seemed not complete, especially when shifted from ovoviviparity condition to oviparity condition (oviparity ratio: 3.4%±3.3% in control and 30.7%±9.8% in treatment of the main experiment; 4.0%±4.0% in control and 31.9%±7.6% in treatment of the supplementary experiment (Tables 2, 4). These results suggest that the maternal stage critical to the second brood could be post-larva IV.

5 CONCLUSION

The critical period inducing oviparity (embryo diapause) might be at the previtellogenesis stage of oocytes during the maternal "early phase of post-larva IV" for the first brood, and post-larva IV for the second brood. During the critical stage, different environmental conditions may induce different patterns of gene expression and thereby the oocytes to develop differently to become either swimming nauplii or diapaused embryos.

6 DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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