

Photoperiod-dependent reproductive plasticity of the brine shrimp *Artemia franciscana*

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Abstract

Photoperiod is a robust seasonal stimulus for phenotypic plasticity in the regulation of reproduction and development. *Artemia* brine shrimps have a unique reproductive mechanism to alter their reproductive mode between ovoviviparity, with the release of free-swimming nauplii, and oviparity, with the production of diapause cysts, in response to differences in photoperiod. However, the existence of relationships between reproductive modes and other reproduction-related traits (e.g., growing period, number of spawns, and litter size) remains elusive. In this study, we show that long-day conditions increase the proportion of free-swimming nauplii and the number of eggs during a lifetime compared to short-day conditions. Histological analysis revealed structural differences in eggs between ovoviviparity and oviparity, providing insight into the alteration of energy allocation: long-day conditions can produce a large number of eggs because a thin egg shell is formed, whereas short-day conditions decreased the number of eggs, because a thick egg shell is formed. The present study provides new insight into the energy trade-off to develop a phenotypically plastic trait in *Artemia*.

Key words: *Artemia*; reproductive modes; photoperiod; trade-off

Introduction

Artemia brine shrimps are extremophile branchiopod crustaceans and the sole macroplanktonic species adapted to hyper saline environments, where they frequently experience desiccation, oxygen deficiency, lack of nutrients, and a large temperature fluctuation. To survive under these harsh environmental stressors, *Artemia* species can alter their reproductive mode between ovoviviparity, with the release of free-swimming nauplii, and oviparity, with the

production of diapause cysts, depending on environmental cues. Diapause cysts exhibit developmental arrest, greatly reduced metabolism, and resistance to multiple physiological stressors (Clegg et al. 1999; Drinkwater and Crowe 1987; Liang and MacRae 1999). When environmental conditions recover, swimming nauplii hatch to build up a new population. Such reproductive plasticity enables survival in drastically changing environments. Previous studies demonstrated that several

environmental physicochemical factors such as photoperiod, temperature, and salinity are stimuli for switching ovoviviparity and oviparity in *Artemia*, especially photoperiod, which is the best stimulus (Dai et al. 2011b; Nambu et al. 2004; Wang et al. 2017).

Artemia species produce free-swimming nauplii by ovoviviparity in favorable environments such as a long-day photoperiod and high temperature but tend to produce dormant cysts in sub-optimal conditions such as short-day photoperiod and low temperature. A free-swimming larva undergoes molting daily to become a sexually matured adult that then starts to reproduce, whereas cysts enter diapause, suppressing their own metabolism (Robbins et al. 2010). Although physicochemical cues that switch reproductive modes have been extensively studied, the relationships between reproductive mode and other reproduction-related traits (e.g., growing period, number of spawns, and litter size) remain elusive.

Here, we conducted a common garden experiment with alterations to photoperiod to comprehensively describe the reproductive plasticity of *Artemia franciscana*, demonstrating that there is a trade-off between reproductive mode and some reproduction-related traits.

Materials and Methods

Artemia culture and experimental design

Commercial dried cysts of the brine shrimp *Artemia franciscana* (Ocean Star International,

Inc., Utah, USA) were kindly provided by Dr. Takashi Ichikawa (Tokyo University of Agriculture, Hokkaido, Japan). Dried cysts were maintained at 28°C in artificial sea water (MARINE ART SF-1, Osaka Yakken Co. Ltd., Osaka, Japan), and hatched nauplii were fed every day with powdered crayfish (Kyorin Co. Ltd., Hyogo, Japan). The culture did not require aeration. We investigated the impact of photoperiod cues on reproductive traits of *A. franciscana*. Details of the common garden experiment (Fig. 1) are described next.

Step 1: Cysts were hatched out at 28°C in artificial sea water with a 12:12 h light:dark regime. Forty nauplii within 24 h after hatching were randomly selected to establish experimental generations and were transferred to 500 ml glass beakers (common garden). A total of eight beakers were prepared. Nauplii were fed daily for 48 h.

Step 2: Four beakers were transferred to incubators conditioned with either a long-day (14:10 h light:dark regime) or short-day (10:14 h light:dark regime) photoperiod at 28°C. Rearing artificial sea water was refreshed every two days and nauplii were fed daily.

Step 3: Mating behavior, which is referred to as “riding” (Fig. 1) because male copulation resembles a riding motion on the female dorsal part, is sustained for about 24 h or longer. When riding pairs were observed, each pair with sustained riding was transferred to a 100 ml falcon tube. A total of 20 pairs were prepared for

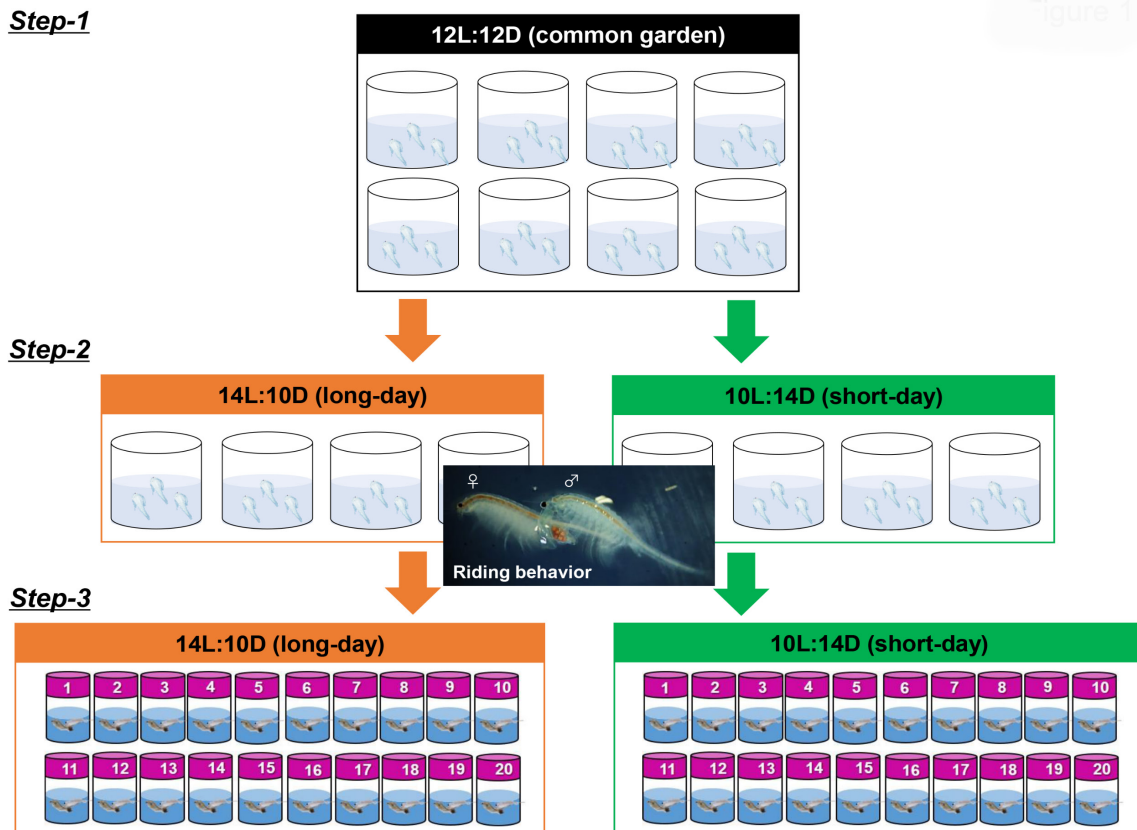


Fig. 1. Experimental scheme of this study.

each photoperiod. All pairs were maintained until one of the members died, after which several reproductive traits were recorded: reproductive habit (oviparity or ovoviviparity), period from nauplius to riding, period from riding to first reproduction, number of lifelong broods, and lifelong number of eggs and nauplii (litter size). The mode of reproduction by each pair was verified daily. A group of dormant cysts spawned on a single day was counted as one clutch of cysts and a group of free-swimming nauplii released on a single day was counted as one clutch of nauplii.

Data analysis

Statistical analyses were performed using the R statistical software (R3.5.3; R Core Team 2019). All two-group comparisons were conducted by either the Student's or Welch's t-test based on the F test. Relationships between photoperiods and reproductive modes were investigated by Fisher's exact probability test. Figures were drawn in the ggplot2 package (Wickham 2016).

Histology and imaging

For tissue sectioning, sexually-matured females ($n = 3$, respectively) reared under long-day or short-day were fixed in Bouin's fluid

overnight. Samples were dehydrated in ethanol and xylene, then embedded in paraffin. Serial sections of 8 μm thickness were subjected to haematoxylin and eosin (HE) staining. Sections were studied under a microscope (BX43, Olympus, Tokyo, Japan) and the images were processed with CellSens Standard (Olympus). For scanning electron microscope (SEM) imaging, dried cysts were observed using a JCM-5000 NeoScope (JEOL Ltd., Tokyo, Japan).

Results

Plasticity of reproductive traits in response to photoperiod cues

We finally used 20 and 15 pairs in the long-day and short-day conditions for the following anal-

yses, because the pairs that died before the first spawning were removed. In this study, female produced either ovoviviparously (producing free-swimming nauplii) or oviparously (producing diapause cysts), meaning that no mixed clutches occurred. All 20 females reared under a long-day photoperiod produced a total of 158 broods (ovoviviparity: 53, oviparity: 105), whereas all 15 females reared under a short-day photoperiod produced a total of 104 broods (ovoviviparity: 5, oviparity: 99), indicating that the proportion of ovoviviparity significantly in the long-day photoperiod was significantly larger than that in the short-day photoperiods (Fig. 2A). No significant difference between the long-day and short-day photoperiods was found

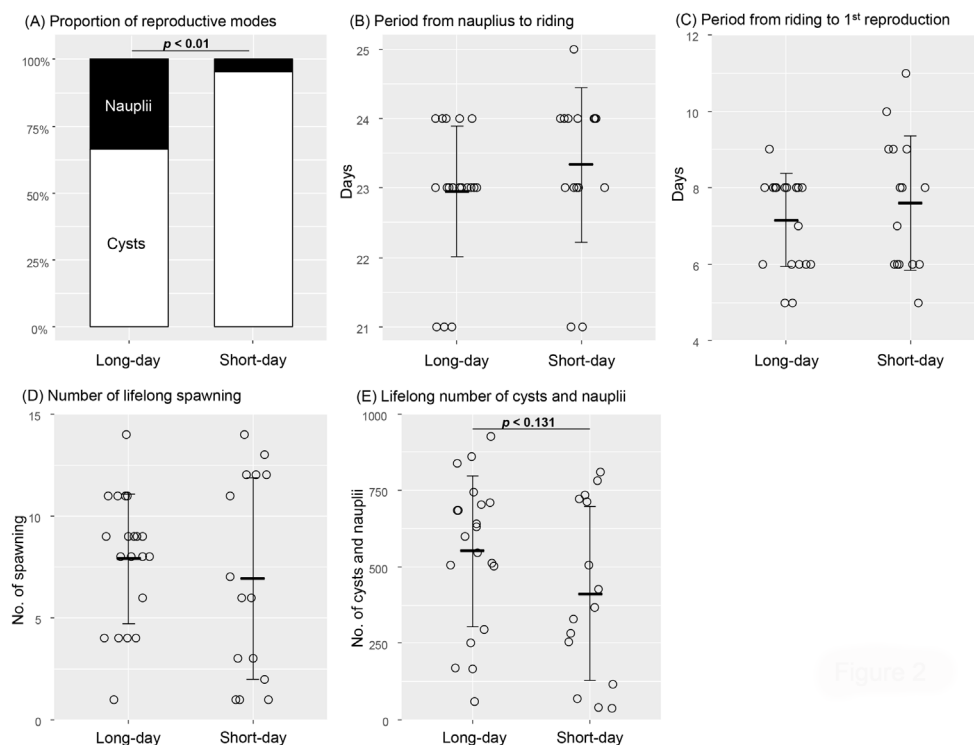


Fig. 2. Comparisons of reproductive traits; proportion of reproductive modes producing nauplii (black) or cysts (white) (Fisher's exact probability test; A), period from nauplius to riding (B), period from riding to first reproduction (C), number of lifelong spawns (D), and lifelong number of cysts and nauplii (Welch's t-test; E), reared under long-day or short-day conditions.

in the other reproductive traits such as growing period from nauplius to riding behavior (Fig. 2B), period from riding to the first reproduction (Fig. 2C), and number of spawns during a lifetime (Fig. 2D). The total lifelong number of cysts and nauplii per individual female were significantly higher in the long-day than in the short-day photoperiod (Fig. 2E).

Relationships between reproductive modes and litter sizes

To understand the relationship between reproductive mode and litter sizes, we focused on the first brood data. Although there were no clear

differences between litter size and both rearing photoperiods, litter size of nauplii was significantly larger than that of cysts (Fig. 3A). Indeed, when comparing litter sizes between both reproduction modes in the long-day condition, litter size of ovoviviparity was significantly larger than that of oviparity (Fig. 3A').

The eggs in the egg pouch (ovisac) were analyzed histologically (Fig. 3B). Females that produced oviparously formed eggs that were enveloped with a thick egg shell to protect them from drought and freezing (Figs. 3D and 3D'). As seen in Fig. 3C, diapause cysts were round

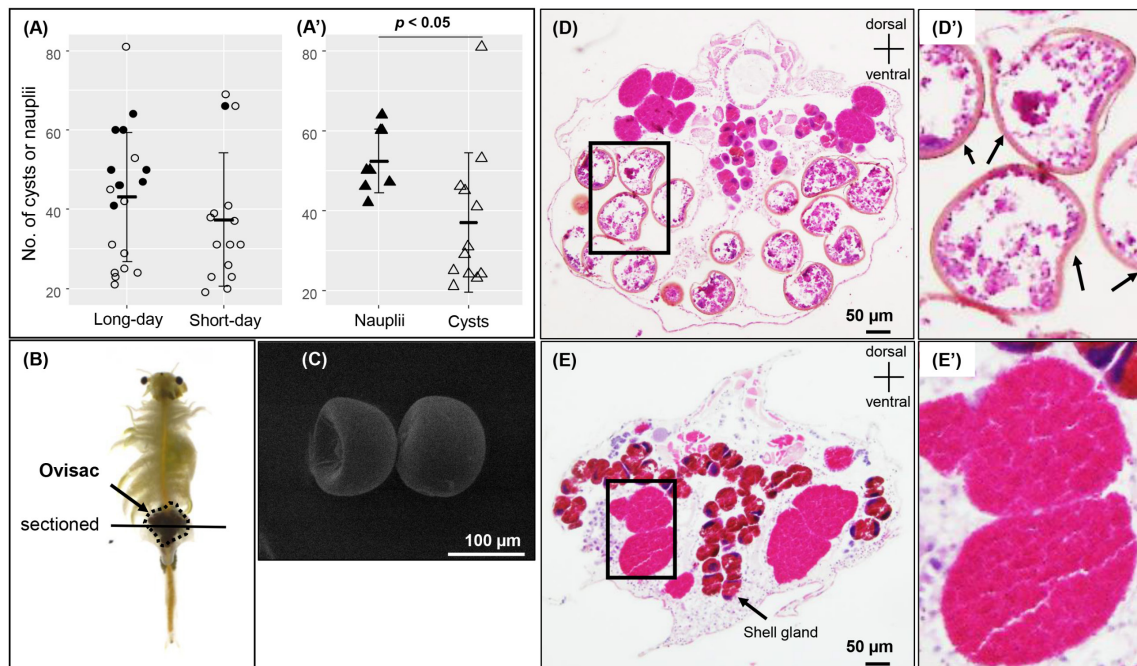


Fig. 3. Relationship between reproductive modes and number of eggs (including cysts and nauplii). (A) Number of cysts or nauplii on the first clutch reared under long-day or short-day conditions. Black and white dots indicate the nauplii and cyst production, respectively. (A') Comparison of egg number when nauplii or cysts produced in the first clutch were reared under a long-day photoperiod (Welch's t-test). (B) Schematic image that was histologically observed. (C) SEM image of cysts. (D) HE-stained image of female with oviparity. (D') Magnified view of the square enclosed in (D). Arrows indicate egg shells. (E) HE-stained image of female with ovoviviparity. (E') Magnified view of the square enclosed in (E).

and had a smooth surface, consistent with a previous report describing the egg shell morphology of Anostraca crustaceans (Fanid et al. 2007). In contrast, females that produced eggs ovoviviparously showed that the entire embryos were full of eosin-positive components (Fig. 3E and 3E'). The shell of a diapause cyst is known to consist of a non-cellular chorion layer and a cellular embryonic cuticle layer: the former is secreted by the shell gland (Fig. 3E) while the latter is formed by blastoderm cells (Morris and Afzelius 1967). The shell glands are located in the ovisac where it was easy to observe when the mode of oviparity took place (Fig. 3E). In contrast, shell glands shrank during ovoviviparous development (Fig. 3D).

Discussion

Trade-off among plastic reproductive traits

Several previous studies focused on the physiological impacts of salinity, temperature, and photoperiod on the alteration of reproductive habits and diapause induction. Since salinity is one of the most influential environmental cues affecting the life of hypersaline animals such as *Artemia*, the effects of salinity on the mode of reproduction were studied more frequently than the other factors. However, low salinity condition induced resting egg production, while high salinity condition had no effects (Wang et al. 2017). Likewise, the effect of temperature as an environmental cue did not have a significant effect on the mode of reproduction of *A.*

franciscana (Nambu et al. 2004), nor was there a dominant effect on the production of nauplii and dormant cysts under higher and lower temperatures (Browne 1980; Wang et al. 2017).

Previous studies demonstrated that photoperiod-based cues are a robust factor to induce ovoviviparity and oviparity in *A. franciscana* (Nambu et al. 2004; Wang et al. 2017). However, in the former attempt (Nambu et al. 2004), dry cysts hatched into larvae under different photoperiod and temperature conditions, and they were reared until they died, whereas in the present study, nauplii just after hatching were reared within 48 hours under an even photoperiod (12:12 h light:dark cycle) to precisely compare the growing period until first mating behavior started under the long-day and short-day photoperiods. Our data showed that ovoviviparity and oviparity were induced by long-day and short-day conditions, respectively, consistent with previous findings (Nambu et al. 2004; Wang et al. 2017). Additionally, we attempted to describe the histological changes in the oviduct between ovoviviparity and oviparity. Some previous studies demonstrated that the color of the oviduct can be used to distinguish the reproductive mode: in oviparous and ovoviviparous reproduction, they are darker and whiter, respectively because the color of the enlarged shell gland is dark brown (Dai et al. 2011b; MacRae 2003). However, histological information has been quite limited. We found that the shell glands had shrunk and that a thick

layer had formed around cysts just before the release of diapause cysts. Recent studies found genes specifically expressed in the shell gland (SGEG1 and SGEG2), coding for peptides that regulate the formation of the cyst layer. Knockdown of both SGEGs caused the cyst shell to become translucent and the chorion layer of the shell to become less compact, resulting in a dramatic decrease in the tolerance against environmental stressors such as extreme salinity, UV radiation, and temperature fluctuations (Dai et al. 2011a; Liu et al. 2009). Furthermore, the current study demonstrated that there is an apparent energy trade-off between litter size and reproductive mode. We hypothesize that the production of dormant cysts requires more energy than the production of nauplii because cysts were enveloped by a thick egg shell.

Critical period to determine reproductive mode

Environmental cues triggering phenotypic plasticity are perceived during a fixed and specific sensitive period (West-Eberhard 2003). In terms of perception of photoperiodic cues in crustaceans, a recent study using a branchiopod water flea *Daphnia* demonstrated that sexual fate is predestined in the oocyte stage (Toyota et al. 2017). Two studies of parthenogenetic *Artemia* suggested that the critical period for determination of reproductive mode (ovoviviparity or oviparity) is at the previtellogenesis stage of oocytes (Dai et al.

2011b; Zhichao et al. 2019). A previous study also demonstrated that many genes, including known diapause-specific genes such as *ArHep22*, are differentially expressed in either diapause- or nauplii-destined oocytes (Dai et al. 2011b). Taken together, the previtellogenesis stage is thought to be that the time when maturing oocytes receive environmental signals, which induce transcriptomic alterations to switch reproductive mode plastically.

Conclusion

Here, we described the reproductive traits of *Artemia* with photoperiod-dependent reproductive plasticity. The long-day photoperiod increased the proportion of free-swimming nauplii and the number of eggs (including dormant cysts and nauplii) during a lifetime more than the short-day photoperiod. Histological analysis revealed structural differences of eggs between oviparity and ovoviviparity, providing insight into energy allocation: the long-day photoperiod produced a large number of eggs because a thin egg shell formed, whereas the short-day photoperiod decreased the number of eggs since eggs were enveloped by a thick egg shell. Our findings provide insight into an energy trade-off to develop a phenotypically plastic trait. A substantial next step using *Artemia* need to investigate the molecular mechanisms underlying reproductive plasticity.

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Author's contributions

All authors considered the experimental design. SM and KT conducted experiments and performed statistical analyses. KT wrote the first draft of the manuscript. All authors conceived the final manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and materials

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

Consent for publication

Not applicable.

Ethics approval consent to participate

The present study complies with current Japanese laws.

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