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An Efficient Method for Hatching Diapausing Embryos of *Daphnia pulex* Species Complex (Crustacea, Anomopoda)

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Abstract

A general method for efficiently hatching sexually produced diapausing embryos of the microcrustacean *Daphnia* is valuable for establishing *Daphnia* as a genetic model system. In this study we examine the effect of ultraviolet light and different amounts of storage time in darkness on the hatching efficiency in two species of the *D. pulex* species complex, *D. pulex* and *D. pulicaria*. We identified a set of lighting conditions that can trigger 80% to ~100% hatching rate for embryos produced through selfing, outcrossing, and obligate parthenogenesis. Furthermore, we found that a storage time of at least two weeks in the dark prior to exposing embryos to ultraviolet light is critical for achieving high hatching rate. The identification of these key factors for hatching diapausing embryos can greatly facilitate future *Daphnia* research involving complex breeding designs as well as investigating the genetic switch that activates the hatching of diapausing embryos.

Keywords

ephippia; zooplankton; diapausing embryos; light activation

Introduction

The feasibility of producing a large number of hybrid offspring from crossing experiments is vital for performing forward genetics research in a model organism. This is true for all classic eukaryotic model organisms - such as the fruit fly *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans* - where forward genetics has contributed to the foundation of modern genetics and continues to play a pivotal role in making new discoveries. As an emerging model organism, prior work on the freshwater microcrustacean *Daphnia pulex* (Crustacea, Anomopoda) has shown that the hatching of sexually produced offspring of this species is often time consuming and with low success rate (Table 1 and references therein). This creates an undesirable obstacle for using *D. pulex* as a model system in genomic research.

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Data availability

All data of this work have been presented in this manuscript.

Conflict of interest

The authors declare no conflict of interest.

The major challenge with hatching sexually produced embryos in *D. pulex* is that these embryos are dormant and hatch only under a strict set of suitable conditions, e.g., light and temperature (Schwartz & Hebert, 1987). *D. pulex* typically engages in a cyclically parthenogenetic reproductive cycle (Ebert, 2005). Under favorable environmental conditions (e.g., abundant food, low population density, suitable temperature), female *Daphnia* produces directly developing diploid embryos through parthenogenesis, generating genetically identical daughters (excluding newly arising mutations). In favorable environments, males rarely appear in the population, as sex is environmentally determined in *Daphnia* (Kato et al., 2011). On the other hand, stressful environmental conditions (e.g., low food, high population density, adverse photoperiod) can trigger the emergence of males in the population and stimulate females to produce haploid eggs through meiosis that need fertilization by sperm to form fertilized embryos. The resulting embryos are arrested in development (i.e., diapausing) and are encapsulated in a protective case called ephippium. These diapausing embryos start to hatch once environmental conditions become suitable (Stross, 1996).

Several previous studies (Davison, 1969; Paes et al., 2016; Pancella & Stross, 1963; Radzikowski et al., 2018; Schwartz & Hebert, 1987; Vandekerkhove et al., 2005) investigated the environmental conditions conducive for diapausing embryo hatching in *Daphnia*, providing insight into how to hatch these embryos in a laboratory setting (Table 1). The set of suitable conditions, which largely simulate the seasonal environmental changes in spring, is reflected in the common practice seen in recent literature (e.g., Heier & Dudycha, 2009; Innes & Hebert, 1988). A period of storing diapausing embryos in darkness and at low temperature is necessary for successful hatching prior to exposing the embryos to ambient temperature and strong white light (Vandekerkhove et al., 2005).

Previous research examined also the effect of temperature for hatching success. For example, Vandekerkhove et al. (2005) suggested that an optimal temperature for hatching is 15 °C for a wide range of cladoceran species. Moreover, Radzikowski et al. (2018) showed that diapausing embryos of *Daphnia* species inhabiting temporary waters can hatch over a broader range of temperatures compared to species living in lakes where hatching success is inversely related to temperature.

Some other techniques have also been examined for facilitating hatching. For example, sodium hypochlorite solution (i.e., bleach solution) can be used to treat ephippia containing diapausing embryos to increase hatching (Pancella & Stross, 1963; Retnaningdyah & Ebert, 2016). In fact, bleach treatment is not a stimulus to hatching. The reason that bleach treatment increases hatching success is because it softens the ephippial case and somewhat decapsulates the embryos. Decapsulation of embryos has been shown to significantly increase hatching rate compared to capsulated embryos (Davison, 1969). Furthermore, bleach treatment shows no significant difference in hatching rate compared to manual decapsulation (Paes et al., 2016), with a hatching rate around 50%.

Despite the different experimental strategies used in previous research, most of these studies used white light as the light source for triggering embryo hatching. In contrast, Davison (1969) found that the most effective light wavelength between 350 nm and 500 nm for

achieving 100% *Daphnia* embryo development is 410 nm. However, the findings of Davison (1969) were derived from the observations of a single *D. pulex* strain. Therefore, it remains to be tested whether light source of wavelength 410 nm is effective for hatching diapausing embryos in other *D. pulex* isolates and closely related species. In this study, we examine the use of ultraviolet light (410 nm wavelength) for activating the development of diapausing embryos of *D. pulex* and *D. pulicaria*, two species of the *D. pulex* species complex. Specifically, we aim to develop a procedure for effectively hatching diapausing embryos for a set of geographically widespread *Daphnia* isolates.

Materials and Methods

Daphnia isolates

We chose five *Daphnia* isolates from two species, three of *D. pulex* and two of *D. pulicaria* (Table 2). Isolates of *D. pulex* include both cyclically parthenogenetic and obligately parthenogenetic clones. These isolates were sampled from varying geographic locations with a broad longitudinal and latitudinal range throughout the United States, with the northernmost location in Washington and the southernmost location in Texas.

Diapausing embryo production

We collected resting embryos produced by selfing or obligate parthenogenesis in three isolates, i.e., DBIV-2, AroMoose, and LakeWash. Each of the *Daphnia* isolates was separately reared in artificial lake water, i.e., lake combo (Kilham et al., 1998). Lake combo was stored in a 20 L polypropylene carboy and aerated for a minimum of 24 hours before use. *Daphnia* cultures were grown in 32 oz. glass mason jars filled with 500–600 mL of lake combo. The cultures were kept at 18 °C under a 18:6 (Light:Dark) light regime and fed twice a week with the green algae *Scenedesmus obliquus*. Once the number of individuals in each culture reached a high level that stressed the animals, males emerged in the population and females switched to sexual reproduction. Because the males and females of the same isolate are genetically identical, the production of diapausing embryos occur through the mating between siblings, which is equivalent to selfing. For the obligately parthenogenetic isolate DBIV-2, the resting embryos are produced through parthenogenesis.

Furthermore, we performed a crossing experiment between *D. pulex* and *D. pulicaria* using males of RLSD26 (*D. pulicaria*) and females of Tex21 (*D. pulex*). For the crossing experiment, 20 males and 20 females were placed in a 250-ml beaker under 12:12 (Light:Dark) photoperiod. To trigger the females to enter sexual reproduction phase, minimal amount of algae was fed. Moreover, asexually produced babies were removed from the beaker within 1–2 days after birth to avoid potential selfing between males and females of the same genotype.

For both types of experiments, ephippia were removed from the jars and rinsed with autoclaved combo before transferring to a beaker with combo. The beakers were wrapped with aluminum foil to simulate dark conditions and were stored in a dark incubator at 18 °C and/or 4 °C for at least 2 weeks.

Embryo hatching experiment

After being stored in the dark for more than two weeks, the decapsulated embryos were then exposed to two photoperiods at 18°C - 18:6 and 12:12 (Light:Dark), respectively. Under each photoperiod, two petri dishes of 20–30 embryos of each isolate was set up. For each photoperiod, four 9 watt UV LED lights were placed adjacent to one another above the petri dishes containing dissected embryos at a distance of ~8 cm (Figure 1). Petri dishes were lidded to avoid introducing contaminants into the petri dishes. The light intensity at this configuration was ~150 foot candle (fc) or 150 lumens per square feet (lm/ft²). We chose this light intensity based on our previous sets of optimization experiments (data not shown) to find a light intensity that can trigger hatching of resting embryos in multiple *Daphnia* clones. It should be noted that the UV light used here is low power and does not cause health problems when viewed directly. These embryos were exposed to two rounds of five days of lighting conditions followed by two days in complete darkness for a total of 2 weeks. The reason for a two-day dark period is to avoid excessive exposure of embryos to light, which could cause degradation of embryos. Embryos were examined every two days for signs of development. Experiments were terminated at the end of two weeks and a cumulative number of developing embryos was calculated.

Furthermore, we also performed hatching experiments of DBIV-2 and AroMoose with white light at 150 foot candle under 18:6 photoperiod to compare the efficiency of white light and UV light. The embryos used for white light hatching experiment were stored in the dark for 3 and 2 weeks for DBIV-2 and AroMoose, respectively.

Results

Development of Embryos

Development was determined in diapausing embryos via several observable cues (Figure 2). First, the initial homogenous dark or greenish color begins to fade from the periphery of the embryo (Figure 2B). The next stage consists of aggregation of yolk towards one side of the embryo. A midline in the yolk can also be observed, creating a bilaterally symmetrical embryo with appendages beginning to form. From then, the embryo continues development until it fully hatches out of its corium and vitelline membrane as a free-swimming neonate (Figure 2D and 2E).

Hatching efficiency

Diapausing embryos of all experiments exposed to 410 nm light reached a hatching efficiency of 80% to ~100% in the span of 2 weeks, demonstrating the strong effect of UV light on stimulating hatching.

For one isolate of *D. pulex* (DBIV-2) and one isolate of *D. pulicaria* (AroMoose), we hatched diapausing embryos that were stored in the dark for 2 and 3 weeks to examine the effect of dark storage on hatching process. For DBIV-2, 3-week dark storage led to the hatching of 87.5% (12:12 photoperiod) and 90% (18:6 photoperiod) of embryos within 3 days after exposure to UV light, with no other embryos hatching in the rest of the 2 weeks (Figure 3). White light had nearly 100% efficiency in triggering the hatching of diapausing

embryos after 3-week dark storage under 18:6 photoperiod (Figure 3). However, this efficiency is not significantly different from that of UV light (t-test $p=0.25$). On the other hand, the diapausing embryos of DBIV-2 with 2-week dark storage started hatching only after 1 week had passed upon exposure to UV light, with the final hatching reaching 80% and 95% for 18:6 and 12:12 photoperiod, respectively (Figure 3).

For AroMoose, opposite to what was observed in DBIV-2, we found that 3-week dark storage caused a delay in the hatching of embryos, with the earliest hatching recorded on day 7 after light exposure (Figure 4). The final hatching for both photoperiods converged at ~80%. In contrast, embryos with 2-week dark storage started hatching in day 3 and hatching rate plateaued around day 8, with a final hatching rate of ~90% and ~85% for 18:6 and 12:12 photoperiod (Figure 4). White light had lower hatching efficiency than UV light under 18:6 photoperiod.

Similar to AroMoose, diapausing embryos of *D. pulicaria* isolate LakeWash with 2-week dark storage started hatching within 3 days after exposure to light, reaching ~100% and 80% hatching rate for 18:6 and 12:12 photoperiod, respectively (Figure 5). Unfortunately, we were not able to collect enough data for 2-week dark storage of LakeWash. Furthermore, the hatching efficiency of RLSD26 and Tex21 crosses resembled the pattern observed in AroMoose and LakeWash, embryos with 2-week dark storage started hatching within 3 days after exposure to light (Figure 6), with a final hatching rate of ~90%

Considering the data above, it is likely that the dark storage period has isolate-specific effect on hatching. However, we caution that a dark storage period less than 2 weeks caused significant reduction of hatching rate in all the above-mentioned isolates in our experiments, with maximal hatching rate reaching ~20% (data not shown). This observation of reduced hatching rate associated with short dark storage time is consistent with the findings of Davison (1969).

Effect of photoperiod and temperature for dark storage

We found that the two photoperiods used in this study (18:6 versus 12:12) have isolate-specific effects on the hatching rate. In the isolates AroMoose and Lakewash (Figures 4 and 5), hatching rate under UV light with 18:6 was higher than that of 12:12, although no significant statistical difference was detected (t-test $p=0.35$), whereas for DBIV-2, 12:12 photoperiod had no significant difference in hatching rate compared to 18:6 (t-test $p=0.28$).

We also observed no difference in hatching efficiency between storing embryos in the dark at 4 °C and at 18 °C in our experiments. More data are necessary for a conclusion on the effects of varying temperatures during dark storage on hatching efficiency.

Discussion

In this work we examined the effect of ultraviolet light and dark storage time on activating the development of diapausing embryos of two species in the *D. pulex* species complex. We identified the parameters of lighting and dark storage time that can trigger the efficient hatching of diapausing embryos in two species of the *D. pulex* species complex.

Consistent with Davison (1969), our high rates of hatching demonstrate the efficacy of UV light on activating *Daphnia* diapausing embryos. The hatching rate in our experiment is much higher than the rates reported in previous work (e.g., Paes et al., 2016) where white light is commonly used. In our own experiments, we also found that UV light performed better than white light in stimulating the hatching of diapausing embryos of AroMoose and had similar performance as white light in DBIV-2. We emphasize that this work is not designed to demonstrate that white light is less effective for hatching *Daphnia* resting embryos than UV light. Even though at low intensity white light may not be as effective for hatching, it would be easy to get white light at much higher intensity to increase hatching rate. The main point of establishing that UV light is effective for hatching diapausing embryos is to set the stage for future work to investigate how UV light effectively triggers the development of diapausing embryos.

Furthermore, we also identified the hatching of diapausing embryos of some *Daphnia* isolates are highly responsive to UV light. For example The *D. pulicaria* isolate LakeWash can consistently reach 80% to nearly 100% hatching rate within 4–7 days under UV light after a storage of 2 weeks in the dark. We suggest that these isolates are ideal for genetic experiments involving selfing. For example, a forward genetic screening where a mutagen, e.g., Ethyl methanesulfonate (EMS), is used to induce mutations requires a round of selfing using the treated animals in order to examine the phenotypic effects of the induced recessive mutations (Jorgensen & Mango, 2002). UV-responsive *Daphnia* isolates are ideal for this kind of experiment to identify the mutations and genes underlying the phenotypes of interests.

We also find that the amount of storage time in the dark plays an important role in the hatching success of a specific *Daphnia* isolate. Davison (1969) identified that a storage period in the dark for more than 9 days is necessary for 100% hatching rate in the single isolate that was examined, whereas a shorter storage period can compromise the hatching rate. We observed the same pattern with the isolates in our experiments, with less than 2-week dark storage severely reducing hatching rate. We further noted that different amounts of dark storage time can have isolate-specific effects on hatching. For example, 3-week storage time can cause a delay in hatching in AroMoose, whereas 2-week storage can trigger hatching within 3 days upon exposure to light (Figure 4). Some other isolates (e.g., DBIV-2) showed exactly the opposite pattern (Figure 3). This observation implies that hatching conditions need to be optimized for the specific isolates of interests, which can be easily done within a few weeks. For example, we suggest that a titration experiment with different amount of storage time in the dark can be performed to find the optimal storage time for a specific *Daphnia* isolate.

We also suggest that the decapsulation of the ephippial case is a large advantage when trying to activate diapausing embryos. Davison (1969) found leaving the ephippial case intact could mechanically hinder the development of diapausing embryos. Intact ephippial case may lengthen the time needed for hatching and dramatically decrease hatching success.

The observation that UV light and a period of storage in the dark play a role in hatching success raises some interesting biological questions. For example, given that the diapausing

embryos are in an arrested stage of a cell cycle (Stross, 1996), what kind of genetic changes occur in the diapausing embryos when exposed to UV light? Also, how does the dark storage facilitate the hatching of diapausing embryos? Unfortunately, little research has been done to examine the genetic mechanisms involved in the activation of resting embryos. Here we propose some simple hypothesis that can be tested in future work. We suggest that UV light may be key for breaking down the proteins blocking the cell cycle or critical for activating some light-dependent proteins that further interact with cell cycle regulators. Although there is no data to support this hypothesis, we could take advantage of the cutting-edge genomic and proteomic tools (e.g., RNA-seq) and examine the transcriptomic and proteomic changes taking place in early hatching embryos to understand the genetic triggers involved in activating the development of diapausing embryos.

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Figure 1.
Experimental design for exposing decapsulated embryos to 410 nm UV light.

Figure 2.

Progression of development in a diapausing embryo of *Daphnia pulex*. (A) An ephippium containing diapausing embryos. (B) An undeveloped diapausing embryo. (C) A developing embryo. (D) A developing neonate with split chorion, while vitelline membrane is still intact. (E) A fully developed neonate.

Figure 3. Hatching efficiency of DBIV-2 (an obligate parthenogen) exposed to 18:6 and 12:12 photoperiods. (A) Hatching efficiency after 2 weeks of dark storage under UV light. (B) Hatching efficiency after 3 weeks of dark storage under UV light and white light. Dotted line (control) represents white light. Error bars represent standard deviation.

Figure 4.

Hatching efficiency of Aromoose (a cyclical parthenogen) exposed to 18:6 and 12:12 photoperiods (A) Hatching efficiency under UV light and white light after 2 weeks of dark storage. Dotted line (control) represents white light. (B) Hatching efficiency under UV light after 3 weeks of dark storage. Error bars represent standard deviation.

Figure 5. Hatching efficiency of LakeWash (a cyclical parthenogen) exposed to 18:6 and 12:12 photoperiods over a two-week span following 2 weeks of dark storage. Error bars represent standard deviation.

Figure 6.

Hatching efficiency of crosses of RLSD26 and Tex21 exposed to UV light at 18:6 photoperiod over a two-week span following 2 weeks of dark storage. Error bars represent standard deviation.

Table 1.

Summary of previous studies on hatching diapausing embryos of *Daphnia*.

Study	Method	Hatching Rates
Pancella & Stross (1963)	Sodium hypochlorite soaking of ephippia prior to exposure to white light	40–50%
Davison (1969)	Manual decapsulation of ephippia prior to exposure to white light and 410 nm (UV) light	100%
Schwartz & Herbert (1987)	Various thermal conditions and manual decapsulation of ephippia prior to exposure to white light	20–100%
Radzikowski et al., (2018)	Various thermal conditions prior to exposure to white light	30–56%
Paes, Rietzler, & Maia-Barbosa (2016)	Sodium hypochlorite soaking and manual decapsulation of ephippia prior to exposure to white light	11–54%

Table 2.

List of *Daphnia* isolates used in this study. OP: obligate parthenogenesis; CP: cyclical parthenogenesis.

Isolates	Species	Reproductive mode	Location
DBIV-2	<i>D. pulex</i>	OP	32°47, –97°7, Village Creek Drying Beds, Texas
Tex21	<i>D. pulex</i>	CP	42°12, –83°12, Textile Road, Michigan
RLSD26	<i>D. pulicaria</i>	CP	44°57, –96°49, Round Lake, South Dakota
Aromoose	<i>D. pulicaria</i>	CP	44°50, –69°16, Sebasticook Lake, Maine
LakeWash	<i>D. pulicaria</i>	CP	47°37, –122°14, Lake Washington, Washington