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Maternal Effects Via Resting eggs in Predator Defense of the Rotifer *Brachionus calyciflorus*

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Maternal effects play important evolutionary and ecological roles. Amictic female mothers of monogonont rotifer *Brachionus calyciflorus* can transmit predatory information of *Asplanchna brightwellii* in their environment to their offspring by changing the offspring’s defensive morphology to increase their fitness. However, it remains unclear whether such maternal effects also exist during sexual reproduction of a mictic mother. This study explored the maternal effect in mictic mothers using the *B. calyciflorus* and *A. brightwellii* as a prey–predator model. We collected resting eggs from two groups of mictic mothers that previously experienced environments with (P environment) or without (NP environment) *Asplanchna* kairomones. Stem females from the resting eggs of each maternal group were also hatched and reared in P and NP environments. The population growth rate of offspring who experienced the same environment as their mictic mothers was significantly higher than those that experienced a different environment. When exposed to a gradient of predator kairomone levels, the posterolateral spine of the offspring elongated with increasing kairomone concentration. Offspring from the P mictic mother showed significantly shorter posterolateral spines than those from the NP mictic mother at each predator kairomone level. Offspring originating from NP mictic mothers clearly elongated their posterolateral spines at low concentrations of predator kairomones, while those from P mothers elongated their posterolateral spines only at the highest levels of predator kairomone. Our findings highlight the existence of anticipatory maternal effects during the sexual process via resting eggs of *B. calyciflorus* in response to predator kairomone.

Key words: *Brachionus calyciflorus*, maternal effect, predator kairomone, inducible defense, resting egg

INTRODUCTION

Maternal effects usually denote the effects of maternal phenotype or environment on offspring phenotypes or life-history traits with non-genetic influences (Martin and Pfennig, 2010). Mothers can transmit environmental information to the next generation to let them prepare for the expected environment. For example, in many univoltine insects, mothers that experienced short photoperiods (a cue for decreasing food resources) tend to produce more diapausing offspring (Mousseau and Fox, 1998). Such maternal effects are adaptive, as they can increase the survival or reproductive success of offspring (LaMontagne and McCauley, 2001; Giesing et al., 2011; Gorbi, 2011). Monogonont rotifer species, which exhibit cyclical parthenogenesis in their life cycle, show phenotypic plasticity (such as morphological parameters and life-history traits) through well-documented maternal effects (Koiso et al., 2009; Sun and Niu, 2012a, b; Gilbert and Mcpeek, 2013). Diploid amictic females typically reproduce diploid daughters parthenogenetically so as to maintain rapid population growth. When mixis stimuli occur in the environment, mictic females appear and produce haploid male offspring if unfertilized (Gilbert, 1977). When mictic females are fertilized by a male during early life-history, they produce resting eggs (Gilbert, 1974). The resting eggs may enter diapause for short or long periods until hatching (Ricci, 2001). Hatched amictic females from resting eggs are known as stem females (Schröder and Gilbert, 2004). Studies examining the transmission of environmental clues via maternal effects usually focus on amictic mothers hatched from amictic eggs. For example, amictic mothers of the rotifer *Brachionus calyciflorus* tend to produce larger eggs at low temperature, and the offspring from larger eggs show higher population growth rates at low temperature than at higher temperature (Sun and Niu, 2012a). Literature on maternal effects relating to sexual reproduction have generally paid closer attention to the maternal effect of stem females of the rotifer *B. plicatilis* (Hagiwara et al., 2005), or the influence of maternal effect on characters of sexual reproduction, such as the proportion of mictic females, and production, morphology, and diapause of resting eggs in daphnia or rotifers (Mikulski and Pijanowska, 2009; Alekseev and Lampert, 2001; Martinez-Ruiz and García-Roger, 2015). In rotifer *B. plicatilis*, starved stem female mothers tend to produce offspring with a higher percentage of mixis than offspring from non-starved stem females (Hagiwara et al., 2005). Recently, Martinez-Ruiz and Garcia-Roger (2015) have showed a maternal effect related to the age of mictic females of *B. plicatilis* affecting...
the hatching phenotype of resting eggs. However, comparing with maternal effects in parthenogenetic reproduction of monogonont rotifer, it remains unclear whether a mictic mother can transmit information about its environment to its offspring via resting eggs during sexual reproduction and how the stem female offspring will respond to maternal effects from a mictic mother.

Resting eggs and their hatched stem females have important ecological and evolutionary consequences, such as gene conservation and establishment of new clone populations in the life history of monogonont rotifers (Gilbert and Thomas, 2004). From an evolutionary point of view, the fate of stem females may be more important than that of amictic females to the long-term fitness of populations (Serra et al., 2004). Furthermore, some studies have demonstrated that transgenerational plasticity differs between stem females and amictic females (Schroeder and Gilbert, 2004; Hagiwara et al., 2005). To date, it remains unclear whether mictic mothers can transmit environmental clues to their stem female daughters via resting eggs and affect the fitness of offspring in diverse environments.

Predation risk is one of the most important environmental factors that profoundly affect natural selection in zooplankton communities (Agrawal et al., 1999). The rotifer *B. calyciflorus* can produce offspring with significantly elongated posterolateral spines to effectively defend against the predatory rotifer *Asplanchna brightwellii* when predator kairomones are present in the environment (Gilbert, 1999). Yin et al. (2015a) reported that an amictic *B. calyciflorus* mother that experienced predatory *A. brightwellii* kairomones could produce offspring with longer posterolateral spines in the current or succeeding generation than those from non-experienced amictic mothers. However, little is known about how offspring from a mictic mother that has been exposed to predatory risk will respond to a similar environment as the mother.

The present study was designed to examine whether mictic mothers can transmit predatory risk clues in their environment to their stem female daughters via resting eggs. We assumed that maternal effects could also exist during sexual reproduction of monogonont rotifer *B. calyciflorus* under predation risk of *A. brightwellii*, manifested as higher population growth and a more sensitive defense response of stem female offspring exposed to a similar predator risk as their mictic mother. Furthermore, stem females from predator environment might prefer to increase the maximal population size in the same environment with predator kairomone and enhance defensive response to low concentration of predator kairomone, such as developing longer posterolateral spines compared with those from non-predator environments.

**MATERIALS AND METHODS**

Rearing conditions and stock rotifer population

The laboratory stock rotifer clone population was parthenogenetically produced from a resting egg collected from a natural lake in Houchai Park, Beijing, China (39°57′N; 116°21′E). Before the experiments, the population was cultured in modified MBL medium (Stemberger, 1981) in 500-mL beakers (containing 400 mL medium) at 20°C with a photoperiod of L:D = 16:8. The cultured medium was totally renewed every day, and the rotifers were fed with fresh green algae, *Chlorella pyrenoidosa*, at a density of 2 × 10^5 cells mL^-1_.

The density of the rotifer population was maintained at 2 inds mL^{-1}. The animals were cultured for one month before use.

**Preparation of Asplanchna-conditioned medium**

We used *Asplanchna*-conditioned medium (ACM) to mimic predation risk. The population of the predatory rotifer, *A. brightwellii*, was derived from a single parthenogenetic female cultured in the laboratory with EPA medium (Pavón-Meza et al., 2007) for at least three months prior to the experiments. Under standard conditions (pH 7.5, temperature 20°C, photoperiod L:D = 16:8), *A. brightwellii* was cultured in 500-mL beakers at low density (< 1.0 inds mL^{-1}) and fed daily with *B. calyciflorus* (with various sizes of basic morphs as shown in Fig. 3).

The ACM was prepared one day before the experiment. Adult *Asplanchna* individuals (with similar body size) were placed in EPA medium at a density of 0.25 inds mL^{-1} (10 predators in 40 mL with 24 hours exposure) without prey. After 24 hours, the predators were removed from the medium. The medium was filtered through a 0.22-μm fiber membrane to give the original ACM solution (concentration 0.25 inds mL^{-1} day^{-1}) (Gilbert, 2000). During experiments, the original ACM was diluted with EPA to the desired lower concentration.

**Experimental design**

Figure 1 shows a synopsis of the experiment. Resting eggs were collected either in predator kairomone-free EPA medium, which was designated the control set from mictic mothers in the NP environment, or in ACM medium with a 0.025 inds mL^{-1} day^{-1} concentration of predator kairomones (the minimum concentration required to induce the maximum defensive posterolateral spine, as determined by our pre-experiment, Supplementary Figure S1), which was designated the treatment set from mictic mothers in a P environment. Each treatment was composed of three replicates. The resting eggs from both NP and P maternal environments were incubated inside either EPA medium or ACM medium, constituting four treatments (NP-NP group, resting eggs collected and incubated in EPA medium; NP–P group, resting eggs collected in EPA medium but incubated in ACM medium; P–NP group, resting eggs collected in ACM medium but incubated in EPA medium and P–P group, resting eggs collected and incubated in ACM medium). The size, hatching rate and hatching pattern of resting eggs was measured. The newly hatched stem females from each of the above treatments were cultured in the same environment as the hatching medium, forming clone populations for the measurement of population growth. The four treatment groups were ranked as NP–NP, NP–P, P–NP, and P–P (letters before the dash denote the mictic maternal medium, and those after the dash denote the medium for population growth of the offspring). Population growth was monitored until day 28, when all populations began to decline in size. The growth rates of populations were calculated for the population’s growth phase (determined from initial to maximum population density). To avoid the initial influence of high concentrations of predator kairomones in the hatching environment, stem females hatched from NP–NP and P–NP treatments were used to assess sensitivity of the offspring’s response to various predator kairomones. Four concentrations of predator kairomones were used as treatments: 0, 0.0025, 0.0125, and 0.025 inds mL^{-1} day^{-1}, from lowest to highest. This experiment lasted for seven days, when the population consisted of mature individuals of the first generation. The lengths of the posterolateral spines of mature descendants (with amictic eggs) were recorded in each treatment group.

**Experimental process for measuring size and hatching strategy of resting eggs**

At the start of the experiment, amictic females carrying parthenogenetic eggs were isolated from stock populations and placed in 40-mL beakers with EPA medium at the density of 1 ind mL^{-1} to
obtain the newborns (age < 4 h). Approximately 60 newborns were randomly pipetted into one of six beakers (two media × three replicates) containing 40 mL of fresh EPA or ACM with fresh green algae, *C. pyrenoidosa* (2 × 10⁶ cells mL⁻¹), and cultured at 20°C (L:D = 16:8). EPA and ACM media were refreshed every 24 hours. After the population reached a rearing density 2 inds mL⁻¹, it was kept at this density for 10 days. All healthy resting eggs were collected daily and stored in 1.5 mL micro-centrifuge tube (one tube for one replicated population) with a small amount of medium at 4°C in the dark. From each replicated population, 50–60 eggs were randomly selected for measuring egg size with an image analysis system (AxioCam MRc5 and Axioskop 2 plus, Carl Zeiss Inc., Germany), connected to a microphotography system (AxioCam image analysis system (Axio Vision 4.6.3.0, Carl Zeiss Inc., Germany). The long and short axis of the outer shell and inner embryo of resting eggs were measured and the volumes were calculated by the equation

\[ V = \frac{4}{3} \pi (a^2b + b^2a) \]

(Sarma and Ramakrishna Rao, 1987) (Fig. 2). After four weeks of storage, 200 healthy resting eggs from each rearing tube were randomly divided into two equal groups, which were respectively hatched in NP and P medium at 20°C (L:D = 16:8) in six-well plates. The medium contained fresh green algae (*C. pyrenoidosa*, 2 × 10⁶ cells mL⁻¹) and was refreshed daily. The numbers of new hatchlings were recorded every 12 h until no more hatching occurred over the subsequent two-days.

**Experimental process for measuring population growth of stem females hatched from resting eggs by mictic mothers**

We randomly collected newborn stem females (age < 12 hours) from each treatment group. Sets of 10 neonates were cultured in a 40-mL beaker containing 20 mL of medium matching the medium in which they hatched (20°C and L:D = 16:8) and fed with 2 × 10⁶ cells mL⁻¹ fresh green algae food, which were marked as NP–NP, NP–P, P–NP, and P–P. The media were refreshed, and population densities were recorded every 24 hours. The population growth experiment was completed after 28 days, when all experimental populations started to decline. Population growth rate (r) was calculated with the following equation:

\[ r = \frac{\ln N_f - \ln N_i}{t} \]

where \( N_f \) and \( N_i \) are initial population density and population density at time t, respectively, and t is the final time of the population's exponential growth. In the current experiment, calculation of population growth rate was from population size at onset (\( N_0 \)) and termination of exponential growth rate (\( N_f \)) in each population.

**Morphological defense of offspring in varied concentrations of predator kairomones**

Each set of 10–15 stem females hatched from NP–NP or P–NP medium (age < 12 h) was randomly placed and reared for seven days in 40 mL medium with four different predator–kairomones concentrations (0, 0.0025, 0.0125, 0.025 inds mL⁻¹ *Asplanchna* ACM) and fed with 2 × 10⁶ cells mL⁻¹ fresh green algae (*C. pyrenoidosa*) at 20°C (L:D = 16:8). The medium was refreshed daily. Thirty mature descendants produced by the stem females of each group were randomly selected and fixed with 5% formaldehyde for later measurements. All measurements were completed 3 hours after fixation. For all treatments, the length of the postero-lateral spine was measured using the same image analysis system mentioned above, and measurement method is shown in Fig. 3.

**Statistical Data**

All data were evaluated for normality and homogeneity of variances using the Kolmogrov-Smirnov test and Leven’s test, respectively, to determine whether they are suitable for parametric
The photographs of (A) the normal morph (without Asplanchna) and (B) Asplanchna-induced morph of Brachionus calyciflorus with posterolateral spine length marked in the image. The method of measurement of posterolateral spine is indicated by the dimension line.

RESULTS

Effect of predator kairomones in the environment of mictic mothers on their resting eggs

Resting eggs produced by P group mictic mothers were distinctly smaller (Student independent t-test, $F = 0.184, df = 342, P = 0.021$) than those produced by NP group mothers and had smaller embryo size (Student independent t-test, $F = 0.821, df = 342, P < 0.001$) and relative embryo size (Kolmogorov-Smirnov, $Z = 1.647, df = 342, P = 0.009$, Fig. 4). However, there was no significant influence of predator kairomones on hatching rate (One-way ANOVA: $F = 1.226, df = 3, P = 0.361$, Supplementary Tables S1 and S2) or hatching pattern (Cox regression: $\chi^2 = 3.356, df = 3, P = 0.34$, Supplementary Figure S3.) of the resting eggs in each treatment.

Effect of predator kairomones in the environment of mictic mothers on clone population growth of their stem female offspring

Population growth curves were significantly affected by interactions between predator kairomones and time (interaction of time and predator kairomones: $df = 6.073, P = 0.016$). Growth of clone populations of the stem females cultured in different environments with their mictic mothers (NP–P and P–NP groups) was significantly slower than that from environments matched to their mothers (repeated measure ANOVA : NP–P vs NP–NP, $P = 0.025$; P–NP vs P–P, $P = 0.015$, Fig. 5). The time to reach peak population density in the P–P group was the 19th day, four days after the NP–NP group (Fig. 5A and B). For the clone population growth rate of stem females, there was no significantly different between stem females reared in the same environments as their mothers’ environments (Least-significant difference: NP–NP vs P–NP, $P = 0.097$). Differences in population growth rates of stem females, cultured in diverse conditions from the mothers’ environments, were not statistically significant (Least-significant difference: NP–P vs P–NP, $P = 0.96$). However, the clone population growth rates of stem females from an environment consistent with their mictic mothers were significantly higher than those from environments different with their mictic mothers (Least-significant difference: NP–NP vs NP–P, $P = 0.001$; P–P vs P–NP, $P = 0.019$, Fig. 6).

Effects of predator kairomones concentration and maternal environment on posteralateral spine length of offspring

There were significant differences in the morphological defense responses between offspring incubated in NP and P maternal environments to various concentrations of predator kairomones (Fig. 7). The Kruskal-Wallis test illustrated that concentrations of predator kairomones had a distinct
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Fig. 4. Effect of predator kairomones on resting egg size, embryo size and relative embryo size (embryo size/egg size) of *B. calyciflorus* (*n* = 344). Data are shown as means ± SE. Note: *: *P* < 0.05; **: *P* < 0.01. NP: Resting eggs produced in an environment without predator kairomones; P: Resting eggs in an environment with predator kairomones.

Fig. 5. Population density trajectories of *B. calyciflorus* in various treatments (mean ± SE). (A) Offspring hatched from resting eggs produced in an environment without predator kairomones and cultured in two environments (filled triangle: without predatory kairomones; hollow triangle: with predatory kairomones). Each group contains three samples. (B) Offspring hatched from resting eggs produced in an environment with predator kairomones and cultured in two environments (filled circle: without predatory kairomones; hollow circle: with predatory kairomones). Each group contains three samples.

Fig. 6. Population growth rate of *B. calyciflorus* offspring hatched from resting eggs of various treatments (*n* = 12). Note: Column with no common superscript letters denotes significant difference (*P* < 0.05).

influence on the posterolateral spine lengths of offspring incubated in both NP (*P* < 0.001) and P (*P* < 0.001) maternal environments. However, the minimum concentrations of predator medium, which is required to significantly increase the length of posterolateral spines of offspring, differed between NP and P maternal environments. Significantly longer posterolateral spines were detected only at the highest concentration of predator kairomones 0.025 inds mL⁻¹ day⁻¹ in the P maternal environment, while these were observed at lower concentrations of predator kairomones (0.0025 inds mL⁻¹ day⁻¹) in the NP maternal environment group (Fig. 7). Furthermore, under predator kairomone induction, offspring originated from NP group mothers developed longer posterolateral spines than those originated from P group mothers (*P* < 0.001, Fig. 7). No significant difference was observed in the lengths of the posterolateral spines of offspring cultured without predator kairomones, regardless of the mother’s environment (NP or P) (Kolmogorov-Smirnov test).
size and embryo size). Generally, larger egg size shows that mictic mothers exposed to predator kairomones, respectively, n = 758). Note: Column with no common superscript letters denotes significant differences (P < 0.05) between groups in the same maternal environment; * and ** indicate significant differences (P < 0.05 or P < 0.01) between groups cultured at the same kairomone level.

**DISCUSSION**

The present study is the first to assess whether mictic mothers can also transfer environmental cues to stem female daughters via resting eggs, as amictic mothers do in monogonont rotifers (Yin et al., 2015a). Our results demonstrate that predator risk experienced by mictic mothers has a significant influence on population growth and morphological defense response of their stem female daughter’s clone populations, indicating that mictic mothers’ environmental cues can be transferred via resting eggs to offspring in the monogonont rotifer *B. calyciflorus*.

In the present study, the stem female offspring clone populations cultured under an environment consistent with their mictic mothers (NP–NP and P–P groups) showed clearly faster population growth than those cultured in an environment different with the mictic mother (NP–P and P–NP groups, Fig. 5A and B). These findings support the hypothesis of an “anticipatory maternal effect,” suggesting that matching to the maternal environment enhances offspring fitness (Marshall and Uller, 2007). The anticipatory maternal effect may be general to monogonont rotifers during both parthenogenetic and sexual reproduction, as long as the mother experiences environmental stress (such as low temperature, starvation, predatory risk, or crowding) when the oocytes are developing inside the mother, a period that is sensitive to maternal effects (Walczyńska et al., 2015; Yin et al., 2015a).

The results of morphological experiments with resting eggs show that mictic mothers exposed to predator kairomones tend to produce smaller resting eggs (resting egg size and embryo size). Generally, larger egg size shows a positive correlation with better offspring quality and performance for population growth (Liu and Niu, 2010; Santo et al., 2001). The clearly higher growth rate of the NP-NP offspring population compared to the P–NP population in our study also demonstrates that the offspring hatched from larger resting eggs produced by NP mictic mothers had distinctly faster clone population growth than those hatched from smaller eggs produced by the P mother when the two offspring populations were cultured in similar NP environments. In insects and birds, mothers prefer to lay smaller eggs under conditions of high predatory risk, either to manage energy for other functions (such as defense response) or to increase clutch size (Fontaine and Martin, 2006; HvanKleef et al., 2007; Kudo, 2001). The reduced resting egg size and embryo size from P mictic mothers may be due to the cost of predator defense. Yin et al. (2015b) found a cost due to inducible predator defense in *B. calyciflorus* in a reduced investment in sexual reproduction, such as decreasing production of mictic females and resting eggs in populations of *B. calyciflorus*. Decreased egg size may also be a result of reducing allocation energy for sexual reproduction. No significant differences in hatching strategy and hatching rate were found between the smaller resting eggs of the P mother and the larger resting eggs of the NP mother, indicating that resting egg size may not be associated with hatching pattern and hatching rate, consistent with the findings reported by Liu and Niu (2010). Yin and Niu (2008) reported that predator kairomones in the hatching medium did not affect hatching rate of the resting eggs. In our results, neither the mother’s environment nor the resting eggs’ hatching environment affected hatching rate or hatching pattern of the resting eggs (Supplementary Figures S2 and S3). More studies need to be done to explore the mechanism of maternal effect on resting eggs.

Our results indicate that neonates produced by the stem female originated from P mothers developed shorter posterolateral spines than those from NP mothers (Fig. 7), at each level of predator kairomone concentration. Unlike groups originating from NP mothers, which extended their posterolateral spines instantly when exposed to predator kairomones, the offspring from P mothers showed significantly longer posterolateral spines than the control only at the highest level of predator kairomones (0.025 inds mL–1 day–1, Fig. 7). For the induction of morphological defense, we sampled amictic females (with amictic eggs) at the seventh day. Although the majority of samples were F1 generational amictic female, a few F2 generation individuals would be included. Yin et al. (2015a) reported that there were no significant differences in defensive morphology (lengths of posterolateral spines) between F1 generation and F2 generation when F0 was exposed to predatory environment at early embryological stage. Thus, we can predict that deduction of maternal effect at day 7 on the measurement of morphological defense may not be significant within our samples. Consulting with the clone population growth results (Figs. 5–6), we speculate that the cost of morphological defense may be reflected in population growth. More sensitivity in defense response, such as producing longer posterolateral spines or responding to lower concentrations of predator kairomones, might be costly by reducing population growth. Aránguiz-Acuña et al. (2010) also found that excessive defense response to low concentrations of kairomones, respectively.
miones might exhibit a cost by reducing population growth. They suggested that incorrect risk alarm about predators may be avoided by delaying the response of prey to develop defensive phenotypes at the appropriate time. Our results for the lower sensitivity in predator kairomone response shown in P mother originated offspring compared with those NP mother originated offspring, might also be a strategy for prey to avoid excessive response to low predatory risk due to the anticipatory maternal effect.

Without elongated posterolateral spines, increased mictic production would be a likely reason why the population growth rate of the P-NP treatment group is slightly lower than the P–P treatment group. Amictic female *Daphnia* has been reported that maternal environmental stimuli (food and predator risk) can stimulate the offspring’s production of resting eggs (Alekseev and Lampert, 2001; Mikulski and Pijanowska, 2009). In the P-NP treatment group, stem females may prefer to produce resting eggs as soon as possible to preserve the population without predator risk. However, when this stem female was incubated in predatory kairomone, delaying production of mictic females would be necessary to increase the success of the fertilizer. The maternal effect may be a polymorphism among different mother–offspring models, and context-dependent (Pliazow and Benton, 2009). Further research should explore the diversity in maternal effects under different environments.

Our findings suggest that mictic females can also transmit predator risk clues in their environment to stem female offspring via resting eggs to affect the offspring’s phenotype, but the strategies might be different from parthenogenetic processes of rotifer. Yin et al. (2015a) found that F1 and F2 generations of amictic females produced in a P environment but grown in a NP environment (P–NP) would prefer to induce significantly longer posterolateral spines than those from NP-NP environment. However, in our study for the mictic mother, no significant difference in posterolateral spine length was detected between the F1 generations of stem females in either the P–0 or NP–0 groups (Fig. 7).

In summary, the present work demonstrates that a *B. calyciflorus* mictic mother can transfer information about current predator pressure to its stem female offspring through resting eggs to benefit the offspring, although there may be a long-term gap for the hibernating resting eggs to hatch. Furthermore, there may be different strategies in response to predator pressure through maternal effects during sexual and asexual reproductive processes.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

LXX and NCJ conceived and designed the study. LXX performed the experiments. LXX performed the statistical analysis and wrote the paper. NCJ reviewed and edited the manuscript. Both authors read and approved the manuscript.
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