TITLE: Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types

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ABSTRACT

Mesostoma ehrenbergii spermatocytes are uniquely useful to study various aspects of cell division. Their chromosomes are large in size and few in number, with only 3 bivalent and 4 univalent chromosomes. During prometaphase, bipolar bivalents oscillate regularly to and from the poles for 1-2 hours. The univalents remain at the poles but occasionally move from one pole to the other. In addition, a precocious cleavage furrow forms during prometaphase and remains partially constricted until anaphase. Attempts to rear these animals indefinitely in laboratory conditions, however, have been mostly unsuccessful because of their reproductive strategy. *M. ehrenbergii* are hermaphroditic flatworms that can produce viviparous offspring (termed S eggs) and/or diapausing eggs (termed D eggs) and they follow either one of two reproductive patterns: (1) they first form S eggs and following the delivery of these eggs produce D eggs, or (2) they only produce D eggs. When only D eggs are formed, which is common under laboratory conditions, the stocks die out until the diapausing eggs hatch, which is irregular and creates unpredictable wait times. As a result, to maintain *M. ehrenbergii* stocks in order to study their spermatocytes, we studied various factors that might influence egg type production. We have found that feeding them daily and keeping them at 25°C favours S egg production. Currently, our cultures have reached the 45th generation. In this article we describe our rearing and dissection methods and describe experiments which led to our present rearing methods.

INTRODUCTION

Mesostoma ehrenbergii are hermaphroditic flatworms that have been used to study chromosome movements during male meiosis. The unique cytological attributes of these cells are described in detail in the accompanying paper (Ferraro-Gideon et al., 2013). Briefly, dividing spermatocytes of these animals have only 3 bivalent and 4 univalent chromosomes (Oakley and Jones, 1982). The bivalents oscillate at regular speeds throughout prometaphase for long periods of time (Fuge, 1987), in contrast to most other cell types where oscillations are more irregular and for much shorter periods of time (Skibbens et al., 1993). The univalents are usually at the poles but sometimes move from pole to pole until correct segregation is achieved (Oakley,1985). *M. ehrenbergii* also forms a precocious cleavage furrow during prometaphase that remains arrested until anaphase finishes (Forer and Pickett-Heaps, 2010), also different from most other cell types (Barr and Gruneberg, 2007). Taken together, the few and large chromosomes that are easily distinguishable, the regular bivalent oscillations, the unique univalent movements, and the precocious cleavage furrows, make these cells uniquely useful to study various aspects of cell division, as elaborated on in (Ferraro et al., 2013).

To study these spermatocytes, one must have animals to dissect. However, the laboratory populations of these worms often die out and, currently, the literature does not describe a way to rear them indefinitely. In previous attempts, Steinmann and Bresslau (1913) were only able to rear *M. ehrenbergii* to 6 generations while De Beauchamp (1924) reared them only to 24 generations before extinction. Fiore and Ioalè (1973) reported that they successfully reached the 100^{th} generation; however, they did not fully describe their rearing methods. We set out to keep a permanent laboratory stock of *M. ehrenbergii*.

The apparent population instability is a consequence of the animal's life strategy. *M. ehrenbergii* can produce viviparous offspring and/or diapausing eggs (Figure 1; Ferguson and Hayes, 1941), similar to other flatworms in the Typhloplanidae family. The viviparous eggs, termed subitaneous eggs (S eggs; Fiore and Ioalè, 1973) are thin-shelled, yolk-poor eggs that develop quickly in uteri; the diapausing eggs, termed dormant eggs (D eggs; Fiore and Ioalè, 1973), are thick-shelled, yolk-rich eggs that have a dormancy period before hatching in the water (Ferguson and Hayes, 1941). As a result, the viviparous S eggs cause a rapid population expansion while the diapausing D eggs allow the population to survive unfavourable conditions (Fiore and Ioalè, 1973). The reproductive cycle of these animals can follow either one of two patterns: they can first form S eggs and following the delivery of these eggs produce D eggs (termed S worms), or they only produce D eggs (termed D worms; Fiore and Ioalè, 1973). When only the D eggs are produced, laboratory stocks extinguish until some D eggs hatch, which has unpredictable wait times and sometimes does not even take place (e.g., Heitkamp, 1977). Consequently, in order to maintain animals for cell division experiments, it is important to raise the animals in conditions that allow for the continuous production of S eggs in successive generations.

Previous research into the conditions that influence egg type production have studied the various factors listed in Table A1, in the appendix, though the results were sometimes unclear or inconsistent between studies. In general, genetic propensity, higher temperatures, lower amounts of food, and rearing animals individually, seem to increase the likelihood of *M. ehrenbergii* bearing S eggs (Table A1, appendix; Fiore, 1971; Fiore and Ioalè, 1973; Heitkamp, 1977;

Beisner et al., 1997). Fiore (1971) also observed that adult worms (defined as worms carrying S eggs or D eggs) inhibited the production of S eggs in juveniles, even when they were separated by a net of nylon mesh, suggesting that a chemical substance produced by adult worms inhibits S egg production.

We wanted to maintain a constant supply of *M. ehrenbergii* for cell division experiments so we reared animals under different regimens of food, temperature and photoperiod and determined their influences on production of the different egg types, on the behaviour and growth rates of the worms, and on whether we could obtain living spermatocytes of the proper stage for study. We have been successful with our ultimate methods and are currently on our 45th generation, with more than 1000 animals in our lab at any given time. In this article, we describe in detail the rearing conditions we use to maintain our present stocks of *M. ehrenbergii* and the methods we use to obtain spermatocytes for cell division studies.

MATERIALS AND METHODS

Our present methods for rearing *M. ehrenbergii* in the laboratory and making preparations of live spermatocytes

Rearing M. ehrenbergii

We started our *M. ehrenbergii* stock by hatching dormant eggs derived from animals collected from Lake Rondeau, Ontario by Hebert and Beaton (1990), from D worms kindly given to us by Dr. Hebert. We store diapausing eggs in water at 4ºC until needed, generally more than several months. Oxygen deprivation aids in breaking diapause (J. Kolasa- personal communication) so we hatch D eggs under anaerobic conditions at room temperature by placing them in the dark in sealed, water-filled plastic jars containing algae, so that respiration from the algae removes oxygen from the water. To break diapause, Heitkamp (1977) put dormant eggs in mud, which also creates anaerobic conditions. Animals hatching from these D eggs constitute the first generation; subsequent generations will continue if the mature worms develop S eggs. We keep the worms in plastic jars (Figure 2A) with 5 animals per 200ml of dechlorinated water, at 25ºC, and in a 16/8hr light/dark photoperiodic cycle, though from our data (described below) we are not sure that the light cycle matters. To control temperature and light cycles, we place jars containing *M. ehrenbergii* into one of two incubators: one from Environmental Growth Chambers (Model: TC-1) and the other from VWR (Model 2005; Serial #: 05037710). Lighting is provided by LED microlights (Microlites, Toronto) mounted in the incubators and programmed to turn on and off by a timer. We feed *M. ehrenbergii* daily (including weekends) with brine shrimp, although they are typically fed *Daphnia* by others (e.g., Table A1) and they also will consume mosquito or chironomid larvae (Blaustein and Dumont, 1990). However, we found it hard to maintain enough *Daphnia* and mosquito larvae to use as daily food for large populations of *M. ehrenbergii* so we switched to brine shrimp, which is relatively easy to prepare in large quantities, as will be described in the next section. There is no set time of the day when the worms are fed and therefore the time between each feeding varies slightly. When the animals die, their bodies disintegrate and their D eggs are released. We collect the D eggs and store them at 4ºC in culture water in vials for at least one month before we try to hatch them.

Our rearing conditions were optimized to raise animals with a high likelihood of producing S eggs and because we have so many animals, not all of them can be accommodated

in the incubators. Consequently, we also keep many at room temperature in dechlorinated water in jars with 30 animals/400ml of water or *en masse* in rectangular tanks; they are fed daily and survive almost as well as animals kept in the more controlled conditions, although not many produce S eggs, so that would not be recommended for maintaining a constant stock. We use animals raised outside the incubators primarily to collect D eggs when the animals die, though we sometimes dissect them for experiments.

Hatching brine shrimps and feeding M. ehrenbergii

We obtain brine shrimp eggs from a local aquarium supply store or from Brine Shrimp Direct (Ogden, UT, USA). We hatch brine shrimp from the eggs in a 'hatchery' consisting of a 2L plastic bottomless pop bottle mounted upside down on a platform (Big Al's) which allows plastic tubing to supply air into the bottle (Figure 2B). To hatch the brine shrimp eggs, we connect the tubing to an air supply and fill the pop bottle with dechlorinated water and 2 tablespoons of Instant Ocean Sea Salt Mix (Big Al's). The air flow is adjusted to give a constant stream of air bubbles to ensure the brine shrimp eggs are properly aerated, and the eggs are allowed to deposit on the walls of the vessel. We typically wait 2 days for enough brine shrimp to hatch before straining them through a filter consisting of a fine nylon mesh (Fabricland) and washing them with dechlorinated water to remove the brine (Figure 2C). The washed brine shrimp are then put into dechlorinated water and we feed *M. ehrenbergii* from this solution of concentrated brine shrimp (Figure 2D) by pipetting a few drops into each jar; this seems to be more than enough food as evident from the full stomachs of the *M. ehrenbergii* and the leftover brine shrimp in the jars. We remove the excess brine shrimp detritus in the jars as necessary: the brine shrimp only survive a few hours in non-ocean water and *M. ehrenbergii* do not eat the dead brine shrimp afterwards. If the brine shrimp detritus accumulates too much, the jars begin to smell and the animals die; prior to that stage we either remove the brine shrimp or transfer *M. ehrenbergii* to a new jar containing fresh dechlorinated water.

Dissection of M. ehrenbergii for preparations of live spermatocytes

We choose individual *M. ehrenbergii* for dissection based on their age and appearance of their testes. We dissect animals as young as when their ovaries first turn white in colour to when they develop D eggs (Figure 1). The age at which these events occur depend on their feeding regime and the temperature they are raised at, but in general it takes around 2 weeks for white-coloured ovaries to appear and around 3 weeks for D eggs to form in animals that do not carry S eggs, are fed daily, and are raised at 23-25ºC. When S eggs develop, the formation of ovaries and D eggs are delayed by a few days to a week. We generally do not dissect S worms because we need their progeny to propagate the stock. For dissection we select animals (under a dissecting microscope) with testes that are clearish-white in colour and look plump and full since these testes seem to contain the most dividing spermatocytes (Figure 3B). An example of an animal with a 'good' pair of testes, likely to contain dividing spermatocytes, is shown in Figure 3B beside an animal with a 'less good' pair of testes, less likely to contain dividing spermatocytes, shown in Figure 3A. To dissect the animals, we first rinse the chosen worm 3 times in *Mesostoma* Ringer's solution (61mM NaCl, 2.3mM KCl, 0.7mM $CaCl_2$, and 1.4mM phosphate buffer, pH6.8) by pipetting them into 3 subsequent Petri dishes filled with Ringer's solution. This is to remove residual culture water. After the rinses, the worm is pipetted into a well that is

formed by vacuum grease squeezed from a syringe onto a microscope slide. Then, most of the Ringer's solution is sucked out from the animal's surroundings to limit their movement during dissection. Next, we use pulled 10µL needles (VWR International) to remove the testes. The wide blunt end of the needle is connected to Tygon tubing with a mouth piece on the other end and cotton wool in between to prevent contamination with mouth fluid, while the sharp end of the needle is inserted into the worm's body where the testes are (Figure 3C; Supplemental Video 1). The testes are then siphoned into the needle and expelled onto a coverslip (Figure 3D; Supplemental Video 1). After we estimate the size of the droplet of testes in Ringer's solution that was expelled, we add an equal amount of 20mg/mL fibrinogen and spread the mixture so a thin layer is formed on the coverslip. The same amount of 50units/ml thrombin as that of fibrinogen is then mixed into the layer to form a fibrin clot and the whole coverslip is mounted on a perfusion chamber containing *Mesostoma* Ringer's solution. The fibrin clot keeps the cells in place and the perfusion chamber allows us to perfuse the cells with Ringer's solution or drugs. Details of the clot and perfusion chamber procedures are given in Forer and Pickett-Heaps (2005).

Methods and procedures we used to study effects of environmental parameters on production of S and D eggs

To investigate factors that influence the type of egg produced by the worms, we reared *M. ehrenbergii* in conditions that varied in temperature, frequency of feeding, and photoperiod. We placed first generation worms (i.e., hatched from D eggs) into jars that housed 5 animals/400ml dechlorinated water in a 21ºC or 17ºC incubator, fed each jar either daily or once every three days, and put the jars into either a 16/8 hr light/dark photoperiod or 8/16hr light/dark photoperiod. (Note: these conditions are different from our current conditions, which are 5 worms/200ml., 25°C and 16/8hr light/dark photoperiod). Subsequent generations continued only when mature worms developed S eggs and delivered progeny. We removed the newborns from their parental jars usually on the day they were born and separated them into the same conditions that their parents were reared under. However, when the population in certain treatment groups went extinct or was low, we sometimes supplemented those groups with animals from other treatments to carry out the experiment for that generation. We sometimes also modified the treatment conditions in subsequent generations as we attempted to increase the number of S worms, as described in the results section. In a small population of *M. ehrenbergii*, we tested the effects of density by keeping some animals at a density of 15 worms/400ml of dechlorinated water and we tested the effect of isolation by keeping worms at the same original density (5 animals per 400 ml) but keeping them in isolation - i.e., 1 worm/ 80 ml of dechlorinated water. These were raised at 21ºC and fed daily.

After the first set of D eggs that was hatched, we hatched a second set of D eggs when the first set was in the 9th generation; these were treated under the same treatment conditions as the animals in the first set, to confirm that the conclusions were not specific to the first isolate.

Data collection and analyses

We observed *M. ehrenbergii* at least twice a week by pipetting all of the animals out of each jar, putting them in a Petri dish, and studying them using a dissecting microscope. We recorded each animal's reproductive state (i.e. whether we saw the formation of S eggs or D

eggs), the age when the first S eggs were observed in the worms, and the lengths of two randomly chosen worms per jar (to determine growth rates). Lengths of worms of known ages were measured in a dissection microscope using a ruler placed under the Petri dish. The bodies of the animals stretch as they move (see Supplemental Video 2) so to standardize the lengths, all worms were measured while they were in a slow gliding motion. Jars with worms carrying S eggs were checked daily for the presence of newborns and we recorded the age of the parent worm when their S eggs hatched. We counted the number of newborns per jar and divided by the number of S worms in the jar to obtain the average number of S eggs produced per individual.

We tested for statistically significant differences in the growth and reproductive parameters between different treatment groups using the Student t-test. We performed linear regression analyses on the growth curves of *M. ehrenbergii* using a commercially available program (Slidewrite). We determined the growth rates as the least mean squares best fit slope of the curves of length versus time during the first 15 days after birth, the period before the growth slowed down and reached a plateau.

RESULTS

Currently, under the conditions in which we feed *M. ehrenbergii* daily and rear them at 25ºC as described above, we have raised animals up to their 45th generation. The percentage of worms producing S eggs provides more than enough animals to dissect and the population does not show any signs of nearing extinction. In addition, we find it easier to obtain spermatocytes when we keep the animals at 25ºC and feed daily compared to when we kept them at lower temperatures and fed them less frequently. Though we are not able to quantify this impression, in previous rearing regimens it sometimes took many days of dissection before we found dividing spermatocytes whereas now we can find them in most worms that we dissect.

Factors that influence egg type production

Several factors influenced the egg type that was produced. The particular generation the animals were in, temperature, and feeding regime all affected whether the juveniles were more likely to become S worms and therefore produced S eggs first and then D eggs, or whether the juveniles were more likely to become D worms and therefore produced D eggs only. Photoperiod, on the other hand, did not seem to affect their reproductive behaviour, at least for the conditions we tried.

Some generational trends in the percentage of S worms occurred in all groups of animals, regardless of rearing conditions. Animals hatching from D eggs and their offspring (i.e. 1st and 2nd generation worms respectively) tended to produce S eggs while animals reaching their 4th or 5th generation tended to produce D eggs only, as indicated by the rapid decline in percentage of S worms in generations 4-5 (Figure 4 & Figure 6). These observations were independent of the environmental factors studied. Keeping these trends in mind, environmental factors that influence egg type production were only evaluated between generations 3-4 and/or after populations stabilized following the rapid decline of S worms (Figure 5 & Figure 6, for the two batches of eggs).

Photoperiod did not seem to have an effect on the egg type produced. In the first 8 generations, animals reared in the 16/8hr light/dark photoperiod (light condition) and animals reared in the 8/16hr light/dark photoperiod (dark condition) had a similar percentage of worms

that produced S eggs. There were no significant differences and as a result, data from these two conditions were combined. In generation 9 however, the percentage of juveniles becoming S worms in the dark condition was significantly higher than in the light condition ($p<0.05$; Figure 5), although we later determined that the effect was actually due to a temperature difference. One of the incubators did not properly regulate the set temperature and experimental tests (switching incubator photoperiod programs for the two incubators, switching animals, changing temperatures, etc.) verified that the differences seen were not due to photoperiod but to temperature.

Both temperature and frequency of feeding on the other hand, were important parameters in determining which egg type was produced. Well fed worms, given food daily, were more likely to produce S eggs compared to poorly fed worms that were given food only every three days ($p<0.05$; Figure 4). Because of the drop in S worms in generation 5, we stopped feeding animals poorly (i.e. every three days) in order to maintain the population. Similarly, temperature influenced egg type production: there was a significantly higher percentage of S worms produced at 21ºC than at 17ºC ($p<0.05$; Figure 4). We obtained further evidence for the importance of temperature when animals reared at 21ºC were switched to 23ºC midway through generation 7 (Figure 4). In generations 4-5, the percent of S worms began decreasing drastically and the first worms with developed ovaries in the beginning of generation 7 all produced D eggs only. We immediately switched the temperature to 23ºC, however, and of animals maturing later in generation 7, after the increase in temperature to 23ºC, 80% became S worms (Figure 4). Populations of worms raised at 17ºC became extinct in the 4^{th} and 5^{th} generation for poorly and well- fed animals respectively, and attempts to use offspring from other conditions to supplement the 17ºC condition in generations 7 and 8 failed as worms produced D eggs only. As a result, we discontinued rearing animals at 17ºC and all animals were reared at 23ºC instead. As previously mentioned, there seemed to be a difference between animals raised in the light and dark conditions but we realized in generation 18 (Figure 5) that the temperature in the incubator with the higher percent of S worms was at 25°C, even though it was set at 23°C. Consequently, we changed the other incubator to 25°C so that from the middle of generation 18 onward, all animals were reared at 25ºC (Figure 5). Following the increase to 25°C, the *M. ehrenbergii* populations in both incubators were stabilized with minimal effects of the different photoperiods and with 40-100% of worms in each generation forming S eggs (Figure 5). This is a relatively large range; the proportion of juveniles that become S worms cycles from high to low through different generations even when they are being raised in constant temperature and feeding regimens (Figure 5).

To confirm that the results we obtained were not unique to the one particular batch of *M. ehrenbergii*, a second batch of D eggs was hatched when the first batch was in the 9^{th} generation. From Figure 6, it apparent that the two batches of worms responded similarly in the same environmental conditions, including the high propensity to become S worms in the first two generations, the rapid decline in generations 4-5, and the fluctuations in the later generations after populations were stabilized.

Effect of temperature and feeding regime on developmental times and subitaneous clutch sizes

We also measured other reproductive parameters such as timing for the start of S egg formation, when the S eggs hatched, and the number of eggs produced by each worm. In all

generations prior to and after the incubator temperature regulation problems, there was no significant difference between animals reared under the photoperiods in any of these parameters; thus the data were pooled. When the worms were fed less frequently, within each temperature group the ages at which the worms developed S eggs and when the S eggs were delivered were significantly delayed ($p<0.01$; Table 1). Similarly, lower temperatures caused significant delay in these events but the difference was only seen when the worms were fed daily ($p<0.01$; Table 1). Worms fed once every three days had similar developmental times at 17°C and 21°C (Table 1). In most cases, the worms first developed S eggs and following the birth of the S eggs, developed D eggs. On very rare occasions (1-2 worms out of 120 worms in each generation), in the current conditions of feeding daily at 25ºC, we observed a second set of S eggs in the same animal following the delivery of the first set, before D eggs appeared. We have also periodically seen instances of both S eggs and D eggs within the same animal (Figure 1).

The temperature and feeding regimens also influenced the subitaneous clutch sizes per individual. Regardless of the temperature, when the worms were fed more frequently, significantly higher numbers of S eggs were obtained ($p<0.05$; Figure 7). Worms fed daily produced on average 5 more S eggs than worms fed once every three days (Figure 7). Moreover, worms reared at lower temperatures tended to produce more S eggs, although this effect was only seen when the worms were well fed ($p<0.05$; Figure 7). Worms reared at 17ºC produced 3 more S eggs than worms reared at 21ºC which produced 1 more S egg than worms reared at 25°C (Figure 7). We did not have enough data per generation to accurately compare the effects of different rearing densities on the propensity to become S worms, but because the clutch sizes were similar between all generations, we were able to combine generational data in order to compare the effects of density on the number of S eggs delivered per individual (Figure 8). From Figure 8, it is evident that the clutch sizes increased as the number of worms in the jar decreased; the greatest number of S eggs produced per individual was when the worms were isolated ($p<0.05$; Figure 8). As described in the introduction, these hermaphroditic worms are capable of self-fertilization when isolated, and they can produce both S eggs and D eggs by self-fertilization.

Effect of temperature and feeding regime on *M. ehrenbergii* growth curves

M. ehrenbergii lengths were measured in the various conditions to determine their growth rates. There were no significant differences in length between the different generations and between the different photoperiodic treatments so those data were combined to generate the growth curves (Figure 9). In all treatments, the animals initially showed a linear increase in length with age (r^2 value from linear regression >0.90) and eventually reached a plateau, or final adult length (Figure 9). The growth rates ± standard errors are given in Table 2 together with the final adult lengths ± standard error. At both 17°C and 21°C, growth rates of individuals fed daily were significantly higher than those of individuals fed once every three days, by about a factor of 2 ($p<0.01$). With the same frequency of feeding, *M. ehrenbergii* growth rates were similar at 17°C, 21°C, and 25°C (Table 2). Similarly, the final adult lengths of the worms that were fed daily were significantly greater than those of worms that were fed once every three days but there were no length differences between worms raised at the three temperatures ($p<0.01$; Table 2). The maximum length of the worms we observed was 12 mm.

Observations of M. ehrenbergii behaviour

Lastly, we would like to provide a general note on *M. ehrenbergii* behaviour. These worms are aggressive predators that secrete a web-like substance to trap their prey (Wrona and Koopowitz, 1998). With brine shrimp, they trap many at once, creating a mini ball of brine shrimp which are brought individually to their mouths to consume (Supplemental Video 2). They typically sink to the bottom of the jar or tank while eating.

We keep large numbers in a rectangular tank, where we noticed that the colonies of *M. ehrenbergii* form an organized net with the web-like sticky substance they produce and tend to aggregate at one corner or side of the tank, or on the surface; they rarely are found swimming in the middle of the tank (Figure 10). The location they aggregate in is not consistent; it varies from day to day and even during a day, and while the currents caused by daily feeding disrupt the integrity of the web, the communities are always reformed the next day. When brine shrimp are put into the aquarium, they get caught in the web and the worms slowly glide to their prey and consume them on the web. They may also display aggressive behaviour to each other when many are crowded together because we have noticed that some animals from the aquarium appear to be missing pieces of their body wall when observed under a dissecting scope (Figure 11).

M. ehrenbergii deliver S eggs through their body wall and the newborns are released from the same position they are in inside the parent (Supplemental Video 3). The parental worm seems to move more actively during release of S eggs and we have not seen any damage or slit in the body wall. On the other hand, D eggs seem to be released only when individual *M. ehrenbergii* die and their body wall disintegrates, leaving behind the D eggs at the bottom of the jar. In quite a few occasions, we observed that when one worm in a jar dies, all of those in the same jar die. The other animals in the jar can be rescued only if we separate them in time from the dying *M. ehrenbergii*.

DISCUSSION

We have described the methods we use to get sufficient stock of animals to dissect, and we described how to dissect the animals to obtain spermatocytes. We present this information to help anyone wishing to study this unique meiotic system, and we are happy to provide animals to anyone who wishes to use them.

In learning how to rear *M. ehrenbergii* for our cell biological experiments we studied some of the parameters that influenced our choices of growth conditions. We now discuss how our observations compare with related observations in the literature.

The *M. ehrenbergii* stock we raised from D eggs usually either produced S eggs and following their delivery produced D eggs, or produced D eggs directly. However, we have periodically seen worms carrying both egg types, a situation never seen by Bresslau (1903), as cited by Fiore and Iaolè (1973). When we have seen this, the S eggs were liberated as babies, unlike the situations described by Fiore and Iaolè (1973) who reported that sometimes the D eggs prevented the release of the S eggs, which degenerated in uteri. Moreover, unlike *M. lingua* which may form S eggs again once D eggs are formed, neither we nor anyone in the literature that we are aware of has observed this in *M. ehrenbergii*. We have, however, seen animals produce a second batch of S eggs following the delivery of the first batch, but, similar to Fiore and Iaolè (1973), this occurs in only a small fraction of the animals that are reared in our current conditions.

The determinants for which egg type these worms produce seem to be a combination of endogenous and environmental factors. Similar to two previous studies, first generation worms

hatching from D eggs had a high propensity to develop S eggs in a wide range of conditions (Heitkamp 1977; Fiore and Iaolè 1973) and in our experiments, this propensity extended to their offspring in the second generation as well. Beisner et al. (1997) on the other hand, reported that *M. ehrenbergii* originating from D eggs were more likely to produce S eggs at lower temperatures of 18ºC and produced D eggs only if given low amounts of food. A strong tendency of D egg-derived worms to become S worms regardless of conditions may be advantageous when the worms are trying to establish their populations in early spring.

Another generation-dependent observation we made was a sharp decline in percentage of S worms in generations 4 or 5, similar to the studies by Steinmann and Bresslau (1913) where they could not maintain stocks for more than 6 generations. As cited by Fiore and Iaolè (1973), Steinmann and Bresslau (1913) suggested that this corresponded to the natural time frame from when the worms would hatch from D eggs in the spring to the extinction of the population in the autumn and thus they concluded that endogenous factors which caused the worms to overwinter after a certain number of generations were mainly responsible for egg type production.

Temperature and frequency of feeding influenced the egg type produced in animals from the 3rd generation onwards, although photoperiod did not, despite being the main cue for diapause induction in most aquatic invertebrates (Alekseev, 2006). This is consistent with Heitkamp's (1972) study on *M. lingua* which showed that length of day did not influence the production of S eggs. Higher temperatures and feeding worms daily, on the other hand, significantly increased the proportion of S worms. Previous studies found similar effects of temperature where animals hatching from S eggs only became S worms in high frequencies when kept at temperatures above 20ºC (De Beauchamp, 1926; Heitkamp, 1977; Beisner et al., 1997). This response to temperature may be advantageous since S-egg derived worms are born in the summer and cooler temperatures may signal the arrival of winter. However, opposite results were reported with respect to the feeding regime. Fiore and Iaolè (1973) and Beisner et al. (1997), studying *M. ehrenbergii,* and Heitkamp (1972), studying *M. lingua,* found that poorly fed worms were more likely to produce S eggs. The discrepancy in results concerning the worms' response to lower food levels may suggest that our frequency of feeding once every three days could be too 'low' since the Beisner et al. (1997) low food level regime is 1 Daphnia per day (the feeding regime Fiore and Iaolè (1973) used was not stated). Alternatively, it may be that *M. ehrenbergii* distinguishes the difference in food quality between the brine shrimp we used as food supply and the *Daphnia* which the other authors used as food supply.

Lastly, in our stable population of worms there is a wide fluctuation in the proportion of animals becoming S worms, ranging from 40-100%, even though they all are raised at consistent conditions of temperature and photoperiod (Figure 5 & Figure 6). Similar observations were reported by Fiore and Iaolè (1973); they raised worms for 100 generations and suggested that the variation may be due either to environmental conditions that are not controlled or to endogenous factors. Because of the regularity of this cycling between high and low proportions of S worms and the observation that both we and another group have the same findings, we think it is possible that intrinsic factors play a role, although as Fiore and Iaolè pointed out, this is not advantageous. Having endogenous factors influence egg type production would decrease *M. ehrenbergii*'s ability to respond to different environments (Fiore and Iaolè, 1973).

M. ehrenbergii developmental times were dependent on temperature (Table 1), as expected for aquatic ectotherms whose body temperatures conform to its surroundings. The development times we observed seem comparable to the times reported in most of the literature, in most of which the animals were reared at slightly different temperatures (Table 4). However,

because we only observed the worms for the presence of S eggs at most three times a week, the observed age for the first appearance of S eggs will be slightly higher than the actual age. The frequency of feeding also affected development times and worms that were fed once every three days developed significantly later than worms fed daily (Table 1). Fiore and Iaolè (1973) made similar observations; the delay most likely is due to the lack of energy and resources.

We also studied the number of S eggs produced in each clutch. Animals that were well fed and reared at lower temperatures produced the highest number of S eggs (Figure 7). *M. ehrenbergii* that were fed once per day at 17ºC produced on average 3 more S eggs (13.3 S eggs/ individual) than those that were fed once per day at 21ºC (10.5 S eggs/ individual; Figure 7). Beisner et al. (1997) also found a difference of 6 more S eggs per animal reared at 18ºC compared to 24ºC. As proposed by Beisner et al. (1997), the effects of temperature may be a result of the faster developmental times at 21ºC compared to 17ºC (Table 1) so that additional eggs do not have as much time to form. In addition, smaller clutch sizes were also observed when the worms were fed less. This is likely because there are fewer resources that can be allocated to form the S eggs. This conjecture is supported by the observation that animals that were fed every three days take longer to develop and do not reach the same length as ones that were fed daily (Table 1 and Table 2). We also found that one animal per jar produced more S eggs compared to 5 animals per jar which in turn produced more S eggs compared to 15 animals per jar (Figure 8). It may be reasonable to suppose that this could be due to the same chemical that is produced by worms that inhibit S egg production as discussed by Fiore (1971). With 15 animals per jar, there may be a higher concentration of the chemical that lowers the number of S eggs produced while isolated worms may not be affected by the chemical produced by themselves, as proposed by Fiore and Iaolè (1973) to explain why the chemical did not inhibit isolated worms from becoming S worms. It is unlikely that the reduction in number of S eggs produced by worms reared in larger numbers is due to the lower levels of food each individual receives because in all three densities excess brine shrimp was present in the jars after feeding.

M. ehrenbergii growth rates were similar between different temperatures but were significantly slower when the worms were fed less (Figure 9; Table 2). Likewise, animals reached a similar maximum body length irrespective of temperature but were smaller when they were fed once every three days (Figure 9; Table 2). Beisner et al. (1997) found a similar effect of food level on growth rate and found that the final body sizes reached by *M. ehrenbergii* were the same at different temperatures. In contrast to our observation that the growth rates were also similar at both temperatures, however, they found that growth was slower at lower temperatures, a relationship that is found in other aquatic invertebrates as well (Angilletta et al. 2004). The final adult lengths of 8 mm reached by daily fed animals at both temperatures (Table 2) were slightly smaller than the usual 10-15 mm reported in literature although Husted and Ruebush (1940) and Ferguson and Hayes (1941) observed that North American species reach the size of only 5-7 mm (Table 3). Beisner et al. (1997) and Elvin and Koopowitz (1994) however, obtained their stock from Alberta and had worms attain a size between 10-15 mm (Table 3). Our stock came from Lake Rondeau in Ontario. The longest length we observed was approximately 12 mm while the reported maximum length of *M. ehrenbergii* in Canada is 15 mm (Elvin and Koopowitz 1994). The smaller size may have been due to the differences in the quality of food that was fed to the animals: *M. ehrenbergii* were fed with brine shrimp in our study but were fed *Daphnia* in the studies which reported the lengths (Table A1).

In conclusion, it seems apparent that these worms have an intrinsic system that causes them to produce S eggs in earlier generations and produce D eggs closer to the 4th or 5th

generation where, in nature, they would encounter autumn. However, this system can be influenced by environmental factors and we found that higher temperatures and daily fed worms were more likely to produce S worms. However, lower temperatures produce the largest numbers of S eggs so there is a trade-off between raising more S worms that carry fewer S eggs or raising fewer S worms that carry more S eggs. Alternatively, if one wishes for more animals, one might grow the worms at 25ºC and once they develop S eggs, transfer them to 17ºC. We have not tried this, however, since with 8 S eggs per worm and 40-80% of the worms producing S eggs, we have more than enough animals to dissect for our experiments.

M. ehrenbergii are both easy to raise and to obtain spermatocytes from. This is a unique system that we believe will provide insight into the forces that cause chromosome movements, insight into control of cleavage furrow formation, and insight into other aspects of cell division in general.

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FIGURES

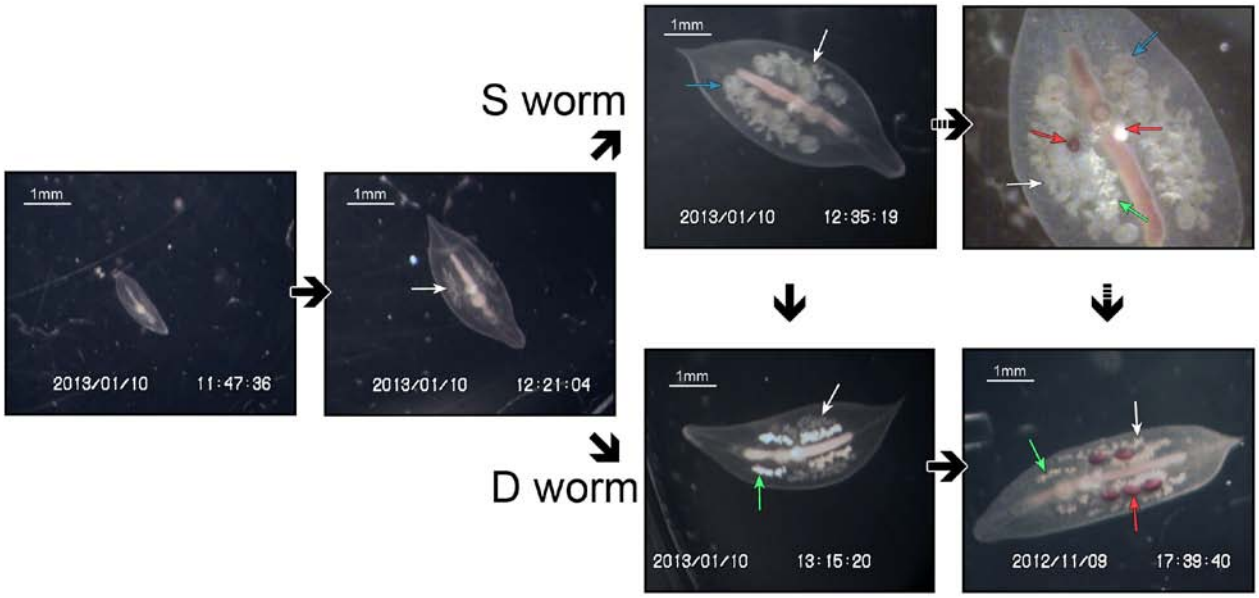

FIGURE 1: Physical appearance of *M. ehrenbergii* at different possible stages of life. The black arrows indicate the progression to a stage in life which usually occurs while the black dashed arrows indicate the progression to a stage in life which only periodically occurs. A newborn animal always first develops testes (white arrows) and then can either follow one of two reproductive patterns: (1) if it is to become an S worm, S eggs (blue arrow) form and ovaries (green arrows) either mature during pregnancy or after S eggs are laid. After ovaries mature, D eggs (red arrows) form. Periodically, ovaries will mature and D eggs will form while S eggs are still in the parental body. (2) if the juvenile is to become a D worm, ovaries mature and only D eggs are formed.

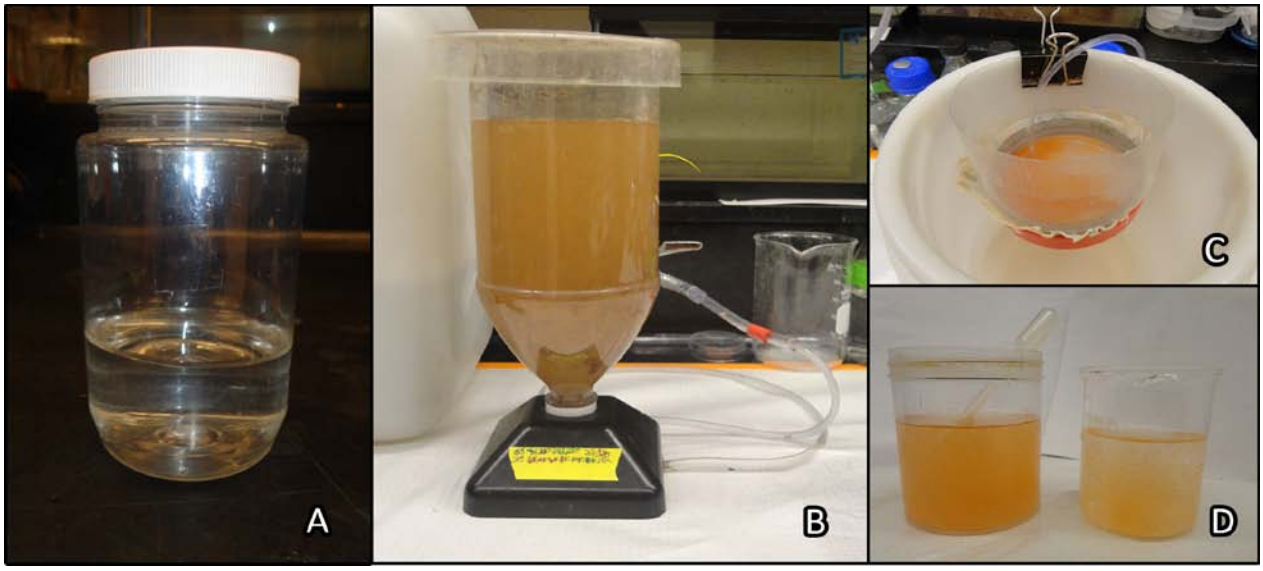

FIGURE 2: **A.** Plastic jars with screw-on lids used to house *M. ehrenbergii*. **B.** Brine shrimp hatchery set-up consisting of a 2L pop bottle inverted onto a platform connected to plastic tubing attached to air supply. **C.** Nylon mesh net filtering brine shrimp from the Oceanic water. **D.** Solution of brine shrimp in dechlorinated water.

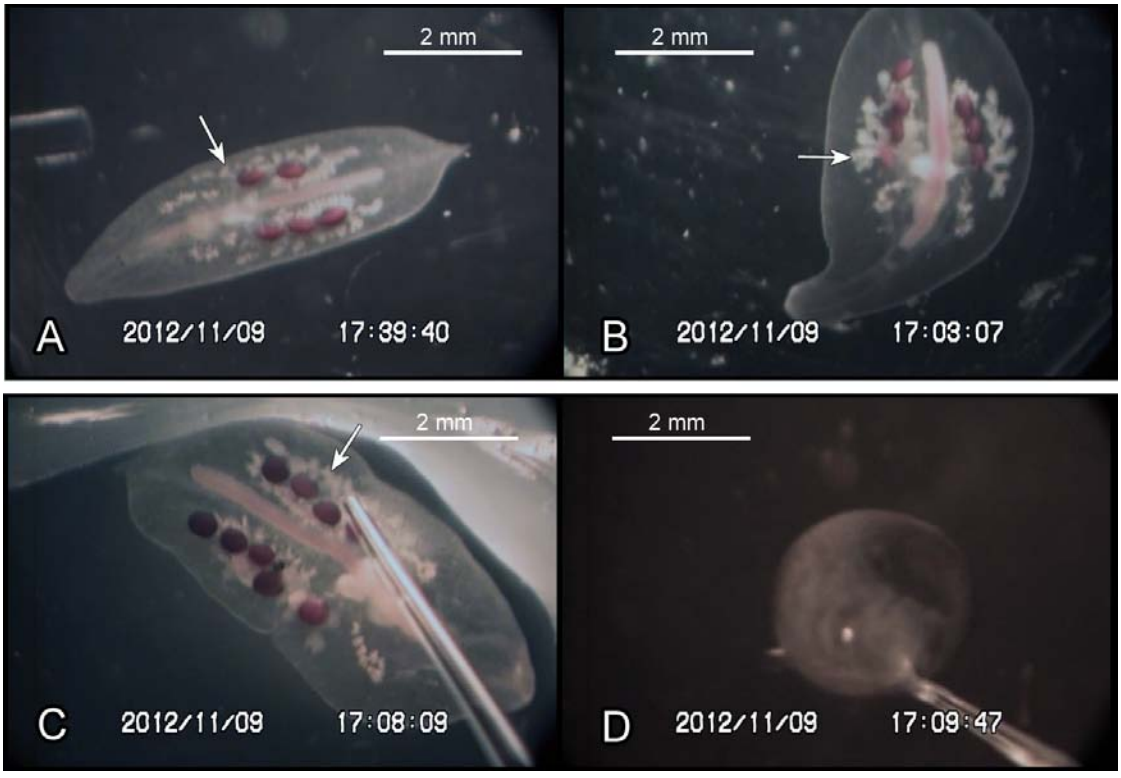

FIGURE 3: **A.** Appearance of *M. ehrenbergii* testes (white arrow) that is less likely to provide dividing spermatocytes. **B.** Appearance of *M. ehrenbergii* testes (white arrow) that is likely to provide dividing spermatocytes. **C.** Needle inserting into *M. ehrenbergii*'s body wall to siphon out the testes during dissection. **D.** Drop of testes and Ringer's solution mixture expelled onto a coverslip.

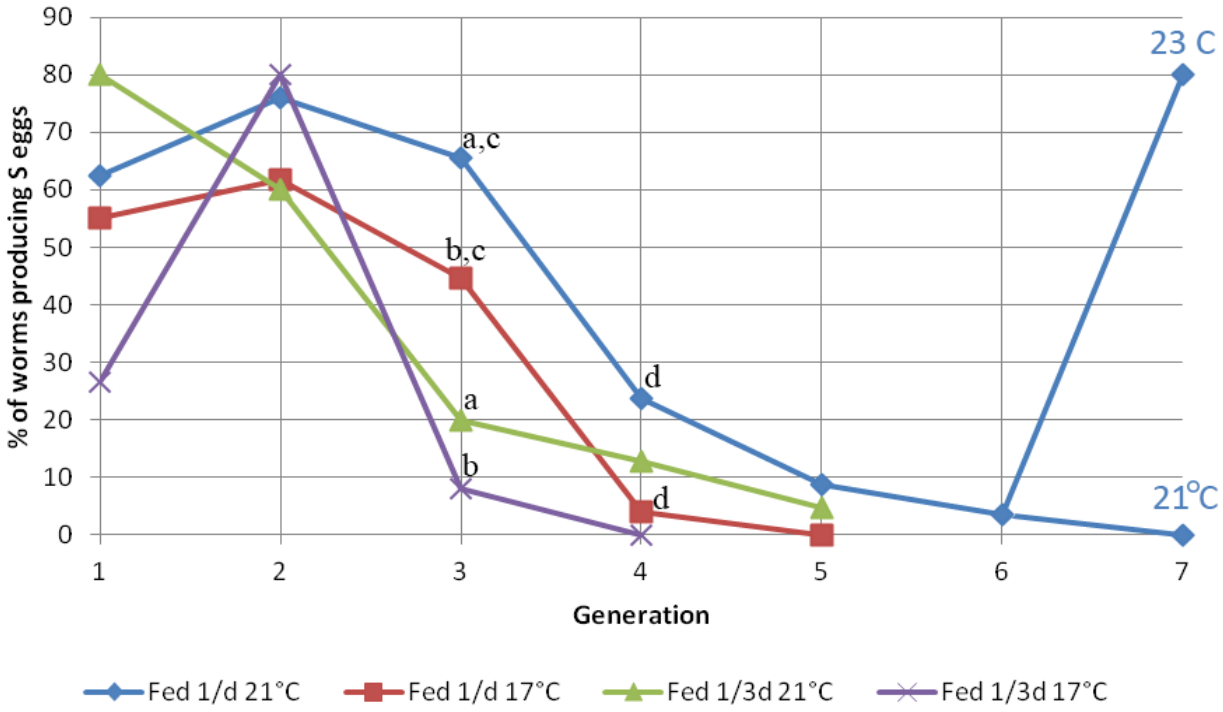

FIGURE 4: The percentage of worms that produced S eggs in different temperature and frequency of feeding conditions from generations 1-7 in the first batch of eggs hatched. Note: data from the two photoperiodic conditions were combined. Significant differences appeared in the third generation between animals fed daily vs. animals fed every three days at 21ºC[a] and 17ºC[b] and between daily fed animals that were reared at 21ºC vs. 17ºC[c] ($p<0.05$). Significance differences appeared in the fourth generation between daily fed animals that were reared at 21ºC vs. 17ºC[d] ($p< 0.05$).

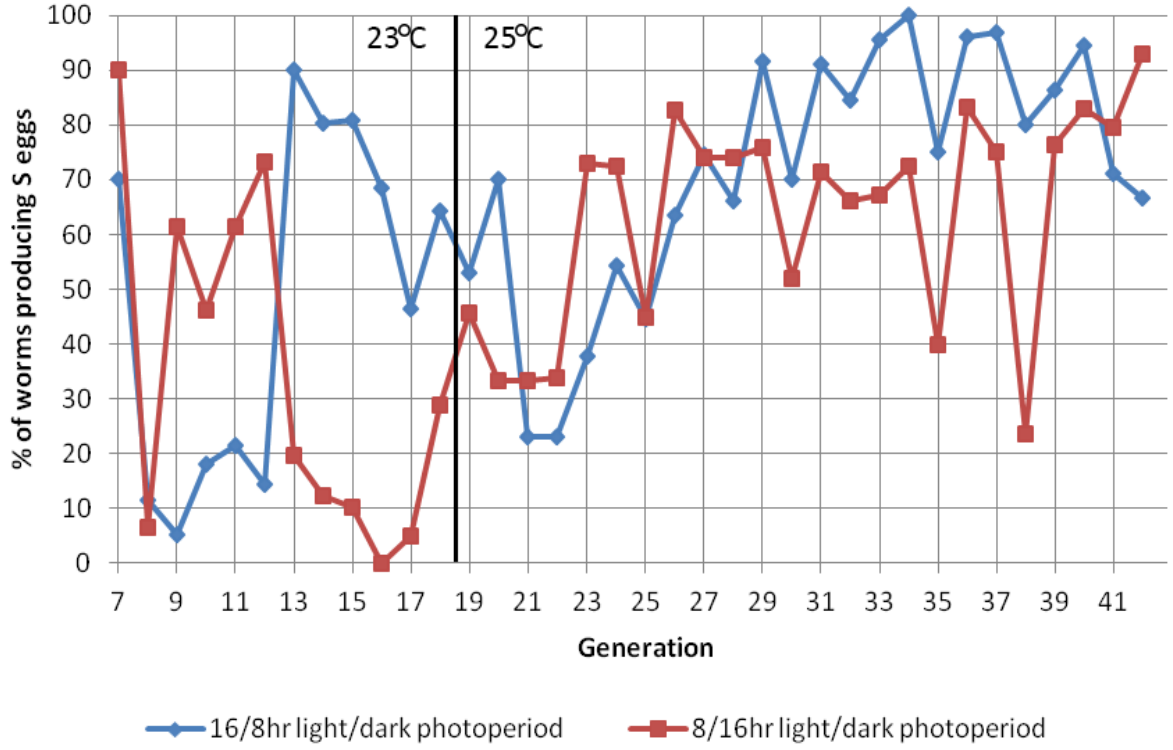

FIGURE 5: The percentage of worms that produced S eggs in different photoperiodic conditions from generations 7-42 in the first batch of eggs hatched. Note the temperature change before generation 19 from 23°C to 25°C. In generation 15 when the % of worms producing S eggs in the 8/16hr light/dark photoperiodic condition decreased to 0%, generation 16 was continued by taking babies born from generation 15 animals raised in the 16/8hr light/dark photoperiod.

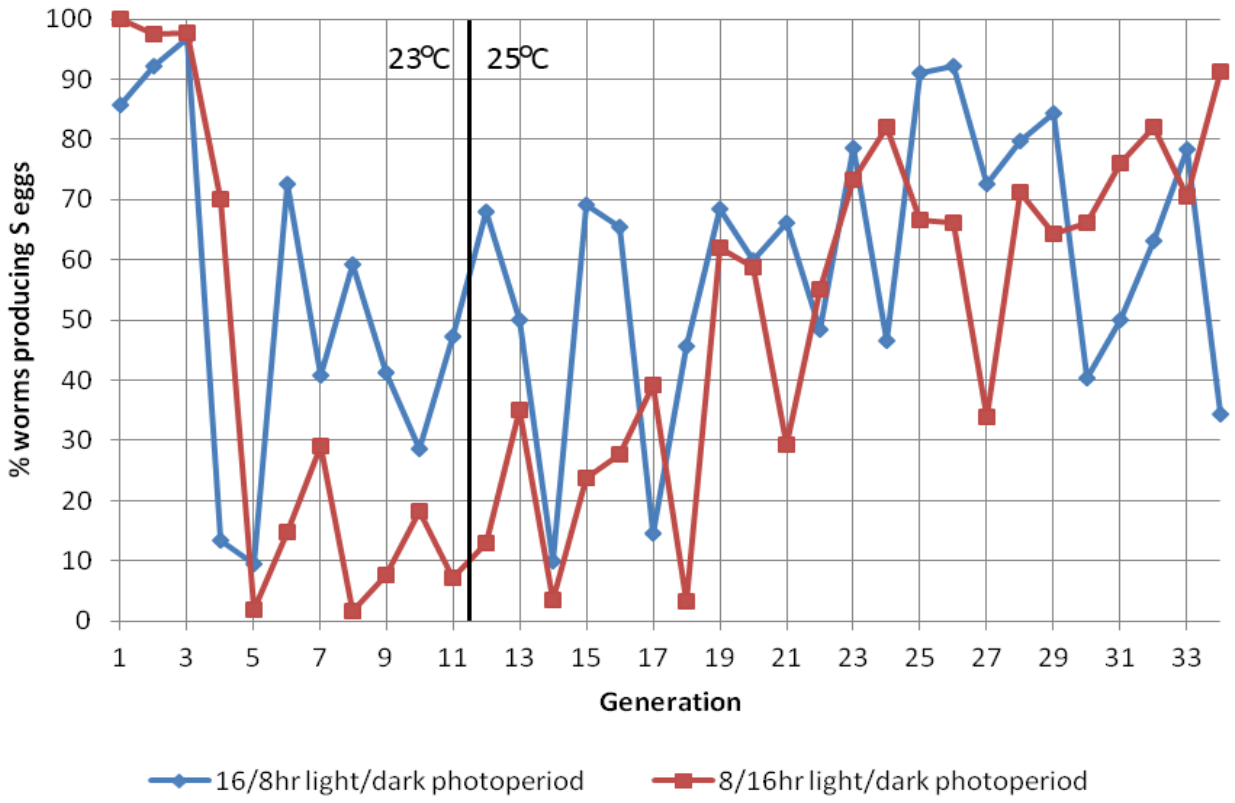

FIGURE 6: The percentage of worms that produced S eggs under different photoperiodic conditions from generations 1-34 in the second batch of hatched eggs. Note the temperature change before generation 12 from 23°C to 25°C.

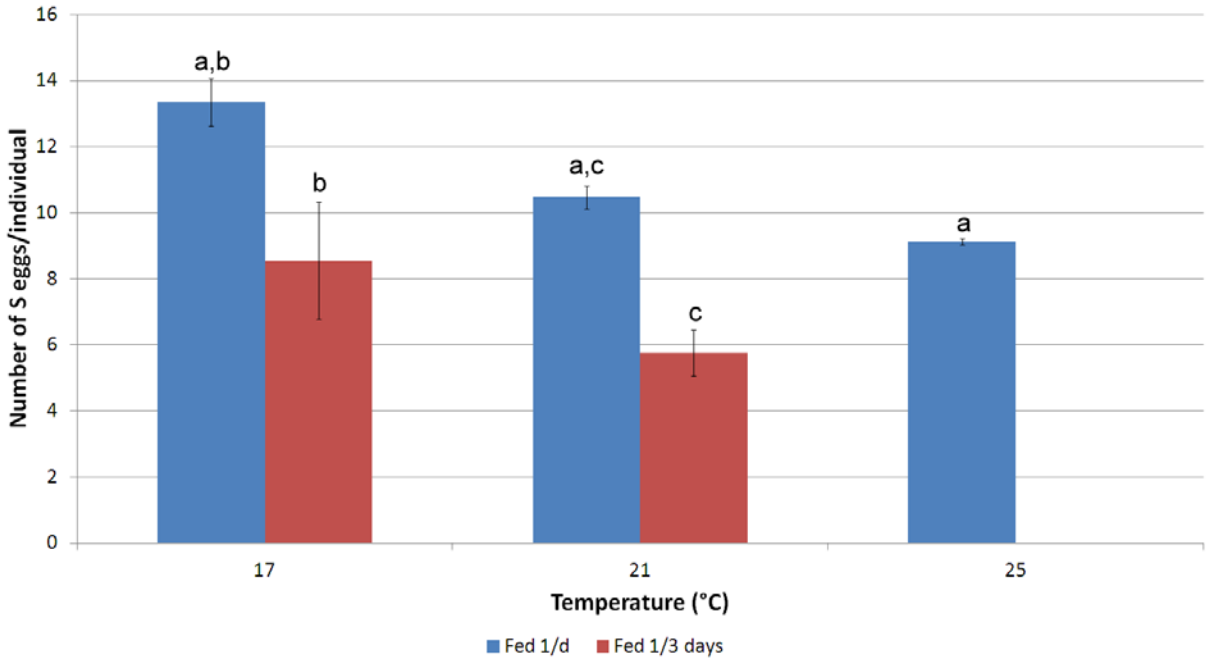

FIGURE 7: The effect of temperature and frequency of feeding on subitaneous clutch size. Note: data from different generations and photoperiodic conditions were combined. [a]Significant difference between daily fed animals at 17ºC vs. 21ºC vs. 25ºC ($p<0.05$). [b,c]Significant difference between animals fed daily vs. once every three days ($p<0.05$).

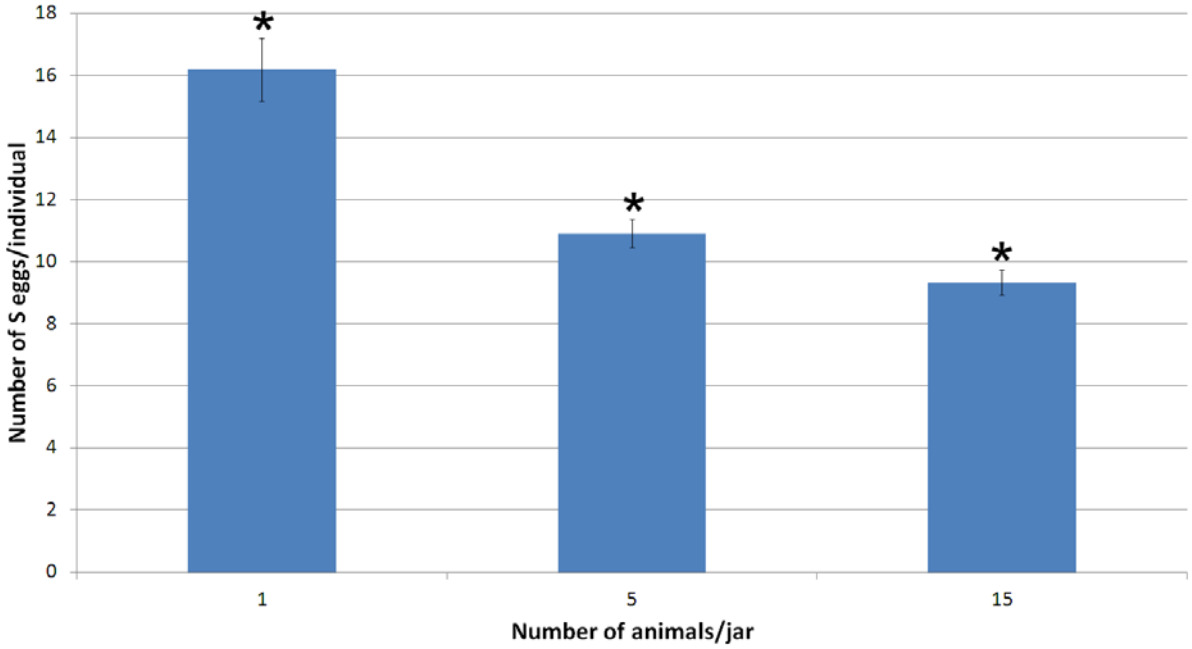

FIGURE 8: The effect of the number of *M. ehrenbergii* reared together per jar on subitaneous clutch size. Note: data from different generations and photoperiodic conditions were combined. *Significant difference in clutch sizes between 1 animal vs. 5 animals vs. 15 animals reared per jar ($p<0.05$).

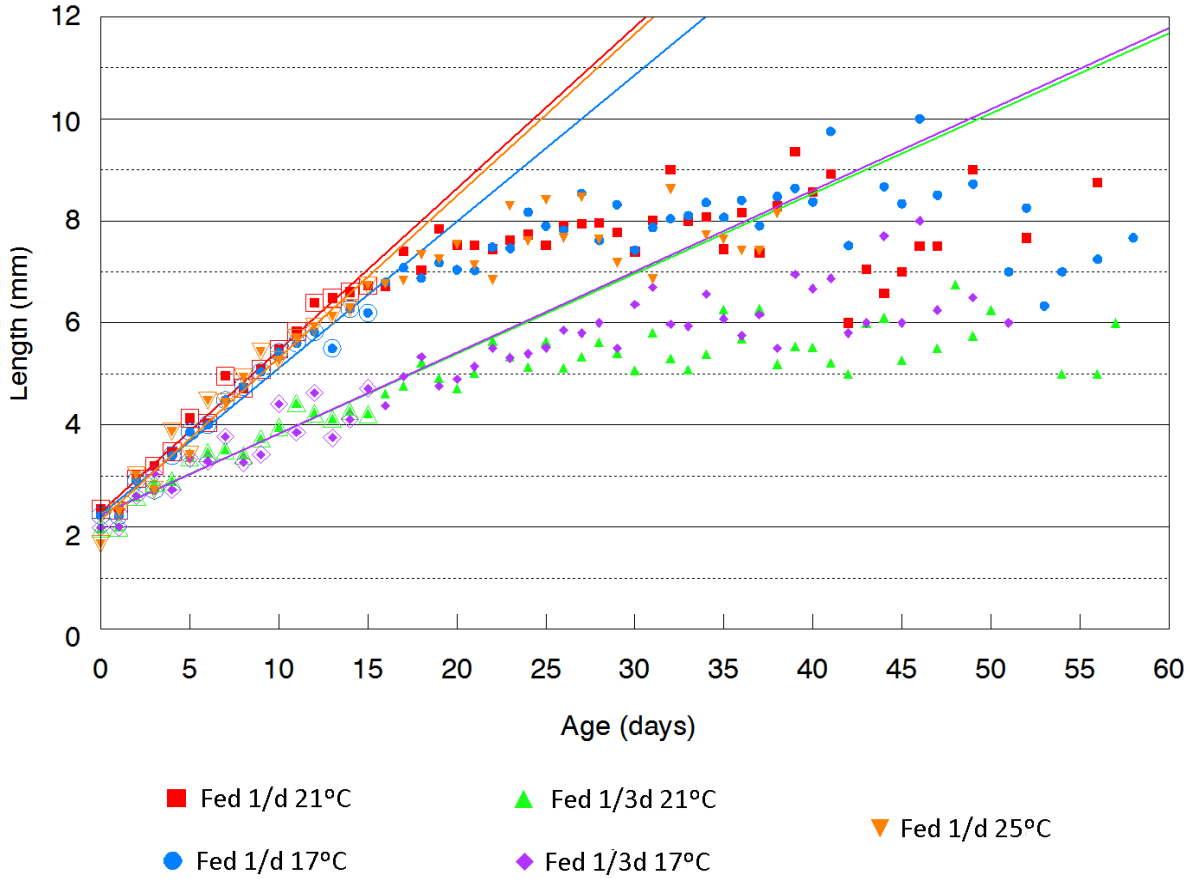

FIGURE 9: The effect of temperature and frequency of feeding on *M. ehrenbergii* growth curves. A linear regression was fit to the lengths corresponding to the first 15 days after birth ($r^2 > 0.9$). Note: data from different generations and photoperiodic conditions were combined. Each data point represents the average length of all animals measured at that condition and age ($n \geq 24$ for each condition). There is a significant difference in linear slopes between animals fed daily vs. animals fed once every three days at both temperatures ($p<0.01$).

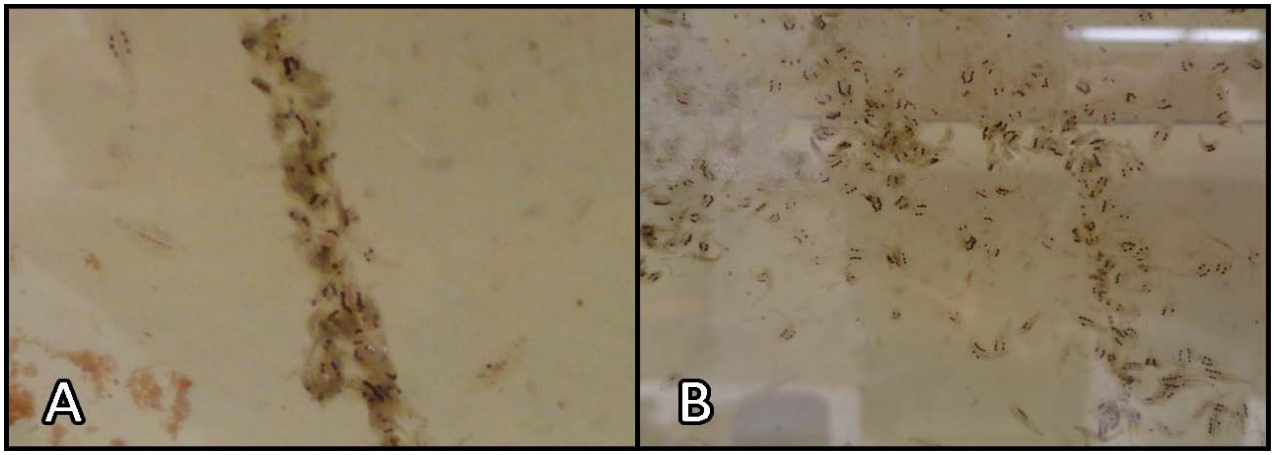

FIGURE 10: **A**. *M. ehrenbergii* aggregating together around the mucus they secrete. **B**. *M. ehrenbergii* spreading only on one side of the tank.

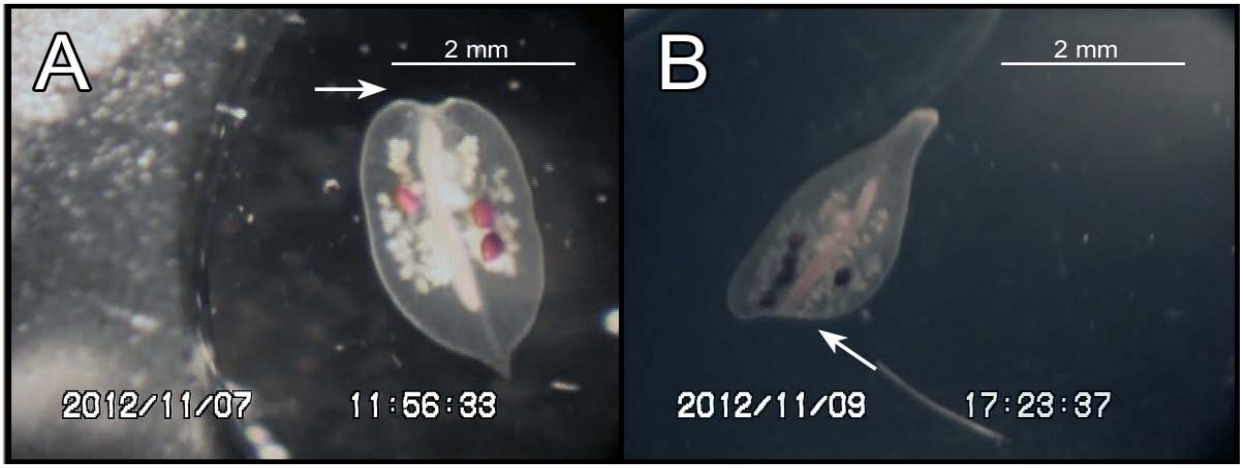

FIGURE 11: A. *M. ehrenbergii* with missing head (arrow). **B.** *M. ehrenbergii* with missing tail (arrow).