SUFFICIENT CHLOROPLAST DNA IS NECESSARY FOR GERM CELL DEVELOPMENT IN VOLVOX POWERSII: AN EMERGING MODEL FOR THE EVOLUTION OF CELLULAR DIFFERENTIATION

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ABSTRACT

Germ-soma cellular differentiation plays a key role in the evolutionary transition from single-celled individuality to multicellular individuality. The volvocine green algae serve as a model system for studying the evolution of multicellularity and cellular differentiation. *Volvox carteri* is the best studied species in its genus and is consequently a well-established model for the evolution of multicellular development. However, *V. carteri* possesses a derived developmental program in which cellular differentiation is determined by differences in cell size that arise through asymmetric division. Compared to other *Volvox* species, *Volvox powersii* is less studied and has ancestral features, including a developmental program in which large gonidia undergo rapid, symmetrical divisions with cellular differentiation occurring after hatching. With the absence of asymmetrical divisions, embryonic cell size cannot be a valid determinant of cell fate. We hypothesized that chloroplast DNA (cpDNA) inheritance may be a determining factor in cell fate due to expression levels of photosynthetic genes varying greatly among somatic and germ cells and greater amounts of chloroplast DNA in germ cells. Using gyrase inhibitors Nalidixic acid and Novobiocin, *Volvox powersii* cultures were manipulated to disrupt the distribution of cpDNA throughout the colonies and analyze the resulting distribution of cell types, the size of each cell, and the number of offspring in consecutive generations. Our results indicate that there is a specific amount of cpDNA that must be present in each cell to be able to fully develop into a germ cell.

INTRODUCTION

Understanding the mechanisms through which simple single-celled organisms evolved into complex multicellular organisms remains a persistent evolutionary puzzle. This transition has evolved over 25 times independently across the tree of life (Grosberg & Strathmann, 2007; Lehtonen et al., 2016). The volvocine green algae are an emerging model system well suited to studying this challenging evolutionary transition, as well as understanding processes of convergent evolution. Collectively, this single clade of algae contains species that represent a gradient of morphological complexity ranging from single-celled to differentiated multicellularity (Coleman, 2012). Even more remarkable is that many complex traits such as cellular differentiation evolved multiple times independently within the volvocine green algae (Hanschen et al, 2018b; Herron & Michod, 2008a).

The volvocine green algae are a group of facultatively sexual eukaryotic Chlorophyte algae in the order Chlamydomonadales. Collectively they span a range of complexity from single-celled to multicellular organisms with two distinct cell-types (Coleman, 2012; Herron & Michod, 2008b; D. L. Kirk, 1998; Michod, 2007). Single-celled algae in the genus *Chlamydomonas* represent the simplest members of the volvocine green algae clade. Flat multicellular genera that lack cellular differentiation in the genera *Tetrabaena* and *Gonium* represent the next level of complexity. Algae in genera *Eudorina*, *Pandorina*, and *Yamagishiella* are yet more complex because they possess more cells (16 - 64) arranged in a three-dimensional sphere or ellipsoid. Cellular differentiation is seen in the genera *Pleodorina* and *Astrephomene* which possess specialized somatic cells and undifferentiated cells, introducing a higher level of complexity. Finally, the most complex genus of volvocine algae is *Volvox* which possesses hundreds to thousands of cells arranged in a sphere, and differentiated into two functionally distinct cell-types, germ and soma. Multicellularity was thought to have evolved once in the volvocine green algae ~240 million

years ago (Herron et al., 2009), although a recent study suggests multicellularity may have evolved twice (Lindsey et al., 2021). Regardless, the origin(s) of multicellularity in the volvocine algae are relatively recent when compared to the origin of multicellularity in plants (748 – 872 mya) and animals (574 – 852 mya) (Sharpe et al., 2014). This diversity in levels of multicellular complexity, recent origin of multicellularity and ease of use in the laboratory make the volvocine green algae an informative model system for studying the transitions from unicellular to multicellular life (Hanschen et al., 2018a; D. L. Kirk, 1998; Matt & Umen, 2016).

In addition, an underappreciated strength of the volvocine green algae is that they are well suited to studying convergent evolution. Most volvocine green algae were named based on morphology alone prior to the arrival of molecular phylogenetics. This established a practice of comparing representative species with different levels of multicellular complexity to one another in order to learn about the developmental and genetic changes that occur during the evolution of multicellularity (Z. Grochau-Wright et al., 2017; Hanschen et al., 2016, 2017; Matt & Umen, 2016; Olson & Nedelcu, 2016). While this approach has certainly been fruitful, the advent of molecular phylogenetics revealed that nearly all genera of volvocine algae are polyphyletic (Nozaki et al., 2000, 2006; Yamada et al., 2008). It is now apparent that many traits associated with the evolution of complex multicellularity such as a large number of cells and germ-soma cellular differentiation evolved multiple times independently within the volvocine green algae through convergent evolution (Herron et al., 2010; Herron & Michod, 2008b).

Species in the genus *Volvox* provide a unique opportunity to study the convergent evolution of differentiated multicellularity. The *Volvox* genus is the most complex of the multicellular volvocine green algae. Characteristic traits of *Volvox* species include: possessing hundreds to thousands of cells, most of which are specialized somatic cells; relatively large size (~0.3 – 1.5 mm); two distinct specialized cell types (germ and soma); and oogamous reproduction with internal fertilization (Hanschen et al., 2018a; Herron et al., 2010). But despite these common characteristics, *Volvox* species have evolved 3 – 4 times independently with the most distantly related species estimated to have diverged ~200 million years ago (Herron et al., 2009, 2010).

Four distinct developmental programs have been described among divergent *Volvox* species. The differences between these developmental programs consider if 1) Germ cells are large or small at the beginning of cleavage, 2) If there is growth between cell cleavages during development or not, 3) If the rate of cell cleavage during development is fast or slow, and 4) If cell division is equal throughout development or if asymmetric cell division occurs during development (Desnitsky, 1995). Despite the characterization of the four different *Volvox* developmental programs over 25 years ago, very little is known about the developmental and genetic mechanisms that control development in all but one program.

The vast majority of research on *Volvox* species has focused on *Volvox carteri* with comparatively little work done on other *Volvox* species. *V. carteri* possesses a type 2 developmental program which is characterized by having large germ cells at cleavage initiation, rapid cell divisions with no growth between divisions, and several rounds of asymmetric division in anterior embryonic cells (Desnitsky, 1995). These asymmetric divisions result in ~16 large germ-progenitor cells that cease dividing early during the cleavage phase of development and will eventually differentiate into germ cells. All other cells continue dividing symmetrically until the end of cleavage and are many times smaller than the germ progenitor cells, these smaller embryonic cells will differentiate into somatic cells. Cell size at the end of cleavage has been shown to be sufficient to determine cell fate in *V. carteri*. Embryonic cells that are < 8µm will differentiate into somatic cells while larger embryonic cells will differentiate into germ cells (Kirk et al. 1993).

In contrast, our focal species *Volvox powersii* evolved characteristics of the *Volvox* body plan independently from *V. carteri* and possesses a type 1 development program, which is thought to be an ancestral mode of development (Desnitski, 1995; Grochau-Wright et al., 2021; vande Berg & Starr, 1971). Unlike *V. carteri* and other D2 species, all cell divisions in *V. powersii* are symmetrical throughout development which is more similar to the development of *Pleodorina* species and undifferentiated volvocine algae. Development in *V. powersii* begins with a large germ cell that divides continuously 8-9 times resulting in an embryo with ~200 – 500 cells of similar sizes (Grochau-Wright, 2019, 2021; vande

Berg & Starr, 1971). Juvenile *V. powersii* colonies then hatch from the mother colony with no distinguishable cell differentiation. As the colonies mature, they expand in size through extracellular matrix deposition and germ cells begin to enlarge in the posterior portion of the colony.
While differences in embryonic cell size play a role in cell fate for *Volvox* species with type 2 development, like *V. carteri*; the lack of asymmetric divisions during cleavage, similarity in cell sizes after cleavage, and differentiation occurring after hatching indicate that *V. powersii* and other *Volvox* species with type 1 development must determine cell fate in a different way. One hypothesis for the signal that determines cell fate in *V. powersii* is differential inheritance of chloroplast DNA (cpDNA) (Grochau-Wright et al 2019). This hypothesis predicts that cpDNA is unevenly distributed in the germ cell of *V. powersii* leading to some daughter cells inheriting more cpDNA than others during cleavage. Those cells that inherit more cpDNA are more likely to differentiate into germ cells during colony maturation while cells that inherit little to no cpDNA will differentiate into soma. This hypothesis was based on several prior observations: 1) Germ cells of *V. carteri* perform significantly more photosynthesis than somatic cells (Matt and Umen 2018), 2) Inheritance of an unknown cytoplasmic factor is thought to be necessary for cell fate determination in other volvocine algae species, specifically *Pleodorina starrii* and *Volvox obversus* (Herron et al. 2014, Ransick 1991 & 1993), 3) The germ cells of *V. carteri* contain a large amount of cpDNA while somatic cells possess little to no cpDNA (Coleman and Maguire 1982), 4) A recent preliminary investigation into *V. powersii* development found an anterior to posterior gradient in cpDNA content in cells of an unhatched juvenile which mirrors the gradient in germ cell density in adult colonies (Grochau-Wright 2019).

To test the hypothesis that differential inheritance of cpDNA content determines cell fate in *V. powersii* we exposed developing *V. powersii* colonies to the gyrase inhibitors Novobiocin and Nalidixic acid. These antibiotics have previously been shown to manipulate cpDNA nucleoid development in *Chlamydomonas reinhardtii* (Odahara et al., 2016). We predicted that exposure to these antibiotics will result in colonies with more somatic cells and fewer germ cells because the antibiotics would cause fewer cells to inherit cpDNA during development. Testing if cpDNA acts as a cytoplasmic factor during cell fate determination in *V. powersii* will help us understand how complex multicellularity evolved multiple times independently in the volvocine green algae.

MATERIALS AND METHODS

Algae growth conditions

Wild-type *Volvox powersii* (Grochau-Wright et al. 2021) was grown in a 6-well tissue culture plate with ~8mL of standard *Volvox* media (SVM) in each well. The well plates were incubated at 25°C on a 16:8 day:night light cycle. All antibiotic experiments described below were initiated with recently hatched juveniles from actively growing semi-synchronous starter cultures.

Antibiotic experiments

To manipulate the cultures of *V. powersii* we used antibiotics Nalidixic acid and Novobiocin, both gyrase inhibitors. Specifically, Nalidixic acid does not allow gyrase to disassociate with DNA when it is covalently bound to it, leading to double-stranded breaks in the DNA. Novobiocin uses a different approach as it prevents ATPase activity, therefore inhibiting the binding of gyrase to DNA through covalent bonds. Previous work treating *C. reinhardtii* with both antibiotics and fluorescence microscopy revealed a decrease in the number of chloroplast nucleoids as well as an increase in size of each nucleoid, dependent on the dosage (Odahara et al., 2016).
Given this effect in *C. reinhardtii* chloroplast DNA (cpDNA), these antibiotics are thought to have the same effect on *V. powersii* in which the distribution of nucleoids is altered resulting in fewer, larger nucleoids. When treating *V. powersii* colonies with Nalidixic acid, however, low concentration did not seem to have an effect while higher concentrations were lethal to the cultures. Therefore, as we continued our experiments, we opted to only use Novobiocin as our treatment.

Experiment 1: Is there a change in somatic cells?

Recently hatched juvenile colonies from an actively growing *V. powersii* culture were transferred into new wells (~10 colonies/well) and antibiotic stock solutions were added to achieve the following concentrations: no antibiotic treatment (Control), 0.2 mM Nalidixic acid (NA0.2), 0.4 mM Nalidixic acid

(NA0.4), 0.6 mM Nalidixic acid (NA0.6), 0.8 mM Nalidixic acid (NA 0.8), 400 µM Novobiocin (Novo400), 500 µM Novobiocin (Novo500), 600 µM Novobiocin (Novo600), 700 µM Novobiocin (Novo700), and 800 µM Novobiocin (Novo800). The inoculated well plates were placed back in the same incubator conditions described above (25°C; 16:8 day:night cycle). By the sixth day all the treatments had hatchlings, except the NA0.6, NA0.8, Novo600, Novo700, and Novo800 treatments which never had offspring.

Images were captured of the mature hatched generation from the control group, NA0.2, NA0.4, Novo400, and Novo500 treatments using a ZEISS AxioLab A1 microscope fitted with an AxioCam ERc 5s camera that was operated with ZEISS ZEN 3.2 Blue Edition software. Images were taken on 50x, 100x and 400x magnification, varying due to the size of the colony being photographed.

Images from the Control, NA0.2, and Novo400 treatments were then analyzed using FIJI (Schindelin et al. 2012). The "Cell Counter" function was then initialized to count the germ, soma, and intermediate cells within a colony. Based on *V. powersii* cell diameter estimates from Grochau-Wright et al. (2021) we defined germ cells as $\geq 25\ \mu m$, somatic cells as $\leq 15\ \mu m$, and intermediate cells $15\ \mu m < x < 25\ \mu m$. To differentiate between the three cell types, the line tool was used to measure the diameter of the cells, and the cells were accordingly labeled by cell type. We counted cell numbers for a total of 22 colonies from the Control, 23 colonies from the NA0.2 treatment, and 21 colonies from the Novo400 treatment. An ANOVA test was performed to determine if there was a significant difference in proportion of each cell type among the treatments.

Experiment 2: What are intermediate cells?

Cultures of recently hatched juvenile *Volvox powersii* were treated with Novobiocin in differing concentrations: 0 µM (control), 100 µM, 200 µM, 300 µM, and 400 µM. The inoculated well plates (~10 colonies/well) were placed back in the same incubator conditions as previously described (25°C; 16:8 day:night cycle). The colonies were monitored daily noting their developmental progress. When the hatched generation showed signs of early-stage embryos, images of colonies from each treatment and control were taken using the same procedure as our previous experiment. The line tool was utilized to measure the diameter of 10 randomly selected cells/embryos from 24 different colonies for each treatment, resulting in 240 randomly selected cell diameter measurements for each treatment. A dip test was performed on each treatment to determine if the distribution of cell sizes fit a unimodal or multimodal distribution.

Experiment 3: Counting offspring across three generations

A total of 24 wells of recently hatched juvenile colonies (~10 colonies/well) were randomly treated with 400 µM Novobiocin or left untreated, resulting in 12 control wells and 12 antibiotic treatment wells. Wells were checked daily to monitor development. Once the next generation had hatched, the well was stirred and a 500 µL sample was taken from the well to count the number of offspring colonies and estimate the total number of offspring in the well. The same procedure was applied to the next generation as well. A t-test was used to determine if there was a significant difference in the number of offspring between the control and antibiotic treatment for each generation.

RESULTS AND DISCUSSION

Experiment 1: Is there a change in somatic cells?

The purpose of this experiment was to investigate cell fate determination mechanisms in *Volvox powersii* to try and understand how these mechanisms differ across *Volvox* lineages with different developmental programs. Previous preliminary research found a gradient in cpDNA nucleoids along the anterior-posterior axis of an unhatched *V. powersii* juvenile that appeared to mirror the gradient in cell fate seen in adult colonies (Grochau-Wright 2019). With this in mind, we proposed that inheritance of chloroplast nucleoids determines cell fate in *V. powersii*. Cells that inherit more cpDNA are expected to be more likely to become germ cells, while those with less cpDNA would be more likely to become somatic cells. The two antibiotics we used have been shown to alter cpDNA development in *C. reinhardtii* (Novobiocin and Nalidixic acid) (Odahara et al., 2016), ultimately resulting in fewer cpDNA nucleoids. We expected to see a decrease in proportion of germ cells and increase in proportion of somatic cells in cultures treated with Novobiocin and Nalidixic acid compared to the control.

Following antibiotic treatment, the total cell number was not significantly different among any of the treatments ($F = 0.848$, $d.f = 2$, $p = 0.433$). Offspring colonies in the control group had an average of 237 cells per colony (SE = 11), those in the 0.2 mM Nalidixic acid treatment had an average of 242 cells per colony (SE = 21), and those in the 400 uM Novobiocin treatment had an average of 216 cells per colony (SE = 8) (Figure 1A). All other treatments died out. The proportion of somatic cells did not significantly differ between treatments ($F = 1.487$, $d.f. = 2$, $p = 0.234$) with the control colonies having 63% soma, the novobiocin treatment 65% soma, and the nalidixic acid treatment 61% soma on average (Figure 1B). In contrast, colonies from the novobiocin treatment had a significantly lower proportion of germ cells (19%) than colonies from the nalidixic acid (37%; $p < 0.001$) and the control treatment (37%; $p < 0.001$). This difference is because colonies from the novobiocin treatment had a significantly higher proportion of intermediate cells (16%) compared to the control (0.2%; $p < 0.001$) and nalidixic acid treatments (2%; $p < 0.001$).

Our prediction that the proportion of somatic cells would be greater in antibiotic treated cultures was not observed. This indicates that a lack of chloroplast DNA is not a signal to the cell to differentiate as soma. Instead, to our surprise, a new type of cell emerged in antibiotic treated cultures that appeared to be intermediate in size between large germ cells and small somatic cells, thus we classified these cells as intermediate cells. To determine what led to the development of intermediate cells we repeated our experiments focusing on novobiocin and the properties of the different types of cells.

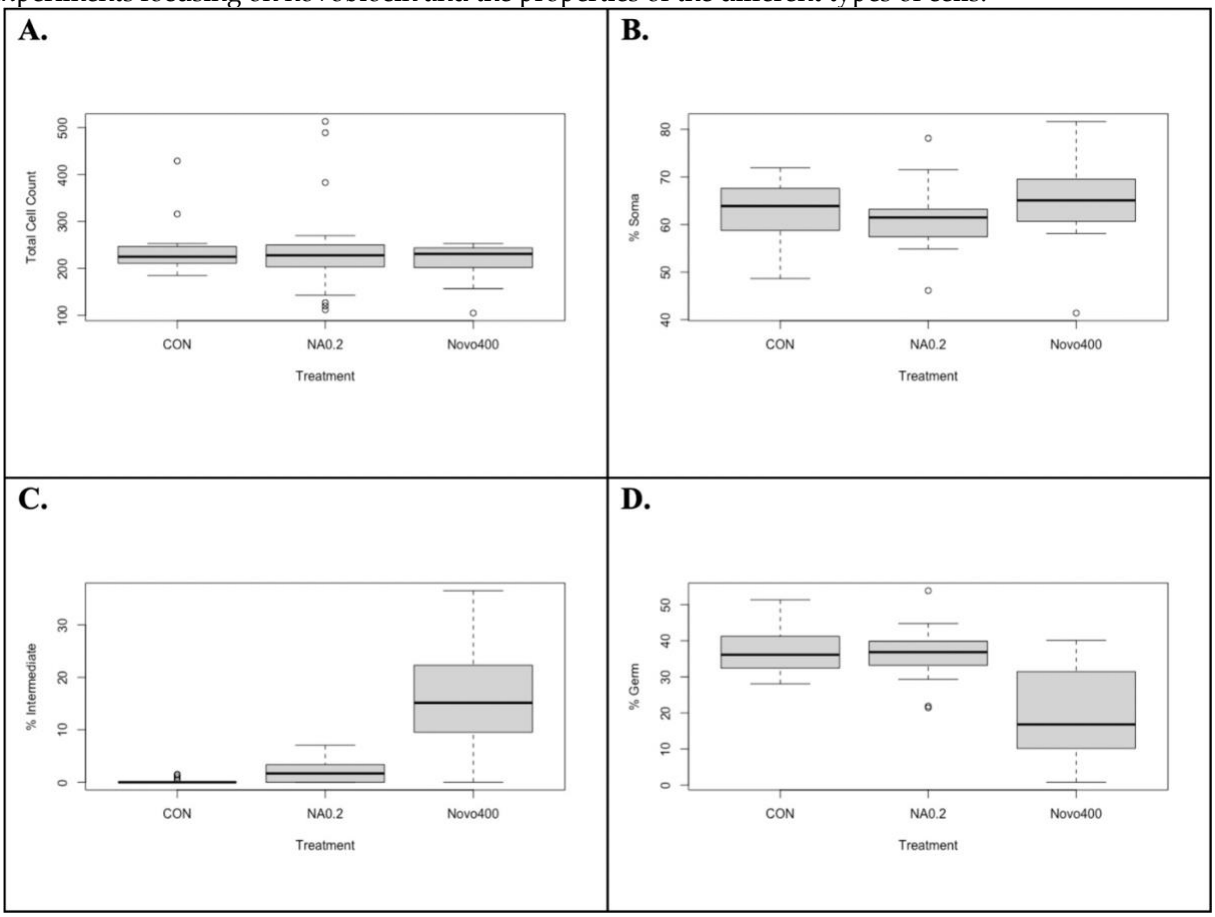

Figure 1. Box plots showing A) no significant difference in the total cell count ($p = 0.433$), B) no significant difference in proportion of soma cells ($p = 0.234$), C) significantly higher proportion of intermediate cells in Novo400 treatment ($p < 0.001$) and D) significantly fewer germ cells in Novo400 treatment ($p < 0.001$).

Experiment 2: What are intermediate cells?

In experiment 1 we found that *V. powersii* colonies treated with novobiocin developed a novel cell type that was larger than typical somatic cells yet smaller than typical germ cells. We hypothesized that these intermediate cells are failed germ cells that were not able to support enough photosynthesis to grow to germ cell size due to a lack of cpDNA. To test this hypothesis, we repeated our novobiocin antibiotic treatment experiment and focused on measuring the distribution of cell sizes in untreated *V. powersii* colonies and colonies treated with different concentrations of antibiotic. Our hypothesis predicts that size of cells in untreated colonies will be bimodal, with a set of small somatic cells and a set of large germ cells while treated colonies will have a different distribution of cell sizes.

As expected, we found that the distribution of cell diameters was not unimodal for colonies grown under our control conditions ($p = 0.043$). The distribution of cell diameters has two distinct peaks, corresponding to germ cells and somatic cells (Figure 2). The Novo100 ($p < 0.05$) and Novo200 ($p = 0.005$) treatments were similarly non-unimodal with two distinct peaks (Figure 2). In contrast, the distribution of cell diameters for the Novo300 ($p = 0.218$) and Novo400 ($p = 0.194$) were not significantly different from unimodality. Visual inspection of the distribution shows this difference is not due to a change in somatic cell size, which is consistent with experiment 1. Instead, the peak for germ cells appears to have shifted left, toward the somatic cell peak (Figure 2).

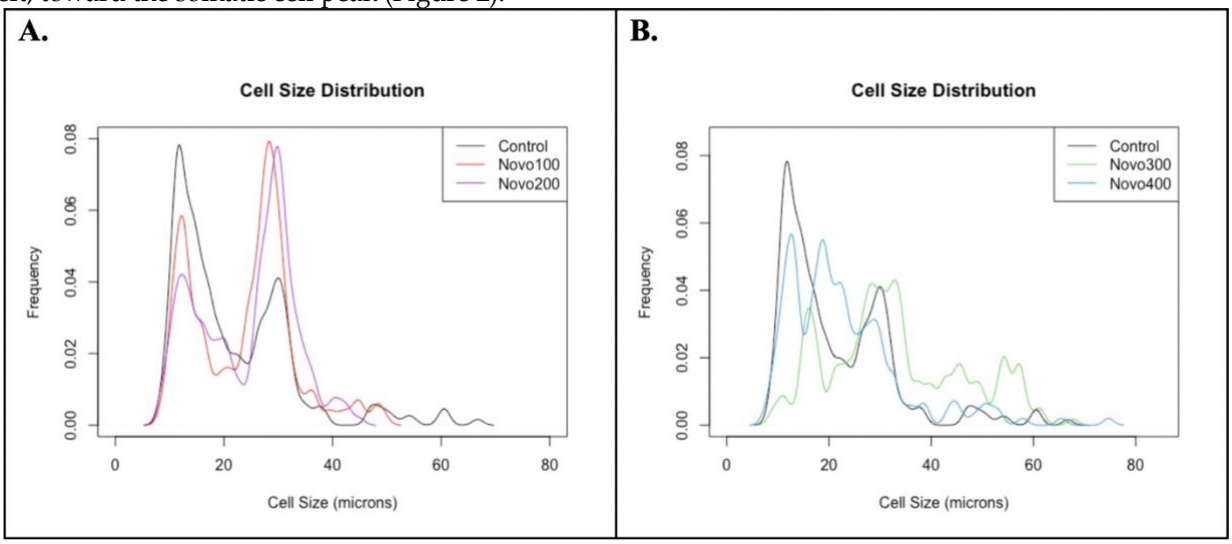

Figure 2. Cell size distribution plot with A) the control and treatments that were significantly different from unimodality (Novo100, red; Novo200, purple), and B) with the control compared to treatments that were not significantly different from unimodality (Novo300, green; Novo400, blue).

Experiment 3: Counting offspring across three generations

Further to test our hypothesis that intermediate cells are failed germ cells we performed an additional experiment tracking reproduction for three generations. Intermediate cells are observed in the offspring of colonies that are exposed to antibiotic treatment. We hypothesized that the intermediate cells are failed germ cells that would have fully developed into reproductive cells except the antibiotic prevented them from inheriting sufficient chloroplast DNA to maintain a high level of photosynthesis. This hypothesis predicts that colonies with intermediate cells will have fewer offspring than untreated colonies with normal proportions of germ and somatic cells.

Generation 0 for both the control and antibiotic treated cultures were established from newly hatched juveniles of the same semi-synchronous starter culture. There was no significant difference in the number of offspring of Generation 0, called Generation 1 ($p = 0.918$, Figure 3). Generation 1 was allowed to continue to grow and reproduce, generating Generation 2 where a significant difference between numbers of offspring was observed between the control and antibiotic treatment conditions ($p = 0.045$). These results support the hypothesis that intermediate cells are failed germ cells. The parental generation (G0) treated with Novobiocin would result in germ cells with an atypical distribution of cpDNA in their

chloroplasts. Both treated and untreated cultures have a normal number of offspring (G1), because these germ cells were already formed with the correct amount of cpDNA. But during DNA replication in the germ cells of the G0 generation, the antibiotic prevents the separation of cpDNA copies from each other resulting in fewer nucleoids to provision the next generation with. The G1 colonies would then have some germ cells that inherited enough cpDNA to be able to complete germ cell development; however, some cells would not have inherited enough cpDNA to complete a germ cell development, therefore resulting in less offspring in the following generation (G2).

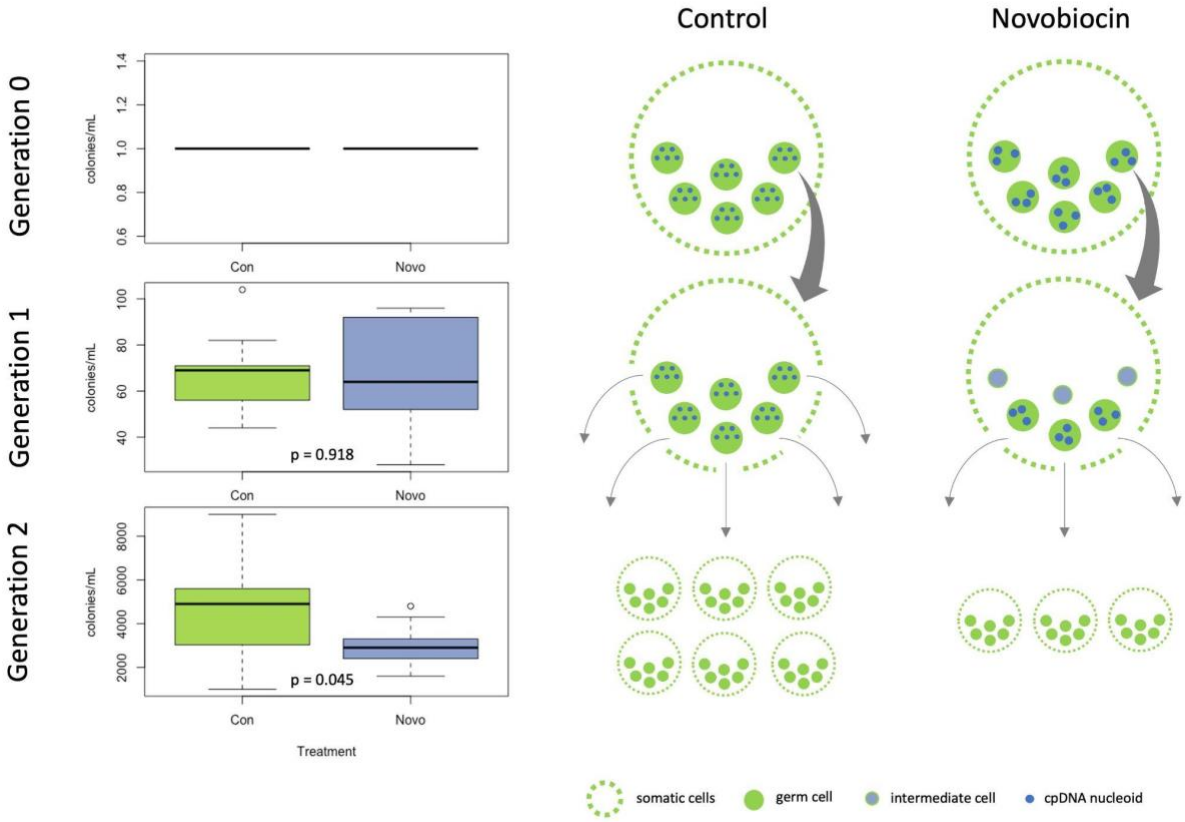

Figure 3. Counting offspring of *V. powersii* colonies across three generations (Generation 0 - 2) in the Control and Novobiocin treatment. In the Control treatment, germ cells have many chloroplast DNA (cpDNA) nucleoids that are concentrated on one end of the cell (G0). The germ cells undergo development and hatch as the next generation of offspring (G1), with germ cells identical to those of the previous generation (G0). These germ cells hatch as the next generation of offspring (G2), once again identical to the previous generations (G0, G1). In the Novobiocin treatment, colonies are treated with 400 µM Novobiocin, resulting in germ cells with fewer, larger cpDNA nucleoids (G0). These germ cells with disrupted cpDNA distribution hatch as the next generation (G1) that contain some fully developed germ cells with fewer, larger cpDNA nucleoids while other cells did not receive enough cpDNA to fully develop into germ cells (intermediate cells). Only the few, fully developed germ cells hatch, therefore the next generation (G2) has less colonies.

CONCLUSION

Through these three consecutive experiments, we have concluded that: 1. Our original hypothesis that lack of chloroplast DNA is a signal to the cell to develop into soma was incorrect because there is no significant difference between somatic cells in control and treatment cultures; 2. There is a specific amount of chloroplast DNA that must be present to be able to fully develop into a germ cell; and 3.

Intermediate cells are failed germ cells that did not reach that threshold of cpDNA due to the effects of the antibiotic novobiocin.

The objective of these experiments was to understand how cell fate is determined in *V. powersii*. This species is uniquely suited to understanding the origin of cellular differentiation and multicellular development because it evolved the *Volvox* body plan independently of the model organism *V. carteri* while still maintaining a less derived developmental program (Herron et al. 2010). While we did not discover what signals embryonic *V. powersii* cells to develop as soma, we did unexpectedly discover that cpDNA plays a major role in the late stages of germ cell development. Further work using fluorescence microscopy might be able to determine the minimal amount of cpDNA needed for germ cell development in *V. powersii*. In addition, what initially triggers a cell to develop into either soma or germ remains to be determined for *V. powersii* but this and previous work help narrow down the list of possibilities. Unlike *V. carteri* embryonic cell size does not appear to play a role in the early stages of cellular differentiation (Grochau-Wright 2019) nor does the amount of chloroplast DNA (this work).

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