CONSEQUENCES OF AN INDUCIBLE DEFENSE: THE ECOLOGICAL AND EVOLUTIONARY
REPERCUSSIONS OF TEMPORARY COLONY FORMATION IN CHLAMYDOMONAS REINHARDTII

By

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ABSTRACT OF THE DISSERTATION:

CONSEQUENCES OF AN INDUCIBLE DEFENSE: THE ECOLOGICAL AND EVOLUTIONARY REPERCUSSIONS OF TEMPORARY COLONY FORMATION IN CHLAMYDOMONAS REINHARDTII

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This dissertation attempts, through four connected experiments, to demonstrate the range of ecological and evolutionary responses to induced anti-predator defenses. The presented works all incorporate the formation of anti-predator colonies by the green alga Chlamydomonas reinhardtii in response to the filter feeding rotifer Brachionus calyciflorus and ciliated protist Euplotes eurystomus. Chapter one demonstrates that while it is an effective defense against micrograzing filter feeders, colony formation allows for opportunistic exploitation of colonies by macrograzers. Chapter two provides evidence that as part of other indirect effects in a simple community, the formation of inducible colony defenses can lead to C. reinhardtii being a superior apparent competitor. Chapter three describes the results of a differential gene expression study comparing C. reinhardtii phenotypes and homologous genes within the multicellular Volvocales. Chapter four considers the evolutionary consequences of colony defense by experimentally manipulating the phenotype to compensate for two distinct modes of predation. Taken together, these results demonstrate the capacity for inducible defenses to have dramatic consequences outside of the predator-prey interaction that induces the defense.
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INTRODUCTION

Community-induced phenotypically plastic traits are abundant in nature, typically observed as changes in morphology, biochemistry, and behavior (Agrawal 2001; Miner et al. 2005). Interest has grown concerning the ecological impacts of these plastic responses on individuals and communities (DeWitt and Scheiner 2004), with effects ranging from the stabilization of microbial communities through anti-predator defenses (Vos et al. 2004) to helping bird species adjust to climate change (Charmantier et al. 2008). In considering phenotypically plastic traits, a more complete (whole organism) understanding can increase appreciation of the broader ecological outcomes resulting from the plasticity (Salinas and Munch 2014; Forsman 2015). Understanding consequences of plasticity requires that we consider a variety of specific types of plasticity to clarify broader implications of plasticity beyond the species expressing the plastic response (Turcotte and Levine 2016). When the pressures that induce this phenotypic plasticity result from community interactions (competition, predation, mutualism, etc.), it offers unique opportunities to study species through ecological and evolutionary lenses.

Inducible defenses are a complex type of plastic response which contribute to diverse natural systems resulting in morphological, behavioral, or biochemical changes in response to ecological stimuli (Adler and Drew Harvell 1990) and can influence a variety of ecological and evolutionary outcomes (Tollrian and Harvell 1999). Models predict that inducible defenses can influence community structure and stability both within and outside the predator-prey interaction involved in the defense (Ramos-Jiliberto 2003; Vos
et al. 2004; Ramos-Jiliberto et al. 2008; Garay-Narváez and Ramos-Jiliberto 2009). These predicted roles of inducible defenses have been supported by a number of empirical studies (van der Stap et al. 2008; Boeing and Ramcharan 2010; Aránguiz-Acuña et al. 2011), though there is a need to expand beyond the focal group of the defense to understand potential short and long-term consequences at the community level (Hammill et al. 2015). Such expansion to more complex community modules creates conditions that alter defense success and can shift the selective pressures acting on either the induced or uninduced phenotype (Fischer et al. 2012). This creates a fascinating opportunity to not only understand how communities might be altered by inducible defenses, but also to incorporate the shifting selective pressures in such communities to understand evolutionary processes through the lens of community-level interactions (Fordyce 2006).

To evaluate the role of inducible defenses, I studied the green alga Chlamydomonas reinhardtii in experimental microcosms where the filter feeding rotifer Brachionus calyciflorus or ciliated protist Euplotes eurystomus induces a defensive multicellular colonial phenotype.

C. reinhardtii is a biflagellated unicellular green alga that has been used as a model species for resolving a variety of biological questions (Harris et al. 2009). Colony formation by C. reinhardtii in response to predation by rotifers such as B. calyciflorus is effective at reducing the influence of predation by the micrograzing predator (Lurling and Beekman 2006), likely through gape limitation (Becks et al 2012) as seen in other algal species (Boraas et al. 1998). Colonies consist of dozens to hundreds of cells encased in an extracellular matrix maintaining the colony. The matrix limits flagellar activity and
motility, and the negative buoyancy of *C. reinhardtii* cells together with the sheer size of
the colonies results in their immobility. This is a direct cost of colony formation, as
sinking potentially removes *C. reinhardtii* colonies from the photic zone and poor
nutrient uptake. Defensive *Chlamydomonas* colonies have been used to answer a variety
of questions about the role of habitat quality on defense success (Fischer et al. 2012), the
functional genomics of inducible defenses (Becks et al. 2012), and the role of
intraspecific variation on defense strategies (Sathe and Durand 2016). The natural history
of the Volvocales and the basal position of unicellular *C. reinhardtii* in that largely
multicellular lineage makes *C. reinhardtii* colony formation an exciting opportunity to
examine the role of inducible defenses in a broader community context. In addition, the
short generation time of *C. reinhardtii* (~5-8 hours) allows examination of how
community level influences on a temporary multicellular phenotype might contribute to
more permanent multicellularity.

The *C. reinhardtii* inducible defense has allowed me to evaluate various
evolutionary and ecological effects of the defense. As in other work investigating protist
microcosms in ecological and evolutionary time, colony formation by *C. reinhardtii* has
proven to be well suited to expanding current knowledge of the behavior of inducible
defenses at those time scales. I have used this system to examine the impacts of colonial
defenses on community interactions such as opportunistic predation (Chapter 1) and
participating in complex networks of both direct and indirect effects that can result from
either positive or negative interactions (Chapter 2). Through examining the genes used by
the colonies in a differential gene expression study, I was able to better characterize the
role of inducible defenses at the molecular level, while exploring the potential role of
differentially expressed genes in an evolutionary context (Chapter 3). Lastly, I was able
to experimentally select for C. reinhardtii colonies which better tolerate predation by two
distinct predator types, and in doing so demonstrate the adaptability of the defense and
the ease with which the transition between temporary and permanent multicellularity
might occur (Chapter 4).

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CHAPTER 1: CONTEXT-DEPENDENT COSTS OF INDUCIBLE DEFENSES: WHEN IS IT BEST FOR *CHLAMYDOMONAS REINHARDTII* TO SINK OR SWIM?

Summary

Inducible defenses can create drastic changes at multiple levels of biological organization, acting on both ecological and evolutionary timescales. As community composition changes over evolutionary time, resulting from both shifting fitness landscapes as well as the emergence of novel predators, the costs or benefits of a particular defense can change immensely. The unicellular green alga *Chlamydomonas reinhardtii* defends itself against small gape-limited filter feeders by expressing an inducible non-motile multicellular phenotype. I examined whether addition of a macrograzer (the snail *Physa fontinalis*) might alter the interaction between *Chlamydomonas* and the filter-feeding rotifer *Brachionus calyciflorus*. The rotifer can coexist with both the unicellular and colonial *Chlamydomonas* phenotypes for many generations. Macrograzers caused populations of both *Chlamydomonas* and *Brachionus* to crash. Consumption of *Chlamydomonas* colonies by macrograzers eliminated the size refuge provided by the colonial phenotype and simultaneously eliminated the defended source of unicells that sustained *Brachionus* populations. In nature, *Chlamydomonas* could potentially overcome these strong contrasting selective pressures in spatially heterogeneous environments, where macro- and micrograzers use different habitats. The combination of selective pressures employed in this study could explain some aspects of
early Volvocale evolution, as *Chlamydomonas*-like ancestors would have found multicellularity coupled with motility to be highly advantageous in communities containing gape-limited micrograzers and macrograzers.

**Introduction**

Inducible defenses can vary in effectiveness due both to costs associated with the pairwise interactions that drive them (DeWitt, Sih & Wilson, 1998), as well as the composition of the community in which they are expressed (Sih, Englund & Wooster, 1998). Opportunities exist to explore the influence of these pairwise interactions on composition and diversity at the community level (Agrawal, 2005; Whitman & Agrawal, 2009; Kishida et al., 2009). An induced defense might alter trophic structure (Vos et al., 2004; Sakamoto et al., 2015), influence community diversity (Harvell, 1990; Miner et al., 2005; Poelman, van Loon & Dicke, 2008; Herzog & Laforsch, 2013) or change evolutionary lineages through creation of alternate phenotypes (Agrawal, 2001; Nunes et al., 2014; Campbell, 2015). An expanding focus on community level impacts of inducible defenses allows for examination of the role and conditions for these plastic responses driving lasting evolutionary change.

Costs of inducible defenses are often assessed as reductions in primary productivity (Valladares, Gianoli & Gomez, 2007), growth rate (Buskirk, 2000), or reproductive success (Harvell, 1986). Changes at the community level can modify these costs, though the complexity of many community assemblages can make studying their role in defense success difficult. Increasing the complexity of community modules beyond the central pairwise interaction can be used to determine effects of defense
systems on broader ecological scales (DeWitt & Scheiner, 2004; Stillwell et al., 2007; Bourdeau, 2009; Walzer & Schausberger, 2013; Hoverman & Relyea, 2016). While the effects of inducible defenses on community interactions vary (Harvell, 1990; Miner et al., 2005), emphasis is often placed on pairwise interactions (e.g. Ramirez & Eubanks 2016) or predation of the defended phenotype by novel predators (Relyea, 2003). While it is possible to examine the consequences of community interactions other than predation (Scheiner, Schlichting & Pigliucci, 1999; Pigliucci, 2001), studies that focus on the exploitation of defensive phenotypes by secondary predators have become more common due to the relative ease of studying relationships between predators and prey.

A number of studies have demonstrated influences of alternate predators on the expression and consequences of inducible defenses (Persons et al., 2001; Beckerman, Rodgers & Dennis, 2010; Hoverman & Relyea, 2016), though few systems explore potential lasting consequences of increasing trophic complexity on the success of inducible defenses. Models of systems containing two predator species and a single prey species with inducible defenses suggest that exposure to disparate predator types can create chaotic dynamics unless the prey can somehow adapt to both predators (Ellner & Becks, 2011). Empirical work by Hirsch (2014) using zebra mussels (*Dreissena polymorpha*) demonstrated that a variety of environmental factors (predator diversity/intraspecific competition/abiotic stressors) ultimately contribute to the prey phenotype, though predation risk was offset through expression of an intermediate phenotype. Many factors influence defense success by affecting costs of defense expression with outcomes that vary as much as the systems in which they occur. Changes
in predator diversity for a given community offer a straightforward means to examine the
effects of defense costs on short-term community stability and long-term defense
effectiveness.

Here I describe the ecological consequences and potential evolutionary
implications of an inducible defense employed by *Chlamydomonas reinhardtii* against
the filter-feeding rotifer *Brachionus calyciflorus* in the presence or absence of a
macrograzer, the snail *Physa fontinalis*. Previous research shows that micrograzers like *B.
calyciflorus* induce the formation of benthic nonmotile multicellular *Chlamydomonas*
colonies (Lurling & Beekman, 2006). These non-motile colonies are too large for small
gape-limited predators, like rotifers, to consume (Figure 1). The colonies, however,
provide an effective refuge for *Chlamydomonas*, resulting in the long-term persistence of
*Chlamydomonas* and *Brachionus* in culture. I examined the added impact of a
macrograzer, the freshwater snail *Physa fontinalis*, in communities containing
*Chlamydomonas* and *Brachionus* (Figure 2) to determine the influence of macrograzers
on the effectiveness of algal colony formation as a defense. This three-species
community module offers a tractable experimental system to examine how predators with
contrasting feeding strategies or prey size constraints may interact to alter the
effectiveness of size-dependent inducible defenses.

Colony formation, while defending against attack by filter feeding micrograzers
like *B. calyciflorus*, potentially creates a major vulnerability to macrograzers that
selectively consume large sessile microalgae while ignoring small motile unicells. I used
experiments with *C. reinhardtii*, *B. calyciflorus*, and *P. fontinalis* to assess whether the
alga’s colony-forming defense would incur costs resulting from macrograzer exposure, or if the combined selective pressures exerted by micrograzers and macrograzers would cause the interaction between the alga and rotifers, which normally coexist, to collapse.

Methods

Organisms and culturing conditions

Cultures of *Chlamydomonas reinhardtii* (CC-1010) were obtained from the *Chlamydomonas* Resource Center (University of Minnesota), and populations were maintained in isolation for a minimum of six months prior to combination with other species. Cultures of *Brachionus calyciflorus* were originally obtained from the laboratory of Nelson Hairston Jr. (Cornell University, Ithaca, New York) and were reared on an alternate algal species (*Ankistrodesmus* sp.) until experimental treatments began. Common bladder snails (*Physa fontinalis*) were collected from Weston’s Mill Pond (Rutgers Gardens, New Brunswick, NJ) and held in sterile medium for 24 hours before addition to treatments described below.

All populations used were maintained in either modified Woods Hole medium (Adapted from Guillard and Lorenzen 1972) (Snails and rotifers) or a 1:1 mixture of TAP medium (Gorman and Levine 1965) and modified Woods Hole medium. Fresh maintenance lines for algae were started every three weeks by adding a 50 mL inoculum of *C. reinhardtii* or *Ankistrodesmus* sp. to sterile medium. An initial inoculum of a dense rotifer culture was introduced to cultures of *Ankistrodesmus* to establish maintenance
lines without recent exposure to *Chlamydomonas*. All cultures were kept at 24° C in an incubator with a 12h light:12h dark photoperiod in 250 mL glass jars.

*Establishment of experimental microcosms*

To examine the impact of macrograzers on the persistence of the *Chlamydomonas*-Brachionus interaction and test whether macrograzers might negate fitness benefits of *C. reinhardtii* colony formation, four different grazing treatments were established: *C. reinhardtii* alone (no grazers, control), *C. reinhardtii* grazed by *B. calyciflorus*, *C. reinhardtii* grazed by both *B. calyciflorus* and *P. fontinalis*, and *C. reinhardtii* grazed by *P. fontinalis*. Each treatment was replicated four times.

All treatments received an initial inoculum of 50 mL of dense *Chlamydomonas* culture (>2 x 10^6 cells per mL) added to 150 mL glass jars. Algae then grew for one week without grazers. In the second week, individuals of *B. calyciflorus* were isolated from maintenance cultures, washed in sterile medium to remove *Ankistrodesmus*, and then transferred 20 at a time using micropipettes to eight separate 20 mL volumes of *C. reinhardtii* culture. These mixtures of *C. reinhardtii* and rotifers were added to the jars from which the 20 mL algal volumes were originally taken and subsequently cultured for four weeks to induce algal colony formation. In the fifth week, jars assigned to the two treatments containing macrograzing snails (with or without rotifers) each received four individual *P. fontinalis*.

Experimental microcosms were maintained in 250 mL jars with initially sterile 150 mL nutrient medium in a 24° C incubator and a 12L:12D photoperiod. Jars were
gently shaken by hand every two days to encourage mixing and gas exchange, and lids were loosely secured to promote gas exchange while limiting contamination.

**Monitoring and maintenance**

Experimental microcosms were maintained for ten weeks and monitored weekly to measure pH, assess changes in volume due to evaporation, observe evidence of bacterial or fungal contamination, and measure herbivore abundance. Cultures were sampled weekly to measure abundances of both unicellular and colonial *Chlamydomonas*, as well as *Brachionus*. Microcosms were gently mixed by manual shaking, and then a 1 mL subsample was removed. Sampled medium was replaced with 1 mL of fresh sterile medium to maintain culture volumes. Data collected included counts (per mL) of unicellular *C. reinhardtii*, multicellular *C. reinhardtii* colonies, and rotifers. Counts of larger colonies and rotifers were made by observing the total sample using a Nikon SMZ-U microscope (Nikon Corporation, Tokyo, Japan) at 20X, while smaller colonies and unicells were counted using a Reichert hemocytometer and a Nikon Eclipse 80i compound microscope (Nikon Corporation, Tokyo, Japan) at 400X (phase contrast). All counts were reported as the logarithm (base 10) of density of each species (for single organisms) or colonies per mL.

**Statistical analyses**

Repeated measures multivariate analysis of variance (SAS institute, Cary, North Carolina) compared the density of *C. reinhardtii* unicells, *C. reinhardtii* colonies, and rotifers among treatments over time.
Results

Impact of micrograzers (rotifers) on algal abundance and colony formation

The number of unicellular *C. reinhardtii* decreased in treatments containing *B. calyciflorus* relative to grazer-free controls within one week after the introduction of *Brachionus* (F3,12 = 524.92, p < 0.0001). There was a corresponding significant increase in the number of *C. reinhardtii* colonies after rotifer introduction (F3,12 = 311.10, p < 0.0001) (Figure 3 and 4). This inverse relationship between the abundance of algal unicells and colonies became more conspicuous over time with significant effects of grazers in the repeated measures analysis of both unicells and colonies for all weeks subsequent to rotifer addition. Rotifer population size fluctuated somewhat over time but did not change significantly prior to snail additions.

Impact of macrograzers on algal abundance and micrograzers

The combined effects of micrograzers (rotifers) and macrograzers (snails) produced a rapid decline in the total abundance of unicellular and colonial *C. reinhardtii* in the first week after macrograzer addition (F3,12 = 298.61 p < 0.004). By two weeks after macrograzer addition, the combination of *B. calyciflorus* and *P. fontinalis* drove *C. reinhardtii* below the limit of detection (Figures 2 and 3). After only two weeks of interaction, rotifers in treatments with *Physa* declined from hundreds of individuals per mL to less than 5 individuals per mL in every replicate (F1,6 = 314.36, p < 0.001) (Figure 5).
Addition of macrograzers (snails) resulted in the total collapse of *Chlamydomonas* and rotifers. Macrograzers eliminated induced algal colonies. Algal colonies are a continuous source of unicells that can sustain rotifer populations. Reduction of algal colony abundance coincided with reduced unicell abundance. The crash of unicellular *Chlamydomonas* abundance was accompanied by rapid declines in rotifers, but only in microcosms also containing *Physa* (p=0.0069). While the effects of either micrograzers or macrograzers are straightforward in isolation, with neither grazer type alone eliminating *Chlamydomonas*, the combined effect of the two grazer types effectively eliminated unicellular *Chlamydomonas*, which in turn eliminated the food supply for the rotifer.

Grazing by macrograzers alone (*P. fontinalis* without *B. calyciflorus*) did not alter the abundance of unicellular algae or induce colony formation relative to the grazer-free controls (see Figures 2-4, grazer-free controls and macrograzer treatment).

**Discussion**

One of the basic assumptions of inducible defense theory is that fitness costs associated with expression of the defense in the absence of natural enemies select against it becoming a fixed trait (Tollrian and Harvell 1999). Such costs typically take the form of reduced reproduction, as energy is diverted from reproduction or resource acquisition to support morphological, chemical, or behavioral defenses. However, other costs can accrue if the induction of one sort of defense against a particular consumer syndrome renders the prey more vulnerable to a different kind of consumer. The reality is that predators and prey interact in complex communities where different kinds of defenses are
effective against different kinds of consumers, but no single defense may be universally effective against the full range of consumers encountered (e.g., Jeffries and Lawton, 1984). The interaction among *Chlamydomonas*, micrograzers, and macrograzers provides an important and tractable model system for the complications that arise from conflicting selective pressures imposed by different kinds of consumers. Overall, the effectiveness of a particular kind of inducible defense, such as increased size created by multicellularity, can be highly context-dependent, where context refers to the array of consumers with which the prey interacts.

The context-dependency of inducible defenses can also inform us about possible scenarios involving the evolutionary transition from inducible to fixed defenses that may incorporate more than one anti-predator adaptation. In the system considered here, large but non-motile multicellular colonies are immune to planktonic micrograzers but become vulnerable to benthic macrograzers when they settle out of the water column. In contrast, small motile unicellular algae are not affected by macrograzers but remain vulnerable to micrograzers. An optimal strategy might involve a combination of increased size with the retention of motility, allowing defended colonies to evade planktonic micrograzers while avoiding benthic macrograzers. As discussed below, this appears to be the case for the multicellular relatives of *Chlamydomonas* in the Volvocales.

*C. reinhardtii* inducible colony formation offers a defense against filter-feeding micrograzers at the cost of facilitating consumption by macrograzers. This study demonstrates a potential pitfall for algal species that employ inducible colony defenses against micrograzers in situations where benthic macrograzers are also present. This
scenario has interesting implications for the community conditions that ancestors of *C. reinhardtii* and members of the Volvocales may have encountered during the evolution of permanent multicellularity.

Although inducible defenses involving colony formation are common in nature (Boraas, Seale & Boxhorn, 1998), the role of these defenses over evolutionary time scales has seldom been explored. The system used here is of special interest, both because of its experimental tractability and the evolutionary significance of *C. reinhardtii* as a unicellular model for the common ancestor of the multicellular Volvocales. Colony formation in response to micrograzers may be an initial step in the evolution of permanent multicellularity (Fisher, Bell & West, 2016), and viewing the results of this experiment in an evolutionary context could provide new avenues for inquiry for study of early multicellular forms.

*C. reinhardtii* may resemble the basal unicellular ancestor that gave rise to the multicellular lineage of Volvocine algae that culminates in *Volvox* (Herron & Nedelcu, 2015). A number of phenotypic changes required to maintain the permanently multicellular Volvocine algae have been proposed (e.g. Kirk 2005) though the selective pressures that would drive the traits seen in these lineages remain largely unexplored (Herron & Michod, 2008). The strong selection against the nonmotile colonial form seen in this experiment, coupled with our knowledge of the natural history of *C. reinhardtii* and its descendants, pose an interesting case for the role of combination of two distinct predator types on this early multicellular transition. Early ancestors of modern *C. reinhardtii* could have overcome predation by small filter feeders through expression of a
colony defense, but as new macrograzers emerged, the defense might become more of a threat than an advantage.

With a proposed divergence from the lineage that gave rise to land plants for *C. reinhardtii* of greater than 1 BYA (Merchant *et al.*, 2007) and the subsequent divergence of the multicellular Volvocales (200-300 MYA (Herron & Michod 2008)), shifting assemblages of predators could influence both the evolution of defenses like the one examined here and the lineages of the populations employing those defenses. Long-term exposure to a mixture of predator feeding modes may have selected ancestors of *C. reinhardtii* either to adapt as part of changing community modules containing benthic macrograzers or to avoid macrograzers entirely via habitat shifts. The former strategy could explain traits seen in the permanently multicellular Volvocales (multicellularity enhanced by motility), while the latter might account for the prevailing notion of *C. reinhardtii* (a species that preferentially inhabits the water column in culture) as a soil dwelling species found in ephemeral waters without macrograzers.

Given that nonmotile cells of Volvocales sink, remaining motile is important for a number of reasons, including the ability to remain in photic zones inaccessible to rapidly sinking non-motile colonies. Upward swimming rates of smaller Volvocales like *Gonium pectorale* are slower than those found in unicellular *C. reinhardtii* (Solari, Kessler & Michod, 2006), leading to conclusions that while escape from filter-feeding micrograzers provided strong selection for multicellularity, motility may not have contributed to the fitness advantages of these early transitional forms (Solari, Galzenati & Kessler, 2015). These studies have considered benefits of motility by comparing hydrodynamic
properties within the Volvocales but have not considered broader community
consequences of being a motile multicellular alga. The contrasting selective pressures
exerted by pelagic micrograzers and benthic macrograzers could explain the ubiquity of
both multicellularity and motility as advantageous traits in the multicellular Volvocales.

The selective pressures acting on *C. reinhardtii* in these experimental microcosms
have implications for understanding the conditions that led to the persistence and success
of multicellular members of the Volvocales. Colonies that form in response to filter-
feeding micrograzers trade motility for a colony size large enough to prevent
consumption. This contrasts with the morphologically similar Volvocales, which
maintain their motility (and flagella) regardless of colony size. While other changes in
control or orientation of the flagella would also be necessary, the favoring of motility
combined with large colony size would provide a better defense against benthic
macrograzers in communities exposed to filter feeders. Exposure to predators that create
these combined pressures in more spatially or structurally complex conditions might
yield intermediate phenotypes between nonmotile *C reinhardtii* colonies and the
permanently multicellular and motile Volvocales.

Defensive strategies should not be evaluated only in terms of success against the
stress that induces them but must be viewed through the lens of the community that will
shape the effectiveness of the response over evolutionary time. As reported here,
interactions between the members of a predator-prey species pair and the larger
community can drastically alter the effectiveness of defenses. The colony-forming
defense of *Chlamydomonas* offers a unique opportunity to apply knowledge about the
natural history of *C. reinhardtii* to the evolutionary lineage of the multicellular Volvocales. The presence of a macrograzer would pose a threat to colonial *C. reinhardtii* and would be a pitfall of most species that attempt to escape predation through colonization. Inducible defenses like the one explored here open new opportunities to assess some of the more striking evolutionary consequences of phenotypic plasticity. Exciting possibilities emerge for considering the *C. reinhardtii* colony defense specifically both because of advantages in the alga’s well described natural history and laboratory biology as well as the increasing amounts of genetic sequence data available for the alga and its close evolutionary relatives (Prochnik *et al.*, 2010; Hanschen *et al.*, 2016)
Figure 1. Comparison photos of *Chlamydomonas reinhardtii* unicells and predator induced colonies (20x Phase Contrast)
Figure 2: Interactions observed in experimental microcosms containing species *Chlamydomonas reinhardti*, *Brachionus calyciflorus*, and *Physa fontinalis*. Solid/dashed lines indicate direct and indirect effects respectively.
Figure 3: Log abundances of *C. reinhardtii* unicells per milliliter. Dotted line denotes snail addition to relevant treatments. Error bars show 95% confidence intervals ($F_{(3,12)} = 524.92 \ p < 0.0001$)
Figure 4: Log abundances of *C. reinhardtii* colonies per milliliter. Dotted line denotes snail addition to relevant treatments. Error bars show 95% confidence intervals ($F_{(3,12)} = 211.10 \, P < 0.0001$)
Figure 5: Log abundances of filter-feeding rotifer micrograzers per milliliter. Dotted line denotes snail addition to relevant treatment. Error bars show 95% confidence intervals ($F_{(1,6)} = 314.36$, $p < 0.001$).
References


CHAPTER 2: FACILITATION PROMOTES PERSISTENCE BETWEEN PREY SPECIES EXPERIENCING APPARENT COMPETITION

Abstract

Apparent competition is one mechanism that can contribute to the complex dynamics observed in natural systems. To better predict the role of indirect effects in complex communities, we must more thoroughly explore the fundamental processes that govern and shape the outcomes of indirect effects, such as apparent competition. Here we describe the results of a factorial experiment using two noncompeting prey (Colpidium kleini, a heterotroph, and Chlamydomonas reinhardtii, an autotroph) consumed by a generalist predator (Euplotes eurystomus) to explore the dynamics of apparent competition. Our results suggest an important role of positive interactions and indirect effects contributing to apparent competition in this system with a marked asymmetrical outcome in favor of Chlamydomonas. Although Chlamydomonas and Colpidium do not directly compete, Colpidium reduces bacteria that may compete with Chlamydomonas. In addition, formation of colonies by Chlamydomonas in response to Euplotes provides an antipredator advantage not available to Colpidium. Taken together, the opportunities provided by considering carefully designed experimental microcosms offer a compelling means of evaluating how asymmetrical apparent competition outcomes are created and how facilitation by apparent competitors might contribute to such interactions in nature.
Introduction

The integration of apparent competition (Holt 1977) into studies considering broader ecological theory is critical to understand how direct and indirect effects interact in theory and in nature (Holt & Lawton 1994; Holt & Bonsall 2017). Theoretical and empirical studies of apparent competition have contributed to a number of vital areas of inquiry such as understanding biological invasions (Strauss et al. 2012) and constructing strategies for conservation and management (Wittmer et al. 2013). While previous work has demonstrated that many natural systems display aspects of apparent competition (Holt & Lawton 1994; DeCesare et al. 2009; Dunn et al. 2012), the complex combination of direct and indirect interactions can often restrict the ability to determine the mechanisms governing outcomes of apparent competition (Stige et al. 2018). Despite the difficulty of observing indirect interactions (Wootton 1994; Orrock et al. 2015), indirect effects may be as important as direct effects in influencing community dynamics (Bonsall & Hassell 1997).

Apparent competition can influence community structure and functioning in a variety of ways (Morris et al. 2004; Frost et al. 2016). Depending on community composition, apparent competition can promote the stable coexistence of prey species (Grover & Holt 1998; Tilman 2007) or the exclusion of one prey species (Holt et al. 1994; Bonsall & Hassell 1997). Limiting our understanding to patterns of prey persistence and excluding broader ecological repercussions can make apparent competition seem misleadingly simple given the number of factors that can promote, modify, or eliminate its effects (Tack et al. 2011). The numerous communities that
display apparent competition, coupled with the suggestion that outcomes are intrinsic to specific community assemblages (Orrock & Witter 2010) and predator behavior (Křívan & Eisner 2006), make understanding broad trends in apparent competition somewhat daunting (Orrock et al. 2015). Given the widespread occurrence of apparent competition in nature, a more thorough understanding of the processes involved is needed to avoid a “sea of exceptions” (Holt et al. 1994).

A major prediction of a number of models (e.g. Holt et al. 1994) is that one prey will be an inferior apparent competitor. Chaneton & Bonsall (2000) found that such cases of asymmetric apparent competition are more common than symmetric cases. They also suggest that the seemingly mysterious patterns of symmetry resulting from apparent competition could be resolved through more robust experimental design. Theoretical work by Stige et al. (2017) indicates that while it can be difficult to infer and interpret indirect effects, evaluating top-down and bottom-up effects in these systems can lead to greater understanding of the role of apparent competition for zooplankton food webs. Their study highlights the need to understand how combinations of processes, such as bottom-up effects and indirect effects like apparent competition, can modulate community-level changes. Given the capacity for apparent competition to influence community-level dynamics, it is imperative that we better understand the links between mechanisms and the resulting patterns of symmetry (DeCesare et al 2010).

The role of certain indirect positive effects, such as facilitation, is being incorporated into broader ecological concepts like apparent competition with increasing frequency (Bruno et al. 2003; Stachowicz 2001; Bulleri et al. 2016). A combination of
direct and indirect facilitation has been observed, though the strength of facilitation appears to be highly context dependent (Cuesta et al. 2010). There is increasing evidence that understanding facilitation is critical for appreciating broader ecological interactions (Michalet & Pugnaire 2016) including apparent competition (Allesina & Levine 2011). Conceptual models predict that interspecific prey facilitation should increase prey abundance (Bruno & Bertness 2001), but that a potential consequence is increased predator abundances (Bulleri et al. 2016). Given the predictions of these models, facilitation and apparent competition seem highly likely to influence one another. These conceptual frameworks, coupled with the observed role of facilitation in regulating community structure (Butterfield 2009; Butterfield & Callaway 2013) and biodiversity (McIntire & Fajardo 2009), make establishing the link between facilitation and apparent competition crucial for understanding apparently competitive outcomes.

Here we describe a factorial experiment in which we evaluated the role of facilitation by prey species in shaping outcomes of apparent competition in a community of protists. The treatments of our experiment allow for consideration of prey and predator abundances resulting from different species combinations to evaluate changes in the strength and sign of an interaction. Two prey species, the heterotrophic ciliate *Colpidium kleini* and the autotrophic green alga *Chlamydomonas reinhardtii*, are unlikely to compete given their different trophic positions, and both prey species are consumed by the ciliated predator *Euplotes eurystomus*. We used our series of factorial treatments to determine: 1) if *Chlamydomonas* and *Colpidium* compete despite the trophic differences between the two species, 2) if each prey can support *Euplotes* in the absence of the other
and measure baseline abundances for each prey coexisting with the predator, 3) if both prey species interacting together with *Euplotes* would lead to increased predator abundance and depressed prey abundances consistent with apparent competition (see Figure 1).

**Methods**

**Organisms and culturing conditions**

To determine the extent and mechanisms by which our two prey species might be influenced by apparent competition, we established treatments with three protist species in a factorial design. The alga *Chlamydomonas reinhardtii* and the bacterivorous ciliated protist *Colpidium kleini* are unlikely to compete directly, and each have the ability to support populations of a shared ciliated protist predator, *Euplotes eurystomus*. Cultures of *Chlamydomonas* (CC-1010) originated from the *Chlamydomonas* Resource Center (University of Minnesota). Populations of *Euplotes* and *Colpidium* were originally obtained from Carolina Biological Supply company and the Adelphia Plant Science Research and Extension Center (Freehold, New Jersey) respectively.

Populations of all three experimental species isolated from one another grew in autoclave-sterilized, loosely lidded 250 mL glass jars. Microcosms contained 100 mL of autoclave-sterilized complex organic medium made by adding 0.4 grams of Carolina Biological supply protozoan pellets (Carolina Biological Supply Company, Burlington, NC) and 0.14 grams Rep-Cal Herptivite nutrient supplement to one liter of filtered well water. Sterile medium received an inoculum containing four bacterial species (*Serratia marcescens, Bacillus subtilis, Bacillus cereus*, and *Proteus vulgaris*) prior to introduction.
of experimental species to standardize bacterial community composition across treatments. *Chlamydomonas* was initially cultured using a 1:1 mixture of TAP medium (Gorman and Levine 1965) and the previously described organic medium.

Experimental set-up

We created seven treatments containing all possible combinations of the three experimental species and a protist-free control to monitor bacterial abundance in the absence of protists (n = 5 for each treatment). Positions of microcosms were randomized in a Percival incubator at 24° C with a 12 hour light:12 hour dark photoperiod. All experimental microcosms contained two sterile wheat seeds for additional nutrients. Treatments containing *Chlamydomonas* were initiated by adding 50 mL of sterile medium to 50 mL of dense algal culture (>1×10⁶ cells/mL). We introduced *Colpidium* and *Euplotes* to experimental cultures by transferring 20 individuals of both species from maintenance lines into appropriate treatments using micropipettes. Nine days of initial growth without predation by *Euplotes* ensured establishment of *Chlamydomonas* and *Colpidium*. We added *Euplotes* to appropriate treatments 9 days after prey introductions and allowed one week to pass before sampling to ensure that predators became established. Throughout the experiment, we monitored microcosms weekly to measure pH, adjust volume due to evaporation, and evaluate bacterial or fungal contamination.

Every two days for three weeks we measured the abundances of all three species by removing a 1 mL subsample after gently shaking each microcosm to ensure contents were well-mixed. Data consisted of counts of *Colpidium* and *Euplotes* using a Nikon
SMZ microscope at 20X and counts of unicellular *Chlamydomonas* using a Reichert hemocytometer and a Nikon Eclipse 80i compound microscope at 400X (phase contrast).

We sampled both *Chlamydomonas* colonies (which form as result of grazing by *Euplotes*) and turbidity of homogenized growth medium (to measure bacterial abundance) in appropriate treatments at the end of our time series. We collected subsamples as described previously and colonial *Chlamydomonas* abundance was measured using the same methods used to count single cells. Turbidity provided an approximation of the relative concentrations of the bacterial communities (Monod 1949). For each replicate, optical density at 590nm of manually shaken homogenized growth medium estimated the approximate abundances of bacteria in each treatment (using a Milton-Roy 601 spectrophotometer). A treatment using the same medium but started outside the original data collection which contained only bacteria was cultured for a period equivalent to the time point where optical density data was collected and used as a protist free control.

Statistical analyses

We calculated mean density per mL for *Chlamydomonas* unicells, *Euplotes*, and *Colpidium* for time points after the establishment of *Euplotes*. Means averaged over time of these values were then log$_{10}$-transformed and analyzed with ANOVAs for each species. Tukey’s Honestly Significant Difference (HSD) comparison test evaluated significant differences in treatment means for each species at the 0.05 level of significance. We performed an ANOVA for turbidity data and a t-test for log$_{10}$-
transformed abundances of *Chlamydomonas* colonies for one time point. All statistical analyses were performed in SAS (SAS institute, version 9.4).

**Results**

**Apparent competition between *Chlamydomonas* and *Colpidium***

*Euplotes* attained much higher abundances when feeding on either of the two prey species compared to its growth where it had access only to bacteria. When *Euplotes* fed on both eukaryotic prey species together its average abundance increased above that seen when grown with either prey species singly (F(3,16) = 51.57, p > 0.0001, Figure 2a). The increase in predator abundance was accompanied by a significant decrease in the mean abundance of one of the apparent competitors, *Colpidium kleini*. *Colpidium* abundance significantly declined relative to predator-free controls only when *Chlamydomonas* was also present (F(3,16) = 6.44, p = 0.0046, Figure 2b). Although different treatments did affect *Chlamydomonas* unicell abundances (F(3,16) = 11.76, p = 0.0003), unlike the pattern displayed by *Colpidium*, the abundance of unicellular *Chlamydomonas* coexisting with *Euplotes* actually increased when *Colpidium* was present (Figure 2c), relative to controls.

**Indirect facilitation of *Chlamydomonas* by *Colpidium***

The positive effect of *Colpidium* on *Chlamydomonas* abundance, with or without predation by *Euplotes*, is associated with the depression of bacterial abundance as assessed by relative turbidity. Treatments containing *Colpidium* were significantly less turbid compared with treatments without it (F(7,32) = 69.09, p > 0.0001). All treatments containing *Colpidium* displayed similarly low levels of turbidity, consistent with lower
levels of bacterial abundance. All treatments without *Colpidium* had higher turbidity and were indistinguishable from a comparison treatment containing bacteria without protists (Figure 3). This indicates that the positive effect of *Colpidium* on *Chlamydomonas* was associated with a reduction in bacteria.

Induction of *Chlamydomonas* defensive colonies by *Euplotes*

Unicellular *Chlamydomonas* formed multicellular colonies in response to *Euplotes*. Colonies did not appear at detectable levels when *Chlamydomonas* grew without *Euplotes* (i.e. in *Chlamydomonas* controls or with only *Colpidium*). There was a slight but significant increase ($t_{(6)} = -2.4109$, $p = 0.042$) in *Chlamydomonas* colonies in the treatment containing *Euplotes* and *Colpidium* compared to cultures containing only *Chlamydomonas* and *Euplotes* (Figure 4).

**Discussion**

As predicted, when *Colpidium kleini* and *Chlamydomonas reinhardtii* occurred together with the predator *Euplotes eurystomus*, we observed results consistent with apparent competition. Patterns of abundance for the two prey species grown singly or together without the predator clearly rule out competition between the two prey species. Instead, there was an asymmetric positive effect of *Colpidium* on *Chlamydomonas* that we attribute to a reduction in bacteria that may have had a negative effect on algal abundance. Abundances of *Euplotes* increased when both prey were present compared with treatments where it fed on either prey species alone (Figure 2a). Interestingly, the impact of increased predator abundance had asymmetric effects on the two prey species
with only Colpidium abundances showing significant reductions (Figure 2b). The pattern of asymmetry observed (Figure 5) is consistent with other commonly observed asymmetrical outcomes between prey in systems displaying apparent competition (Chaneton & Bonsall, 2000). It also raises the possibility that when one apparent competitor facilitates another, this may drive the asymmetry in the apparent competition outcome. Microcosm systems like ours, in which researchers can directly manipulate interacting species, offer important opportunities to explore interactions which create such asymmetrical outcomes and how apparent competition can contribute to population and community level stability.

The lack of reduced Chlamydomonas abundance in response to Colpidium and Euplotes was unanticipated. Because Chlamydomonas and Colpidium are equally capable of supporting Euplotes populations when either prey occurs alone, and Chlamydomonas abundances are significantly higher in the presence of Colpidium, the results suggest that the outcome is not determined by negative interactions (e.g., apparent competition, predation) alone. When considering the source of asymmetric outcomes, a number of additional indirect interactions may explain why Chlamydomonas is a superior apparent competitor. Two factors help explain the asymmetry in our system: 1) the facilitation of Chlamydomonas by Colpidium and 2) the enhanced persistence of Chlamydomonas with Euplotes as a result of the inducible anti-predator defense of colony formation.

Algae and bacteria frequently compete for nutrients such as phosphorus (Grover 2000; Løvdal et al. 2007), and high turbidity could also potentially depress algal growth through reducing light available for photosynthesis (Wang 1974). The observed
facilitation of *Chlamydomonas* was associated with decreased bacterial abundance, measured as decreased turbidity, in cultures containing *Colpidium* (Figure 3). This is especially interesting since by facilitating the alga, *Colpidium* likely contributes to its own reduction by *Euplotes*. Work demonstrating the role of such non-trophic positive interactions structuring and modifying communities (*e.g.* Filazzola *et al.* 2017) makes this an exciting opportunity to explore interplay between positive effects and apparent competition.

While cases of predator facilitation of prey species have been considered previously (*e.g.*, Pope *et al.* 2008), our results highlight a need to explore cases where facilitation between prey can influence apparent competition in ways that affect the fitness consequences for both prey species (Schöb *et al.* 2014). Indirect facilitation like that observed in our system has been linked to negative interactions previously (Adams *et al.* 2003; Flory & Bauer 2014), but the roles of such indirect effects are far from clear (Cuesta *et al.* 2010). Consideration of indirect facilitation can broaden understanding in complex systems of ecological interactions (Saccone *et al.* 2010). Our findings support previous evidence that facilitation can modify the strength of negative interactions between species (Bulleri *et al.* 2016) and contribute to changes in species abundances by modifying interactions at the community level (Levine 1999). The fact that both facilitation and apparent competition contribute to shared processes at the community level (modifying interaction strength, community structure, invasions and conservation, etc.) indicates that understanding the interplay between the positive effect of facilitation and the negative effect of apparent competition should be a focus of future work.
In addition to facilitation of *Chlamydomonas* by *Colpidium*, there was a significant increase in the abundance of multicellular defensive colonies formed by *Chlamydomonas* in the three species treatment in response to predation by *Euplotes* (Figure 4). *Chlamydomonas* forms colonies comprised of dozens to hundreds of cells in response to predators which reduce predation by small filter feeding micrograzers (Lurling & Beekman 2006). This defense has been documented previously for other predators, including ciliates, but to our knowledge not in response to *Euplotes eurystomus*. Combined with the facilitation of *Chlamydomonas* by *Colpidium*, the formation of defensive colonies by *Chlamydomonas* suggests an additional mechanism that may contribute to asymmetrical apparent competition.

There is a need to relate food web dynamics and processes to the various outcomes of apparent competition (Holt and Bonsall 2017) to better understand how these outcomes arise. Our results allow us to integrate previous work on predation, apparent competition, and the role of inducible defenses in community dynamics. Models of species coexistence support the idea that predator-prey interactions are stabilizing (Allesina & Tang 2012), and many examples of top-down drivers for maintaining diversity have been found in natural systems (Terborgh 2015). In addition to the more general role of predation for enabling species coexistence, defensive phenotypes can also be important in driving persistence of focal groups (e.g. Aranguiz-acuna *et al.* 2010) or communities as a whole (e.g. Boeing & Ramcharan 2010). The role of *Chlamydomonas* defensive colonies in our experimental systems provides a case like that predicted by Grover and Holt (1998), in which coexistence results from one prey being better
defended from predation (*Chlamydomonas* forming colonies which favor defense over growth rate) and one prey specializing on resource acquisition (*Colpidium*). The interplay of direct and indirect effects creates a fascinating chain reaction where: 1) *Colpidium* reduces bacterial abundances and consequently promotes *Chlamydomonas* abundance. 2) The combined presence of *Colpidium* and *Chlamydomonas* increases *Euplotes* abundance and results in apparent competition, though only the facilitating prey seems to suffer the consequences, perhaps because- 3) The formation of *Chlamydomonas* colonies scales with increased predator abundance and potentially facilitates persistence of the *Euplotes* and *Chlamydomonas* interaction, perpetuating the apparent competition and resulting in the asymmetrical pattern we observed.

This work allowed us to explore role of mixed direct and indirect effects, including facilitation and trait mediated indirect effects, in shaping the outcomes of apparent competition. Our finding that *Colpidium* indirectly facilitates *Chlamydomonas*, which can then defend itself and further modify negative interaction strengths within the community, suggests that the combination of positive and negative effects can influence persistence of species in the system. This raises a number of interesting questions for fields such as invasion and conservation biology, where both apparent competition and facilitation have been observed to play a pronounced role. More work is required to disentangle the combination of direct and indirect effects that ultimately shape the outcomes observed from apparent competition and how effects may be promoted or diffused in natural systems. By exploring how these interactions are shaped and modified,
however, we hope to expand our understanding of the role of positive interactions in
governing the outcomes of apparent competition.
Figure 1. Conceptual diagram of the possible interactions between the three species in our experimental microcosms. Panel A shows the three species with symmetrical apparent competition. Panel B shows asymmetrical apparent competition with *Colpidium* as a superior apparent competitor. Panel C shows asymmetrical apparent competition with *Chlamydomonas* as a superior apparent competitor.
Figure 2. Mean (over time and across replicates) log_{10} transformed abundances for *Euplotes eurystomus* (A), *Chlamydomonas reinhardtii* unicells (B), and *Colpidium kleini* (C). Letters above boxes indicate treatments which group significantly in Tukey’s HSD. Box plots: middle line, median; box, interquartile range; whiskers, 10th and 90th percentiles.
Figure 3. Absorbance data of all experimental treatments measured at 590nm at end of experiment. Reduced absorbance implies reduced bacterial abundance. Letters above boxes indicate treatments which group significantly in Tukey’s HSD. Box plots: middle line, median; box, interquartile range; whiskers, 10th and 90th percentiles.
Figure 4. Log10 transformed abundances of *Chlamydomonas* colonies from relevant treatments. Box plots: middle line, median; box, interquartile range; whiskers, 10th and 90th percentiles.
Figure 5. Observed dynamics in our experimental microcosms expanded to include the bacterial community and both *Chlamydomonas* phenotypes.
References


CHAPTER 3: MAKING DUE WITH WHAT YOU HAVE: EVALUATING ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES OF THE C. REINHARDTII COLONIAL DEFENSIVE PHENOTYPE USING RNA-SEQ

Abstract

Despite the potentially extensive ecological and evolutionary ramifications for species which deploy inducible defensive phenotypes, the genetic basis of these defenses has only recently begun to be explored. Here I describe a transcriptomic (RNA-seq) assessment of the temporary defensive colonial phenotype of the green alga Chlamydomonas reinhardtii. Isogenic populations of C. reinhardtii were grown with and without the filter feeding predatory rotifer Brachionus calyciflorus and were sampled across four weekly time points and pooled by treatment to characterize variation in gene expression associated with the production of the colonial phenotype. Differential expression analyses identified 417 upregulated and 412 downregulated genes in populations expressing the defense phenotype with 286 genes being of particular interest for evaluating the defense. The enriched gene ontological categories for differentially expressed genes support the ecological constraints experienced by C. reinhardtii forming defensive colonies, most notably reducing expression of flagella, increasing uptake of trace elements, and regulating the cell cycle. Of particular evolutionary importance, certain genes previously investigated for their co-option in the transition between temporary and permanent multicellularity in the Volvocine algae see differential expression in the defensive phenotype, and there is a trend of enrichment and constriction of up and downregulated genes in the permanently multicellular Volvocale Volvox
carteri. These results not only provide a more complete sense of the transcription events underlying C. reinhardtii colony formation, but also allow us to assess whether similar pathways have been conscripted to maintain permanent multicellularity in the Volvocales.

Introduction

Inducible defense phenotypes can have dramatic ecological and evolutionary consequences (Harvell 1990). Understanding how these plastic phenotypes can influence community structure (Boeing and Ramcharan 2010), eco-evolutionary dynamics (Becks et al. 2010), and major evolutionary transitions (Tollrian and Harvell 1999) contributes greatly to our appreciation of the role of these defenses in nature. While evaluation of such defensive phenotypes in community level experiments has clarified outcomes for the predator-prey defense focal groups or larger community modules (Hammill et al. 2015), the differentially expressed genes underpinning these phenotypes are less often explored.

Expression of some defensive phenotypes can drastically alter an organism’s fitness (Buskirk 2000; Brönmark et al. 2012). The shifting landscape of selective pressures acting on the defended and undefended phenotypes (Kishida et al. 2009; Friman et al. 2016) makes appreciating how these defenses are elicited genetically of particular interest. Surveys of genes associated with defense phenotypes can inform us of
their short-term ecological consequences and potential for contribution to major evolutionary transitions (Maynard Smith and Szathmáry 1997; Fordyce 2006).

A variety of studies have related phenotypically plastic traits to the genes involved in their expression (Scheiner 1993, 2014), though only a fraction of these studies involve inducible defenses. One powerful means of determining genetic components of these defense phenotypes is to employ transcriptomic analyses, hereafter termed RNA-seq (Aubin-Horth and Renn 2009; Smith et al. 2013). RNA-seq compares RNA transcripts across environmental conditions of interest to quantify and describe changes in transcriptomic expression (De Wit et al. 2012). Evaluating how gene expression changes using RNA-seq allows determination of whether differentially expressed genes relate to informative changes in gene ontology (e.g. identifying biological functions or molecular pathways of interest for resulting phenotypes) or exploration of changes in homologs of those differentially expressed genes in evolutionarily related species (Conesa et al. 2016). While the information gained from RNA-seq does not account for post transcriptional modification, it can provide a wealth of information about the active steps involved in crafting defensive phenotypes.

The green alga *Chlamydomonas reinhardtii* forms a colonial phenotype in response to a variety of biotic and abiotic stimuli (Harris et al. 2009). For example, increasing size and insulating interior cells provide a means of moderating negative effects of increased salinity (Khona et al. 2016), organic acids (Iwasa and Murakami 1969), or predation (Lurling and Beekman 2006). In addition to being a frequent stress response by *C. reinhardtii*, novel stressors which select for larger size have been shown
to result in novel heritable colony formation (Becks et al. 2010; Ratcliff et al. 2012; Boyd et al. 2018), suggesting that colonial phenotypes can develop rapidly with little genomic modification. While temporary multicellular phenotypes might be a common response by *C. reinhardtii* to stress, it is unlikely only one static set of genes is responsible for inducing and maintaining the phenotype. A microarray study by Becks et al. (2012) found that populations expressing colonial phenotypes in response to predation differentially express genes when producing the unicellular and colonial phenotypes, and eco-evolutionary dynamics between predator and prey led to corresponding changes in gene expression. This suggests that expression of genes involved in defensive phenotypes can vary both over time and in response to shifting environmental conditions.

*C. reinhardtii* will form colonies when exposed to a number of filter feeding predators ranging from ciliated protists (such as *Euplotes eurystomus*) to small filter feeding metazoans (such as the rotifer *Brachionus calyciflorus*). Colonies are an effective means of limiting the effects of gape-limited micrograzers, though the broader impacts of the defense are less well described. Algal anti-predator defenses have been proposed as a potential evolutionary origin for permanent multicellularity in green algal lineages (Boraas et al. 1998), and *C. reinhardtii* shares many similarities with the putative unicellular ancestor of the multicellular Volvocales (Herron et al. 2009). Investigation of the differentially expressed genes responsible for facultative multicellularity can potentially identify the regulatory and evolutionary mechanisms that may have contributed to permanent multicellular phenotypes.
Studies comparing the genomes of *C. reinhardtii* and the multicellular Volvocales *Gonium pectorale* (Hanschen et al. 2016) and *Volvox carteri* (Prochnik et al. 2010) demonstrate that the genomes of the unicellular and multicellular species are highly similar. *V. carteri* in particular, as a highly diverged member of the lineage, has a similar number of coding genes (17,741 for *C. reinhardtii* and 14,247 for *V. carteri*) and total genome length (118 million base pairs for *C. reinhardtii* and 131 million base pairs for *V. carteri*) with most of the gain in genome size for *V. carteri* representing non-coding DNA. The apparent lack of major modifications in the development of multicellularity implies that exploration of *C. reinhardtii* may provide insight into how (and how rapidly) permanent multicellularity emerged (Herron and Nedelcu 2015).

The genomic similarity observed in the Volvocine algal lineage, coupled with experimental selection for novel multicellular phenotypes, suggests that *C. reinhardtii* may be capable of transitioning to temporary and then permanent multicellularity in surprisingly short periods of evolutionary time. While there has been description of the morphological modifications required for multicellularity (e.g., Kirk 2005) few studies have used the temporary colonial phenotypes of *C. reinhardtii* to explore the development of permanent multicellularity.

Here I describe the results of a transcriptomic analysis of *C. reinhardtii* colony formation resulting from predation by the rotifer *Brachionus calyciflorus*. To better understand transcriptomic expression between the two phenotypes I cultured *C. reinhardtii* in the presence and absence of *B. calyciflorus* to determine which genes are differentially expressed by populations of *C. reinhardtii* experiencing predation. I then
determined enrichment of gene ontological groups for the differentially expressed genes and explored patterns in the *V. carteri* genes homologous to those differentially expressed by *C. reinhardtii*.

**Methods**

*Organisms and culturing conditions*

To compare genes expressed by the *Chlamydomonas reinhardtii* colonial phenotype with unicellular *C. reinhardtii*, I cultured *C. reinhardtii* in replicated batch cultures with and without the filter feeding rotifer *Brachionus calyciflorus*. Cultures of *C. reinhardtii* (strains CC-1010 and CC-1009) were obtained from the *Chlamydomonas* Resource Center (University of Minnesota). Cultures of *B. calyciflorus* were originally obtained from the laboratory of Nelson Hairston Jr. (Cornell University, Ithaca, New York) and were reared on an alternate algal species (*Ankistrodesmus sp.*) until experimental treatments began. Populations of *C. reinhardtii* used in the experiment were grazed by *B. calyciflorus* for two weeks before the algae were isolated and kept until the start of the experiment.

All populations used were maintained in either modified Woods Hole medium (Adapted from Guillard and Lorenzen 1972, experimental treatments) or a 1:1 mixture of TAP medium (Gorman, D.S. and Levine 1965) and modified Woods Hole medium (*C. reinhardtii* stock cultures). Fresh maintenance lines for algae were started every week by adding a 50 mL inoculum of *C. reinhardtii* or *Ankistrodesmus sp.* to sterile medium. An initial inoculum of a dense rotifer culture (>50 individuals per mL) was introduced to cultures of *Ankistrodesmus* to establish maintenance lines without recent exposure to
*Chlamydomonas*. All maintenance cultures were kept at 24° C in an incubator with a 12h light:12h dark photoperiod in 250 mL glass jars.

**Establishment of experimental batch cultures**

I sampled grazed and ungrazed *Chlamydomonas* at four time points to capture as much temporal variation in gene expression of the *C. reinhardtii* populations as possible (n=3). Cultures containing a total volume of 600 mL of initially sterile modified Woods Hole medium in 1 L Erlenmeyer flasks were stored in a 24° C incubator and 12L:12D photoperiod. Flasks were arranged in a randomized design and were loosely lidded to promote gas exchange while limiting contamination. All batch cultures were mixed by hand daily to promote gas exchange.

Both treatments were initiated by adding 500 mL of sterile medium to 100 mL of dense *C. reinhardtii* culture (>1x10⁶ cells/mL). Populations of *B. calyciflorus* were washed of *Anistrodesmus* and kept in sterile medium for one day before use. I added 150 individuals of *B. calyciflorus* to the predated treatment using micropipettes and observed batch cultures for three days to ensure predator establishment. Cultures from both treatment conditions had samples collected weekly for one month.

**Population monitoring, RNA extraction, and sequencing**

Experimental batch cultures were maintained for four weeks and monitored weekly for pH, changes in volume due to evaporation, evidence of bacterial or fungal contamination, and population collapses. Counts (number / mL) of *B. calyciflorus* individuals and *C. reinhardtii* colonies were obtained using a Nikon SMZU microscope.
at 40X; counts of *C. reinhardtii* unicells were made using a Reichert hemocytometer and a Nikon Eclipse 80i compound microscope at 400X (phase contrast).

Total RNA samples from each treatment replicate were collected for each weekly time point and pooled within replicates. Batch cultures were mixed vigorously and passed through 50-micron Nitex membrane to remove *B. calyciflorus*. Filtered *C. reinhardtii* samples were gently centrifuged (2,000 x g) to produce cell pellets. Cells were lysed using Trizol (Life Technologies) and bead beating using Qiagen TissueLyser (version II). Total RNA was extracted using the Qiagen RNeasy PlantMini kits including the DNase on column digestion. Samples were submitted to the Rutgers Genome Cooperative for cDNA library preparation and sequencing. An additional poly(A) enrichment step was performed to target eukaryotic mRNA. cDNA libraries were prepared for single end sequencing using Illumina TruSeq RNA library kits (version 2) following standard protocols. Sequencing of libraries was performed on an Illumina MiSeq platform using 150-cycle kits (version 3) to produce >10 million reads per library.

*Analyses and Bioinformatics*

Reads from each replicate were processed prior to alignment using sortmeRNA (version 2.1, Kopylova et al. 2012) to remove ribosomal RNA sequences compared to an index created from Silva databases for *C. reinhardtii* and Trimmomatic (version 0.36, Bolger et al. 2014) to remove Illumina adapter sequences. Copies of the *C. reinhardtii* reference genome and annotation files (version 5.5) were retrieved from the Phytozome platform maintained by the Joint Genome institute (Goodstein et al. 2012).
Sequences were aligned using STAR (Dobin et al. 2013, version 2.4) using default parameters for indexed single end alignment with samples being included in analyses if they had higher than 85% mapping reads. Differentially expressed genes were determined using DESeq2 (Love et al. 2014, version 1.18) to compare genes mapped between treatments. A cutoff requiring both FDR adjusted p-values calculated by DESeq2 below a threshold of 0.05 and a minimum log-fold change of 2 was used to identify differentially expressed genes.

Supplementary sequence (nucleotide and peptide) data, gene identifiers and descriptions, relationships of orthologous genes between *C. reinhardtii* and *V. carteri*, and gene ontological (GO) annotations were retrieved from JGI using biomaRt (version 3.6, Durinck et al. 2009). For sequences lacking sufficient annotation through JGI for analysis, I queried the Algal Functional Annotation Tool (Lopez et al. 2011) and retrieved any remaining annotation information using Blast2GO (version 5.0.13, Gotz et al. 2008). Queries in Blast2GO were run using default settings and the GO identifiers for the top scoring BLAST search to the non-redundant protein database were included for genes lacking annotation through either Phytozome or the Algal Functional Annotation Tool. For enrichment analysis of overrepresented and underrepresented GO categories, I used goseq (version 1.3, Young et al. 2010) using a 0.05 FDR cutoff against the Wallenius distribution to determine enriched GO categories. Differential expression and GO analyses were performed using the R Bioconductor package (version 3.5, Gentleman et al. 2004) in R version 3.4 (R Core Team 2017). To find *V. carteri* orthologs of
differentially expressed *C. reinhardtii* genes without existing data on Phytozome I used eggNOG (version 4.5.1, Huerta-Cepas et al. 2016).

To detect observed changes in abundance by treatment over the four weeks of culturing, I analyzed Log$_{10}$ transformed abundance data using a repeated measures analysis of variance for *C. reinhardtii* unicells, *C. reinhardtii* colonies and rotifers. To evaluate potential importance of differentially expressed *C. reinhardtii* colony genes for permanent multicellularity I determined the number of orthologs for each gene included in the differential expression analysis in the *V. carteri* genome. A sign test was used to determine if the number of *V. carteri* orthologs for upregulated, downregulated, and non-differentially expressed *Chlamydomonas* genes differed significantly from zero, indicating expansion (positive difference) or constriction (negative difference) in *V. carteri*. Repeated measures ANOVAs and sign tests were performed in SAS (version 9.4, SAS institute).

**Results**

*Expression of the C. reinhardtii colonial phenotype*

As expected, the exposure of *Chlamydomonas reinhardtii* to the filter feeding rotifer *Brachionus calyciflorus* resulted in the formation of defensive colonies. This corresponded to marked increases in the abundances of colonies as well as significantly different gene expression in grazed populations. The results of repeated measures ANOVAs detected differences in abundances both between treatments and over time for *C. reinhardtii* unicells ($F_{(2,3)}$ =447.025, $p$>0.002) and colonies ($F_{(2,3)}$ =10.9, $p$=0.042),
indicating that variation in colony formation occurred between treatments and time points (Figure 1). Results of preliminary analyses performed in DeSeq2 demonstrate a genetic component to this variation with the grazed and ungrazed populations more closely resembling one another with some amount of observable differences in mRNA expression within treatments (Figure 2). The grazed treatment varied more both in C. reinhardtii abundances for either phenotype as well as gene expression indicating that the grazed trial might capture a good deal of variation of gene expression for the colonial phenotype.

**Differential expression and GO enrichment analysis**

Results of a differential expression analysis performed using DeSeq2 show that out of 15,497 total genes with any expression 677 (4.4% total genes expressed) upregulated genes and 455 (2.9% total genes expressed) downregulated genes were differentially expressed at the 0.05 degree of significance (Figure 3). Of these, 417 upregulated genes and 412 downregulated genes saw at least a 2-fold change in expression and were included in GO enrichment and homologous gene analyses.

Existing annotation data for GO enrichment analysis was retrieved for more than 87% of the dataset, but limitations of current functional annotation create a subset of differentially expressed genes which require future study. Differentially expressed genes had particularly low annotation with 36% of upregulated genes and 65% of downregulated genes having sufficient existing annotation for inclusion in the analysis. The limited amount of existing annotation of differentially expressed gene function in C. reinhardtii creates a set of 286 genes (153 upregulated/123 downregulated) of particular
interest for future evaluation of the colonial defense phenotype. While the available annotation data limits interpretations from the current dataset, especially for upregulated genes, enriched categories based on available annotation point to sensible changes in enriched GO categories for the phenotypic transition seen in this study.

GO enrichment analyses conducted using goseq indicates enrichment of 37 GO categories from upregulated genes and 22 from downregulated genes (Table 1). A combination of factors (poor annotation of differentially expressed genes for *Chlamydomonas*, assignment of GO terms which are non-relevant to *Chlamydomonas* [e.g. pollen maturation], low number of differentially expressed genes in the category) led to the exclusion of some GO categories which were indicated as being enriched by goseq. Enriched categories for downregulated genes are almost all related to motility or flagellar expression (Figure 4A). Upregulated categories are more varied in their function with nucleic acid binding/modification and nutrient assimilation comprising roughly three-quarters of enriched categories (Figure 4B). The inability to properly annotate nearly 70% of upregulated genes indicates that targeting these genes for further study will only increase our understanding of temporary colonial phenotypes in *C. reinhardtii*.

*Evaluation of homologs for differentially expressed defense genes in Volvox carteri*

To better establish a link between the *C. reinhardtii* temporary multicellular phenotype and the permanently multicellular phenotypes of the Volvocales, I evaluated whether the genes differentially expressed by grazed *C. reinhardtii* have expanded or constricted numbers of orthologs (genes in different species evolved from a shared ancestral genes) in the genome of *V. carteri*. As expected based on the high degree of
genomic similarity between *C. reinhardtii* and *V. carteri*, I observed a 1:1 relationship between the number of orthologs for over 94% of non-differentially expressed genes. While many genes which are differentially expressed by grazed *C. reinhardtii* also have a 1:1 relationship in terms of numbers of *V. carteri* orthologs, 45 upregulated and 43 downregulated genes show some degree of expansion or constriction in the multicellular species. Interestingly, in cases where there is modification for differentially expressed genes in the *V. carteri* genome, 82% of upregulated genes are expanded while 73% of downregulated genes are constricted (Figure 5). Sign tests performed to determine gain or loss of orthologs in *V. carteri* detected significant change for non-differentially expressed genes (*Z* = -7.494, *p* < 0.001), downregulated genes (*Z* = -3.731, *p* < 0.001), and upregulated genes (*Z* = 3.3436, *p* < 0.001). This finding suggests that when modifications to the *V. carteri* was necessary in the development of permanent multicellularity, the genes expressed for temporary multicellularity were particularly important.

While the most strongly expanded and constricted genes from this dataset are not differentially expressed by grazed *C. reinhardtii*, the pattern of expansion and constriction for those orthologs in the *V. carteri* genome suggests that genes important for the temporary multicellular phenotype contributed to the development of permanent multicellularity. The fact that some genes which are not differentially expressed see such dramatic increase or decrease in numbers of *V. carteri* orthologs indicate that while genes for temporary multicellularity are of interest, further evaluation of non-differentially expressed genes may also provide insights into making multicellularity a fixed trait.

**Discussion**
The results described here provide insights into the ecological and evolutionary ramifications of defensive colony formation of *Chlamydomonas reinhardtii*. The variation of abundances for the grazed treatment (Figure 1) and the reduced similarity between the genes expressed by that treatment and the ungrazed treatment (Figure 2) suggest that sampling across time and replicate lines captured variation in gene expression for the populations experiencing predation and induced colony formation. Comparing the two treatments with differential expression analysis identifies a set of 286 (153 upregulated and 123 down regulated) *C. reinhardtii* genes of interest out of a total of 829 differentially expressed genes. These 286 genes are of particular interest largely due to poor previous description of function for the genes in the species, as well as the lack of any significant ability to find homologs for these genes outside the Volvocales. Differentially expressed genes currently possessing sufficient annotation for GO enrichment analysis indicate interesting ecological consequences for *C. reinhardtii* in expressing the colonial phenotype.

Enriched GO categories for populations with high abundances of colonies suggest that the colonies modified their expression of flagella as movement becomes too cumbersome, and compensate for the negative effects of settling out of the water column. Summarized functional changes for grazed populations (Figure 4) indicate that one of the most distinctive changes is the reduction in activity of genes responsible for flagella and motility by colonies and associated reduction in positive phototaxis. These modifications of structures associated with motility make sense given that *C. reinhardtii* is negatively buoyant colonies become too large and irregularly shaped to support themselves. This
coincides with an upregulated enriched GO category responsible for flagellar excision
(Quarmby et al. 1992) indicating both upregulated and downregulated genes are
contributing to the reduction in flagella.

The pattern in upregulated GO enrichment is less straightforward both due to a
wider variety of enriched categories and far fewer upregulated genes having sufficient
functional annotation to be readily interpreted. The upregulated categories that can be
identified based on existing annotation indicate that as colonies form and settle, they
increase gene regulation and modification (44% upregulated enriched GO categories),
increase nutrient metabolism and uptake (22%), respond to stressors including increased
bacterial presence and breakdown of cellular waste (21%), and modify regulation of the
cell cycle (9%). These findings are not only consistent with a previous microarray study
by Becks et al. (2012) which observed modifications to similar types of gene sets to those
observed here (nitrogen deprivation, cell cycle regulation, modification to the
extracellular matrix) but also suggest sensible reactions for an alga making such a
dramatic change in phenotype.

Considering that only 36% of upregulated genes could be included in my GO
analysis due to limitations in existing functional annotation, improving knowledge of the
functions of these genes will greatly advance our understanding of how C. reinhardtii
compensates for grazing stress. Regardless, the enriched GO terms for upregulated genes
show that maintaining the colonial phenotype (cell cycle regulation and nucleic acid
modification) and dealing with the change from a motile to sessile lifestyle (stress
response and nutrient uptake) result in sensible changes based on the annotation data currently available.

While temporary colonial phenotypes have been suggested as a first step toward the evolution of permanent multicellularity (e.g. Boraas et al. 1998), functional ties between temporary and permanent multicellularity have not been resolved. The significant relationship between genes differentially expressed in my study to ortholog number in *Volvox carteri* (Figure 5), with upregulated genes seeing expanded numbers of orthologs and downregulated genes seeing constriction, support mounting evidence for rapid transition toward multicellularity being possible in the Volvocales using genes already available to *C. reinhardtii* (Prochnik et al. 2010; Hanschen et al. 2016). While there were also significant losses for the non-differentially expressed genes, this is likely explained by the 3,500 genes lost in the hypothetical transition between *C. reinhardtii* and *V. carteri* despite (or perhaps in light of) the ~11 million basepair increase to the *V. carteri* genome. The fact that upregulated genes in *C. reinhardtii* colonies result in increased orthologs in *V. carteri* does suggest that, at the very least, there is overlap between genes important for temporary and permanent multicellularity in this lineage.

A number of genes have been proposed as being of particular interest in the transition of multicellularity including those regulating the cell cycle, the extracellular matrix between cells (ECM), and modifying genes associated with motility (Olson and Nedelcu 2016). Out of 32 genes of interest for Volvocine multicellularity surveyed from the literature, 13 see differential expression in this study (Table 2). Though not all of these genes are upregulated in grazed populations, since co-option of genes and
regulatory pathways is a common strategy for development of multicellularity in the Volvocales (Nedelcu and Michod 2006; Hanschen et al. 2016; Olson and Nedelcu 2016), upregulation might not be required to promote genomic expansion for those genes of interest.

In *V. carteri*, the genes in Table 2 contribute to cell cycle regulation, the extracellular matrix, and flagellar-related motility, but the function of some in *C. reinhardtii* varies significantly. On the one hand, differential expression of pherophorins and glycoproteins of the ECM and subsequent change in *V. carteri* homologs for those genes make sense given the importance of the ECM in connecting cells in both temporary and permanent multicellular phenotypes (Godl et al. 1995; Hallmann 2006). Interestingly, not all the pherophorins see upregulation, and some other classes of glycoproteins present in *C. reinhardtii* (i.e. *Volvox* metalloproteases) are not differentially expressed in colonies at all, suggesting that there is some specialization for ECM glycoproteins in either the unicellular, temporary multicellular, or permanently multicellular phenotypes.

Conversely to the pattern seen in ECM-related genes, there is differential expression of some genes involved in regulation of the *V. carteri* cell cycle that have been co-opted from *C. reinhardtii* orthologs, such as the RLS1 gene. In *C. reinhardtii*, the RLS1 gene is expressed under nitrogen or light limitation (Nedelcu 2009) but is the closest relative of the critical cell growth regulating regA gene in *V. carteri* (Duncan et al. 2007). The expression of RLS1, along with the other genes responding to nitrogen deprivation, are not only sensible in the context of the *C. reinhardtii* temporary colonial phenotype, but might also help resolve conditions that favored the expanded role of regA
in *V. carteri*. As stress conditions restricted the growth of temporarily multicellular *V. carteri*-like ancestors, they may have co-opted RLS1 as they transitioned to permanent multicellularity.

This study has provided tantalizing insights into the ecological and evolutionary ramifications of inducible defenses like those expressed by *C. reinhardtii*. The enriched genes in this study paint a clear (but incomplete) picture of the considerations required for the transition toward temporary multicellularity with a focus on morphological changes and triaging resulting stresses. Future work evaluating the functions of genes in the dataset lacking sufficient annotation will only expand our understanding of the ecology of *C. reinhardtii*. In addition, when this dataset is viewed through the lens of the genome of *V. carteri*, the combination of expansion of *V. carteri* orthologs for upregulated genes and the differential expression of genes noted to be important for the development of permanent multicellularity solidify the evolutionary significance for temporary *C. reinhardtii* colonies in the development of permanent multicellularity. While this is clearly only the first step down the long road toward multicellarity, establishing this connection between the temporary and permanent multicellular phenotypes in the Volvocales opens new avenues of inquiry for studying early multicellular transitions through exploration of existing temporary colonial phenotypes.
Figure 1) Log_{10} transformed per mL abundances for *Chlamydomonas reinhardtii* unicells (Panel A, F_{(2,3)} =447.025, p>0.002), *Chlamydomonas* colonies (Panel B, F_{(2,3)} =10.9, p=0.042), and *Brachionus calyciflorus* (Panel C) over the four weeks of batch culture growth. Bars indicated 95% confidence intervals.
Figure 2. Heat map showing the Euclidean distances between biological replicates within treatments. Relative shading of cells indicate similarity between replicates. Differences between treatment lines suggest similarity in expression according to treatment with some increase in variation in the predated treatment.
Figure 3. Scatterplot (MA plot) where each dot represents a gene expressed in either treatment. The x axis is the number of normalized counts for each gene as calculated by DeSeq2. The y axis is the calculated log 2-fold change. The green line represents a 1:1 degree of expression between treatments, with genes above the line being differentially over expressed and genes under the line being differentially under expressed. Genes with at least a 2-fold change in expression and a FDR adjusted p-value of 0.05 are marked in green. Genes which see greater than 2-fold change in either direction are denoted with a triangle.
Table 1. The enriched Gene Ontology terms produced by goseq and their annotation organized by direction of regulation.

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<td>GO:0045505</td>
<td>Down</td>
<td>1.72E-12</td>
<td>dynein intermediate chain binding</td>
<td>Molecular Function</td>
</tr>
<tr>
<td>GO:0008569</td>
<td>Down</td>
<td>1.72E-12</td>
<td>ATP-dependent microtubule motor activity, minus-end-directed</td>
<td>Molecular Function</td>
</tr>
<tr>
<td>GO:0003774</td>
<td>Down</td>
<td>5.96E-09</td>
<td>motor activity</td>
<td>Molecular Function</td>
</tr>
<tr>
<td>GO Accession Number</td>
<td>Regulation</td>
<td>P value</td>
<td>Annotation</td>
<td>GO Class</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------</td>
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</tr>
<tr>
<td>GO:0031514</td>
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<td>6.19E-39</td>
<td>motile cilium</td>
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<tr>
<td>GO:0030286</td>
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<td>8.72E-14</td>
<td>dynein complex</td>
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</tr>
<tr>
<td>GO:0036156</td>
<td>Down</td>
<td>6.74E-11</td>
<td>inner dynein arm</td>
<td>Cellular Component</td>
</tr>
<tr>
<td>GO:0005929</td>
<td>Down</td>
<td>3.56E-10</td>
<td>cilium</td>
<td>Cellular Component</td>
</tr>
<tr>
<td>GO:0042995</td>
<td>Down</td>
<td>4E-09</td>
<td>cell projection</td>
<td>Cellular Component</td>
</tr>
<tr>
<td>GO:0005856</td>
<td>Down</td>
<td>8.52E-09</td>
<td>cytoskeleton</td>
<td>Cellular Component</td>
</tr>
<tr>
<td>GO:0005874</td>
<td>Down</td>
<td>1.14E-07</td>
<td>microtubule</td>
<td>Cellular Component</td>
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<tr>
<td>GO:0030991</td>
<td>Down</td>
<td>1.09E-05</td>
<td>intraciliary transport particle A</td>
<td>Cellular Component</td>
</tr>
<tr>
<td>Other (Membrane Coat, Response to light)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0009638</td>
<td>Down</td>
<td>0.029693</td>
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<td>Biological Process</td>
</tr>
<tr>
<td>GO:0030117</td>
<td>Down</td>
<td>0.054521</td>
<td>membrane coat</td>
<td>Cellular Component</td>
</tr>
</tbody>
</table>
Figure 4. Functional groupings of GO categories observed for upregulated and downregulated genes. Numbers associated with each segment are the number of differentially expressed genes in that category.
Figure 5. Scatter plot showing genes differentially expressed by grazed *Chlamydomonas reinhardtii* versus the number of orthologs of those genes in *Volvox carteri*. Upregulated genes are shown in green while downregulated genes are shown in red. Genes not differentially expressed are black. A positive value on the y-axis indicates more orthologs of a particular gene in *V. carteri* while a negative one indicates more orthologs in *C. reinhardtii*. The blue line indicates a 1:1 relationship in the number of orthologs. Downregulated ($Z = -3.731$, $p < 0.001$) and non-differentially expressed genes ($Z = -7.494$, $p < 0.001$) both see ortholog constriction in the *Volvox* genome while upregulated genes see expansion ($Z = 3.3436$, $p < 0.001$).
Table 2. Genes which are differentially expressed in grazed *Chlamydomonas reinhardtii* which have been demonstrated to have played a role in the development of permanent multicellularity in the Volvocales.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Genes of Importance Differentially Expressed</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Cycle Regulation</strong></td>
<td>Genes modified in <em>V. carteri</em> to regulate cell cycle either between phases or during division</td>
<td>CDKA1, RLS1, E2FR1, GAR1, HSP70A</td>
<td>Hanschen et al. 2016; Olson and Nedelcu 2016; Jong et al. 2017; Matt and Umen 2018</td>
</tr>
<tr>
<td><strong>ECM and cell exterior</strong></td>
<td>Genes modified in <em>V. carteri</em> that comprise the extracellular matrix</td>
<td>Pherophorins (C7, C11, Cre11.g481750, Cre06.g301806, Cre05.g245950)</td>
<td>Hanschen et al. 2016; Olson and Nedelcu 2016; Klein et al. 2017</td>
</tr>
<tr>
<td><strong>Microtubules and flagellar components</strong></td>
<td>Genes associated with movement and regulation of flagella</td>
<td>tubA1, tubB1, dyhA</td>
<td>Bisova et al. 2005; Olson and Nedelcu 2016; Matt and Umen 2018</td>
</tr>
</tbody>
</table>
References


CHAPTER 4: A FIRST TURN OF THE RATCHET: ARTIFICIALLY SELECTING FOR MACROGRAZING TOLERANCE OF *CHLAMYDOMONAS REINHARDTII* DEFENSIVE COLONIES IN THE PRESENCE OF MULTI-PREDATOR COMMUNITIES

Abstract

Temporary multicellular phenotypes of predominantly unicellular species offer a unique opportunity to examine the types of selective pressures that favor the evolution of multicellularity. Here I describe a selection experiment that first induced multicellular colony formation in *Chlamydomonas reinhardtii* by alternately allowing the alga to interact with rotifers (*Brachionus calyciflorus*), and then allowing colonies to interact with freshwater snails (*Physa* sp.) that selectively prey on colonies. Ten months of alternating selection by the two different kinds of predators, micrograzers and macrograzers, led to the evolution of a novel modification of the *C. reinhardtii* colonial phenotype. Some cells of the normally non-motile colonies began to develop flagella in as few as 550 generations after selection began. Colonies with flagella-bearing cells make up only a fraction of the total colonial population, with only a subset of cells in each colony possessing flagella. The colonies with flagella do, however, persist in the absence of macrograzers for a minimum of four weeks, suggesting that this was a non-plastic adaptation rather than a transient manifestation of phenotypic plasticity. While the colonies containing flagella-bearing cells are similar to their non-flagellated counterparts in many ways (in terms of size, cell number, and micrograzer resistance), they remain suspended in the water column longer than their non-flagellated counterparts. Longer settling time creates a potential advantage in terms of macrograzer avoidance for colonies.
with flagella. This modification of the colonial phenotype, and the rapidity with which it develops, supports the idea that the evolution of traits seen in the permanently multicellular and motile Volvocales could have developed rapidly with few novel mutations from unicellular ancestors. The emergence of this transitional phenotype provides an exciting example of how combinations of different community interactions over evolutionary time may have shaped the natural history of *C. reinhardtii* and its ancestors and led to phenotypes that are permanently both multicellular and motile.

**Introduction**

Studying the initial stages of major transitions in evolutionary lineages, such as the emergence of permanently multicellular phenotypes from previously unicellular lineages, is a difficult task (Michod 2000). It is often impractical to reconstruct the conditions and selective pressures that favor large changes in morphology and ecology. While great advances have been made by evaluating extant species and their evolutionary relationships, much of our understanding is incomplete due to an inability to directly observe such transitions (Maynard Smith and Szathmáry 1997).

The development of permanently multicellular species from previously unicellular lineages has attracted a great deal of attention due to the biological impact and evolutionary success of increasingly complex multicellular species. Multicellularity appears to have emerged independently a number of times across (and within) different groups (Knoll 2011), ranging from as few as one event giving rise to an entire
multicellular clade — as in the metazoa (King 2004; Richter and King 2013) — to multiple cases of multicellular emergence in bacteria, fungi, and plants (Medina et al. 2003; Grosberg and Strathmann 2007; Umen 2014). Even the simplest forms of multicellularity present a number of ecological advantages compared with single cells, ranging from opportunities for occupying novel niches by reducing limitations in size and complexity (Beardall et al. 2009; Umen 2014) to reductions in predation risk by small gape limited predators (Boraas et al. 1998; Lass and Spaak 2003). The most rudimentary expression of multicellular phenotypes, the temporary or reversible formation of multicellular colonies, can provide an inducible defense against grazing and has been suggested as a likely origin for some multicellular lineages (Boraas et al. 1998; Pfeiffer and Bonhoeffer 2003).

While the advantages of adopting permanent multicellularity are retrospectively evident, studying the eco-evolutionary relationships between extant multicellular organisms and their unicellular relatives can determine the types of genotypic and phenotypic changes required for developing these advantageous phenotypes. However, evaluating the earliest transitions in maintaining successful multicellular phenotypes can be difficult, partially due to a lack of extant clear transitional forms. These key organisms would provide much needed information on how early facultatively multicellular species made the changes toward permanently multicellular phenotypes, and the suites of traits which favored the persistence of those phenotypes.

Early transitions between temporary multicellular forms and more permanent ones might be relatively easy to accomplish (Knoll 2011), with a number of studies
suggesting that some lineages with unicellular and multicellular members can maintain permanent multicellularity with relatively little modification (Merchant et al. 2007; Prochnik et al. 2010; Hanschen et al. 2016). In addition to, or perhaps resulting from, the apparent ease of the unicellular-multicellular transition, experimental studies on yeast and green algae have demonstrated that artificial selection can create novel multicellular phenotypes (Ratcliff et al. 2013a, b), highlighting the possibility that the evolution of multicellular phenotypes might be far less difficult than previously thought (Ratcliff et al. 2012).

Despite the speed with which multicellularity can develop, the environmental requirements and constraints on species undergoing the earliest unicellular-multicellular transition can lead to reversion to unicellularity without some form of stabilization (James et al. 2006; Becks et al. 2010; Geng et al. 2013). Modifications which reduce reversion to unicellularity, sometimes referred to as ratcheting, have been predicted to lead to the persistence of more complex forms by increasing fitness of more permanent multicellular phenotypes compared with temporary ones (Libby and Ratcliff 2014). Modeling such cases of early multicellularity suggests that reversion can be avoided as fitness for each member cell of these multicellular organisms increases compared with the fitness of cells in temporary phenotypes. This suggests that iterative increases in fitness could lead to permanent multicellularity through modifications that make complex forms more ecologically feasible (Shelton and Michod 2014; Maliet et al. 2015; Libby et al. 2016).
The Volvocine green algae offer an excellent system for studying the evolution of permanent multicellularity (Herron and Nedelcu 2015). This lineage is well described phylogenetically and contains extant members which range from predominantly single cellular phenotypes (e.g. *Chlamydomonas reinhardtii*) to permanently multicellular species (e.g., *Volvox carteri*). The lineage has been used in the past to conceptually outline the necessary transitions toward fixed multicellularity (Kirk 2005; Hanschen et al. 2016), while also demonstrating that novel multicellular forms can arise in *C. reinhardtii* under favorable conditions (Ratcliff et al. 2013a). However, the selective pressures and conditions that favor the ratcheting of temporary multicellular phenotypes to more permanent one are poorly described.

The high degree of genomic similarity between *C. reinhardtii* and multicellular members of the lineage (*Gonium pectorale* and *Volvox carteri* (Prochnik et al. 2010; Hanschen et al. 2016)), as well as the proposed similarity between *C. reinhardtii* and the unicellular ancestor that gave rise to the Volvocales (Kirk 2005; Herron and Michod 2008), demonstrate the suitability of this lineage for studying unicellular-multicellular transitions. *C. reinhardtii* expresses a variety of non-permanent colonial phenotypes in response to organic acid exposure (Iwasa and Murakami 1969), increased salinity (Khona et al. 2016), and perhaps most notably predation (Lurling and Beekman 2006; Ellner and Becks 2011; Sathe and Durand 2016). Consequently it is an ideal candidate for evaluating how increased fitness of temporary colonial phenotypes can become more advantageous and develop into fixed multicellularity.
This study explores the effects of fluctuating selection by micrograzers and macrograzers on populations of the green alga *Chlamydomonas reinhardtii*. The introduction of micrograzing predators to cultures of *C. reinhardtii* is known to cause the expression of a colonial algal phenotype, which is highly effective at defending against predation by micrograzers but has been previously shown to create an opportunity for macrograzing species to exploit the colonial phenotype. The combined exposure of *C. reinhardtii* to micrograzers and macrograzers can lead to the exclusion of the alga in fewer than 50 generations (see Chapter 1). The experiment described here alternated the exposure of *C. reinhardtii* to micrograzers to macrograzers to alternately select for and against the temporary multicellular phenotype. By alternately relaxing each mode of selection, it might be possible for the algae to persist long enough to evolve additional traits, such as motility in the multicellular phenotype. Algal populations were monitored to determine if abundances of the colonial phenotype, and traits involved in its expression, might change when given time to adapt. Additionally, it was of interest to evaluate if these fluctuating selective pressures and led to the development of a colonial phenotype more typical of the multicellular Volvocales. The emergence of colonies that display a varying degree of flagella expression and a reduced tendency to settle out of the water column would suggest that modifications of this multicellular phenotype are tractable and may have important ecological and evolutionary consequences.
Methods

Design overview

To determine if traits involved in *Chlamydomonas reinhardtii* defense expression change as a result of pulsed selection by macrograzers on the colonial phenotype this experiment employed three distinct phases (see Figure 1). 1) Phase 1 involved a ten month period of selection during which populations containing micrograzers were exposed to macrograzers followed with periods of recovery without macrograzers in two day cycles. (Figure 1 Phase 1). 2) After completion of the initial 10 month selection regime I assessed potential changes in the ability of *C. reinhardtii* colonies to resist predation by either micrograzers or macrograzers separately (Figure 1 Phase 2). 3) Lastly I determined if evolved *C. reinhardtii* colonies were better able to tolerate combined predation by both micrograzers and macrograzers concurrently (Figure 1 Phase 3).

Pre-selection regime setup

Organisms and culturing conditions

Cultures of *Chlamydomonas reinhardtii* (strain CC-1010) were obtained from the *Chlamydomonas* Resource Center (University of Minnesota), and were maintained in isolation for a minimum of six months prior to interaction with other species. Cultures of the micrograzing rotifer *Brachionus calyciflorus* were originally obtained from the laboratory of Nelson Hairston Jr. (Cornell University, Ithaca, New York) and were reared on an alternate prey species (*Ankistrodesmus sp.*) until experimental treatments began. Common bladder snails (*Physa spp.*) were collected from Weston’s Mill Pond (Rutgers
Gardens, New Brunswick, NJ) and stored in initially sterile medium for 24 hours before addition to treatments described below.

All populations were maintained in either modified Woods Hole medium (Adapted from Guillard and Lorenzen 1972) (Snails and rotifers) or a 1:1 mixture of TAP medium (Gorman and Levine 1965) and modified Woods Hole medium. Fresh lines of algae used to feed predator populations were started every three weeks by adding a 50 mL inoculum of *C. reinhardtii* or *Ankistrodesmus sp.* to sterile medium. An initial inoculum of a dense rotifer culture was introduced to cultures of *Ankistrodesmus* to establish maintenance lines of predators without recent exposure to *Chlamydomonas*. All maintenance and experimental cultures were kept at 24° C in a Percival incubator with a 12h light:12h dark photoperiod in 250 mL glass jars.

**Phase 1 – Selection Regime**

*Selection regime design*

A periodically relaxed selection regime by macrograzers was used to maintain selection against the nonmotile colonial phenotype while preventing complete extinction that can result from continuous predation by micrograzers and macrograzers (see Chapter 1). After initially establishing cultures containing algal prey and micrograzers to induce colony formation, macrograzers (snails) were added to each replicate for two days alternating with macrograzer removal for two-days. This alternating selection regime continued for ten months.
Selection regime cultures consisted of 100 mL of initially sterile media kept in 250mL glass jars. All experimental treatments received an initial inoculum of 100 mL of dense *C. reinhardtii* culture (>2 x 10^6 cells per mL) that then grew for one week without grazers. *B. calyciflorus* were isolated from maintenance cultures, washed in sterile medium to remove *Ankistrodesmus*, and then transferred 20 at a time using micropipettes to cultures of *C. reinhardtii*. Algae and rotifers interacted for two weeks to induce algal colony formation. After monitoring to confirm colony formation, forty replicates were subjected to the alternating selection regime outlined above. Cultures received two snails transferred from sterile algal free medium, then snails were removed after two days by transferring the culture to a new sterile jar through a sterile 250 micron Nitex® membrane that retained macrograzers.

Subculturing every three weeks involved transferring 50 mL from each replicate to 50 mL of dense un-predated *C. reinhardtii* cultures (>2 x 10^6 cells per mL) to supply fresh medium and prevent micrograzer population collapse. Jars were gently shaken continuously on a rotary shaker at 300 rpm and lids were loosely secured to promote gas exchange while limiting contamination.

*Monitoring for changes in colony expression*

Experimental microcosms were monitored weekly for pH, changes in volume due to evaporation, evidence of bacterial or fungal contamination, and herbivore abundance. Cultures were sampled weekly to measure abundances of unicellular and colonial *Chlamydomonas* and *Brachionus*. Microcosms were gently mixed by manual shaking, and a 1 mL subsample was taken with a sterile pipette. Sampled medium was replaced
with 1ml of sterile medium. Data collected included counts (per mL) of unicellular *C. reinhardtii*, multicellular *C. reinhardtii* colonies, and rotifers. Counts of larger colonies and rotifers were made by observing the total number in the sample using a Nikon SMZ-U microscope at 20X, while smaller colonies and unicells were counted using a Reichert hemocytometer and a Nikon Eclipse 80i compound microscope at 400X (phase contrast). Morphological changes were monitored by visual observation at 40x magnification.

Following the first observation of colonies with cells bearing flagella, the abundance of these colonies was measured using the methods described previously for colonies lacking flagella. All counts were reported as the logarithm (base 10) of density of each species (for single organisms) or colony per milliliter.

**Phase 2 – Characterization of evolved lines**

*Persistence of the flagellated colonial phenotype*

Four replicates which developed colonies with flagella were subcultured by filtering 25 mL from each replicate through 250 micron Nitex® membrane into 3000 L Erlenmeyer flasks containing 1000 mL of TAP medium. Fresh unselected populations obtained from the *Chlamydomonas* resource center were obtained to create maintenance lines of naïve *C. reinhardtii* using the same methods. After establishment of *C. reinhardtii* in these maintenance lines, biweekly subculturing was performed by adding 100 mL of dense algal culture (>2 x 10⁶ cells per mL) to 900 mL of sterile TAP medium. Cultures were maintained in the absence of any grazers for a minimum of two weeks before testing.
Potential plasticity of the colonies with flagella was assessed by establishing ten 1000 mL populations of *C. reinhardtii*, sourced from each population from the selection regime with colonies with flagella, in sterile Woods Hole medium. After allowing one week for algal growth, 20 individual micrograzers were introduced and cultured for four weeks in all populations. Populations were monitored weekly as described previously to assess effects of grazers on selected and naïve populations. The ten replicate populations for each of the lines containing colonies with flagella were used for subsequent comparison with naïve populations.

*Comparison of colonies with and without flagella*

To determine if *C. reinhardtii* colonies with or without flagella differ in other characters, especially those enhancing tolerance against micrograzing or macrograzing predators, colonies from populations were compared for maximal dimensions (length and width) and number of cells per colony to determine changes in potential micrograzer tolerance. I also used the rate at which populations with and without evolved colonies settled after disturbance (and were consequently outside areas available to macrograzers) to determine if colonies with active flagella increase resistance to macrograzing.

To characterize colonies between evolved and unevolved lines I sampled ten colonies with flagella (roughly the number of evolved colonies per mL) from each of ten replicate populations created at the end of the four-week time course. For comparison, ten colonies were also taken from each of the naïve populations of *C. reinhardtii* established previously. This yielded 100 colonies for the naïve population and 100 colonies with flagella for each of the lines from the selection regime containing the modified
phenotype. Each colony was taken from a 3mL subsample and moved to a concave glass microscope slide using micropipettes. I counted the number of cells (with and without flagella) making up each colony. Maximal length and width of each colony were determined by photographing each colony at 20x magnification and using ImageJ (version 1.51j8).

To assess if populations containing colonies with flagella take longer to settle in the water column each population used for colony comparison was gently shaken before a 10mL subsample was collected and placed in a sterile test tube. Abundances of unicells and colonies were determined before diluting each subsample to an approximate density of 100 colonies per milliliter. The subsamples were gently homogenized by repeated inversion. Each test tube was monitored until it visually resembled an undisturbed reference from the same population and carefully pipetted samples from the water column contained no colonies. The total elapsed time for settling by the populations was recorded after no colonies remained suspended.

**Phase 3 – Determination of macrograzer resistance in evolved lines**

**Comparison of fitness between colonies with and without flagella with macrograzing**

To evaluate the macrograzer tolerance of populations containing colonies with flagella, populations of the four evolved lines were pooled to create a treatment containing colonies with flagella. This was compared with a treatment containing naïve *C. reinhardtii*. Initial cultures of *C. reinhardtii* for these two treatments were established either from fresh cultures obtained from the *Chlamydomonas* resource center or by
pooling 20 mL of homogenized samples taken from selection regime cultures which
contained colonies with flagella (80 mL total) filtered through 250 micron Nitex®
membrane. Initial cultures were grown on TAP medium for a minimum of two weeks
prior to use in establishing experimental cultures. Both treatments were replicated five
times.

All treatments received an initial inoculum of 10 mL of dense algal culture (>2 x
10⁶ cells per mL) added to 140mL of modified Woods Hole medium in glass jars and
then grew for one week without grazers. After one week individuals of B. calyciflorus
were added as previously described and grown for four weeks to induce algal colony
formation. In week five, four individual snails were added to all cultures. Experimental
microcosms were maintained and algal and rotifer abundances were sampled as described
previously.

Statistical analyses

Log transformed densities of C. reinhardtii colonies from macrograzed and
control treatments from the selection regime were compared using a repeated measures
analysis of variance (ANOVA). Populations containing colonies with flagella were
compared before and after macrograzer removal to determine the log transformed
abundances of colonies with flagella changed in the absence of macrograzers using a
repeated measures ANOVA for the four weeks of monitoring.

A multivariate analysis of variance (MANOVA) compared the log transformed
abundances of micrograzers, log transformed abundances of all phenotypes of the algal
prey, average colony settling time, number of cells per colony, and average maximal dimensions for colonies for populations having colonies with or without flagella.

Repeated measures multivariate analysis of variance compared the log transformed abundances of *C. reinhardtii* unicells, *C. reinhardtii* colonies, and rotifers among treatments over time in the continuous macrograzing trials. All statistical tests were performed in R version 3.3.3 (R Core Team 2017).

**Results**

*Selection against the colonial phenotype by macrograzers*

Alternating selection imposed by macrograzers and micrograzers significantly reduced the abundances of *C. reinhardtii* colonies compared with treatments without macrograzers (Repeated Measures ANOVA, $F_{19,180} = 29.34$, $p < 0.0001$). The alternating addition and removal of the macrograzers resulted in the long-term persistence of algae and micrograzers in experimental microcosms (Figure 2). Subculturing temporarily increased the abundances of algae and micrograzers but all populations returned to long term trends after one week.

*Emergence of flagellated colonial phenotype*

After roughly 120 days of selection colonies with cells bearing flagella first appeared. Individual colonies with flagella maintained that phenotype throughout a 72-hour observation period (Figure 3) without predation. Out of 40 total replicates undergoing selection, only four replicates contained colonies with cells bearing flagella. Once colonies with flagella developed they were observed to persist in the absence of
macrograzers for at least four weeks at the population level (Repeated Measures ANOVA, $F_{19,60} = 0.847$, $p = 0.66$).

_Characterization of colonies with flagella_

Populations of _C. reinhardtii_ colonies sampled from evolved and unevolved lines were compared for traits potentially related to their ability to tolerate micrograzing (colony size dimensions and cell number) and macrograzing (settling time). The results of a multivariate analysis of variance indicated a significant difference between evolved and unevolved lines based on the variables included (MANOVA, $F_{8,41} = 3.098$, Wilks $\lambda = 0.291$, $p < 0.0001$). Abundances of colonial phenotypes and unicells, colony dimensions, cell number per colony, and population settling time, are statistically distinct between selected and naïve lines. However, colony dimensions and cell number did not differ significantly between colonies with and without flagella (Table 1). In lines established using an inoculum from each replicate containing colonies with flagella, there was up to a five-minute increase in the amount of time it took to for populations to settle (ANOVA, $F_{4,45} = 31.57$, $p < 0.0001$, Figure 4).

The modified colonial phenotype is far from pervasive in populations containing it, with colonies bearing flagella making up between seven and thirty-five percent of the total colony population. In addition, colonies with flagella remain irregular in structure and only between seventeen and fifty-three percent of cells in colonies from selected lines have observably beating flagella. The increase in population settling time in colonies with flagella suggests that while the modified phenotype is irregular and
unevenly expressed, it might differ from the naïve phenotype in avoidance of macrograzing.

**Effects of continuous macrograzing on colonies with and without flagella**

Microcosms of micrograzed *C. reinhardtii* which were exposed to consistent macrograzing after the appearance of colonies produced significantly higher abundances of *C. reinhardtii* unicells (Repeated Measures ANOVA, $F_{2,37} = 44.29, p < 0.0001$), colonies (Repeated Measures ANOVA, $F_{3,36} = 50.56, p < 0.0001$), and micrograzing *B calyciflorus* (Repeated Measures ANOVA, $F_{2,27} = 4.082, p = 0.03453$) after one week. Macrograzing caused algal population collapses after only one week in 80% of the replicates of the unevolved treatments, while populations with colonies that developed flagella had higher abundances of both unicellular and colonial phenotypes (Figure 5 & Figure 6). Despite this initial advantage, populations of both selected and naive lines of *Chlamydomonas* did collapse after two weeks of sustained exposure to macrograzers.

**Discussion**

Numerous examples of opportunistic exploitation of induced defense phenotypes have been documented (Persons et al. 2001; Beckerman et al. 2010; Hoverman and Relyea 2016). The possible evolutionary ramifications for the defended species are difficult to explore because susceptibility can result in rapid elimination by opportunistic predators before evolution can occur. In addition, long generation times of some defended species limit the ability for such consequences to be explored due to intractability. This selection experiment made use of a clearly detrimental opportunistic
exploitation of the defensive *Chlamydomonas reinhardtii* colonial phenotype to circumvent these problems by using a periodically relaxed selection regime imposed on an organism with a short generation time.

After roughly 550 *C. reinhardtii* generations, colonies that possessed flagella emerged in a fraction of the selected lines. These colonies, while rare, persist for a minimum of four weeks suggesting that while the transition to colonies with flagella under this selection regime is far from an absolute consequence of macrograzers acting on *C. reinhardtii* colonies it is not the result of plasticity. The rarity of the modified phenotype across selection regime replicates is not surprising as a number of studies involving experimental evolution through artificial selection regimes have noted that even employing strong selection regimes can often result in little if any observable change in phenotype (Blount et al. 2008; Ratcliff et al. 2013a). The speed with which the populations of *C. reinhardtii* evolved macrograzer resistant colonies, coupled with the fact that the modified phenotype was not limited to only one experimental replicate, could have interesting implications for understanding the ecological interactions contributing to the evolution of permanent multicellularity in the Volvocales. This also suggests that while the frequency of colonies with actively beating flagella increased here, some degree of standing variation for flagellar expression within the micrograzer resistant colonies likely exists. While significant numbers of colonies did not form in the absence of micrograzers, the continued expression of the colonies with flagella in the absence of macrograzers suggests the observation of evolutionary change instead of plasticity.
Interestingly, while colonies with flagella maintain traits that will retard micrograzing, populations containing them take significantly longer to settle fully after disturbance compared with naïve populations. This modification to the colonial phenotype demonstrates that \textit{C. reinhardtii} colonies can adapt to a form more similar to the multicellular Volvocales as a result of a combination of community level interactions. The observed increase in settling time for colonies with flagella, coupled with the increase in abundances for both \textit{C. reinhardtii} colonies and unicells in evolved populations, suggests a direct ecological benefit to colonies with flagella. The colonies containing cells with flagella observed here offer an exciting insight into how a variety of factors at the community level can contribute to maintaining permanent multicellularity through creating phenotypes that indirectly reduce some of the primary costs of being temporarily colonial (such as reduced photosynthetic rate).

Over the nearly 1 billion years following the divergence of the first unicellular member of the Volvocales (Michod 2007; Herron et al. 2009), exposure to multiple grazing strategies may have selected for traits like the addition of flagella to colonies to reduce the impacts of benthic herbivory. As the early Volvocales experienced growing community-level grazing pressures, \textit{C. reinhardtii} may have found refuge in ephemeral waters in exchange for a less ideal growing environment. Contrasting with this proposed cause for habitat restriction in \textit{C. reinhardtii}, multicellular species of the lineage combined retention of flagella and a colonial phenotype (investment in multicellularity) which allowed them to simultaneously minimize the effects of predation by micrograzers and benthic macrograzers. Such early multicellular species, better able to maintain their
position in the water column compared with the immobile *C. reinhardtii* colonial phenotype, would see the added benefit of not being restricted to less productive areas of their habitat. This combination of macrograzer avoidance and increased productivity (compared with temporary colonial phenotypes in *C. reinhardtii*) poses an interesting conceptual framework for the initial transition from temporary multicellular forms into more permanent ones. The rapid appearance of retention of flagella by colonial *C. reinhardtii* in response to artificial selection suggests that similar processes may have been one of the first turns of the evolutionary ratchet to tighten the cooperative link between the members of the colony.

Previous work on settling rates in the Volvocales suggest a relative reduction of mobility in simple multicellular members of the lineage, such as *Gonium pectorale*, resulting in simple multicellular Volvocales settling more quickly than *C. reinhardtii* unicells (Solari et al. 2006, 2015). This consideration of only the hydrodynamics of the Volvocales has led to speculation that motility may not have been an integral factor (or may have been coincidental) in the early development of the lineage, though the results I report here suggest otherwise. While the most rudimentary multicellular Volvocales may not have experienced a significant increase in motility compared to their unicellular ancestors, forms like those resulting from this experiment which increase settling time as a form of predator avoidance could explain why the less complex multicellular members of the Volvocales possess flagella when the temporary colonial phenotype in *C. reinhardtii* often do not. As these slower settling colonies gained an advantage against multiple predator types by being able to maintain a higher position in the water column
for longer periods following a disturbance, they would also be closer to the photic zones which are more favorable for photosynthesis. This combination of increased macrograzer avoidance and energy production compared with colonies without flagella would provide two major advantages which, barring major changes at the ecosystem level, could make remaining a simple multicellular more favorable than separating.

The results of this selection experiment demonstrate several novel findings. First, modification of the temporary colony defense of *C. reinhardtii* to include the expression of at least some cells with flagella is possible in relatively short periods in response to strong community level selection. Second, these colonies provide at least one ecological benefit. At the population level in the form of increased macrograzer avoidance and persistence time in the presence of consistent macrograzing, 3) Populations containing these modified colonies are likely better suited to maintaining a position in the photic zone (as the ability to avoid macrograzers incorporates maintaining a higher position in the water column). While I did not observe increases in abundances of micrograzed populations of colonies with flagella directly, the development of further advantages of this phenotype are likely to be contingent on a diverse array of conditions and selective pressures. Regardless, exploring early evolutionary transitions in this fashion provides us with thrilling opportunities to study how such transitions are accomplished as part of a shifting ecological environment. Expanding exploration of selective pressures acting on temporary colonial forms, observing changes to genomic architecture and gene expression, and the subsequent changes to fitness will doubtlessly improve our ability to
examine what factors tighten the evolutionary link between members of a multicellular organism.
Figure 1: Conceptual Diagram of Experimental Design. **Phase 1** 10 month period of selection in which snails were added to cultures of *C. reinhardtii* and micrograzers every two days. Out of 40 total replicates four eventually contained colonies with active flagella. **Phase 2** Batch cultures of each of the four replicates from Phase 1 were grown to measure characters of evolved and unevolved colonies. **Phase 3** Evolved and unevolved lines were exposed to consistent grazing by micrograzers and macrograzers to determine resistance of evolved colonies to macrograzing.
Figure 2: Comparison of abundances of *C. reinhardtii* colonies over the course of the selection regime in populations exposed to fluctuating macrograzing (Squares) and those with only micrograzers (Circles). Bars indicate 95% confidence intervals. (Repeated Measures ANOVA, $F_{19,180} = 29.34$, $p < 0.0001$)
Figure 3: Color photograph of a modified *C. reinhardtii* colony at 20X.
Table 1. Averages of measured characters for replicates derived from the four selected lines containing colonies with flagella compared with naïve populations. Average abundances and settling times were determined for each replicate population while average colony length and width were calculated for 100 colonies from each replicate line. Only colonies with flagella were selected for dimension measurements for evolved lines.

<table>
<thead>
<tr>
<th>Replicate of Origin</th>
<th>C. reinhardtii unicells/mL</th>
<th>C. reinhardtii colonies/mL</th>
<th>C. reinhardtii colonies with flagella/mL</th>
<th>Colony Length (microns)</th>
<th>Colony Width (microns)</th>
<th>Settling time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unselected Line</td>
<td>185,100</td>
<td>160</td>
<td></td>
<td>132.3</td>
<td>83.8</td>
<td>14.4</td>
</tr>
<tr>
<td>Evolved Line 1</td>
<td>200,600</td>
<td>182</td>
<td></td>
<td>140</td>
<td>61.6</td>
<td>16.2</td>
</tr>
<tr>
<td>Evolved Line 2</td>
<td>177,370</td>
<td>159</td>
<td></td>
<td>117.7</td>
<td>79.8</td>
<td>19.7</td>
</tr>
<tr>
<td>Evolved Line 3</td>
<td>192,200</td>
<td>177</td>
<td></td>
<td>134.6</td>
<td>86.4</td>
<td>18.5</td>
</tr>
<tr>
<td>Evolved Line 4</td>
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<td></td>
<td>124</td>
<td>72.9</td>
<td>17.5</td>
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</table>
Figure 4: Comparison of population settling times between unevolved populations (white) and the four experimental replicates which evolved colonies with flagella (gray). Bars indicate 95% confidence intervals (ANOVA, F$_{4,45}$=31.57, p < 0.0001)
Figure 5: Values of log transformed abundance data for *C. reinhardtii* colonies. Populations started from unevolved lines are represented by circles, evolved lines with squares. Bars represent 95% confidence intervals. (Repeated Measures ANOVA, $F_{3,36} = 50.56$, $p < 0.0001$)
Figure 6: Values of log transformed abundance data for *C. reinhardtii* unicells. Populations started from unevolved lines are represented by circles, evolved lines with squares. Bars represent 95% confidence intervals. (Repeated Measures ANOVA, $F_{2,37} = 44.29, p < 0.0001$)
References


CONCLUSION

The research presented in my dissertation not only expands current knowledge regarding the ecology and evolutionary biology of *Chlamydomonas reinhardtii*, but also highlights the importance of evaluating the consequences of inducible defenses. I have been able to demonstrate that formation of temporary colonial phenotypes can both be beneficial by providing a competitive advantage in certain community modules while the same defense can have its benefit entirely negated in others. I have shown that the identities of member species in community modules where the defense phenotype is expressed are extremely important to evaluating defense success and may have influenced the natural history of both *C. reinhardtii* and the multicellular Volvocales. In exploring the differential expression of genes in the colonial phenotype I was able to evaluate the *C. reinhardtii* defense phenotype on at the transcriptomic level to better understand the strategies it employs in response to grazing while also creating a set of 286 candidate genes of interest for future study. In evaluating orthologs of the genes from my differential expression analysis in the genome of the multicellular Volvocale *V. carteri* I was able to more firmly relate the proposed position of temporary multicellular phenotypes to permanent ones for this lineage. I was also able to further demonstrate that the *C. reinhardtii* defense phenotype is capable of adapting rapidly to combined predation under experimental conditions using fluctuating selection in ways that favor maintaining a colony instead of separating into single cells.