The genetic basis of cooperative aggregation in the green alga *Chlamydomonas reinhardtii*

by

CHRISTOPHER MICHAEL BERGER

B.S., Kansas State University, 2013

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

Major Professor
Bradley JSC Olson

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Abstract

Unicellular organisms alter their behavior and morphology in response to environmental stresses, particularly in response to immediate threats to their survival. A common tactic of predator avoidance for unicellular green algae is to aggregate to form groups. We have found that the model unicellular green algae *Chlamydomonas reinhardtii* forms aggregates in response to the presence of the filter feeding zooplanktonic predator, *Daphnia magna. Chalmydomonas* is a member of the volvocine algae, a morphologically diverse group of closely related green algae that is often used to study multicellular development. We have characterized aggregation in *Chlamydomonas reinhardtii* and found that it is rapid, transient and induced by signals originating from the *Daphnia* predators. To understand the genetic basis of cooperative aggregation we used an RNA-seq approach. RNA-seq characterized the transcriptomic response by *Chlamydomonas* during aggregation, and we identified 131 genes are significantly differentially expressed between predated and unpredated cultures of *Chlamydomonas*. Several candidate genes were characterized based on existing annotations, evolutionary history and expression profile. Evolutionary relationships between candidate aggregation genes in *Chlamydomonas* and their orthologs in multicellular Volvocales suggest a possible role of aggregation genes in multicellular development. Our results demonstrate that *Chlamydomonas* dynamically alters its morphology based on its environment and identify several candidate genes for aggregation and multicellular development.

Table of Contents

List of Figures vi
Acknowledgements vii
Dedication viii
Chapter 1 - Size Dependent Predator Defenses In Microbes 1
Introduction 1
Chapter 2 - Predator Induced Cooperative Aggregation In *Chlamydomonas reinhardtii* 6
Introduction 6
Material and Methods 7
Algal Strains and Growth Conditions 7
Daphnia Rearing Conditions and Decontamination 7
Growth Rate Analysis 8
Cooperative Aggregation 9
Short and Long Term Aggregation 9
Transmission Electron Microscopy 10
Aggregate Dispersal 10
Cell Lysates 10
Supernatant Experiments 11
Cycloheximide Treatment 12
RNA sequencing and quantification of genome-wide expression 12
Candidate Gene Cloning 14
Functional Testing of *g18292* 14
Statistics 16
Results 16
Organismal size correlates with fitness 16
Predators cause *Chlamydomonas* to rapidly aggregate 17
Aggregates are variable in size, adhered by cell wall contacts and their formation is dose dependent. 18
Aggregation occurs in multiple species and strains of unicellular green algae and is independent of *Daphnia* species. 18

Identification of genes that transcriptionally respond to predation 19
A predicted transmembrane protein *g18292* from *Gonium* causes *Chlamydomonas* to be multicellular 20
Discussion 20
Conclusions 40
References 41
Appendix A – Gene Table And Enrichment Data 48
Table A.1 Significant Genes And FPKM Values 48
Table A.2 Functional Enrichment Data 54

List of Figures

Figure 2.1 Algal Growth Rates in the Presence and Absence of Predators…….31
Figure 2.2 Predator Induced Aggregation……32
Figure 2.3 Mechanism of Aggregation……33
Figure 2.4 Aggregate Morphology……..34
Figure 2.5 Characteristics of Aggregation……...35
Figure 2.6 Conservation of Aggregation…….36
Figure 2.7 Transcriptional Response to Predation…….……..37
Figure 2.8 Time Course of Expression in Candidate Genes……38
Figure 2.9 *g18292* Functional Testing……..……...……39

Acknowledgements

I would like to thank my adviser, Brad Olson, for his support and mentorship during my graduate school career and enabling me to pursue a project that I am passionate about. Thanks to my committee Ted Morgan and Mike Herman for their input and guidance during my time at K-State. I would also like to thank current and former members of the Olson Lab, especially Sarah Cossey, Breanna Canning, Jaden Anderson and Tara Marriage for their involvement in this project.

Having attended K-State for both undergraduate and graduate school I have been fortunate enough to work with many people in the Division of Biology through research, teaching and in class. I would like to thank all the outstanding faculty members and graduate students I have had the privilege to work alongside. I would like to thank Dr. Dan Boyle for his assistance with electron microscopy and the NSF for funding during my time at K-State. My time in the department has expanded my skill as a scientist and equipped me for life and work outside of school.

I am also grateful for my family for their support over the years. I have been fortunate to have amazing parents who have valued my education and supported my aspirations through my entire academic life. Finally, I would like to thank my wife, Stevie, for her wonderful support and patience during my time in graduate school.

Dedication

I would like to dedicate this work to all my grandparents for their love, guidance and care throughout my entire life.

Chapter 1 - Size Dependent Predator Defenses In Microbes

Introduction

Organisms alter their behavior, metabolism and morphology in response to biotic and abiotic stimuli in order optimize their fitness in challenging environments. Organisms are particularly responsive to environmental threats that immediately impact their survival, especially the effect of predation (Boersma, Spaak, & De Meester, 1998; Fisher, Bell, & West, 2015; Johnsson, Höjesjö, & Fleming, 2001; Lima & Dill, 1990). Thus, species have evolved diverse strategies to evade predators. For microbial organisms, evading predation presents a challenge; thus most species evade predation by increasing their size making them more difficult to consume (Becks et al., 2012; Fisher et al., 2015; S Lass & Spaak, 2003; Lüring & Van Donk, 1997). Increasing size is especially effective for microbial organisms evading consumption by filter feeders that cannot easily capture large prey (Burns, 1968; Hansen, Bjørnsen, & Hansen, 1994). Cellular growth and organismal size comes with a cost, thus there is strong selective on organisms for optimal organismal size (Banse, 1976). This selective pressure for microbial eukaryotes is especially intense, as cells must balance the cell surface area to cell volume (Lewis, 1976). Thus, unicellular organisms aggregate as a method to increase total size rather than increasing individual cell size (Fisher, Bell, & West, 2015; Lurling & Van Donk, 2000; Sathe & Durand, 2015). Because cooperative behavior in microbial organisms is not well studied, little is known about the molecular basis of microbial predator evasion.

The evolution of cooperation is well established in animals, but often overlooked in microbial organisms where it is thought to be unique to “unusual” organisms such as social amoeba. However, an increasing body of literature demonstrates that microbial cooperation is more common than originally thought (Alegado et al., 2012; Damore & Gore, 2012; Fisher et al.,

2015; Foster, Parkinson, & Thompson, 2007; Lüring & Van Donk, 1997; Nadell, Xavier, & Foster, 2009; Sebé-Pedrós et al., 2013; Strassmann, Zhu, & Queller, 2000; West, Diggle, Buckling, Gardner, & Griffin, 2007). For example, aggregative cooperation is well understood in social amoeba such as *Dictyostelium*, where aggregation forms multicellular groups that make up a fruiting body used in spore dispersal (Abedin & King, 2010; Strassmann et al., 2000). Aggregative behavior is not restricted to eukaryotes; multiple species of prokaryotic genus *Myxococcus* exhibit multicellular group formation when preying on bacterial cells and forming fruiting bodies (Kraemer & Velicer, 2011; Travisano & Velicer, 2004; G J Velicer, Kroos, & Lenski, 2000). Similar phenotypic responses have been observed in unicellular relatives of metazoa such as *Capsaspora owczarzaki* (Sebé-Pedrós et al., 2013). Cellular aggregation has also been observed in several genera in the eukaryotic subgroup Rhizaria (Brown, Kolisko, Silberman, & Roger, 2012).

Predation is another environmental stress that is known to induce aggregation in microorganisms (Becks et al., 2012; Fisher et al., 2015; Lüring & Van Donk, 1997; Sathe & Durand, 2015). Cooperative aggregation in response to grazing by zooplanktonic predators has been observed in numerous species of unicellular green algae when subjected to *long-term* predation, usually after 24h or more hours (Fisher et al., 2015). Aggregation has been observed in various species of normally unicellular *Scenedesmus* during direct predation by zooplanktonic grazers such as *Daphnia* or rotifers (Elert & Franck, 1999; Lurling & Van Donk, 2000; Wu, Zhang, Qin, Cui, & Yang, 2013).

Appearance of multicellular morphologies has also been observed in mixed cultures of *Chlamydomonas reinhardtii* and the rotifer *Brachionus calyciflorus* (Becks et al., 2012; Lurling & Beekman, 2006). Aggregate formation in the green alga *Chlorella vulgaris* upon predation by

the ciliate *Tetrahyma thermophila* has also been observed (Fisher et al., 2015). Aggregation has also been induced in *Scenedesmus* by exposure to media that previously contained predators (Elert & Franck, 1999; Lüring & Van Donk, 1997; Lürling & Von Elert, 2001). *Daphnia* are thought to release small molecule "kairomones" that induce phytoplankton to aggregate, though there is disagreement as to the identify of these molecules (Lass & Spaak, 2003; Uchida et al., 2008; van Gool & Ringelberg, 1996; Wiltshire, Boersma, & Meyer, 2003; K Yasumoto et al., 2006; Ko Yasumoto et al., 2008; Yokota, 2007). Thus, it is unclear if or how algae detect the presence of filter feeding predators.

Widespread reporting of aggregation in the literature suggests that unicellular green algae have a mechanism for detecting signals released by predators and respond by forming aggregative colonies. However, these studies have focused on morphological changes observed after extended periods of predation (>24 h) and not on the short-term immediate response, its genetic basis or its relation to multicellular evolution.

In addition to being a known microbial predator response, cooperative aggregation also has implications in multicellular development and evolution. It has long been hypothesized that predation drove the evolution of undifferentiated multicellularity. Predation presented a novel and strong selection pressure for size that my have driven the evolution of multicellularity in groups including the volvocine algae (Michod & Herron, 2006; Michod & Nedelcu, 2003; Niklas, 2014). The volvocine algae are a monophyletic group of closely related green algae that includes the unicellular *Chlamydomonas reinhardtii*, the undifferentiated multicellular *Gonium pectorale* and the large multicellular *Volvox carteri* with germ soma tissue differentiation (Kirk, 2005). These three species represent vast diversity in multicellular complexity and orders of magnitude differences in size. This morphological variation coupled with remarkable genetic

similarity makes them a powerful tool for understanding the genetic basis of major evolutionary transitions particularly the evolution of multicellularity (Hanschen et al., 2016; Kirk, 2005; Merchant et al., 2007; Prochnik et al., 2010).

Selective pressures that drive the transition from unicellularity to undifferentiated multicellularity have been investigated. Stable multicellular strains of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* have been produced in the lab by utilizing gravitational selection that favors colonial morphologies. Because multicellular morphologies settle faster in liquid culture after homogenization, they can be selected for by sub-culturing from the bottom volume of culture containers (Moulton & Bell, 2013; Ratcliff, Denison, Borrello, & Travisano, 2012). However, these experiments are unrepresentative of real size-dependent selective pressures faced by microorganisms in nature. Experiments investigating long-term effects of size selection have also been performed in laboratory settings using more ecologically relevant selective pressures. Constitutively multicellular strains of *Chlorella vulgaris* have been produced by continuous predation by phagocytosing protists (Boraas, Seale, & Boxhorn, 1998). Groups formed by gravitational or long-term predator selection differ from transient, induced aggregates as they remain after the selective pressure is removed.

The plastic, colonial phenotypes observed in organisms capable of cooperative aggregation may represent an evolutionary stepping-stone to multicellularity (Olson, 2013; Sebé-Pedrós et al., 2013). The benefits of unicellularity, such as high growth rate (Banse, 1976), are well understood as are its costs, including increased predator threat. Likewise, there are costs and benefits to multicellularity. Costs of multicellularity include lower growth rate and the potential for "selfish" individuals that do not invest resources to benefit the group (Michod & Herron, 2006; Gregory J Velicer & Vos, 2009), but multicellular groups benefit from higher predator

resistance. Cooperative aggregation may allow for exploitation of the benefits unicellularity or multicellularity depending on the environment. In high predator environments microbes forgo the benefits of unicellularity in favor of predator resistant aggregates but revert to their unicellular lifestyle when predators are absent and unicellularity is favored again. Under constant and intense predator selection the necessity of transient aggregation is lost. In this context, selection may favor permanent colonies and thus modifications to aggregation genes that promote constitutive multicellularity. We hypothesize that the evolution of undifferentiated multicellularity in groups like the volvocine algae was driven by modification to genes regulating aggregation.

While group formation by unicellular organisms in response to the presence of predators or their exudate has been described, the mechanism of this response and its genetic basis in the prey organism are not well understood. The ability of organisms to rapidly respond to changing environments, especially when it poses an immediate threat to its existence as does predation, is essential for survival. Here, we have investigated the dynamics of a complex predator-prey interaction and found that the unicellular green algae, *Chlamydomonas reinhardtii,* has a surprising ability to detect presence or absence of predator threats and rapidly alter its morphology to best suit its current environment. We have also investigated the transcriptomic response during aggregate formation and found several candidate aggregation genes. We analyzed the evolutionary history of these genes in the volvocine algae to investigate if they were involved in the evolution of multicellularity in this group. This work provides insights into the complex nature of microbial predator-prey interactions and may have broader implications for multicellular evolution and development.

Chapter 2 - Predator Induced Cooperative Aggregation In *Chlamydomonas reinhardtii*

Introduction

Predation is a strong environmental stress especially for microorganisms which and poses an immediate threat to survival. A tactic for predator avoidance in microorganisms is to increase their total size (Boersma et al., 1998; Fisher et al., 2015; Johnsson et al., 2001; Lima & Dill, 1990). Because filter feeding and phagocytosing predators have limits to particles they are able to consume, larger size may help microorganism evade predation (Burns, 1968; Hansen et al., 1994). There are limits to cell viability as total size increases (Lewis, 1976), thus, prey organisms will form cooperative groups to increase their collective size and counter predation.

While it has been known that microorganisms form aggregate groups as an anti-predator defense tactic, the dynamics of this response and its genetic basis have not been closely examined. In this work, we utilized *Chlamydomonas reinhardtii*, a model green algae, and *Daphnia magna* as a model system to investigate the short and long-term dynamics of aggregate formation in response to predation and its genetic basis. We utilized RNA sequencing to determine transcriptomic responses to predation in *Chlamydomonas reinhardtii*. Since predation has been hypothesized as a selective pressure that drove the evolutionary transition from unicellularity to undifferentiated multicellularity (Michod & Herron, 2006; Michod & Nedelcu, 2003; Niklas, 2014), we also investigated implications these results may have in multicellular development and evolution.

Material and Methods

Algal Strains and Growth Conditions

Chlamydomonas reinhardtii (CC-1691 and CC-4533), *Chlamydomonas moewussii* (CC-1480), *Chlamydomonas eugametos* (CC-1419), *Scenedesmus obliquus* (2h, Jiang et al., 2014), *Gonium pectorale* (K4, NIES 2863) and *Pleodorina starrii* (UTEX 1362) were cultured in standard *Volvox* media (SVM) at 25ºC under 24 hour ~100 µE of light for all experiments. *Chlamydomonas* and *Scenedesmus* culture density was ~$1.0x10^5$ cells mL^{-1} unless otherwise noted. Culture density was determined using a Beckman Coulter Counter Z1 with a 50 µm aperture gating between 5 and 1000 fl similar to previous work with *Chlamydomonas* (Fang, De Los Reyes, & Umen, 2006). For all experiments the wildtype strain (CC-1691) of *Chlamydomonas reinhardtii* was used unless otherwise noted. All references to "*Chlamydomonas*" refer to wildtype *C. reinhardtii* (CC-1691). *Gonium pectorale* (K4, NIES 2863) and *Pleodorina starrii* (UTEX 1362) will hereafter be referred to as *Gonium* and *Pleodorina* respectively.

Daphnia Rearing Conditions and Decontamination

Daphnia pulex and *Daphnia magna* were reared in 18 MΩ water 10L aquaria, illuminated with 100µE of light and aerated with an air stone connected to an aquarium air pump. Tanks were fed with ~300 mL of wildtype *Chlamydomonas reinhardtii* at a density of ~$1.0x10^5$ cells mL^{-1} every 2-3 days, including their standard *Volvox* growth media. Axenic *Daphnia magna* and *Daphnia pulex* were prepared by suspension in an antibiotic and clay particle cocktail containing trimethoprim (10 µg/mL), tetracycline (15 µg/mL), erythromycin (100 µg/mL), cefotaxime (90 µg/mL), chloramphenicol (15 µg/mL), ciprofloxacin (10 µg/mL) and carbenicillin (50 µg/mL) for 2-12 hours. DCMU (10 µM) was also included to kill any

residual algae transferred from the *Daphnia's* tank rearing environment. Clay particles were included to induce purging the gut of any microbes. After treatment, *Daphnia* were washed with ~250 mL of sterile media. *Daphnia magna*, referred hereafter to as "*Daphnia*", were used for all experiments unless otherwise noted.

Growth Rate Analysis

Growth rates of three algal species (*Chlamydomonas, Gonium* and *Pleodorina*) were measured in the presence and absence of *Daphnia* by measuring changes in chlorophyll content in the culture over 24 hours. Three replicate cultures of each algal species were exposed to predator by decontaminated *Daphnia* at a concentration of 1 Daphnid mL^{-1}. Three additional replicate cultures were not subjected to predation to serve as a control. To measure growth rate 1 mL samples were collected at 0, 8, 16 and 24 hours post predation. Samples were centrifuged at 5000 **g** for 10 minutes and cell growth media was decanted. Pellets were re-suspended in 80% acetone and mixed using a VWR Vortex Genie 2 for OD reading by photo spectrometry (Arnon, 1949). OD readings were performed at A645 and A663 using a Milton Roy Spectronic 501. Chlorophyll concentration was calculated by the formula:

$$\mu g\ mL^{-1}\ chlorophyll\ 8.02\ x\ A663\ +\ 20.2\ x\ A645$$

as described in Arnon 1949. Biomass accumulation rate was calculated by the change in chlorophyll content per hour by the equation:

$$\frac{mg\ \ mL^{-1} chlorophyll\ t_0\ -\ mg\ mL^{-1} chlorophyll\ t_1}{t}$$

where mg *chlorophyll* $mL^{-1} t_0$ is the chlorophyll content at time 0, *mg chlorophyll* $mL^{-1} t_1$ is the chlorophyll content at time 1 and *t* is the total time elapsed. Previously it has been determined that chlorophyll is a proxy for biomass in algae (Carlson, 1977). Growth rates were measured over a period of 24 h.

Cooperative Aggregation

For all experiments where *Daphnia* were used, they were decontaminated according to the procedure above. In experiments where aggregation was reported, it was determined by the percent of cells present in aggregates and was determined by counts using a light microscope. Aggregate size distribution was determined by counting aggregates and assigning them to bins based on the number of cells per aggregate. All morphological observations were made by microscopy with live cell mounts, or after fixation with 2% glutaraldehyde, 0.01% tween-20 Wildtype and mutant strains of *Chlamydomonas reinhardtii, Chlamydomonas moewussii, Chlamydomonas eugametos* and *Scenedesmus obliquus* were exposed to predation by *Daphnia* and/or active supernatant in SVM media for various times after which a 1 ml aliquot was fixed in 2% glutaraldehyde 0.01% tween-20 followed by phenotype quantification. *Daphnia* were used at a density of 1 Daphnid mL^{-1} unless otherwise noted. Phenotypes were measured at 1 h post predator/supernatant addition unless otherwise noted and compared to un-predated and pre-predator treated controls.

Short and Long Term Aggregation

Aggregation was measured over a period of 24 hours and over 60 minutes. In these experiments 3 replicate cultures were exposed to predation at 1 Daphnid mL^{-1} and 3 replicate cultures were left unpredated to serve as a control. 1 mL of culture was collected and fixed at each time point to be used for phenotyping. Aggregation was determined by the method described above.

Transmission Electron Microscopy

Cultures of *Chlamydomonas* were subjected to predators at a density of 1 Daphnid mL^{-1} for 1 h at a prior to fixing. Samples were fixed and mounted as described by Arakaki at al. 2013. Imaging was performed at the Kansas State University Microscopy Facility.

Aggregate Dispersal

Aggregate dispersal was investigated by measuring the phenotype after the removal of predators. Three replicates for performed for three different treatments. In one treatment cultures were subject to predation by *Daphnia* but had their *Daphnia* were removed by filiation after 30 min of exposure. The culture was centrifuged at 600 **g** for 5 minutes and suspended in fresh SVM to remove any aggregation inducing factors in the media. In another treatment, cultures of *Chlamydomonas* were subjected to continuous predation by *Daphnia* over a period of 12 hours to function as a positive control. In a third treatment, cultures were left unpredated to serve as a negative control. To control for effects of centrifugation, both control cultures were centrifuged at the same time point as the culture that had its predators removed. However, these cultures were re-suspended in their original media post centrifugation. Additionally, after re-suspension the positive control cultures had their *Daphnia* returned for the duration of the experiment. Time points were collected prior to predator removal and over a 12-hour period post *Daphnia* removal for phenotyping.

Cell Lysates

Heat lysates were prepared by heating *Chlamydomonas* cultures at a density of $1.0x10^6$ cells mL^{-1} to ~90° C for 5 minutes. After heating, lysates were allowed to cool to room temperature. Freeze-thaw lysates of *Chlamydomonas* were prepared by first centrifuging 15 mL of culture at a density of $1.0x10^6$ cells mL^{-1} L at 3000 **g** for ten minutes. Media was decanted

before the pellet in its centrifuge tube was submerged in liquid nitrogen for 3 minutes. It was then removed from liquid nitrogen and immediately incubated in a water bath at 37° C until completely thawed. These steps were repeated two additional times. After the final freeze-thaw, the pellet was re-suspended in 15 mL of SVM. Lysates were observed under a light microscope to confirm cell destruction. To determine if cell lysates from *Chlamydomonas* induce aggregation, 5 mL of either freeze-thaw or heat lysates was added to 5 mL of *Chlamydomonas* cultures. A control had 5 mL of SVM added. Each treatment was performed with three replicate cultures. Aggregation was recorded at 1 h post treatment by the method described above.

Supernatant Experiments

To determine the source of aggregation inducing signals, supernatants were prepared from mixed cultures of *Chlamydomonas* and *Daphnia*, cultures containing only *Daphnia* and cultures of *Chlamydomonas* only. For the *Chlamydomonas/Daphnia* supernatant, triplicate 50 mL cultures of *Chlamydomonas* had *Daphnia* added at a concentration of 1 Daphnid mL^{-1} for 12 hours. *Daphnia* were removed from the culture by filtering through a sterile 50 µm mesh. The filtrate, containing the *Chlamydomonas* cells and their media, was then centrifuged for 10 min at 3000 **g**. The supernatant was then poured off into a new sterile microcentrifuge tube. 10 mL of each replicate supernatant was added to 10 mL of naïve *Chlamydomonas* culture are a density of ~$1.0x10^5$ cells mL^{-1}. The second *Daphnia* only supernatant was prepared identically, except *Daphnia* were kept in 50 mL of sterile SVM instead of 50 mL of *Chlamydomonas* culture. A final control supernatant from unpredated cultures of *Chlamydomonas* was prepared in the same manner as the first treatment except no *Daphnia* were added to the culture. Three replicates were performed for all treatments. Aggregation was recorded at 1-hour post supernatant addition.

Cycloheximide Treatment

5 mL cultures of *Chlamydomonas* were treated with the translational inhibitor cycloheximide for 30 minutes at 10 μg mL^{-1}. Two treatments received no cycloheximide and were treated with an equal volume of ethanol vehicle. All treatments were centrifuged at 600 **g** and re-suspended in 5 mL of SVM 30 minutes after the addition of cycloheximide. After re-suspension, the cycloheximide treated and untreated culture were treated with 5mL of *Daphnia-Chlamydomonas* supernatants prepared as described above. Another culture not treated with cycloheximide was treated with *Chlamydomonas*-only supernatant. Three replicates were performed for all treatments. Cultures were phenotyped by microscopy for aggregation two hours after supernatant addition.

RNA sequencing and quantification of genome-wide expression

Triplicate replicate cultures of wildtype *Chlamydomonas* strain CC-1691 were cultured as described above in Standard *Volvox* Media (SVM). Cultures were grown asynchronously under 24 hour light. Flasks were inoculated 12 hours prior to the experiment and culture density was approximately $1.0x10^5$ cells mL^{-1} at the first time-point taken. Two time points were taken 1 and 2 h prior to the addition of *Daphnia*. Time points were also taken at 1, 2, 3, 4, 5, 6, 8, 10 and 12 h post *Daphnia* addition. *Daphnia* were added to each culture at a concentration of approximately 1 *Daphnia* per 1 ml of culture. A control replicate was treated identically except no *Daphnia* were added.

Each predated and unpredated replicate consisted of two flasks containing 900 ml of culture. For each replicate at each time point, 50 ml was taken from each flask and combined. The pooled culture was then pelleted by centrifugation at 3000 **g** for 10 minutes, had its media decanted and flash frozen in liquid nitrogen to be used for RNA extractions. To maintain a

concentration of 1 *Daphnia*/ml through the duration of the experiment ~50 *Daphnia* were removed from each flask after each collection. One mL of culture was also collected at each time point and fixed with 2% glutaraldehyde 0.01% tween-20 for phenotyping. Total RNA was extracted using Omega Plant RNA Kits (Omega R6827-01). Total RNA was DNase I treated (NEB #M0303S). mRNA was collected using the NEB poly(A) selection kit (NEB # E7490S). Time-points were pooled prior to library preparation by combining equal amounts of mRNA determined by reading on a Nanodrop 1000. The two time-points taken at 1 and 2 h prior to *Daphnia* addition were pooled. Time points taken post *Daphnia* addition were pooled as follows: 1 h and 2 h, 3 h and 4 h, 5 h and 6 h. The final time point pool consisted of the 8 h, 10 h and 12 h collections. RNA libraries were prepared from mRNA using the NEB RNA library prep kit (NEB #E7530L) and 24 unique barcodes (NEB # E7335 and NEB # E7500). Libraries were quantified by quantitative PCR followed by sequencing on one lane of an Illumina HiSeq 2500 (Genome Sequencing Core, University of Kansas).

Sequencing reads were aligned to the *Chlamydomonas* genome and analyzed for differential expression utilizing the Tuxedo suit of tools. TopHat2 (version 2.0.12, Kim et al., 2013) was used to align reads to the *Chlamydomonas* reference genome v5.3 (Merchant et al., 2007). Cufflinks (version 2.1.1, Trapnell et al., 2012) was used for read mapping and CuffDiff (version 2.1.1 Trapnell et al., 2012) was used to determine differential expression between predated and unpredated treatments. Only genes with a significant change in expression determined by Benjamini-Hochberg correction ($q < 0.05$) and a log2 fold change in expression between treatments were considered significant. Expression was compared at specific time points between predated cultures and unpredated cultures using the time series option in CuffDiff. Expression clusters were determined and heat maps generated by performing hierarchical

clustering on z-normalized expression values (Cheadle, Vawter, Freed, & Becker, 2003) using Multi Experiment Viewer (version 4.9.0, http://www.tm4.org/mev.html). The dN/dS relative to *Gonium pectorale* was previously calculated in Hanschen et al. 2016.

Functional enrichment was preformed for GO (Gene Ontology, Ashburner et al., 2000), KEGG (Kyoto Encyclopedia of Genes and Genomes, Ogata et al., 1999), and PFAM (Protein Family Database, Finn et al., 2007) on the genes in each cluster using the Phytomine tool on Phytyozome (https://phytozome.jgi.doe.gov/pz/portal.html, Goodstein et al., 2012). Bonferroni test correction was performed and annotations with a p-value > 0.05 were excluded. GO, KEGG and PFAM IDs and descriptions for all significant genes were obtained for manual inspection using Biomart (version 0.7) with the Phytozome database (Goodstein et al., 2012).

Candidate Gene Cloning

The *Gonium pectorale* ortholog of the *Chlamydomonas* transmembrane gene *g18292*, scaffold00055.g258 gene sequence (NCBI Accession Number: LSYV01000056) including the putative endogenous promoter (~650 nts upstream) and its putative terminator (~550 nts downstream), was PCR amplified with primers 5' CGCACTAACCACACTGAGCT 3' and 5'ATCGTTCTTGCCCTTCTTGT 3' from *Gonium* genomic DNA. After gel purification (Zymoclean Gel Recovery kit D4001), it was cloned into the pJET1.2/blunt vector (Clonejet PCR Cloning Kit #K1231). Plasmids were grown in Turbo Competent High Efficiency *E. coli* (NEB # C2984I) and purified with Qiaprep spin mini prep kit (Qiagen #27104).

Functional Testing of *g18292*

Chlamydomonas was co-transformed with a plasmid containing the full *Gonium* genomic sequence *g18292* (pJET1.2-*g18292*) and with pSI103, a paromomycin resistance vector (Sizova, Fuhrmann, & Hegemann, 2001). Cultures to be used for transformation were grown in flasks in

tris acetate phosphate (TAP) media to a density of $5.0x10^6$ cells mL^{-1}. 300 mL of culture was then transferred to a large conical vial and 75 µL of 20% tween-20 was added to facilitate pelleting. Cultures were centrifuged at 600 **g** for 5 minutes and supernatant was immediately poured off. Cells were then re-suspended to a density of $3.5x10^8$ cells mL^{-1} in TAP media supplemented with 50 mM sorbitol and transferred to a sterile 50 mL conical vial. The cell suspension was then incubated at room temperature on a light shelf. After 1-4 hours of incubation, 300 µL of the cell suspension, 500 ng of pSI103 and 500 ng of pJET1.2-*g18292* was transferred to a 2 mm electroporation cuvette and incubated for 30 minutes at room temperature. Electroporation was performed using the NEPA21 electroporater. The entire 300 µL volume of the cuvette was then transferred to 10 mL of TAP containing 50 mM sorbitol in a sterile glass tube and incubated at room temperature over night on a shaker at 75 RPM. The next day, the 10 mL volume was centrifuged at 600 **g** for 3 minutes and re-suspended in 1 mL TAP containing 50 mM sorbitol in a sterile microcentrifuge tube. 250 µL of the suspension was transferred to and spread on TAP plates containing 15 µg/mL of paromomycin. Plates were incubated on a light shelf for 3 days. Single colonies were then picked from plates using sterile toothpicks and transferred to well of 96-well plate containing 200 µL of SVM media. Plates were incubated two days on a light shelf at room temperature.

To identify transformants expressing a multicellular phenotype, each well was visually inspected by light microscope. Three transformed strains with a multicellular phenotype were identified and subcultured in glass tubes containing 25 mL of SVM. DNA extractions were performed on the three multicellular strains using a DNA extraction kit (Omega D3485-01). Insertions of both pSI103 and pJET1.2-*g18292* were verified by PCR on extracted DNA using the primers 5'-TTGTGAGTGGGTTGTTGTGG-3' and 5'-CAACACGAGGTACGGGAATC-3'

for pSI103 and 5'-CGCACTAACCACACTGAGCT-3' and 5'-ATCGTTCTTGCCCTTCTTGT-3' for pJET1.2-*g18292*. The percent of the culture that was multicellular was determined by fixing a 1 mL aliquot of cells in 2% glutaraldehyde, 0.01% tween-20, then counting unicells and cells within multicellular groups using a light microscope. Wildtype cultures of *Chlamydomonas reinhardtii* and *Gonium pectorale* were also cultured and phenotyped as controls. For all reported phenotypes, the average and standard error of three replicas are reported.

Statistics

Student's t-tests were performed using Prism 7. Significance for functional enrichment was determined by Bonferroni test correction using the Phytomine tool on Phytozome (Goodstein et al., 2012) as described above. Genes with significant changes in expression were determined by Benjamini-Hochberg correction using Cuffdiff (Trapnell et al., 2013) as described above.

Results

Organismal size correlates with fitness

To determine if algal size provides a benefit under predation, we subjected three species within the Volvocales, *Chlamydomonas reinhardtii*, *Gonium*, and *Pleodorina,* to predation by *Daphnia* and measured the each species' biomass accumulation rate measured by change in chlorophyll continent over 24 hours.

We found that the smallest species tested, *Chlamydomonas,* had decreased growth rate under predation by *Daphnia* compared to an unpredated control (Fig 2.1a, *t*-test : $t = 2.947$, $df = 4$, $p = 0.0421$). Intermediate sized *Gonium* (Fig 2.1a, *t*-test : $t = 0.95324$, $df = 4$, $p = 0.3945$) and the much larger *Pleodorina* (Fig 2.1a, *t*-test : $t = 1.22436$, $df = 4$, $p = 0.2880$), had similar growth rates under predation and in the absence of predators.

Predators cause *Chlamydomonas* to rapidly aggregate

To investigate how *Chlamydomonas* responds to predation both immediately and after longer predator exposure times, axenic *Daphnia* were added to cultures of *Chlamydomonas.* Upon the addition of *Daphnia* to liquid cultures of *Chlamydomonas,* visible aggregates of *Chlamydomonas* were observed that were sustained at least for 12 h in the presence of predators (Fig 2.2a). Aggregation is maximal at approximately 1h, and then is reduced over a period of 24h of predator exposure (Fig 2.2a). The aggregation of *Chlamydomonas* was rapid; aggregates can be observed as early as 5 minutes after predator exposure with maximum aggregation being ~70% (Fig 2.2a and Fig2.2b).

Aggregation is a transient response that is dependent on the presence of *Daphnia*. Aggregates begin to disperse within an hour in cultures when *Daphnia* are removed but are sustained for 12 hours in cultures that were subjected to continuous predation (Fig 2.3a). Aggregation does not require *Daphnia* to be alive. Cultures exposed to freshly killed Daphnia had a similar phenotype to cultures exposed to predation by live *Daphnia* (Fig 2.3b, *t*-test, $t = 0.05319$, $df = 4$, $p = 0.9602$). Freeze/thaw lysates (Fig 2.3b, *t*-test, $t = 0.2965$, $df = 4$, $p = 0.7816$) and heat lysates (Fig 2.3b*, t*-test: $t = 0$, $df = 4$, $p > 0.9999$) from *Chlamydomonas* cultures do not induce aggregation when added to naïve cultures of *Chlamydomonas* when compared to unpredated controls (Fig 2.3b). Supernatants from cultures containing *Daphnia* only and containing a mix of *Daphnia* and *Chlamydomonas* induce a similar aggregation phenotype when added to naïve cultures of *Chlamydomonas*; ~56% for *Daphnia* and *Chlamydomonas* supernatants and ~57% for *Daphnia* only supernatants (Fig 2.3c *t*-test: $t = 0.7928$, $df = 4$, $p = 0.3991$). Treatment of *Chlamydomonas* cultures with the translational inhibitor cycloheximide

significantly reduces aggregation from exposure to *Daphnia* supernatant to 19% compared to 55% in untreated controls (Fig 2.3d, *t*-test: $t = 3.945$, $df = 4$, $p = 0.0169$).

Aggregates are variable in size, adhered by cell wall contacts and their formation is dose dependent.

Aggregates are readily visible when predated cultures are observed by light microscope (Fig 2.4a-b). Transmission electron microscopy revealed that cells within aggregates lack cytoplasmic connections and appear to be adhered by extracellular complexes (Fig 2.4c-d). *Chlamydomonas* aggregates are highly variable in size ranging from 2 to over 16 cells with an average of 5 cells/aggregate (Fig 2.5a). Aggregation in *Chlamydomonas* is dose dependent with 5 Daphnia mL^{-1} inducing aggregation at 47.6%, 30.8% at 2 *Daphnia* mL^{-1}, 29.1% at 1 *Daphnia* mL^{-1}, and 5.5% at 0.5 *Daphnia* $mL^{-}1$ (Fig 2.5b).

Aggregation occurs in multiple species and strains of unicellular green algae and is independent of *Daphnia* species.

In addition to *Chlamydomonas reinhardtii*, we found three other species of green algae, *Chlamydomonas moewussi*, *Chlamydomonas eugametos* and *Scenedesmus obliquus,* respond to predation by aggregating. After 1 hour of predator exposure *Chlamydomonas reinhardtii* aggregates at 62% under predation compared to 1% in unpredated controls (Fig 2.6a *t*-test: $t = 38.48$, $df = 4$, $p < 0.0001$). *Chlamydomonas moewussi* aggregates at 20% under predation compared to 1% in unpredated controls (Fig 2.6a *t*-test: $t = 7.712$, $df = 4$, $p = 0.0015$). *Chlamydomonas eugametos* aggregates at 5% under predation compared to 1% in unpredated controls (Fig 2.6a *t*-test: $t = 4.752$, $df = 4$, $p = 0.009$). *Scenedesmus obliquus* aggregates at 25% under predation compared to 5% in unpredated controls (Fig 2.6a *t*-test: $t = 11.47$, $df = 4$, $p = 0.0003$).

Aggregation is induced by multiple *Daphnia* species. *Daphnia pulex* and *Daphnia magna* both induce aggregation at similar rates of 61% and 53% respectively (Fig 2.6b *t*-test : $t = 1.252$, $df = 4$, $p = 0.2787$). Aggregation is enhanced in a mutant strain of *Chlamydomonas.* A *Chlamydomonas* strain harboring an unknown mutation affecting cell wall attachment, CC-4533, was subjected to predation where 92% of cells in this strain aggregated compared to 53% in wild-type strains with intact cell walls (Fig 2.6c *t*-test : $t = 12.45$, $df = 4$, $p = 0.00002$).

Identification of genes that transcriptionally respond to predation

RNA-seq analysis found 131 genes, or ~0.78% of the genome, was significantly differentially expressed between predated and unpredated cultures of *Chlamydomonas*. Hierarchical clustering revealed 5 distinct expression patterns (C1-C5, Fig 2.8b). We found one cluster of genes whose expression increased at 8-12 h of predation (C1), one cluster where expression of genes was off prior to predator treatment, but in the presence of predators steadily increased during the entire 12 h predation (C2), a cluster where gene expression was rapidly upregulated in response to predators at 2-4h, but then rapidly turned off (C3), a cluster that was down-regulated after 4 h of treatment (C4) and a cluster where genes were up-regulated in the absence of predators and were down regulated immediately after treatment (C5). Functional enrichment of GO terms, pathway, and PFAM domains revealed several enriched functions.

C1 is enriched for genes with GO terms known to be important in protein kinase activity, and calcium/calmodulin signaling activity. Interestingly, a single protein domain, a Steroid delta5-4-isomerase, was also enriched in this cluster, a domain known to be important for to production of steroid hormones in a wide variety of eukaryotes and prokaryotes and is important for quorum sensing in bacteria (Hughes & Sperandio, 2009).

The immediate response of the algae in 1-2 h reflected in Cluster 3 to the presence of predators, when aggregation is the highest is to significantly increase core cellular metabolism involved in aerobic respiration the generation of precursor molecules reflected in both GO code and protein domain enrichment. Likewise, early during predation, genes in Cluster 5, protein domains involved with sodium and solute symporting are significantly down regulated (Fig 2.7c). C2 and C4 had no significant functional enrichment.

Manual inspection of annotations obtained using the Phytomine tool on Phytozome uncovered several promising candidates. A cellulase, septin, flagellar associated protein, a pherophorin and a transmembrane protein were identified as candidates by this analysis. dN/dS analysis of *Chlamydomonas* and *Gonium* genomic sequences found the predicted transmembrane gene *g18292* had a dN/dS > 1.0.

A predicted transmembrane protein *g18292* from *Gonium* causes *Chlamydomonas* to be multicellular

Chlamydomonas expressed a constitutive colonial multicellular phenotype after transformation with the *Gonium* ortholog of *g18292*. In wildtype *Chlamydomonas* >5% of cells are present in multicellular groups (Fig 2.9a). In wildtype *Gonium* ~90% of cells are multicellular in groups (Fig 2.9a). *Chlamydomonas* expressing the *Gonium* ortholog of *g18292* has a multicellular phenotype of ~72%, significantly higher than the wildtype *Chlamydomonas* parent strain (Fig 2.9a, *t*-test : $t = 33.71$, $df = 4$, $p < 0.0001$).

Discussion

Past studies have examined cooperative aggregation in response to zooplanktonic predators, but have not closely investigated the dynamics of aggregation or its genetic basis. This work has contributed both to the understanding of the mechanism of microbial group formation

and its genetic basis. Our work is unique in that it characterizes the mechanism of aggregation and utilizes RNA-seq to identify the genetic basis of prey response.

There have been long hypothesized benefits and costs to both unicellular and multicellular lifestyles. Unicellularity may allow for rapid growth and direct access to resources in the environment, however small size is costly and carries increased susceptibility to predation (Banse, 1976; Lurling & Van Donk, 2000; Michod & Herron, 2006). For multicellularity, the costs and benefits are reversed. Groups of cells are larger in size and more resistant to predation but suffer a cost to growth rate (Banse, 1976; Lurling & Van Donk, 2000). Aggregation may be an evolutionary stepping-stone to multicellularity, allowing organisms to reap the benefits of unicellularity when the environment is free of predators, but also resist consumption by increasing their total size through group formation when predators are present. For aggregation to be a reliable method for predator evasion, individual cells must detect predators in their environment and form groups rapidly to prevent consumption. Our results support our hypothesis that aggregation is a response that requires two steps: recognition of the presence of *Daphnia* via a signal and subsequent genetic response of the algae to facilitate aggregation. The goal of this project was to define the response mechanistically and to investigate its genetic basis.

We hypothesized that *Chlamydomonas* forms aggregates to increase its total size making it inedible to *Daphnia*. We first wanted to demonstrate that size provides a benefit under predation. Our results show that the multicellular species tested, *Gonium* and *Pleodorina*, do indeed have a reduced cost to growth rate under predation compared to the unicellular *Chlamydomonas* (Fig 2.1a). These three species represent the vast morphological diversity in the Volvocales. *Chlamydomonas* is unicellular, *Gonium* is an undifferentiated colonial multicellular species and *Pleodorina* is the largest and exhibits cell type specialization. They also represent an

order of magnitude difference in size, with *Chlamydomonas* cells being typically ~10 μm and diameter and *Pleodorina* colonies being ~100 μm in diameter. Because growth rates were measured over a period of 24 h, *Chlamydomonas* aggregates would have dissipated in the latter half of the experiment (Fig 2.2a) making the culture primarily unicellular and susceptible to predation. Our data supports our hypothesis that multicellularity reduces predator-induced reductions in growth in the Volvocales.

Our results demonstrate that aggregation is dynamic and rapidly reversible upon predator removal. Aggregates begin to form as soon as five minutes after predator exposure and are maintained for 12 hours. We have also found that aggregation is a transient state. Removing *Daphnia* and re-suspending cells in fresh media resulted in dispersal of aggregated *Chlamydomonas* cells within 30 minutes, while re-suspension in the original media containing predators, continued to elicit aggregation for up to ~12 h (Fig 2.3a). This result suggested that *Chlamydomonas* detects the presence or absence of predators and dynamically alters its phenotype in response to its predator environment. This allows *Chlamydomonas* to form groups when necessary to evade predation, but resume its unicellular life cycle when its environment is free of predators to avoid potential costs associated with group formation.

To determine what induces aggregation, we prepared lysates from *Daphnia* and *Chlamydomonas*. Heated lysates of *Daphnia* continued to induce naïve *Chlamydomonas* cells to aggregation, while heated lysates from *Chlamydomonas* did not. To ensure that heating of the cells was not denaturing the signal from *Chlamydomonas* we also prepared a freeze-thaw lysate of *Chlamydomonas* and this also did not elicit aggregation. The absence of an aggregation response to *Chlamydomonas* lysates suggested that the aggregation-inducing signal is not produced and stored by cells under normal conditions. Heat killed *Daphnia* induced a response

similar to that of live *Daphnia* suggesting that biological activity or active feeding by *Daphnia* is not required to induce aggregation. However, continuous production of a signal by live *Daphnia* is likely required to sustain the response. We also found that when supernatant from cultures of *Daphnia* feeding on algae were added to naïve cultures of *Chlamydomonas*, aggregation was induced (Fig 2.3c). Supernatants from cultures containing only decontaminated *Daphnia* also induced aggregation (Fig 2.3c). Because supernatants from *Daphnia* only cultures induced aggregation, we reasoned that the signal originates from *Daphnia* alone, not from the algae or an interaction between the *Daphnia* and algae. In sum, these results suggested that a signal was released into the media by *Daphnia* and was perceived by *Chlamydomonas* by a specific receptor, ultimately inducing production of adhesion factors.

This was tested by treating *Chlamydomonas* cells with the translational inhibitor cylcoheximide (CHX) prior to subjecting them to active supernatant from cultures of *Daphnia* feeding on *Chlamydomonas*. Cycloheximide treatment significantly reduced aggregation (Fig 2.3d), suggesting that the response is dependent on translation of a protein or proteins. We attempted to inhibit translation of the aggregation-inducing signal in *Daphnia,* but we were unable to identify sub-lethal concentrations of CHX that could demonstrate statistically significant reduction in *Chlamydomonas* aggregation. In sum, these data suggest that a signal originating from *Daphnia* is perceived by a receptor in *Chlamydomonas* that subsequently induces translation of new protein, likely adhesion factors, to promote aggregation.

Aggregates of *Chlamydomonas* are flagellated, where their cells walls are attached to each other (2.4a and 2.4b). Transmission electron microscopy of aggregates revealed that cytoplasmic bridges, a characteristic multicellular relatives of *Chlamydomonas* (Arakaki et al., 2013; Bisalputra & Stein, 1966), are not present in aggregates (Fig 2.4c and 2.4d). The lack of

cytoplasmic bridges was evidence that aggregates do not form by adhesion post mitosis as do colonies in multicellular Volvocales like *Gonium*. Cells appear to be attached extracellularly, likely through their cell walls. When viewed under high magnification, there appears to be extracellular structures composing the connections between cells (2.4c and 2.4d). Visualization of what appear to be extracellular connections cells within aggregates suggested that the extracellular matrix might be expanded or otherwise modified to facilitate aggregation.

Aggregates range in size from 2 to over 16 cells (2.5a). Aggregates are likely formed during G1 phase of their cell cycle, because cells undergo a multiple fission cell cycle where the flagella are retracted so that the basal bodies can be used for cytokinesis (Johnson & Porter, 1968). The observed aggregates remain flagellated suggesting they are vegetative in G1 phase. Likewise, aggregates are not in limited to multiples of 2^n cells (Fig 2.5b) and begin to form as soon as five minutes after exposure to predators (Fig 2.2b), insufficient time for *Chlamydomonas* to enter and complete its cell cycle. If aggregates are formed by post-division adhesion it is expected that aggregate size would be limited to groups of 2^n cells. Together, these results suggest that aggregates are not formed by post-cytokinesis cellular adhesion, but by aggregation formerly independent single cells.

Our observations of cell-cell connections by transmission electron microscopy led us to hypothesize that the mechanism of cell-cell adhesion in response to predators might be mediated through their cell walls. A *Chlamydomonas* strain harboring an unknown mutation affecting complete cell wall attachment was found to have enhanced aggregation compared to the wildtype strain. The lack of complete cell wall attachment in CC-4533 may enhance aggregation by either allowing more rapid insertion of the adhesion protein into the membrane, or in normal strains, their cells walls may antagonize aggregation and increase the threshold amount of aggregation

proteins that are required to promote efficient aggregation. Additionally, these results suggest that loosening or modification of the cell wall may be required to facilitate aggregation. Regardless, these results demonstrate that cell-cell adhesion in aggregate likely involves the cell wall.

Next, we investigated whether aggregation was a defense mechanism specific to *Chlamydomonas reinhardtii* or a tactic widely utilized by green algae. When *Daphnia* are added to various species of distantly related unicellular algae, both in the genus *Chlamydomonas* and a Chlorophycaen relative, *Scenedesmus obliquus*, aggregation is observed (Fig 2.6a). The *Chlamydomonas* genus is polyphyletic and *C. moewussi* and *C. eugametos* are considered distant relatives of *C. reinhardtii* (Jupe, Chapman, & Zimmer, 1988; Pröschold, Marin, Schlösser, & Melkonian, 2001). The observation of aggregation in multiple distantly related species suggested that it is either a broadly conserved mechanism for predator evasion in unicellular algae or has evolved multiple times. Interestingly, *C. eugametos*, which is adapted for growth on plates and does not swim well in liquid culture, has a reduced ability to aggregate. This suggests that swimming or movement may be an essential component of aggregation. We also found that predation by *D. pulex* elicits similar levels of aggregation in *C. reinhardtii* as *D. magna* (Fig 2.6b) supporting that aggregation is independent of *Daphnia* species and may be a general predator response.

Because aggregation was inhibited by the translational inhibitor cylcoheximide (Fig 2.3d), we hypothesized that the response was facilitated by production of adhesion factors downstream of a receptor. To further understand the molecular basis of aggregation, we next quantified the genome-wide response of *Chlamydomonas* to predation using a quantitative RNA-seq approach in *Chlamydomonas*.

Rapid production of cell-cell adhesion factors is likely required to facilitate aggregation, so we focused on genes that rapidly increased in expression after the addition of predators represented by clusters C2 and C3. Modifications to the cell wall may be necessary for aggregation to occur so genes that may be involved in cell wall alterations were also considered. Using the Phytozome 11 annotations and the Phytomine tool (Goodstein et al., 2012), we then determined if any classes of genes were enriched in each of the clusters (Fig 4.7c). From this analysis, we identified a limited number of significantly enriched cellular functions.

Enrichment of calmodulin-dependent protein kinase activity in C1 may be related to signaling cascades necessary to facilitate and maintain aggregation. GO terms related to the TCA cycle and metabolism are enriched in C3 and are heavily down-regulated after 2 h post predator exposure. It is possible that widespread down-regulation of genes related to growth and metabolism is associated with the costs related to aggregate formation.

The GO code analysis as useful in understanding the major well characterized biological processes that were changing when *Chlamydomonas* aggregates in response to predation, especially the potential role of calcium-calmodulin signaling and metabolic processes. However, this analysis did not immediately identify candidate genes that mediated cell-cell adhesion. We manually inspected the list of 131 candidate predator responsive genes to identify those that may be involved in facilitating cell-cell adhesion, cell wall and extracellular matrix modification or signaling. We also considered their expression profile during predation and their evolutionary history in the Volvocales to investigate whether aggregation genes may have implications in multicellular evolution.

We noted that a flagellar associated protein, FAP139, was immediately activated in response to predation. The flagella in *Chlamydomonas* has long been known to be important for

recognition and adhesion prior to plasmogamy during mating (Snell & Roseman, 1979). FAP139 has no known biological function other than having been identified as a component of the flagellum. However, FAP139 has an array of 6 coiled-coil domains that have patches of strong hydrophobicity and strong charge on their predicted alpha-helices similar to known coiled-coil domain receptor proteins that mediate cell-cell adhesion such as integrins. It is also has a IgA endopeptidase domain that is known to be released by certain bacteria to loosen the ECM of human cells to facilitate bacterial adherence and pathogenesis (Plaut, 1983; Qiu, Brackee, & Plaut, 1996). The known function of the flagella makes the flagellular-associated protein found in our analysis a strong candidate gene for cooperative aggregation. We hypothesized that if the flagella is involved in adhesion during mating that it may play a similar role during aggregation and may be facilitated by FAP139 expression.

We identified a putative cell wall component, a pherophorin, which was activated during predation (Fig 2.8b). This gene could potentially play a role in solidifying the cell wall in response to predation, or making the cell wall more amenable to cell-cell adhesion. Pherophorins are known extracellular matrix proteins in the Volvocales and have been expanded in multicellular members of the group, suggesting a possible role in multicellular development (Hanschen et al., 2016). The known function of this gene family and its expansion in multicellular the Volvocales make it a strong candidate gene for cooperative aggregation.

We also identified an ortholog of septin (Fig 2.8c), proteins that originally were identified by their importance for cytokinesis, but are now known to be critical for other developmental process such as sporulation and conjugation. The presence of a septin ortholog is interesting because septins are part of the cytoskeleton that localize at cell-cell connections during cytokinesis (Douglas & Alvarez, 2005; Gladfelter, 2006; Weirich, Erzberger, & Barral, 2008).

A cellulase ortholog was also considered a candidate gene. Cellulase is a highly conserved enzyme in eukaryotes and has long been known to be involved in cell wall modifications in plants (Campillo, 1999; Levy, Shani, & Shoseyov, 2002; Minic & Jouanin, 2006). Recall that cell wall defective mutants have a stronger aggregation response (Fig 2.6c). From this data in combination with known functions of cellulases, we reasoned it might be involved in modification or loosening of the cell wall to facilitate aggregation. Interestingly, the expression of the cellulase was up-regulated at the 8, 10 and 12 h time point (Fig 2.8e), corresponding with a reduction in aggregation (Fig 2.7a). We hypothesize that upregulation of the cellulase may play a role in facilitating aggregate dispersal at this time point. Aggregate size does not appear to be regulated (Fig 2.5a) and cells on the interior of aggregates may have a reduction in cell surface area exposed to nutrients in their environment. Since *Chlamydomonas* cells are not known to share nutrients, cells on the interior of aggregates may become nutrient starved making continued aggregation deleterious.

C. reinhardtii is closely related to the volvocine algae, a group of multicellular algae that exhibit a stepwise increase in multicellularity and developmental complexity (Kirk, 2005). Because of this, we thought that perhaps genes important for predator responsive aggregation in *Chlamydomonas* could have been co-opted as multicellularity genes in the volvocine algae. To investigate this, we analyzed the expression pattern of genes undergoing positive selection, those with a dN/dS ratio greater than 1.0 (Yang & Bielawski, 2000) between the *Chlamydomonas* and the *Gonium* genomic sequences (Hanschen et al., 2016; Merchant et al., 2007). We found one predicted transmembrane protein, *g18292*, that was both predator responsive in *Chlamydomonas* and had a dN/dS > 1.0 when comparing the *Chlamydomonas* and *Gonium* sequences (Fig 2.7d). Because this gene has a dN/dS > 1.0 between *Chlamydomonas* and its close colonial

multicellular relative *Gonium*, we hypothesized that it may have evolved a novel function related to multicellular development or cell adhesion. Surprisingly, the transmembrane protein was not present outside the volvocine algae suggesting that its function in aggregation and multicellular development may be family specific or that it may be involved in species-specific recognition between cells.

We hypothesized that genes involved in aggregation in *Chlamydomonas* may have been modified to facilitate permanent multicellular colonies in *Gonium*. Because *g18292* was predator responsive, a predicted transmembrane protein and showed evidence of positive selection between the *Chlamydomonas* and *Gonium* sequences, we reasoned that it might be involved in multicellular development in *Gonium*. We expected that if *g18292* played a role in multicellular development in *Gonium* we would see a gain of colonial multicellularity in *Chlamydomonas* strains expressing the *Gonium* ortholog. Our functional testing showed that *Chlamydomonas* when transformed with the *Gonium* ortholog of *g18292* expressed a multicellular phenotype, with multicellular groups accounting for ~70% of cells in the culture, significantly higher than in wildtype cultures of *Chlamydomonas* (Fig 2.9a). These multicellular groups were permanent and maintained after multiple subcultures suggesting that they differed from plastic predator induced aggregates. Notably, colonies in *Chlamydomonas* expressing *Gonium g18292* do not appear to be regulated in size or shape (Fig 2.9b) suggesting that *g18292*'s function in *Gonium* may be limited to simple cell-cell adhesion and other genes regulate colony morphology in the *Gonium*. If *g18292* is involved both in aggregative cooperation in *Chlamydomonas* and in permanent multicellular colony development in *Gonium*, it is evidence that multicellularity in the Volvocales evolved by cooption of genes involved in aggregation. However, because the transformed *Chlamydomonas* strains retained and presumably normally expressed the

endogenous copy of *g18292*, copy number effects cannot be ruled out. Regardless, the gain of function in *Chlamydomonas* strains expressing the *Gonium g18292* ortholog demonstrates at a minimum that the gene plays some role in cell-cell adhesion in the Volvocales.

We have defined the short and long term dynamics of cooperative aggregation. Based on these results we hypothesize that aggregation occurs by recognition by *Chlamydomonas* of a specific signal originating from the *Daphnia,* inducing a transcriptomic response resulting in the production of cell-cell adhesion factors. In summary, these data uncover the robust transcriptomic response to predation and potential signaling pathways in *Chlamydomonas* that respond to predation resulting in the expression of specific cell-cell adhesion genes.

Figure 2.1 Algal Growth Rates In The Presence Absence Of *Daphnia* (a) Mean biomass accumulation rates measured over 24 hours for *Chlamydomonas*, *Gonium* and *Pleodorina* in the presence and absence of *Daphnia*. (b) Light microscope images of *Chlamydomonas*, *Gonium* and *Pleodorina*.

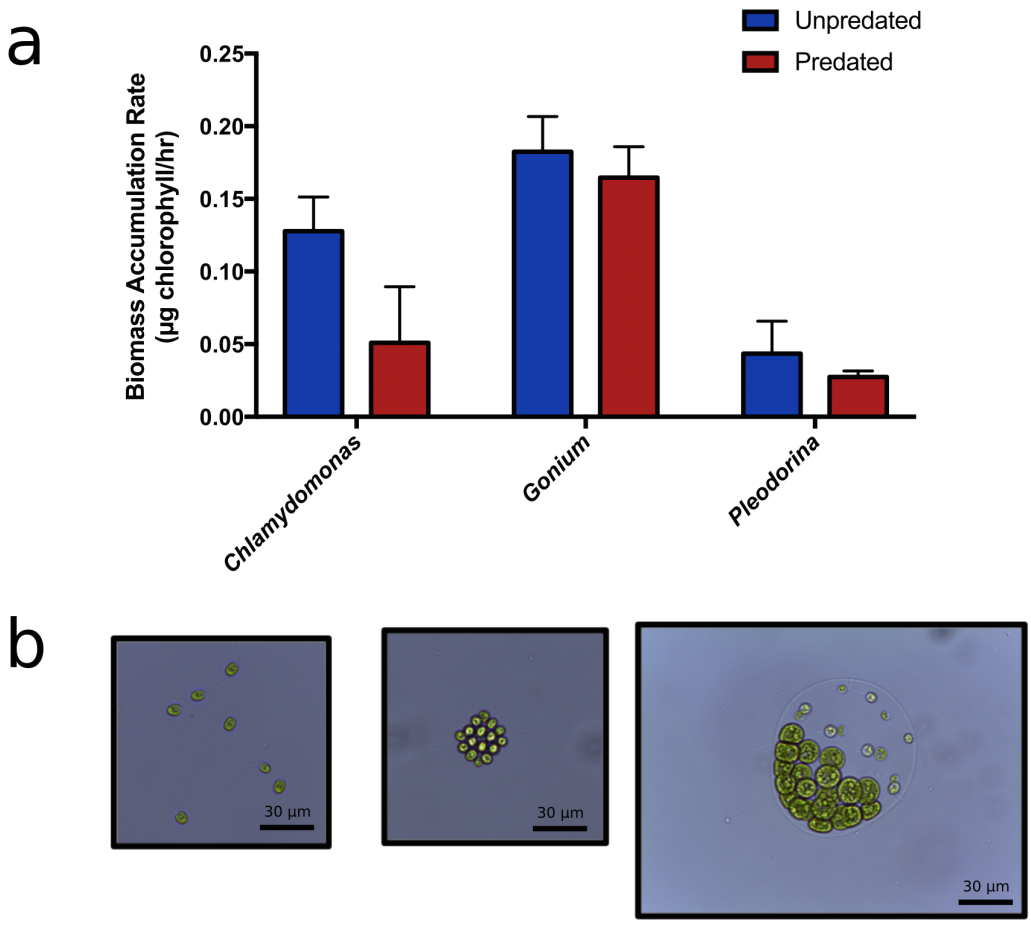


Figure 2.2 Predator Induced Aggregation (a) Mean aggregation in *Chlamydomonas* in the presence (solid line) and absence (dashed line) of predators over 24 hours. (b) Mean aggregation in *Chlamydomonas* over 1 h of predation. N = 3 for both experiments.

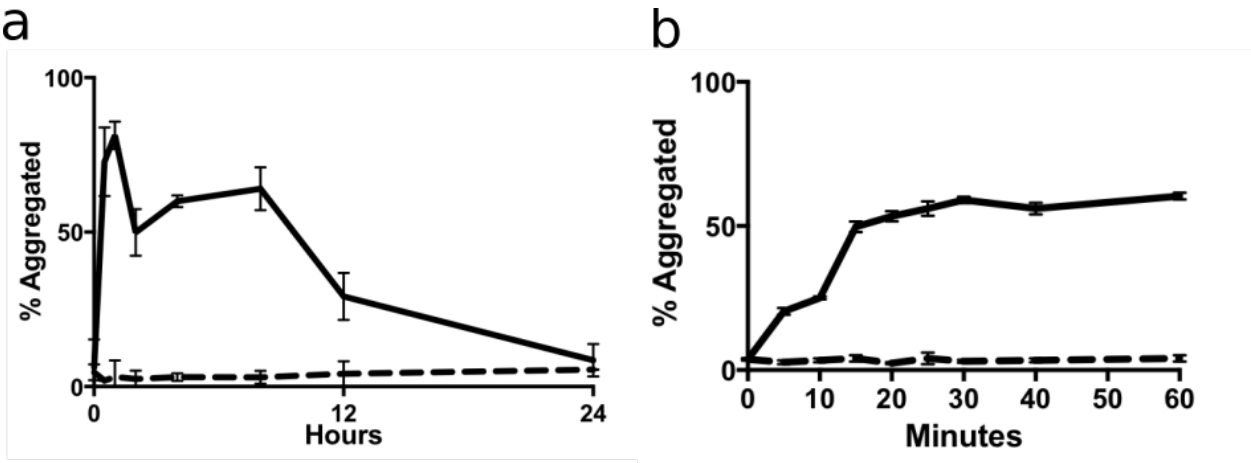


Figure 2.3 Mechanism of Aggregation (a) Dispersal of *Chlamydomonas* aggregates after the removal of predators (solid line). Aggregates are maintained in cultures where predators are left (wide dashed line). An unpredated control was also included (narrow dashed line). The arrow indicates time of predator removal. (b) Aggregation in response to exposure to *Chlamydomonas* lysates, killed *Daphnia* and live *Daphnia*. An unpredated control is also included (c) Aggregation in *Chlamydomonas* cultures treated with supernatants from unpredated cultures of *Chlamydomonas* (left), predated cultures of *Chlamydomonas* (middle) and *Daphnia* only cultures (right). (d) Aggregation response to *Daphnia* supernatant after translational inhibition by cylcoheximide (right). An unpredated control (left) and vehicle only control (middle) are also included. Means and standard errors are shown. N = 3 for all treatments and time points.

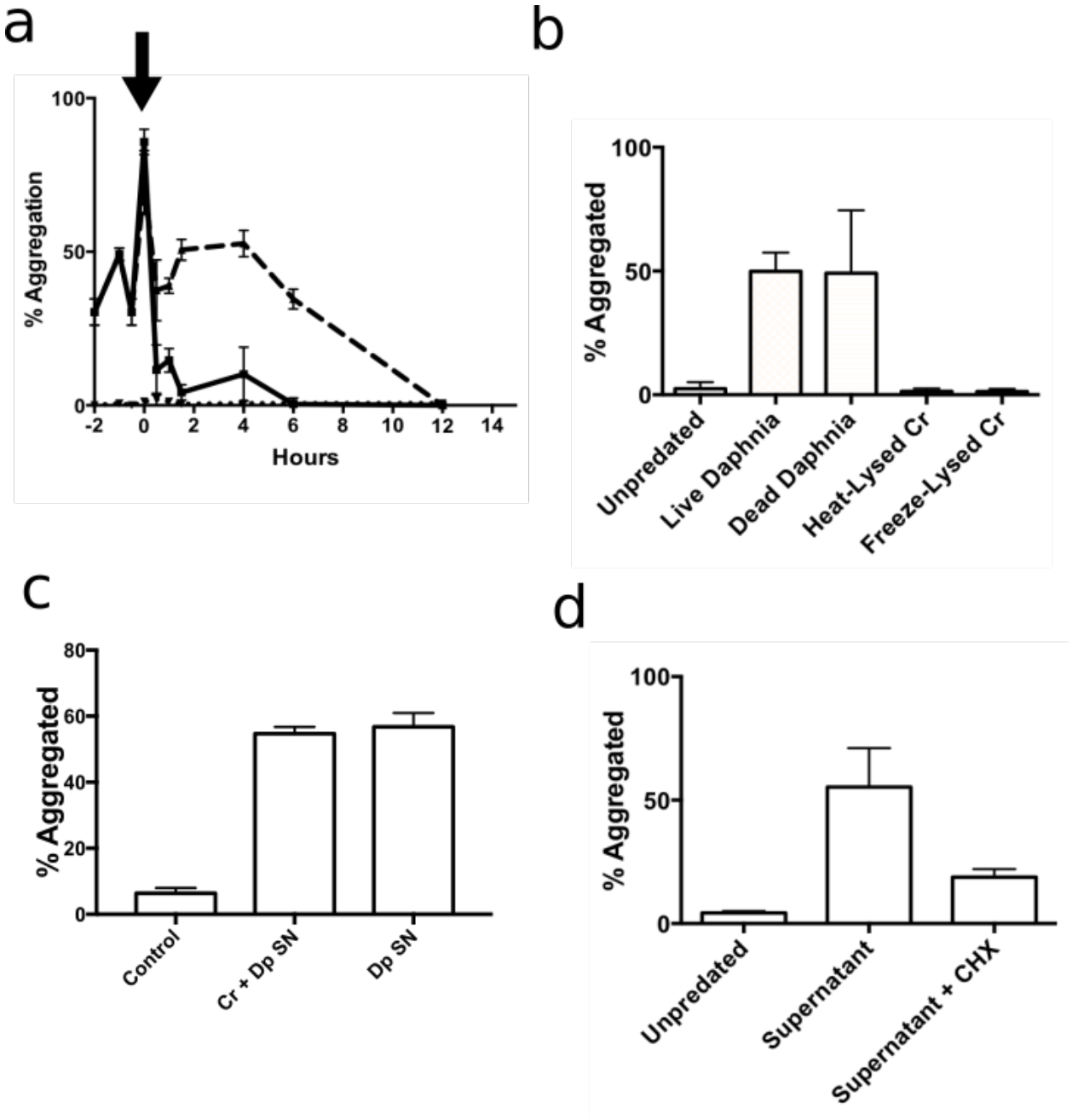


Figure 2.4 Aggregate Morphology (a and b) Light microscope image of a culture of *Chlamydomonas* aggregates. (c) Scanning electron image of two cells in a *Chlamydomonas* aggregate. (d) Extracellular adhesion complex (arrow) between two *Chlamydomonas* cells in an aggregate.

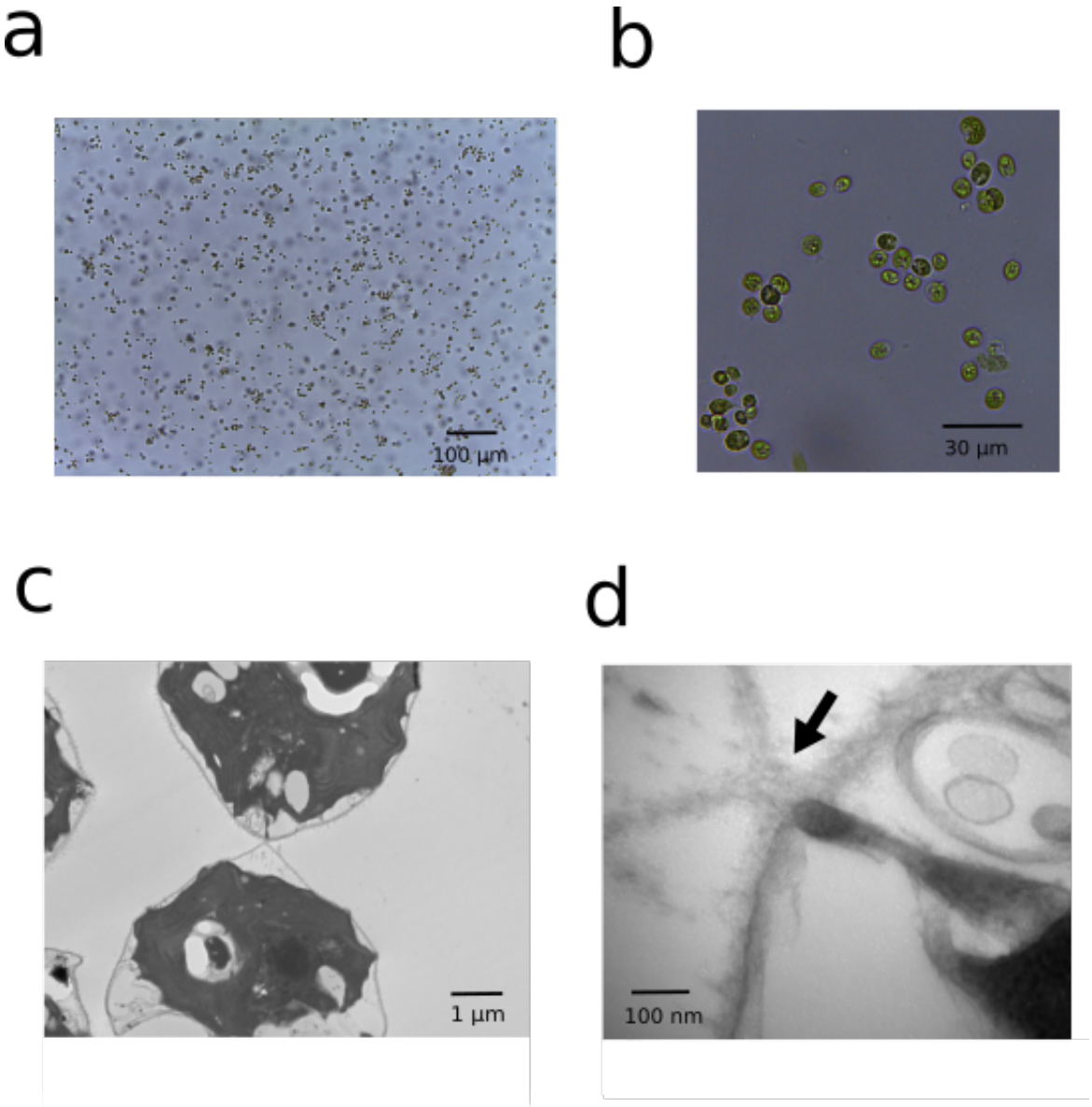


Figure 2.5 Characteristics of Aggregation (a) Histogram of aggregate size. Mean aggregate size is ~5 cells/aggregate. (b) Variation in aggregation based on predator concentration. Means and standard errors are shown for concentration dependent aggregation. N = 3 for each treatment.

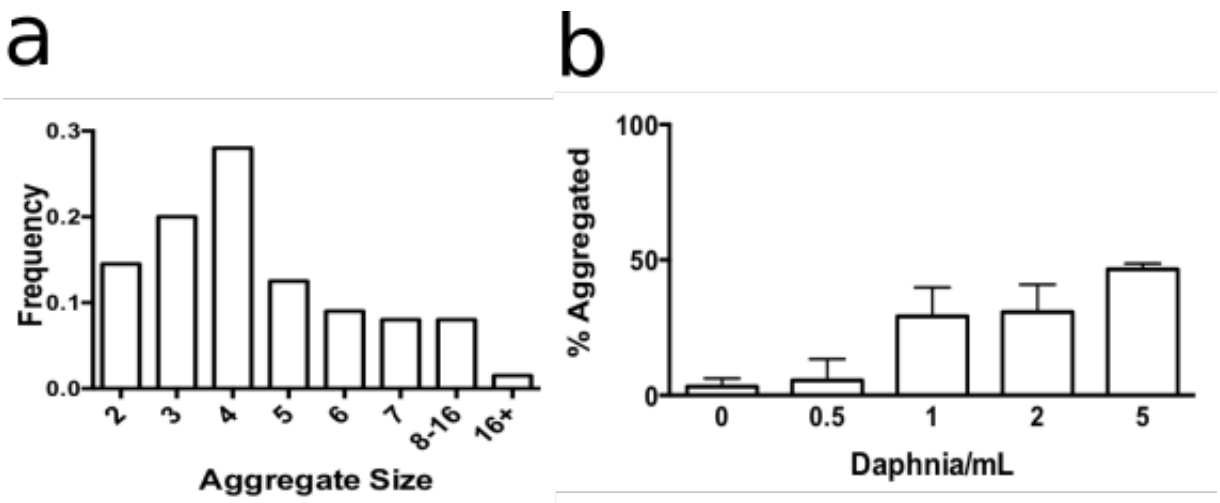


Figure 2.6 Conservation of Aggregation (a) Aggregation in several species of unicellular green algae during predation by *Daphnia*. (b) Aggregation in *Chlamydomonas* from predation by *D. magna* and *D. pulex*. And unpredated control is also included (c) Aggregation during predation in a wildtype *Chlamydomonas* strain (CC-1691) and a mutant cell wall strain (CC-4533). Means and standard errors are shown. N = 3 for all treatments.

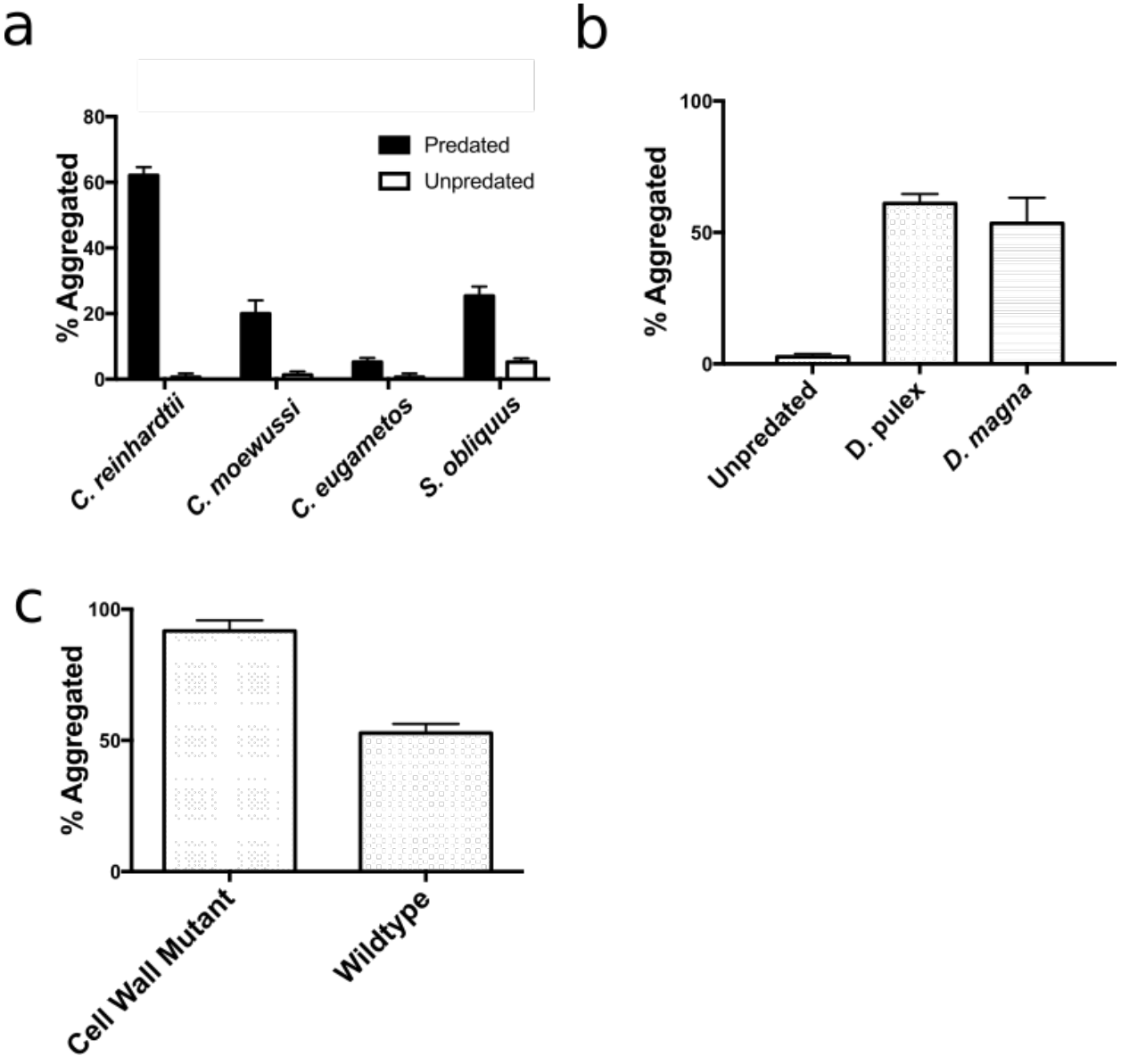


Figure 2.7 Transcriptional Response to Predation (a) Aggregation over 24 h in cultures used for RNA-seq. Lines below x-axis group represent time point pooling for RNA-seq. Means and standard errors are shown. N = 3 for each time point. (b) Heatmap of significantly differentially expressed genes during predation. Blue represents low expression and yellow represents high expression (c) Functional enrichment of genes by cluster.

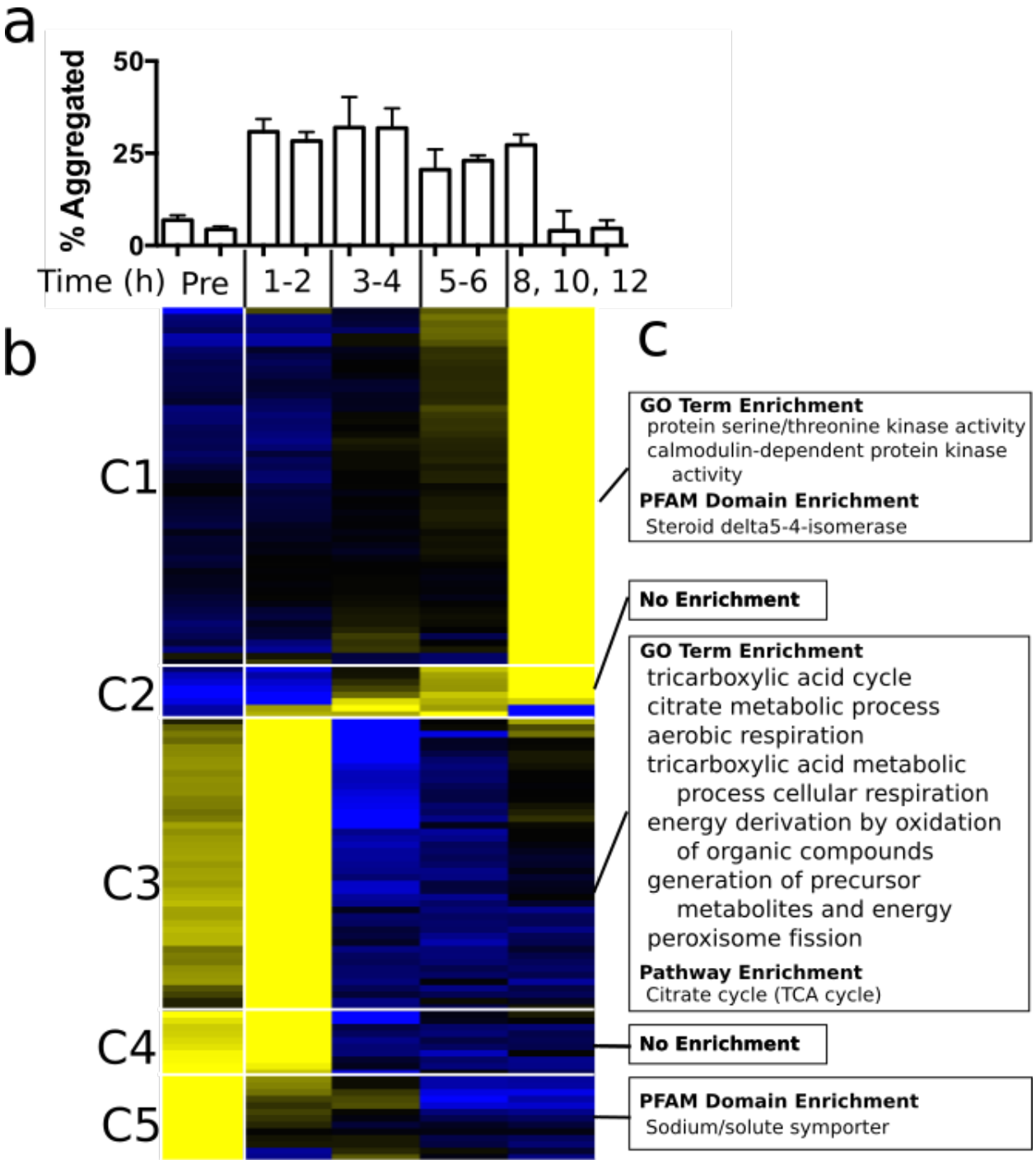


Figure 2.8 Time Course of Expression in Candidate Genes. (a-e) FPKM values for candidate genes over of 12 hours of predation by *Daphnia*. Means and standard errors are shown.

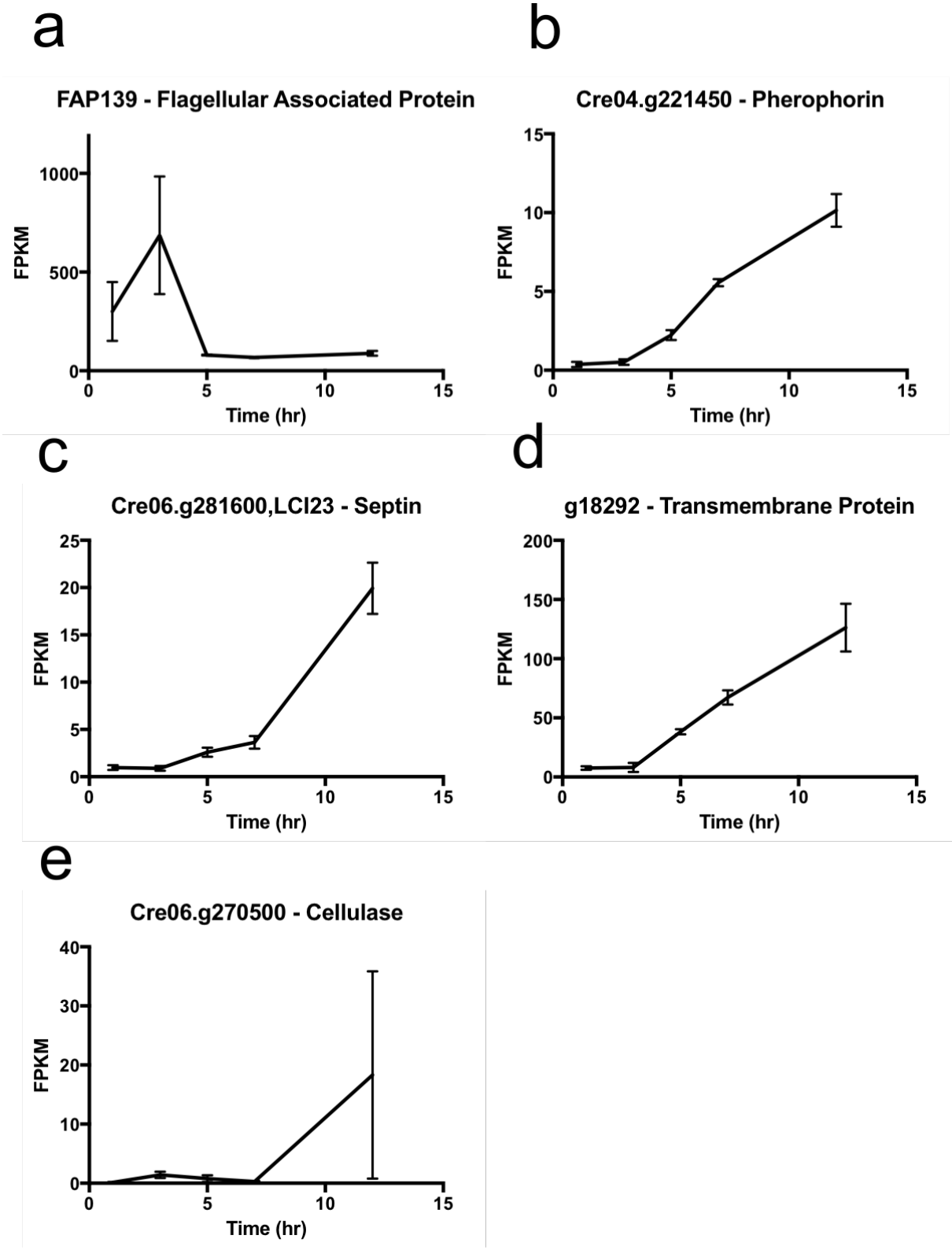
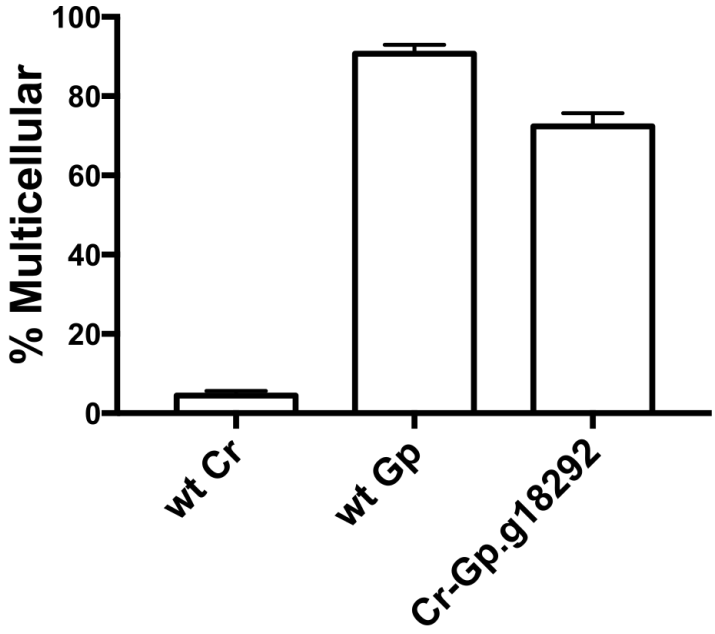
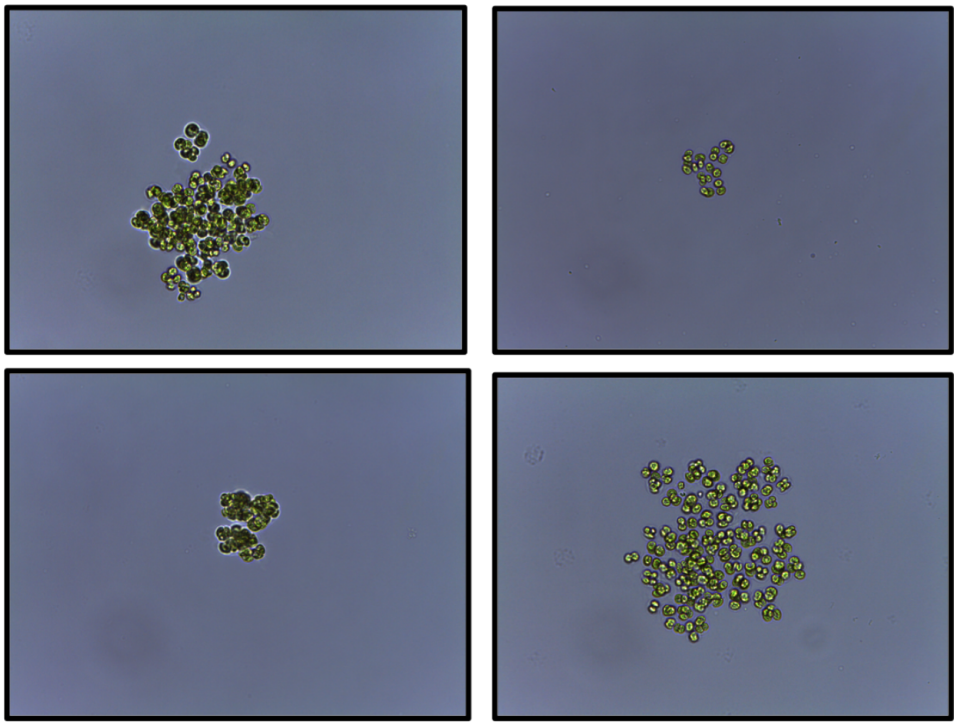


Figure 2.9 *g18292* Functional Testing (a) Phenotype in wildtype *Chlamydomonas* (left), wildtype *Gonium* (middle) and wildtype *Chlamydomonas* transformed with the *Gonium g18292* ortholog (right). Means and standard errors are shown. N = 3 for each strain. (b) Light microscope images of multicellular groups in a *Chlamydomonas* strain transformed with the *Gonium g18292* ortholog.

a



b



Conclusions

In sum, this work has contributed to our understanding of complex microbial-predator interactions. Aggregation has been known as a predator response in microbial organisms, however, the complex nature of the mechanism has not been described. Even less was known about the genetic basis of aggregation.

Here, we have characterized aggregation and found that it is rapid, transient and occurs in multiple green algal species. We also have found evidence that aggregation is induced by *Chlamydomonas* detecting a cue released into the environment by the *Daphnia* predators.

The RNA-seq experiment revealed a robust transcriptomic response by the algae during aggregation and several promising candidate genes for aggregation. We found candidates for cell wall modification with the cellulase. We also found several candidate adhesion genes in our analysis. The septin, the flagellar associated protein and the pherophorin are promising candidates genes for cell-cell adhesion. Evolutionary analysis and functional testing of the transmembrane protein, *g18292,* provided evidence that aggregation genes may play a role in multicellular development, challenging the conventional knowledge that complex multicellular organisms evolved by clonal development.

This work has expanded on our understanding of the mechanism by which unicellular green algae aggregate to form groups under predation by *Daphnia*. It has also shed light on the genetic basis of this response and its implications in multicellular evolution in the Volvocine algae.

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Appendix A – Gene Table And Enrichment Data

Table A.1 Significant Genes And FPKM Values

Gene IDs and FPKM values for all 131 significantly differentially expressed genes between predated and unpredated cultures of *Chlamydomonas* identified in the RNA-seq experiment.

gene_id	Pre FPKM	1,2 hr FPKM	3,4 hr FPKM	5,6 hr FPKM	8, 10 , 12 hr FPKM
ACH1	496.5483333	951.1776667	125.523	128.816	146.0129333
ADK4	52.71363333	93.32373333	17.48013333	11.07242	9.687783333
AKC3	2.240123333	1.885406667	3.730063333	5.94184	23.71013333
AMI1	229.9199333	68.21293333	80.9534	18.33812667	19.70018
AST1	166.357	296.6706667	32.7801	29.17036667	47.48603333
CAH4	4.718533333	3.68519	7.108026667	12.46766667	93.62203333
CAH5	4.389283333	3.00919	6.247496667	10.36187333	78.30943333
CCP1	0.952987333	1.99818	3.422666667	7.535793333	92.29716667
CIS2	314.342	583.486	104.9080667	134.2026667	173.1236667
COX18	3.499286667	6.6354	1.540316667	2.332873333	2.519823333
CPN60C	16.71059333	33.14281	4.317106667	4.989316667	4.37521
Cre01.g013450	1.37011	1.573623333	3.12653	1.393311667	10.63148667
Cre01.g013500	2.074786667	1.789653333	3.210866667	3.725233333	19.11897
Cre02.g080600	5.499436667	0.202193333	1.193553667	0.911876133	0.575207333
Cre02.g097800	11.7606	15.90067667	50.42166667	139.7003333	940.1756667
Cre02.g112700	0.2260189	0.165551333	0.246511133	0.452807	2.042273333
Cre03.g149050	1.166415667	1.064382	4.896796667	6.693673333	8.767113333
Cre03.g159254	0.297649	0.077603667	1.182213333	7.302594667	24.26273
Cre03.g172250	2.910683333	4.85516	1.16679	1.504593333	1.060871333
Cre03.g172300	64.56066667	135.0952667	11.2816	22.4439	37.0718

Cre03.g179500	13.19726633	2.416796167	0.823122667	0.248426667	0.103641333
Cre03.g183300	6.770003333	6.072166667	9.907313333	16.1313	69.9786
Cre03.g193900,SCL1	70.4564	117.9785667	20.0177	24.18746667	30.00206667
Cre03.g199050	0.0306809	0.016786467	0.035427667	0.1841054	3.595624667
Cre03.g199150	7.595786667	5.398193333	0.839343	1.996325333	1.532602333
Cre03.g204500	0.209678333	0.250987667	0.279761	0.237110633	4.51458
Cre03.g205100	4.088176667	2.10507	9.37531	14.4225	70.02986667
Cre04.g214600	0.723191667	0.905690667	1.869646333	1.583539333	12.63461
Cre04.g221450	0.372405967	0.514969	2.236763333	5.55491	10.14099667
Cre05.g236039	22.37985067	3.88983	3.964066667	1.87298	1.156403333
Cre05.g237800	0.229540467	0.472595333	0.416331	0.650135667	5.850926667
Cre06.g258350	0.973769	1.618863333	0.360759333	0.553850667	0.679335
Cre06.g263200	1.832653333	1.387214	6.454283333	5.735013333	6.2884
Cre06.g263300	171.7522333	327.2307667	27.72373333	38.28743333	52.944
Cre06.g266100	2.052763333	2.210134333	0.498519333	0.376677333	0.738321333
Cre06.g266150	1.967836667	3.06	0.639276333	0.332245	0.541514667
Cre06.g267850	3.082353333	5.346013333	1.1853	2.47823	2.917873333
Cre06.g270500	0.1515173	0.537968467	0.7674074	0.276759	18.30602867
Cre06.g281600,LCI23	0.968952667	0.879660667	2.584236667	3.639876667	19.92863333
Cre06.g307750	8.82812	10.97876333	17.45563667	12.93237333	67.68034333
Cre07.g315550	1.623559667	2.977547	0.520507667	0.793899	1.253123333
Cre07.g327950	0.006922533	0.041683733	0.032433867	0.121342233	1.737269467
Cre07.g334750	6.319716667	8.599246667	5.684746667	5.280443333	22.28506667
Cre07.g346900	289.0521667	39.83741	43.15293333	13.53683667	9.811953333
Cre08.g360050	105.4743333	44.5094	17.45903533	1.2323754	1.770195333
Cre08.g364100	0.682506267	3.037847333	0.159274233	0.096100967	0.091831167
Cre08.g375400	0.119505567	0.172640333	0.177006	0.668058333	3.986652
Cre08.g384650	1.235933667	0.814770333	1.509546667	1.98887	9.143333333

Cre09.g393250,HSP70C	28.53262667	50.6595	5.715206667	5.85444	10.14816333
Cre09.g395750	1.238327	3.943043	0.736567667	0.846977667	1.154245667
Cre09.g404900	14.25552667	12.45802	1.898716667	0.564681	0.403685667
Cre11.g477350	0.146890933	0.374896333	0.922328333	3.024296667	12.43282667
Cre12.g491950,g12151,g12152	0.238651333	0.574872	0.969725333	0.831339333	5.92241
Cre12.g514400	0.139479333	0.127699	0.223433333	0.467238	3.417626667
Cre12.g527250	0.117639767	0.184045033	0.136939733	0.420660333	4.037
Cre12.g540500,g13764	118.4103333	193.523	38.20693333	49.07886667	58.7987
Cre12.g550750	6.5118	12.26254333	1.225433667	2.423633333	3.008693333
Cre12.g551350	1.709836667	1.60955	4.05145	16.75079	45.04467
Cre12.g560300	5.227586667	11.03143667	2.41469	4.65784	7.542213333
Cre13.g585000	0.726357	1.053182333	5.407183333	7.779976667	14.55801667
Cre13.g588271	2.576259667	0.982144	4.635665	11.58925333	16.885496
Cre13.g591400	6.646303333	12.03596	1.7916	3.479203333	3.943916667
Cre14.g610250	0.117909333	0.320707	0.585627667	0.445296333	6.783498333
Cre14.g625650	35.2574	58.38229	6.981386667	3.407473333	5.890653333
Cre14.g626000	9.760333333	18.57037333	2.623663333	2.447856667	4.428653333
Cre15.g637761	23.5393	42.11303333	9.29735	13.1262	14.94906667
Cre15.g641200	58.64513333	98.5393	20.04776667	25.62253333	35.7734
Cre16.g659300	7.867003333	12.23459333	2.01771	1.573023333	1.282573
Cre16.g659800	0.548553333	0.668391667	1.039944667	2.162123333	10.05118667
Cre16.g661750,Cre16.g661800	11.70159667	12.56719667	52.48523333	92.03763333	301.4366667
Cre16.g661850	4.409613333	5.85794	37.2693	76.37763333	224.0803333
Cre16.g664700	5.46125	6.37804	1.502123333	2.472316667	2.573316667
Cre16.g669800	0.020292633	0.160798133	0.167164933	0.975702	6.597045667
Cre16.g683350	5.147316667	12.17836333	2.916103333	2.607433333	3.006813333
Cre16.g686350	0.21775	0.098420167	0.279799667	0.416250333	2.267526667
Cre16.g690950	2.221413333	3.376916667	3.636813333	2.738653333	26.33649

Cre16.g691150	0.485575333	1.291622333	1.857733333	1.190830333	33.80111267
Cre17.g711150	3.548215	3.652021	0.702581333	1.545902333	1.79785
Cre17.g742750	0.235871667	0.181351667	0.478460333	0.549862	2.663833333
DUR2	32.25703333	14.6674	5.809256667	0.895935333	1.161613333
DUR3	586.577	235.3165333	168.2882333	11.63937833	10.13850433
DUR5	283.5639667	99.2617	101.8387033	17.51347333	37.1603
FAP139,IPY3,g9597	300.691	686.5853667	79.0733	67.05913333	88.5987
FBP2	99.9948	224.9322	33.892	35.2328	45.95876667
FDX2	50.93289667	117.9239333	159.7821333	117.4685333	28.27935
FDX5	320.9251037	7.658265333	0.767559	0.094376	0
FEA2	24.9472	101.1496333	73.06816667	107.5701	220.7676667
g11482	1.104306333	2.344905333	0.252787	0.514587667	0.770677333
g11524	4.582046667	4.48564	3.299326667	2.916783333	16.22137
g12149	0.129915633	0.257623667	0.513391333	0.693438333	4.260413333
g12871	0.365276	0.375087733	0.4704379	0.668404667	3.297923333
g1436	0.499720667	0.425818667	0.478285	0.587468333	2.626306667
g14920	169.8956667	247.3533333	47.71393333	54.3828	64.2103
g16366	0.635672	0.425622333	0.717755	0.777284667	5.48096
g18292	7.510966667	8.097896667	38.27936667	67.17733333	126.233
g1910	3.45156	0.676709	0.274237667	0.328275333	0.271756
g2912	15.13767	26.11326333	1.461273333	1.215655667	1.66294
g4852	0.707695	0.631075333	1.787693333	1.447072667	5.920183333
g4912	0.981506667	2.589329667	0.122599	0.079441833	0.119031133
g5109	0.061451633	0.182616167	0.363390667	0.168623833	8.654134
g5303	0.499929667	0.402436933	0.787353667	0.570856333	2.46835
g5605	2.130576667	3.183523333	0.743230333	1.219181	1.314083333
g5732	0.0210358	0.011291567	0.057535433	0.2743676	2.112474467
g6293	42.35076667	72.1405	16.79363333	21.29523333	24.54486667

g9501	0.098366667	0.171762867	0.157463933	0.176711667	1.857909667
GOX7,g11470	8.530846667	2.163482333	1.187927	0.339511333	0.241613
GSN1	36.4356	45.33536667	9.902543333	12.14663333	11.58334
HSP22F	3.878076667	0.912516667	1.735636667	1.159395	0.942119
HSP70A	277.11	367.038	78.1964	71.40026667	83.31813333
HSP70E	23.58976667	28.27933333	5.160153333	5.580363333	6.053256667
HSP90A	172.6366667	234.3515333	52.63546667	46.00526667	44.2052
ICL1	1041.545	1950.841667	169.5416667	210.9076667	415.703
IDH2	68.40023333	110.1273333	24.851	33.97356667	35.91306667
IRT1	0.309185333	1.187542633	1.334436333	3.615978333	19.36605333
LAO1,g13518	12.47842667	2.71774	4.824982333	260.7504567	836.7998323
LCI1	0.396174	0.275229333	0.823748667	0.897586	3.990666667
LHCSR2	3.6929	3.298893333	13.0737	23.11753333	102.2973333
LHCSR3	2.108126667	2.439153333	9.709743333	16.50563333	79.94646667
MAS1	569.4556667	919.0356667	92.30753333	100.5315667	207.179
MFT10	4.160374	12.20064333	2.64902	3.731126667	3.269933333
MTA2	1.139378667	1.25008	1.89448	1.7704	7.941003333
NAR1.2	4.57594	5.88541	21.72773333	48.6093	306.2686667
NAR1.5	10.92839	18.22923	3.583863333	5.14153	7.078896667
OGD1	118.2216333	195.6713667	31.70366667	41.08543333	46.37686667
OGD2	131.5882333	221.3807	43.1871	44.27133333	45.47473333
PCK1	514.5736667	859.319	210.8116667	247.3436667	304.512
PDC3	26.85756667	42.11556667	6.69231	10.63422333	11.70225
PHO1	55.6087	59.9521	9.100833333	5.733703333	3.561233333
RLS7	0	0.048527833	0.0861116	0.1964963	1.953707667
TEF18	2.380846667	4.335113333	0.594723667	0.835725	2.308793333
THB1	4.035206	8.665093333	8.53716	10.37288	2.548692333

Table A.2 Functional Enrichment Data

Gene IDs, annotations and clusters for functional enrichment of significantly differentially expressed genes.

Annotation	p-value	Gene IDs	ID	Cluster	Type
protein serine/threonine kinase activity	0.000994489	Cre03.g199050, Cre16.g661750, Cre16.g661850, Cre16.g669800	GO:0004674	C1	GO
calmodulin-dependent protein kinase activity	0.012121373	Cre16.g661750,Cre16.g661850	GO:0004683	C1	GO
steroid delta5-4-isomerase	0.041581846	Cre16.g661750,Cre16.g661850	IPR011944	C1	Protein Domain
tricarboxylic acid cycle	1.6044E-06	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0006099	C3	GO
citrate metabolic process	2.73974E-06	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0006101	C3	GO
aerobic respiration	4.4348E-06	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0009060	C3	GO
tricarboxylic acid metabolic process	4.4348E-06	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0072350	C3	GO
cellular respiration	2.10167E-05	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0045333	C3	GO
energy derivation by oxidation of organic compounds	0.000140942	Cre01.g042750, Cre02.g143250, Cre07.g343700, Cre12.g537200, Cre14.g619133	GO:0015980	C3	GO
generation of precursor metabolites and energy	0.010989509	Cre01.g042750 ,Cre02.g143250, Cre07.g343700, Cre08.g367500, Cre12.g537200, Cre14.g619133	GO:0006091	C3	GO
peroxisome fission	0.042506784	Cre06.g263300,Cre12.g540500	GO:0016559	C3	GO
Citrate cycle (TCA cycle)	1.76E-08	Cre01.g042750, Cre02.g141400, Cre02.g143250, Cre03.g149100, Cre03.g193850, Cre07.g343700, Cre12.g537200, Cre14.g619133	cre00020	C3	Pathway
Sodium/solute symporter, subgroup	0.017260309	Cre08.g360200, Cre17.g703800	IPR019900	C5	Protein Domain