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Consequences of Kleptoplasty on the Distribution, Ecology, and Behavior of the

Sacoglossan Sea Slug, *Elysia clarki*

by

Michael L. Middlebrooks

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

I would like dedicate this dissertation to my friends and family who have been very supportive of me while I completed this work, especially my little sisters Jenna and Margaret and my girlfriend Elizabeth. Thank you!

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ABSTRACT

The sacoglossan sea slug *Elysia clarki* is able to photosynthesize for three to four months using chloroplasts sequestered from its algal food sources. Furthermore, the slug is able to store multiple chloroplasts from different algal species within the same cell. This research, consisting of several related studies, explores the role that provision of organic nutrients via photosynthesis plays in the biology of the slug. The first chapter demonstrates that, under conditions of starvation, photosynthetic activity in *E. clarki* remains fully functional for one month after which it then declines. During the first month of starvation the slug exhibits similar feeding behavior as slugs provided a continuous supply of food, suggesting that photosynthesis delays the onset of starvation-induced behavioral changes. The second chapter explores *E. clarki's* spatial relationships with algae known to be food sources in the field. In areas with high slug density, edible algal populations were very low. DNA barcoding was employed to demonstrate that the algae found near slugs were poor predictors of which foods were actually consumed by slugs. Generally, there was a mismatch between algae available in the field and slug diets. The third chapter explores how *E. clarki* is able to maintain photosynthesis. After labeling with a C^{14} ALA incubation process, then chlorophyll was extracted from slugs and purified using HPLC. Results indicate that recently collected *E. clarki* are able to synthesize chlorophyll, whereas slugs starved for 3 months were not. Photosynthesis plays a very important role for *E. clarki* and its relationships with food algae.

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GENERAL INTRODUCTION

Sacoglossan sea slugs (Opisthobranch: Mollusca) are a group of herbivorous gastropods that feed on coenocytic green algae. An interesting characteristic of is that a number of species are able to sequester chloroplasts, ingested from their algal food, inside of specialized cells in the digestive tubules (Pierce and Curtis 2012). These chloroplasts are then maintained and remain photosynthetically active. This process, often called "kleptoplasty", provides the slug with an alternative energy source to heterotrophy (Trench et al. 1969, Clark et al. 1990, Pierce et al. 2012). Recent studies have discovered that kleptoplasty may increase sacoglossan survival during starvation (Gimènez-Casalduero and Muniain 2008)

My study focuses on *Elysia clarki*, a sacoglossan endemic to the Florida Keys, USA, typically found in near-shore shallow water habitats. *Elysia clarki* is able to maintain photosynthesis for over 3 months (Pierce et al. 2006) without feeding and can even sequester chloroplasts from multiple algal species within a single cell (Curtis et al. 2006). Specifically, the work presented below investigates how the relationship between *E. clarki* and its algal food sources and sequestered chloroplasts affects the distribution, ecology, and behavior of the slug.

This dissertation is divided into three chapters. The first chapter focuses on how *E. clarki*'s photosynthetic ability modifies the behavior of the slug when it is faced with starvation, given that kleptoplasty provides the slugs with an extra energy source. This

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laboratory-based study measured changes in the slugs' fluorescence and chlorophyll concentration, as proxies of measuring photosynthesis, at different intervals of starvation. Additionally, behavioral feeding experiments were conducted to determine if slugs display a prolonged satiation effect from photosynthetic activity.

The second chapter is primarily field-based and examines the spatial distribution of *E. clarki* relative to its algal food sources. Of primary interest was determining if there was a strong spatial relationship between *E. clarki* and its algal food sources and if algal availability can be used to determine *E. clarki*'s diet. In 2008-2010, detailed field surveys were conducted at 4 sites across the Florida Keys, USA during which the location of slugs and potential food sources were recorded. Slugs were also collected from each of the 4 sites in 2008 and were further examined to determine which foods were consumed in the field, based upon the identity of chloroplasts sequestered by the slugs. Here molecular techniques were utilized to identify the algal species consumed through the *rbcL* chloroplast gene. Molecular results were then compared with observations from the field surveys to gather unique information on actual resource use in relation to relative availability.

The third and final chapter was designed to explore the mechanism by which *E. clarki* is able to maintain photosynthesis over a time period of up to many months. In a laboratory setting, field collected slugs were exposed to an isotope of C^{14} ALA as a label. Then, High Performance Liquid Chromatography was used to separate chlorophyll and evaluate whether *E. clarki* is able to synthesize both chlorophyll a and b for use in maintaining chloroplasts and photosynthesis. Furthermore this experiment provided data

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to evaluate whether *E. clarki* can continue to synthesize chlorophyll after photosynthetic activity declines.

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CHAPTER ONE:

FORAGING BEHAVIOR UNDER STARVATION CONDITIONS IS ALTERED VIA PHOTOSYNTHESIS BY THE MARINE GASTROPOD, *ELYSIA CLARKI*

Note to Reader:

Portions of these results have been previously published (Middlebrooks, et al. 2011) and are utilized with permission of the publisher.

Introduction:

Foraging behavior of animals, defined here as actively searching for and consuming food, encompasses diverse processes such as dispersal, predator-prey interactions, predation risk, and resource optimization (Houston 1991, Vadas et al. 1994, Jeschke 2007, Bonte et al. 2008). Animals deprived of food are more likely to travel further and invest more time in search of food than satiated conspecifics (Torres et al. 2002), thereby utilizing energy that might otherwise have been allocated to purposes such as growth or reproduction. Additionally, starved animals may exhibit reduced anti-predatory behavior to gain increased access to a food source (Vadas et al. 1994). Some models based upon risk-sensitive foraging theory suggest that animals will choose a more variable array of energy-yielding food items during periods of starvation (Stephens 1981, Houston 1991). Specific behavioral displays in response to starvation can be highly variable, as environmental conditions (e.g. predators, refuge) (Wojdak 2009)or physiological traits, such as fat storage, (Jacome et al. 1995, Prop et al. 2003) might

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modulate responses. Altogether, starvation generally modifies the actions related to food acquisition (Vadas et al. 1994, Avila et al. 1998, Prop et al. 2003, Biesinger and Haefner 2005) which in turn may have consequences for an organism's survival and interspecific interactions (Vadas et al. 1994).

Most animals which forage are limited to acquiring energy by ingestion (Venn et al. 2008). However, a number of species from diverse taxa not only acquire energy through assimilation of ingested food but also by photosynthesis conducted by endosymbiotic zooxanthellae or stored algal chloroplasts and are termed mixotrophic (Trench and Ohlhorst 1976, Stoecker et al. 1989, Venn et al. 2008). Depending on light conditions, these photosynthetic animals can derive a significant portion of energy yielding metabolites via photosynthetic pathways (Anthony and Fabricius 2000). As a result, photosynthetic animals may have different behavioral responses to a lack of food than are displayed by non-photosynthetic capable species. Thus, despite food limitation, if photosynthesis provides sufficient metabolites, then the incidence of food searching behavior, and the associated elevated risk of mortality, might be correspondingly reduced or even eliminated. Although studies have demonstrated the effect of photosynthesis on feeding rates in corals (Titlyanov et al. 2000, Piniak 2002), the foraging behavior of starved photosynthetic-capable animals which are motile has not been well studied.

Most animal species capable of utilizing photosynthesis as an energy source are aquatic and sessile (e.g. corals, sponges, giant clams) or have limited motility (e.g. benthic jellyfish), and thereby do not forage at all (Venn et al. 2008). However, the sacoglossan (Opisthobranchia: Mollusca) sea slugs are highly motile and actively forage on algae, which is usually siphonaceous and typically found in shallow water (Clark et al.

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1990). Importantly, many sacoglossans are capable of kleptoplasty, a process by which slugs photosynthesize using chloroplasts which are sequestered from the algal food by specialized cells of the digestive tubules (Figure 1.1) (Greene 1970, Greene and Muscatin.L 1972, Trench and Ohlhorst 1976, Clark et al. 1990). Nuclear genes horizontally transferred from algae into the slugs likely play an important role in the slugs' ability to photosynthesize (Pierce et al. 2008, Pierce et al. 2009). These combined features suggest that kleptoplastic sea slugs are a specialized group of herbivores which can be utilized to examine how the level of satiation/starvation affects foraging behavior in a photosynthetic animal. Although increased foraging efforts under starvation conditions and a decrease during satiation is usual for many species (Torres et al. 2002, Jeschke 2007), the foraging behavior of photosynthetic sea slugs may be different, if food is withheld, provided that photosynthesis continues.

Previous studies of kleptoplastic slugs have considered the evolutionary benefit of supplemental energy provided by sequestered chloroplasts. A relatively large amount of sequestered chloroplasts could allow less time to be spent foraging (Marin and Ros 1993). Kleptoplasty might also increase slug survival during times of famine (Gimènez-Casalduero and Muniain 2008). However, among sacoglossan species, the length of time that algal chloroplasts remain functional is quite variable, ranging from only several hours to up to 9 months (Clark et al. 1990, Händeler et al. 2009). Therefore the impact of photosynthetic metabolites must also vary among species, and kleptoplastic slugs experiencing food limitations may only manage to delay behavioral changes related to food gathering, rather than completely avoid modifying their behavior. Although kleptoplasty provides metabolites to slugs, ultimately most slug species require ingested

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food at least to replace degraded chloroplasts (Clark et al. 1990, Händeler et al. 2009) with the notable exception of *Elysia chlorotica* which can complete the entire adult portion of its life cycle (up to 9 months) relying only on photosynthesis (Pierce et al. 1996, Mondy and Pierce 2003). Thus, the benefits of kleptoplasty are time limited. It is likely that, for most kleptoplastic organisms, as photosynthetic function declines foraging behavior will increase.

We describe here a set of experiments examining the incidence of foraging behavior displayed by a kleptoplastic slug subjected to different conditions of starvation. Our hypothesis was that starved slugs would not change their foraging behavior while able to photosynthesize. However, as starvation continues and photosynthetic activity decreases due to chloroplast failure (Clark et al. 1990, Händeler et al. 2009) foraging behavior likely is triggered. Therefore once photosynthesis has ceased, an increase in starvation time should also increase the probability of slugs exhibiting behavior linked to food acquisition.

Methods:

Source and Maintenance of Slugs: *Elysia clarki* (Figure 1.2), a sacoglossan species (Pierce et al. 2006), is an excellent organism for evaluating the relationship between kleptoplasty and feeding behavior. The slug lives in the Florida Keys in near-shore, low wave energy habitats, such as mangrove swamps, borrow pits, and mooring canals. It feeds suctorially on several species of siphonaceous green algae including *Penicillus capitatus, P. lamourouxii, Halimeda incrassata, Bryopsis plumosa,* and *Derbesia tenissima* (Curtis et al. 2004, Curtis et al. 2006, Curtis et al. 2007). The slug

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sequesters the chloroplasts from all of these species and uses them for photosynthesis. Furthermore, *E. clarki* photosynthesizes using the stored chloroplasts for up to three months without ingesting food and attains relatively large size (up to 35mm)(Pierce et al. 2006). Unlike the case for many *Elysia* species, which are tiny, observations of feeding behavior and measurements of photosynthesis are possible with *E. clarki*.

Project Overview: In order to assess the effects of starvation on photosynthesis and feeding behavior of *E. clarki,* slugs were collected from the field and assigned to feeding trials representing different levels of provided food. Once desired time of starvation for the experiment had been established, the slugs' feeding behavior was assessed. Finally, the amount of sequestered chlorophyll and photosynthetic rates of the slugs were measured and any display of feeding behavior was noted to determine whether photosynthesis was associated with delayed onset of foraging.

Specimens of *E. clarki* approximately 2cm in length were collected by snorkel from a borrow pit on Grassy Key, FL (24°44'56.07"N, 80°58'42.77"W) with permission from the Florida Fish and Wildlife Conservation Commission (permit # SAL-11-0616-SR) and then transported to the laboratory at the University of South Florida in Tampa, FL during July 2009. Twenty-four groups of 4 slugs were randomly assigned to 10L aquaria filled with artificial seawater (Instant Ocean ™) and treated one time before the start of the experiments with the antibiotics, Penicillin and Streptomycin (100µg/ml) to control bacteria. Aquaria were maintained at room temperature (~20°C). Photoperiod was maintained by alternating 12 hr light/dark cycles provided by overhead cool-white fluorescent lights. All slugs were initially starved for 4 weeks to ensure empty guts before the start of the experiment. Then, for a 2 week period, all aquaria were stocked

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with the algae *P. capitatus* (Figure 1.2), collected from a local seagrass bed near Tarpon Springs, FL (28° 8' 38.73" N, 82° 47' 26.49" W), and slugs were allowed to feed *ab libitum*.

After the 2 week feeding period, slugs were randomly assigned to one of three starvation level treatments, or a satiation control. The experiment was designed to represent a gradient of food availability and associated physiological state ranging from no food (starvation) to continuous access to food (satiation). Completely starved slugs had all algal food removed at the start, and were starved for the entire 12 week trial. Other slugs received food for 4 or 8 weeks respectively, and were then starved for the rest of the 12 week trial. The control group received a continuous supply of food for the entire 12 week experiment and slugs in this treatment were considered satiated. Each of the feeding treatments was replicated in 6 aquaria (n=24 total). All slugs were provided light (overhead cool white fluorescent lights) on the 12hr light/dark cycle to allow photosynthesis. After 12 weeks, the slugs from each feeding treatment were tested for their response to food.

Feeding Behavior: In order to examine the impact of starvation on feeding behavior of *E. clarki*, we tested feeding behavior of slugs as follows: Observations of slugs were made in 2L glass beakers filled with 1L of artificial seawater (Instant Ocean™), and each containing an individual cap and stipe of *P. capitatus,* placed on the bottom. *Penicillus capitatus* was the only food source presented to slugs during the trials. The beakers were maintained in ambient light conditions during the feeding trials which took place during daylight hours. Individuals of *P. capitatus* were only used once and beakers were then emptied and cleaned for subsequent trials. Individual slugs were

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placed into containers with their anterior end ~1cm from the alga to ensure slugs would have quick access to the food. Preliminary trials determined that this design represented the most effective arrangement to determine whether a slug initiated the feeding response.

After placement into an experimental container, slugs were observed for 5 min to determine if any feeding behavior occurred, defined as moving to and remaining on the alga. Although the physical presence of the slug on the alga does not necessarily indicate feeding, it reflects the position of the slug when it naturally feeds in the field. Because the slugs feed by sucking contents out of algal filaments, the densely packed architecture of filaments (Jensen 1993) prevents actual confirmation that the slug fed on the alga within the 5 min observation period. The length of feeding observations was constrained to 5 min because slugs were subsequently analyzed for chlorophyll concentration and additional feeding time could increase the amount of chlorophyll present in an individual slug.

To test the prediction that the proportion of slugs displaying feeding behavior (yes/no) would increase as time of starvation (continuous predictor) increased, we analyzed the results of experimental trials using the general linear model (GLM) via Statistica™. For the feeding experiment, slugs were analyzed as the of individuals that fed for each starvation treatment. To obtain a direct comparison between treatments, each possible combination of pairs of the 4 treatment groups was tested separately to evaluate differences using a Chi-squared goodness of fit test. For example, the control group and the 12 week starvation group were analyzed together without the 4 and 8 week starvation groups.

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Changes in Photosynthetic Ability: Once behavior had been evaluated, we measured two aspects of photosynthetic capabilities of sequestered chloroplasts within the slugs used in behavioral observation: Pulse Amplitude Modulated (PAM) Fluorometry for quantifying chlorophyll fluorescence and chlorophyll a (Chl a) extraction to determine the amount of Chl a remaining in the slugs. We used both fluorescence and Chl a concentration [Chl a] because each provides a different measure of photosynthesis. The amount of Chl a serves as an estimate of the amount of sequestered chloroplasts within a slug (Stirts and Clark 1980, Hoeghguldberg and Hinde 1986, Gimènez-Casalduero and Muniain 2008), while fluorescence measures the photosynthetic activity (Wägele and Johnsen 2001). The latter may or may not be related to the amount of algal chloroplasts depending on the condition of the chloroplasts. Therefore, although photosynthetic activity and [Chl a] are often correlated, high [Chl a] can be present despite low photosynthetic activity (Trench and Ohlhorst 1976).

PAM Fluorescence: Photosynthetic activity of each slug used in the feeding trial was measured using a PAM Fluorometer, which measures chlorophyll a fluorescence originating from photosystem II by emitting a strong pulse of light and measuring the returning fluorescence (Hader et al. 1997, Wägele and Johnsen 2001) (Diving PAM, Walz, Germany). PAM has also been used successfully by others to measure photosynthesis in sea slugs (Wägele and Johnsen 2001). Slugs were first dark adapted for 20 min in a dark room, and then measured for maximum quantum yield of fluorescence ($\square_{IIe}$) using the PAM and the following equation:

$$\Phi IIe = (Fm - Fo) / Fm$$

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where F_m is the maximum fluorescence during the light pulse and F_0 is the fluorescence measured in dark-acclimated tissues before the light pulse is emitted(Wägele and Johnsen 2001). Each slug was measured three times to ensure an accurate reading of $\square_{IIe}$ and a mean value of all three $\square_{IIe}$ readings was determined.

Chl a measurement: Upon completion of the PAM measurements, slugs were freeze-dried and weighed (g). The freeze-dried slugs were each homogenized in acetone and then centrifuged (~12,000 X G). The supernatant was saved and absorbance determined at 423 nm (Beckman Coulter DU 640^{TM} Spectrophotometer), the wavelength at which Chl a absorbs (Joyard et al. 1987). [Chl a] were then calculated from a standard Chl a curve and normalized (µg chl a/g dry weight of slug) (Joyard et al. 1987).

A. priori we expected to find higher maximum quantum yield and [Chl a] in slugs which had been feeding more recently. Maximum quantum yield was analyzed across treatments using a one way analysis of variance (ANOVA) followed by a Tukey HSD *post hoc* test with starvation length as the predictor and $\square_{IIe}$ as the dependent variable. [Chl a] was analyzed across starvation treatments using a one way ANOVA and Tukey HSD *post hoc* analysis after being log-transformed to meet ANOVA assumptions of homoscedasticity. For these analyses, starvation length was the predictor and [Chl a] the dependent variable.

Results:

Significant differences in slug feeding behavior and length of starvation were clearly evident. Slugs from all treatments displayed some feeding behavior; however, slugs from the continuously fed control, as well as those starved for 4 weeks, were less

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likely to feed than slugs starved for 8 and 12 weeks (Figure 1.3). Thirty-three percent of slugs in both the control and 4 week starvation group displayed feeding behavior, compared to 73% and 69% of the slugs in the 8 and 12 week starvation period, respectively. The length of time that slugs were starved was significantly associated with the proportion of slugs displaying feeding behavior ($p<0.001$, $F=13.67$) (GLM). Pair-wise Chi-squared goodness of fit tests indicated that slug foraging behavior in both the control and 4 week starvation treatments were not significantly different from each other, but both were different from slugs in the 8 ($p<0.05$, $\chi^2=4.311$) and the 12 ($p<0.05$, $\chi^2=4.200$) week starvation groups.

The length of starvation significantly reduced Chl a fluorescence (PAM) ($F_{3,9}=33.81$, $p<0.001$). The mean $\square_{IIe}$ in the continually feeding control slugs was approximately 2.5 x higher than slugs starved for 8 weeks and over 3 x higher than slugs starved for 12 weeks. Both the controls and slugs from the 4 week starvation group had a significantly higher mean $\square_{IIe}$ than that of slugs starved for either 8 or 12 weeks ($p<0.001$) (Figure 1.4).

[Chl a] (µg chl a/g dry weight of slug) in slugs declined as the length of starvation increased. After 12 weeks of starvation, [Chl a] was less than 1/3 that recorded for control slugs. An overall decrease in [Chl a] was detected in all starvation groups ($F_{3,9}=10.10$, $p<0.001$) (Figure 1.5). Tukey HSD *post hoc* analysis showed that [Chl a] from both the 8 and 12 week starvation treatments were significantly lower than that for the continuously fed control ($p<0.01$) (Figure 1.5). The [Chl a] in slugs starved for 4 weeks was only significantly different from the slugs starved for 12 week ($p<0.01$).

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Discussion:

Eventually, an increased length of starvation increased the likelihood of foraging behavior in *E. clarki*, as usual with starvation (Vadas et al. 1994, Biesinger and Haefner 2005, Jeschke 2007). However unlike earlier studies, our findings highlight that *E. clarki's* foraging behavior is further modified by its photosynthetic capability. The onset of the foraging behavior in *E. clarki* in response to prolonged starvation was delayed during the time period that the slugs' photosynthetic capabilities remain relatively high. Some slugs foraged although satiated, under conditions of a continuous food supply, so foraging is not eliminated for all members of the population. However, the incidence of foraging behavior increased as a decrease in photosynthetic ability occurred. In effect, the presence of kleptoplasts and their photosynthetic ability allow *E. clarki* to behave, even after a month of starvation, in a similar way to slugs that have been satiated. This is the first report to demonstrate the role of photosynthesis in modifying the foraging behavior in a starved mixotrophic animal.

Although reducing movement to conserve energy in times of famine or starvation might seem like a viable strategy, many species actually increase foraging effort under starvation conditions (Torres et al. 2002, Jeschke and Tollrian 2005). Nutritional state has long been implicated in behavioral changes of starved animals traveling more often and farther distances in search of food than satiated animals (Torres et al. 2002). For example, starved nymphs of *Podisus nigrispinus* are more likely to disperse and move further from a starting location than satiated nymphs (Torres et al. 2002). Other species such as the sea star, *Leptasterias polaris*, are more likely to move when starved, and are also more likely to orient towards a water current, increasing the likelihood of

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encountering prey, than satiated conspecifics (Rochette et al. 1994). Behavioral changes, such as consuming chemically-defended food that typically would have been avoided, have also been documented in starved herbivorous urchins (Hart and Chia 1990, Cronin and Hay 1996). If our laboratory study predicts field behavior, then a large percentage of the population of *E. clarki* likely exhibits a delay of weeks after food sources become scarce before at least some animals display a behavioral change and commence foraging. Moreover, by not feeding slugs can remain located in a suitable habitat, in regards to conditions such a light availability and wave energy. Reduced feeding activity by slugs which photosynthesize may also allow the algal food resources sufficient relief from grazing pressure to regrow.

One other consequence of starvation is that it may induce behavioral changes in an animal that increase the risk of predation. For example, the whelk, *Acanthina monodon,* normally reduces feeding in the presence of a predator, but when starved, will continue to feed despite the proximity of the predator (Soto et al. 2005). The hermit crab, *Dardanus pedunculatus,* hosts symbiotic sea anemones on its shell which protects the crab from predation. However, when starved, the crab will eat its anemones, decreasing its camouflage and correspondingly increasing the risk of predation (Imafuku et al. 2000). Although *E. clarki* is a relatively cryptic species, delayed foraging may still provide benefit from reduced exposure to predation as foraging behavior becomes less likely. While not specifically examined in *E. clarki*, predation has been demonstrated on several other cryptic sacoglossan species (Trowbridge 1994). However, the advantage of avoiding predation in relation to foraging may be difficult to demonstrate in sacoglossans

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because the sequestered chloroplasts that provide metabolites also modify the slugs' color and serve as a camouflage (Williams and Walker 1999).

Although the ability to photosynthesize delays foraging changes in slugs of *E. clarki* starved 8 to 12 weeks, it is not clear if similar patterns occur in other photosynthetic animals. Studies on corals have had mixed results. Some species show an increase in feeding when light conditions, and thus photosynthesis, are reduced (Titlyanov et al. 2000), while others show no indication that the rate of feeding changes in relation to the condition of their symbiotic zooxanthellae (Piniak 2002). However, corals are non-motile and thus do not engage in foraging behavior, as defined above, making them poor candidates for demonstrating the effects of starvation on the foraging of photosynthetic animals. It is more likely that other motile, photosynthetic slug species will behave similarly to *E. clarki*. The timing of a behavioral change will likely depend on the duration of photosynthesis in a given species. Species with short-lived photosynthesis, such as *Elysia ornata* (Händeler et al. 2009), may have a rapid response to starvation with little delayed feeding while others like *E. chlorotica* (Pierce et al. 1996), which never lose photosynthetic ability once symbiotic plastids are acquired, will likely exhibit little to no difference in feeding behavior between starved and fed individuals. Therefore, the ability to sustain photosynthesis, which may be related to horizontally transferred-algal genes (Pierce et al. 2008, Pierce et al. 2009), is likely to determine the effect of starvation on foraging behavior.

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Conclusion:

The onset of a behavioral shift coinciding with a decline in photosynthesis by *E. clarki* demonstrates an aspect of foraging behavior and starvation not previously considered. *Elysia clarki* starved for 8 or 12 weeks and displaying a reduced photosynthetic ability increased the incidence of foraging behavior compared to continuously fed controls and slugs starved for 4 weeks. Our work uniquely demonstrates that foraging behavior in starved photosynthetic animals is likely to remain unchanged while photosynthesis remains functional. However, our experiments revealed that a change in foraging behavior is more likely to occur when photosynthetic activity declines under conditions of starvation.

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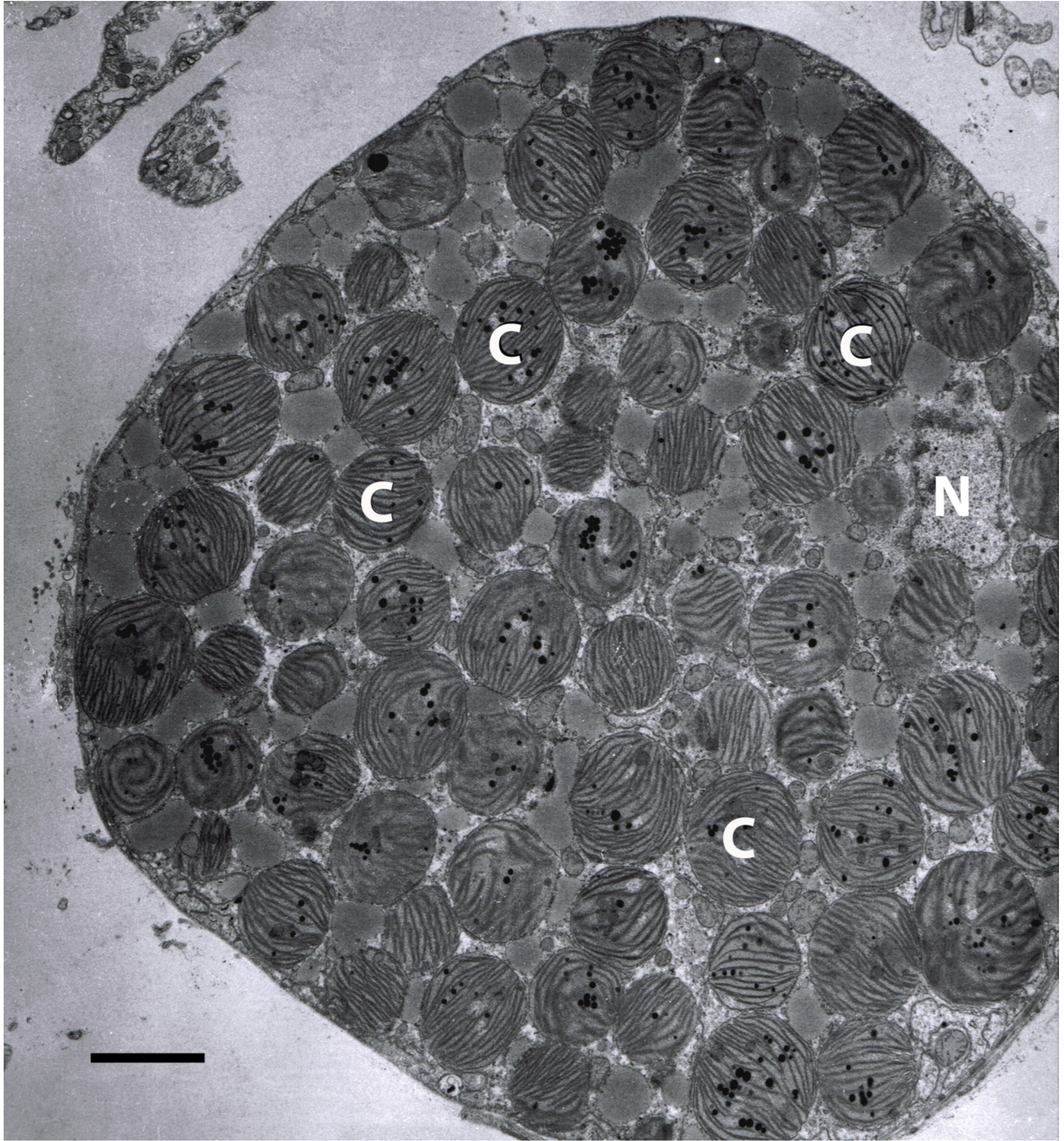

Figure 1.1: Electron micrograph of a digestive tubule cell of *E. clarki.* The digestive tubule cell is densely packed with sequestered chloroplasts. C= chloroplast, N= nucleus. Scale bar represents 3µm. Image taken by Nicholas Curtis.

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Figure 1.2: *Elysia clarki* on the algal food source, *Penicillus capitatus.* Photo reprinted with permission from Curtis et al. (2006) Scale bar represents 500mm.

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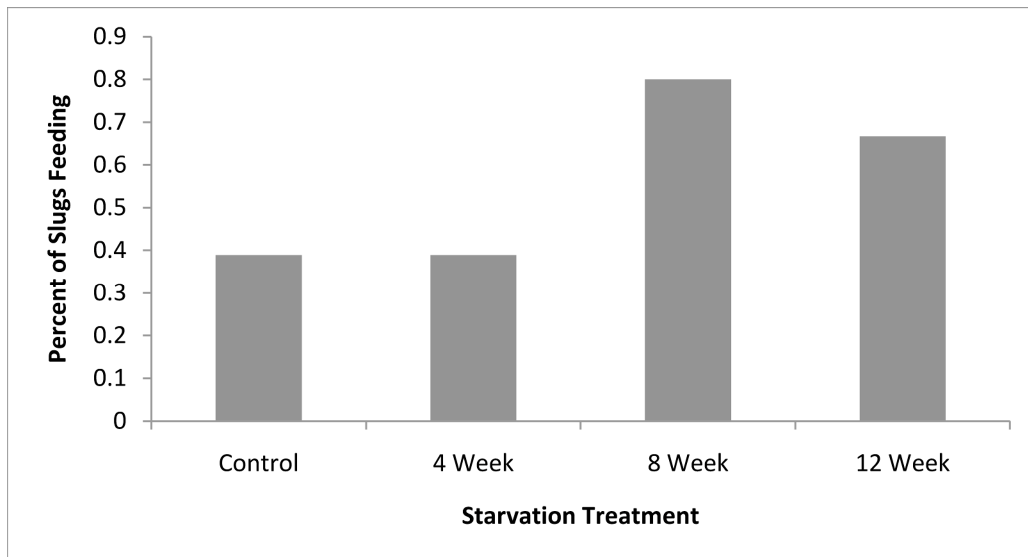

Figure 1.3: Percentage of slugs displaying feeding behavior after each starvation period. Data represent all slugs tested for each starvation treatment group.

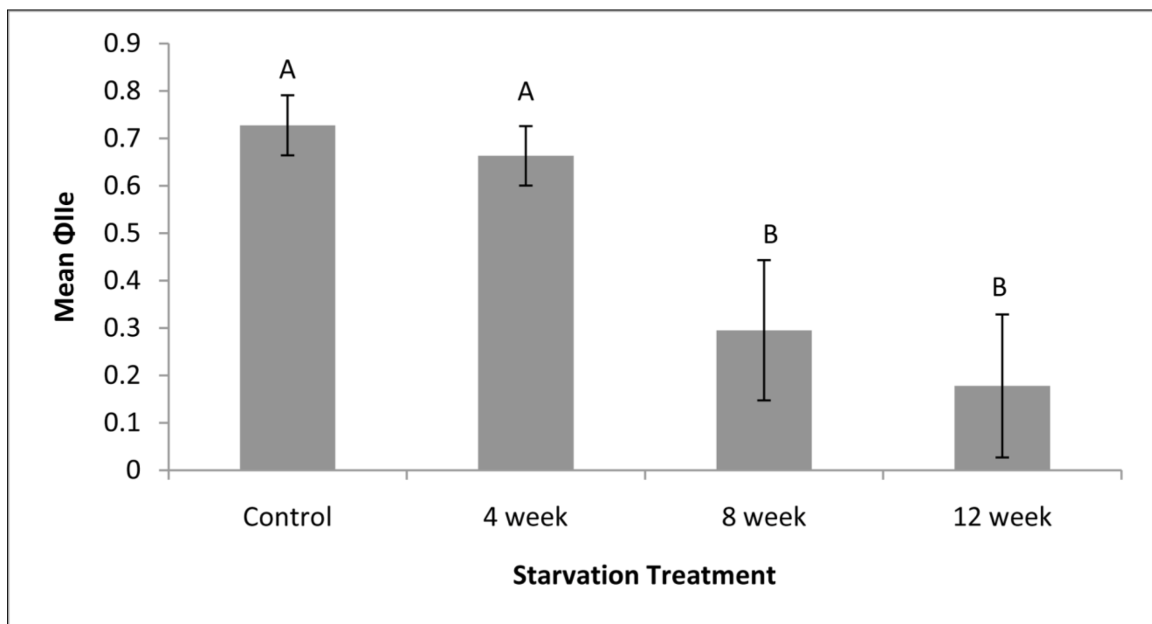

Figure 1.4: Mean maximum quantum yield (+/- standard deviation) for slugs from each starvation treatment. Letters indicate significant differences among treatments.

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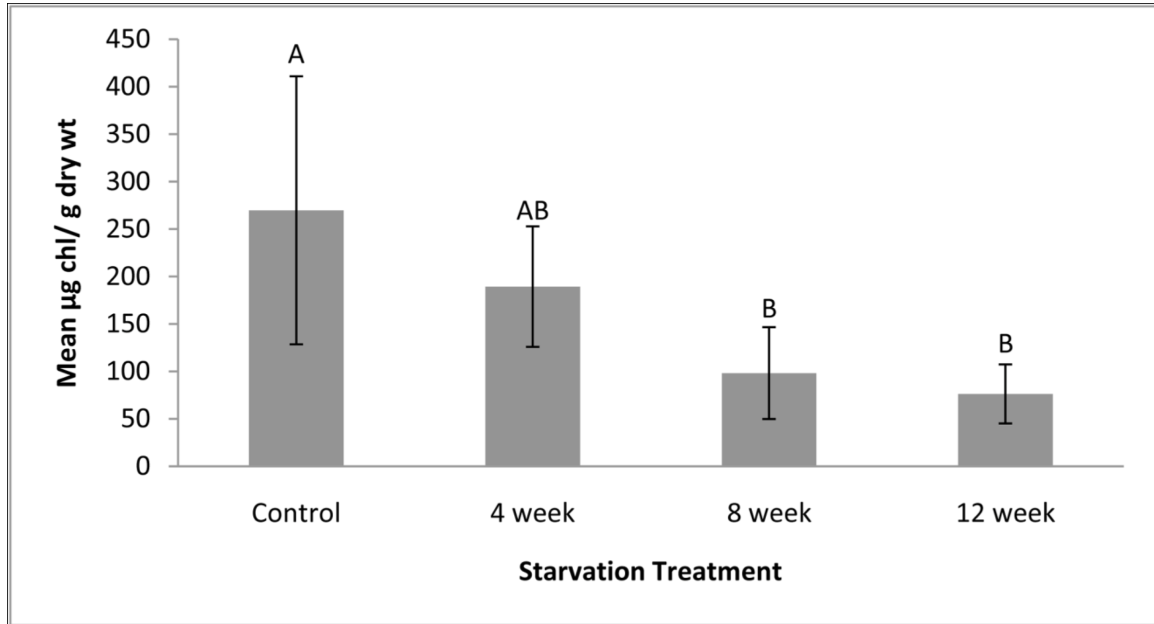

Figure 1.5. Mean [Chl a] (+/- standard deviation) for slugs for each starvation treatment. Letters indicate significant differences among treatments. Presented data were back-transformed after a logarithmic transformation to meet assumption of homoscedasticity.

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CHAPTER 2:

SPATIAL AND FEEDING RELATIONSHIPS BETWEEN A PHOTOSYNTHETIC MARINE HERBIVORE AND ITS ALGAL HOSTS

Introduction:

Herbivores are a taxonomically diverse group of animals, varying greatly in size and occurring in a wide range of terrestrial and aquatic habitats. These plant consumers range from large generalist feeders, such as elephants, which graze over large expanses, to tiny obligate specialists, such as aphids, which live and feed directly on host plants. Typically, specialist herbivores have a stronger spatial relationship with their plant food sources and are more likely to occur in high densities in areas with plentiful food sources compared to that reported for generalists (e.g., Tahvanai and Root 1972, Root 1973, Duffy and Hay 1994).

In marine systems, specialist herbivores are thought to be relatively rare, in contrast to the many specialists, predominantly insects, found in terrestrial systems (Hay et al. 1990). In fact, a subset of marine herbivores might more accurately be classified as omnivores (Miller and Hay 1998, Stachowicz and Hay 1999). The reasons for the paucity of herbivore specialists in marine settings are not clear, but may be related, in part, to a variety of ecological and evolutionary constraints which favor plastic life history traits (Warner 1997, Sotka 2005). Both pelagic dispersal displayed by many marine invertebrates and the ephemeral nature of numerous marine plants may present a considerable challenge to the survival of a marine herbivore specialist. Other factors

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contributing to the lack of specialist marine herbivores may be related to benefits obtained from feeding on multiple plant species such that exposure to high concentrations of plant- produced toxins is reduced (="non-additive toxin hypothesis") (Freeland and Janzen 1974) or growth rate of a herbivore is increased by eating a diverse diet (= "complementary resources hypothesis") (Pennings et al. 1993). Several marine herbivore specialists have nonetheless been identified, with many exhibiting a spatial distribution that is strongly associated with that of their food sources (e.g., Choat and Black 1979, Pennings and Paul 1993, Trowbridge and Todd 2001, Sotka 2007).

Sacoglossan (Opisthobranch: Mollusca) sea slugs display many features typically associated with a feeding specialist. They feed suctorially on siphonaceous, coenocytic algae by puncturing cell walls with a highly modified radular tooth and then extract the contents (Jensen 1993, 1994, 1997). Most species are oligophagous (eat a few algal species) if not monophagous (eat only one algal species) (Jensen 1980, Händeler and Wägele 2007). Often, in field settings, sacoglossans have been observed atop of their host algae. Sacoglossans often are tolerant of, or immune to, defensive secondary metabolites produced by their host algae and, in some cases, slug species sequester these secondary compounds (Paul and Van Alstyne 1988, Becerro et al. 2001, Marin and Ros 2004, Baumgartner et al. 2009). However, the small size and cryptic coloration of sea slugs has limited direct *in situ* observation of both feeding and spatial distribution and therefore even basic information of slug and host algal co-occurrence is generally lacking.

A striking feature of many sacoglossan herbivores is their ability to sequester chloroplasts from ingested algae, storing them inside of specialized cells of the digestive

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tubules. Slugs can maintain these chloroplasts and utilize them for photosynthesis in a process termed “kleptoplasty” or chloroplast symbiosis (Trench et al. 1969, Clark et al. 1990, Pierce and Curtis 2012). Although the chloroplasts will be replaced when new food is consumed, many species can maintain sequestered chloroplasts and continue photosynthesizing for up to many months (Gallop et al. 1980, Clark et al. 1990, Evertsen and Johnsen 2009, Pierce and Curtis 2012).

The implications of kleptoplasty for slug physiology are beginning to be considered in discussions of feeding behavior (Middlebrooks et al. 2011). Exposure to light will eventually degrade sequestered chloroplasts and photopigments (Pierce and Curtis 2012) and sacoglossans display a wide variety of mechanisms to reduce chloroplast degradation.. Some sacoglossans have morphological and behavioral shading of photosynthetic cells for combating photo-degradation (Jesus et al. 2010, Schmitt and Wägele 2011). Other species have biochemical adaptations, such as chlorophyll synthesis as well as synthesis of thylakoid proteins mediated by horizontally transferred genes from the algal genome (Pierce et al. 2009, Schwartz et al. 2010, Pierce et al. 2012). Although these photosynthetic sacoglossans are ultimately dependent upon their host alga as a source of chloroplasts, the presence of the plastid reduces dependency on the algae for energetic needs. This was demonstrated in a recent study that found that when this alternative energy source, provided by chloroplasts, was available to a slug via kleptoplasty, a corresponding reduction, but not elimination, in feeding behavior occurs (Middlebrooks et al. 2011). Importantly, because of this photosynthetic capability, kleptoplastic sea slugs might not be expected to maintain a strong pattern of spatial

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concurrence with their algal hosts unlike what has been commonly predicted for most specialist herbivores (Tahvanai and Root 1972, Root 1973).

So, an intriguing question is whether slugs that display kleptoplasty occur on or in close proximity to their food. Investigating the spatial association between photosynthetic slugs and their food resources however requires, at the very least, that information on the field distribution of both slugs and their food resources. However, information on food items ingested by slugs in field settings is generally lacking. Although the extent to which a photosynthetic sacoglossan is found on, or near, a host alga can be documented via observation *in situ* surveys of distributional patterns, such surveys cannot confirm consumption of an alga. Other studies have discussed how animals may co-occur with plants or algae for other reasons such as camouflage, chemical defense, or physical protection from abiotic factors (Hay et al. 1990, Duffy and Hay 1994, Sotka et al. 1999, Sotka 2007). In fact the algal food source of some sacoglossans has been misidentified when only co-occurrence of slug and algae has been used as a basis for inferring diet (Händeler et al. 2010). Given the unique characteristics of kleptoplastic slugs (e.g. photosynthesis, suctorial feeding, and specialization), assembling the most complete or accurate description of slug-algal feeding relationships would require documenting the availability of resources, as well as validating resource use in natural settings.

Unfortunately techniques typically available investigate animal feeding/diet composition have limited applicability for the study of sacoglossan slugs. But, molecular techniques (DNA bar-coding), have recently been employed to identify accurately the species composition of diets of target organisms and thus offer an alternative approach when other methods of dietary analysis prove logistically unfeasible (Symondson 2002,

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Bourlat et al. 2008, King et al. 2008, Valentini et al. 2009, Bucklin et al. 2011). These techniques have been used successfully to evaluate the diets of "difficult to observe" species such sea lions (Tollit et al. 2009), bats (Bohmann et al. 2011, Zeale et al. 2011), and deep sea invertebrates (Blankenship and Yayanos 2005). Although previous studies have mostly focused on carnivores, molecular dietary analysis has also been successfully implemented in a limited number of studies on plant-herbivore relationships (Miller et al. 2006, Jurado-Rivera et al. 2009, Raye et al. 2011) providing information on the presence/absence of ingested prey items. However, gene sequences for potential prey species must be available, if DNA bar-coding is to be employed to assess rapidly diet composition. Conceivably, incorporation of field data on relative abundance of food items with results from DNA bar-coding of ingested food could provide unique ecological information about actual feeding behavior.

Here we investigate feeding ecology of a sacoglossan herbivore, *Elysia clarki*, with photosynthetic capability and document herbivore distribution relative to that of host plants. Then, using results from detailed field surveys in combination with molecular analyses we tested: 1) Does the photosynthetic specialist herbivore *E. clarki* display a strong spatial association with its algal food; 2) Does the diet of *E. clarki* correspond to patterns of algal food occurrence; and 3) Does *E. clarki* display selective feeding on any algal taxa?

Methods:

Study Organism: *Elysia clarki* is a kleptoplastic sea slug that can photosynthesize for 3-4months (Pierce et al. 2006). *Elysia clarki* is endemic to the Florida Keys in low wave energy, near-shore habitats such as mangrove swamps,

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mooring canals, and borrow pits. *Elysia clarki* is oligophagus, feeding on several closely related species of algae and can sequester chloroplasts from several macroalgal species within a single cell (Curtis et al. 2006, Curtis et al. 2007). While such a diet might be considered relatively wide for a sacoglossan, the diet is quite narrow compared to that characteristic of most marine herbivores (Hay et al. 1990, Curtis et al. 2006, Curtis et al. 2007). *Elysia clarki* is relatively large (up to ~3cm) and can occur in high densities, making visual field surveys feasible. Importantly, *E. clarki* shows a reduced feeding response during the first month of starvation when photosynthetic rates remain high compared to slugs that have not been fed for 8-12 weeks and photosynthetic rate is much reduced (Middlebrooks et al. 2011). Thus *E. clarki* may not be constrained to remain near its host alga especially during the first month after a feeding event. Combined, these characteristics make *E. clarki* an excellent organism for examining the distribution and diet of a specialist herbivore.

Site Descriptions: Field investigations of herbivory by *E. clarki* were conducted at 4 sites along the Florida Keys, USA (Figure 2.1). These sites were generally <2.0m in depth with good visibility and supported populations of *E. clarki* and diverse assemblages of macroalgae. But, each site had some distinguishing features which a briefly presented below.

The Pit site is an old limestone quarry borrow pit, and is well protected from wave action by a mangrove (*Rhizophora mangle*) fringe with near vertical walls. The walls are inhabited by a fouling community of invertebrates including anemones, tube worms, mussels, oysters, and sponges. The macroalgae eaten by *E. clarki* are typically low in abundance or absent on the walls of the Pit, but other algal species, *Caulerpa spp.*in

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particular, are present. This site served as the source population for earlier studies on *E. clarki* (Clark 1994, Curtis et al. 2006, Pierce et al. 2006).

The Swamp site is a shallow mangrove swamp immediately seaward (North) of the Pit surrounded by a mangrove (*R. mangle*) fringe. The bottom is primarily hard limestone, but also contains patches of softer sediment dominated by seagrass **(***Halodule wrightii*). Most of the hard substrate is covered by a multi-species microalgal mat consisting primarily of diatoms, but macroalgae (primarily *Penicillus spp.* and *Halimeda spp.*) are also present in the area. The mangrove jellyfish, *Cassiopeia xamachana* and *C. andromeda,* are found in large numbers on the substrate.

The Mote site is a mooring canal located on the ocean (South) side of Summerland Key at the Mote Tropical Research Laboratory. Despite moderate boat traffic, the walls are fairly well protected from wave energy. The canal has vertical limestone walls which support a fouling community of invertebrates, mostly anemones, tunicates, sponges, and tube worms as well as a high abundance of *Caulerpa spp.* and *Halimeda spp*.

The Salt Pond site on the ocean side of Key West is heavily fringed with mangroves (*R. mangle*) and access to the sea is limited by a narrow canal entrance and shallow depth. Unlike the Swamp site, the bottom here is primarily soft sediment and mostly without any visible algal matt. Seagrass (*H. wrightii*), mangrove jellyfish (*C. xamachana* and *C. andromeda),* and macroalgae (primarily *Penicillus spp.* and *Halimeda spp.*) are also present in the Salt Pond.

Distribution Surveys: At each site. 4, non-overlapping, belt transects were established within areas containing *E. clarki*. Because the slugs can be found on steep or

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vertically-oriented substrate, transects were oriented with the terrain. Each transect (20m x 1.5m) was divided into 20 (1m x 1.5m) quadrats. Each quadrat was visually surveyed on snorkel and the number of slugs in each quadrat was recorded along with the substrate on which each individual slug was found (e.g., bare substrate, *Caulerpa* etc.). Additionally, presence/absence of all macroalgae observed in the quadrats was recorded to the lowest taxonomic level possible. All sites were sampled in either July or August 2008 and again in July and August of 2009. One additional survey was conducted at the Pit in July 2010.

Slug density was calculated for each site, along with the percentage of quadrats containing each algal species and the percentage of total slugs found on each substrate type. Algal frequency, measured as number of quadrats containing each species per transect, was analyzed using PRIMER 6 statistical software (Primer E ltd, Lutton UK). Data were log transformed and then used to create a Bray-Curtis similarity matrix. The similarity matrix was used in an analysis of similarity (ANOSIM) using site and year of survey as treatment factors. A similarity percentage (SIMPER) analysis, based upon the similarity matrix, was then utilized to examine the contribution of each algal species to the algal community at each site.

Molecular Study: In kleptoplastic sea slugs, the diet can be determined by examining the species composition of sequestered chloroplasts, which can be identified via molecular bar-coding of chloroplast-encoded genes such as ribulose bisphosphate carboxylase/oxygenase (*rbcL*) (Curtis et al. 2006, Curtis et al. 2007) or the *tufA* gene (Händeler et al. 2010). We used the *rbcL* gene here, which can be used to identify even closely related algal species, because it has already been successfully utilized in previous

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work on this species (Curtis et al. 2006, Curtis et al. 2007), and because of the diversity of *rbcL* sequences available for comparison in GENBANK. Also, because *E. clarki* can retain intact chloroplasts within its cells for several months, the molecular identity of the chloroplast genes can be used to verify the species of algae consumed by slugs over a similar time period.

Slugs were collected for molecular analysis on the same dates as the transects were surveyed in 2008. Six slugs were haphazardly collected from each site for molecular analysis, preserved immediately in 95% ethanol, and returned to the laboratory. DNA was extracted from slugs using a Phytopure DNA extraction kit (Gen Probe, San Diego CA USA) (Pierce et al. 2009). The *rbc*L gene was amplified by polymerase chain reaction (PCR) from the slug DNA using degenerate primers designed by hand from conserved regions determined by aligning 31 rbcL sequences from species of the Chlorophyta found in GENBANK (Curtis et al. 2008) (forward primer sequence: 5' AAAGCNGGKGTWAAAGAYTA3' ; reverse primer sequence: 5'CCAACGCATARADGGTTGWGA3'). Reaction mixtures were made with IDPROOF reaction buffer (IDLabs, London, ON, Canada) [2 mM Tris–HCl (pH 8.8) 1 mM KCl, 1 mM $(NH_4)_2 SO_4$, 0.01% Triton-X 100, and 0.01 mg mL^{-1} BSA], 125 μM dNTP, 0.25 mM rbcL 1, 0.25 mM rbcL 2, 1 ng μL^{-1} DNA, and 0.1 U μL^{-1} DNA polymerase (IDPROOF; IDLabs). PCR touchdown reactions were performed using a Gene Amp PCR 2400 thermocycler (Applied Biosystems, Foster City, CA, USA) with a denaturing temperature of 94°C for 30 s, annealing temperature starting at 45°C for 30 s, and an extension temperature of 72°C for 1 min for 35 cycles, with the annealing temperature lowered by 0.5^{o}C on each subsequent cycle (Curtis et al. 2006, 2008).

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The PCR products were purified with a Qiaquick gel extraction kit (Quiagen Valencia, CA USA) and cloned using a Topo TA cloning kit (Invitrogen, Grand Island NY USA). Ten clones per animal were then screened with PCR for inserts close in size to the PCR product, miniprepped using the Nucleospin Plasmid kit (Clontech, Mountain View, CA, USA), and the resulting purified PCR product DNA was sent to MWG (Huntsville, AL USA) for sequencing (Curits et al. 2006, 2008).

To determine the sequence identity of sequestered chloroplasts, all slug *rbc*L sequences identified were aligned with all available *rbcL* sequences from representatives of the Bryopsidales, Dasycladales, and Cladophorales using Clustal X (Larkin et al. 2007). For all obtained sequences, the 5' and 3' primer sequences were discarded, and sequences obtained from GENBANK were edited to correspond to the remaining region, with all noncoding regions removed. Initially, a comprehensive Maximum Likelihood phylogeny was constructed from the resulting alignment using Mega 5.05 (Tamura et al. 2011) with the following parameters: GTR + G + I model, using 5 discrete gamma categories, all sites included in analysis, with 100 bootstrap replicates. *Chaetomorpha linum* (Cladophorales) was designated as the outgroup. Because previous analysis has shown a strong phylogenetic signal at the third codon position in *rbc*L (Curtis et al. 2008), all codon positions were included in the analysis. Slug sequences which showed moderate to strong nodal support (Bootstrap value > 50%) with specific algal species were then individually aligned with that particular algal species sequence by Clustal X to confirm species identity. Sequences were considered to match a species if they diverged by 0.50 % or less from the native algal sequence. Raw sequences were also examined to determine if any sequence differences could be the result of sequencing error. A final

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cladogram was constructed by trimming nonessential branches, and combining clades with identical sequences [Appendix A].

Field and molecular comparison: In order to determine whether the algae consumed by *E. clarki* reflect the availability of algal food in the field at a given site, molecular and field data for 2008 were compared within each of the sites using the Sørensen similarity index (QS) (Sørensen 1948) and the following equation:

$$QS = \frac{2C}{A + B}$$

where A is the number of algal species found throughout all transects at a given site, B is the number of algal species identified by PCR for slugs from the same site, and C is the number of algal species that co-occur in both the field survey and the molecular results. The Sørensen similarity index ranges from 0-1 with 1 representing identical similarity and 0 representing complete dissimilarity. For calculating A and C above, only algal species that are known food sources for *E. clarki* based upon results from this or other studies (e.g. Curtis et al. 2006) were used. Therefore, some algae (e.g. *Caulerpa spp.*) present in the survey were omitted from this calculation because *E. clarki* does not feed on them. A high similarity score at any site indicates that the diet of *E. clarki* largely reflects the species composition of possible food items in the field for a given area. Thus calculations of QS should indicate whether, even with a narrow range of possible foods, *E. clarki*'s diet matched apparent food sources.

Selectivity: In order to examine further the feeding selectivity of *E. clarki*, the frequency of algae in the field transects was compared to the frequency of algal molecular sequences obtained for each algal species at all 4 sites using 2008 data and utilizing Pearre's selectivity index (C) (Pearre 1982):

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$$C = \pm \left[\frac{\left(| a_d b_e - b_d a_e | - \frac{n}{2} \right)^2}{(a_d + a_e)(b_d + b_e)(a_d + d_d)(a_e + b_e)} \right]^{\frac{1}{2}}$$

where a_d is the sum of *rbcL* sequences that match a given algal species (1-60 in this study), b_d represents all other *rbcL* sequences, a_e is the number of quadrats per site (0-80 in this study) containing the same given alga, b_e is the sum of all other algal frequencies, and n is the sum of a_d, b_e, b_d, and a_e. The C index ranges from -1 to +1 with 0 signifying no selection by the slug, 1 signifying highly positive selection, and -1 signifying highly negative selection. The index was tested for significance using a X^2 contingency table; indices with $p<0.05$ were considered significant. As with the S□rensen index, only algae that are known food sources were included in the analysis. Again

Results:

Field Surveys: The density of *E. clarki* ranged from a low of 0.33 x m^{-2} in the Swamp in 2008 to a high of 2.92 x m^{-2} in the Pit in 2009 (Table 2.1). Densities were the highest during the three surveys when the lowest amounts of edible algae were also recorded in the Pit during 2008 and 2009 and in Mote during 2008 (Table 2.1). In these two sites the most frequently encountered edible alga was *Acetabularia sp.*, an alga which appears, however, to be a low preference food source based on the low frequency with which it was consumed (see below). However, at all sites slugs were most commonly found on bare substrate, except in the Salt Pond where slugs were most often positioned on the cap of the alga, *Penicillus capitatus,* (Figure 2.2). The algae, *Caulerpa spp.*, which was also a commonly encountered taxa, was the 2nd most common substrate occupied by slugs in both the Pit and Mote sites.

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The species composition and frequencies of algal occurrence varied significantly among sites (Table 2.2, Figure 2.3, ANOSIM p=0.001, global R-0.968). Algal assemblages from the Pit site were grouped together in 2008 and 2009, indicating strong a similarity between years, but this was not true for algal assemblages in 2010 when several new algal species were found in field surveys at the site. An unusually cold winter in 2009 coincided with decreases in *E. clarki* density in 2010 at the Pit (Table 2.1). SIMPER analysis (Table 2.3) indicates that the species identified as the largest contributor to community composition across all years varied by site with *Caulerpa mexicana* the most important at the Pit, *C. verticillata* at Mote, and *P. capitatus* at the Swamp and Salt Pond. Among these species, only *P. capitatus* is consumed by *E. clarki* (Curtis et al. 2006).

Sequence Analysis: The *rbc*L sequences from all *E. clarki* samples were 562 bp in length. The total sequence length of aligned characters (slug and algal) was 520 bp. Sixty *rbc*L sequences were obtained from slugs at each site. A total of 17 unique *rbc*L sequences were obtained from *E. clarki* in this study, with 9 sequences representing previously unreported algal food sources (Figure 2.4). Interestingly, the diet of *E. clarki* varied among the sites examined in our study. In 2008, the *rbcL* results indicated a wide variation in the algae consumed among slugs (Table 2.4 and Figure 2.4). Specifically, in the Pit, *E. clarki* was eating four different algal species: *H. monile, H. incrassata, P. capitatus,* and *P. lamourouxii,* with *H. monile* being the most commonly recorded *rbcL* sequence. Slugs from the Swamp had the same *rbcL* sequences as slugs from the Pit. But, in the swamp *P. capitatus* was the most commonly encountered *rbcL* sequence. At the Salt Pond site all *rbcL* sequences matched *P. capitatus*. Molecular analysis revealed

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that slugs from the Mote site displayed the most diverse diet (15 species) and *H. incrassata* being the most common sequence recorded.

While the molecular analyses revealed unique information on algal consumption by *E. clarki*, a complete profile was not obtained. Some of the *rbcL* sequences from slugs collected at the Mote site, including three *Halimeda* and four *Bryopsis* species, could only be identified to genus level, based upon available *rbcL* sequences in the database. Sequences for several algal species not found before as a food of *E. clarki* including *P. pyriformus*, *Acetabularia sp.,* and a sequence closest matched to *Pseduochlorodesmis sp.,* a genus which has never been reported in Florida were also recorded at the Mote site. However, the latter sequence diverged from *Pseduochlorodesmis sp.* by over 10% so it may represent a species not yet in the sequence database. Notably the detection of *Acetabularia sp.* provided the first report of an alga of the Dasycladales clade in the diet of *E. clarki*.

Field and Molecular Comparison: Relative abundance of algal food sources, as determined from all field surveys combined at a site, was not a good indicator of food choice by *E. clarki* at most survey sites. Sørenson indices, comparing similarity of the algae identified in field surveys to algae consumed by slugs as identified by the molecular analyses of the *rbcL* gene, were generally low at all sites ranging from 0.125 at Mote to 0.89 at the Swamp (Table 2.5). The exception was the swamp where a high value was calcualated. The majority of low Søresnson indices suggest that field surveys of the presence/relative abundance of local algae are not aligned with patterns of algal species utilized by as food *E. clarki*. However, it is possible that some smaller filamentous species, such as *Bryopsis spp.,* could have been missed in visual surveys and would

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influence Sørenson values. When Pearre's C index was compared across all sites *Elysia clarki* was found to display positive selectivity for algal species at all sites except the Swamp. Those algal species selected by slugs were not consistent across sites (Table 2.6). In the Pit, *E. clarki* showed significant positive selectivity for *H. monile* (Pearre's C=+0.72) and *H. incrassata* (Pearre's C=+0.27). Slugs collected from Mote also displayed positive selectivity for *H. incrassata* (Pearre's C=+0.26), while those collected from the Salt Pond displayed positive selectivity for *P. capitatus* (Pearre's C=+0.37). Thus, *E. clarki* demonstrates selective feeding, although the slugs are not often found on or near algal food, and the alga selected is inconsistent between locations.

Discussion:

Despite the oligotrophic diet displayed by *E. clarki* at most study sites, the slug did not have a close spatial relationship, as evidenced by Søresnson index scores and the location of slugs (Figure 2.2), with its algal food sources in contrast to typical patterns exhibited by specialist herbivores (Tahvanai and Root 1972, Root 1973). In fact, at 3 of 4 sites, *E. clarki* was most commonly found on either bare substrate (over 90% of slugs at the Pit) or fronds of *Caulerpa spp.* which, *E. clarki* does not eat (Curtis et al. 2006, Curtis et al. 2007). In general, a considerable mismatch was detected between those food items which were available in the field and those ingested. At sites supporting the highest densities of *E. clarki,* algae that are food sources were present in low abundance and, in most cases, slugs only fed on a small subset of potential food items. At the Pit in 2008 and 2009, and at Mote in 2008, the majority of the available edible algae was *Acetabularia sp.* While this taxa is occasionally consumed by *E. clarki,* curiously this abundant alga was only detected as a food item in 2 of the 240 available sequence results.

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Given that *E. clarki* can maintain high levels of photosynthetic activity for at least one month without feeding (Middlebrooks et al. 2011), and also continue to photosynthesize for 3-4 months (Pierce et al. 2006) a lack of close correspondence between distributional patterns of the slug and food items may only be a very short lived association.

One of the most surprising results from the examination of slug diets was that, in 3 of 4 sites, common food items were missing from field surveys. Our finding that the two sites with the highest densities of *E. clarki* (The Pit and Mote) also supported the lowest abundance of edible algal species, may suggest that slugs are exerting top down grazing effects on the algae, leading to the absence of edible algal species. Likewise the coordinated appearance of typically rare taxa *H. incrassata* at Mote an*d H. incrassata, P. capitatus, P. lamourouxii,* and *Bryopsis sp.* at the Pit when slug populations declined may also be explained by high densities of *E. clarki* controlling the algal community via grazing. While such suggestions require more detailed study, information does exist to support the idea that slugs can be effective grazers. For example, several species of sacoglossans can reduce local algal populations (Trowbridge 1992, 2002) and grazing by *Elysia viridis* has even been suggested as a biological control agent for invasive algal species (Trowbridge 2004). Alternatively, it is also possible that some *E. clarki* migrated to study site had after feeding in another area which supported a different algal community. Most herbivores, faced with a depleted food supply, would either switch food sources (for generalist herbivores) (Martins et al. 2002), move to a new location that contained the target algae (Schops 2002) (for specialists), or starve. However, even if *E. clarki* effectively grazed down algal food populations, the slugs might still be able to maintain high densities because of their photosynthetic capability. Importantly under this

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scenario, "relaxed" grazing by slugs may allow algae to recover in an area or for the slugs to relocate to more luxurious algal fields because of their ability to photosynthesize.

While the focus of this study emphasized feeding relationships between slugs and algae, spatial associations between slugs and algae may also be molded by non-trophic interactions. Earlier studies on slugs from the Pit reported that *E. crispata* [slugs matching this description were changed to *E. clarki* (Pierce et al. 2006)] fed on *Caulerpa sp.* (Clark 1994), but this was later demonstrated to be incorrect (Pierce et al. 2006, Curtis et al. 2007). Enigmatically, *E. clarki* is often found on, or near, *Caulerpa spp.* (Table 2.1, Table 2.2). This association of slug and algae may be coincidental or it may reflect the use of *Caulerpa spp.* by *E. clarki* as a source of camouflage or other modes of protection from predation. Indeed, it is not unusual for animals to make use of algae for non-feeding purposes (Sotka et al. 1999) and some documented cases of predation on sacoglossans may support the need for refuge (Trowbridge 1994, Rogers et al. 2000). Yet the need for refuge is questionable, as sacoglossans often have fairly effective chemical defenses (Hay et al. 1990, Becerro et al. 2001, Marin and Ros 2004, Baumgartner et al. 2009) and some predators learn to avoid slugs (Gimenez-Casalduero et al. 2002). Another possibility for *E. clarki*'s apparent non-feeding association with *Caulerpa spp.* is that all of the other algae in the area may have been consumed, leaving *Caulerpa spp.* as the most abundant algae remaining. It is even possible that feeding on other algae by *E. clarki* benefits *Caulerpa spp.* by reducing competition between *Caulerpa spp.* and other macroalgae taxa.

Because many of the seemingly apparent aspects of herbivore feeding can be misleading (Moore 1977, Nicotri 1980), determining resource use by herbivores remains

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challenging. The low Sørenson indices for most sites clearly demonstrate that algal availability cannot be used to predict accurately the diet of *E. clarki*. At only one site, the Swamp, was a high degree of similarity between the algae present in the habitat and algae consumed recorded. These results warn of potential errors with inferring the diets of sacoglossans and other herbivores based on proximity to nearby plants/algae (Händeler et al. 2010).

The use of DNA bar-coding for dietary analysis not only revealed new insights into slug feeding behavior, but also provides strong support for using this approach in conjunction with field observations. Although all algal identities were not possible to the level of species, as more species sequences are added to databases, this tool should become even more powerful. DNA barcoding can be particularly useful for investigating diets of species where no prior feeding data are available. For example, Händeler et al. (2010) used similar techniques with the *tufA* gene to decipher the diet of 7 sacoglossan species, three of which had no dietary information available. In our study, although *E. clarki*'s diet from one locale had already been examined by DNA bar-coding (Curtis et al. 2006, Curtis et al. 2007), the use of *rbcL* sequences to explore feeding in multiple sites confirmed the presence of additional algal taxa in *E. clarki*'s diet and provided a broad perspective of highly variable but specialized feeding by the slug when viewed across all sites. However, just as field surveys alone may provide an inaccurate impression of herbivore feeding, DNA barcoding, like gut content analysis, may, in isolation, also offer an incomplete record of herbivore feeding because it lacks the context of which food sources are chosen.

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Elysia clarki displayed differences in feeding selection among sites. In the Swamp slugs were in addition to ingesting *P. capitatus*, a common alga in the area, slugs also utilized other algal food sources. Thus, no significant positive selection was demonstrated by *E. clarki* for *P. capitatus* at the Swamp. On the other hand, at the Salt Pond, a high abundance of *P. capitatus* was also recorded, but this was the only alga consumed by slugs at this site, thereby demonstrating a significant positive selection for the alga. It is not clear why slugs fed only on *P. capitatus* in the Salt Pond, but it may be the result of a general feeding preference for *P. capitatus* when available, or alternatively could be an example of local adaption (Sotka 2005). For example an earlier investigation showed that, *Placida dendritica*, collected from different host plants, displayed different feeding preferences based on the original host from which slugs were collected (Trowbridge 1991). It is possible that *E. clarki* behaves in a similar fashion and that feeding selectivity could be a plastic trait for the species, which becomes fixed at the level of the individuals.

In summary, the spatial distribution of *E. clarki* at our study sites did not show strong correspondence with the plant food consumed. Moreover, the fact many slugs were found instead on bare substrate suggests that the kleptoplastic nature of *E. clarki* makes the relationship between slugs their food sources a complex one. The photosynthetic capability of *E. clarki* appears to be release the slugs from the constraint of having to remain near its food source, although this may only be true over the duration of time when chloroplasts are functional. Our study highlights the major benefit of pairing of DNA dietary analysis of an animal with field surveys, especially when surveyed across multiple sites. While this approach was of particular interest here

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because of *E. clarki's* photosynthetic ability, these methods should be applicable to other systems. Combining dietary DNA information and field surveys, as demonstrated here, is currently one of the best available techniques for uncovering the complex ecological relationships that exist between herbivores and plants when actually observing the herbivore feeding is not possible.

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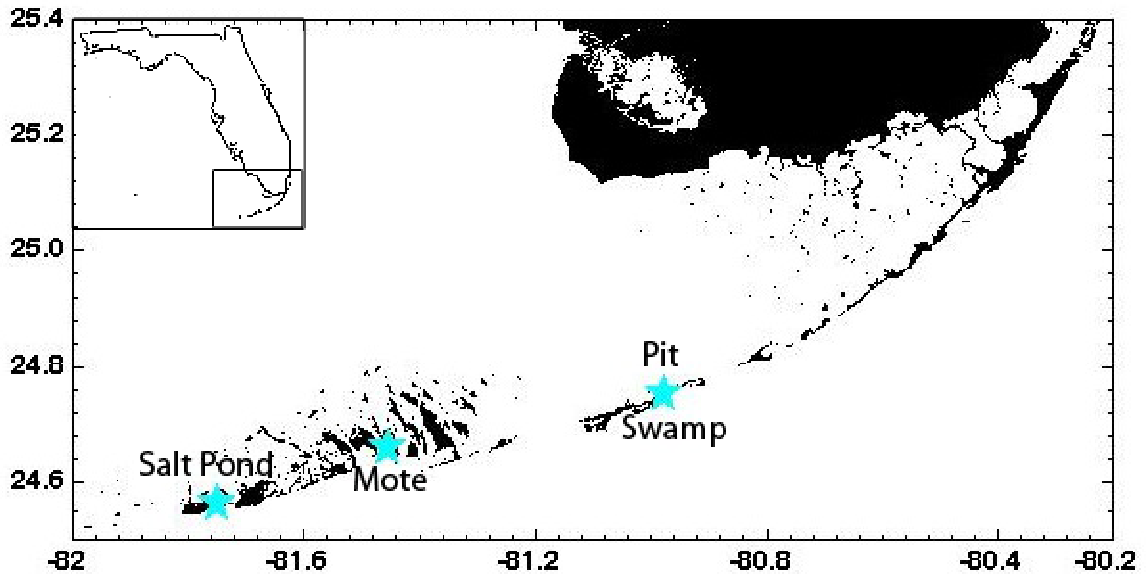

Figure 2.1: Map of study sites in the Florida Keys, USA

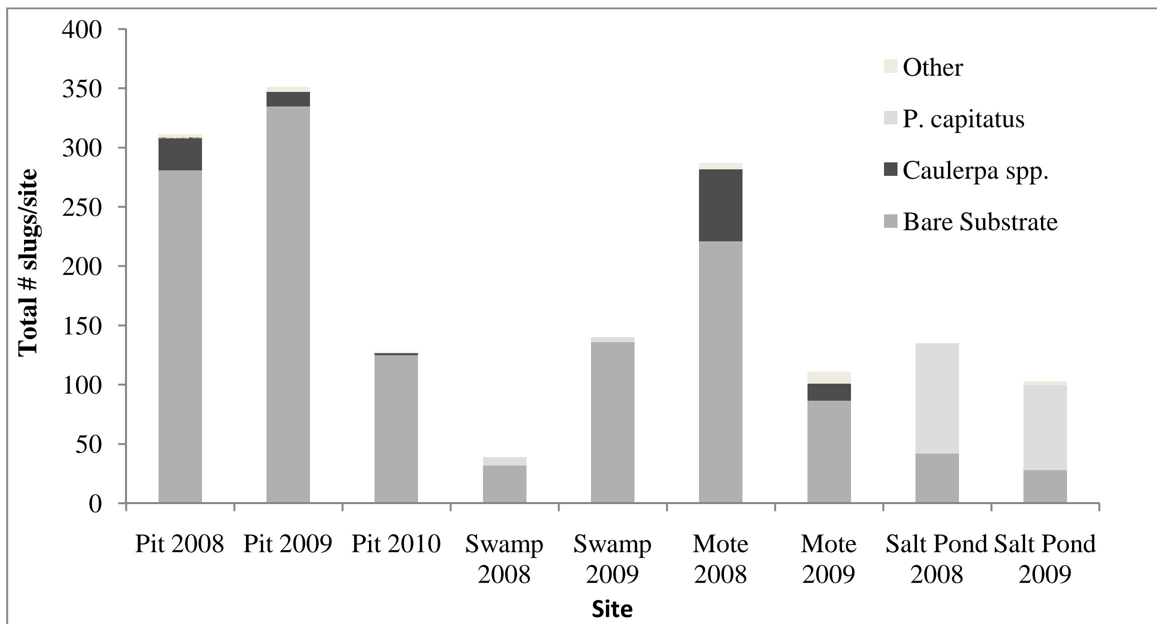

Figure 2.2: Total slugs surveyed at each study site at each respective time period and the substrate on which slugs were found.

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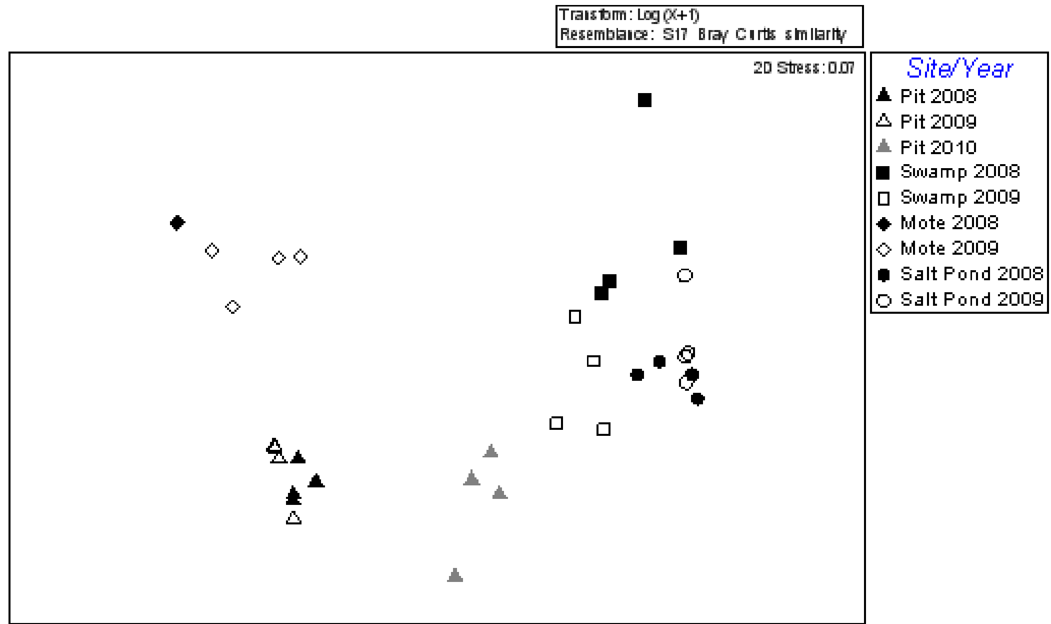

Figure 2.3: MDS plot of the log transformed algal populations of each study site surveyed for each time period. Plot was made using PRIMER 6 software.

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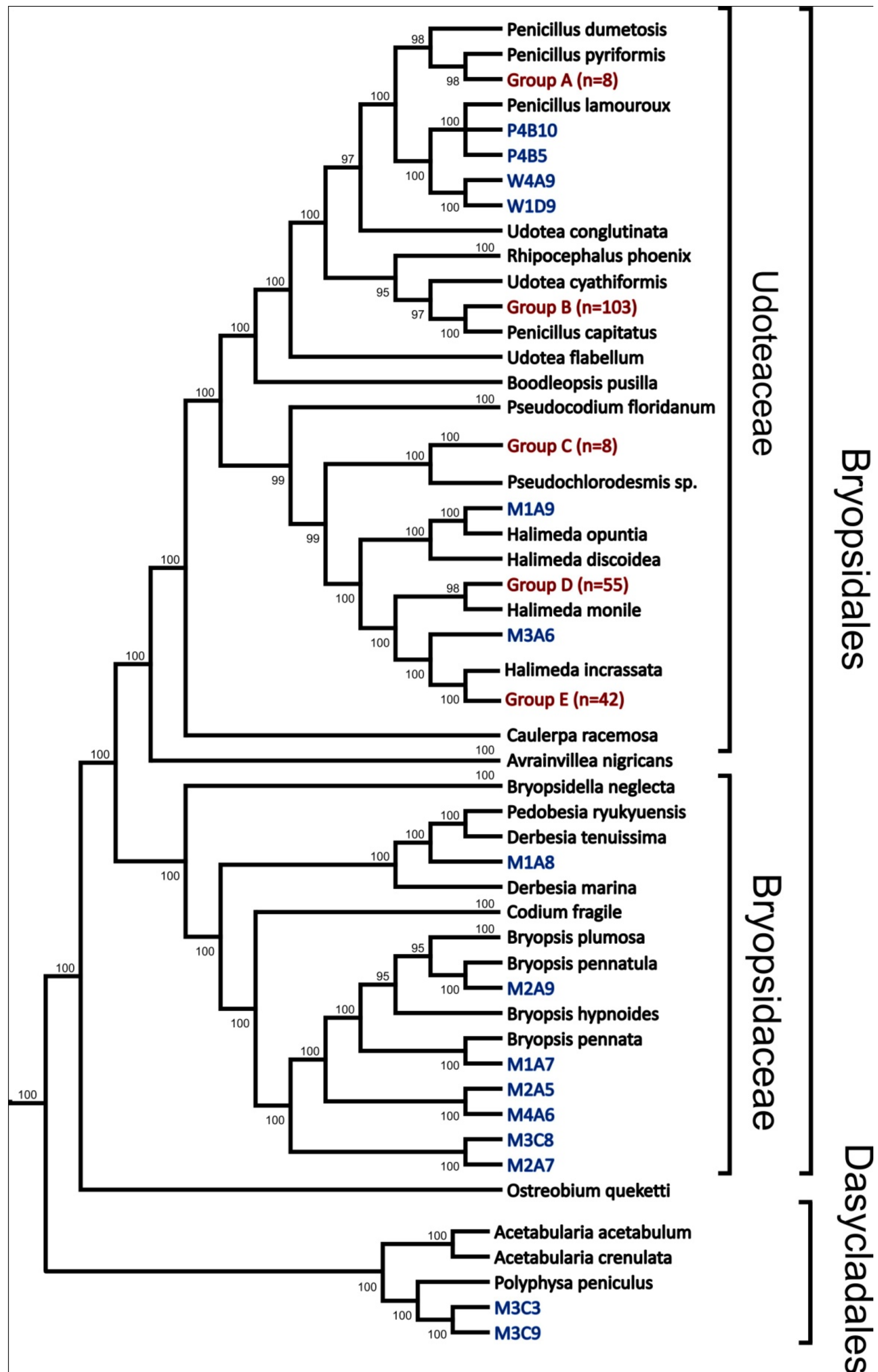

Figure 2.4: Maximum likelihood cladogram show the sequence identity of collected samples based on the *rbcL* gene across all sites.

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Table 2.1: Algae presence and slug density during field surveys. Algae in bold are food sources for *E. clarki*. Numbers inside of parentheses represent the number of slugs found on a given substrate and the percentage of slugs found on that same substrate.

Site	Algae present in survey	Total Slugs	Slugs/m^2	Substrate Slugs Are Found On
Pit 2008	***Acetabularia sp., Halimeda monile***, *Batophora sp., Caulerpa mexicana, C.verticillata, Udotea sp.*	310	2.58	Bare Substrate (281, 90.6%), *C. mexicana* (20, 6.5%), *C. verticallata* (7, 2.3%), *Acetabularia sp.* (2, 0.6%)
Swamp 2008	***Acetabularia sp., H. monile, H. incrassata, Penicillus capitatus, P. lamourourxii,***	39	0.33	Bare Substrate/Microalgal Mat (32, 82.1%), *P capitatus* (7, 17.9%)
Mote 2008	***Acetabularia sp, H. opuntia,*** *C.verticillata*	285	2.38	Bare Substrate (221, 77.0%), *C. verticillata* (61, 21.3 %), *Halimeda sp.* (5, 1.7%)
Salt Pond 2008	***Acetabularia sp., H. monile, H. incrassata, P. capitatus, P. lamourourxii,*** *Udotea sp.*	135	1.13	Bare substrate (42, 31.1 %), *P. capitatus* (93, 68.9%)
Pit 2009	***Acetabularia sp.,*** *Batophora sp., C. mexicana, C. verticillata, Udotea sp.*	350	2.92	Bare Substrate (335, 95.4%), *C. mexicana* (6, 1.7%), *C. verticillata* (6, 1.7%), *Acetabularia sp.* (4, 1.1%)
Swamp 2009	***Acetabularia sp., H. monile, H. incrassata, P. capitatus, P. lamourourxii,*** *Udotea sp.*	140	1.12	Bare substrate (136, 97.1%), *P. capitatus*(4, 2.9%)
Mote 2009	***Acetabularia sp, H. opuntia, H. incrassata,*** *Batophora sp., C. verticillata*	116	0.97	Bare substrate (87, 78.3%), *C. verticillata*(14, 12.6%), *Halimeda sp.* (10, 9.1%)
Salt Pond 2009	***Acetabularia sp., H. monile, H. incrassata, P. capitatus, P. lamourourxii***, *Udotea sp.*	103	0.86	Bare substrate (28, 27.2%), *P. capitatus* (72, 69.9%), *H. incrassata* (3, 2.9%)
Pit 2010	***Acetabularia sp., H. monile, P. capitatus, P. lamourourxii, Bryopsis sp.***, *C. verticillata, C. mexicana, Batophora sp., Udotea sp.*	122	1.02	Bare substrate (119, 97.7 %), *C. mexicana* (2, 1.6%), *H. incrassata* (1, 0.7%)

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Table 2.2A: The frequency of algal species occurrence within quadrats at study sites in 2008. Algae in bold are confirmed food sources for *E. clarki*.

Site/Year	Algae present in survey	# quadrats containing alga	# slugs in quadrat w/ alga	% slugs in quadrats w/ alga	% quadrats w/ alga
Pit 2008	***Acetabularia sp.***	57	211	68.1	71.25
	Halimeda monile	1	7	2.2	1.25
	Batophora sp.	14	26	8.4	17.5
	Caulerpa mexicana	50	170	54.8	62.5
	C. verticillata	35	112	36.1	43.75
	Udotea sp.	6	39	12.6	7.5
Swamp 2008	***Acetabularia sp.***	5	3	7.7	6.25
	H. monile	1	1	2.6	1.25
	H. incrassata	15	8	20.5	18.75
	Penicillus capitatus	46	21	53.8	57.5
	P. lamourourxii	6	16	41	7.5
Mote 2008	***Acetabularia sp.***	6	26	9.1	7.5
	H. opuntia	25	55	19.2	31.25
	C. verticillata	79	277	96.5	98.75
Salt Pond 2008	***Acetabularia sp.***	3	4	2.9	3.75
	H. incrassata	31	46	34.1	38.75
	H. monile	1	2	1.5	1.25
	P. capitatus	79	133	98.5	98.75
	P. lamourourxii	11	18	13.3	13.75
	Udotea sp.	5	14	10.4	6.25

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Table 2.2B: The frequency of algal species occurrence within quadrats at study sites in 2009 and 2010. Algae in bold are confirmed food sources for *E. clarki*.

Site/Year	Algae present in survey	# quadrats containing alga	# slugs in quadrat w/ alga	% slugs in quadrats w/ alga	% quadrats w/ alga
Pit 2009	***Acetabularia sp.***	77	329	93.8	96.25
	Batophora sp.	3	27	7.7	3.75
	C. mexicana	65	280	79.8	81.25
	C. verticillata	50	213	60.1	62.5
	Udotea sp.	9	63	18.9	11.25
Swamp 2009	***Acetabularia sp.***	44	88	62.9	55
	H. monile	2	1	0.7	2.5
	H. incrassata	9	18	12.9	11.25
	P. capitatus	67	123	87.9	83.75
	P. lamourourxii	17	15	10.7	21.25
Mote 2009	***Acetabularia sp.***	6	7	6.3	7.5
	H. opuntia	61	88	79.3	76.25
	H. incrassata	3	6	5.4	3.75
	C. verticillata	73	109	98.2	91.25
	Batophora sp.	1	0	0	1.25
Salt Pond 2009	***Acetabularia sp.***	2	6	5.8	2.5
	H. incrassata	16	19	18.4	20
	H. monile	2	0	0	2.5
	P. capitatus	78	103	100	97.5
	P. lamourourxii	14	27	26.2	17.5
	Udotea sp.	5	4	3.9	6.25
Pit 2010	***Acetabularia sp.***	25	53	41.4	31.25
	H. incrassata	16	26	20.3	20
	Batophora sp.	1	6	4.7	1.25
	C. mexicana	71	106	82.8	88.75
	C. verticillata	4	5	3.9	5
	Bryopsis sp.	5	2	1.6	6.25
	P. lamourourxii	2	7	5.4	2.5
	P. capitatus	16	34	26.6	20
	Udotea sp.	4	6	2.6	5

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Table 2.3: Algal species contribution to community composition at each study site as revealed by similarity analysis (SIMPER). Analysis was completed using PRIMER 6 software.

Algae	Contribution %	Cumulative %
Pit		
Caulerpa mexicana	40.7	40.7
Acetabularia sp.	31.93	72.63
C. verticillata	17.49	90.12
Swamp		
Penicillus capitatus	60.55	60.55
Acetabularia sp.	18.18	78.73
Halimeda incrassata	16.16	94.89
Mote		
C. verticillata	94.51	94.51
Salt Pond		
P. capitatus	49.17	49.17
H. incrassata	27.23	76.4
P. lamourourxii	13.82	90.22

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Table 2.4: Dietary sequences confirmed for *Elysia clarki* via molecular analysis of the *rbcL* gene at each study site for slugs collected in 2008. Numbers is parenthesis represent the proportion of *rbcL* sequences that matched each algal species.

Site	Algae present in *rbcL* sequences
Pit	*Penicillus capitatus* (1/60) , *P. lamourouxii* (2/60), *Halimeda monile* (42/60), *H. incrassata* (15/60)
Swamp	*P. capitatus* (42/60), *P. lamourouxii* (2/60), *H. incrassata* (15/60), *H. monile* (1/60)
Mote	*Pseudochlorodesmis sp.* (8/60), *P. pyriformus* (8/60), *Acetabularia sp.* (2/60), *Bryopsis pennatula* (1/60), *B. pennata* (1/60), *Bryopsis sp. 1* (1/60), *Bryopsis sp.* 2 (1/60), *Bryopsis sp.* 3 (1/60), *Bryopsis sp.* 4 (1/60), *H. monile* (10/60), *Halimeda sp. 1* (1/60), *Halimeda sp.* 2(8/60), *Halimeda sp.* 3 (1/60),*H. incrassata* (15/60), *Derbesia sp.* (1/60),
Salt Pond	*P. capitatus* (60/60)

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Table 2.5: Comparison of potential food sources available to *Elysia clarki* at each site to algae confirmed as a food source via DNA barcoding. Algae in bold indicate species that were confirmed via DNA barcoding, but not found during visual surveys.

Site	Edible algae present in survey	Algae present in *rbcL* sequences	Algae eaten but not in survey	Algae in survey but not eaten	Sørensen Index
Pit	*Acetabularia sp., Halimeda monile*	***Penicillus capitatus***, ***P. lamourouxii***, *H. monile*, ***H. incrassata***	3	1	0.33
Swamp	*Acetabularia sp., H. monile, H. incrassata, P. capitatus, P. lamourourxii,*	*P. capitatus, P. lamourouxii, H. incrassata, H. monile*	0	1	0.89
Mote	*Acetabularia sp, H. opuntia*	***Pseudochlorodesmis sp.***, ***P. pyriformus***, *Acetabularia sp.*, ***Bryopsis pennatula***, ***B. pennata***, ***Bryopsis spp.***, ***Halimeda spp.***, ***H. monile***, ***H. incrassata***, ***Derbesia sp.,***	14	1	0.125
Salt Pond	*Acetabularia sp., H. monile, H. incrassata, P. capitatus, P. lamourourxii,*	*P. capitatus*	0	4	0.33

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Table 2.6: Sequence frequency, environmental frenquency and Pearre's C selectivity indices for each algal species confirmed via *rbcL* DNA barcoding for *Elysia clarki* at each of the study sites. Only significant ($p<0.05$) Pearre C values are reported.

Algae	Sequence Frequency	Environment	Pearre's C
Pit			
Penicillus capitatus	1	0	NS
P. lamourourxii	2	0	NS
Halimeda monile	45	1	+0.72
H. incrassata	12	0	+0.27
Swamp			
P. capitatus	42	46	NS
P. lamourourxii	2	6	NS
H. incrassata	15	15	NS
H. monile	1	1	NS
Mote			
H. incrassata	15	0	+0.26
H. monile	10	0	NS
Halimeda sp. 1	1	0	NS
Halimeda sp. 2	8	0	NS
Halimeda sp. 3	1	0	NS
P. pyriformus	8	0	NS
Pseudochlorodesmis sp.	8	0	NS
Bryopsis pennata	1	0	NS
B. penatula	1	0	NS
Bryopsis sp. 1	1	0	NS
Bryopsis sp. 2	1	0	NS
Bryopsis sp. 3	1	0	NS
Bryopsis sp. 4	1	0	NS
Acetabularia sp.	2	6	NS
Derbesia sp.	1	0	NS
Salt Pond			
Penicillus capitatus	60	79	+0.37

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CHAPTER 3:

ELYSIA CLARKI PROLONGS PHOTOSYNTHESIS BY SYNTHESIZING CHLOROPHYLL (CHL) A AND B

Introduction:

Autotrophy is not a defining characteristic of multi-cellular animals, although a variety of heterotrophic animal species obtain substantial nutrition from photosynthesis (Stoecker et al. 1989, Venn et al. 2008), usually from semi-obligate symbiotic relationships with zooxanthellae or zoochlorellae. Alternatively kleptoplasty, another mechanism of animal photosynthesis, is utilized by some species of sacoglossan sea slugs (Opisthobranch, Mollusca) (Greene 1970, Greene and Muscatin1972, Clark et al. 1990, Pierce and Curtis 2012). These slugs sequester chloroplasts from their algal food sources inside specialized cells of their digestive tubules. The captive chloroplasts continue to photosynthesize and provide the slugs with at least a supplemental energy source (Trench et al. 1969, Pierce and Curtis 2012). A few slug species can survive for months on the products of photosynthesis alone (Pierce and Curtis 2012). The benefits of kleptoplasty have yet to be fully explained, but should have implications for allocations of energy to food acquisition. As a consequence of their photosynthetic ability, in contrast to organisms that must feed continuously, kleptoplastic slugs may be more able to maintain populations even under low food or famine conditions (Gimènez-Casalduero and

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Muniain 2008) and spend less time foraging when food resources are abundant (Marin and Ros 1993, Middlebrooks et al. 2011).

Although several sacoglossan species are kleptoplastic, the intensity and duration of photosynthesis is quite variable among species (Clark et al. 1990, Händeler et al. 2009, Pierce and Curtis 2012) ranging from several days or less to over 10 months in the case of *Elysia chlorotica* (Clark et al. 1990, Mondy and Pierce 2003). In the slugs with long term photosynthesis a maintenance mechanism is necessary to offset the organellar damage caused by light harvesting and subsequent electron transport. Although energy from sunlight is a required component of photosynthesis, light exposure degrades both thylakoid structure and photopigments, especially chlorophyll (Chl). Some slug species use mechanical or behavioral adaptations to simply shade the symbiotic plastids to slow damage caused by exposure to ultraviolet light (Jesus et al. 2010, Schmitt and Wägele 2011). However, a few other slug species have partially integrated the symbiotic plastids into the host cell biology and synthesize some of the chloroplast proteins and pigments consumed during photosynthesis (Pierce et al. 2009). For example, *Elysia chlorotica* synthesizes Chl a as well as several proteins in the light harvesting complex (Pierce et al. 2009). This surprising capability is due to the presence and function of several horizontally-transferred nuclear genes from its algal food source present in the slugs' genome (Pierce et al. 2007, Pierce et al. 2009, Schwartz et al. 2010, Pierce et al. 2012). Once it has fed and obtained chloroplasts, *E. chlorotica* is able to survive and complete its life cycle exclusively using photosynthesis (West 1979). While the biochemical mechanisms for maintaining kleptoplasty in *E. chlorotica* are beginning to be understood, the mechanisms used by other slug species which photosynthesize for lesser, but still

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impressively long (months) periods of time, remain unclear. Examining the mechanisms employed by this latter group with intermediate term kleptoplasts is critical to understand the evolution of kleptoplasty and evaluate its physiological consequences for photosynthetic animals.

Elysia clarki is a kleptoplastic sacoglossan which is able to photosynthesize for over 3 months of starvation (Pierce et al. 2006), a duration that falls in the middle of the time course of known photosynthetic slugs. This species is unusual in that it is capable of sequestering chloroplasts from several algal species within the same digestive cell (Curtis et al. 2006, Curtis et al. 2007). However, after about two months of starvation both photosynthetic activity and [Chl a] significantly decline, while feeding behavior increases (Middlebrooks et al. 2011). Although not as long as *E. chlorotica*, the duration of the *E. clarki* association suggests that some plastid maintenance may be present in the host cell. Therefore, we investigated whether or not *E. clarki* is able to synthesize Chl and, if so, does synthesis continue when slugs' photosynthetic activity declines. We found that recently collected *E. clarki* synthesizes Chl a and Chl b, but that slugs starved for a prolonged duration were not able to synthesize either type of Chl.

Methods:

Animals: Specimens of *E. clarki* were collected from a borrow pit on Grassy Key, FL, USA (Middlebrooks et al. 2011) and then transported to the laboratory in Tampa, FL, USA where they were housed in aquaria in artificial seawater (Instant Ocean) (1000 mosm) (ASW) without access to algal food. They were kept at room temperature (~23°C) and on a 14/10h light/dark cycle under cool white florescent lights. One group of slugs was starved for 2 weeks, to ensure their guts were empty, before use in the

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experiment. A second group of slugs was starved for 14 weeks before use, by which time photosynthetic activity significantly declines (Middlebrooks et al. 2011).

Chlorophyll synthesis: At the end of a 10h dark cycle, the 2 week starved slugs were exposed to a radioactive Chl precursor, 15µCi ^{14}C *aminolevulinic acid* (ALA) (^{14}C-4, 55 µCi/mmol, American Radiolabeled Chemical, St.Louis, MO) in ASW for 2 h in the dark at constant temperature (25° C), in an agitator. Then, the slugs, still in the ^{14}C ALA in ASW, were exposed to light from two 75 W halogen flood lamps for the next 22 hours in the agitator (Pierce et al. 2009). Afterwards Chl was immediately extracted (see below). Slugs from the 14 week starvation group were treated similarly to the two week group except that aeration of the incubation medium had to be added during the 22 h of light exposure. Interestingly, preliminary experiments found that the starved slugs, which have very reduced photosynthetic ability (Middlebrooks et al. 2011), would not survive the procedure without additional aeration of the small volume (20ml) of medium used.

After the 22 h incubation, Chl a was extracted on ice in the dark by homogenizing slugs in HPLC grade acetone and then centrifuging (~12,000x G) the homogenate. The supernatants were kept at -20°C in the dark until chromatography to prevent photo-degradation of Chl a. Then Chl a was purified from the acetone supernatant using High Performance Liquid Chromatography (HPLC) (System Gold, Beckman Coulter, Fullerton, CA) on two C18 columns (Microsorb 100-3, 100x4.6mm Varian, Lake Forest CA , and Vydac 201 TP 105x4.6mm, Vydac, Hespire, CA) connected in series, with a mobile phase starting with 80%MeOH: 20% NH_4CH_3COOH (0.5 M, pH 7.2) changing to 80% MeOH : 20% (Pinckney et al. 1998). NH_4CH_3COOH was added to all samples as an ion pair. Detection was done at 438 nm, the wavelength that Chl absorbs (Joyard et al.

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1987). This protocol successfully separates Chl a from its metabolites as well as other photopigments (Pinckney et al. 1998).

Fractions were collected from the analytical column eluent as it exited the detector, starting at 40 min from injection until the end of the end of run (50 min)-the region where the Chl elutes (Pinckney 1998-see also Figure 3.1), at 12 sec intervals. Since we were limited to using an analytical HPLC and, as result, could only inject 50µl per run without overloading the columns, multiple runs of each homogenate had to be pooled to obtain sufficient radioactivity to measure. The collected fractions from each run were pooled, dried under a stream of N_2, dissolved in acetone and added to a scintillation cocktail (Ready Safe^{TM} Beckman Coulter, Fullerton, CA). The radioactivity in the fractions was then determined using a scintillation counter (LS6500 Multipurpose Scintillation Counter, Beckman Coulter, Fullerton, CA). Finally to eliminate any quenching effect caused by Chl a, samples were spiked with 2000 cpm ^{14}C ALA and the data converted to dpm [(Pierce et al. 2009) modified from (Pinckney et al. 1998)].

Results:

Comparison of the HPLC chromatograms of the acetone extracts of *E. clarki* that had been starved for 2 weeks with that of a standard mixture of a variety of plant pigments (Pinckney et al. 1998, Pierce et al. 2009), as well as with an extract from *Penicillus capitatus*, one of the *E. clarki* plastid sources, (Fig.3.1) indicated the presence of 2 prominent peaks that exactly co-eluted with the position of Chl a and Chl b (compare Fig. 3.1, Fig. 3.2). The corresponding pattern of radioactivity in the post column eluent from the same slug extract indicated activity peaks co-eluting with the Chl a and Chl b peaks (Fig. 3.2). In this separation protocol, C^{14}-ALA elutes in the column void volume

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and radioactivity in the Chl a fraction is shifted downsteam to the elution position of phaeophytin, following the photoconversion of Chl a into phaeophytin, indicating that the radioactive label is in Chl a, not just co-eluting (Pierce et al. 2009). Therefore, the 2 week starved *E. clarki* are synthesizing both Chl a and Chl b. However, chromatography of the extracts of *E. clarki* after 14 weeks of starvation revealed no detectable Chl a and b peaks (Fig. 3.3). Radioactivity in the eluent did not rise above background levels in any fraction (Fig. 3.3). So for the slugs subjected to the the longer term starvation, Chl a and Chl b were depleted and its synthesis had ceased.

Discussion:

During the early stages of starvation, *E. clarki* synthesizes Chl a and Chl b. The synthesis of Chl within an animal is unusual, but the months long photosynthesis by the symbiotic plastid could not be accomplished unless Chl was somehow replaced. After some period of starvation, synthesis of Chl ceases. The longer time point we tested, 14 weeks of starvation, coincides with a significant reduction in both photosynthesis and Chl concentration in slugs (Middlebrooks et al. 2011). At present, it is not clear why Chl synthesis stops in *E. clarki* by 14 weeks. Although the decline of photosynthetic activity in *E. clarki* suggests that the decline in Chl synthesis may be responsible for the loss of photosynthetic activity, other aspects of photo-degradation, such as thylakoid degradation, could be the cause for the loss of photosynthesis and production of Chl a and Chl b.

The mechanism that is used for Chl synthesis in *E. clarki* is unclear. One possibility is that during ingestion of the plastid, a sufficient amount of precursors are also taken up, which accordingly would allow for Chl synthesis to persist for some period

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of time. While this is possible, it seems unlikely that sufficient precursors to last for months of photosynthesis would be present in the slugs. Another possibility is that the slug digestive cell provides the precursors using algal biochemistry provided by horizontal gene transfer between the source species and the slug. A handful of non-botanical species are able to produce photo-pigments using genes horizontally transferred from various fungi or algae. For example, the aphid, *Acyrthosiphon pisum*, is able to synthesize carotenoids using genes transferred into its genome from a fungus (Moran and Jarvik 2010), although these pigments are not involved in photosynthesis in the insect. But, several sacoglossans possess pigments used for photo-regulation. *Elysia viridis* synthesizes xanthophyll, but not Chl a (Trench et al. 1973). *Elysia timida* also has an active xanthophyll cycle (Jesus et al. 2010), although gene transfer has not been documented in this species *(*Wägele et al. 2011*).* *Elysia chlorotica* synthesizes Chl a using horizontally transferred algal genes present in the host cell genome (Pierce et al. 2009, Pierce et al. 2012). So, similar to *E. chlorotica*, the synthesis of Chl a by *E. clarki* may suggest the presence of horizontally transferred genes. However, the ultimate failure of Chl synthesis may indicate that *E. clarki* possesses fewer transferred genes than *E. chlorotica* and may therefore lack a gene critical for long term maintenance of Chl synthesis, or that another mechanism is responsible for the synthesis. Complicating the issue is that, unlike *E. chlorotica* which feeds exclusively on *Vaucheria litorea*, *E. clarki* feeds on and sequesters chloroplasts from multiple algal species (Curtis et al. 2006, Curtis et al. 2007) so the underlying biochemistry and its specificity in *E. clarki* may play an important role in the longevity of chloroplasts from particular algal species.

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Maintaining functional chloroplasts is a unique challenge for kleptoplastic animals. Without a maintenance mechanism exposure to light will rapidly degrade Chl and chloroplast structure. Many sacoglossan species with short term kleptoplasty simply turn over the plastids, slowly digesting them, and replace them by relatively continuous feeding (Pierce and Curtis 2012). Even slugs capable of long term photosynthesis will replace most of their sequestered chloroplasts when food is available (Gallop et al. 1980, Evertsen and Johnsen 2009, Pierce and Curtis 2012). However, by synthesizing Chl and other plastid proteins some sacoglossans are able to prolong the duration of photosynthesis. *Elysia chlorotica*, which is able to complete its entire 10 month life cycle using only photosynthesis (West 1979), synthesizes Chl and a variety of plastid proteins (Pierce et al. 2009). *Elysia crispata,* closely related to *E. clarki* (Pierce et al. 2006), synthesizes several chloroplast proteins, but not Chl (Trench and Ohlhorst 1976). Other sacoglossan species use behavioral and morphological adaptations to reduce photo-degradation of sequestered chloroplasts. For example, *E. timida* has opaque parapodia with which it shades the cells that contain chloroplasts when exposed to bright light. This shading behavior starts following starvation, when plastid turnover declines and the slugs' dependence on photosynthesis increases (Jesus et al. 2010, Schmitt and Wägele 2011).

Regardless of the mechanism, extending the duration of sequestered chloroplast function has clear benefits to the host. As long as photosynthesis continues, kleptoplasty aids sacoglossans by decreasing mortality during times of famine (Marin and Ros 1993, Gimènez-Casalduero and Muniain 2008). Also it follows that, kleptoplastic slugs may not need to feed as frequently as non-photosynthetic animals. For example,

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photosynthetically capable *E. clarki* are less likely to feed than conspecifics with lower photosynthetic-activity (Middlebrooks et al. 2011). Additionally, reducing foraging behavior may, in turn, reduce predation risk (Vadas et al. 1994). Sequestered chloroplasts may also provide camouflage, whether photosynthetic or not (Clark et al. 1990, Wägele and Klussmann-Kolb 2005).

Finally, our experiment produced some unanticipated results suggesting that *E. clarki* benefitted from the O_2 produced as a byproduct of photosynthesis, in addition to the benefits of photosynthetic carbon fixation. In preliminary experiments, the slugs starved for 14 weeks all died during the overnight incubation process (see Methods). However, the starved slugs provided with aeration all survived, suggesting that anoxia in the small volume (20ml) of incubation medium may have been the cause of death. In contrast, all of the slugs starved for 2 weeks survived without aeration perhaps due to O_2 produced during photosynthesis. Although not thoroughly tested here, these results suggest that, in addition to the central role of photosynthesis in energy metabolism, kleptoplastic O_2 production by the slug can increase tolerance of low oxygen conditions for *E. clarki* and other sacoglossans, (Taylor 1971, Trench et al. 1972, Gibson et al. 1986).

In conclusion, clearly *E. clarki* synthesizes Chl for some time period during starvation. This is the first demonstration of Chl synthesis by a sacoglossan with a 3-4 month kleptoplasty duration. The capacity to synthesize Chl likely increases the longevity of chloroplast function in *E. clarki,* which, in turn, reduces the slugs' reliance on a continuous supply of algal food. However, the mechanism responsible for the slugs'

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ability to synthesize Chl is unknown and may be complex in *E. clarki* as a result of this slug's ability to sequester symbiotic plastids from several algal species.

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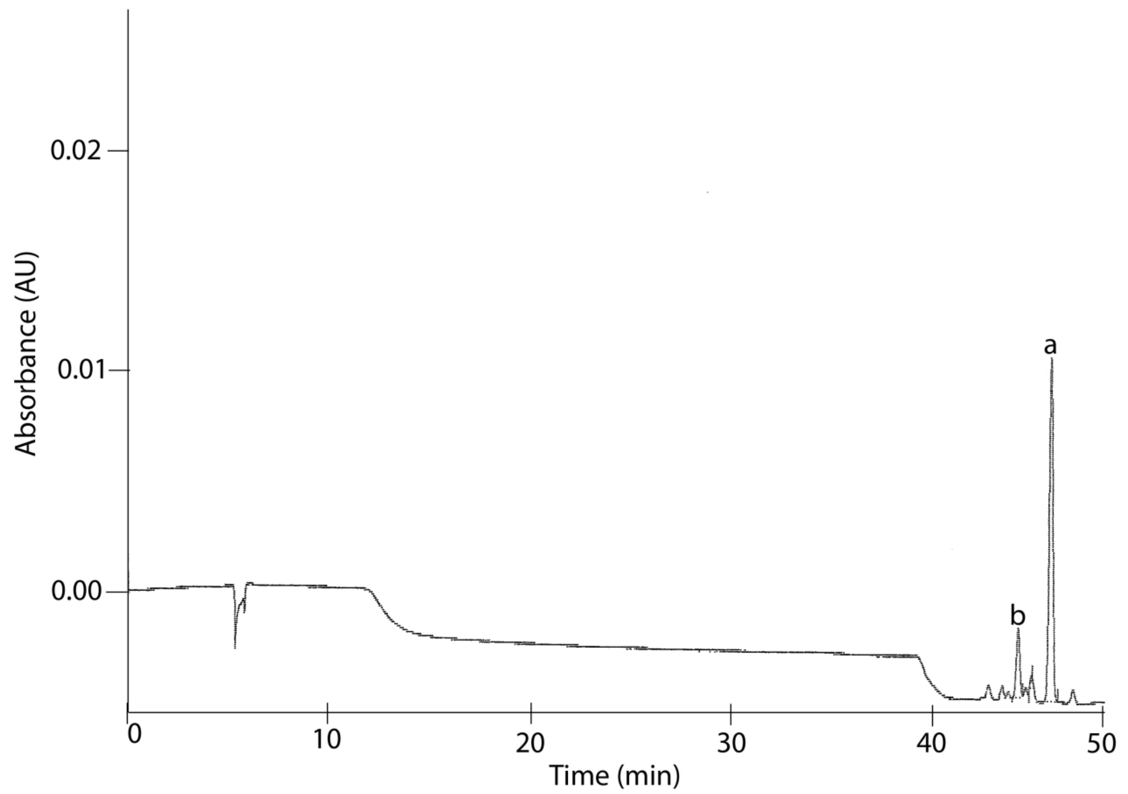

Figure 3.1: Typical HPLC chromatogram at 438nm of an acetone extract of *Penicillus capitatus*, one of the chloroplast sources of *E. clarki*. The peaks marked “a” and “b” label the peaks of Chl a and Chl b, respectively, in chromatograms of authentic pigments (Pierce et al. 2009 Pinckney et al. 1998) .

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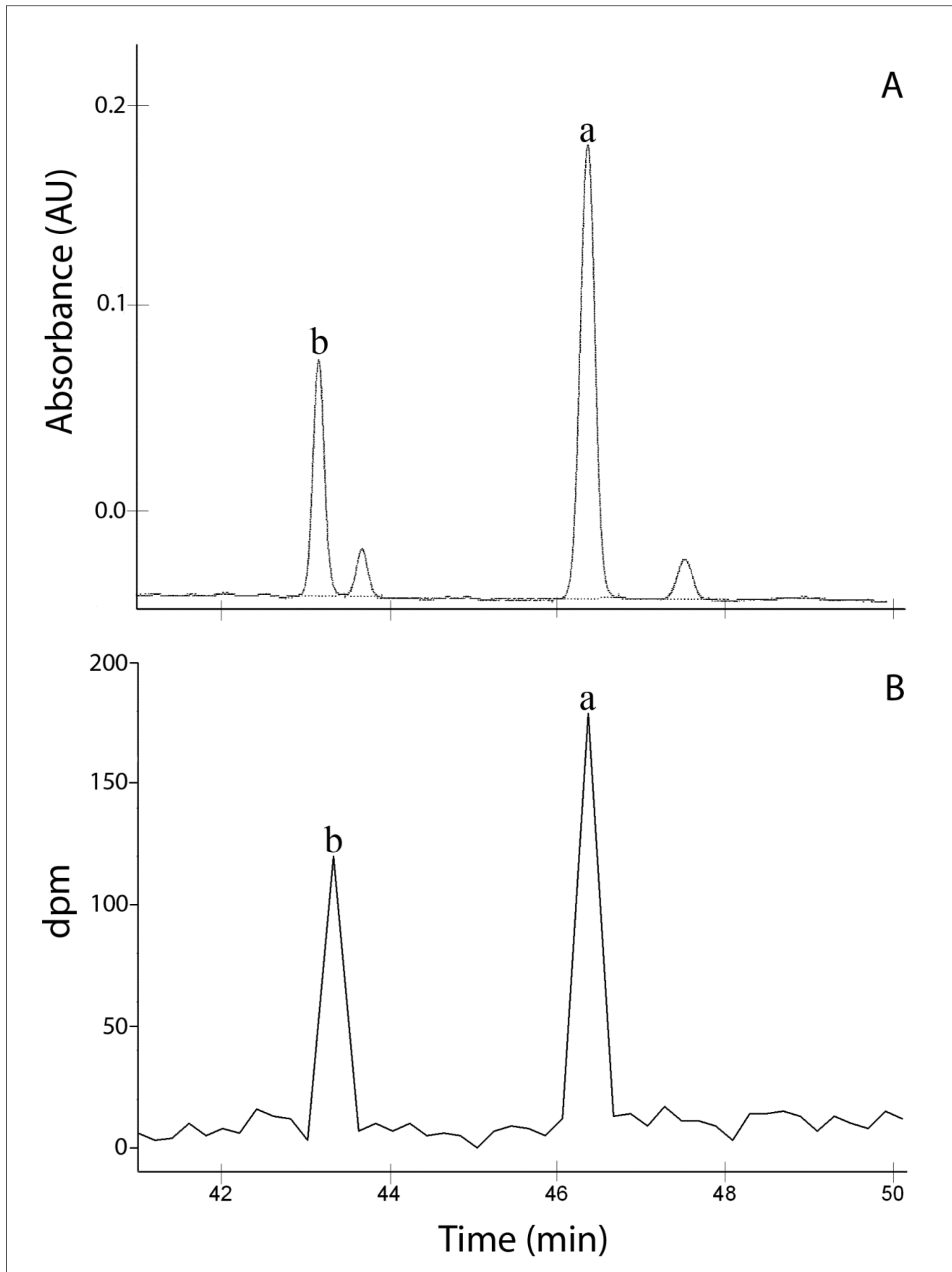

Figure 3.2: A) Typical HPLC chromatogram at 438 nm of the Chl eluting region of an acetone extract from a 2 week starved *E. clarki*. Lower case “a” and “b” represent the peaks of Chl a and Chl b respectively. B) Radiation counts in the column effluent from the same region of the chromatogram in A. The radiation peaks “a” and “b” exactly co-elute with the Chl a and Chl b peaks, indicating incorporation of radioactivity into both compounds.

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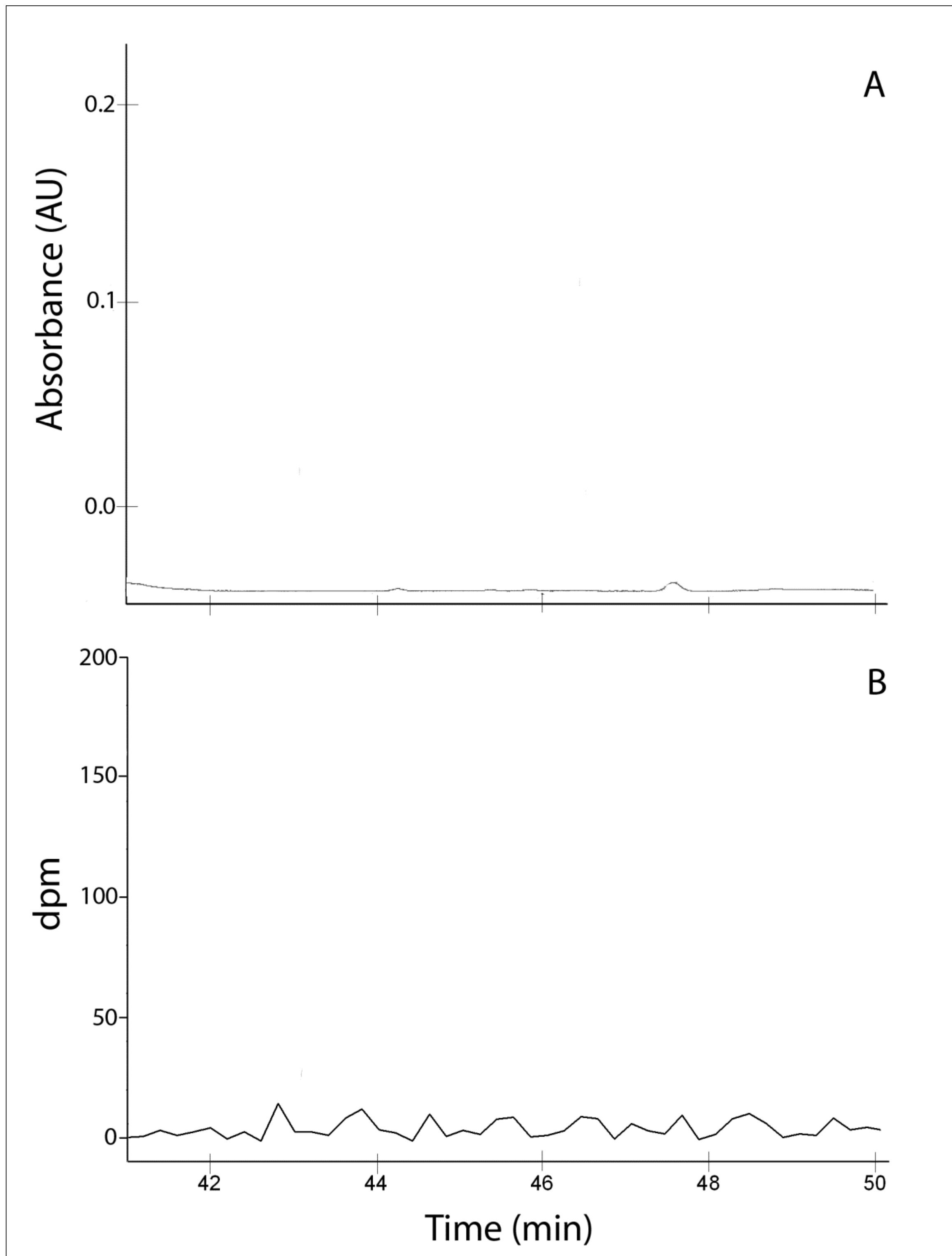

Figure 3.3: A) Typical HPLC chromatogram at 438 nm the Chl eluting region of an acetone extract from a 14 week starved *E. clarki*. B) Radiation counts in the column effluent from the same region of the chromatogram in A. The lack of Chl peaks and corresponding radioactivity demonstrate that *E. clarki* is no longer synthesizing Chl by this point in starvation.

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Appendix A: Algal Sequence Identities from Chapter 2

Sequence	Site	Best Algal Match	Group
M1A1	Mote	*P. pyriformis*	A
M1A10	Mote	*Halimeda sp. 2*	
M1A2	Mote	*Halimeda sp. 2*	
M1A3	Mote	*Halimeda monile*	D
M1A4	Mote	*Halimeda incrassata*	E
M1A5	Mote	*P. pyriformis*	A
M1A6	Mote	*Halimeda sp. 2*	
M1A7	Mote	*Bryopsis pennata*	
M1A8	Mote	*Derbesia sp.*	
M1A9	Mote	*Halimeda sp. 2*	
M2A10	Mote	*Halimeda incrassata*	E
M2A10	Mote	*P. pyriformis*	A
M2A2	Mote	*Pseudochlorodesmis*	C
M2A3	Mote	*Halimeda incrassata*	E
M2A4	Mote	*Pseudochlorodesmis*	C
M2A5	Mote	*Bryopsis sp. 4*	
M2A6	Mote	*Halimeda sp. 1*	
M2A7	Mote	*Bryopsis Sp. 2*	
M2A8	Mote	*Halimeda sp. 2*	
M2A9	Mote	*Bryopsis pennatula*	
M3A10	Mote	*Halimeda incrassata*	E
M3A2	Mote	*Halimeda incrassata*	E
M3A3	Mote	*P. pyriformis*	A
M3A4	Mote	*Halimeda incrassata*	E
M3A5	Mote	*Halimeda incrassata*	E
M3A6	Mote	*Halimeda sp. 3*	
M3A7	Mote	*Halimeda incrassata*	E
M3A8	Mote	*Halimeda monile*	D
M3A9	Mote	*Halimeda sp. 2*	
M3C1	Mote	*Pseudochlorodesmis*	C
M3C10	Mote	*Halimeda sp. 2*	
M3C2	Mote	*Halimeda sp. 2*	
M3C3	Mote	*Acetabularia sp*	
M3C4	Mote	*Pseudochlorodesmis*	C

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M3C5	Mote	*Pseudochlorodesmis*	C
M3C6	Mote	*Pseudochlorodesmis*	C
M3C7	Mote	*Halimeda incrassata*	E
M3C7	Mote	*Halimeda incrassata*	E
M3C8	Mote	*Bryopsis Sp. 3*	
M3C9	Mote	*Acetabularia sp*	
M4A1	Mote	*Halimeda incrassata*	E
M4A10	Mote	*P. pyriformis*	A
M4A2	Mote	*Halimeda monile*	D
M4A3	Mote	*Halimeda monile*	D
M4A4	Mote	*Halimeda incrassata*	E
M4A5	Mote	*Halimeda incrassata*	E
M4A6	Mote	*Bryopsis Sp. 1*	
M4A7	Mote	*Halimeda monile*	D
M4A8	Mote	*Pseudochlorodesmis*	C
M4A9	Mote	*P. pyriformis*	A
M4B1	Mote	*P. Pyriformis*	A
M4B19	Mote	*P. pyriformis*	A
M4B2	Mote	*Halimeda monile*	D
M4B3	Mote	*Halimeda monile*	D
M4B4	Mote	*Halimeda incrassata*	E
M4B5	Mote	*Halimeda monile*	D
M4B6	Mote	*Pseudochlorodesmis*	C
M4B7	Mote	*P. pyriformis*	A
M4B8	Mote	*Halimeda incrassata*	E
M4B9	Mote	*Halimeda monile*	D
P2B1	Pit	*Halimeda monile*	D
P2B10	Pit	*Halimeda monile*	D
P2B2	Pit	*Halimeda monile*	D
P2B3	Pit	*Halimeda monile*	D
P2B4	Pit	*Halimeda monile*	D
P2B5	Pit	*Halimeda monile*	D
P2B6	Pit	*Halimeda monile*	D
P2B7	Pit	*Halimeda monile*	D
P2B8	Pit	*Halimeda monile*	D
P2B9	Pit	*Halimeda monile*	D
P4A1	Pit	*Halimeda monile*	D
P4A10	Pit	*Halimeda monile*	D
P4A2	Pit	*Halimeda incrassata*	E
P4A3	Pit	*Halimeda monile*	D
P4A4	Pit	*Halimeda monile*	D
P4A5	Pit	*Halimeda incrassata*	E
P4A6	Pit	*Halimeda incrassata*	E

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P4A7	Pit	*Halimeda monile*	D
P4A8	Pit	*Halimeda incrassata*	E
P4A9	Pit	*Halimeda incrassata*	E
P4B1	Pit	*Halimeda monile*	D
P4B10	Pit	*P. lamourouxii*	
P4B2	Pit	*Halimeda monile*	D
P4B3	Pit	*Halimeda monile*	D
P4B4	Pit	*Halimeda monile*	D
P4B5	Pit	*P. lamourouxii*	
P4B6	Pit	*Halimeda incrassata*	E
P4B7	Pit	*Halimeda monile*	D
P4B8	Pit	*Halimeda monile*	D
P4B9	Pit	*Halimeda monile*	D
P4C1	Pit	*Halimeda monile*	D
P4C10	Pit	*Halimeda monile*	D
P4C2	Pit	*Halimeda monile*	D
P4C3	Pit	*Halimeda monile*	D
P4C4	Pit	*Halimeda monile*	D
P4C5	Pit	*Halimeda monile*	D
P4C6	Pit	*Halimeda monile*	D
P4C7	Pit	*Halimeda monile*	D
P4C8	Pit	*P. capitatus*	B
P4C9	Pit	*Halimeda incrassata*	E
P4D1	Pit	*Halimeda incrassata*	E
P4D10	Pit	*Halimeda incrassata*	E
P4D2	Pit	*Halimeda monile*	D
P4D3	Pit	*Halimeda incrassata*	E
P4D4	Pit	*Halimeda monile*	D
P4D5	Pit	*Halimeda monile*	D
P4D6	Pit	*Halimeda incrassata*	E
P4D7	Pit	*Halimeda monile*	D
P4D8	Pit	*Halimeda incrassata*	E
P4D9	Pit	*Halimeda monile*	D
P5A1	Pit	*Halimeda monile*	D
P5A10	Pit	*Halimeda monile*	D
P5A2	Pit	*Halimeda monile*	D
P5A3	Pit	*Halimeda monile*	D
P5A4	Pit	*Halimeda monile*	D
P5A5	Pit	*Halimeda monile*	D
P5A6	Pit	*Halimeda monile*	D
P5A7	Pit	*Halimeda monile*	D
P5A8	Pit	*Halimeda monile*	D
P5A9	Pit	*Halimeda monile*	D

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S2A1	Salt Pond	*P. capitatus*	B
S2A10	Salt Pond	*P. capitatus*	B
S2A2	Salt Pond	*P. capitatus*	B
S2A3	Salt Pond	*P. capitatus*	B
S2A4	Salt Pond	*P. capitatus*	B
S2A5	Salt Pond	*P. capitatus*	B
S2A6	Salt Pond	*P. capitatus*	B
S2A7	Salt Pond	*P. capitatus*	B
S2A8	Salt Pond	*P. capitatus*	B
S2A9	Salt Pond	*P. capitatus*	B
S2B1	Salt Pond	*P. capitatus*	B
S2B10	Salt Pond	*P. capitatus*	B
S2B2	Salt Pond	*P. capitatus*	B
S2B3	Salt Pond	*P. capitatus*	B
S2B4	Salt Pond	*P. capitatus*	B
S2B5	Salt Pond	*P. capitatus*	B
S2B6	Salt Pond	*P. capitatus*	B
S2B7	Salt Pond	*P. capitatus*	B
S2B8	Salt Pond	*P. capitatus*	B
S2B9	Salt Pond	*P. capitatus*	B
S2E1	Salt Pond	*P. capitatus*	B
S2E10	Salt Pond	*P. capitatus*	B
S2E2	Salt Pond	*P. capitatus*	B
S2E3	Salt Pond	*P. capitatus*	B
S2E4	Salt Pond	*P. capitatus*	B
S2E5	Salt Pond	*P. capitatus*	B
S2E6	Salt Pond	*P. capitatus*	B
S2E7	Salt Pond	*P. capitatus*	B
S2E8	Salt Pond	*P. capitatus*	B
S2E9	Salt Pond	*P. capitatus*	B
S3B1	Salt Pond	*P. capitatus*	B
S3B10	Salt Pond	*P. capitatus*	B
S3B2	Salt Pond	*P. capitatus*	B
S3B3	Salt Pond	*P. capitatus*	B
S3B4	Salt Pond	*P. capitatus*	B
S3B5	Salt Pond	*P. capitatus*	B
S3B6	Salt Pond	*P. capitatus*	B
S3B7	Salt Pond	*P. capitatus*	B
S3B8	Salt Pond	*P. capitatus*	B
S3B9	Salt Pond	*P. capitatus*	B
S4A1	Salt Pond	*P. capitatus*	B
S4A10	Salt Pond	*P. capitatus*	B
S4A2	Salt Pond	*P. capitatus*	B

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S4A3	Salt Pond	*P. capitatus*	B
S4A4	Salt Pond	*P. capitatus*	B
S4A5	Salt Pond	*P. capitatus*	B
S4A6	Salt Pond	*P. capitatus*	B
S4A7	Salt Pond	*P. capitatus*	B
S4A8	Salt Pond	*P. capitatus*	B
S4A9	Salt Pond	*P. capitatus*	B
S4C1	Salt Pond	*P. capitatus*	B
S4C10	Salt Pond	*P. capitatus*	B
S4C2	Salt Pond	*P. capitatus*	B
S4C3	Salt Pond	*P. capitatus*	B
S4C4	Salt Pond	*P. capitatus*	B
S4C5	Salt Pond	*P. capitatus*	B
S4C6	Salt Pond	*P. capitatus*	B
S4C7	Salt Pond	*P. capitatus*	B
S4C8	Salt Pond	*P. capitatus*	B
S4C9	Salt Pond	*P. capitatus*	B
W1A1	Swamp	*P. capitatus*	B
W1A10	Swamp	*P. capitatus*	B
W1A2	Swamp	*P. capitatus*	B
W1A3	Swamp	*P. capitatus*	B
W1A4	Swamp	*P. capitatus*	B
W1A5	Swamp	*P. capitatus*	B
W1A6	Swamp	*P. capitatus*	B
W1A7	Swamp	*Halimeda incrassata*	E
W1A8	Swamp	*Halimeda incrassata*	E
W1A9	Swamp	*P. capitatus*	B
W1D1	Swamp	*Halimeda incrassata*	E
W1D10	Swamp	*Halimeda incrassata*	E
W1D2	Swamp	*Halimeda incrassata*	E
W1D3	Swamp	*Halimeda incrassata*	E
W1D5	Swamp	*Halimeda incrassata*	E
W1D4	Swamp	*Halimeda incrassata*	E
W1D6	Swamp	*P. capitatus*	B
W1D7	Swamp	*Halimeda incrassata*	E
W1D8	Swamp	*P. capitatus*	B
W1D9	Swamp	*P. lamourouxii*	
W1E1	Swamp	*P. capitatus*	B
W1E10	Swamp	*P. capitatus*	B
W1E2	Swamp	*P. capitatus*	B
W1E3	Swamp	*P. capitatus*	B
W1E4	Swamp	*P. capitatus*	B
W1E5	Swamp	*Halimeda monile*	D

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W1E6	Swamp	*P. capitatus*	B
W1E7	Swamp	*P. capitatus*	B
W1E8	Swamp	*P. capitatus*	B
W1E9	Swamp	*P. capitatus*	B
W3A1	Swamp	*P. capitatus*	B
W3A10	Swamp	*P. capitatus*	B
W3A2	Swamp	*P. capitatus*	B
W3A3	Swamp	*Halimeda incrassata*	E
W3A4	Swamp	*P. capitatus*	B
W3A5	Swamp	*Halimeda incrassata*	E
W3A6	Swamp	*P. capitatus*	B
W3A7	Swamp	*P. capitatus*	B
W3A8	Swamp	*P. capitatus*	B
W3A9	Swamp	*P. capitatus*	B
W3B1	Swamp	*P. capitatus*	B
W3B10	Swamp	*Halimeda incrassata*	E
W3B2	Swamp	*P. capitatus*	B
W3B3	Swamp	*Halimeda incrassata*	E
W3B4	Swamp	*Halimeda incrassata*	E
W3B5	Swamp	*P. capitatus*	B
W3B6	Swamp	*P. capitatus*	B
W3B7	Swamp	*P. capitatus*	B
W3B8	Swamp	*P. capitatus*	B
W3B9	Swamp	*Halimeda incrassata*	E
W4A1	Swamp	*P. capitatus*	B
W4A10	Swamp	*P. capitatus*	B
W4A2	Swamp	*P. capitatus*	B
W4A3	Swamp	*P. capitatus*	B
W4A4	Swamp	*P. capitatus*	B
W4A5	Swamp	*P. capitatus*	B
W4A6	Swamp	*P. capitatus*	B
W4A7	Swamp	*P. capitatus*	B
W4A8	Swamp	*P. capitatus*	B
W4A9	Swamp	*P. lamourouxii*	

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