Examining the Retention and Digestion of Functional Kleptoplasts in Solar-Powered Sea Slugs

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"If you are looking for alternatives to Darwinism,
don't waste your time on opisthobranchs."

-G. Cimino and M.T. Ghiselin (2001)

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Declaration

I hereby declare that I am the sole author of this thesis and that no other sources or learning aids, other than the ones listed, have been used. Furthermore, I declare that I have acknowledged the work of others by providing detailed references of said work.

Hereby I also declare, that this thesis has not been prepared for another examination or assignment, neither wholly nor partially.

Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst habe, keine anderen als die angegebenen Quellen/Hilfsmittel verwendet habe und alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen wurden, als solche kenntlich gemacht habe. Ich versichere außerdem, dass ich die beigefügte Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine entgültig gescheiterten Promotionsverfahren vorausgegangen sind.

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Summary

Sacoglossan sea slugs are the only metazoans known to sequester and retain functional chloroplasts within their digestive gland tubules, a process known as functional kleptoplasty. This ability coincides with the capacity to withstand extended starvation periods – some species even achieving 3-12 months without access to food. Since these chloroplasts remain photosynthetically active and multiple different slug-derived compounds contain photosynthetically fixed carbon, the ability to sequester and retain plastids has been linked to the ability to withstand extended starvation periods. Despite the correlation between functional chloroplasts and starvation survival, almost nothing is known about how these plastids help the slug survive and when their photosynthates become available to the starving slug.

In this study, I examined sequestered chloroplasts throughout the starvation periods of two sister species, one that can withstand extended starvation and one that cannot, finding that only the long-term plastid retaining species contains chloroplasts that produce starch. Starch, the main photosynthate produced in chlorophytes, accumulates in the sequestered chloroplasts for the first half of the starvation period and then disappears gradually, likely due to digestive processes (Chapters 2,3). Contrastingly, chloroplasts do not accumulate starch in the short-term surviving species and are observed in the animal's excrement throughout the starvation period (Chapter 5). The ability to retain plastids without excreting them while they build up starch is likely the key to withstanding starvation in these species.

The decrease in starch concentration is likely due to digestion, but previous studies on intracellular digestion in these animals are virtually non-existent. To examine digestive processes regarding the decrease in functional chloroplasts and decrease in starch within those chloroplasts, I tried a myriad of staining techniques finally finding success with acridine orange, a stain that reveals lysosomes in living tissues. Using acridine orange staining in multiple species, I present here a first look at intracellular digestion in these animals. I observed different digestive

activity trends for a species capable of long-term plastid retention and multiple species unable to withstand extended starvation, providing evidence as to how some species regulate their own digestive activity to withstand starvation (Chapters 2,4).

The last aspect of the slug/plastid interaction that I present here concerns the development of this ability in juveniles. Functional kleptoplasts are not passed from parents to offspring, rather each generation must acquire its own plastids. During early development, these slugs cannot sequester and retain plastids for extended periods, nor can they survive prolonged starvation. I examined the acquisition of functional chloroplasts by juveniles and their digestive activity throughout different developmental stages, to determine when functional kleptoplasty is established and the starvation capacity experienced at each developmental stage (Chapter 6).

Overall, these studies provide new insight into how these remarkable animals maintain foreign organelles in their tissues and profit from the photosynthates produced to withstand extended starvation periods. Each investigation required the development of new methodologies that can be used in a variety of future endeavors. The conclusions presented here reshape our hypotheses about functional kleptoplasty in metazoans, and provide new theories as to how this amazing ability evolved in within the Sacoglossa.

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Chapter 1 Introduction

1.1 Sacoglossan Slugs

Sacoglossan slugs (Gastropoda: Heterobranchia), otherwise known as sap-sucking slugs, are a relatively small group comprising roughly 400 described species with a wide-variety of forms (Figure 1.1). Most species are small, ranging from about 0.5 – 3 centimeters in length. They occur in a wide variety of nearshore habitats, from salt marshes to sandy and rocky subtidal zones throughout the temperate and tropical oceans where they mostly feed on chlorophyte species (Cruz et al. 2013). Sacoglossan slugs have numerous qualities that have attracted interest from both, the public and researchers in numerous fields. Certain species have been observed sequestering toxic metabolites from their algal food and using these compounds for their own chemical defense (Cimino and Ghiselin 1998). These metabolites are currently being examined as to their medical and pharmaceutical applications (Ashour et al. 2006; Ciavatta et al. 2016; Manzo et al. 2005). Sacoglossans are most famous, however, for some species ability to steal and retain chloroplasts from their algal food, using these chloroplasts to survive extended starvation periods. This process, termed "functional kleptoplasty" has earned them the nicknames "solar-powered sea slugs", "leaves that crawl" and "photosynthetic slugs".

Previously assigned to the now defunct "Opisthobranchia", the clade Sacoglossa is currently included in the Panpulmonata. The basal-most group, Oxynoacea contains the shelled sacoglossans: Families Cylindrobullidae Oxynoidae H. Adams & A. Adams, 1854 and Volvatellidae Pilsbury, 1885 have a single shell, while Family Juliidae E. A. Smith, 1885 has a double or bivalve shell. The remaining sacoglossans are members of the Plakobranchacea, comprised of two clades, the cerata-bearing Limapontiidae and the parapodia-bearing, Plakobranchiodea, (Katharina Händeler and Wägele 2007; K. R. Jensen 1996) although the monophyly

of the Limapontioidea is doubted (K Händeler 2011; Katharina Händeler and Wägele 2007; K. R. Jensen 1996; Jörger et al. 2010; Maeda et al. 2012; Neusser et al. 2011). Cerata (Greek: horn) are extensions of the mantle that protrude from the lateral and dorsal body wall (Marín and Ros 2004), while parapodia are lateral extensions of the foot (Greek: subsidiary foot). Both cerata and parapodia contain digestive gland tubules and likely serve to increase the surface area of these tubules (Graves et al. 1979; Rumpho et al. 2010).

Numerous characters unite Sacoglossa as a group and its monophyly is strongly supported by morphological (K. R. Jensen 1996; P M Mikkelsen 1996; Paula M. Mikkelsen 1998) as well as molecular methods (Jörger et al. 2010; Wägele et al. 2003). As in all gastropods, sacoglossan teeth are found on a radular ribbon, however the sacoglossan radula contains two autapomorphic features, hollow, pointed teeth and an ascus (synonym saccus) – a pouch-like structure used for storing old teeth (Figure 1.2A). Sacoglossans (synonym Ascoglossa) derive their name from this ascus, which is located behind their tongue-like (glossus) radula (Figure 1.2B-C) (von Ihering 1879). Their pointed radular teeth previously characterized Sacoglossa (K. R. Jensen 1980, 1993, 2012), however it is now accepted that the basal-most sacoglossan, genus *Cylindrobulla* lacks this tooth morphology (Christa, Händeler, Kück, et al. 2014; Jörger et al. 2010; E. Laetz et al. 2014; Maeda et al. 2010).

The radula contains two limbs, the upper containing new teeth waiting to be used and the lower consisting of previously used teeth ending in the ascus (Figure 1.2A). Only one tooth is used at a time, called the leading tooth. Sacoglossan tooth morphology falls into one of four categories: 1) broad multi-denticled, 2) triangular-shaped, 3) blade-shaped and 4) sabot-shaped teeth (Figure 1.2D-G) (K. R. Jensen 1993; E. Laetz et al. 2014). While genus *Cylindrobulla* has a type 1 radula used for scraping, all other sacoglossan species have Types 2-4, which are used for piercing (Fretter 1940; K. R. Jensen 1993). Correlation between the radular tooth type and the algal species ingested has been debated, with some studies suggesting correlation and other presenting evidence to the contrary (Katharina Händeler and Wägele 2007; K. R. Jensen 1993, 1997).

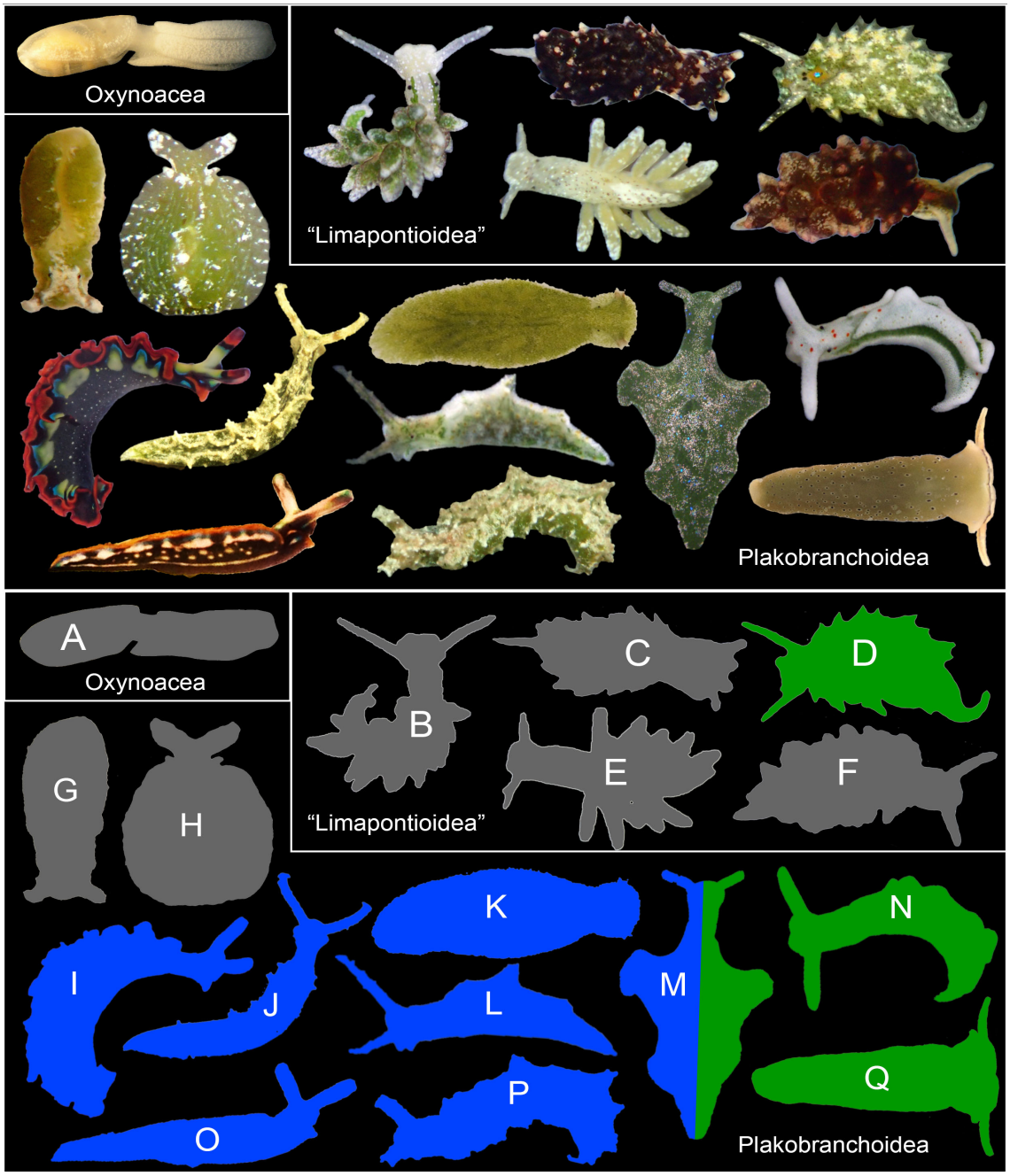

Figure 1.1 Examples of sacoglossan diversity and the different photosynthetic abilities of the species represented. The top graphic shows the species habitus' while the side shows their photosynthetic abilities – grey for non-chloroplast-retaining species (NR), blue for short-term retaining species (StR) and green for long-term retaining species (LtR). A: the oxynoacean, *Cylindrobulla schuppi* [~5 mm long]. B-F: Limapontioidean species. B: *Placida dendritica* [~3 mm]. C: *Costasiella nonatoi* [~2 mm]. D: *Costasiella ocellifera* [~4 mm long]. E: *Ercolania fuscata* [~3 mm]. F: *Ercolania viridis* [~4 mm]. G-Q: Plakobranchoidean species. G: *Elysia tuca* [~4 mm]. H: *Elysia marcusi* [~2 mm]. I: *Thuridilla picta* [~11 mm]. J: *Elysia patina* [~5 mm] Photo used with permission from Gregor Christa. K: *Bosellia mimetica* [~4 mm]. L: *Elysia cornigera* [~6 mm]. M: *Elysia viridis*, classified sometimes as LtR and sometimes StR [~4 mm]. N: *Elysia timida* [~6 mm]. O: *Thuridilla hopei* [~8 mm]. P: *Elysia papillosa* [~6 mm]. Q: *Plakobranchus ocellatus* [~10 mm].

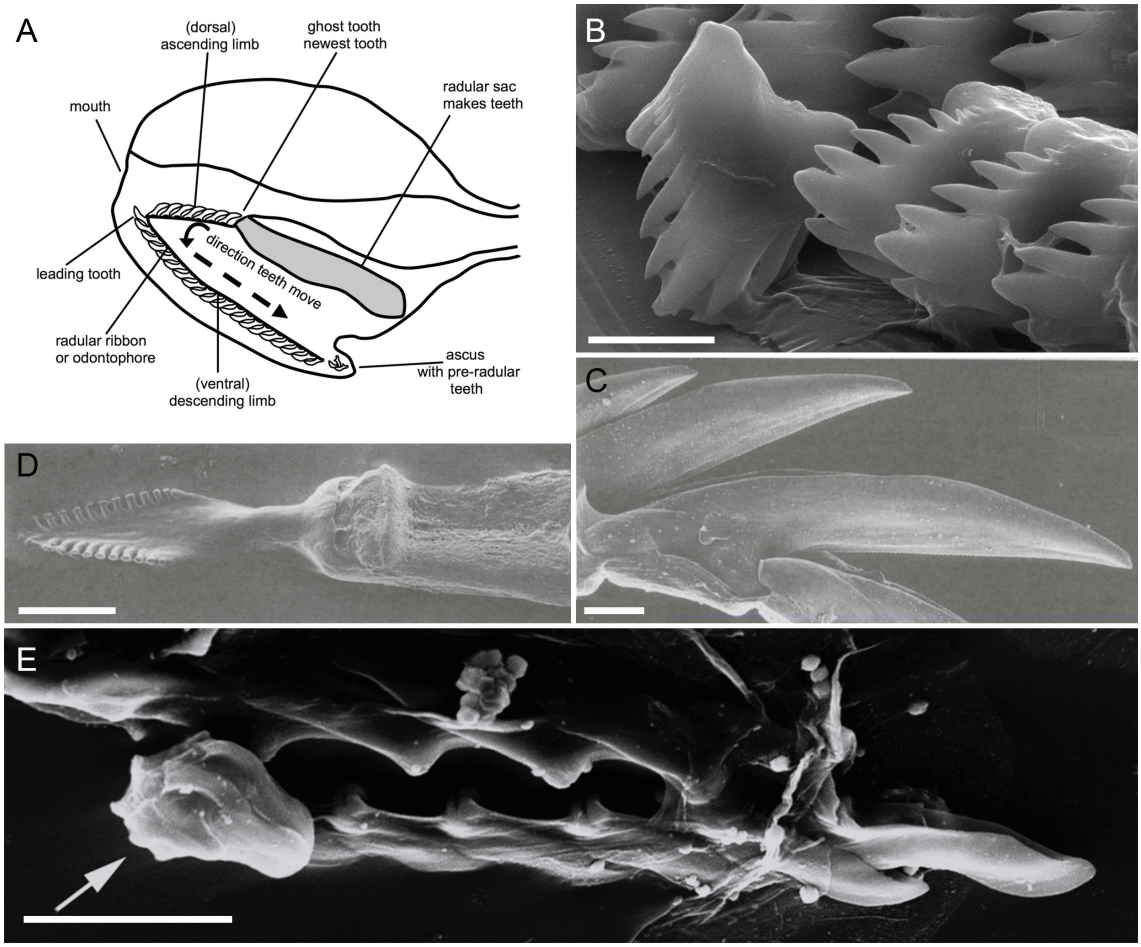

Figure 1.2 Buccal anatomy and radula morphology. A: This schematic shows the anatomy of the buccal apparatus (median lateral view) as drawn by Krug et al. 2016. B: Broad multi-denticled tooth from *Cylindrobulla schuppi* (modified from Laetz et al. 2014). C: Blade-shaped tooth from *Elysia trisinuata* (modified from Jensen 1993). D: Triangular-shaped tooth from *Cyerce antillensis* (modified from Jensen 1993). E: Scanning electron micrograph shows *Ercolania annelyleorum's* radular ribbon with sabot-shaped teeth, which ends in an ascus (arrow indicates the ascus) modified from Wägele et al. 2010. Scale bars: B – 8µm; C – 20µm; D – 10µm; E – 50µm.

1.2 Sacoglossan Diets

Almost all sacoglossan slugs feed on green algal species (Chlorophyta: Ulvophyceae) by piercing the algal cell wall and sucking out the algal cell contents including organelles and cytosol (Figure 1.3) (Christa, Gould, et al. 2014; Christa, Händeler, Schäberle, et al. 2014; Christa, Wescott, et al. 2013; N E Curtis et al. 2005; Nicholas E Curtis et al. 2006; Katharina Händeler et al. 2010; Katharina Händeler and Wägele 2007; K. R. Jensen 1980, 1993, 1994; Maeda et al. 2012; S. K.

Pierce et al. 2006; Williams and Walker 1999). Some sacoglossans feed on many species (polyphagy) while others feed on a few (stenophagy) (Christa, Gould, et al. 2014; Christa, Wescott, et al. 2013; Nicholas E Curtis et al. 2006; K. R. Jensen 1980, 1994; S. K. Pierce et al. 2006). Determining which slug species feed on exactly which algal species has been the focus of many investigations.

The algal species ingested by some sacoglossan slugs have been identified due to a number of different methods including but not limited to: feeding experiments, laboratory culturing, and DNA barcoding. Feeding experiments, which usually involve a researcher presenting a slug with numerous algal species and then observing which it consumes, have resulted in the identification of numerous sacoglossan food sources (notable examples: K. Jensen 1975; K. R. Jensen 1981, 1983, 1994). Jensen (1997) summarize these results into four conclusions regarding sacoglossan food preference and diet radiation: 1) oxynoacean species feed on the algal genus *Caulerpa* 2) most Sacoglossa only feed on one clade of green algae, Ulvophyceae; 3) within the Plakobranchoidea, most diet radiation occurs in the large genus *Elysia*; and 4) limapontioidean species mostly feed on ulvophyceaen genera other than *Caulerpa*. Despite these advances, feeding experiments only present some of the possible algal species found in a slug's natural habitat, so they only confirm some algae on which the slug may feed (K. R. Jensen 1980, 1993, 1994; Trowbridge 1991; Trowbridge and Todd 2001).

Successful laboratory cultures have also identified algal preference in sacoglossan slugs, additionally revealing which algal cultures are capable of sustaining a slug throughout its lifespan. Multiple generations grown in laboratory cultures of *Elysia timida* and *Elysia cornigera* have been raised on *Acetabularia acetabulum* alone (de Vries et al. 2015; Schmitt et al. 2014). *Elysia nigrocapitata* were bred their entire lives feeding on *Chaetomorpha moniligera* and *Cladophora sakaii*, however they were also observed feeding on *Bryopsis plumosa* and *Phyllodictyon orientale* although the later food sources were not sufficient for cultured animals to reach maturity (Klochkova et al. 2013). *Elysia chlorotica* have been raised on their heterokontophyte food, *Vaucheria litorea* (Pelletreau et al. 2012).

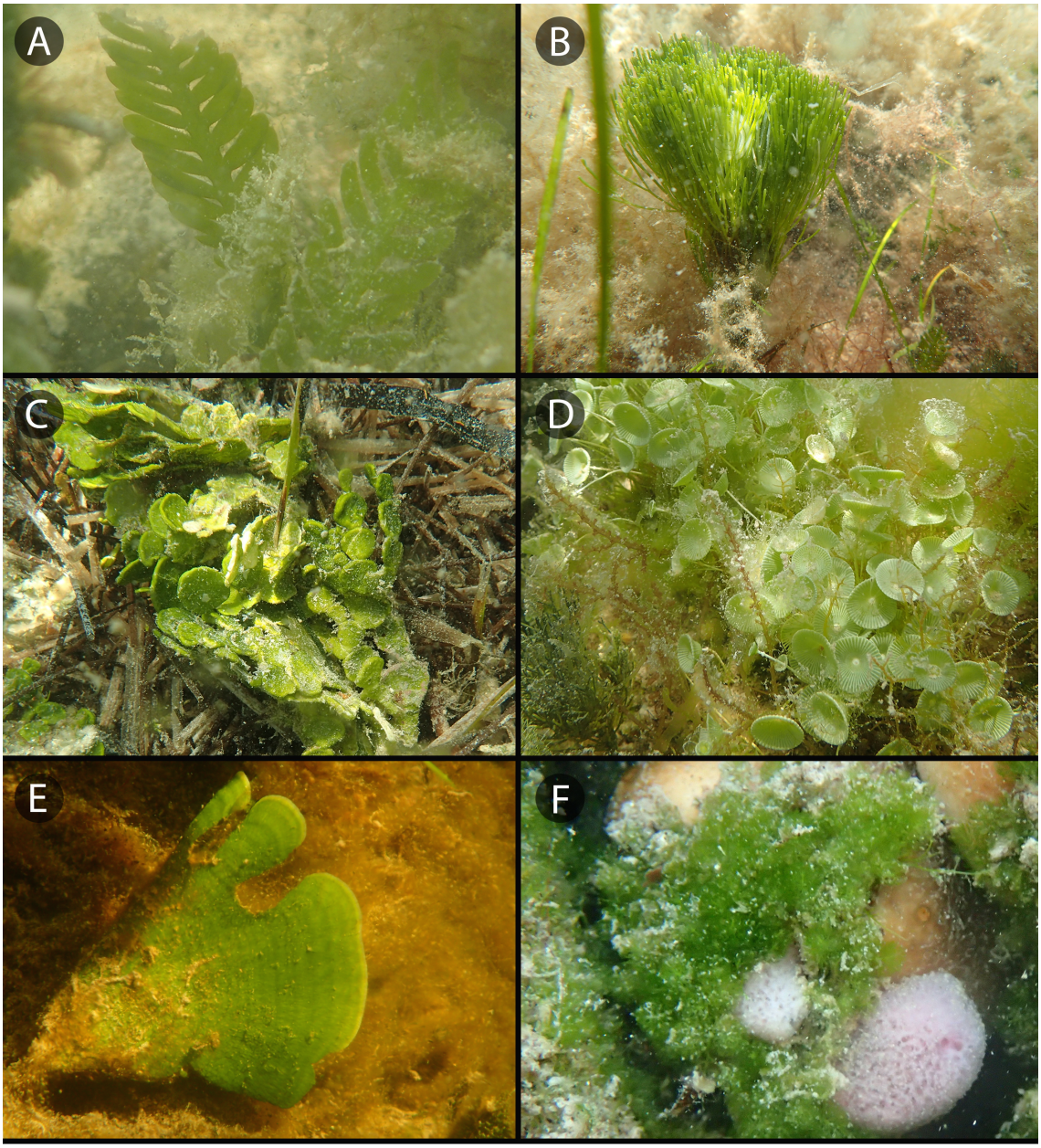

Figure 1.3 Examples of sacoglossan food algae. A: *Caulerpa taxifolia* (frond ~10cm). B: *Penicillus capitatus* (frond ~25cm). C: *Halimeda opuntia* (clump ~35cm). D: *Acetabularia crenulata* (each cap ~8-1.3mm diameter). E. *Udotea* sp. (frond ~8cm). F: *Bryopsis* cf. *plumose* (clump ~12cm).

The advent of molecular technologies had enabled plastid identification by DNA barcoding, a method that compares differences in DNA sequences to make species level determinations. Chloroplasts have their own DNA (cpDNA)– unrelated to the algae's nuclear DNA, which contains genes that are

transcriptionally active. As in nuclear DNA barcoding, differences in plastid DNA can be used for species level identifications. Numerous studies have used this method to considerably further the list of confirmed sacoglossan food sources. Händeler et al. (2009) identified algal species in 37 sacoglossans and 7 the following year (Katharina Händeler et al. 2010). Christa et al. (2013) identified 7 algal species amongst different *Plakobranchus ocellatus* populations. Christa et al. (2014) combined the known food sources from multiple molecular analyses revealing 24 algal taxa found as food sources for 68 sacoglossan species. *Elysia clarki* has the highest recorded number of ingested algal species (17) according to Curtis et al. (2006, 2007) and Middlebrooks et al. (2014). Multiple other studies have also reported sacoglossan food sources based on chloroplast barcoding (Christa 2014; N E Curtis et al. 2015).

Despite these advances, plastid barcoding has limitations at the present time. Many algal species are presumed species complexes because of two common issues: 1) the difficulty in identifying differences in their morphological characters or 2) molecularly similar specimens exhibiting drastically different morphologies due to other factors such as wave action or current strength (Bakker et al. 1995; Belton et al. 2014; Blomster et al. 1999; Pillmann et al. 1997; R. L. Taylor et al. 2017). Additionally, many species have yet to be barcoded so there is no reference sequence available for comparison to the slug's digestive contents or the algae on which it was observed feeding (Christa, Wescott, et al. 2013).

1.3 Functional Kleptoplasty

Functional kleptoplasty is the ability to steal (Greek: klepto) functional chloroplasts from an algal food source and accumulate them within the host's own cells. This capability is not limited to sacoglossan sea slugs and has been found in a few other organisms including foraminifers, ciliates, and dinoflagellates (Johnson 2011; Lindholm and Mork 1989; Stoecker et al. 2009), however sacoglossan slugs are the only known metazoans to have this ability. Functional kleptoplasty differs from other metazoan/algae photosymbioses (such as corals / zooxanthellae, some

poriferans / zooxanthellae, the salamander species *Ambystoma maculatum* Shaw, 1802 / *Oophila amblystomatis* F.D. Lambert, 1909, the platyhelminth *Symsagittifera roscoffensis* Graff, 1891 / *Tetraselmis convolutae* Parke and Manton, 1967 and others), because it involves the sequestration of organelles (chloroplasts) rather than an entire organism (a single-celled alga).

The first reports regarding functional kleptoplasty in sacoglossan slugs describe green pigment found in *Elysia viridis* (De Negri and De Negri 1876), which were later identified as chlorophyll (Brandt 1883). Despite their original identification as zooxanthellae (Yonge and Nicholas 1940), these chlorophylls were only identified as belonging to isolated chloroplasts in 1965, when Kawaguti and Yamasu and (later) Taylor applied electron microscopy, revealing the lack of entire algal cells within the slugs (Kawaguti and Yamasu 1965a; D. L. Taylor 1968). Trench (1969) also demonstrated the same pigment profiles in both the algal species and the slug indicating the chloroplasts found in slugs are identical to those in their food algae. This included a lack of peridinin, the main xanthophyte pigment found in zooxanthellae, in *Elysia* (then called *Tridachia*) *crispata*, further proving the incorporated chloroplasts do not originate from zooxanthellae and instead match the Siphonales clade of green algae (Robert K Trench 1969).

Radiolabelling studies using either ^{14}C or ^{13}C isotopes demonstrate that carbon is still fixed by incorporated *Codium fragile* (Suringar) Hariot ,1889 plastids in *Elysia viridis*, suggesting they still function inside the animal (Raven et al. 2001; Teugels et al. 2008; M. E. Trench et al. 1970; R K Trench et al. 1973; Robert K Trench 1969; Robert K Trench et al. 1969; Robert K Trench and Smith 1970). These studies also report algal-derived carbon in numerous slug-produced compounds such as their mucus (M. E. Trench et al. 1970), glucose, galactose and an unidentified third compound (R K Trench et al. 1974), pigments (Robert K Trench and Smith 1970), amino acids, organic acids and others (not specified in (Hinde and Smith 1974)) renopericardium, lipid-like sheaths surrounding the cephalic ganglia, and pedal mucus gland (Robert K Trench 1969). These experiments imply that the slug does actually benefit from its incorporated chloroplasts and that these plastids allow a slug to withstand long starvation periods.

McLean (1972) analyzed *Placida dendritica* J. Alder & A. Hancock, 1843, finding that it cannot survive long starvation periods nor retain functional chloroplasts although it also feeds on *C. fragile.* This indicates that distinctive slug species have differing degrees of functional kleptoplasty, the slug species determines if kleptoplasty is possible and more research was needed to determine how and why kleptoplasts from the same alga could be maintained in one species (*E. viridis*) but not the other (*P. dendritica*). Each additional species investigated was shown to have differing life histories, habitats, larval types and food preferences (Thompson and Brown 1976). The differences in food preference were of particular interest, since different chloroplast species may determine whether or not the plastid can be incorporated and how well the slug can withstand starvation.

1.4 Algal Species and Functional Kleptoplasty

The examination of algal food sources in sacoglossan slugs has impacted our understanding of functional kleptoplasty by demonstrating that the algal species involved is also responsible for the successful sequestration of plastids. Two main hypotheses have been examined in the last twenty years, 1) certain algal genes needed to maintain a chloroplast were transferred from the algal cell nucleus to the slug's nucleus allowing the slug to maintain its incorporated plastids and 2) factors inherent to the plastid itself allows their retention in certain sacoglossan species.

A horizontal (also called lateral) transfer of algal genes (HGT) to the slug, would elegantly explain how a foreign, algal organelle is maintained in a metazoan tissue, because it implies the slug has the algal genes to support this organelle already incorporated into it's DNA. While numerous studies originally provided evidence that showed this to be a possibility, they were often based on single-gene PCR techniques (Hanten and Pierce 2001; Mujer et al. 1996; S. Pierce et al. 1996; S. K. Pierce et al. 2003; Rumpho et al. 2001a, 2008; Schwartz et al. 2010). Since separating slug tissue from it's incorporated chloroplasts is nearly impossible due

to the highly branched structure of the digestive gland tubules that run throughout their bodies, the algal genes that were "identified" are likely contamination rather than evidence of algal genes inside the slug's nucleus. Recently, deep-sequencing and egg sequencing approaches have revealed a lack of algal genes in slug tissues, confirming that algal genes were not transferred to *Elysia timida, Plakobranchus ocellatus* (Wägele et al. 2011) nor *E. chlorotica* (Bhattacharya et al. 2013; Pelletreau et al. 2011). Although some researchers continue to search for evidence of HGT in sacoglossan slugs, a majority of sacoglossan researchers now reject this hypothesis (reviewed in Rauch et al. 2015; Heike Wägele and Martin 2014).

The second hypothesis recently examined explores whether algal plastids have inherent factors allowing their retention. This is supported by investigations into kleptoplast retaining species, *Plakobranchus ocellatus,* which does not have the same plastid composition after two months in starvation, as it did when it began starving (Christa, Wescott, et al. 2013). This supports the hypothesis that plastid viability inside the slug differs from algal species to algal species. Further supporting this hypothesis, *Elysia viridis* specimens that are fed *Codium fragile* have higher growth rates than those fed *Cladophora rupestris* (Baumgartner et al. 2015), and can only retain chloroplasts from *C. fragile*, directly digesting those ingested from *C. rupestris* (G. Christa unpublished results). Botanists have long speculated that some algal lineages have plastids that are more robust than others (Giles and Sarafis 1972), and the accurate identification of sacoglossan species' food sources is allowing the examination of this theory. Genes such as *ftsH* and *tufA* are involved in the repair of D1, a crucial and easily photo-damaged protein belonging to photosystem II (PS II). These genes are absent in higher plant chloroplasts but present in *Acetabularia acetabulum* and *Vaucheria litorea*, two species that are ingested by long-term plastid retaining slugs (de Vries et al. 2013). This means that *A. acetabulum* and *V. litorea* plastids may be able to partially repair themselves once incorporated into the slugs digestive gland, allowing them to function longer in the slug, also this requires confirmation (de Vries et al. 2013). Other factors that may facilitate the sequestration of some plastid species over others have yet to be described.

1.5 Chloroplast Functionality in the Slugs

In 2001, Wägele and Johnsen introduced a method of measuring and monitoring photosynthetic activity in sacoglossan tissues, called Pulse Amplitude Modulated (PAM) fluorometry. PAM values express an estimated proportion of the number of absorbed photons that can drive photochemistry in PS II using chlorophyll a autofluorescence (Perkins et al. 2002; Wägele and Johnsen 2001). The effective quantum yield values derived from PAM fluorometry are relative values, comparing the number of available PSII reaction centers to the number that could be actively involved in photosynthesis. As qualitative measurements, PAM values do not reflect how many reaction centers there are in the examined tissue, instead providing an estimate of how efficient any photosynthesis in the tissue is and confirming that there is chlorophyll present.

PAM Fluorometry has greatly increased our understanding of photosynthetic activity with numerous sacoglossan species. Numerous studies have applied this technique throughout sacoglossan starvation periods revealing a range in photosynthetic abilities depending on the both the slug and algal species (Christa, Händeler, Kück, et al. 2014; N E Curtis et al. 2015; de Vries et al. 2015; K Händeler 2011; Krug et al. 2012; Schmitt et al. 2014; Ventura et al. 2013, 2013; Vieira et al. 2009; Wägele and Johnsen 2001). Händeler et al. 2009 classified slugs based on PAM values, showing three distinct groupings, Non-plastid-Retaining (NR), Short-term Retaining (StR) and Long-term Retaining (LtR). NR species are slugs that incorporate chloroplasts from their algal food and then immediately digest them, whereas StR and LtR species retain functional plastids for over a week (StR) or over a month (LtR). Multiple investigations have now furthered the list of species investigated by PAM measurements revealing about 36 species as StR forms, 6 as LtR forms and 45 as NR (Christa, Händeler, Kück, et al. 2014; Katharina Händeler et al. 2009; Klochkova et al. 2010, 2013; Schmitt et al. 2014; Ventura et al. 2013; Vieira et al. 2009). When examined regarding phylogeny, the NR- StR- LtR classification reveals that functional kleptoplasty likely evolved more than once. While every investigated oxynoacean species has been designated NR, both the

Limapontioidea and Plakobranchoidea have LtR and StR species (Christa 2014; Christa, Gould, et al. 2014). A recent overview depicting most of the species investigated and their food preferences, taken from Christa et al. (2014) can be seen in Figure 1.4.

1.6 The Development of Functional Kleptoplasty

Adding to the complex nature of functional kleptoplasty, juveniles of both long- and short-term retention species must individually acquire this ability, meaning something in the digestive glands of these animals changes during development to facilitate kleptoplast retention. This line of inquiry began when Trench et al. (1969) reported a lack of chloroplast pigments in juvenile slugs indicating that chloroplasts must be acquired by every generation and are not passed from parent to offspring. This has been confirmed by numerous studies that observed egg masses and newly hatched juveniles (de Vries et al. 2015; Pelletreau et al. 2012, 2014; Schmitt et al. 2014), as well as transcriptomic studies that investigated whether or not algal genes were expressed in egg masses (Bhattacharya et al. 2013; Han et al. 2015). These experiments not only point out a change in the digestive gland cells of a developing slug, they also confirm that the slug is involved in plastid retention. This means that the right algal species must be ingested by the right sacoglossan species at the right time in its development for functional kleptoplasty to occur.

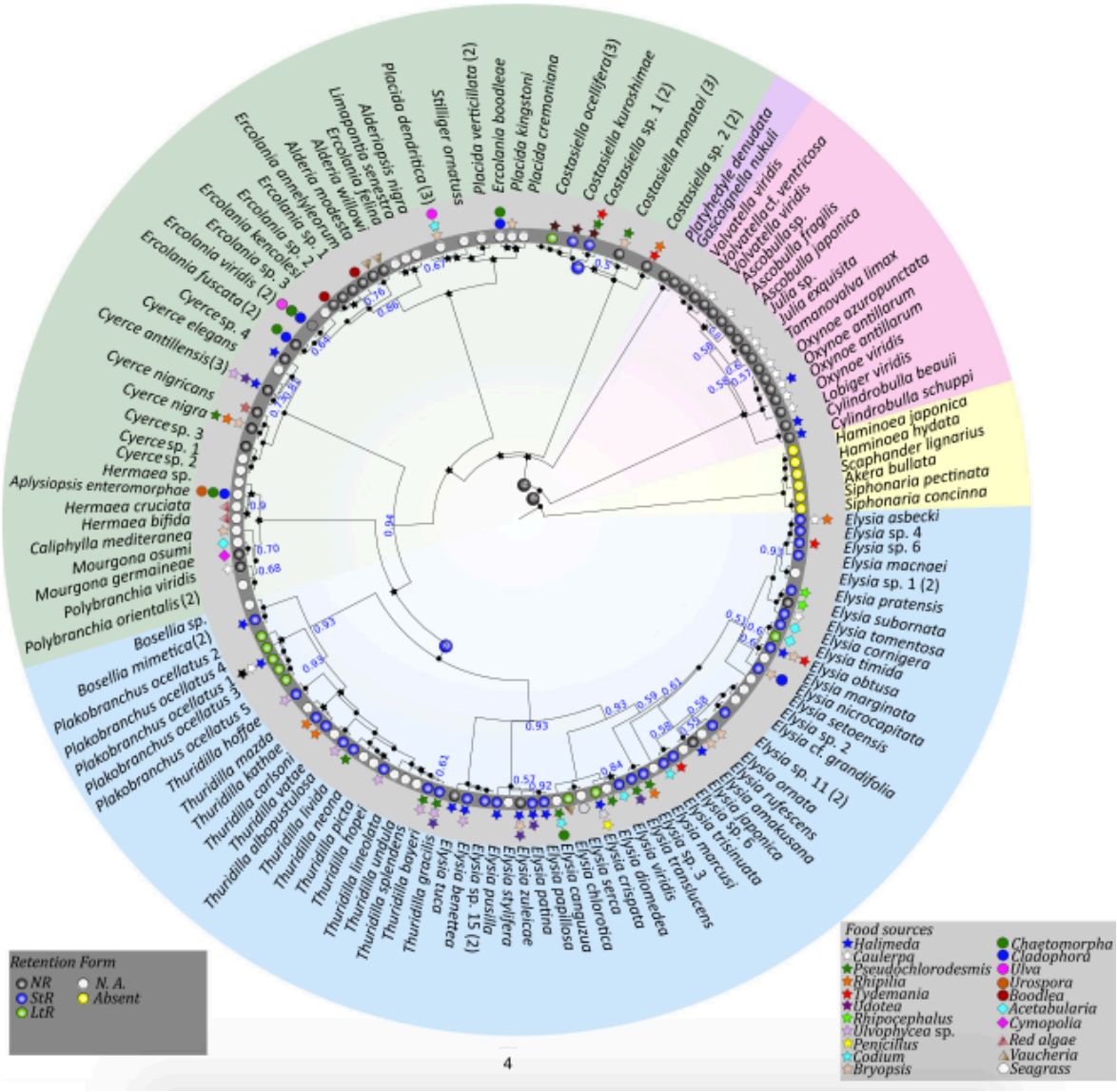

Figure 1.4 Sacoglossan phylogeny, food sources and chloroplast retention grouping (non-, short- and long-term retention) taken from Christa, et al. 2014. The original caption accompanying this figure is: Phylogeny of the Sacoglossa based on Bayesian analysis (50 % majority rule consensus tree). Numbers at nodes indicate Posterior Probability (PP), black circles indicate PP=100, and black asterisks indicate PP=95–99. The inner dark gray circle borders functional-retention information of taxa, the outer food sources (displayed are only three major food sources per species). The scale bar displays substitutions per site. Yellow highlighted is the "outgroup", pink the Oxynoacea, purple the Platyhedylidae, green the "Limapontioidea", and blue the Plakobranchoidea. Numbers in brackets behind species names indicate number of investigated individuals for this species. Gray circle in the tree displays the evolution of non-functional retention at the base of the Sacoglossa obtained by Ancestral Character State reconstruction (ASR); blue circles display the multiple, independent evolution of short-term retention. Information on food sources and retention form was taken out of literature (Christa et al. 2014d) or generated in this study.

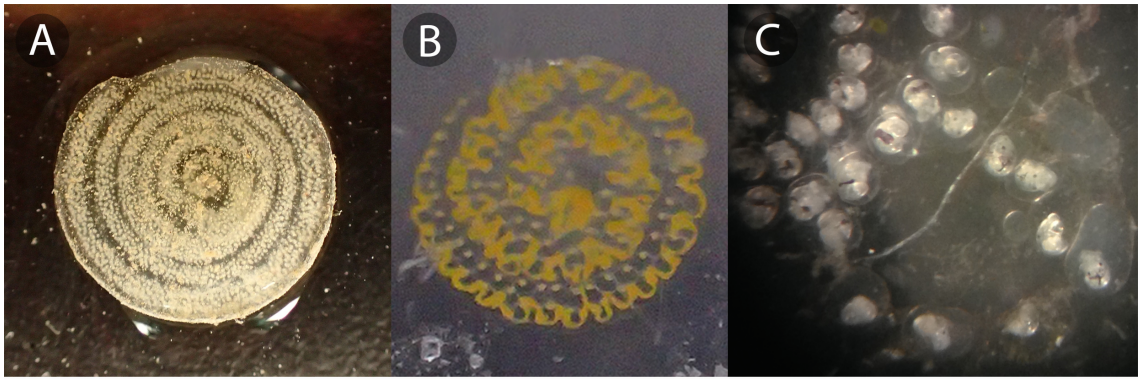

Figure 1.5 Eggs and embryos lacking chloroplasts. A: *Elysia clarki* egg mass – 1.6cm in diameter. B: *E. timida* egg mass – 6mm in diameter. C: *E. timida* developing embryos – each egg has ~100µm diameter. Neither the egg masses nor the embryos have green pigment indicating chloroplasts are present.

To summarize, the last 125 years of sacoglossan research have reached a number of generally accepted conclusions regarding functional kleptoplasty.

1. Most sacoglossans do not retain chloroplasts and survive starvation for extended time spans (more than a few weeks).
2. Retained chloroplasts are capable of fixing carbon.
3. Photosynthetically derived carbon can be found in numerous slug-produced compounds, meaning the slugs are receiving photosynthetic products.
4. The six confirmed long-term retention species are not closely related.
5. Chloroplast-retaining species can be stenophagous or polyphagous.
6. Long-term functional kleptoplasty likely evolved at least two times, in two independent lineages.
7. Both the right algal species and the right slug species are needed for long-term functional kleptoplasty to occur.
8. Every generation must acquire it's own plastids - they are not transferred from parent to offspring.

1.7 Ongoing Debates Regarding Functional Kleptoplasty

Despite recent advancements in our understanding of which slug species conduct functional kleptoplasty, which algal species are involved, how long plastids remain photosynthetically active, which genes may be involved and how often it has evolved, almost nothing is known about how functional kleptoplasty actually occurs. Functional kleptoplasty is a multiple step process, and almost all of the steps remain uninvestigated. I determined the following steps based on ultrastructural observations from *E. timida, E. viridis, P. dendritica* and *P. ocellatus* (Hirose 2005; Martin et al. 2013; McLean 1976; R K Trench et al. 1973). These steps are presumed to hold true for species other than *E. timida, P. ocellatus, P. dendritica* and *E. viridis,* but cannot be confirmed until functional kleptoplasty is investigated in other species.

Functional kleptoplasty begins with 1) the ingestion of algal cytoplasm by the slug. This sap travels through the esophagus, stomach and into the digestive gland lumen where it is 2) endocytosed into the digestive gland cells. The chloroplasts are then considered incorporated (3), while the other cell contents are digested. (4) Kleptoplasts are then retained for over a week in short-term retention species or over a month in long-term retention species. 5) Kleptoplasts begin to break down and are eventually no longer found in the digestive gland tissue leaving large vacuoles where they previously were. While Step 1 has been thoroughly investigated as described in Chapter 1.4, the rest have hardly been investigated for any species. Step 2, the method by which plastids enter digestive gland cells has been referred to as phagocytosis (Giménez-Casalduero and Muniain 2006; Hirose 2005; Martin et al. 2013; McLean 1976; S. K. Pierce et al. 2015), however this is merely hypothesized since this entry method has not been confirmed by experimentation. Step 3 involves selected digestion, and has not been investigated to date. The fourth and fifth steps involve chloroplasts incorporated in the algal cell and their eventual breakdown, a subject that has been the course of much debate with very little resolution.

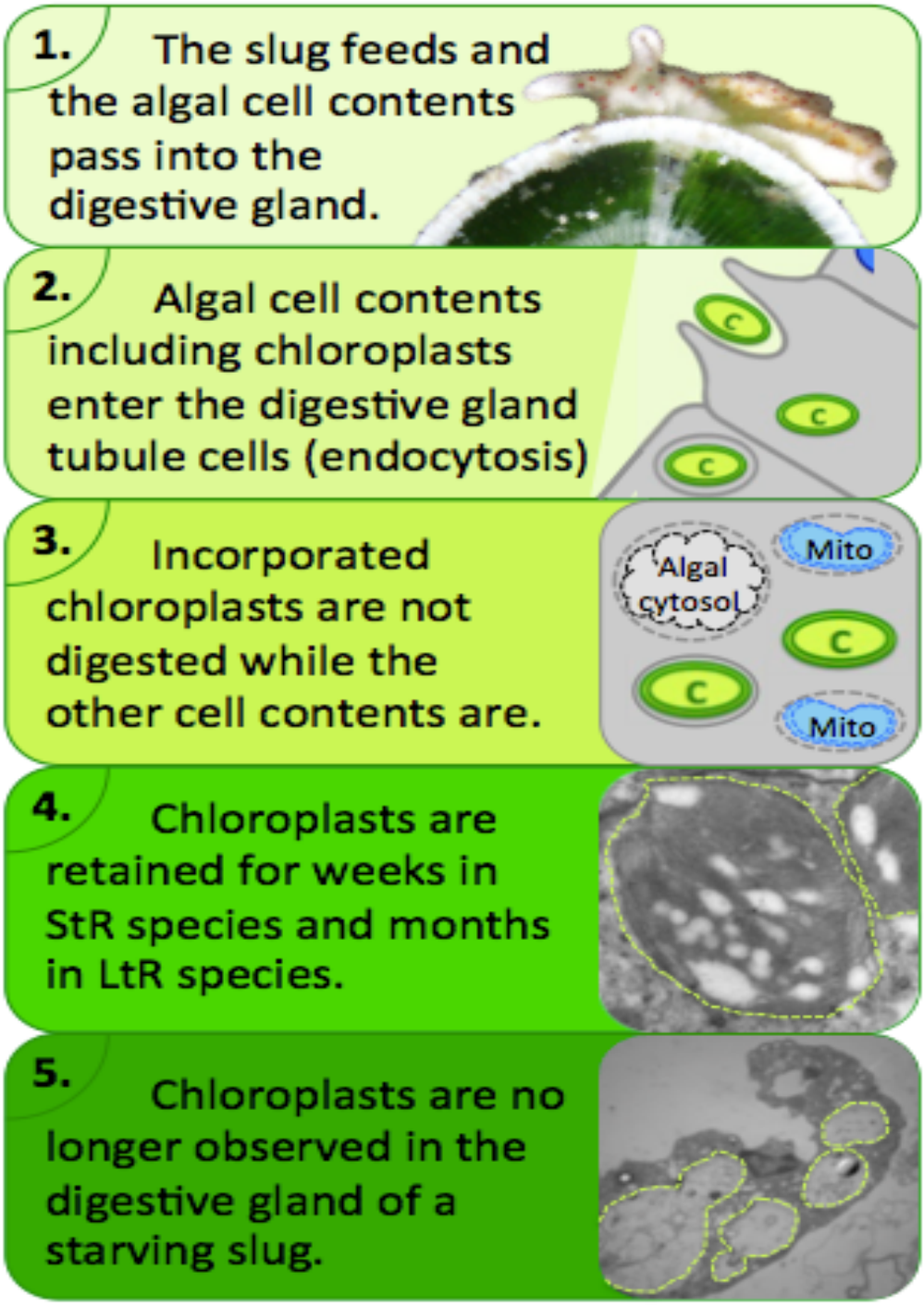

Figure 1.6 Steps involved in functional kleptoplasty. Photos – 1: *E. timida* feeding on *A. acetabulum.* 2: Chloroplasts are endocytosed into the digestive gland tubule cells from the digestive gland lumen. 3: Non-chloroplast cell contents (algal cytosol and mitochondria depicted here) are digested (indicated by dashed lines) but the chloroplasts are not. 4: TEM micrograph of *E. timida* chloroplasts inside the digestive gland cells (outlined with dashed line). 5: Empty vacuoles in the *E. timida* digestive gland showing where chloroplasts previously were (outlined with dashed line).

When a chloroplast is endocytosed into a slug's digestive gland cell, it gains a membrane from the endocytosis itself. In chlorophyte plastids, which are naturally surrounded by a double membrane, the number should increase to three membranes surrounding a newly incorporated plastid. The presence of this membrane at both, the moment of incorporation and during periods of starvation, has been highly debated with no clear consensus. TEM micrographs of chloroplasts throughout starvation periods sometimes clearly reveal a third membrane, although other micrographs show a fragmented third membrane or the absence of a third membrane altogether. Authors presenting fragmenting membranes suggest the third membrane is initially present but disintegrates after a certain time leaving the chloroplast freely floating in the slug's digestive gland cell. Since fragmented membranes could be due to imperfect fixation and/or artifacts, this hypothesis is still not adequately supported and requires further investigation.

Another ongoing debate regarding incorporated plastids (Step 4) discusses the benefits to having incorporated chloroplasts. As described in Chapter 1.2, radiolabeling studies have revealed algal-derived carbon in slug tissues, but it remains unclear whether the plastids themselves are the source of the carbon (energy) a starving slug uses, or if any photosynthates they produce support the starving slug. Previous investigations support the photosynthate theory more, since ^{14}C was found in slug tissues within hours of exposure (R K Trench et al. 1973, 1974; Robert K Trench et al. 1972), when the chloroplasts still appear intact in TEM micrographs. Starch grains have also been observed in numerous slug and algae species (R K Trench et al. 1973, 1974). Pelletreau et al. (2012, 2014) investigating *E. chlorotica* feeding on the heterokontophyte *V. litorea* found accumulations of lipid droplets during starvation suggesting the lipids were photosynthetically produced and may benefit the starving slug. Lipids can derive from metazoans too, however, so this conclusion may be premature and it requires further examination. Contrary to these studies, a few reports hypothesize that the kleptoplasts themselves are the food source that sustain a starving slug rather than the photosynthates they produce. This is supported by light/dark experiments where each treatment survived the same duration, indicating that light-powered photosynthesis may not be responsible for starving slug longevity (Christa,

Zimorski, et al. 2013a). With neither of these hypotheses confirmable with current data, further investigations are needed to resolve this issue.

1.8 Aims and Contents of this Investigation

This thesis aims to clarify some of the ongoing debates detailed in the previous chapters by examining digestive activity, photosynthate production and juvenile development throughout starvation periods in sacoglossan sea slugs. I focused on the model species *Elysia timida* and *Elysia cornigera* because they are confirmed sister-taxa that eat only one algal genus, *A. acetabulum* and only *E. timida* is capable of long-term kleptoplast retention. Other sacoglossan species were also examined in some experiments to better understand the long-, short- and non-retention categories and to compare species within each group.

Specifically, I am attempting to answer the following questions throughout the subsequent eight chapters:

1. How many kleptoplasts are present in sacoglossan tissues throughout the starvation period? Does this differ between LtR and StR species?

The number of kleptoplasts within sacoglossan tissues has never been assessed and could reveal differences amongst species that support kleptoplasty in some.

2. Is it possible to monitor digestive processes in living sacoglossan digestive gland cells? If so, what patterns of digestive activity occur and do they relate to starvation longevity?

Understanding digestive processes in sacoglossan slugs could reveal why plastids from the same algal species are digested in some species and retained in others.

3. Do sacoglossan slugs actually digest chloroplasts? Does this differ between species?

This has never been examined despite being assumed for many years now, and sacoglossan slugs may not actually digest chloroplasts at all, instead excreting them.

4. Are photosynthates produced by incorporated plastids? If so, which photosynthates are produced and when do they become available to the slug? Does this differ between long-term plastid retaining species and short-, non-retaining species?

Carbon fixation indicates photosynthetic products, but if/when these photosynthates become available to the slug has never been assessed in a chlorophyte-feeding model system. Since the main photosynthate produced by chlorophytes is starch, and metazoans do not produce starch, any starch observed is of algal origin.

5. Does temperature account for the differences in functional kleptoplasty between *E. timida* and *E. cornigera*?

E. cornigera is found in the Caribbean Sea in waters averaging 25°C while *E. timida* is found in colder Mediterranean waters. Temperature affects the rate of all chemical reaction, including those governing functional kleptoplasty and this could be a crucial factor in determining any differences between these species.

6. Do juvenile *E. timida* have different digestive activities that could account for their inability to retain incorporated plastids?

Juvenile *E. timida* are not capable of functional kleptoplasty and they directly digest incorporated chloroplasts. Changes in digestive activity may be responsible for the transition from direct digestion to functional kleptoplasty.

These questions are addressed in the subsequent five chapters taken from individual studies, a subsequent chapter reviewing experiments that were unsuccessful and/or still in progress and a general discussion.

Chapter 2

Examining the Retention of Functional Kleptoplasts and Digestive Activity in Sacoglossan Sea Slugs

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Abstract

Solar-powered sea slugs (Sacoglossa: Gastropoda) have long captured the attention of laymen and scientists alike due to their remarkable ability to steal functional chloroplasts from their algal food, enslaving them to withstand long starvation periods. Recently, a wealth of data has shed insight into this remarkable relationship; however, the cellular mechanisms governing this process are still completely unknown. This study explores these mechanisms, providing insight into the chloroplast retention and delayed digestion, occurring within the slug's digestive gland. We examine the relation- ships between functional chloroplast and lysosome abundances during starvation, in live material, for the long-term retaining species *Elysia timida*, the ambiguous long/short- term retaining *Elysia viridis*, and the short-term retaining *Thuridilla hopei*, to elucidate digestive differences that contribute to the development of functional kleptoplasty. Functional chloroplast and lysosome abundance are measured using chlorophyll a autofluorescence and the pH-dependent stain acridine orange. In each species, the number of chloroplasts and lysosomes is indirectly proportional, with the plastid density decreasing when starvation begins. We also present a new FIJI/Image J Plugin, the 3D—Accounting and Measuring Plugin, 3D-AMP, which enables the reliable analysis of large image sets.

2.1 Introduction

Sacoglossan sea slugs (Gastropoda: Heterobranchia) have gained the nicknames “solar-powered sea slugs” and “leaves that crawl” due to some species’ ability to acquire and retain functional chloroplasts (cps) for months on end (Greene 1970; Rumpho et al. 2000; D. L. Taylor 1968). Although acquired phototrophy, the use of photosynthates from incorporated photoautotrophic organelles, has been described in ciliates, foraminifers, and dinoflagellates (Johnson 2011; Lindholm and Mork 1989; Stoecker et al. 2009), sacoglossan sea slugs are the only known metazoans with this ability. Recent research has focused on the evolution and development of this extraordinary ability from an ecological, and more recently, genetic standpoint (Christa, Händeler, Kück, et al. 2014; Christa, Wescott, et al. 2013; de Vries et al. 2014, 2015; Katharina Händeler et al. 2009; Pelletreau et al. 2014; Wägele et al. 2011; Wägele and Johnsen 2001). These studies show the development of long-term retention (LtR) and functional kleptoplasty in several sacoglossan lineages and suggest this ability evolved independently within different clades (Christa, Händeler, Kück, et al. 2014; Christa, Wescott, et al. 2013; de Vries et al. 2014, 2015; Katharina Händeler et al. 2009; Pelletreau et al. 2014; Schmitt et al. 2014; Wägele et al. 2011; Wägele and Johnsen 2001), meaning there is likely a difference in the cp-storing digestive glands of slug species capable of LtR, versus those incapable (StR and NR). Furthermore, these digestive gland cells digest non-plastid algal cell contents while leaving the cps intact, suggesting selected digestion (Christa, Zimorski, et al. 2013a). Since both LtR slugs and StR slugs have stenophagous and polyphagous members, and amongst the polyphagous LtR forms, the survival duration of differing algal cp species is highly variable; the algal species contributing cps also influences functional kleptoplasty (Christa, Händeler, Kück, et al. 2014; Christa, Händeler, Schäberle, et al. 2014; Christa, Wescott, et al. 2013; N E Curtis et al. 2015; Nicholas E Curtis et al. 2006; Katharina Händeler et al. 2009). This reveals that functional kleptoplasty is a complex system that must include the right species of slug acquiring the right algal cp species in order to occur.

Kleptoplast functionality has been measured within sacoglossan tissues for many years, using pulse amplitude modulated (PAM) fluorometry, a system that measures the chlorophyll a autofluorescence originating from photosystem II (Christa 2014; Christa, Zimorski, et al. 2013a; N E Curtis et al. 2015; de Vries et al. 2015; Schmitt et al. 2014; Wägele and Johnsen 2001; Wägele and Martin 2014). The effective quantum yield values obtained from PAM fluorometry provide a relative value of the number of reaction centers actively involved in photosynthesis, an index of how efficiently photosystem II is working in a given tissue. This qualitative measurement does not actually indicate the quantity of photosynthesis reaction centers occurring within this sample, although chlorophyll a must be present and functioning to yield a signal. Recently, pigment analyses have attempted to bridge this gap, providing quantitative measurements of pigment concentrations in sacoglossan tissues; however, this data does not assess pigment functionality, is therefore unable to be compared to digestion rates within these tissues, and these studies have not accounted for starvation time periods (Baumgartner et al. 2015; Rauch et al. 2015; Ventura et al. 2013).

Functional kleptoplasty is a complex process involving a number of steps, each of which is crucial to its development and performance: (1) the slug must sequester algal cell contents from an algal species with cps capable of being retained within the slug's digestive gland, (2) these algal cell contents must be taken up into the slug's digestive gland cells, (3) incorporated cps must not be digested while the other cell contents are, and (4) cps are then retained for many months where they may remain active in some slugs, contrasting NR forms where they do not (N E Curtis et al. 2015). However, incorporated active cps do lose their functionality over time (Christa 2014; Christa, Zimorski, et al. 2013a; N E Curtis et al. 2015; de Vries et al. 2015; Schmitt et al. 2014; Wägele and Johnsen 2001; Wägele and Martin 2014) and may or may not still be active when they are (5) finally digested. The mechanisms behind these steps have yet to be investigated with clear interpretable results. Many studies have examined the kleptoplast incorporation, retention, and later digestion processes using transmission electron microscopy, to identify the method by which cps enter digestive gland cells and

examine cp breakdown at a few time points during starvation (Martin et al. 2013; Wägele and Martin 2014). Other studies have focused on enzymatic analyses (D. L. Taylor 1968) and more recently on gene expression (de Vries et al. 2015); however, none of these methods surveyed enough time points during the starvation process to accurately detail how and when intracellular digestion occurs within these tissues, nor do they provide images that show exactly what is occurring inside the digestive gland cells. To assess these problems, we developed and present here the first intracellular glimpse into digestion in these cells, by using confocal scanning laser microscopy to monitor functional kleptoplasts and lysosomes, as indicators of active digestion, in living digestive gland cells.

We examined starved animals at numerous time points, to determine functional kleptoplast abundance throughout a starvation period. This is compared to animals that are first starved and then later reintroduced to food to determine the difference in kleptoplast abundance. Lysosome abundance and location were also measured to explore a correlation between starvation point and digestion. The LtR species *Elysia timida* is compared to the LtR/StR species *Elysia viridis* Montagu, 1804 (the ingested algal species determines how long *E. viridis* retains cps, causing its classification as either LtR or StR, depending on the study (Baumgartner et al. 2015; Katharina Händeler et al. 2009)(Baumgartner et al. 2015; Händeler et al. 2009)) and the StR species *Thuridilla hopei* Vérany, 1853 to determine if digestion patterns differ in species performing different degrees of kleptoplasty. A large data set was analyzed comprising hundreds of specimens and thousands of images taken at different time points during each species' respective starvation period. The size of the data set produced required us to develop a new FIJI plugin, 3D-AMP (3D—Accounting and Measuring Plugin) to automatically and reproducibly analyze our images and handle the sheer volume of generated data.

2.2 Materials and Methods

2.2.1 Live Material

E. timida adults (Figure 2.1A) were individually collected in Fetovaia on the island of Elba, Italy, May 2015 (population 1), and Blanes, Spain, in August 2015 (population 2) alongside *T. hopei* (Figure 2.1C). Stones covered in *Acetabularia acetabulum* were also collected in Fetovaia and Cavoli, Elba, Italy. *E. viridis* and *Codium tomentosum* Stackhouse, 1797 were collected in July 2015 in Porto, Portugal (Figure 2.1B). All specimens were kept at 18 °C with artificial lighting providing 220 $\mu E^{-2}s^{-1}$, full spectrum light for 12 h L : 12 h D. Twenty to 25 animals shared a 5 L aerated tank filled with about 3 L of freshly prepared artificial seawater. An acclimatization week with feeding was provided before the animals were removed from the algae and placed in empty tanks for the duration of their starvation periods. During starvation, all animals were housed in the similar tanks and only removed for tank cleaning and experimentation. When reintroduced to food, specimens were monitored until feeding commenced (as indicated by buccal contractions which indicate sucking) and then allowed 30 min to feed. Since 30-min feedings resulted in no visible change in lysosomal or chloroplast density, a second population was collected, starved, and allowed 2 h to feed. They were then removed from the algae and processed using the same methods outlined for the starving animals. Specimens were randomly selected for re-feeding and staining. *E. timida* population 1 was sampled at 0, 1, 3, 7, 14, 30, and 42 days; *E. timida* population 2 was sampled at 0, 3, 7, 14, 21, 30, 42, 60, and 89 days, *E. viridis* at 0, 1, 7, 14, 21, and 30 days, and *T. hopei* at 0, 7, 14 and 21 days. There were no permissions or permits required to collect any of the species involved.

2.2.2 Chloroplast and lysosome abundance in starving slugs

Lysosomes were visualized with the stain acridine orange (AO), a vital stain occurring in two forms when in solution. These two forms stain different structures in living and dead tissues and emit photons at different wavelengths when excited by blue laser at 488 nm. In living tissues, monomer type AO stains

RNA (533 nm emission) and dimer-type AO aggregates in extremely acidic organelles (lysosomes) (656 nm emission). In dead tissues, monomer type AO intercalates into double-stranded DNA (533 nm emission) and dimer-type AO intercalates into RNA (656 nm emission) (Kusuzaki et al. 2014; Moriyama et al. 1982). Adult *E. timida*, *E. viridis*, and *T. hopei* were sampled at different starvation points according to the time series seen in Table 2.1. Overall, 56 *E. timida* were starved from population 1, 62 from population 2, 33 *E. viridis*, and 12 *T. hopei*. The slugs were stained with acridine orange (a 5 µmol solution was prepared with filtered seawater) for 30 min.

Each sample was then pinned to a silicone-covered petri dish, decapitated, and vivisected (Figure 2.2A). Each cross-section was roughly 0.5 mm thick, mounted on a microscope slide, covered (Figure 2.2B,C), and imaged on a Leica SPE CLSM. Five scans were taken from different sections for each slug. Each scan contained eight images, each 1 µm thick, and therefore covered a depth of 8 µm to capture the entire digestive gland cell while avoiding the digestive gland lumen (Figure 2.3). Acridine orange fluorescence and chlorophyll a autofluorescence were measured using the blue laser (excitation 488nm), with 645–670 nm (AO dimer-type optimum 656) and 600– 640nm (chl a optimum 633) as the accepted emission ranges.

This allows dimer-type staining while excluding anything stained by the monomer type. Control animals that were not stained with acridine orange verified that the emission range 645–670 did not yield any noteworthy background signal. Once the method was established, a preset was created in the software so every image was captured using the exact same settings and parameters. All specimens were sampled at 11:00, in the exact middle of the daily light period, to avoid the daily size changes that occur in *A. acetabulum* cps (Driessche 1966). This process was repeated with animals from each starvation point that were reintroduced to food for 30 min and 2 h before staining and slicing to see if there was a change in lysosome quantity in response to the first feeding after starvation.

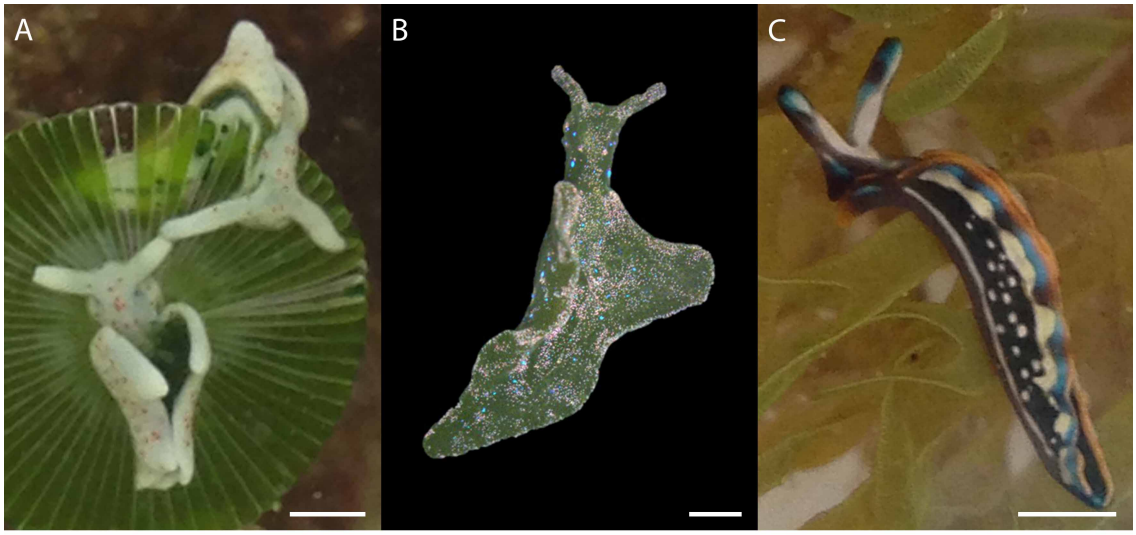


Figure 2.1 Investigated species. A: *Elysia timida* adults crawling on *Acetabularia acetabulum*. B: *Elysia viridis*. C: *Thuridilla hopei*. Scale bars ~2mm.

2.2.3 Image analysis—3D-AMP

The huge volume of images produced due to the high number of sampling points inhibited manual image analysis. We tested numerous available software packages and found their results to be inaccurate and irreproducible due the close proximity of the organelles to one another in our images (both plastids and lysosomes). This is due to the algorithms underlining these software packages. Rather than relying on object recognition software as these tools do, which needs clear boundaries around each object in order to discern one from another, we developed and presented here a script that relies on pixel-based counting. This method assesses each pixel in the image, evaluating whether it should be counted because it is not black indicating signal, or it should not be counted. While evaluating each pixel, 3D-AMP ignores every other pixel in the image.

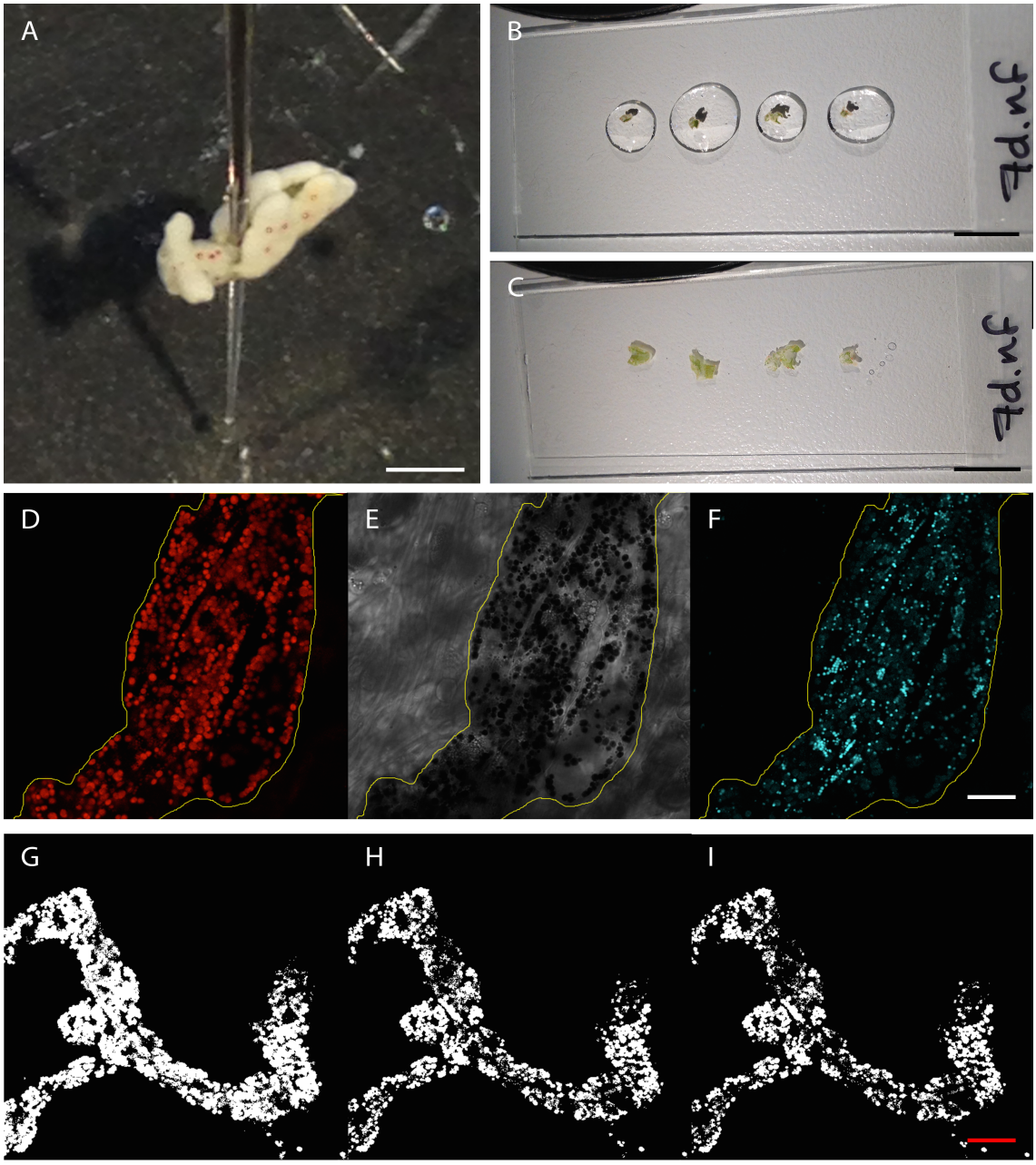


Figure 2.2 Vivisection method and 3D-AMP analysis. A: Acridine orange stained slugs (*E. timida* shown here) were pinned to a silicone-covered petri dish to immobilize them before decapitation and slicing. B: Individual slices were placed on a microscope slide in seawater before C: covered with a coverslip. D: Chloroplasts were imaged on one channel and false colored red. E: A transmission channel was used to help delineate non-digestive gland tissue (NDGT) from the digestive gland tissue (DGT) and was not used in the analysis. The dark spots seen in the digestive gland are individual chloroplasts. F: Lysosomes were observed using a third channel and falsely colored blue. The yellow line surrounding the digestive gland tubule was drawn in using FIJI and outlines the region of interest (ROI) used in the 3D-AMP analysis. G: A raw image before any thresholds have been applied. H: The same image, however a chloroplast threshold of 24, the value used in this analysis was applied to remove some background signal and noise. I: The same image with the threshold set to 36 to show an over-filtered image. Scale bars – A: 2 mm; B & C: 2 cm; D – F: 50µm; G – I: 50µm.

Table 2.1 - Specimen List. This table shows the number of specimens used in each experiment, for each time point sampled.

	Time point in days	# Animals starved	# of Animals starved & reintroduced to food
Elysia timida Series 1 Food Reintroduction 30 Minutes	0	8	n.a.
	1	3	3
	3	3	3
	7	3	3
	14	3	3
	21	3	3
	30	3	3
	42	3	2
Elysia timida Series 2 Food Reintroduction 2 Hours	0	8	n.a.
	1	3	3
	3	3	3
	7	3	3
	14	3	3
	21	3	3
	30	3	3
	42	3	3
	60	3	3
	89	3	3
Elysia viridis	0	6	n.a.
	1	3	3
	7	3	3
	14	3	3
	21	3	3
	30	3	0
Thuridilla hopei	0	3	n.a.
	7	3	n.a.
	14	3	n.a.
	21	3	n.a.

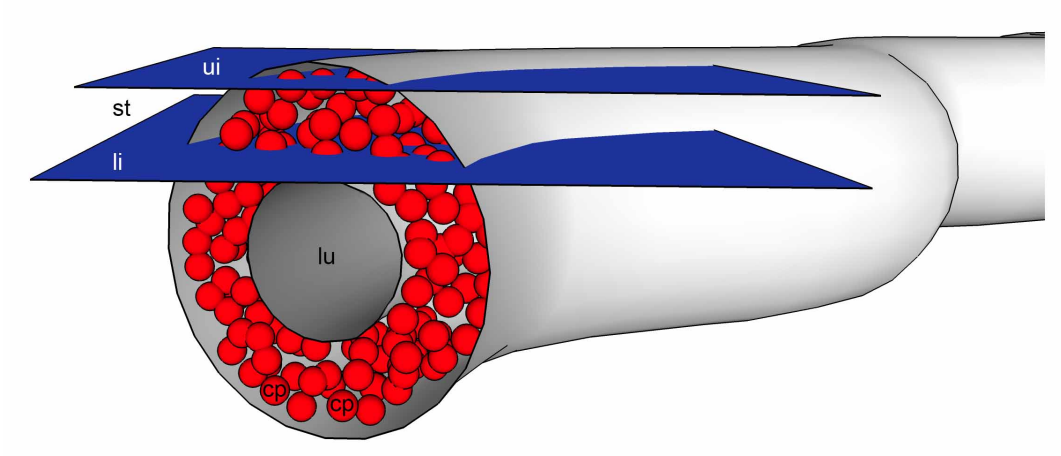
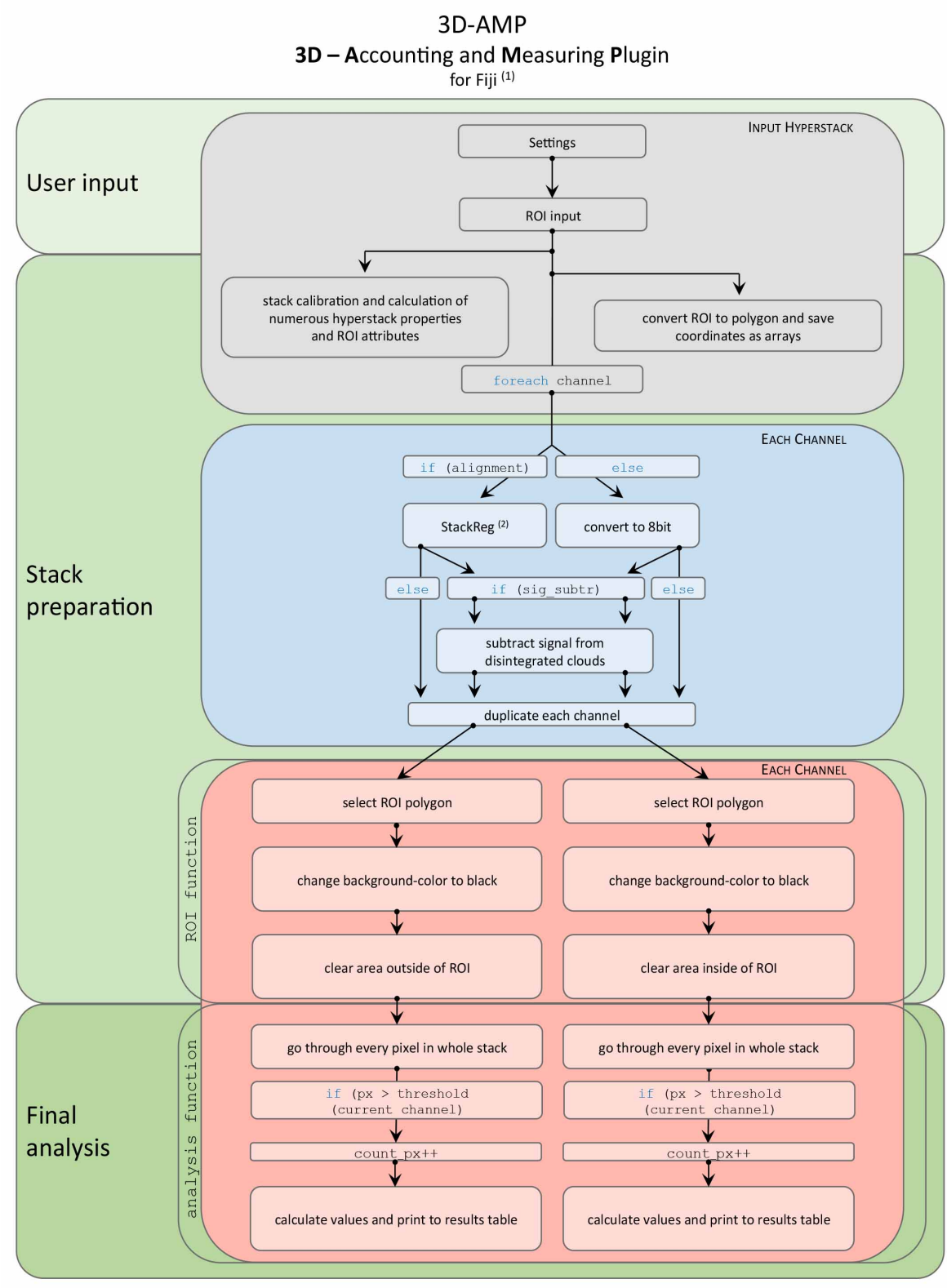


Figure 2.3 Digestive tubule schematic with imaging plane. Chloroplasts and lysosomes within the branched digestive gland were imaged by taking stacks of images transversally through the digestive gland cells. Efforts to avoid digestive gland lumen and surrounding tissues meant the stacks of eight images fit between the two planes depicted here and were each 8 µm thick or 1 µm/per image. Abbreviations: cp - chloroplast, li - lower image boundary, lu - digestive gland tubule lumen, st - surrounding tissue, ui - upper image boundary.

The plugin we present here is named 3D-AMP. 3D refers to the three-dimensional nature of image stacks and AMP stands for Accounting and Measuring Plugin. 3D-AMP was based on scripting functions already built into the FIJI software package (ImageJ version 2.0.0-rc-15) and counts pixels on a user-provided number of channels within and outside a user- defined region of interest (ROI). To filter out background fluorescence and any other less intense signals, thresholds can be set for each channel and only those pixels more intense than this threshold are counted. This means that some white pixels are not counted if the author chooses to filter them out using this function. The image scale can be entered to provide area calculations in square microns (μm^2). An alignment tool aligns all images in a stack to account for any sample movement while the image was being captured. The final ROI and non-ROI areas, pixel counts, and calculated pixel coverage areas are exported into a Microsoft Excel table for further analysis. The plugin layout is explained in Figure 2.4.

The images produced in this study were recorded using two emission channels and one transmission channel. Each ROI was defined as the entire digestive gland tubule (DGT) and the area outside the ROI was deemed non-

digestive related tissue (NDGT) (Figure 2.2D–F). The transmission channel images were used to help delineate DGT tissue from NDGT tissue and were not used for quantification (Figure 2.2E). Cp and lysosome (lys) abundance inside DGTs and NDGT were measured. As cps only occur in the digestive tubules, the non-ROI value was always 0. Lysosome abundance was calculated by subtracting the average area covered by lysosomes in NDGT from the average area in DGT area for each image, to account for the average lysosome abundance related to background cell maintenance rather than the digestive process. Numerous threshold values were analyzed and the optimal values (values that most closely aligned with the manually counted number of cps and lys) were used. Since 3D- AMP records pixels rather than objects, chloroplasts containing decreased chlorophyll content were still measured. The final thresholds were set to 24 for the chloroplast channel and 36 for the lysosome channel, to reduce back- ground noise while preserving fluorescent signal and the alignment function was used although very little tissue movement was observed (Figure 2.2G–I).



(1) SCHINDELIN, J.; ARGANDA-CARRERAS, I.; FRISE, E.; KAYNIG, V.; LONGAIR, M.; PIETZSCH, T.; PREIBISCH, S.; RUEDEN, C.; SAALFELD, S.; SCHMID, B.; TINEVEZ, J.Y.; WHITE, D.J.; HARTENSTEIN, V.; ELICEIRI, K.; TOMANCAK, P.; CARDONA, A. **(2012)**: Fiji: An Open-Source Platform for Biological-Image Analysis. *Nature Methods* **9**, 676-682.

(2) THÉVENAZ, P.; RUTTIMANN, U.E.; UNSER, M. **(1998)**: A Pyramid Approach to Subpixel Registration Based on Intensity. *IEEE Transactions on Image Processing* **7(1)**, 27-41.

Figure 2.4 3D-AMP design and logical flow chart.

2.3 Results

2.3.1 3D-AMP

3D-AMP was designed to facilitate a time efficient analysis of the thousands of images compiled in this study. While numerous tools already exist to quantify objects in CLSM images, they rely on blob analysis, which proved unreliable for the images produced here since our cps and lysosomes are densely packed and often lie next to one another. To accurately analyze these images, 3D-AMP utilizes pixel counting rather than blob detection, and the resulting values reflect an area measurement instead of an abundance value. The abundance can later be estimated using an average object size, which is easily computed in FIJI, by drawing ROIs around individual plastids and measuring the area inside. 3D-AMP's accuracy and reliability were measured by comparing manual counting to the automated values it calculated. One hundred chloroplasts were first measured manually, yielding an average diameter of 2.75 µm ± 0.44 and an average area of 5.94 μm^2 ± 1.02. 3D-AMP measured an average cp diameter of 2.93 µm ± 0.13 with a mean area of 6.74 μm^2 ± 0.35. Manually measuring lysosomes yielded an average diameter of 1.09 µm ± 0.72 and area of 0.93 μm^2 ± 0.19 compared to the automatically measured (3D-AMP) 1.3 µm ± 0.6 diameter and 1.32 μm^2 ± 0.12 area. A Student's t-test revealed no significant difference in these values (for cps, $p = 0.44$; lys, $p = 0.29$) meaning the accuracy of 3D-AMP's accounting and measuring is comparable to that achieved manually.

This software and a user manual can be downloaded from https://www.zfmk.de/en/3D-AMP.

2.3.2 Functional chloroplast degradation and lysosomal activity

The degradation of functional cps, as presumed by the de- creased chlorophyll a content, was observed in all of the tested species and specimens. In both *E. timida* (LtR) adult populations, this degradation was best modeled by exponential functions and supported by the r^2 value 0.9 for both populations 1 and

2 (Figures 2.5A, B and 2.6A, D, G, J). *E. viridis* (StR/LtR) cp degradation was best modeled by a quadratic function with $r^2 = 0.70$ (Figures 2.5C and 2.7A, D, G, J). *T. hopei* was only slightly best modeled with a quadratic function ($r^2 = 0.98$) compared to the exponential $r^2 = 0.92$ (Figures 2.5D and 2.8A, D, G, J). All three species showed rapid decline in their functional chloroplast abundance, regardless of whether they were LtR or StR.

Lysosomes were observed throughout the starvation period in all of the species examined. The average area covered by lysosomes in NDGT was compared to DGT to examine the baseline levels of digestion occurring inside and outside the digestive system. NDGT lysosomal activity was consistently low in *E. viridis*, rising only slightly from a NDGT mean of 2.4 % coverage for time points 0–7 days to 2.8 % for 14– 30 days (Figure 2.9C). Both *E. timida* populations also had low NDGT means, 0.17 and 1.5 % in populations 1 and 2, respectively (Figure 2.9A, B). *T. hopei* specimens, however, had low NDGT lysosome densities until the 14-day time point (mean 2.8 %) when this quantity rose substantially (mean 6.6 %); however, it was still always substantially lower than the DGT lysosome density (mean 20.8 %) (Figure 2.9D).

The lysosome abundance recorded within the DGT was higher than in NDGT for all of the species examined. In *E. timida* populations 1 and 2, DGT lysosomal abundance is low during the first time points, having an average percent area coverage of 1.4 and 1.03 %, respectively, and then quickly increasing after the 21-day time point (P1 $r^2 = 0.91$, P2 $r^2 = 0.96$) to an average of 61.4 and 69.7 % at 60 and 89 days of starvation (Figures 2.6B, E, H, K and 2.9A, B). *E. viridis* also displays an exponential increase in lysosome abundance throughout the starvation period ($r^2 = 0.66$); however, there are significantly more lysosomes at the beginning (9.7 %) and the increase is far greater (52.1 %) (Figures 2.7B, E, H, K and 2.9C). *T. hopei* shows an almost constant lysosomal abundance having a mean 22.2 % lysosome coverage at the beginning and 23.6 % in the later time points and only 0.41 r^2 support (Figures 2.8B, E, H, K and 2.9D).

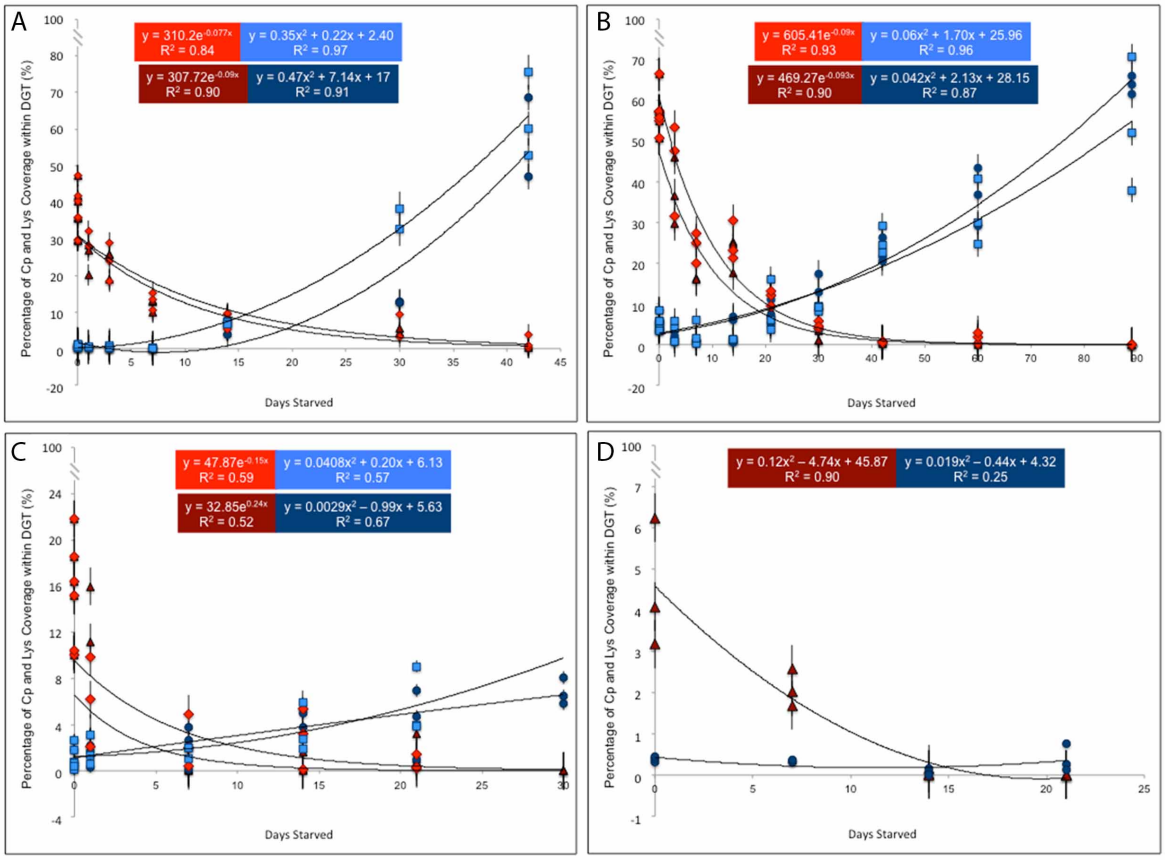


Figure 2.5 Average chloroplast and lysosome density per specimen. The percentage of digestive gland tubule covered by lysosomes and chloroplasts. Chloroplasts are depicted in dark red triangles for the initial experiments where starved animals were examined and in light red diamonds where starved animals were allowed to feed again before staining and imaging. The lysosome coverage measured in starved animals are shown by dark blue circles and the light blue squares correspond to re-fed animals. Each value was obtained by measuring the areas covered by chloroplasts and by lysosomes, independently, as well as the area covered by DGT and the area in NDGT tissue. The cp and lys areas were each divided by the DGT area (ROI in 3D-AMP) to produce the relative values graphed here. A: *Elysia timida* (population 1, spring) adults were starved for various time periods (the maximum starvation length achieved was 42 days) and half of the surveyed specimens were re-fed *Acetabularia acetabulum* for 30 min. B: *Elysia timida* (population 2, autumn) were starved for the same time points surveyed in population 1; however, specimens achieved a maximum 89 days starving and the half that were re-fed were provided 2 h. C: *Elysia viridis* starvation period with specimens re-fed for 2 h on Co*dium tomentosum*. D: *Thuridilla hopei* starvation period, no re-feeding experiments were possible due to a lack of reliable information on food preferences. The chloroplasts and lysosomes in each species showed an inversely proportional relationship, with the number of chloroplasts decreasing as the animals starve and the number of lysosomes increasing.

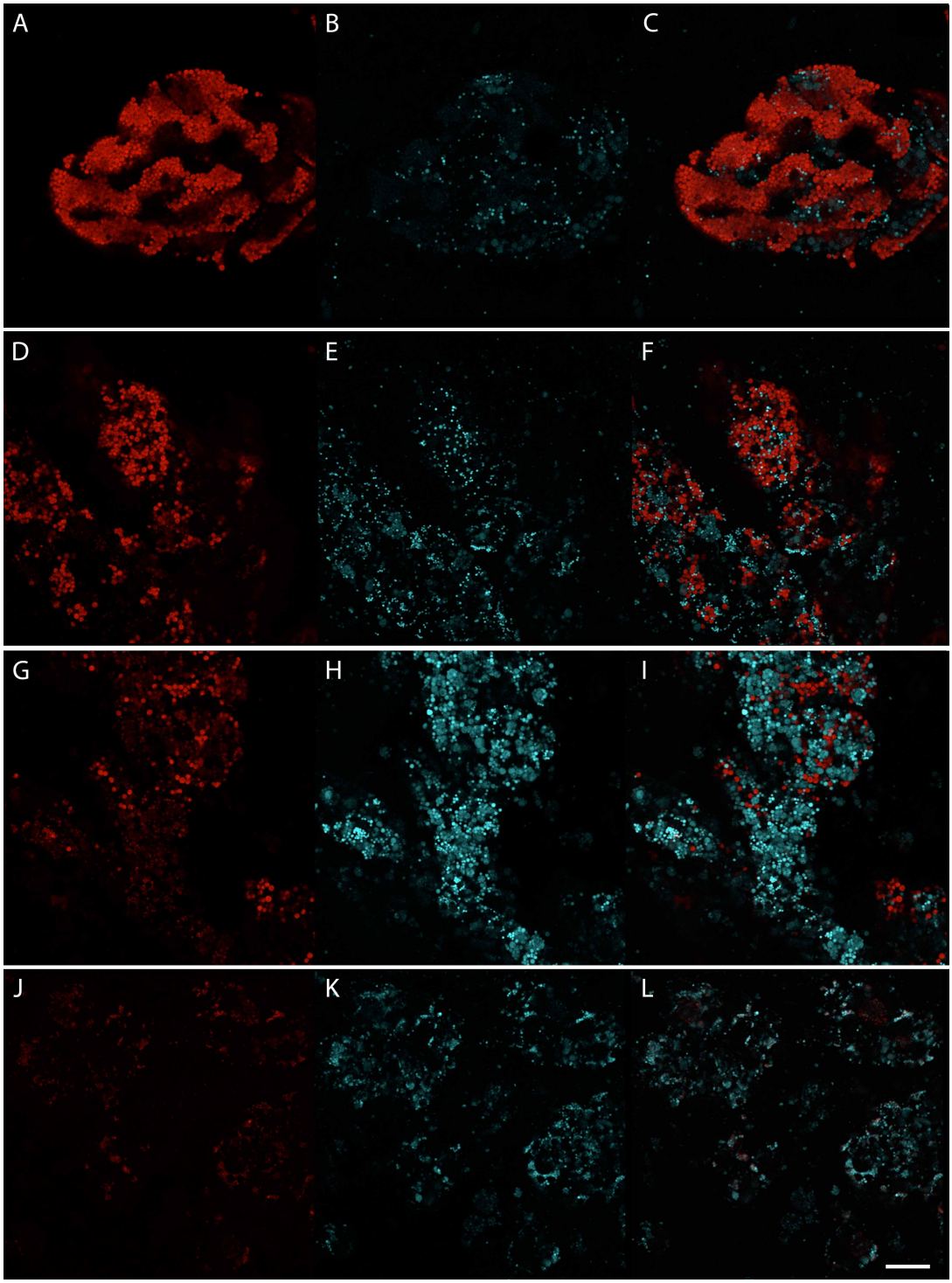


Figure 2.6 Chloroplast and lysosome density in *E. timida*. A: Chloroplasts (cps) (falsely colored red) in an unstarved specimen. B: Lysosomes (lys) (blue) in the same specimen. C: Composite of chloroplasts and lysosomes. D-F: Cps, lys, and composite after 14 days starvation. G–I: Cps, lys, and composite after 30 days starvation. J–L: Cps, lys, and composite after 60 days starvation. Scale bar: 50 µm.

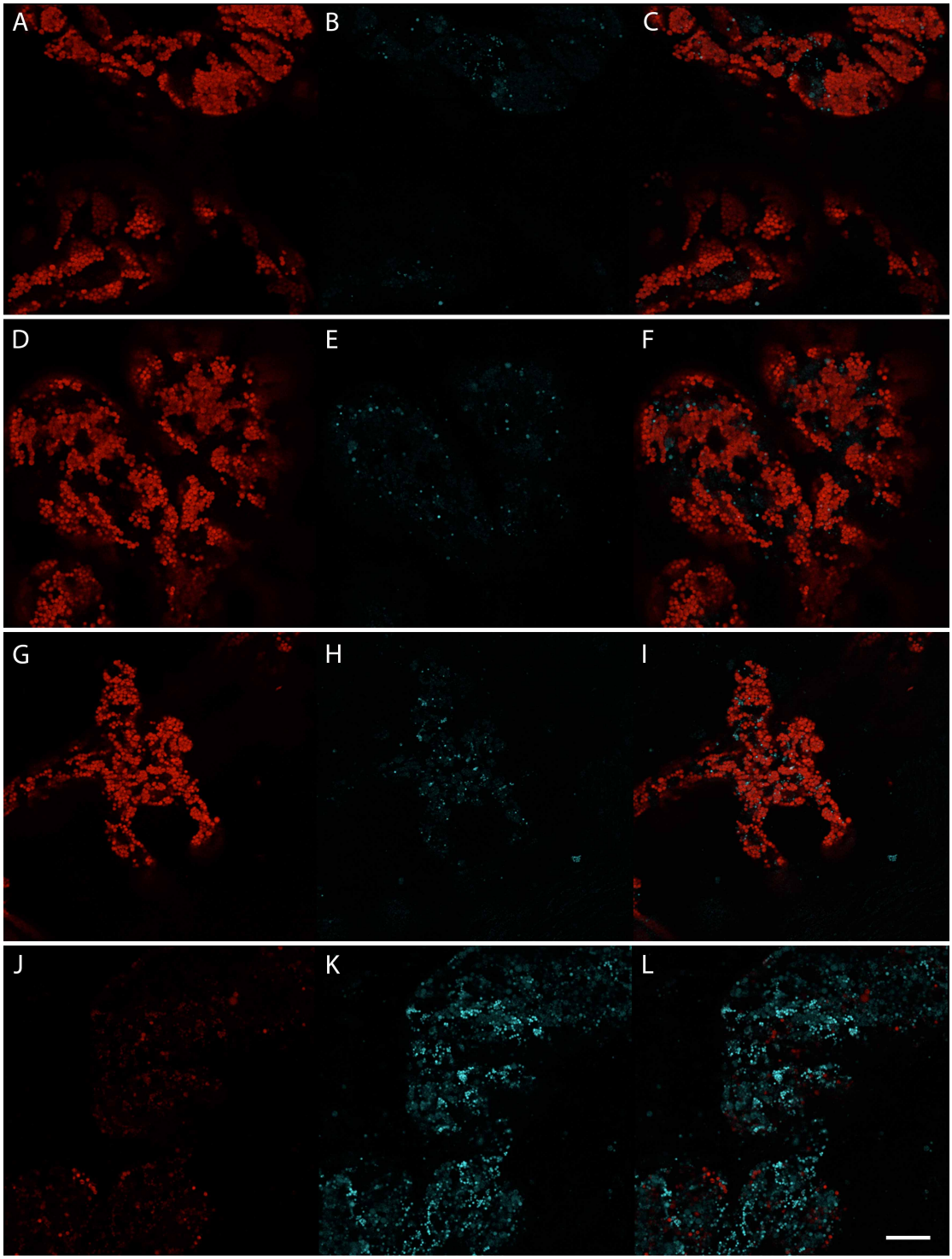


Figure 2.7 Chloroplast and lysosome density in *Elysia viridis*. A: Chloroplasts (falsely colored red) in an unstarved specimen. B: Lysosomes (blue) in the same specimen. C: Composite of chloroplasts and lysosomes. D-F: Cps, lys, and composite after 14 days starvation. G–I: Cps, lys, and composite after 21 days starvation. J–L: Cps, lys, and composite after 30 days starvation. Scale bar—50 µm.

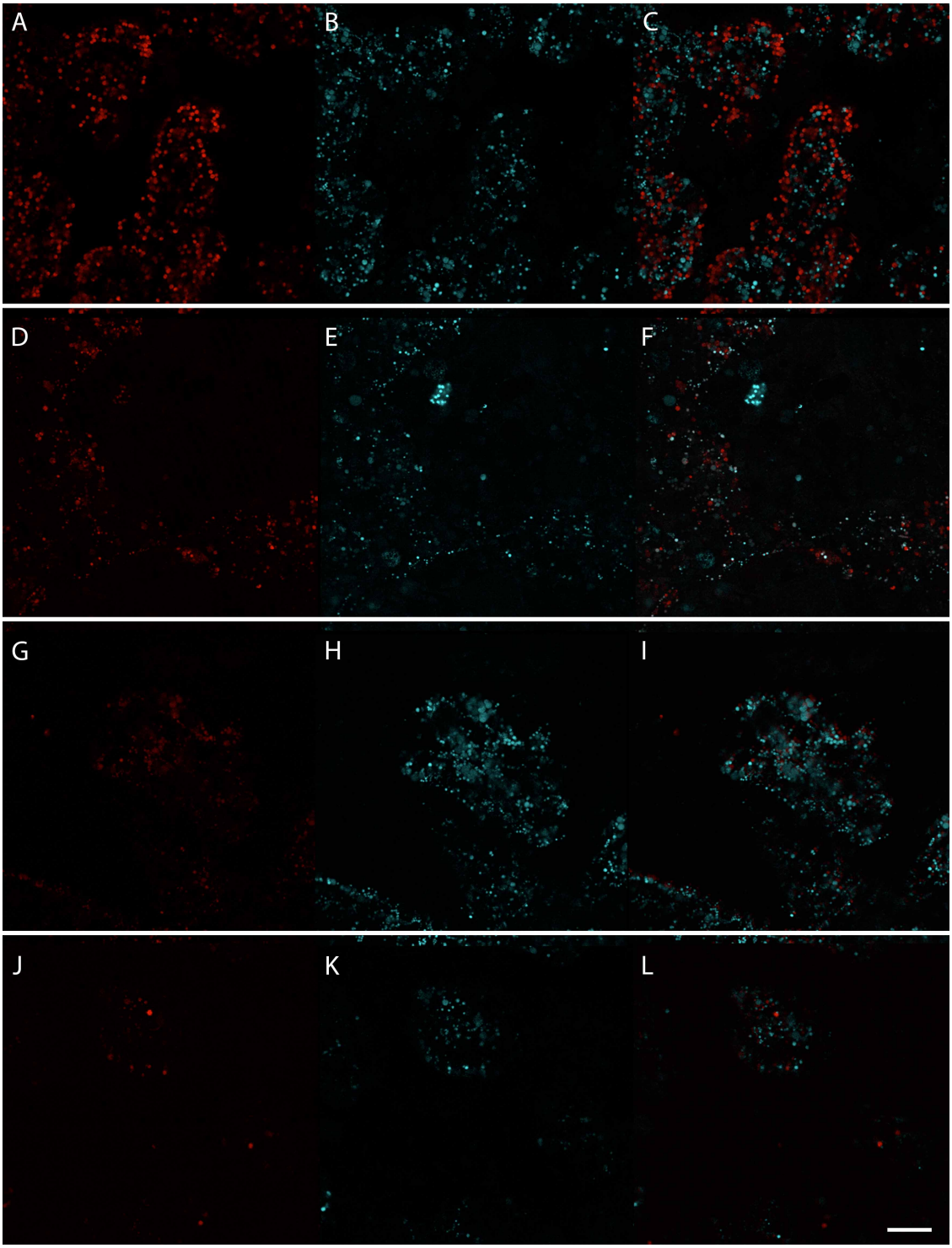


Figure 2.8 Chloroplast and lysosome density in *Thuridilla hopei*. A: Chloroplasts (cps) (falsely colored red) in an unstarved specimen. B: Lysosomes (lys) (blue) in the same specimen. C: Composite of chloroplasts and lysosomes. D-F: Cps, lys, and composite after 7 days starvation. G–I: Cps, lys, and composite after 14 days starvation. J–L: Cps, lys, and composite after 21 days starvation. Scale bar—50 µm.

The chloroplast and lysosome abundances show an inverse relationship, although neither is modeled by a linear function and the particular relationship between these two variables could not be determined. In both *E. timida* populations, cps and lysosome abundance are equal around the 21-day time point (Figure 2.5A, B). *E. viridis* displays an equal abundance at the 5-day time point (Figure 2.5C) and *T. hopei* occurs at 14 days (Figures 2.5D, 2.6, 2.7, and 2.8C, F, I, L).

2.3.3 Food reintroduction experiments

Half of the adult *E. timida's* from P1 were re-fed for 30 min, at each sampling point as described in Table 2.1, to measure any difference in lysosomal activity when the slug is reintroduced to food. The predicted increase in starving slugs' lysosomal abundance due to the availability of food was observed; however, significance tests conducted with multiple programs yielded no discernable result. The near parallel status of the lysosome abundance curves suggests the same digestive pattern occurs in both animals not reintroduced to food, and those fed for 30 min. The cp abundance curve is higher in the animals allowed to feed again. The parallel nature of the cp abundance curves suggests that 30 min is too short a period of time and the cps gained in this time are too few in number, to actively change the digestive processes in these tissues (Figure 2.5A).

E. timida P2 specimens were also separated into two treatments (starved and starved then re-fed); however, the animals allowed to feed were given 2 h. The predicted increase in lysosomal activity was not seen however, rather a small decrease in lysosome abundance. The significance of this decrease could not be determined, but is likely non-existent since both curves are modeled by highly similar functions (Figure 2.5B). Cp abundance was higher in the animals allowed to feed and contrary to the results from P1, the curves are not parallel, indicating a difference in the DGT likely related to time the animal was allowed to feed. P1 and P2 animals starved up to 42 days showed a high degree of interest when presented with food, feeding almost immediately and continuing for the entire time allowed. P2 slugs starved longer than 42 days; however, they showed few attempts to feed

and required various amounts of time before they began (the 2-h time period only began after they first appeared to feed). This likely explains the intersection of the two curves in Figure 2.5C and indicates that although slugs starved for more than 42 days appeared to feed, they may not have actually ingested cps and therefore functionally contain the same cp amount as un-fed samples.

E. viridis re-feeding treatments report slightly different trends. While the number of cps is always higher in the animals allowed to feed (2 h), the lysosome abundance was lower than the non-fed treatment for time points before 18 days and higher after 18 days. The significance of this difference could not be established; however, it is likely insignificant.

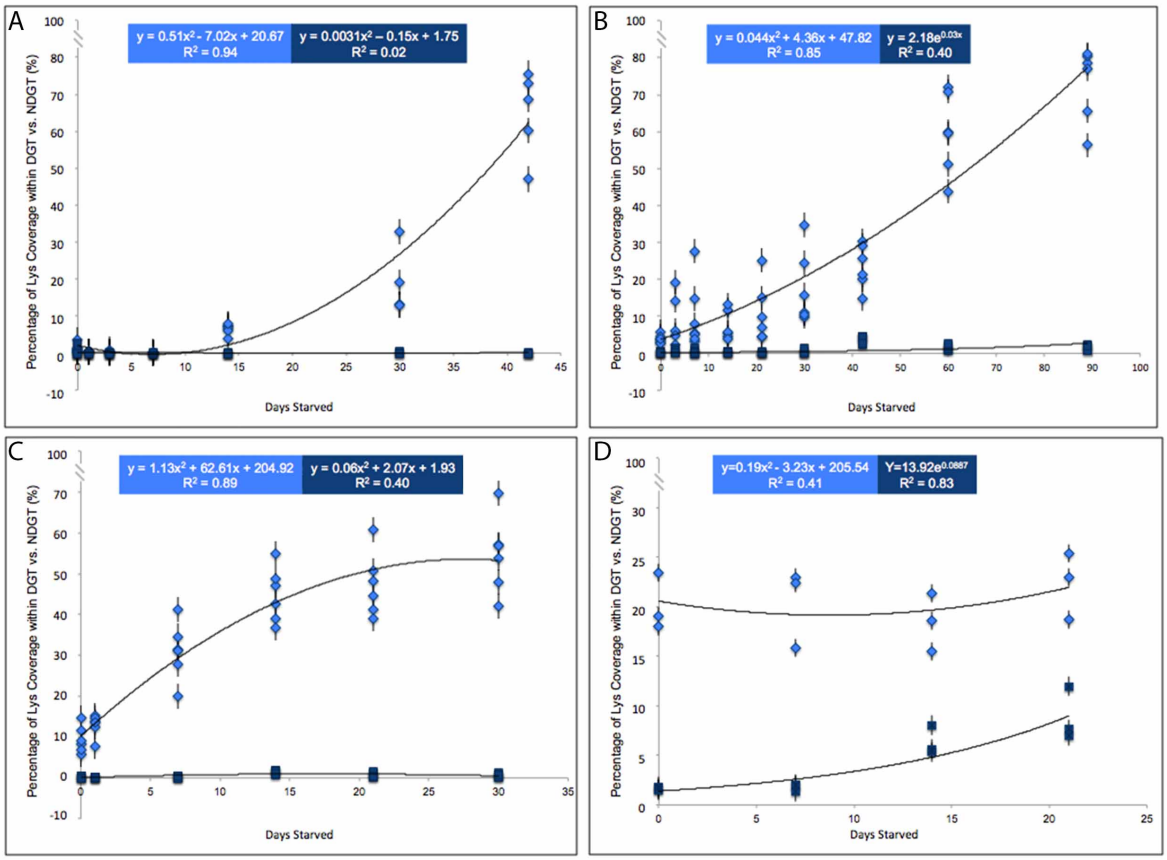


Figure 2.9 Lysosome density in digestive tissue versus non-digestive tissues. The percentage of DGT tissue covered by lysosomes within the digestive gland tubule is indicated with light blue diamonds and compared to the average area covered by lysosomes outside the DGT (shown in dark blue squares), at each time point sampled, for each population investigated. A: *Elysia timida* population 1 from Elba (spring). B: *Elysia timida* population 2 from Blanes (autumn). C: *Elysia viridis*. D: *Thuridilla hopei*. Specimens that were re-fed were not included.

2.4 Discussion

For the first time, the degradation of functional chloroplasts within the digestive gland is detailed and compared to lysosomal abundance to reveal the relative amounts of digestion occurring throughout starvation periods. Thus, functional chloroplast abundance, as indicated by the presence of chlorophyll a and measured by recording its autofluorescence, is now reported in situ. PAM fluorometry data has shown significant declines in the photosynthetic capability of chloroplasts within sacoglossan digestive glands; however, the relative nature of these yield values does not quantitatively explain the decrease in chloroplast abundance (Christa 2014; Christa, Zimorski, et al. 2013a; N E Curtis et al. 2015; de Vries et al. 2015; Schmitt et al. 2014; Wägele and Johnsen 2001; Wägele and Martin 2014). Pigment analyses have described the dry weight chlorophyll abundance in unstarved *E. timida*; however, they fail to assess the decline in plastids during a starvation period and do not examine the location and density of these plastids (Baumgartner et al. 2015; Rauch et al. 2015; Ventura et al. 2013). This study details the decline in functional plastids over time, in situ, showing that the decrease in *E. timida* is not modeled linearly as seen in the PAM yield value, but rather shows a sharp decrease and subsequent softening.

The functional chloroplast abundance and the rates at which plastids are digested amongst the different species examined here support the hypothesis that LtR and StR species handle retained chloroplasts differently. The LtR species, *E. timida*, is able to retain chloroplasts for up to 100 days (author unpublished results), and during this period, the number of functional plastids decreases substantially; however, it decreases slower than in the StR species. While the decrease occurs mostly at the beginning of the starvation period, between 0–30 days, functional plastids are still found in abundance until around 60 days. After this, functional plastids are rarely observed and those still present emit a very weak fluorescent signal, indicating the breakdown of chlorophyll a and likely the breakdown of the plastid itself. Further work must be conducted to determine the relationship between chlorophyll a breakdown and plastid longevity in

sacoglossan digestive glands. Transmission electron micrographs show some cps are still embedded within DGT, even after the chlorophyll a has broken down; however, these cps are less abundant and show structural abnormalities, further supporting the idea that these plastids are being actively digested during starvation (Martin et al. 2013; Schmitt and Waegele 2011).

The ambiguous LtR/StR slug *E. viridis* and *T. hopei* (StR) both display a rapid functional plastid decrease, with most of the breakdown occurring between 0 and 15 days and a very low functional plastid density afterward. Despite ingesting *C. tomentosum*, the algae that should allow *E. viridis* to achieve LtR and therefore presumably behave similarly to *E. timida* when examined intracellularly (Baumgartner et al. 2015; Christa, Wescott, et al. 2013; Gallop et al. 1980), the specimens observed here more closely matched trends seen in *T. hopei* and suggest that *E. viridis* may actually function like other StR forms when kleptoplast digestion is concerned. The relative cp density observed in all species also differs between LtR and StR forms. Unstarved animals from both E. timida populations have cp coverage of over 40 %, while *E. viridis* has roughly 16 % and *T. hopei* has only 4.5 %. Due to this difference, chloroplast abundance within these tissues may be a leading factor in a slug's ability to survive starvation. More research will need to be conducted to properly quantify the number of chloroplasts in these animals and determine if the number of plastids correlates to slug longevity.

As expected, the number of functional chloroplasts de- creased with an increased lysosome density indicating that functional digestion is indeed occurring within DGT tissue and that lysosomes are involved in this breakdown. The specific type of lysosome (autophagosome, late endosome, etc.) could not be determined however, since acridine orange is a pH-dependent stain and each of these types shares a similar pH range, all of which are visible with AO staining (Moriyama et al. 1982). The highly variable lysosome sizes indicates a mixture of different lysosomal types, maturities, and stages, meaning the specific nature of these organelles will require further investigation in order to delineate and properly classify the specific steps in the retention and subsequent kleptoplast digestion process (De Duve 1963). While AO could have also stained another structure in the

same pH range, since it does not discriminate based on anything other than pH, the size of our stained objects (perfectly within the expected size for lysosomes) and location (heavily aggregated in the digestive gland cells and rarely observed outside the digestive gland) provide indirect evidence that lysosomes themselves were stained. The area inside thylakoid membranes is very acidic (at the lowest, pH 4), even before they begin to break down, so AO could theoretically aggregate within these compartments; however, AO was never observed aggregating within chloroplasts. We examined the chloroplast boundaries using both the transmission channel (light micros- copy) and the chlorophyll autofluorescence and found no trace of AO aggregating within these boundaries. This suggests that while AO could aggregate there due to the pH, it does not penetrate into the acidic thylakoid membrane areas.

Neither LtR nor StR species showed a significant digestive response to newly sequestered chloroplasts, even after starving for a few weeks. Since algal material is taken up into digestive gland cells within minutes of ingestion for other species (Martin et al. 2013; McLean 1976) (Martin et al. 2013; McLean 1976), a cellular response within 30 min or even 2 h was expected. No significant lysosomal response was measured at any time point, in any species, so understanding the reestablishment of kleptoplast retention will require further attention and the application of different methods.

When reintroduced to algae, specimens starved for shorter periods of time did ingest new material, indicating the possibility of either increased digestion or plastid retention whereas animals starved longer were not reliably observed feeding even when observed for up to 5 h. The lack of ingestion may indicate that the animals are no longer capable of feeding due to autophagy. This however was not seen in the NDGT tissue measured. Autophagosomes occurring in the NDGT would have been stained with acridine orange, and no significant increase in the number of stained bodies was observed even in the later starvation periods, except for *T. hopei*, which had a significant in- crease in the number of NDGT lysosomes after 14 days. In this respect, *E. viridis* matches the LtR species E. timida by having no recognizable signs of autophagy, rather than aligning with the StR form *T. hopei*

which clearly shows autophagy-like digestive activity in cells outside the digestive system. Numerous genes associated with autophagy were also investigated with no apparent pattern emerging, suggesting further work will need to be conducted to properly investigate autophagy in these animals.

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2.6 Author contributions

EMJL and HW devised the project concept and EMJL conducted the lab work. PTR and EMJL designed 3D-AMP and PTR wrote this software. AP and TB help troubleshoot staining and all authors contributed to the analysis and manuscript.

Chapter 3

Photosynthate Accumulation in Solar-powered Sea Slugs – Starving Slugs Survive Due to Accumulated Starch Reserves

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Keywords

Sacoglossa, Amylose, *Elysia*, Lugol's Iodine, Kleptoplast, Starch

Abstract

Background

Solar-powered sea slugs are famed for their ability to survive starvation due to incorporated algal chloroplasts. It is well established that algal-derived carbon can be traced in numerous slug-derived compounds, showing that slugs utilize the photosynthates produced by incorporated plastids. Recently, a new hypothesis suggests that the photosynthates produced are not continuously made available to the slug. Instead, at least some of the plastid's photosynthetic products are stored in the plastid itself and only later become available to the slug. The long-term plastid-retaining slug, *Elysia timida* and its sole food source, *Acetabularia acetabulum* were examined to determine whether or not starch, a combination of amylose and amylopectin and the main photosynthate produced by *A. acetabulum*, is produced by the stolen plastids and whether it accumulates within individual kleptoplasts, providing an energy larder, made available to the slug at a later time.

Results

Histological sections of *Elysia timida* throughout a starvation period were stained with Lugol's Iodine solution, a well-known stain for starch granules in plants. We present here for the first time, an increase in amylose concentration, within the slug's digestive gland cells during a starvation period, followed by a sharp decrease. Chemically blocking photosynthesis in these tissues resulted in no observable starch, indicating that the starch in untreated animals is a product of photosynthetic activity.

Conclusion

This suggests that kleptoplasts function as both, a nutritive producer and storage device, holding onto the polysaccharides they produce for a certain time until they are finally available and used by the starving slug to withstand extended starvation periods.

3.1 Introduction

Sacoglossan sea slugs (Heterobranchia: Gastropoda) are also known as "solar-powered sea slugs" and "leaves that crawl" due to some members' ability to steal chloroplasts (kleptoplasts) from their algal food and retain them for many months (Greene 1970; Greene and Muscatine 1972; Kawaguti and Yamasu 1965b; D. L. Taylor 1968; Robert K Trench et al. 1969). As the only described metazoans with this ability, they stand out as study subjects, captivating the interest of both researchers and laymen alike. Most sacoglossans feed on chlorophyte species by piercing the cell wall and sucking out the cell content including the chloroplasts (Katharina Händeler et al. 2009; K. R. Jensen 1980, 1994). While other organelles such as mitochondria are digested in all slug species, some slug species can incorporate and maintain chloroplasts in their digestive gland tubule cells. Because these stolen chloroplasts remain photosynthetically active, this incorporation phenomenon is called functional kleptoplasty (Wägele and Martin 2014).

Chlorophytes, the algae most often ingested by sacoglossans, produce numerous photosynthates including carbohydrates, lipids and proteins (Greene 1970; Greene and Muscatine 1972; Hinde and Smith 1975; Muscatine et al. 1975; Shephard and Levin 1972; R K Trench and Gooday 1973). The main photosynthate produced however is starch, a combination of amylose and amylopectin (Shephard and Levin 1972). To establish whether or not photosynthates are made available to a sacoglossan slug, radiolabeling studies with ^{14}C or ^{13}C were conducted (Raven et al. 2001; Teugels et al. 2008; M. E. Trench et al. 1970; R K Trench et al. 1973; Robert K Trench 1969; Robert K Trench et al. 1969; Robert K Trench and Smith 1970), finding that both photosynthetically and heterotrophically fixed carbon are present in multiple slug-derived compounds for some species. These include glucose, galactose and an unidentified third compound (R K Trench et al. 1974), pigments (Robert K Trench and Smith 1970), mucus (M. E. Trench et al. 1970), amino acids, organic acids and others (not specified in (Hinde and Smith 1974)). Since the total amount of incorporated ^{14}C was lower in specimens kept in the dark, which presumably has no effect on heterotrophically derived ^{14}C, the amount

of photosynthetically derived ^{14}C was determined, demonstrating the metabolic movement of photosynthetically derived carbon into multiple slug-produced compounds [15, 20-22, full review available in 9]. Recently two long-term plastid retaining (LtR) species, *Elysia timida* Risso 1818 and *Plakobranchus ocellatus* van Hasselt 1824, were shown to fix $^{14}CO_2$ further showing that photosynthetically active plastids are fixing carbon, which is then incorporated into slug tissues (de Vries et al. 2015). Although CO_2 fixation requires light, LtR species do not necessarily need light and can survive several months in complete darkness, although their longevity is not equal to that of specimens kept in the light. They also survive when photosynthesis is chemically blocked (e.g. monolinuron), although their longevity may be compromised (Christa, Zimorski, et al. 2013b).

These last observations question the necessity for kleptoplast functionality and the nature of the benefits gleaned by starving sacoglossans. Recent hypotheses consider sequestered chloroplasts as a source of stored food reserves rather than as a source of directly and continuously available photosynthates (Christa, Zimorski, et al. 2013b; Pelletreau et al. 2014).

Only one LtR form has been investigated to determine if photosynthates accumulate, the misfit species *Elysia chlorotica*, a slug that feeds on the heterokontophyte, *Vaucheria litorea* Agardh 1832 rather than chlorophyte species like most other sacoglossans. Pelletreau et al. (2014) observed lipid droplet accumulation in *E. chlorotica's* digestive gland tubules during a starvation period, and they state that these lipid droplets were likely "of algal origin". Lipid droplets are known to be the main photosynthate storage molecule produced by *V. litorea*, so this accumulation is not surprising and lends evidence to the hypothesis regarding chloroplasts as photosynthate larders. It remains unknown whether chlorophyte-feeding sacoglossans profit from stored photosynthates in a similar manner or not. Starch grains have been seen in numerous electron micrographs (Hirose 2005; Arnaldo Marín and Ros 1993; Martin et al. 2013; D. L. Taylor 1968; R K Trench et al. 1973; Robert K Trench et al. 1969), however no information on starch quantity, production or accumulation has been reported.

In this paper, we examine the accumulation of starch within kleptoplasts in the digestive gland tubules of stenophagous, LtR *Elysia timida*. Starch, the main

storage polysaccharide produced by the chlorophyte *Acetabularia acetabulum* Linneaus is a complex carbohydrate comprising glucose units bound together to form amylose and amylopectin (Levine 2011; Love et al. 1963; Werz and Clauss 1970). This starch is stored in the form of granules within the chloroplast's stroma. When needed by the algal cytosol, it is broken down to maltose and glucose units and transported to the algal cytosol, a process that occurs nightly when photosynthesis ceases (Levine 2011). Whether or not these processes function in kleptoplasts stored in the slug's digestive gland remain uninvestigated. It is also still unclear whether any photosynthates produced are immediately transported to the slug or if the slug lacks the feedback system found in the algae, therefore directly causing a build up of starch within the kleptoplast. Any starch build up would support the more recent ''larder hypothesis'' (Christa 2014; Pelletreau et al. 2014) - that photosynthesis does contribute to meeting a starving slug's metabolic needs, but not through the continuous release of the photosynthates produced, but rather by storing them inside the plastid where they later become available to the slug when the plastids are finally digested at a later time point.

In order to test if the starch granules stained here were really produced by photosynthesis inside the slug and were not for instance thylakoid membrane degradation inside the tubules (Vetterman 1973), we used the photosynthesis blocker, monolinuron (Algol), during the starvation process. Phenylureas such as monolinuron bind at the site of the quinone B in the D1 protein of photosystem II (P680) and thus block the electron flow (Oettmeier 2003). Therefore, by adding this inhibitor from the beginning of the starvation process, we hypothesized that we would not observe any starch increase if starch is indeed a product of ongoing photosynthesis in the slug. Pulse Amplitude Modulated (PAM) fluorometry values were also measured in both, monolinuron treated and in untreated samples, to monitor photosynthetic efficiency during the starvation process (Christa 2014; Katharina Händeler et al. 2009; Wägele and Johnsen 2001). Since *Acetabularia acetabulum* availability in the Mediterranean is highly variable (A Marín and Ros 1992), both a spring and fall population were investigated to account for seasonal differences due to temperature, food availability or other factors.

3.2 Materials and Methods

Elysia timida and *Acetabularia acetabulum* were collected in Blanes, Spain (November 2014) and Fetovaia, Elba, Italy (April 2015) at one to two meters depth. Specimens were kept in groups of 25, in aerated tanks containing roughly 3L freshly prepared artificial seawater (changed every other day). Artificial lighting for 12 hours each day provided full-range lighting measuring about 220 $\mu E^{-2}s^{-1}$ at the water's surface to mimic natural conditions and the optimal irradiance (A Marín and Ros 1992; Monselise and Rahat 1980) . Both populations were kept in the laboratory at different temperatures, 20-22°C average for the spring population, and 18°C for the fall to match the water temperature at collection and mimic natural conditions. All specimens were kept in the tanks with *A. acetabulum* for two weeks to acclimate to laboratory conditions and to assure feeding.

Prior to fixation, the maximum quantum yield (PAM or F_v/F_m) was measured three times with a Pulse Amplitude Modulated fluorometer (Diving PAM) for each animal in order to compute a mean value (for detailed methods, see 35). This method assesses photosynthetic efficiency in living tissues. PAM values were also measured for specimens treated with the photosynthetic inhibitor Monolinuron – diluted to 2 μg.ml-1 with filtered seawater, in order to determine if photosynthesis was still occurring in the slugs and how it decreased with the continuous presence of the blocker. The monolinuron and seawater mixture was prepared freshly and changed with the water every other day to ensure continuous exposure.

Forty individuals were starved for each time series: the fall population, spring population and monolinuron. Two specimens (fall population) or three specimens (spring population) were first photographed (Figure 3.1) and then fixed in 4% formaldehyde for the following starvation time points given in days: 0, 3, 10, 15, 21, 30, 42, 88. The 0-day individuals were preserved as a control group, to measure the amount of starch in the incorporated chloroplast when the slug is normal, unstarved conditions. These animals were removed directly from the algae

for fixation. Each specimen was fixed at 17:00 to maximize the amount of starch produced before it is broken down at night (Levine 2011). After fixation, specimens were embedded in hydroxymethacrylate (Kulzer Technovit® 7100) and sectioned at 2.5µm thick. Every third section was stained with toluidine-blue in order to observe the overall anatomy of each specimen and assure it was not a

Figure 3.1 Starvation in *E. timida* populations. A: Series of photos showing starving *E. timida* from Blanes, Spain. B: Starvation photos of *E. timida* from Elba, Italy. C: Starvation in monolinuron-treated specimens. A cross (†) indicates that animals from this population did not survive starvation up to the day indicated.

juvenile (Figure 3.2A, B) while the next sections were stained with a 1:10 Lugol's Iodine Solution (Fisher Scientific) diluted in distilled water in order to quantify amylose (Figures 3.2C, 3.3A-I). Lugol's solution has long been used by botanists to identify starch in plants and algae, with Werz and Clauss (1970) successfully identifying it in *A. acetabulum.*

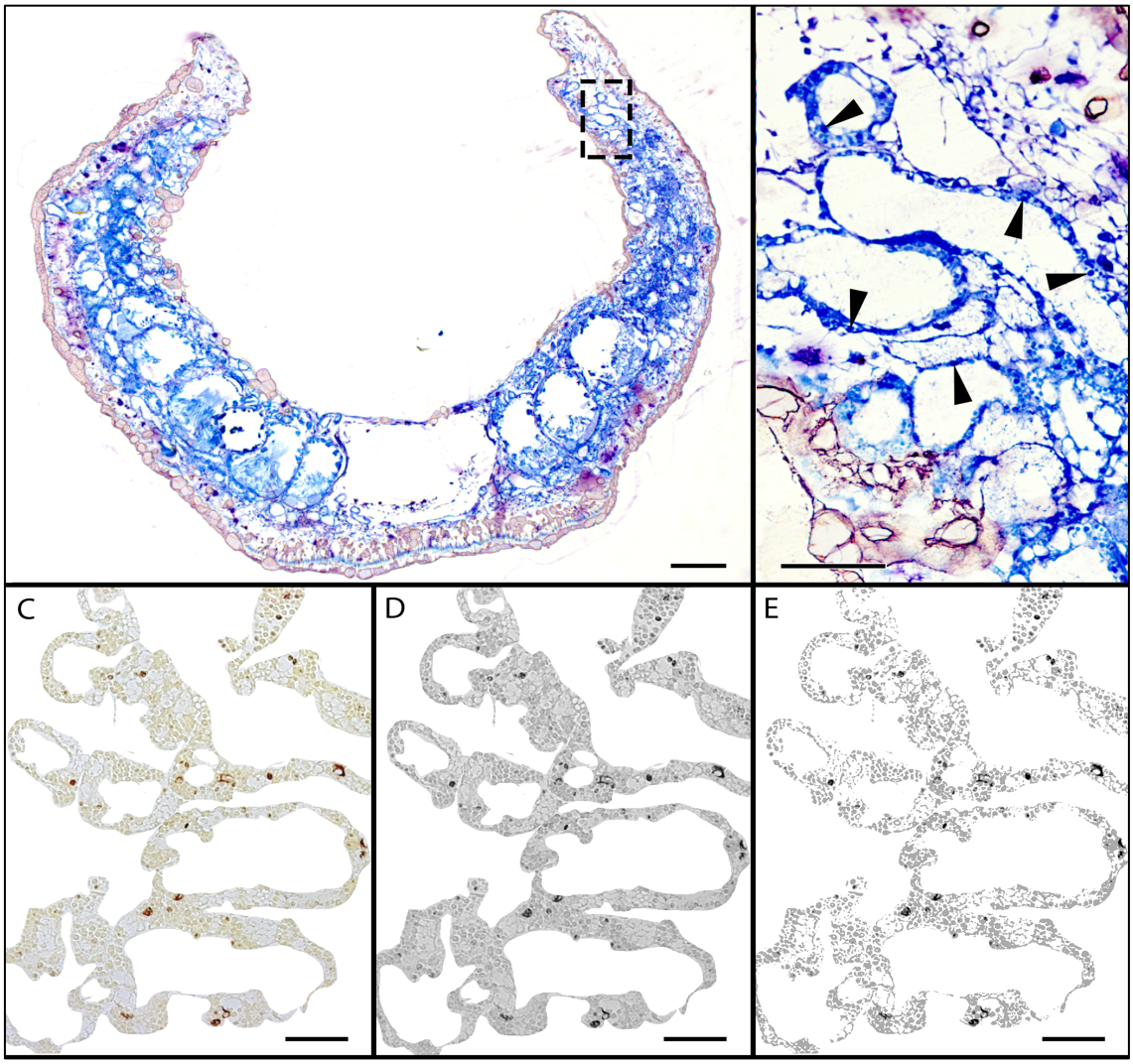

Figure 3.2 Methods. A: Toluidine-blue stained cross-section of 42 days starvation specimen of *E. timida* from Blanes population, the rectangle indicates the area seen in B; B: magnified area showing digestive tubule epithelium with chloroplasts (tubules indicated with arrowheads) in the parapodia at higher magnification; C: Lugol's solution-stained section of a 0 days starved *E. timida* from the Elba population, the digestive gland tubules have been isolated by deleting the background, non-digestive tissue; D: Conversion to greyscale image in Adobe Photoshop; E: Grey shades reduced to 5 for final starch counting, the dark black coloration shows the Lugol's stained starch. Scale bars: A – 50µm; B – 25µm; C-E – 20µm.

Ten regions were chosen throughout the slug, in order to analyze different areas containing digestive gland tubules. For each region, two pictures were taken and analyzed in order to estimate the mean relative starch percentage value (RSP). This percentage value was computed using Adobe Photoshop (version CS5.1) by first deleting all non-digestive gland tubule tissue and recording the total digestive gland tubule area (Figure 3.2C). Then each image was converted to greyscale (Figure 3.2D), reduced to 4-5 shades depending on the staining intensity (Figure 3.2E), and the two darkest colors – those corresponding to the amylose stained tissue – were measured in pixels. Each image was adjusted individually to avoid overestimating the relative starch percentage (RSP). These measurements were then converted back to area measurements from pixels based on the original image scale.

3.3 Results

Both populations surveyed here show first an increase in amylose followed by a decrease compared to the 0-day, unstarved control specimens (Figures 3.3,3.4). Only the specimens from Blanes (Spain, collected in November) survived 88 days of starvation. The *E. timida* specimens from Elba (Italy, collected in April) did not survive more than 30 days of starvation (except for some specimens that managed 42 days) (Figures 3.4, 3.5).

In the case of the Blanes fall population, the relative starch percentage (RSP) increases until 42 days (Figure 3.3A,B), reaching a maximal 23 % tubule area coverage. This value drops to a mean of 2.2 % after 88 days of starvation (Figure 3.3C). In the same specimens, the maximum quantum yield of PSII values, which averaged 0.75 at the beginning, decreased to an average 0.30 (Figure 3.4A). The Elba spring population also showed an increase in the RSP until 15 days of starvation (Figure 3.3D-F). Although the maximal value, 25.91 is reached after 10 days of starvation in one specimen, the mean value is at 15 days of starvation (17.95), and is higher than the average for 10 days (15.1). After 21 days of starvation, the RSP decreases and reaches a mean of 2.5 after 30 days. The

maximum quantum yield of PSII (PAM) follows this trend. These PAM values are stable at the beginning of the starvation period and oscillate around 0.70 up to 21 days of starvation when they decrease to a mean of 0.55 and finally drop to values close to 0 (in two specimens) (Figure 3.4B).

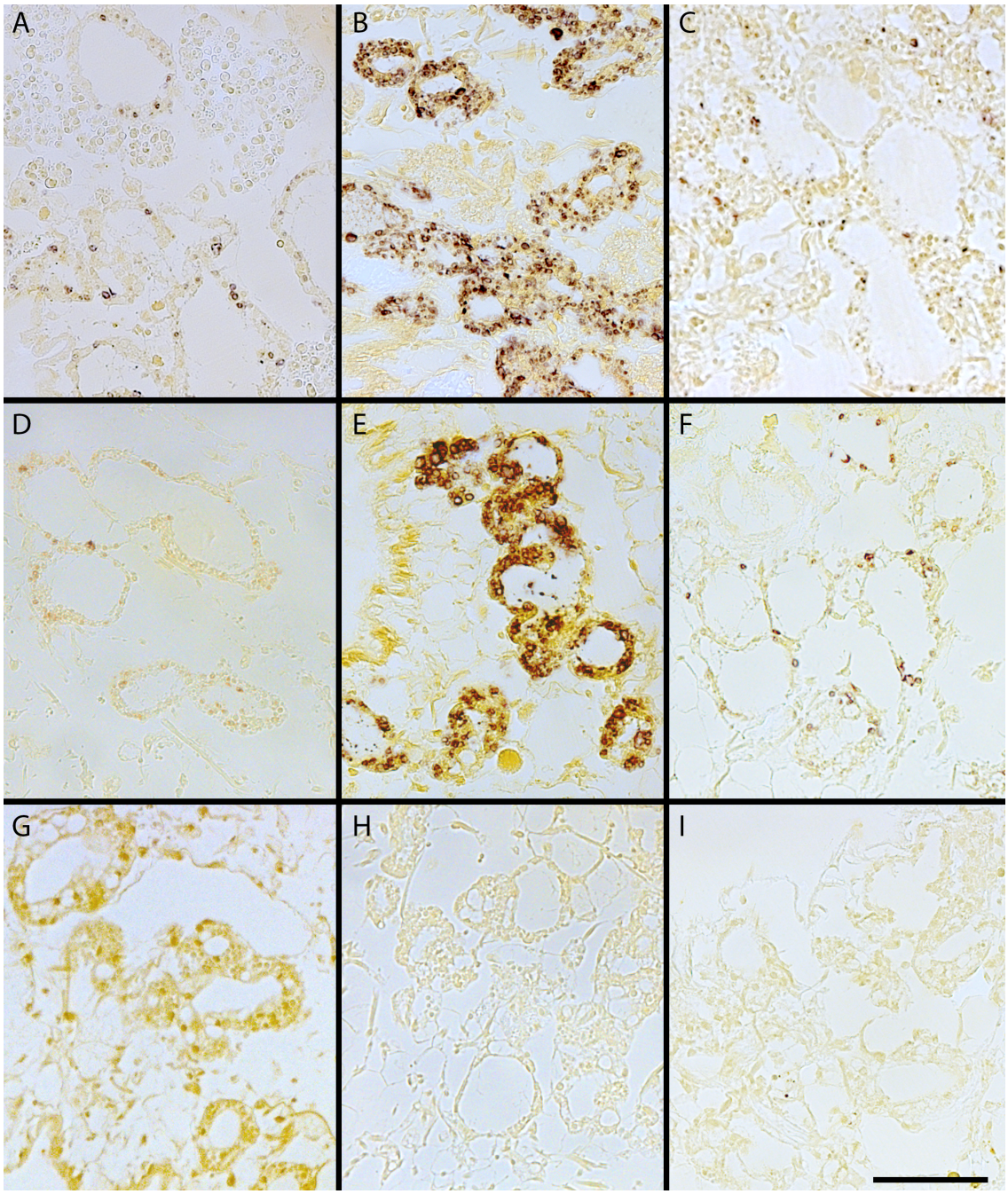

Figure 3.3 Starch accumulation and degradation in various *E. timida* Specimens. A-C: *E. timida,* fall population – Blanes, A: starved for 0 days; B: starved 42 days; C: starved 88 days; D-F: *E. timida,* spring population – Elba, D: starved 0 days; E: starved 10 days; F: starved 30 days; G-I: monolinuron-treated *E. timida,* spring population – Elba, G: starved 3 days; H: starved 10 days; I: starved 21 days. Scale bar 50µm.

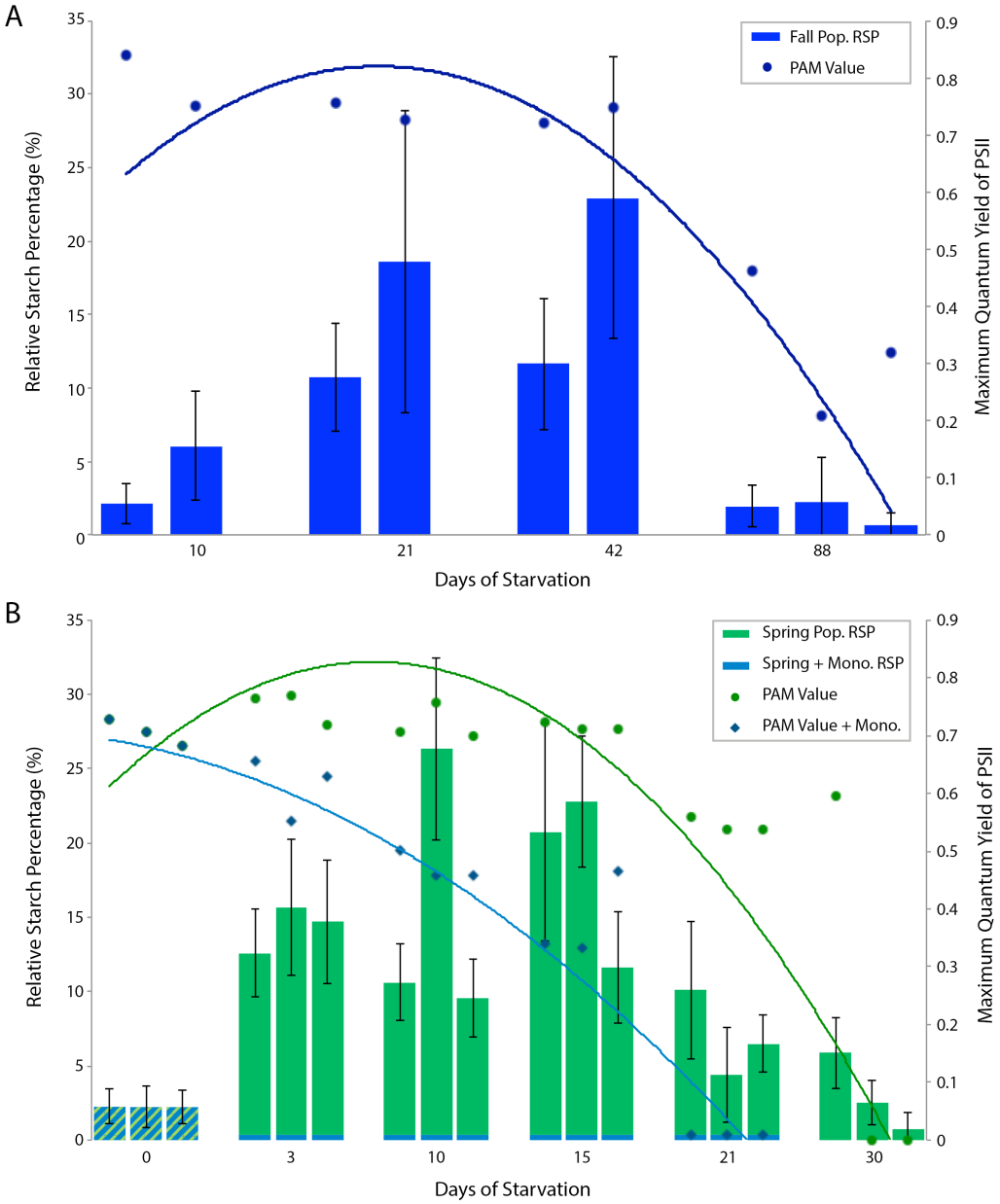

Figure 3.4 Histogram of the Mean Relative Starch Percentage Values (MRSP) ± standard error. A: *E. timida* specimens of the Spain fall population (MRSP on the first y axis, left side) at different starvation time points, 10 days, 21 days, 42 days and 88 days and their relative maximum quantum yield of PSII with a parabolic trend line (on the second y axis, right side). B: MRSP ± standard error for each *E. timida* specimens of the Elba spring population represented as the histogram (MRSP on the first y axis, left side) at different starvation time points, 0 day, 3 days, 10 days, 21 days, and 30 days and their relative maximum quantum yield of PSII with a parabolic trend line represented by the line (on the second y axis, right side). Specimens under starvation conditions (S) are represented in green, while those in starvation and monolinuron treatment (S+M) at the same time points are represented in blue for both RSP and maximum quantum yield of PSII. Specimens for 0 day of starvation are the same S and for S+M and therefore are hatched with blue and green. The specimens treated with monolinuron under starvation have a zero relative starch percentage value.

Monolinuron-treated samples did not contain amylose, at any time point during the starvation period (starting after the 0-day, control time point) (Figure 3.3G-I, 3.4B). Moreover, the maximum quantum yield of PSII (PAM) values decreased faster during starvation in specimens treated with monolinuron, than in those without. After 10 days the mean value was 0.47 and after 21 days most of them had a zero value and began to die (Figure 3.4B).

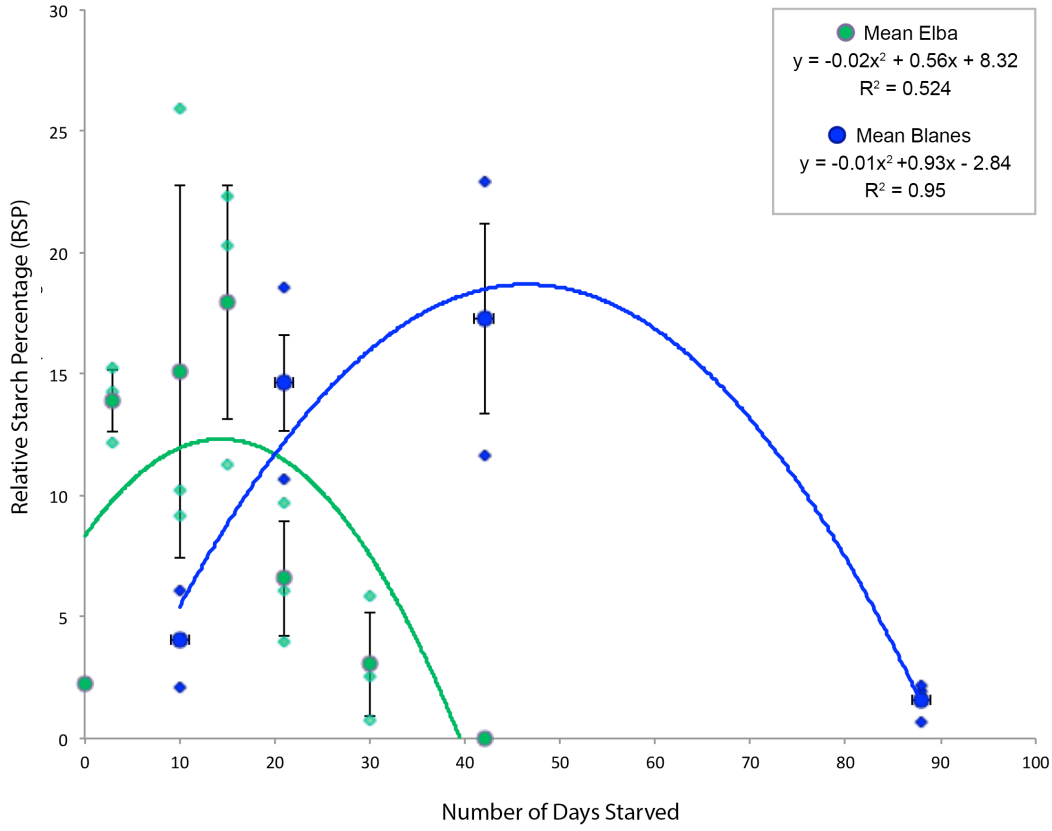

Figure 3.5 Overall Mean Relative Starch Percentage (MRSP) for both populations. The MRSP for the Blanes, fall population slugs is shown in blue and the Elba, spring population is shown in green (including two specimens which starved for 42 days). Best fit curves show the shift in total days starved between the two populations, and show a higher overall RSP in the Blanes fall population.

3.4 Discussion

This study clearly reveals starch granules in starving *E. timida* adults, showing that this photosynthate is produced and maintained by *A. acetabulum* kleptoplasts. Unstarved (control) slugs contained only small amounts of starch. After 3 days (in the case of the Elba spring population) or 10 to 21 days (Blanes fall population), incorporated chloroplasts had the time to produce photosynthates, which accumulated as starch within the plastid (Figure 3.5). After 21 days (Elba population) and 42-88 days (Blanes population), the RSP decreased, indicating that more starch is degraded than produced. Since the PAM values decrease at the same times as the RSP for both populations, a decrease in the electron chain transfer and therefore in the photosynthetic activity can be inferred. Moreover, the small amount of starch remaining after extended starvation indicates that this molecule is likely degraded and consumed by either the slug or the plastid itself, to meet its own metabolic needs. A quantitative number of chloroplasts at each time point is unavailable in published literature, although Laetz et al. (2016) show a decrease in the number of functional plastids, based on chlorophyll a autofluorescence, throughout a starvation period. While they could not interpret the decrease in functional chloroplasts as a decrease in plastids themselves, since the plastids could still be there without intact chlorophyll a, the combination of this information and the decrease in starch presented here suggests the chloroplasts are no longer there and therefore likely being digested (Laetz et al. 2016).

Even though the two populations analyzed show the same trend, an increase in starch followed by a decrease, the RSP maxima and minima do not occur at the same time point (Figure 3.5). The fall population produced starch slowly and was able to survive longer starvation periods, whereas the spring population produced a high amount of starch very quickly at the beginning of the starvation process and died sooner. This was likely caused by temperature differences due to the time of year. The population from Blanes was starving during the fall with a lower temperature in the laboratory (18°C) than the

population from Elba (20-22°C). Therefore, the chloroplasts in specimens from Elba probably demonstrated a higher metabolic rate, producing starch faster. This higher activity could also explain the shorter lifespan of the starved specimens from Elba compared to those from Blanes, since the chloroplasts may be degraded more quickly making them less functional overall.

Another effect due to the sampling period is that *A. acetabulum* was not found in the same quantity and not in the same point in its life cycle. The fall population (Blanes) was found amongst *A. acetabulum* that were young and completely uncalcified whereas the spring population (Elba) was collected from algae that had already grown long stalks and were already forming caps (Casalduero and Muniain 2008). This almost aligns with the results from Marin and Ros (1992), who described cap-formation and lower stalk calcification in Mazarrón Bay, Spain during February. We observed cap formation on partially calcified *A. acetabulum* during April, on the island of Elba, Italy. Calcified *A. acetabulum* stalks present a physical barrier that prevents *E. timida* from feeding (Casalduero and Muniain 2008). Cap-formation is the last step in the *Acetabularia* lifecycle and is followed by a planktonic stage that presumably cannot be eaten by *E. timida.* Calcified stalks and cap-formation indicate a lower amount of food meaning slugs take in fewer kleptoplasts within the spring population (Elba) slugs, which may also account for a faster decrease in the spring RSP. Kleptoplast abundance has not been described from adult specimens collected in different seasons, so this hypothesis warrants further investigation.

The immediate decrease in amylose observed in monolinuron-treated specimens shows that starch production is the result of the ongoing photosynthesis in incorporated chloroplasts. Furthermore, the slugs are not able to face longer starvation periods and show drastically reduced longevity, dying after 21 days. This contradicts previous findings (Christa, Zimorski, et al. 2013b; de Vries et al. 2015),which suggested that monolinuron-treated animals and those kept in the dark lost weight and showed survival rates almost indistinguishable from those starving in illuminated conditions. However, the specimens used in Christa et al. (2013) were bred and raised in a climate-controlled system rather than wild-caught animals kept in a temperature fluctuating environment reflecting

the natural habitat, and this or other unknown factors may have influenced our results. Monolinuron may also have unknown side effects, which might compromise the longevity of marine animals, however no peer-reviewed investigations into this issue were found and the manufacturer of this compound states it is safe for animals. The validity of this statement should be verified in future studies involving organisms that do not depend on photosynthesis for their nutrition.

Christa et al. (2014) state that monolinuron-treated slugs have a "marginally reduced" life expectancy where chemical blocking is likely incomplete. They continue by saying that "some plastids may still be able to fix carbon to a small extent, contributing energy-rich polymers that are available to the slugs", but this was not seen to be the case in this report, for *A. acetabulum* plastids and starch production in *E. timida.* The exact effects of monolinuron in these tissues is still unknown, and it is possible that these plastids may still be producing simple sugars such as fructose and glucose, the prerequisite components of amylose and amylopectin, but not producing starch itself. If starch was produced in some of the unblocked plastids, as Christa et al. (2014) suggest, it should have been detected in the samples presented here. We therefore conclude that starch is not produced in monolinuron-treated slugs. The decreased longevity seen in our samples treated with monolinuron leads us to conclude that starch produced in these kleptoplasts does help prolong a starving slug's life. Yet, the specific effects of monolinuron on all photosynthetic production will require further research.

By demonstrating that *E. timida* specimens were able to incorporate photosynthetically active chloroplasts, which increase their lifespan during starvation via starch production, we conclude that these incorporated chloroplasts provide nutrition and appear essential for slugs in longer starvation periods. The slug as a direct recipient of these photosynthates is a more likely scenario, since the isotopic radio labeling studies showed ^{14}C labeled carbon in numerous slug produced compounds (Raven et al. 2001; Teugels et al. 2008; M. E. Trench et al. 1970; R K Trench et al. 1973; Robert K Trench et al. 1969; Robert K Trench and Smith 1970), however, it remains difficult to know exactly when the slugs have access to this additional source of energy during starvation. It is likely that the slug

facing starvation will have access to these photosynthates when the kleptoplasts start to degrade on their own, or are actively digested by the slug itself, at a certain time during starvation. Regarding our results, it seems likely that this degradation process of chloroplasts and their photosynthates starts at least when we observed the decrease in amylose and probably already beforehand. In order to fully understand this degradation process, the expression of the genes involved in digestion of chloroplasts and photosynthates during starvation should be investigated.

3.5 Conclusions

This study is the first to examine starch production by the chloroplasts stolen and stored in long-term retaining sacoglossan slugs. Previous findings have uncovered algal-derived carbon in slug tissues unrelated to digestion, however it was unknown when and in which form fixed carbon was made available to the starving slug. We now know that not all of the photosynthates produced are immediately transported to slug tissue, rather some accumulate in the kleptoplasts as starch grains. These starch grains increase in size until about halfway through the maximum starvation time experienced by the slugs and subsequently decrease rapidly. This suggests that starving slugs receive the nutritive benefits of the enslaved plastids after an extended time period and that these chloroplasts do indeed help the slug withstand long starvation periods. By using the photosynthetic blocker monolinuron and finding the chloroplasts lack starch, we confirm here that the starch buildup is in fact due to photosynthesis occurring in these chloroplasts.

While many aspects of this enigmatic interaction between slugs and kleptoplasts remain unknown, this study directly indicates a major benefit the slug receives from its sequestered chloroplasts. This directly supports the causative hypothesis that some species of sacoglossan slugs can withstand extended starvation due to their sequestered plastids.

3.6 Acknowledgements

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3.7 Author Contributions

EMJL and HW devised the project concept and EMJL developed the method. LM and ANH conducted preliminary lab work as a proof of concept. VM conducted the lab work presented in this manuscript and analyzed the images. VM and EMJL interpreted the results. EMJL wrote the manuscript and all authors contributed to its revision. None of the authors have a competing interest.

Chapter 4

The Effects of Temperature on Functional Kleptoplasty in the Solar-powered Sea Slug Sister-taxa *Elysia timida* Risso, 1818 and *Elysia cornigera* Nuttall, 1989 (Gastropoda: Sacoglossa).

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Keywords: Kleptoplasty; *Elysia*; Intracellular Digestion; Endosymbiosis; Sacoglossa; Sea Slug

Abstract

Background

Despite widespread interest in solar-powered sea slugs (Sacoglossa: Gastropoda), relatively little is know about how they actually perform functional kleptoplasty. Sister-taxa *Elysia timida* and *E. cornigera* provide an ideal model system for investigating this phenomenon, since they feed on the same algal genus and only *E. timida* is capable of long-term kleptoplasty. Recent research has explored factors regarding functional kleptoplasty in *E. timida*, including their starvation longevity, digestive activity, autophagal response and photosynthetic efficiency under two different temperature conditions (18°C and 21°C) revealing both, the trends *E. timida* displays regarding each factor during starvation and influences temperature has on some aspects of functional kleptoplasty. This study examines *E. cornigera* regarding each of these factors in an attempt to elucidate differences between each species that could explain their differing kleptoplastic abilities. Since both species naturally occur in 25°C seawater (*E. timida* peak summer temperature, *E. cornigera* low winter temperature), each species was acclimatized to 25°C to facilitate comparison and determine if these species exhibit physiological differences to starvation when under the same environmental conditions.

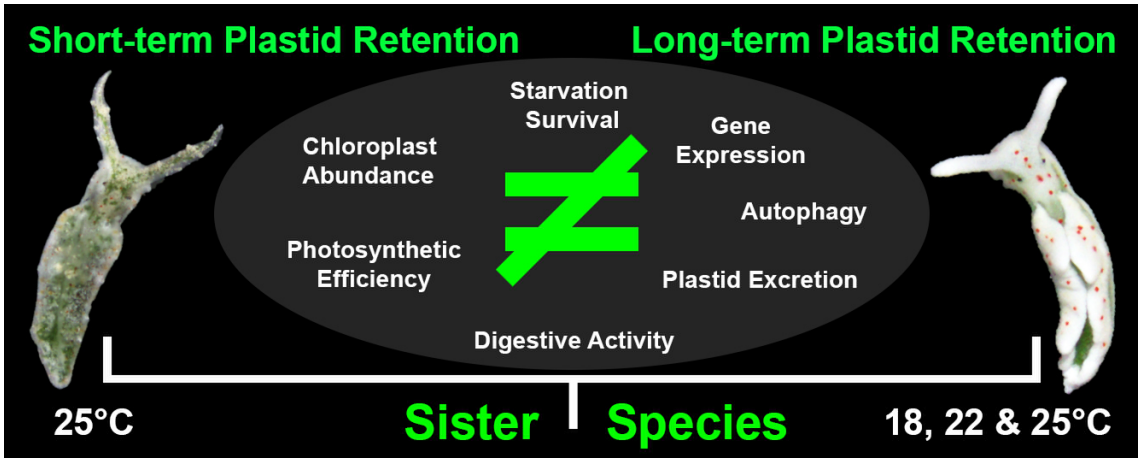
Results

When comparing the different *E. timida* temperature treatments, it becomes clear that increased temperatures compromise *E. timida*'s kleptoplastic abilities. Specimens acclimatized to 25°C revealed shorter starvation longevities surviving an average 46 days compared to the 92 day average observed in specimens exposed to 18°C. Each temperature treatment displayed a significantly different decrease throughout the starvation period in both, the rate of photosynthetic efficiency and in the decreasing functional kleptoplast abundance. Lysosomal abundance is assessed here as an indicator of different aspects of metabolic activity, neither of which could be correlated to temperature. *E. cornigera,* also

acclimatized to 25°C did not display significantly similar patterns as any of the *E. timida* temperature treatments, having fewer incorporated kleptoplasts, a higher lysosomal response to starvation, a faster decrease in photosynthetic efficiency and a lower starvation longevity.

Conclusions

These results confirm that each species has different physiological reactions to starvation and kleptoplast retention, even under the same conditions. While temperature affects aspects of functional kleptoplasty, it is likely not responsible for the differences in kleptoplastic abilities seen in these species.



Graphical Abstract

4.1 Introduction

Some members of the enigmatic sea slug clade, Sacoglossa (Gastropoda: Heterobranchia) are the only known metazoan taxa capable of enslaving functional chloroplasts from their algal food in a process entitled functional kleptoplasty [1–5]. *Elysia timida* Risso, 1818 has long been used as a model organism in investigations regarding functional kleptoplasty due to the extended period they can remain alive without access to food (averaging about 90 days) and their widespread distribution in high densities across the Mediterranean Sea [6–14]. The high functionality of their sequestered chloroplasts and the duration these kleptoplasts remain active, as revealed by Pulse Amplitude Modulated Fluorometry data, groups *E. timida* with a few other species as a Long-term (plastid) Retaining – LtR species [7, 15–20].

LtR species are found throughout the world's temperate and tropical oceans. *E. chlorotica* is probably the most well-known sacoglossan slug and holds the record for chloroplast retention with up to 14 months, depending on the report [15, 21–26]. This stenophagous slug, feeding only on *Vaucheria litorea* Agardh, 1873, is found in the western Atlantic coast, between Nova Scotia, Canada and Florida, USA [23]. *Plakobranchus ocellatus* Hasselt, 1824 is found throughout the Indo-Pacific where it feeds on numerous food algae and can survive starvation for up to 12 months, [21, 27–29]. *E. crispata* and *E. clarki,* currently debated as to whether they are two distinct species or one species with two morphotypes living in different ecosystems, are found in the Caribbean where they eat a multitude of algal species and last up to 40 days in starvation [15, 19, 20, 30–32]. The limapontioidean species *Costasiella ocellifera* Simroth, 1895 also survives over 50 days [17]. *E. viridis* has been attributed to the LtR group, however reports on its starvation longevity and photosynthetic efficiency vary, leading some to remove it from this group [33–38]. Another species, *E. asbecki* may also retain functional kleptoplasts for extended durations, however this is only suggested due to PAM activity in the first few weeks of starvation and requires confirmation before it can be considered a LtR form [39].

Recent phylogenetic analyses have confirmed *E. timida* and *E. cornigera* Nutall, 1989 as sister-species [15, 31, 32, 40, 41]. These organisms are anatomically very similar, and were even synonymized as one species [42] before the name *E. cornigera*

was resurrected and reassigned to the Caribbean populations, limiting *E. timida* to the Eastern Atlantic and Mediterranean Sea populations [40]. Although they are again considered separate species, *E. timida* and *E. cornigera* occur in similar habitats - the sublittoral zone at 0-5 meters depth. Both species feed by sucking the cell contents out of *Acetabularia* sp. (Dasyclades: Chlorophyta) cells: *E. timida* feeds on the Mediterranean algal species *A. acetabulum* Silva, 1952, while *E. cornigera* feeds on Caribbean *Acetabularia* species [31]. Despite not encountering it in the field, *E. cornigera* is capable of feeding on *A. acetabulum* and has not been reported to have a diminished longevity when the food is switched (de Vries et al. 2015; Schmitt et al. 2014). Interest in *E. timida* and *E. cornigera* as a model system is beginning to gain a foothold because *E. cornigera* cannot withstand the extended starvation periods *E. timida* can [43, 44]. While *E. timida* has a slow decline in photosynthetic efficiency throughout its long starvation period, *E. cornigera* kleptoplasts also begin high in efficiency, but then drop rapidly as the animal starves, leading to their classification as a Short-term Retaining (StR) form [31].

E. timida and *E. cornigera* inhabit similar habitats with a few notable differences. Mediterranean salinity is higher with an average 37.8-38.6 practical salinity units (psu) in Blanes (Spain) where *E. timida* was collected, whereas Caribbean species *E. cornigera* occurs in seawater averaging 35.5-36.5 psu (NOAA). *E. cornigera* is naturally found in warmer waters than *E. timida*: the Caribbean surface temperature along the Florida Keys ranges between 25-30° C each year [45], while the Mediterranean ranges from 14-25° C at Blanes, Spain (Marullo et al. 2007). The range in temperatures *E. timida* naturally experiences has already been shown to influence a number of factors related to functional kleptoplasty [8]. Despite both species naturally occurring at 25°C, these species have never been investigated under the same conditions with regard to functional kleptoplasty and their ability to withstand extended starvation.

Digestive activity and starch production in *E. timida* have been previously assessed for two different populations, each of which displayed significantly different results [35, 47]. A spring population was kept in a warmer environment (20-22°C) reflecting the surface temperature where they were collected, while the fall population was collected and cultured in 18°C matching the temperature from which they were collected. The trends observed in each of these investigations were the same, but the

time frames differed, with the warmer population showing increases in digestive activity, starch maxima and death occurring earlier in the starvation period. This revealed that temperature may be responsible for this shift and that its effects warrant further study [47]. Additionally, the increased temperatures and photosynthetic rates almost align with published *E. cornigera* data, leading us to hypothesize that the lower temperatures naturally experienced by *E. timida* may facilitate long-term plastid retention and a long survival duration in starvation.

In this study, the following factors were compared in order to elucidate differences between these species and determine which factors are influenced by temperature: longevity in starvation, photosynthetic efficiency (PAM values), functional kleptoplast abundance, digestive activity (lysosome activity within the digestive gland), autophagy (lysosomal activity outside the digestive gland) and excrement content for *E. cornigera* and *E. timida*. This is the first report to examine functional kleptoplast abundance, digestive activity, and excrement content for *E.* cornigera. *E. timida* were compared under three temperature regimes reflecting natural conditions (18°C, 21°C, and 25°C) to elucidate the effect temperature has on metabolic processes regarding functional kleptoplasty in this species. Furthermore, the 25°C *E. timida* population reflects natural conditions this species experiences and mirrors those experienced by *E. cornigera*, which allows the direct comparison of these species. Our working hypothesis therefore is, that *E. timida* and *E. cornigera* will display significant differences for each of the factors examined here despite experiencing the same environmental conditions, which supports the idea that each species has physiological differences regarding functional kleptoplasty and starvation. To exclude the effects of irradiance - which varies highly in each natural environment, both species were placed under the same artificial lighting conditions. *E. cornigera* were also transitioned to *A. acetabulum* to avoid any effects of the algal species.

4.2 Materials and Methods

4.2.1 Field Collection and Lab Conditions

Four aquaria containing lab-cultured *Acetabularia acetabulum* were transferred from an 18°C culture room to a 22°C room in March, 2016. The

temperature was slowly increased using tank heaters until it reached 25°C. Two of these tanks were gradually introduced to water with a lower salinity to match that of the Caribbean Sea: 36.5 psu seawater (from Mediterranean average 37.8-38.6 psu (NOAA). This was done to ensure that the algae consumed by *E. cornigera* did not suffer any effects of a sudden change in salinity.

Adult *Elysia cornigera* were collected on Spanish Harbor Key in April, 2016. They were removed from rocks covered in *Acetabularia crenulata* at a depth of 0.3-1 meters. The surface water temperature was 25°C. Animals were relocated to Bonn, Germany for experimentation. In Bonn, they were first acclimatized to artificial seawater and a 12 h L: 12 h D artificial light cycle (220 µmol quanta $m^{-2}s^{-1}$) for a week. They were then transitioned to the food alga *A. acetabulum* (previously acclimatized to 25°C) and allowed a further three acclimatization weeks to ensure most if not all of their sequestered chloroplasts derived from *A. acetabulum* rather than *A. crenulata*.

Adult *E. timida* were collected in July, 2016 in Blanes, Spain on rocks covered in *A. acetabulum* at 3-7 meters depth. The water temperature was 23.3°C when measured (measured at 7 meters depth, surface temperature 24.2°C). Animals were transported to Bonn, Germany and acclimatized to laboratory conditions for a week. Artificial lighting provided 220 µmol quanta $m^{-2}s^{-1}$ light for 12 h L: 12 h D. Large tanks (50-90L) containing 50 specimens and abundant food algae were then slowly warmed to 25°C over the course of a week, by placing the tank in a 22°C room and using tank heaters. They were then given a week to feed and acclimatize to the higher temperature before experimentation begun. To better reflect the natural acclimatization to a broader range of temperatures, data already available from specimens in the same wild population (Blanes, Spain) are included here [35]. These animals were collected during fall and spring seasons and kept according to their actual and natural acclimatization at 18°C and 21°C respectively, in the same room, same tanks, under the same light regimes as the 25°C *E. timida* (summer population) and *E. cornigera* populations.

E. cornigera were not subjected to multiple temperature treatments for multiple reasons including such as: our investigation set out to examine these species under natural conditions and *E. cornigera* has not been reported from water colder than ~24°C (Fig. 4.1); 2) subjecting *E. cornigera* to temperatures lower than those which they are naturally adapted could have a variety of different effects and would not reflect the natural conditions in which they are found and to which they are adapted; 3) collection limits necessitated prioritizing a limited number of animals for each experiment and prohibited studying artificially induced colder temperatures for *E. cornigera*.

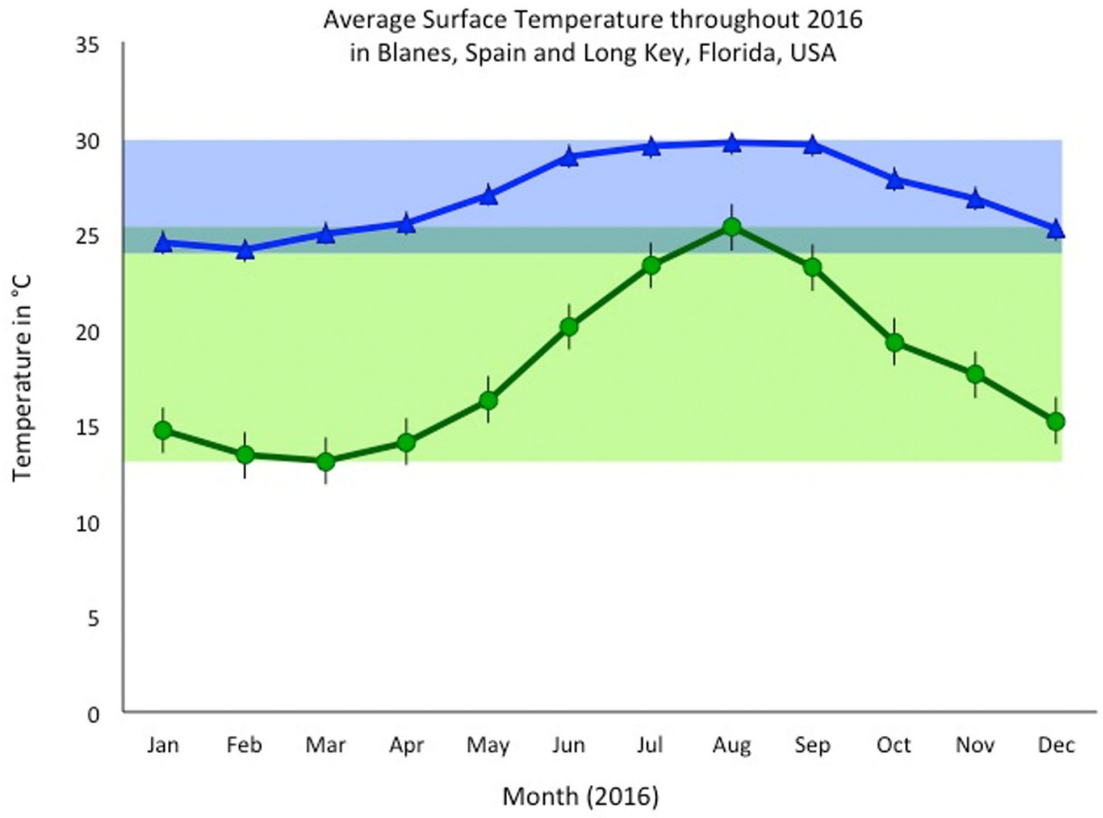


Figure 4.1 The average sea surface temperature in Blanes, Spain and Spanish Harbor Key, Florida, USA for 2016 according to satellite maps provided by NOAA. The monthly average for Blanes, Spain is depicted with a green line and the range is shaded in green showing *E. timida's* natural temperature range. *E. cornigera's* natural temperature range is shown by blue shading and the blue line depicts the monthly average temperature. The overlap in these ranges occurs at 25°C and is depicted by bluish-green shading.

4.2.2 Laboratory Procedures

The sample time points chosen for *E. timida* 25°C were 0, 7, 14, 21, 30, 42 days in starvation, to match the time points from the fall and spring season surveyed in Laetz et al. (2016). 60 and 89 days were planned but the slugs did not survive past 42 days. *E. cornigera* were sampled at 0, 7, 14, 21 days. For both *E. timida* and *E. cornigera,* three Pulse Amplitude Modulated Fluorometry (PAM) measurements were taken for each specimen, after 15 minutes of dark acclimatization, for each time point, according to the protocol established by [16]. Pulse Amplitude Modulated fluorometry (PAM) is a technique that allows the calculation of values, which reflect the overall photosynthetic efficiency of chloroplasts within a tissue (maximum quantum yield of photosystem II). These values are expressed as a percentage (the relative value F_V/F_M). Each specimen was then photographed (Fig. 4.2), and subsequently stained for lysosomal activity with acridine orange (AO). Staining was conducted by placing each specimen in a 5 µmol AO and filtered seawater solution for 30 minutes [35]. They were then vivisected and placed on a microscope slide for confocal microscopy on a Leica SPE Confocal Laser Scanning Microscope (CLSM). Five different cross-sections were scanned to gather an impression of the digestive activity throughout the entire animal. Each scan was 8µm deep, encompassing the entire thickness of the digestive gland tubule wall cells, while avoiding digestive gland lumen and tissue outside the digestive gland. Acridine Orange is excited by the blue laser (488 nm) and emits photons at 645-670 nm (dimer type optimum 656 nm) in extremely acidic environments such as lysosomes. Functional chloroplast abundance was also measured during these scans, by capturing chlorophyll a autofluorescence with blue laser excitation (488nm) and 600-640nm as the accepted emission range (chlorophyll optimum 633nm) [35].

Throughout the experimental processes, *E. timida* and *E. cornigera* were kept in tanks of 25 animals, without access to food. Weekly cleaning inhibited the growth of algae in these tanks. Excrement samples from each tank were examined weekly to determine if chloroplasts were present, indicating the decreases in functional chloroplast abundance is due to excretion rather than digestion. This

was accomplished using the CLSM to look for chlorophyll a autofluorescence in intact chloroplasts for each of the excrement samples.

4.3 Results

4.3.1 Starvation times

Three *E. timida* temperature conditions are compared here, to determine if any differences in survival rate are likely due to temperature. *Elysia timida* starved at 25°C survived for an average 42 days, the same duration the 21°C animals withstood. Neither of these populations survived as long as *Elysia timida* starved at 18°C – 89 days. *Elysia cornigera* survived an average 29.4 days in starvation (maximum 32 days) at 25°C, a shorter time span than any of the *E. timida* populations.

4.3.2 PAM values

Each *E. timida* temperature treatment, 18°C, 21°C and 25°C, and *E. cornigera* begin their starvation periods with high PAM values (F_VF_M), having averages of 0.749, 0.74, 0.749, and 0.729 respectively. A one-way ANOVA reveals an F-value of 1.09 and a p-value of 0.36, which confirms that there is no significant difference in the mean starting value for each population. After only 7 days however, the *E. timida* populations group with high PAM values: 0.723 (18°C), 0.721 (21°C) and 0.760 (25°C), while *E. cornigera* diverges, dropping rapidly to and average 0.401. While each of the *E. timida* treatments and *E. cornigera* decrease, the rate of PAM value decrease differs. *E. timida* (18°C) decreased slowest at 0.0065% per day (R^2 = 0.99), *E. timida* (21°C) by 0.012% per day (R^2 = 0.90), *E. timida* (25°C) by 0.013% per day (R^2 = 0.97), and *E. cornigera* by 0.023% per day (R^2 = 0.92) (Fig 4.3A). One-way ANOVAs reveal that after the 0-day time point, *E. cornigera* PAM values are always significantly lower than each of the *E. timida* treatments, for example: at 14 days, $F = 83.1$ and $p < 0.0001$. Tukey HSD tests show no significant difference between the 18°C and 21°C *E. timida* populations at this point, but significant differences between these treatments and *E. timida* 25°C ($p < 0.05$) as well as *E. cornigera* ($p < 0.01$). By 30 days in starvation, all of the treatments diverge

significantly: $F = 118.62$, $p < 0.0001$ (ANOVA) and comparison between each treatment $p < 0.01$ (Tukey HSD). PAM averages throughout their starvation periods for each of the species examined are summarized in Fig. 4.3A.

4.3.3 Functional Kleptoplast Abundance

Kleptoplast abundance was also recorded for each time point during the starvation period for each *E. timida* temperature treatment and *E. cornigera.* While both species decline in the number of functional chloroplasts, the number of incorporated plastids at the beginning of the starvation period and rates at which they decrease differ (Fig. 4.3B). *E. cornigera* has the lowest functional chloroplast retention time and some specimens died with some functional kleptoplasts in their bodies after 21 days (Fig. 4.4A, H). Both 18°C and 21°C *E. timida* populations contained functional chloroplasts until 42 days, while the warmer *E. timida* (25°C) lost almost all functional chloroplasts earlier, after only 30 days (Fig. 4.4J, Q). An overview of chloroplast decrease in 25°C *E. timida* and *E. cornigera* can be seen in Fig. 4.4. Miniscule amounts of chlorophyll a were found in *E. timida* 18°C until 60 days, however the amounts measured are so low they are likely remnants found in degraded thylakoids.

4.3.4 Lysosomal Activity

Lysosomal activity inside the digestive gland tubules is presumably involved in digestion, and used here as an indication of digestive activity in these animals. Each of the *E. timida* populations surveyed here followed the same trend, a very low lysosomal abundance at the beginning of the starvation period (0-5 ± 0.22% of the digestive gland total area covered by lysosomes) and a large increase (48 – 60 ± 3.4% total lysosome coverage) in the second half of the starvation period (differing time spans depending on the temperature acclimatized population) (Fig. 4.4J, 5A). These populations differ however, in the rate of lysosomal increase. 25°C *E. timida* have an almost linear increase, modeled best by the function: $y = 1.16x - 0.51$ ($R^2 = 0.99$). The 18°C and 21°C *E. timida* relative abundances do not increase linearly, instead modeled best by the quadratic functions: $y = 0.0054x^2 + 0.23x +$

0.99 (R^2 = 0.99) and y = 0.052x^2 - 1.07x + 3.69 (R^2 = 0.95). The *E. cornigera* specimens examined display a slight increase in lysosome coverage, from 3.9 - 9.4 ± 1.1% over the 21 days they starved (Fig. 4.5A).

While lysosomes are an integral part of normal cellular machinery, activity increases above the baseline normal background machinery level, outside the digestive gland, may be indicative of autophagy. Only *E. cornigera* had changes in the relative percent coverage of lysosomes outside the digestive gland. *E. cornigera* began the starvation period with a 1.5 ± 0.31% non-digestive gland tubule percent coverage, and increased to 4.7 ± 1.1% during the 21-day starvation period. The different *E. timida* populations displayed minor fluctuations in their percent coverage (0 -0.5 ± 0.02%), but never increased to even 1% percent throughout the entire starvation period. (Fig. 4.5B).

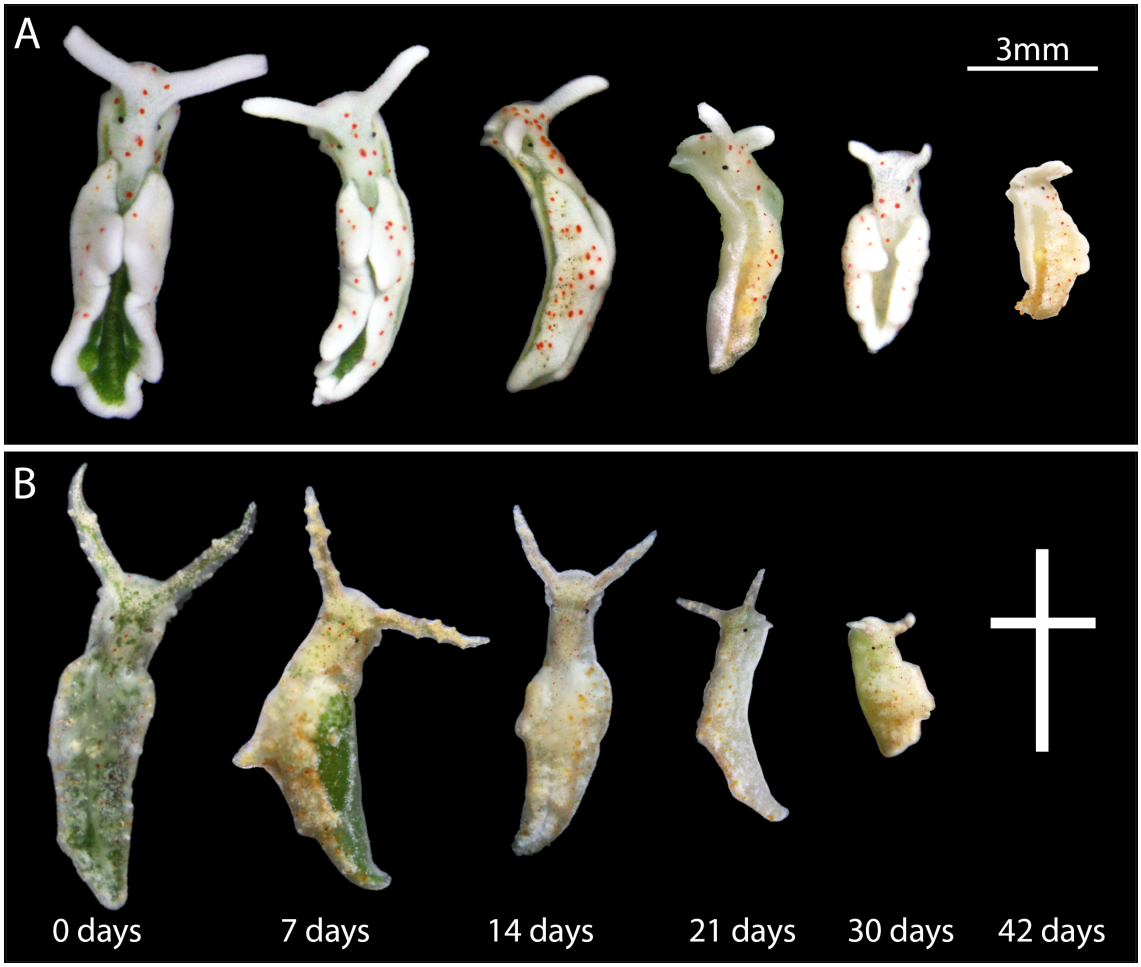


Figure 4.2 **Species investigated during starvation. A: *E. timida* during starvation at 25°C. The loss of green pigmentation is due to the loss of chlorophyll a within the slug. B: *E. cornigera* during starvation. The cross (†) at the end of the *E. cornigera* series indicates that this species did not survive starvation up to 42 days.**

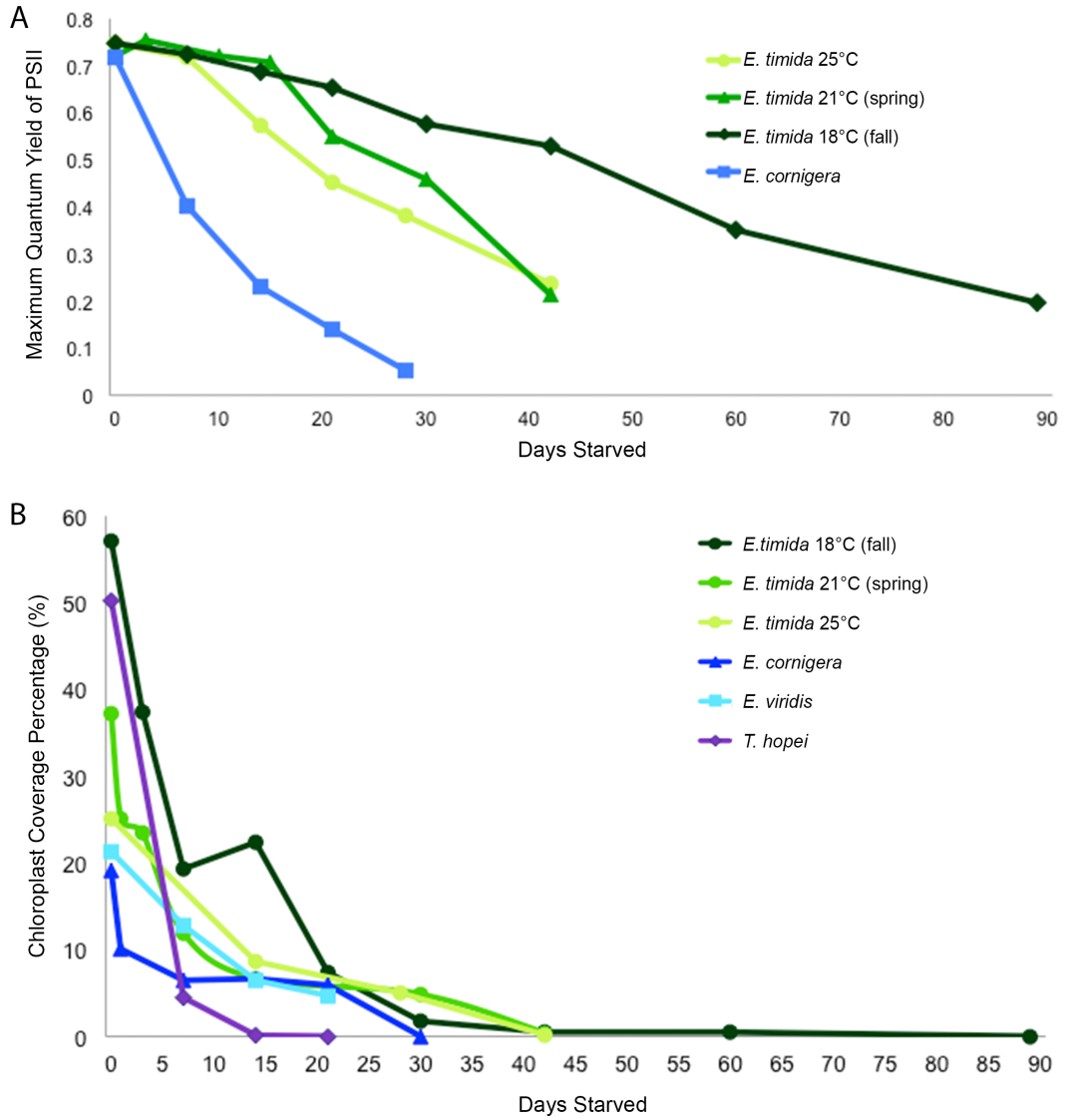


Figure 4.3 Chloroplast efficiency and abundance. A: The chloroplast efficiencies (PAM Values) of *E. timida* in each temperature treatment and *E. cornigera*. *E. cornigera* drops in photosynthetic efficiency more rapidly than any of the *E. timida* treatments. Each point is the average of three independent measurements following dark acclimation. *E. timida* at 25°C is denoted by light green circles, at 21°C by medium green triangles and at 18°C by dark green diamonds. *E. cornigera* at 25°C is represented by blue squares. Lines connecting each point display the rate of decrease and increase the visibility of each trend. B: Percent chlorophyll coverage throughout starvation for each examined species. This was computed by measuring the area (in each image, for each stack) that was covered in Digestive Gland Tubule (DGT) and the remaining area – designated as Non-Digestive Gland Tissue. The area covered by functional chloroplasts inside the DGT was divided by the total DGT producing a relative value, a percent of the DGT that is filled with chloroplasts. This value was averaged for each measurement taken (8 images/stack, 5 stacks/specimen, 3 specimens/time point totaling 120 measurements for species at each time point) and graphed here to show the overall trend for each species at each time point. *E. timida* 18°C is shown by dark green circles, 21°C by medium green circles and 25°C by light green circles. *E. viridis* is depicted by light blue squares, *E. cornigera* by dark blue triangles and *T. hopei* by purple diamonds.

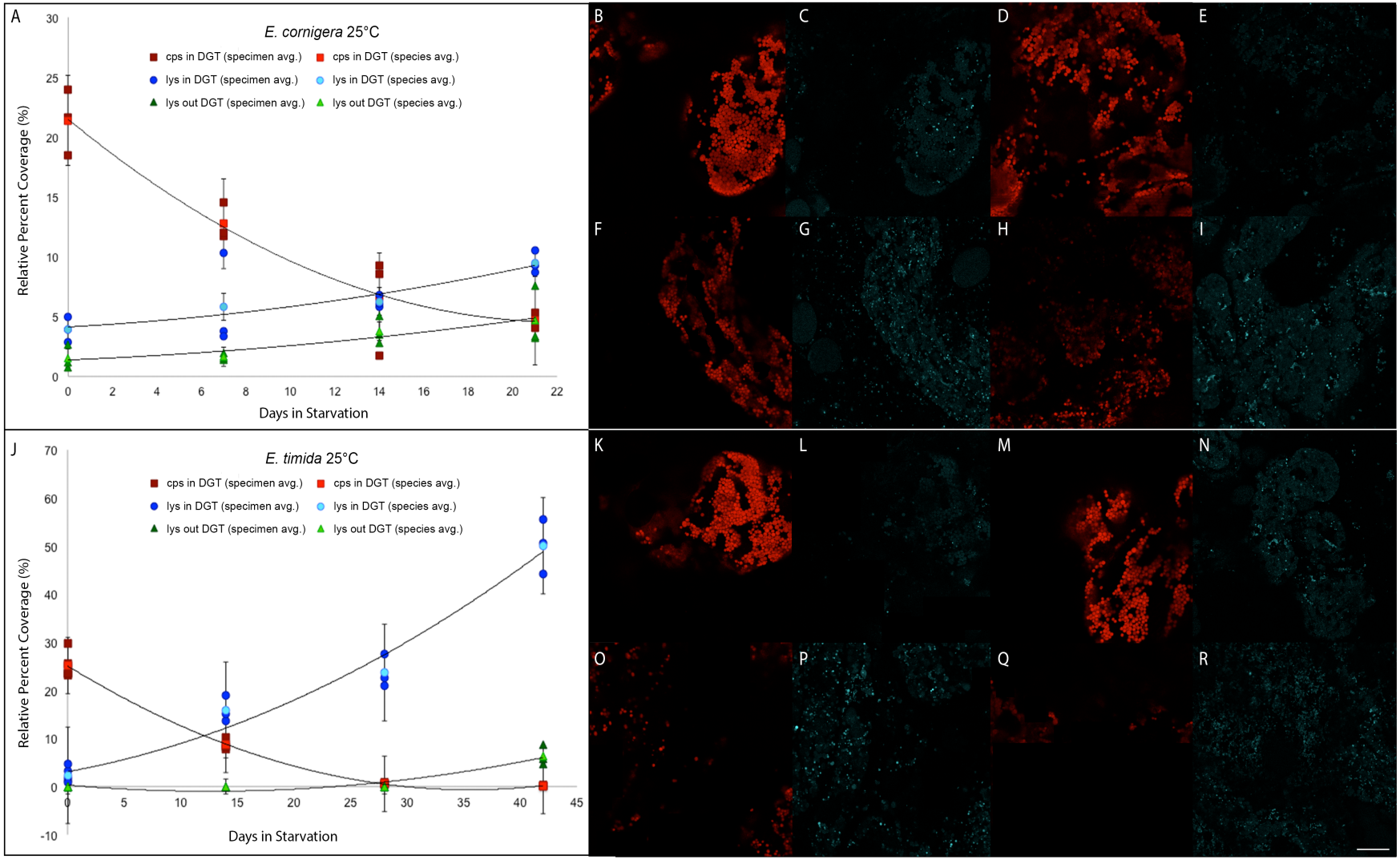

A
E. cornigera 25°C
cps in DGT (specimen avg.)
cps in DGT (species avg.)
lys in DGT (specimen avg.)
lys in DGT (species avg.)
lys out DGT (specimen avg.)
lys out DGT (species avg.)
Relative Percent Coverage (%)
Days in Starvation
B
C
D
E
F
G
H
I
J
E. timida 25°C
cps in DGT (specimen avg.)
cps in DGT (species avg.)
lys in DGT (specimen avg.)
lys in DGT (species avg.)
lys out DGT (specimen avg.)
lys out DGT (species avg.)
Relative Percent Coverage (%)
Days in Starvation
K
L
M
N
O
P
Q
R

Figure 4.4 (previous page) Functional chloroplast and lysosome abundance in *E. cornigera* and *E. timida* 25°C. A: *E. cornigera.* Functional chloroplasts are depicted by red squares, the dark red ones showing the average per specimen (n=3) and the light red squares showing the average of these three specimens representing the species average at each time point. Lysosomes within the digestive gland tubules (DGT) are illustrated with blue circles, the dark circles again showing the specimen average and the light circles revealing the species average. Lysosomes outside the DGT are demonstrated by green triangles, the dark depicting the specimen average and the light green indicating the species averages. For clarity, error bars (standard error) are only shown for the species average. B: *E. cornigera* cp abundance at 0-days starved. Cps are falsely colored red. C: *E. cornigera* lys abundance at 0-days starved. Lys are falsely colored blue. D, E: *E. cornigera* – 7-days starved. F, G: *E. cornigera* – 14-days starved. H, I: *E. cornigera* – 21-days starved. J: Cp and Lys Abundance in *E. timida* starved at 25°C. Functional cps and lys in/out DGT are depicted as described above for (A). K: Cps in *E. timida* 25°C starved for 0 days. L: Lys in *E. timida* 25°C starved for 0 days. M, N: *E. timida* 25°C – 14 days starved. O, P: *E. timida* 25°C – 21 days starved. Q, R: *E. timida* 25°C – 42 days starved. Scale bar – 50 µm.

4.3.5 Excrement

Excrement samples were examined to determine whether or not functional chloroplasts were being excreted throughout the starvation period. Excreted chloroplasts were discovered in *E. cornigera* samples throughout the starvation period although none were ever observed in samples form any of the *E. timida* populations (Fig. 4.6A, B). Excreted plastids were still round in shape, appearing intact. Nothing about the chloroplasts found in the *E. cornigera* excrement appeared different than those found within the slug, except for the debris surrounding them (Fig. 4.6A).

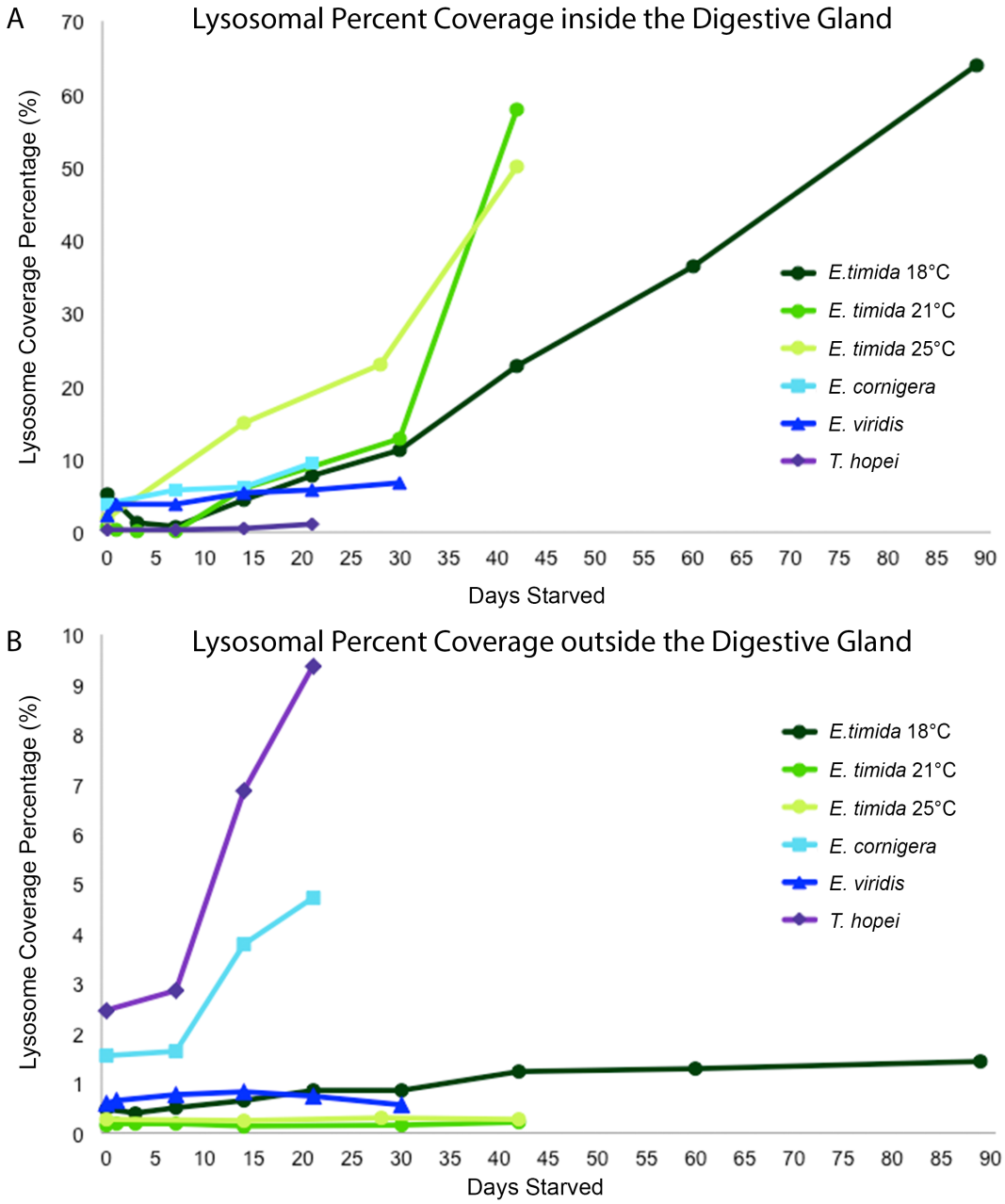


Figure 4.5 Lysosome abundance in each species examined throughout a starvation period. A: Percent Lysosome Coverage inside the Digestive Gland Tubule (DGT). B: Percent Lysosome Coverage outside the DGT. The data in A and B were computed following the same procedure as described in Figure 4.2 for functional chloroplasts. *E. timida* 18°C is shown by dark green circles, 21°C by medium green circles and 25°C by light green circles. *E. viridis* is depicted by light blue squares, *E. cornigera* by dark blue triangles and *T. hopei* by purple diamonds.

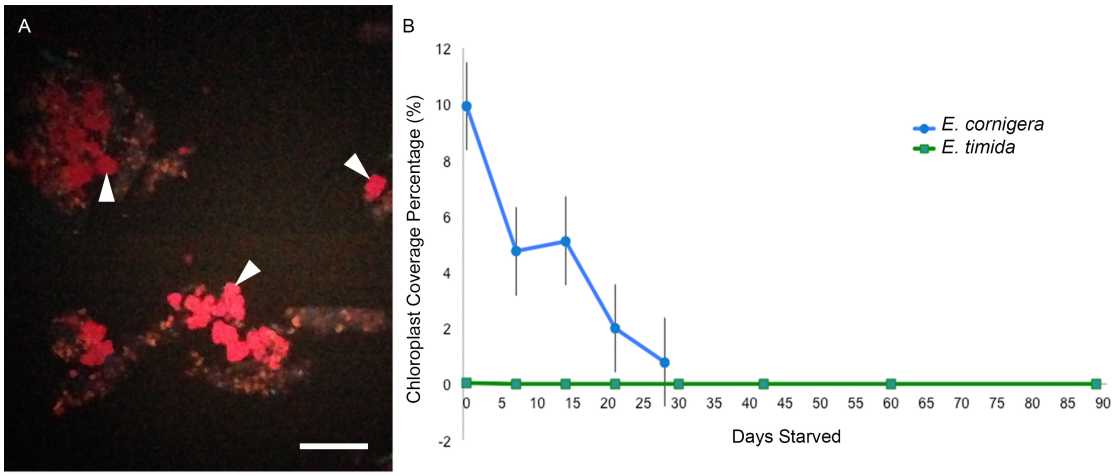


Figure 4.6 Functional chloroplasts in *E. timida* and *E. cornigera* excrement. A: *E. cornigera* excrement containing a high density of functional kleptoplasts (red circles, a few indicated by arrowheads), scale bar – 25µm. B: Functional chloroplast density within *E. timida* and *E. cornigera* excrement. Since *E. timida* at each temperature treatment lacked chloroplasts in their excrement, they were all graphed as a single *E. timida* line (green squares). *E. cornigera's* excreted plastid abundance is depicted by blue circle.

4.4 Discussion

The duration each *E. timida* population could survive depends on the temperature conditions to which it was acclimatized. Both *E. timida* populations that experienced elevated temperatures (21°C and 25°C), lived for a significantly shorter time span, only reaching 42-50 days, while *E. timida* at 18°C, the temperature recorded in their natural habitat during the fall, survived over 100, with some specimens even surviving to 120 days (EMJL unpublished result). Interestingly, *E. timida* at 21°C do not survive longer than those at 25°C. Despite this, temperature may still explain the reported discrepencies for *E. timida* longevity in starvation, chloroplast abundance, starch abundance and photosynthetic efficiency [35, 47]. *E. cornigera* only survived a maximum of 32 days in the laboratory, suggesting that elevated temperatures shorten *E. timida's* life expectancy, but not to the same degree that *E. cornigera* naturally experiences. This indicates that lower temperatures may facilitate *E. timida's* long-term survival

but temperature is not the only factor distinguishing these species from one another regarding longevity in starvation.

The photosynthetic efficiencies of *E. cornigera* and *E. timida* have been reported in previous investigations, and while water temperatures were often reported, these experiments were conducted under a variety of different conditions (different field sites, lab setups, collection times) so comparison to our study risks unintended error [6, 8, 15, 48]. In this study, PAM values are recorded for *E. timida* acclimatized naturally according to environmental temperatures and then accordingly cultivated at 18°C, 21°C and 25°C and *E. cornigera* at 25°C under standardized conditions. Each *E. timida* temperature group and the *E. cornigera* began with a statistically similar value but diverged rapidly, suggesting both *E. timida* and *E. cornigera* that are fed the same food algae and kept under the same conditions will sequester chloroplasts with the same photosynthetic efficiencies, however once starvation begins, the photosynthetic efficiencies change depending on the temperature and species. Since algae from the same culture was provided each species and the algae for 25°C *E. timida* and *E. cornigera* was also acclimatized to 25°C, the differing declines in photosynthetic efficiency between 25°C *E. timida* and *E. cornigera* indicate a difference in the way chloroplasts function and survive in the digestive gland cells of these two species. This suggests the slug species itself is at least partially responsible for the health and longevity of the chloroplasts within its digestive gland and the ability of a chloroplast to be retained is not solely due the algal species involved.

The declines in *E. timida* photosynthetic efficiency presented in this study differ from the photosynthetic efficiencies measured by Schmitt et al. (2014), which also examined *E. timida* under different temperature regimes. In that study, the temperatures were not closely controlled (fluctuating between ~19-24°C depending on the season), and this may explain why their populations did not reveal distinct grouping based on the temperature like they did in this study. Despite the lack of a standardized temperature, both of these studies confirm that temperature leads to a faster decline in photosynthetic efficiency during starvation.

Functional kleptoplast abundance within the digestive gland tissue of multiple species has now been analyzed [35, 49–51]. This analysis adds *E. cornigera* and *E. timida* starved at 25°C to determine if there are differences in chloroplast metabolism inherent to the slugs' cells that are independent to the natural temperatures these animals encounter and the results here indicate this to be the case. Each of the species investigated here reveal a different rate of functional chloroplast decrease, with no two species sharing a similar rate, although they all decrease. Despite consuming the same algal species and living in side-by-side aquaria under the same lab conditions (light, temperature…), *E. timida* 25°C and *E. cornigera* do not display the same decrease in functional chloroplasts. *E. cornigera* were even observed dying with functional chloroplasts inside their digestive gland tubules, while none of the *E. timida* temperature treatments were observed containing functional plastids at their time of death. The only discernable abiotic factor that was different in each tank was the salinity of the water, which was always matched to the natural conditions each species encounters in the field. The effects of salinity on functional kleptoplasty require further investigation to determine whether or not it influences chloroplast vitality.

Two other kleptoplast retaining species, *Thuridilla hopei* (StR) and *Elysia viridis* (StR or LtR depending on the ingested algae) have been investigated regarding digestive activity, kleptoplast abundance and the potential autophagal response [35]. Despite confirmation that both species are StR forms, *T. hopei* and *E. cornigera* do not display the same pattern regarding functional chloroplast abundance in their tissues. They survive about the same time in starvation, however *T. hopei* is devoid of functional chloroplasts after only 14 days (confirming observations made by Martin et al. (2015)) whereas *E. cornigera* dies containing functional chloroplasts. *E. viridis,* which is sometimes considered a StR species also differs in functional chloroplast abundance, having no sign of functional chloroplasts by the end of its starvation period, but lasting and average of 10 days longer in starvation. *E. viridis* is enigmatic however, having various starvation longevities that may depends on the algae ingested, and exhibiting various photosynthetic efficiencies in starvation, so further research is required to facilitate a proper comparison to this species [33, 38, 52].

Two of the *E. timida* populations (21°C and 25°C) showed a faster decline in the number of functional chloroplasts, lower number of sequestered plastids at the beginning of the starvation period and faster death by starvation, however they still survived starvation longer than *E. cornigera* (this study), *E. viridis* and *T. hopei* [35]. A comparison of all of the *E. timida* populations here also shows that temperature does not seem to affect the rate of functional chloroplast decrease in the tissues, since 25°C *E. timida* have a lower rate of decrease than the 21°C and 18°C groups. While 18°C *E. timida* lived the longest, their functional plastid abundance was almost non-existent after 42 days, the same time span observed in 21°C *E. timida* and 25°C *E. timida.* These results seem to contradict results that show the maximum amylose concentration inside kleptoplasts occurring at 42 days in 18°C starved *E. timida,* which also indicates the presence of these plastids since amylose is accumulated within the plastid [47]. However, this discrepancy is likely due to the means by which kleptoplast abundance/presence was assessed. Upon examining histological sections, it is clear that kleptoplasts are still present until after 63 days [47], however if they do not contain structurally intact chlorophyll a, they will not be detected using chlorophyll a autofluorescence. This highlights the need for a method that accurately quantifies kleptoplast abundance in these slugs and does not rely on autofluorescence.

Lysosomal activity within the digestive gland tubule network was monitored as an indicator of digestive activity in these tissues. Two different patterns were observed amongst the species examined. Species with low longevities in starvation, *E. cornigera, E. viridis* and *T. hopei,* display a steady increase in lysosomal abundance during their starvation periods [35]. *E. timida*, however, can withstand a much longer duration without food and exhibits a two part pattern: the first half of the starvation period is marked by a slight increase in the number of lysosomes while the second half of the starvation period reveals a faster rate and significantly higher lysosome abundance. This pattern occurs within each of the temperature treatments investigated here suggesting temperature and lysosome production are not correlated in this species.

Lysosomal activity outside the digestive system is not involved in an organisms digesting foreign material as food, but rather comprises an organism's

internal digestion of its own cellular components as part of normal cellular functioning and as autophagy. There was no observable difference between *E. timida* at 18°C and 21°C when compared to *E. timida* 25°C and these populations closely resembled those observed for *E. viridis* [35]. Neither *E. cornigera* nor *T. hopei* displayed a trend that aligned with *E. timida* or *E. viridis,* indicating each of these species has a different autophagal responses during starvation. The strong increase in lysosomes produced by *E. cornigera* and *T. hopei* after 7 days in starvation suggests an activation of autophagal procedures in these animals. This furthers conclusions made by de Vries et al. (2015) when they examined metabolic transcription in *E. timida* and *E. cornigera,* and revealed elevated expression rates of autophagal genes in *E. cornigera* after 7 days in starvation, which were not present in *E. timida.* Interestingly, *E. timida* and *E. viridis* do not exhibit a visible increase, suggesting the autophagal processes behind their decreased size are not detected using the acridine orange method. Fully understanding the autophagal processes in these species will require further investigations.

The excrement surveys conducted here reveal that *E. cornigera* do not digest all of the chloroplasts they sequester. The chloroplast abundance within the excrement samples deceases throughout the starvation period and since no new chloroplasts were introduced to these animals, it is clear that some chloroplasts were retained for weeks within the digestive gland before finally being excreted. The cause for this delay is unknown, and requires further investigation, but may be due to Reactive Oxygen Species (ROS) buildup around these chloroplasts. ROS accumulation was examined by de Vries et al. (2015) in *E. cornigera* and *E. timida*, the former showing increases in both hydrogen peroxide (H_2O_2) and superoxide (O_2^-) during starvation. Plastid excretion and autophagy (as seen with the lysosomes in- and outside the digestive gland) by *E. cornigera* may be a response to the buildup of these cytotoxic molecules. Each of the *E. timida* temperature groups lacked plastids in their excrement, showing that *E. timida* do digest the chloroplasts they sequester. *E. timida* also lack an increase in ROS during starvation [43], although this was only monitored until the 30-day starvation point, which likely explains the first half of the *E. timida* starvation period, where lysosomal activity is low. Further investigations are needed to confirm if ROS

levels increase in *E. timida* after 30 days, correlating to the lysosomal activity increases observed here. This fundamental difference in the way *E. timida* and *E. cornigera* react to their sequestered plastids may be exactly what allows *E. timida* to survive 3-4 times as long as *E. cornigera* in starvation and ROS could be implicated in the digestive activity differences observed here.

The overall differences observed in each of the species examined here suggest the complexity behind functional kleptoplasty. *E. cornigera, T. hopei* and *E. viridis* have all been previously labeled StR forms, which is based on photosynthetic efficiency [15]. Despite this, when another factor is examined, such as functional kleptoplast abundance, this category no longer appropriately groups these organisms. When each of the species examined here is reduced to a trend and compared to the other's trends, it is clear how the LtR, and StR category labels fail to group these organisms in consistent groups. Figure 4.7 (specifically C, E and H) compares each species with each of the factors investigated here, to show how this grouping proves inadequate when examining something other than photosynthetic efficiency amongst these animals. Based on this, we therefore suggest that these labels only be used when referring to photosynthetic efficiency amongst sacoglossan slugs. For other factors that have been or may be investigated, such as functional plastid abundance and lysosomal activity, the lack of natural clustering should be kept in mind and these categorical labels avoided.

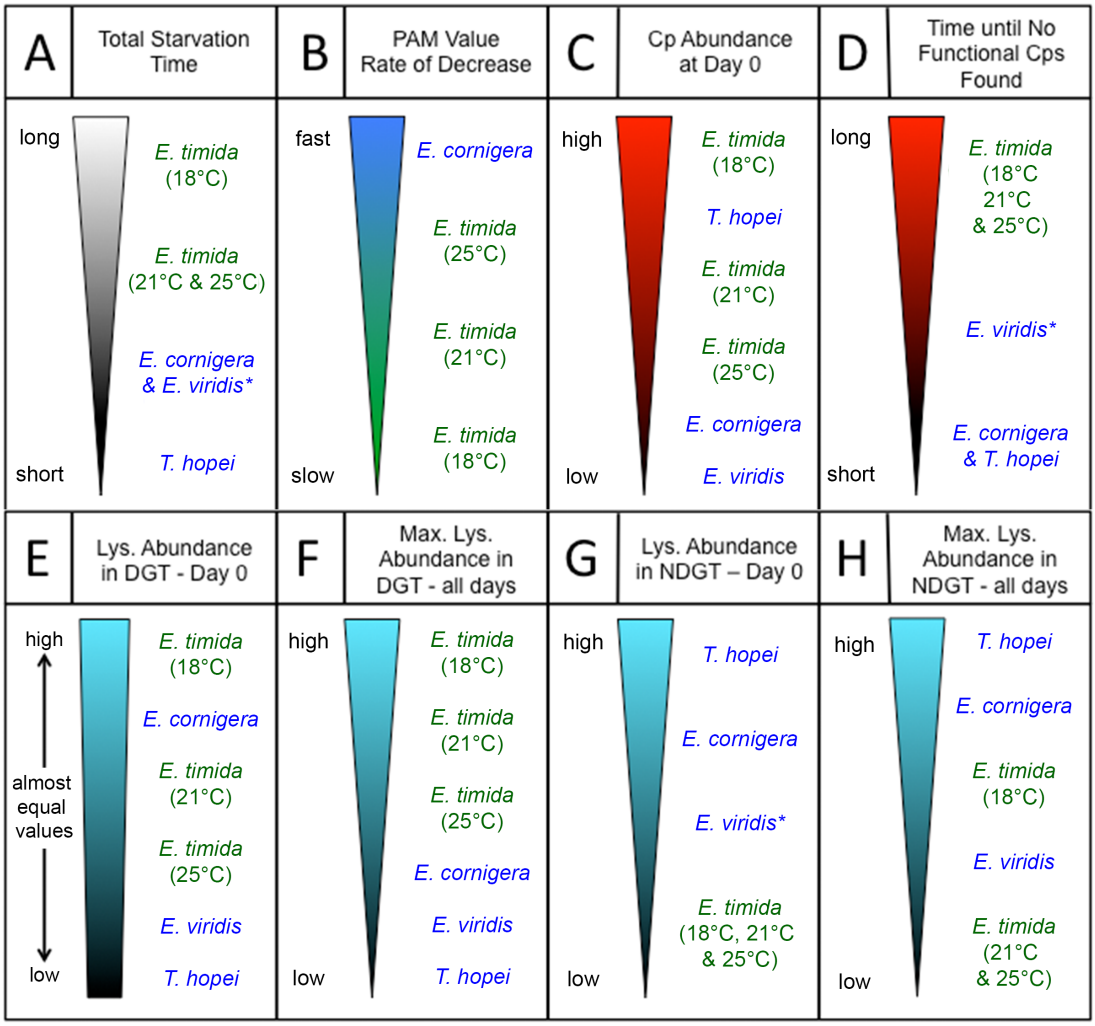


Figure 4.7 Overview of the digestive trends in the examined species. To visualize the overall trends observed in each of these populations, for each experiment, the numbers were removed and each population was ranked. The colored triangles represent the quantity of each factor (for example: number of chloroplasts in unstarved animals) from large to small. Each population is listed according to rank. Species written in green are the traditional long-term chloroplast retaining species whereas those in blue are the short-term retaining species. *E. viridis* is designated in blue with an asterisk because it more closely resembles an StR form in this investigation, but has been classified as LtR in the past. A: Total longevity in starvation from a long duration to short. *E. timida* 18°C have the longest longevity in starvation and *T. hopei* have the shortest. The triangle is colored black and white to represent alive/dead. B: PAM value rate of decrease. *E. cornigera* have the fastest decrease in photosynthetic efficiency during starvation. The triangle is blue/green because StR is often depicted in blue and LtR in green. C: Functional chloroplast abundance in unstarved (Day 0) slugs. Throughout this investigation, chloroplasts are indicated in red because chlorophyll a fluoresces red. D: The number of days until functional chloroplasts are no longer observed in the digestive gland. E: Lysosome abundance within the digestive gland tubule for unstarved specimens (Day 0). A quadrilateral rather than triangle is drawn because the values are very similar. It is colored blue aligning with the pictures of lysosomes presented in this investigation. F: The maximum lysosome abundance throughout the entire starvation period. These maximums occur at different time points for each species. G: Lysosome abundance in non-digestive gland tissue (NDGT) at Day 0. H: Maximum lysosome abundance in NDGT throughout the starvation period.

4.5 Conclusions

Put simply, *E. cornigera* are not simply warmed up *E. timida* that do not survive long starvation periods due to increased temperatures. Physiologically, these two species are not the same when it comes to responding to starvation, even under the same temperature conditions, and these dissimilarities require further investigation. Temperature did affect *E. timida's* photosynthetic efficiency and total starvation time, but could not be correlated to increased digestive rates, autophagy or the decline of functional chloroplasts in these tissues. When the comparison is broadened to include all of the species examined here, it is clear that each species reacts to starvation differently, some excreting their chloroplasts - others digesting, some showing signs of autophagy - others not, which defies our ability to assume similarity between sacoglossan species and reveals the complex nature of functional kleptoplasty in these animals.

4.6 Acknowledgments

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Chapter 5

A Comparison of Photosynthate Production in the Solar-powered Sister-taxa *Elysia timida* Risso, 1818 and *Elysia cornigera* Nuttall, 1989

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Abstract

Sacoglossan sea slugs are the only known metazoans capable of functional kleptoplasty, the ability to sequester functional chloroplasts from an algal food source and withstand extended starvation periods due to the photosynthates they produce. Sister-taxa *Elysia timida* and *E. cornigera* are increasingly used as model species when examining this phenomenon, since they feed exclusively on the same algal genus, *Acetabularia,* and only *E. timida* is capable of long-term plastid retention. Recent investigations have used Lugol's solution and semi-thin histology to examine photosynthate production in *E. timida* throughout a starvation period, revealing an increase and subsequent decrease in starch abundance. This study confirms these results by examining starch accumulation in starving *E. timida*, using transmission electron microscopy at frequent intervals throughout the starvation period. We also present differences found in *E. cornigera* kleptoplasts, found by both semi-thin histology and Lugol's Iodine as well as transmission electron microscopy. This provides a first glimpse into photosynthate production in a sacoglossan species not capable of long-term kleptoplast retention. Despite coming from the same algal culture, *E. cornigera's* incorporated chloroplasts do not show the same increase in starch abundance nor subsequent decrease. Unstarved *E. cornigera* plastids contain low amounts of starch, which are no longer present when starvation begins. By comparing photosynthate production in these species, after they have ingested the same algal culture, it becomes clear that *E. cornigera* does not profit from accumulated starch like *E. timida* does, and this is likely what defines each species' ability to withstand starvation.

5.1 Introduction

Solar-powered sea slugs (Heterobranchia: Gastropoda) are the only known metazoans capable of stealing functional chloroplasts from their algal food, retaining them for months while staving off death by starvation presumably due to these stolen plastids (Kawaguti & Yamasu, 1965; Taylor, 1968; Robert K Trench, Greene & Bystrom, 1969; Greene, 1970; Greene & Muscatine, 1972). This enigmatic process, called functional kleptoplasty has garnered the attention of both, researchers and laymen, interested to know how this phenomenon occurs. Most sacoglossan species feed by piercing chlorophyte cell walls with a highly derived radular tooth and sucking out the cell contents (Jensen, 1980, 1994; Händeler *et al.*, 2009). Once ingested, these cell contents pass into the slug's stomach and then the digestive gland where they are endocytosed for digestion (Hirose, 2005; Martin, Walther & Tomaschko, 2012; Wägele & Martin, 2014). Sacoglossan species capable of kleptoplasty, however, do not digest these chloroplasts, but rather incorporate them into their digestive gland cells where they are retained for a few weeks to 12 months depending on the species (Maeda *et al.*, 2012; Christa, Wescott *et al.*, 2013).

Sacoglossans primarily feed on the chlorophyte order Ulvophyceae, a morphologically diverse group of macroscopic, large-celled algae (Jensen, 1980, 1997; Williams *et al.*, 1999; N E Curtis *et al.*, 2005; Nicholas E Curtis, Massey & Pierce, 2006; Pierce *et al.*, 2006; Händeler & Wägele, 2007; Händeler *et al.*, 2010; Klochkova *et al.*, 2010; Maeda *et al.*, 2012). Chlorophyte plastids produce a number of different photosynthates including carbohydrates, proteins and lipids (Greene, 1970; Greene & Muscatine, 1972; Shephard & Levin, 1972; R K Trench & Gooday, 1973; Hinde & Smith, 1975; Muscatine, Pool & Trench, 1975). Numerous studies have identified ways in which the slug benefits from these photosynthetic products, linking photosynthate production to their ability to withstand extended starvation periods. Radiolabelling studies have demonstrated algal-derived carbon isotopes in multiple slug-produced compounds, showing the slug does receive photosynthetic products (summarized in E. M. J. Laetz et al. 2017; Wägele and

Martin 2014). Since compounds containing photosynthetically fixed carbon isotopes were found almost immediately after exposure to labeled carbon, in numerous investigations (Robert K Trench, 1969; Robert K Trench, Greene & Bystrom, 1969; M. E. Trench, Trench & Muscatine, 1970; Robert K Trench & Smith, 1970; R K Trench, Boyle & Smith, 1973; Raven *et al.*, 2001; Teugels *et al.*, 2008), it was hypothesized that the slug directly benefits from its incorporated chloroplasts – here called the direct benefit hypothesis.

Recent reports have challenged this hypothesis, proposing instead the larder hypothesis, which suggests the nutritional support chloroplasts provide starving slugs is only made available to the slug later in the starvation period, when the plastid itself is digested (Christa, Gould *et al.*, 2014; Pelletreau *et al.*, 2014). This is supported by starvation experiments where animals starved in the light had the same starvation longevity as those starved in the dark, which questions the role of photosynthesis in slug survival overall (Christa, Zimorski *et al.*, 2013). The larder hypothesis is also supported by recent investigations into *Elysia chlorotica* Gould, 1870, which show an accumulation of lipid droplets surrounding their sequestered chloroplasts during a starvation period (Pelletreau *et al.*, 2014). The accumulation of a high energy molecule while the slug is starving and losing mass due to autophagy suggests the slug is not directly profiting from these photosynthates and that any photosynthates that are being directly digested are not enough to meet the slug's metabolic demands (Christa, de Vries *et al.*, 2014).

Both the examination of starvation longevity in darkness and the increase in lipid droplets are debated, however, since the conclusions in both of these examinations have received scrutiny and/or are contradicted by other findings. Other light/dark starvation experiments show a reduction in longevity amongst specimens kept in the dark supporting the theory that photosynthesis helps maintain a starving slug (Hinde & Smith, 1972, 1975; Casalduero & Muniain, 2008; Yamamoto *et al.*, 2013) and one of the species examined in Christa et al. (2013) is known to starve up to 12 months but was only examined for 50-60 days. The accumulation of lipid droplets has also received scrutiny since their origin was not confirmed as algal (Laetz *et al.*, 2017). These lipids could be photosynthetically derived as the authors suggest, since the algal food source ingested by *E. chlorotica*

is the heterokontophyte *Vaucheria litorea* Agardh, 1823, which produces lipids as it's main energy storage molecule (Pelletreau *et al.*, 2014), however they could also be of slug origin, since metazoans also utilize lipids as energy storage molecules.

To clarify if the larder hypothesis has any merit, Laetz et al. (2017) examined *Elysia timida* Risso, 1818, a long-term plastid retaining and starvation withstanding species that only feeds on the chlorophyte *Acetabularia acetabulum* Linneaus (Händeler & Wägele, 2007; Christa, Händeler, Kück *et al.*, 2014; Schmitt *et al.*, 2014). *A. acetabulum* produces starch as it's main photosynthetic product (Love *et al.*, 1963; Werz & Clauss, 1970; Levine, 2011). Starch is a carbohydrate comprised of amylose and amylopectin chains (Shephard & Levin, 1972) that is not produced in metazoans and therefore conclusively of algal origin. Starch granules have been identified inside sacoglossan chloroplasts in numerous electron micrographs (Taylor, 1968; Robert K Trench, Greene & Bystrom, 1969; R K Trench, Boyle & Smith, 1973; Arnaldo Marín & Ros, 1993; Hirose, 2005; Martin, Walther & Tomaschko, 2012), however quantitative information was never previously reported nor was starch abundance throughout a starvation period. To monitor starch abundance throughout *E. timida's* starvation period and investigate the larder hypothesis, Laetz et al. (2017) applied Lugol's Iodine, a stain that intercalates in amylose helices allowing the visualization of starch granules in semi-thin histological sections. This revealed first an increase in the relative starch abundance followed by a decrease, confirming that photosynthates are first accumulated and then eventually broken down. This aligns with Pelletreau et al. (2014)'s lipid analysis and supports the larder hypothesis.

Despite these conclusions, almost nothing is known about how *E. timida* postpones digestion of its incorporated chloroplasts and why its sister species *Elysia cornigera* Nuttall, 1989 cannot. Multiple analyses have confirmed these species as sister-taxa (Händeler *et al.*, 2009; Carmona *et al.*, 2011; Krug, Händeler & Vendetti, 2012; Christa, Händeler, Kück *et al.*, 2014; Krug, Vendetti & Valdes, 2016) and while they are superficially very similar, even having been synonymized in the past (Ortea, Moro & Espinosa, 1997), they have little in common regarding functional kleptoplasty (Krug, Händeler & Vendetti, 2012). Similarities only include the algal species ingested and the subsequent plastid incorporation. Every

other aspect of functional kleptoplasty that has been investigated has revealed differences between these species. Pulse Amplitude Modulated (PAM) fluorometry data shows a faster decline in *E. cornigera* kleptoplast photosynthetic efficiency than the values reported for *E. timida* (Christa, Händeler, Kück *et al.*, 2014). Longevity in starvation also differs between these species, with *E. timida* surviving 42-100 days depending on temperature (18-25°C) and *E. cornigera* failing to survive more than 30 days in starvation (Christa, Händeler, Kück, et al. 2014; de Vries et al. 2014, 2015). Transcriptomic data reveals different responses to starvation in these species: *E. cornigera* entering starvation upregulate metabolic gene transcription experiencing a panic-like state whereas *E. timida* at first downregulate metabolic processes as if hibernating and only later upregulate transcription related to metabolism (de Vries *et al.*, 2015).

It is due to the differences in all of these factors that *E. timida* and *E. cornigera* are increasingly used as a model system in the attempt to understand functional kleptoplasty. The investigation presented here examines another factor, starch production, in *E. cornigera* during starvation. This is compared to published data on starch accumulation in *E. timida* (Laetz *et al.*, 2017). Both species were exclusively fed on *A. acetabulum* to reduce the risk of error related to the algal species ingested. To further examine starch production in both species, transmission electron micrographs were captured from both species throughout their starvation periods. While starch grains have been previously identified in transmission electron micrographs of sacoglossan kleptoplasts, this investigation is the first report to quantitatively detail the amount of starch found in these kleptoplasts, for each of these species. The conclusions revealed here provide a possible explanation as to how long-term functional kleptoplasty allows *E. timida* to survive extended starvation and why *E. cornigera* cannot.

5.2 Materials and Methods

Elysia cornigera were collected on Spanish Harbor Key, Florida USA in April, 2016. They were abundantly found in an *Acetabularia crenulata* Lamouroux, 1816 meadow at 0.3 - 1 meter depth. Living specimens were transported to Bonn,

Germany and given 10 weeks to acclimate to laboratory conditions. The tanks were placed in a climate controlled room (22°C) and tank heaters increased the temperature to 25°C. Changing *E. timida* and *E. cornigera* diets to other *Acetabularia* species has not had a demonstrable effect on starvation longevity nor photosynthetic efficiency in either species (Schmitt et al. 2014), so *E. cornigera* were switched to *A. acetabulum* to standardize any impact algal species has on chloroplast retention and validate comparison between both slug species. The transition to *A. acetabulum* 10 weeks prior to experimentation ensured that any chloroplasts observed in *E. cornigera* were *A. acetabulum* plastids rather than *A. crenulata.* This facilitates direct comparison to the chloroplasts observed in *E. timida* since the ingested chloroplasts stem from the same algal culture. *E. timida* were collected in Blanes, Spain from rocks covered in *A. acetabulum* at a depth of 2-7 meters in July, 2016. These animals were also brought back to the laboratory in Bonn where they were placed in an 18°C room and allowed two weeks of acclimation time feeding on *A. acetabulum*. Both species were provided with freshly prepared artificial seawater twice a week and exposed to 220 µmol quanta $m^{-2}s^{-1}$ light for 12 h L: 12 h D.

Three *E. cornigera* specimens were preserved in 4% formaldehyde for starch quantification with Lugol's Iodine throughout the starvation period at 0, 7, 14, 21 and 28 days each, to match the time points surveyed for *E. timida* (Laetz *et al.*, 2017). The unstarved specimens function as a control, displaying the starch quantity in specimens that are feeding normally. To account for daily fluctuations in starch abundance and provide an equal basis for comparison with *E. timida*, each specimen was fixed at 17:00, revealing the maximum daily starch content (Levine, 2011; Laetz *et al.*, 2017). Each specimen was embedded in hydroxymethacrelate (Kulzer Technovit® 7100) and sectioned into 2.5µm thick cross-sections. Ten sections were chosen throughout each slug, to accurately reflect the starch quantity throughout each animal. Sections were stained with a 1:10 Lugol's Iodine Solution (Fisher Scientific) diluted in distilled water. Two measurements were recorded for each of the ten sections analyzed, 1) the area covered by digestive gland tubule tissue and 2) the area covered by starch (stained black by Lugol's Iodine). Dividing the area covered by starch by the total digestive

gland tubule area reveals the relative starch percentage and allows the comparison of starch quantities between samples. This method is detailed in Laetz et al. (2017).

E. timida starved for 0, 7, 14, 21, 28, 42, 63, and 89 days were preserved in 2.5% glutaraldehyde buffered in 1 M sodium cacodylate at room temperature for one hour. *E. cornigera* were also fixed at 0, 7, 14, 21, and 28 days, however they did not survive starvation long enough to be examined at the later time points. Samples from both species were post-fixed in a 1% OsO_4 and 1M sodium cacodylate buffer, dehydrated in an ascending acetone series, transferred to propylene oxide and then Araldite. Polymerization was initiated with benzyldimethylamine (BDMA). Ultrathin sectioning (70nm thick) was accomplished using a Reichert Ultracut E microtome with a diamond knife. Bands of 4-6 sections were transferred to formvar-covered single-slot copper grids and subsequently stained in an automated TEM stainer with uranyl acetate and lead citrate (QG-3100, Boeckeler Instruments). Imaging was conducted using a Zeiss Libra 120kV transmission electron microscope. Each section was examined for ring-shaped cross-sections of digestive gland tissue and each of the chloroplasts inside these rings was imaged revealing the starch grain morphology during starvation. For additional comparison between chloroplasts, *A. acetabulum* was also embedded and imaged.

5.3 Results

Starch grains were not observed using Lugol's Iodine in any of the *E. cornigera* time points after the control, 0 days starved, animals. The relative starch percentage (RSP) for unstarved specimens averaged 1.3% for the first specimen, 0.64% for the second and 0.87% for the third. This decreased to 0% in each of the subsequent time periods. Both *E. timida* populations surveyed in Laetz et al. (2017) did display a starch accumulation and subsequent loss throughout the starvation period, following a negative parabolic curve. The fall population's minimums occurred at the beginning and end of the starvation period. At 0 days starved, the average RSP amongst these specimens was 4.6% and specimens

starved 88 days had a mean 2.2%. The maximum measured RSP occurred after 42 days in starvation and covered approximately 23% of the digestive gland tubule area (Laetz *et al.*, 2017). The *E. timida* spring population investigated in Laetz et al. (2017) survived a shorter time span but followed the same trend, having minimum RSP values in unstarved specimens (RSP = 3.7%) and 30 days starved specimens (RSP = 2.5%) and a maximum average RSP (18%) at 15 days starved (Fig. 5.1).

Electron microscopical investigations of *A. acetabulum* reveal oblong and discoid chloroplasts averaging 20.6μm^2 in area, with relatively few thylakoid membranes (Fig. 5.2a). Starch grains are visible, however they are almost always small in size, having an average area of 0.79μm^2, or 3.83% of the total chloroplast area. Only 13 of the total 50 chloroplasts surveyed contained visible starch granules (26%).

A. acetabulum chloroplasts inside unstarved *E. timida* are circular or only slightly elongated (mean area: 22.9 μm^2), and contain much more densely packed thylakoid membranes. Table 1 summarizes the number of chloroplasts surveyed for both *E. timida* and *E. cornigera,* the average plastid area and the average area covered by starch for each of the time points surveyed here. In unstarved *E. timida,* small starch granules are visible in the chloroplast stroma. Most of the starch granules are centrally located between the thylakoid membranes, however two of the ten surveyed chloroplasts have starch grains that are located on the chloroplast's outer edge causing a protrusion of the tightly wrapped outer membranes and distorting the circular shape (Fig. 5.2b). Protruding starch grains are also visible in some of the chloroplasts surveyed from 7 and 14-day starved slugs, however in the 14-day specimens, the chloroplast membranes are no longer tightly stretched around each protruding starch granule (Fig. 5.2c). After 28 days, starch granules were only observed between the thylakoid membranes and no longer occurred in proximity to the chloroplast outer and inner membranes (Fig. 5.2d-f). The starch grains present increase in abundance but remain the same average size, throughout the starvation period. By 42 days in starvation, the average kleptoplast starch coverage is 65%. After 42 days however, the digestive gland tubule cross-sections are characterized by large vacuoles containing fluid and/or chloroplast fragments rather than intact plastids (Fig. 5.2g-h). The average

chloroplast areas and percent starch coverage throughout the starvation period are compared in Figure 5.3a-b.

Throughout the starvation period, the average chloroplast size decreases slightly, starting at an average 22.9 μm^2 and ending at a mean 21.8 μm^2. Two-tailed T-tests comparing the means between unstarved and 88-day starved specimens show this decrease is not statistically significant ($p = 0.32$). The area occupied by starch granules increases significantly from 1.4 μm^2 in unstarved specimens to 14.2 μm^2 in 42-day starved specimens ($p < 0.001$). Dividing by the average total chloroplast area reveals a relative percentage of chloroplast area occupied by starch (6.11% - 65.74% throughout the *E. timida* starvation period).

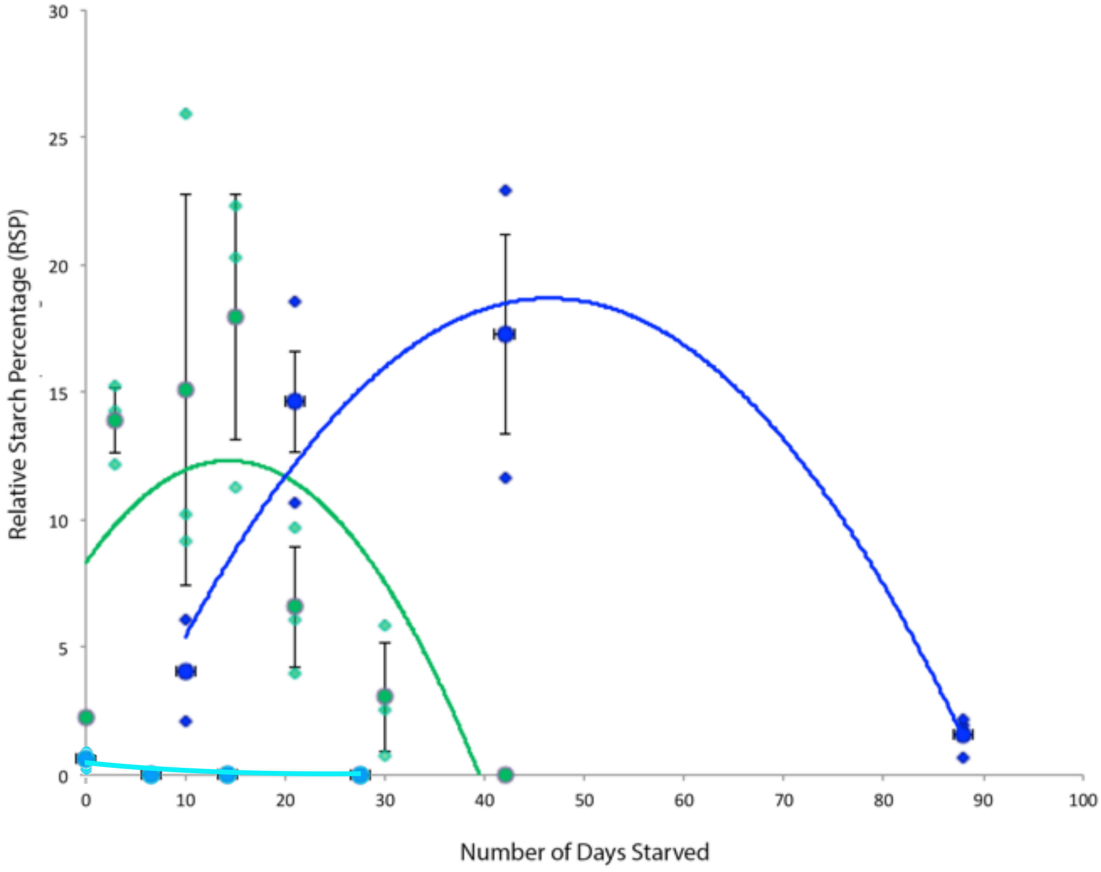

Figure 5.1 The mean relative starch abundance in both *E. timida* and *E. cornigera* during starvation (modified from Laetz et al. 2017 to add *E. cornigera*). Two populations (fall and spring) were monitored throughout starvation regarding the accumulation of starch in their incorporated chloroplasts. The *E. timida* spring population is shown in green, the fall in dark blue and *E. cornigera* in light blue. Best fit curves show the negative parabolic trend and the temporal shift in starch maxima for *E. timida*.

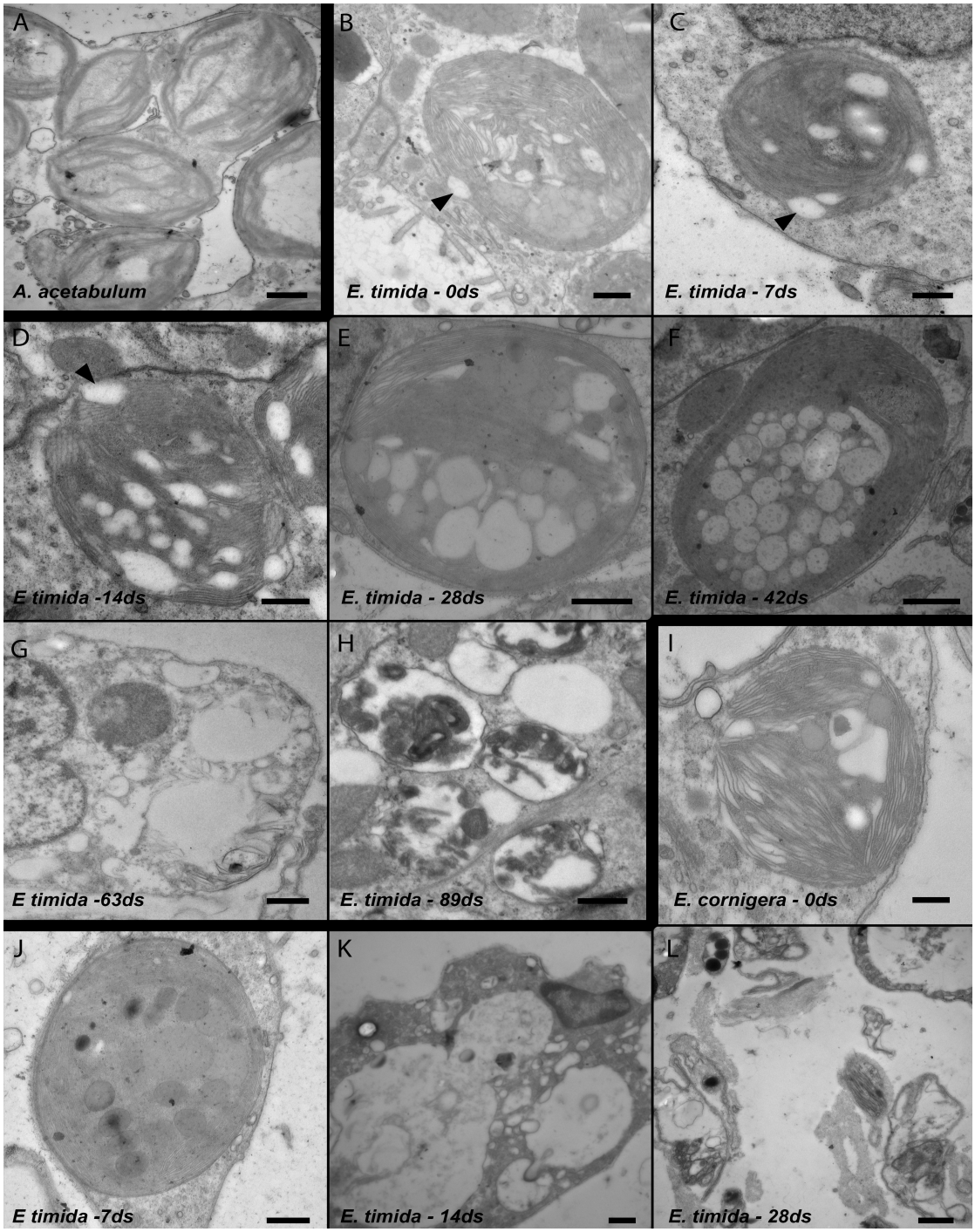

Figure 5.2 Transmission electron micrographs of each species. A: *A. acetabulum* chloroplasts inside the cytosol of *A. acetabulum.* B: *A. acetabulum* chloroplasts in unstarved *E. timida* (0 days starved (0ds)). C: 7 days starved *E. timida.* D: 14 days starved *E. timida.* E: 28 days starved *E. timida.* F: 42 days starved *E. timida.* G: 63 days starved *E. timida* showing only one chloroplast and two large vacuoles. H: 89 days starved *E. timida* displaying plastid remnants and large vacuoles. I: *A. acetabulum* chloroplasts in unstarved *E. cornigera.* J: 7 days starved *E. cornigera.* K: 14 days starved *E. cornigera* lacking intact chloroplasts and only containing plastid remnants. L: 28 days starved *E. cornigera* showing fragmented tissues and no discernable organization. Arrowheads indicate a starch granules pushing out the chloroplast outer and membranes, and distorting the oval outline. Scale bars – A-F: 1µm; G-H: 2.5µm; I-L: 1µm. Abbreviation: ds=days starved.

Ultrastructure investigations examining *A. acetabulum* chloroplasts in unstarved *E. cornigera* revealed circular chloroplasts, densely packed thylakoid membranes and small starch grains (only covering 5.06% of the average total plastid area) in the chloroplast stroma (Fig. 5.2i, Table 1). After 7 days in starvation, few starch grains are visible comprising an average 0.09% starch coverage (Fig. 5.2j). After 14 days in starvation, only partially digested chloroplast fragments and large vacuoles were visible (Fig. 5.2k). By 28 days in starvation, most of the slugs' own tissues were destroyed and almost all cell structure was impossible to discern (Fig. 5.2l).

Two-tailed T-tests confirm that unstarved slugs of each species have sequestered chloroplasts of the same size and the same average starch content (chloroplast total area: $p = 0.46$), starch grain percentage $p = 0.24$). After 7 days in starvation however, *E. timida* had an increase in starch abundance while *E. cornigera* showed a decrease in starch abundance, and these values significantly diverged ($p < 0.01$). The chloroplast size however was not significantly different ($p = 0.11$). Differences in later time points could not be assessed since structurally intact plastids could not be found in *E. cornigera.*

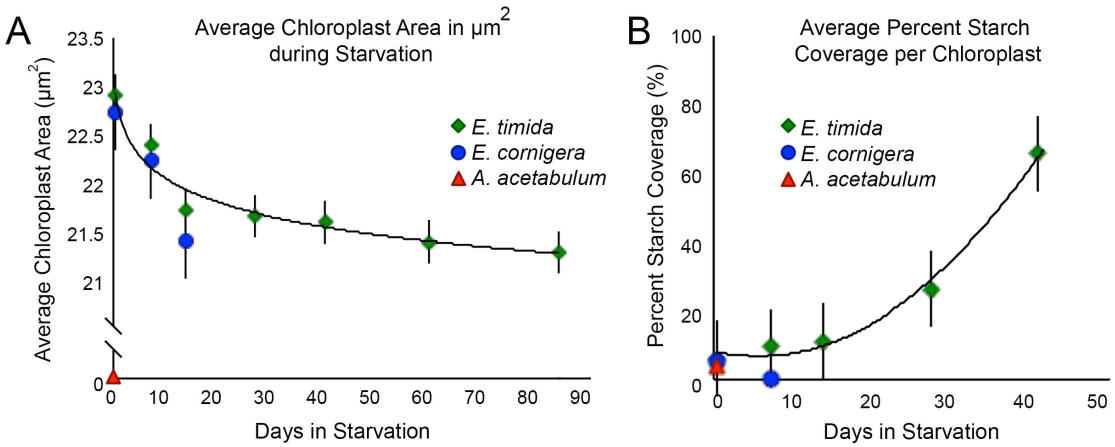

Figure 5.3 The average chloroplast area in µm² and percent starch coverage throughout the starvation period for each species based on transmission electron microscopy. A: The average chloroplast area in µm² for *A. acetabulum* (displayed by a red triangle) *E. timida* (indicated by green diamonds) and *E. cornigera* (blue circles). *E. timida* was best modeled by the quadratic function $y=0.04x^2 - 0.55x + 8.5$. Best-fit curves could not be determined for *E. cornigera* (since only three sampling points were observed) nor *A. acetabulum* (as only one time point was observed because the algae do not enter the starvation period). B: Average percent starch coverage per chloroplast for each species. This was calculated by dividing the

area covered by starch granules by the total plastid area for each observed chloroplast. Best-fit curves could not be determined for neither *A. acetabulum* nor *E. cornigera.*

5.4 Discussion

Starch production by sequestered chloroplasts has already been demonstrated in the long-term plastid retaining species *Elysia timida* (Laetz et al. 2017). This species has been documented as surviving over 110 days in starvation depending on the temperature in which they are cultivated, with an average starvation time of around 90 days at 18°C [26, 52, Laetz and Wägele submitted]. During the first 42 days of this starvation period, starch accumulates inside kleptoplasts, as evidenced by Lugol's Iodine stained granules (Laetz et al. 2017). This is confirmed here by transmission electron microscopy (TEM), which details an increase in the number of granules within the chloroplasts' stroma. When cross-sectioned, a digestive gland tubule appears ring-shaped, with a lumen in the middle surrounded by the plastid-bearing cells. In all of the time points up to and including 42 days, these rings were easily distinguished and full of chloroplasts. After 42 days, the amount of starch stained by Lugol's Iodine decreases coinciding with the lower plastid abundance observed in the TEM images. At 63 days in starvation, few intact chloroplasts are present and they are characterized by disintegrating thylakoid membranes and very low starch presence, which could not be accurately quantified with morphometric measurements. The digestive gland tubule cross-sections are still easily identified, however no longer by intact chloroplasts but rather by numerous chloroplast-sized vacuoles, many appearing empty or with plastid remnants inside, and very few intact plastids. By 89 days in starvation, no intact chloroplasts were observed in TEM micrographs and the relative starch percentage was lower than 2%, meaning almost all of the starch had disappeared.

The actual number of chloroplasts within these tissues has yet to be investigated, however the number of functional kleptoplasts in starving *E. timida* has been, revealing a high functional chloroplast abundance (50-68% of the total digestive gland tubule area) in unstarved specimens which drops rapidly until 42

days in starvation, after which functional chloroplasts are only found in very low abundance (<1%) (Laetz et al. 2016). This aligns with the starch abundances observed in both the Lugol's Iodine (Laetz et al. 2017) and TEM (this study) experiments, since starch is produced by photosynthetic activity and functional chloroplasts are required for photosynthesis to occur. The TEM images presented here for 63 days in starvation reveal only a few intact chloroplasts, most of which were partially degraded and contained damaged thylakoid membranes. These observations support the results from Laetz et al. (2016), who suggested that chloroplasts may still be present after 42 days in starvation but they are likely nonfunctional.

The accumulation of starch and subsequent decrease observed by Lugol's Iodine staining (Laetz et al. 2017) and TEM (this investigation) correlate with digestive activity and metabolic gene regulation observed in previous investigations. Lysosomal activity, an indicator of intracellular digestion, in unstarved *E. timida* (18°C), is low (4%), increasing exponentially throughout the starvation period (22% at 42 days in starvation and 61% in 89 day starved specimens) (Laetz et al. 2016). This trend is corroborated by gene expression analysis, which reported downregulation in metabolic genes for the early part of the starvation period, followed by upregulation at 30 days in starvation (de Vries et al. 2015). These observations suggest that incorporated chloroplasts are not digested early in the *E. timida* starvation period (low lysosomal abundance and low metabolic gene transcription), but are digested later on when signs of high digestive activity are observed. These observations refine our understanding of functional kleptoplasty and the benefits received by a long-term plastid retaining species, suggesting that chloroplasts continue to photosynthesize inside the slug, building up starch reserves that are later received when the chloroplast itself is digested. These results support the larder hypothesis, that at least some of the photosynthates produced are not immediately available to the slug and instead accumulate, only contributing to slug nutrition later in starvation.

The benefits of incorporating chloroplasts are not apparent for the short-term plastid retaining species *E. cornigera.* Contrary to our expectations, incorporated *A. acetabulum* chloroplasts did not function similarly in *E. timida* and

E. cornigera despite coming from the same algal culture. *E. cornigera's* incorporated chloroplasts did not show starch accumulation throughout the starvation period and displayed signs of digestion far earlier in the starvation period, after only 7 days. Since *E. timida* ingest the same chloroplasts, which continue to function and were observed structurally intact until 42 days in starvation, the digestive cell environment is clearly different in *E. cornigera.* It is still unclear however if all *Acetabularia* species have chloroplasts that would demonstrate the same trends, or if the lack of starch observed here also occurs in *E. cornigera's* natural food source, *A. crenulata.* Further investigations are required to ascertain the degree to which different *Acetabularia* species influence photosynthate production in these species.

Regarding digestive activity, *E. cornigera* displayed a slow increase in percent lysosome coverage (4.2% in unstarved animals to 9.2% in 21 day starved specimens) (Laetz and Wägele submitted), suggesting they do not suspend chloroplast digestion until a later time point like *E. timida*. It is doubtful whether *E. cornigera* even digests its incorporated and still functional chloroplasts, since they were found in excrement samples throughout the starvation period (Laetz and Wägele submitted). The regulation of genes involved in metabolism for *E. cornigera* reveals upregulation of these genes when the slug enters starvation, although data is only presented up to 7 days so later time points cannot be compared. Each of the factors examined in these investigations reveal that *E. cornigera* probably does not receive energetic benefits from its incorporated plastids, and certainly does not receive photosynthetically derived starch produced by sequestered chloroplasts after incorporation. This may be exactly why *E. cornigera* cannot withstand the extended starvation periods its sister-species *E. timida* can.

From an evolutionary perspective, functional kleptoplasty likely evolved in numerous steps. Previous investigations have shown that only certain algal species are involved in functional kleptoplasty, however these species are also fed on by short-term plastid retaining species and non-retaining species, so the food source cannot be the only factor facilitating this phenomenon (Christa, Händeler, Kück, et al. 2014; Christa, Wescott, et al. 2013; Katharina Händeler et al. 2009, 2010).

Evolutionary steps made within the slug lineages are necessary toward the development of this ability, and previous investigations have suggested where these steps may have occurred. Short-term kleptoplasty has likely evolved two or more times, once in the *Costasiella* lineage and one in the Plakobranchoidea, although a lack of taxon sampling, particularly within the Limapontioidea and Oxynoacea means this is merely hypothesized (Christa, Händeler, Kück, et al. 2014). Long-term plastid retention (LtR) has been found in at least five independent lineages, since each of the confirmed long-term plastid retaining species has short- (StR) and/or non-plastid retaining (NR) sister-taxa. For example, according to recent phylogenies (Christa et al. (2014) – Sacoglossa, and Krug et al 2016 – *Elysia*), LtR species (complex) *Plakobranchus ocellatus* shares a sister-taxa relationship with the genus *Thuridilla*, which has both NR and StR species (Christa, Wescott, et al. 2013). *E. timida* also independently acquired the ability to conduct LtR, since sister-species *E. cornigera* is only capable of StR.

The physiological differences in *E. timida* and *E. cornigera* have yet to be fully understood, although investigations such as this one, that examine one factor involved in functional kleptoplasty at a time are beginning to shed light on some of the steps that must have occurred. Based on the factors examined so far, it is clear that a shift occurred in the *E. timida* lineage that suppresses digestive activity when these animals enter starvation. This suppression leads to long-term plastid retention and allows the accumulation of starch. Why *E. cornigera* excretes chloroplasts throughout starvation while *E. timida* does not is still unknown, but the mechanism behind either *E. timida's* retention or *E. cornigera's* identification and subsequent removal of plastids had to have developed in one of these lineages, further differentiating these species. It is also still unclear why *E. cornigera* contains fewer incorporated chloroplasts irrespective of whether it is feeding ad libitum or starving and if this is due to plastid excretion. By identifying differences in each of the factors examined in these sister-taxa, future investigations may discover the actual mechanisms behind these factors and reveal how functional kleptoplasty actually functions in *E. timida* and consequently the other LtR species.

5.5 Acknowledgements

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Chapter 6

Chloroplast Digestion and the Development of Functional Kleptoplasty in Juvenile *Elysia timida* Risso, 1818 as Compared to Short-term and Non-Chloroplast-Retaining Sacoglossan Slugs

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Abstract

Sacoglossan sea slugs are the only metazoans known to perform functional kleptoplasty, the sequestration and retention of functional chloroplasts within their digestive gland cells. Remarkably, a few species with this ability can survive starvation periods of 3–12 months likely due to their stolen chloroplasts. There are no reports of kleptoplast transfer from mother slug to either eggs or juveniles, demonstrating that each animal must independently acquire its kleptoplasts and develop the ability to maintain them within its digestive gland. We present here an investigation into the development of functional kleptoplasty in a long-term kleptoplast retaining species, *Elysia timida*. Laboratory-reared juvenile slugs of different post-metamorphic ages were placed in starvation and compared to 5 known short-term retaining slug species and 5 non-retaining slug species. The subsequent results indicate that functional kleptoplasty is not performed by *E. timida* until after 15 days post-metamorphosis and that by 25 days, these animals outlive many of the short-term retention species. Digestive activity was also monitored using lysosomal abundance as an indicator, revealing different patterns in starving juveniles versus adults. Starved juveniles were reintroduced to food to determine any differences in digestive activity when starvation ends, resulting in an increase in the number of kleptoplasts, but no overall change in lysosomal activity. By revealing some of the changes that occur during early development in these animals, which begin as non-kleptoplast-retaining and grow into long-term retaining slugs, this investigation provides a basis for future inquiries into the origin and development of this remarkable ability.

6.1 Introduction

The heterobranch (Gastropoda) clade Sacoglossa is well known for some member's ability to steal functional chloroplasts from their algal food, incorporate them and stave off death by starvation when new food sources are unavailable [1–3]. This phenomenon, termed functional kleptoplasty [4] due to the chloroplasts continued fixation of carbon, occurs in multiple sacoglossan lineages although only six species are confirmed as long-term (two to ten months) retention (LtR) [5–8]. Numerous other species are capable of varying degrees of kleptoplasty ranging from those capable of short-term (two to eight weeks) retention (StR), to those that don't retain kleptoplasts (NR) [5]. Most sacoglossan species have not been investigated regarding kleptoplast retention.

Much of the recent research on the evolution of kleptoplasty has centered on *Elysia timida* (Risso, 1818) [9–19], an LtR species living in shallow Mediterranean waters [5,9,20–22]. Most evidence shows *El. timida* to be a stenophagous species, feeding only on the chlorophyte *Acetabularia acetabulum* Linnaeus, 1758 which is abundantly found in large meadows across the sublittoral zone [9,23–28], although other reports suggest it may have another food source [12,21,22]. A secondary food source has not been identified, however by barcoding or any other distinctive identification method [23]. *A. acetabulum*'s bi-phasic life cycle leaves barely any food for *El. timida* during the late summer and early autumn months when the algae enter a generative phase consisting of microscopic, planktonic gametes [21]. This lack of available food may have driven *El. timida* to develop long-term retention. Whether or not these slugs benefit from the photosynthates produced by their enslaved chloroplasts is debated [29–33] although photosynthates are produced and they may be what allows a slug to survive extended starvation periods [17,34–36]. Thus, food availability may have shaped the *El. timida* life cycle [21,25].

Juvenile *El. timida* slugs hatch as either veliger larvae or shell-less juveniles depending on food availability and temperature [9,21,37,38]. Regardless of the developmental stage in which they hatch, there are no reports of kleptoplasts

within their tissues before they begin to feed, showing that kleptoplasts are not inherited from the parent slugs, rather they must be newly acquired by each new generation [9,24,39,40]. Young *El. timida* are assumed incapable of long-term kleptoplast retention, directly digesting chloroplasts for a few weeks until functional kleptoplasty can develop, however little evidence exists to substantiate this claim. Marín and Ros [1993] state that kleptoplasts are retained in the digestive gland of 12 Days Post-Metamorphosis (DPM) juveniles, although a detailed description of how this was determined is missing. A juvenile non-retention period has been demonstrated in *Elysia chlorotica* Gould, 1870, a LtR form that feeds on the heterokontophyte *Vaucheria litorea* Agardh, 1823 [41,42], however most sacoglossans feed on chlorophytes so *El. chlorotica* may not be the best model species to represent the entire group.

Recent studies have provided evidence suggesting kleptoplasts from different algal species have varying fitness levels, meaning the algal species is also important to the development of functional kleptoplasty [14,23,43,44]. *Elysia viridis* (Montagu, 1804) is either classified as a LtR or a StR, with its longevity in starvation and fitness depending on the algal species ingested [6,45]. Juvenile *El. timida* feed on the same algae as adult *El. timida*, where the kleptoplasts are known to be robust (functional and not showing signs of degradation for up to 2 months). This means any difference between adult *El. timida* and juvenile *El. timida* likely stems from a difference in the slug's digestive system and not algal or chloroplast type. Little is known about juvenile longevities in starvation and whether *El. timida* displays a transient kleptoplasty phase as observed in *El. chlorotica*, defined by Pelletreau et al. (2012) as the transitional stage between non-retention and long-term kleptoplast retention [42]. Laetz et al. (2016) measured functional kleptoplast and lysosome abundances in starving adults, finding an inverse relationship. The increase in lysosomes within the digestive gland - but not outside in other tissues - as the number of functional kleptoplasts decrease, suggests that these animals are digesting their incorporated kleptoplasts.

This study assesses whether the functional kleptoplast decrease and presumed digestion observed in adults [46] is the same in juveniles, if ingested chloroplasts (cps) are actually digested in juveniles, and when exactly juvenile

slugs gain the ability to maintain cps in their tissues. Chlorophyll *a* autofluorescence is used as an indicator of functional chloroplast presence. Individual juveniles of different ages were measured every day during starvation to examine the digestion of functional kleptoplasts within their digestive glands and uncover when functional kleptoplasty is established. Longevity tests were conducted on both, the animals used in testing and in unhandled animals (animals not subjected to staining and microscopy), to determine if handling stress affected the outcomes. Lysosomal activity, as an indicator of functional digestion, was also monitored throughout various starvation points to determine if chloroplast decrease and lysosomal abundance display the same patterns in juveniles as observed in adults [46]. The StR species *Thuridilla hopei* (Vérany, 1853) and LtR/StR *Elysia viridis* were assessed in Laetz et al. (2016), showing different kleptoplast digestive patterns than those seen in LtR form *Elysia timida.* This study also presents starvation data on five StR forms, *Elysia patina* Ev. Marcus, 1980, *Elysia papillosa* Verrill, 1901, *Elysia cornigera* Nuttall, 1989*, Bosellia mimetica* Trinchese, 1891 and *Thuridilla hopei*, as well as five NR forms, *Placida dendritica* (Alder & Hancock, 1843), *Elysia tuca* Ev. Marcus & Er. Marcus, 1967*, Elysia marcusi* (Ev. Marcus, 1972), *Ercolania fuscata* (Gould, 1870) and *Ercolania viridis* (A. Costa, 1866) as a comparison to juvenile *Elysia timida.* Some of these species were measured for photosynthetic activity (PA) and / or starvation longevity (SL) in previous reports (PA: *Er. fuscata, Er. viridis;* PE+SL: *El. patina, El. papillosa, El. tuca, El. marcusi, El. cornigera;* SL: *P. dendritica* [5,47,48]) and the results found there are compared to those presented here, whereas other species are reported here for the first time (SL: *Er. fuscata, Er. viridis;* PA: *Placida dendritica;* PA+SL: *T. hopei* and *B. mimetica*).

6.2 Materials and Methods

Elysia timida, Placida dendritica, Thuridilla hopei and *Bosellia mimetica* adults were individually collected in May 2014/2015 from Fetovaia on the island of Elba, Italy. Stones covered in young *Acetabularia acetabulum* were also collected at the

same locality. *Elysia marcusi*, *El. tuca, El. cornigera, El. papillosa, El. patina, Ercolania viridis* and *Er. fuscata* were all collected at Spanish Harbor Key and Long Key, Florida USA (Fig. 6.1 A-K). All specimens were kept at either 18°C (Mediterranean specimens) or 21°C (Caribbean specimens) under full spectrum light (220 µmol quanta m^{-2} s^{-1}) for 12h L: 12h D (day and night rhythm) and provided fresh water every 3 days. Table 1 further explains where each species was collected and the algae on which it was collected. Table 2 gives an overview of how many *El. timida* juveniles, from which age groups, were used for each of the experiments presented here. All adult animals were measured with a Diving PAM Fluorometer (Walz Heinz GmbH, Germany) at each time point during starvation and their starvation longevity was recorded. Juvenile animals were too small to be measured with this device.

To obtain juveniles for the experiments, adult *El. timida* were allowed to feed freely, their egg masses were removed from the stones or tank walls and cultivated separately in small dishes. Embryos hatched after 21-24 days in the egg mass as shelled larvae (Type 2 according to Thompson and Brown 1976). They metamorphosed, discarding their shells after 3-5 days. Directly after metamorphosing, they were presented with food and were allowed to feed for differing lengths of time before experimentation began: 4, 10, 15 and 25 Days Post-Metamorphosis (DPM). These intervals were chosen to surround the 12-day time point reported by Marín and Ros [1993].

Juvenile slugs of each age (4, 10, 15, 25 DPM) were used in four different experiments. The number of specimens and overview of each experiment is detailed in Table 2. For each experiment, animals were placed in a separate bowls without access to algae. In the first experiment (juvenile survival without handling, see Table 2), ten animals were selected from each age group (4, 10, 15, 25 DPM) and monitored during starvation as a control to the various handling, e.g., with $MgCl_2$ and /or cooling, effects on juvenile viability. They were examined under a microscope daily, in their individual bowls while starving, to assess vitality. This revealed the base rate of starvation for each age group. Their longevity was recorded and compared to those in the Experiments 2 to 4, as outlined in Table 2.

In the second experiment, another ten animals for each time point, were imaged daily under the confocal microscope in order to observe chlorophyll *a* degradation (see Table 2). They were first removed from the algae and placed in 7% $MgCl_2$ for up to three hours until they stopped moving. The animals that did not stop were then cooled until movement slowed enough for imaging. Once still, they were transferred to slides containing a well. They were then scanned using the blue laser 488nm excitation (600-640nm accepted emission range, chl *a* optimum: 633) on a Leica SPE confocal laser scanning microscope. The entire animal was scanned in order to view any chlorophyll *a* autofluorescence present. They were then returned to individual bowls. This process was repeated every day for each animal until death.

In a third experiment, lysosomal abundance and activity (during starvation) were recorded by staining 18 animals from each age group (allowed to feed for 4, 10, 15, 25 Days Post-Metamorphosis DPM), with Acridine Orange, a fluorescent stain that aggregates in extremely acidic regions (pH < 4.5) [46,49,50] (Table 2). As Acridine Orange stains acidic organelles in living tissues, living animals were first stained for 30 minutes at room temperature (diluted with filtered seawater to a 5µmol solution) and then vivisected, mounted and imaged on the same microscope using the blue laser (excitation 488nm), with 645-670nm (AO dimer type optimum: 656) as the accepted emission range [46]. These animals were also examined for their functional chloroplast abundance. Three animals were investigated at the following time points: 1, 3, 7, 10, 15 and 25 days in starvation, although the number of days survived for the younger juveniles was very low and increased in number with increasing age of juveniles (see Table 2).

The final (fourth) experiment comprised individuals from each age group that were first starved and then re-introduced to food before Acridine Orange staining to determine the increase in chlorophyll *a* gained by one feeding and explore any changes in digestive activity when reintroduced to food. They were not subjected to $MgCl_2$, but were cooled before imaging. Six specimens were placed in starvation for each point surveyed. Three individuals were measured at each starvation time point (Table 2, S), while the other the three specimens were starved under the same condition but received food for 2 hours directly before

Figure 6.1 Species investigated. A: *Bosellia mimetica*, specimen length: ~5mm (when stretched out as pictured here). B: *Elysia marcusi*, specimen length: ~4mm (when stretched out rather than disk-shaped as seen here). C: *Elysia tuca*, specimen length: ~7mm. D: *Elysia papillosa*, specimen length: ~8mm. E: *Elysia cornigera*, specimen length: ~7mm. F: *Elysia timida*, specimen length: ~8mm. G: *Ercolania fuscata*, specimen length: ~3mm. H: *Ercolania viridis* (both color morphs found in Florida), specimen length: ~3-4mm. I: *Placida dendritica*, specimen length: ~4mm. J: *Elysia patina*, specimen length: ~6mm. Photo by Gregor Christa, used with permission. K: *Thuridilla hopei*, specimen length: ~8mm.

Table 6.1 Specimen collection information.

Species	# Used in Starvation Experiments	Collection Location	Associated algae	Collection Notes
Bosellia mimetica	10	Mediterranean – Elba – Fetovaia Bay (April 2014)	*Halimeda tuna*	Many differently sized individuals found in high abundance on *H. tuna* from 1-15m depth.
Elysia cornigera	10	Caribbean – Florida Keys, USA – Spanish Harbor Key (April 2016)	*Acetabularia penniculus*	Found on subtidal rocks in *A. penniculus* meadows, 0-2m depth. Observed feeding on *A. penniculus.*
Elysia marcusi	10	Caribbean – Florida Keys, USA – Long Key, Bayside (April 2016)	*Halimeda opuntia*	Many differently sized individuals found on *H. opuntia* from 1-4m depth.
Elysia papillosa	8	Caribbean – Florida Keys, USA – Spanish Harbor Key (April 2016)	*Acetabularia penniculus*	Found on subtidal rocks in *A. penniculus* meadows, 0-2m depth. Not observed feeding on *A. penniculus.*
Elysia patina	10	Caribbean – Florida Keys, USA – Spanish Harbor Key (April 2016)	*Acetabularia penniculus*	Found on subtidal rocks in *A. penniculus* meadows, 0-2m depth. Not observed feeding on *A. penniculus.*
Elysia timida	10	Mediterranean – Elba – Fetovaia Bay (April 2014)	*Acetabularia acetabulum*	Found on subtidal rocks in *A. acetabulum* meadows 0-5m depth. Observed feeding on *A. acetabulum*.
Elysia tuca	10	Caribbean – Florida Keys, USA – Spanish Harbor and Long Keys (April 2016)	*Halimeda opuntia, H. macroloba*	Many differently sized individuals found in high abundance on *Halimeda* sp. from 1-10m depth.
Ercolania fuscata	10	Caribbean – Florida Keys, USA – Spanish Harbor Key (April 2016)	*Cladophora liniformis* cf?	Found on large *C. liniformis* mats covering rocks from 0-0.3m depth.
Ercolania viridis	8	Caribbean – Florida Keys, USA – Long Key Bayside (April 2016)	Either *Halimeda* sp. or *Avrainvillea* sp.	Found in tanks containing *Halimeda* sp. and *Avrainvillea* sp. Both algal species were collected at 2-3m depth.
Placida dendritica	10	Mediterranean – Elba – Fetovaia Bay (April 2014)	*Codium fragile*	Found on *C. fragile* at 2-18m depth. Observed feeding on *C. fragile.*
Thuridilla hopei	10	Mediterranean – Elba – Fetovaia Bay (April 2014)	unknown	0-15m depth. Found in *A. acetabulum* meadows and deeper rocks. Not observed feeding on *A. acetabulum*.

Table 6.2 Overview of each experiment detailing the number and age of the juvenile *E. timida* used in this study. Parentheses refer to animals that were included at the beginning of the experiment and placed in starvation, but did not survive until the date they were to be surveyed. They are therefore excluded from all analyses.

Experiment Number and Description	Time points surveyed (after starvation begins)	Number of 4 DPM juveniles used	Number of 10 DPM juveniles used	Number of 15 DPM juveniles used	Number of 25 DPM juveniles used
1 Juvenile longevity without handling	Every specimen, every day until death	10	10	10	10
2 Chlorophyll degradation	Every specimen, every day until death	10	10	10	10
3 Digestive activity at different time points in starvation	0 days starved (control)	3	3	3	3
	3 days starved	(3)-	3	3	3
	7 days starved	(3)-	3	3	3
	10 days starved	(3)-	(3)-	3	3
	15 days starved	(3)-	(3)-	(3)-	3
	25 days starved	(3)-	(3)-	(3)-	3
4 Food Reintroduction - Starved juveniles and Starved then Reintroduced to Food (S+RF) juveniles	0 days starved (control)	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF
	3 days starved	(6) total, (3) S and (3) S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF
	7 days starved	(6) total, (3) S and (3) S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF
	10 days starved	(6) total, (3) S and (3) S+RF	(6) total, (3) S and (3) S+RF	6 total, 3 S and 3 S+RF	6 total, 3 S and 3 S+RF
	15 days starved	(6) total, (3) S and (3) S+RF	(6) total, (3) S and (3) S+RF	(6) total, (3) S and (3) S+RF	6 total, 3 S and 3 S+RF
	25 days starved	(6) total, (3) S and (3) S+RF	(6) total, (3) S and (3) S+RF	(6) total, (3) S and (3) S+RF	6 total, 3 S and 3 S+RF

being measured (Table 2, S+R). Staining began directly after feeding and lasted 30 minutes, so the observed lysosome abundances provided enough time for both chloroplast uptake and cellular response to newly sequestered kleptoplasts [19].

Image stacks were analyzed using the Fiji/ImageJ plugin, 3D-AMP [46]. Lysosome and chloroplast abundance were estimated using the area measurements provided by the FIJI – ImageJ Plugin, 3D-AMP [46]. Normality for each experiment was assessed using a Kolmogorov-Smirnov test, since some of the samples contained many identical values. After confirming normality, two-tailed T-tests were used to compare the means in each treatment, for each age group.

6.3 Results

6.3.1 Handled Juveniles Compared to Un-handled Specimen Longevity (Experiments #1 & #2)

The juveniles imaged in these experiments were always alive when imaged, so steps were taken to reduce sample movement and therefore the error associated with it. This involved first subjecting each animal to a 7% $MgCl_2$ and seawater mixture and then cooling each slide on a block of ice until movement stopped (usually 2-3 minutes). $MgCl_2$ did not appear to affect the mobility of juveniles younger than 25-days-old when they began the starvation period, although it did slow many of the animals after 7 days of starvation. The 25 DPM animals were always slowed, if not completely immobilized by the $MgCl_2$. Higher concentrations were not applied due to any potential toxic effects, and juveniles treated with lower concentrations showed no decrease in movement.

To determine any detriment to the slugs' longevities, a separate population, containing each of the age groups was not treated with $MgCl_2$, put on ice nor imaged. The longevities of these animals were recorded, and while they appear to live slightly longer than the treated animals in each group, two-tailed T-Tests reveal no significant difference in the life span of the animals in each group (4 DPM slugs: $p=1$, 10 DPM slugs $p=0.88$, 15 DPM slugs $p=0.71$ and 25 DPM slugs $p=0.53$.

Each treatment was normally distributed according to a Kolmogorov-Smirnov test: 4 DPM max difference [md] – 0.08 (critical difference [cd] – 0.27); 10 DPM md – 0.23 (cd – 0.25); 15 DPM md – 0.14 (cd – 0.18); 25 DPM md – 0.03 (cd – 0.09).

6.3.2 Juvenile *Elysia timida* Longevity (Experiment #2)

After hatching, *El. timida* veliger larvae were presented with *Acetabularia acetabulum*, although they were not observed feeding for 1-3 days until they crawled out of their larval shells. Once in starvation, many of these animals died with intact chloroplasts filling their digestive glands. The 4 DPM juveniles starved for an average 1.4 ± 0.84 days, with one animal dying within the first 24 hours and the longest surviving animal reaching 3 days. The 10 DPM population survived an average 2.3 ± 1.57 days, having 2 animals dying within the first 24 hours and 5 days as a maximum starvation time. Of the 15 DPM animals, the shortest lifespan was 3 days, the average 5.6 ± 2.01 days and the maximum 9 days. The 25 DPM juveniles reached a maximum starvation time of 22 days, and average of 18.4 ± 2.17 days and a minimum of 14 days.

The number of deaths per day was also recorded, with no more than 3 deaths occurring on a single day in any of the age groups, and a multi-day span when each group's deaths occurred. The 4 DPM group died throughout a four-day time span, the 10 DPM group spanned six days, from one death in the first 24 hours to the sixth starvation day (Fig. 6.2). The 15 DPM deaths were spread over seven days (the first occurring after three days and the last on the ninth day) and the 25 DPM animals spanned ten days (starvation days 21-30) (Fig. 6.2). Comparing the average life span of each age group with a Tukey HSD test revealed significant differences between 4 and 15 DPM juveniles ($p=0.006$), 4 and 25 DPM juveniles ($p=0.001$), 10 and 25 DPM juveniles ($p=0.001$), 15 and 25 DPM juveniles ($p=0.001$). No significant difference was revealed between 4 and 10 DPM individuals ($p=0.71$), and 10 and 15 DPM juveniles ($p=0.08$).

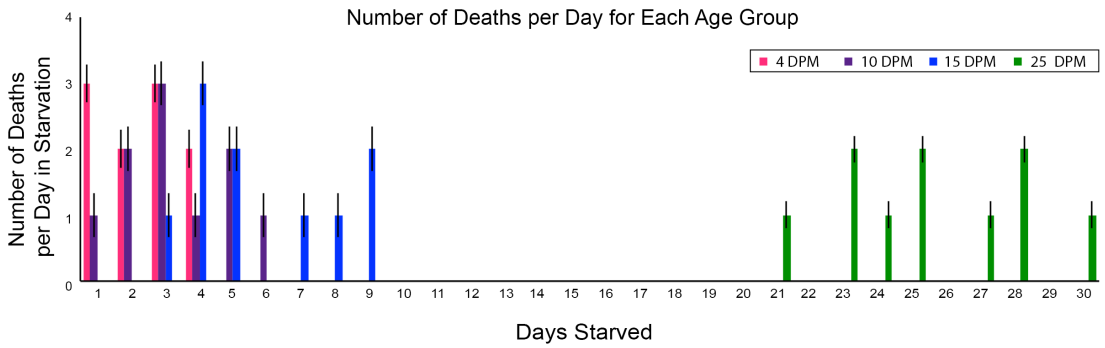


Figure 6.2 The number of deaths per day in starvation. 4 DPM deaths are indicated in pink, 10 DPM deaths in purple, 15 DPM in blue and 25 DPM in green. The relatively even distribution suggests a range of fitness levels amongst specimens of each age.

6.3.3 Chloroplast Abundance and Degradation in Juvenile *Elysia timida* (Experiment #2)

Ten juveniles from each age group were imaged daily with the CLSM, to determine the decline in functional chloroplasts within each animal every day (Fig. 6.3A-L). Chloroplast abundance was assessed using 3D-AMP, a FIJI plugin that reports the number of pixels in an image stack, so the final chloroplast abundances are reported here in percent coverage areas: the area covered by functional chloroplasts compared to the animal's overall area. The 4 DPM juveniles showed varying percent coverage, with 4 animals having a mean 0.3% and the rest ranging from 2.2-5.8% on the day they began starving (Fig. 6.4A). Each 10 DPM animal ranged from 1.2-8% (Fig. 6.4B), the 15 DPM animals ranged from 3-30.8% (Fig. 6.4C) and the 25 DPM animals spanned 19-43% chloroplast coverage at the beginning of the starvation period (Fig. 6.4D). While some animals had 0% chloroplast coverage upon death, many died with undigested functional kleptoplasts within their bodies. Six 4 DPM slugs and all 10 DPM slugs fall into this category, whereas the older age groups (15- and 25- day-old) each only had 1 animal die containing functional kleptoplasts – every other animal was devoid of functional chloroplasts. The average kleptoplast coverage throughout the starvation period for each group is shown in Fig. 6.4E.

6.3.4 Juvenile Digestion and Lysosomal Activity (Experiment #3)

Lysosomal abundances inside and outside the digestive gland tubules were measured for each of the age groups at the following time points: 0, 3, 7, 10, 15, and 25 days when they survived long enough to be measured. Inside the digestive gland tubule, lysosomal activity serves as an indicator of intracellular digestion, whereas outside the digestive gland tubule, lysosomal activity may indicate autophagy. The 4 DPM slugs were only measured at one time point (0-days), since they did not starve long enough to be measured at the three-day time point. Since both the 4- and 10 DPM juveniles had less than three measurements, no best-fit curves could be applied. The average lysosome coverage for 4 DPM slugs was 6.38 ± 0.8% in the digestive gland tubule (DGT), and 0.11 ± 0.1% outside the DGT (Fig. 6.5A). The average coverage within the DGT for 10-day old animals was relatively constant throughout the starvation period, at 5.5 ± 1%, and was always between 0 and 1% outside the DGT (Fig. 6.5B). Starving 15 DPM slugs had slightly more fluctuating lysosomal coverage, starting at 4.21 ± 2.1%, dipping at 7 days of starving to 2.6 ± 0.9% and then returning to 4.5 ± 0.2%. Outside the DGT, their percent coverage was close to 0 (Fig. 6.5C). Only the 25 DPM animals started the starvation period with very low values inside the DGT (0.2%), which stayed low until 15 days in starvation when it rose to 2.3 ± 0.7% and finally 5.6 ± 1.3% at the 25-day time point (Fig. 6.5D).

6.3.5 Re-fed *E. timida* Juveniles (Experiment #4)

Six juvenile slugs from each age group were starved (Table 2 (S)) and three of them were then reintroduced to food (Table 2 (S+R)) before their chloroplast and lysosome abundances were imaged to determine the increase in chloroplasts after a single feeding. Lysosomal activity of starved and re-fed specimens (S+F) were compared to those that were only starved (S). A Kolmogorov-Smirnov test revealed normally distributed data for each group compared. In the 4 DPM unstarved slugs (control), there were significantly more chloroplasts inside the digestive gland tubules (DGT), (S: 29.9%, S+R: 36.2%) (two-tailed T-test: p=0.005), however lysosome abundance inside and outside the DGT were not significantly different in the slugs provided with food after starving when compared to the un-

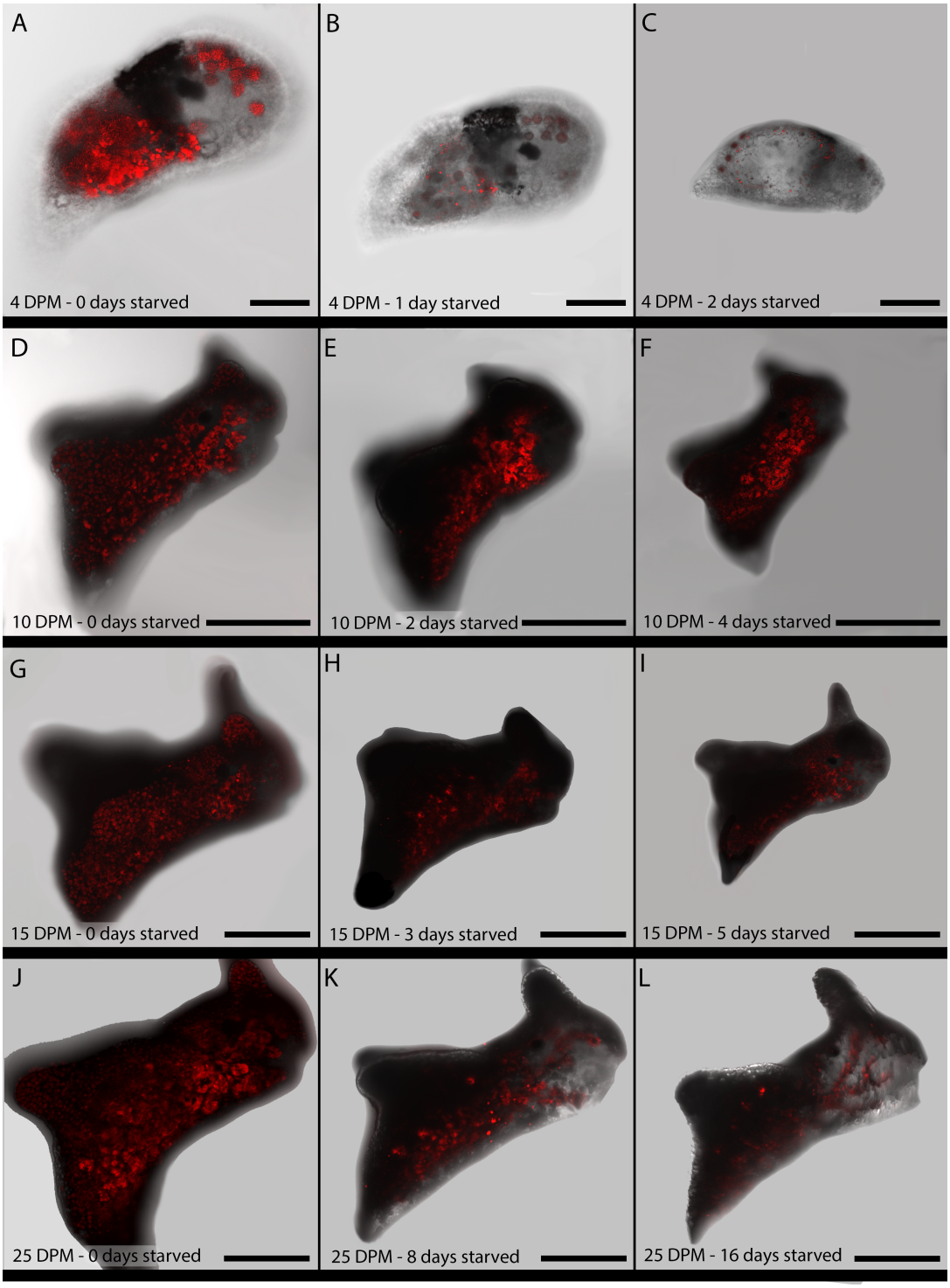


Figure 6.3 Confocal microscopy images of juvenile *E. timida* and their chlorophyll content during digestion. A: 4 DPM juvenile before starvation (day 0). B: The same 4 DPM juvenile after 1 day of starvation. C: The same 4 DPM juvenile after 2 days of starvation. D: 10 DPM juvenile before starvation (day 0). E: The same 10 DPM juvenile after 2 days of starvation. F: The same 10 DPM juvenile after 4 days of starvation. G: 15 DPM juvenile before starvation (day 0). H: The same 15 DPM juvenile after 3 days of starvation. I: The same 15 DPM juvenile after 5 days of starvation. J: 25 DPM juvenile before starvation (day 0). K: The same 25 DPM juvenile after 8 days of starvation. L: The same 25 DPM juvenile after 16 days of starvation. Scale bars: A-C: 70µm; D-F: 250µm; G-I: 350µm, J-L: 450µm.

fed slugs (S: 6.4%, S+R: 6.9%; two-tailed T-test: p=0.4 lysosomes in DGT), (S: 0.2%, S+R: 0.2%; two-tailed T-test: p=0.66 lysosome outside DGT) (Fig 6.5A).

The 10 DPM unstarved slugs followed a similar trend, having no significant difference in the lysosome abundance outside or inside the DGT (S: 0.7%, S+R:0.8%; p=0.88 and S: 5.9%, S+R:6.1%; p=0.75 respectively) and a significant difference in chloroplast abundance (S: 31.1%, S+R:44.6%; p=0.01). After 3 days of starvation however, the chloroplast difference after feeding decreases and is no longer significant (S: 9.7%, S+R: 10.1%; p=0.1) and the lysosome values remain insignificant (S: 6.0%, S+R: 6.1% inside DGT; p=0.59 and S: 0.8%, S+R: 0.9%; p=0.43 outside DGT) (Fig. 6.5B).

The first two measurements (unstarved and 3 days starving) for 15 DPM slugs had significant differences in the number of functional chloroplasts when comparing slugs that were allowed to feed to those not given food (S: 43.5%, S+R: 49.0%; p=0.05 unstarved and S: 17.2%, S+R: 32.5%; p=0.001 after 3 days). Lysosomal abundances inside and outside the digestive gland tubule were never significant (S: 8.2%, S+R: 8.4%; p=0.74 inside DGT and S: 0.3%, S+R: 0.2%; p=0.81 outside DGT) (Fig. 6.5C). The same trend was seen in 25 DPM slugs, although the first 4 measurements had significant differences in the number of chloroplasts (unstarved S: 74.2%, S+R: 81.8%; p=0.03; 3 days S: 54.9%, S+R: 57.8%; p=0.05; 7 days S: 50.5%, S+R: 57.2%; p=0.001, 10 days S: 41.7%, S+R: 48.8%; p=0.01). At 15 and 25 days of starvation though, the number of chloroplasts were no longer significant (S: 20.8%, S+R: 19.7%; p=0.78 and S: 10.3%, S+R: 13.6%; 0.59 respectively). The difference in lys. abundance inside and outside the DGT also lacked significance (Fig. 6.5D). The trends for each group are summarized in Fig. 6.4F.

The first two measurements for 15 DPM slugs had significant differences in the number of functional chloroplasts when comparing slugs that were allowed to feed to those not given food (p=0.05 after 1 day of starving and p=0.001 after 3 days). Lysosomal abundances inside and outside the digestive gland tubule were never significant (Fig. 6.5C). The same trend was seen in 25 DPM slugs, although the first 4 measurements had significant differences in the number of chloroplasts (1day of starvation p=0.03; 3 days p=0.05; 7 days p=0.001, 10 days p=0.01). At 15

and 25 days of starvation though, the number of chloroplasts were no longer significant (p=0.78 and 0.59 respectively). The difference in lysosome abundance inside and outside the DGT also lacked significance (Fig 6.6.5D). The trends for each group are summarized in Fig. 6.6.4F.

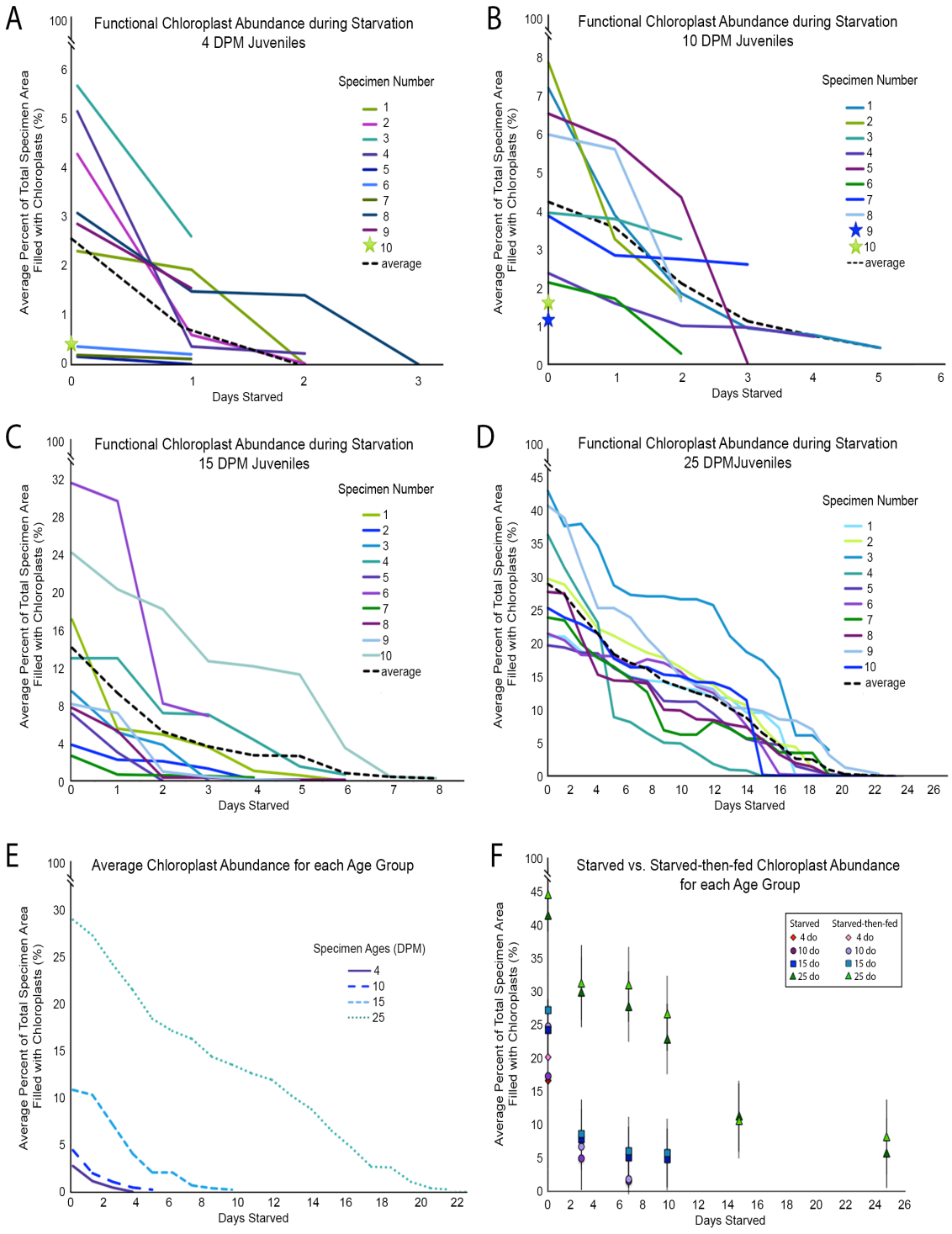
6.3.6 Comparison to Non- and Short-term Retaining Species

Five short-term kleptoplast retaining species (StR), and five non-retaining (NR) species were measured with the Pulse Amplitude Modulated Fluorometer (PAM) to determine their photosynthetic activity during starvation and their longevity in starvation. The StR and NR designation were assigned according to published reports [5,48]. Since this study uses these NR and StR species to provide context for the argument that functional kleptoplasty develops after 15 days in juvenile *El. timida*, we wanted to verify that the specimens observed here had photosynthetic activities similar to those recorded in previous reports and be able to state any discrepancies.

NR species *Ercolania viridis* (n=8) and *Ercolania fuscata* (n=10) starved an average of 5.8 ± 1.1 days and 7.25 ± 1.69 days respectively (Fig. 6.6A). Both species had measurable average photosynthetic activity values (F_v/F_M) on the first day they were measured, *Er. viridis* with 0.111 ($y = -5.14x^3 + 63.87x^2 - 248.13x + 296$, $R^2 = 0.95$) and *Er. fuscata* had 0.173 ($y = -1.59x^3 + 28.87x^2 - 169.65x + 320.76$, $R^2 = 0.98$), however after 24 hours, no autofluorescence was measured in either species (Fig. 6.7A-B). *Elysia marcusi* (n=10) averaged 4.5 ± 0.9 days in starvation (Fig. 6.6C), while *Elysia tuca* (n=10) and *Placida dendritica* (n=10) starved longer, with 13.3 ± 1.9 and 8.6 ± 1.3 days respectively (Fig. 6.6B). Photosynthetic activity was measured in unstarved specimens for each species: *El. marcusi* having an F_v/F_M = 0.471 on the first day in starvation and 0 by the fifth day ($y = -15.17x^2 + 21.83x + 441$, $R^2 = 0.92$) (Fig. 6.7A); *El. tuca* starting starvation with F_v/F_M = 0.438 and continuing to have photosynthetic activity after death ($y = 1.1433x^2 - 40.434x + 507.49$, $R^2 = 0.91$) (Fig. 6.7B), and *P. dendritica* which began starvation with an F_v/F_M = 0.533 and showed little to no photosynthetic activity upon death (0.0 - 0.1) ($y = 4.22x^2 - 103.95x + 610.26$, $R^2 = 0.97$) (Fig. 6.7A).

StR species *Bosellia mimetica* survived 13.7 ± 1.4 days (n=10) in starvation, starting starvation with an F_v/F_M = 0.567 and ending with 0.0 ($y = 1.26x^2 - 62.16x + 615.03$, $R^2 = 0.97$) (Figs. 6.6C, 6.7D). *Elysia cornigera* had an F_v/F_M = 0.688 when unstarved and 0.0 after 14 days, despite living an average 22.1 ± 2.3 days (n=10) ($y = 1.9132x^2 - 74.939x + 718.98$, $R^2 = 0.97$) (Figs. 6.6D, 6.7D). *Elysia patina* started starvation with F_v/F_M = 0.614, ending after an average 6.1 ± 1.7 days (n=10) with F_v/F_M = 0.386 ($y = -35.591x + 660.95$, $R^2 = 0.88$) (Figs. 6.6D, 6.7C). *Elysia papillosa* starved an average of 22.1 ± 1.3 (n=8), having a beginning F_v/F_M = 0.562 and ending at 0.0 after 22 days ($y = -0.553x^2 - 7.5443x + 514.6$, $R^2 = 0.94$) (Figs. 6.6E, 6.7C). *Thuridilla hopei* (n=10) began starvation with F_v/F_M = 0.548, with no detectable autofluorescence after 13 days, despite surviving an average 23.6 ± 1.8 days ($y = 1.48x^2 - 60.37x + 577.04$, $R^2 = 0.97$) (Figs. 6.6E, 6.7C). The average longevity in starvation for each species and the different *E. timida* age groups is summarized in Fig. 6.6F.

Figure 6.4 (next page) Functional chloroplast abundance during starvation for each age group (DPM). A-D: The average juvenile longevity is shown by the black dotted lines. A: Each juvenile E. timida (n=10), aged 4 days post-metamorphosis (DPM) was measured daily to determine the decrease in functional chloroplast abundance throughout the starvation period until death. The green star indicates an animal that died within the first 24 hours and could not be represented by a line. B: The longevity of each juvenile E. timida specimen aged 10 DPM (n=10). The green and blue stars indicate individual animals that died within the first 24 hours and could not be represented by a line. C: 15 DPM juvenile longevities in starvation (n=10). D: 25 DPM individual longevities in starvation (n=10). E: The average chloroplast abundance for each age group depicted in A-D, plotted together for comparison with the same axes scaling. F: Functional chloroplast abundance in starved and starved then re-fed E. timida juveniles of each age group for each time point surveyed. Juveniles of each age group were starved and their functional chloroplast abundances recorded at 0, 3, 7, 10, 15 and 25 in starvation. Additional animals were re-introduced to food and allowed to feed for 2 hours before their chloroplast abundances were recorded. 4 DPM juveniles are indicated by diamonds: red for starved and pink for starved-then-fed; 10 DPM juveniles are shown by circles: purple for starved and lavender for starved-then-fed; 15 DPM juveniles are denoted by squares, dark blue for starved and light blue for starved- then-fed; 25 DPM juveniles are designated by triangles: dark green for starved and light green for starved-then-fed.


A
Functional Chloroplast Abundance during Starvation
4 DPM Juveniles
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Specimen Number
1
2
3
4
5
6
7
8
9
10
average
B
Functional Chloroplast Abundance during Starvation
10 DPM Juveniles
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Specimen Number
1
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3
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5
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7
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average
C
Functional Chloroplast Abundance during Starvation
15 DPM Juveniles
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Specimen Number
1
2
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4
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9
10
average
D
Functional Chloroplast Abundance during Starvation
25 DPMJuveniles
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Specimen Number
1
2
3
4
5
6
7
8
9
10
average
E
Average Chloroplast Abundance for each Age Group
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Specimen Ages (DPM)
4
10
15
25
F
Starved vs. Starved-then-fed Chloroplast Abundance
for each Age Group
Average Percent of Total Specimen Area Filled with Chloroplasts (%)
Days Starved
Starved
4 do
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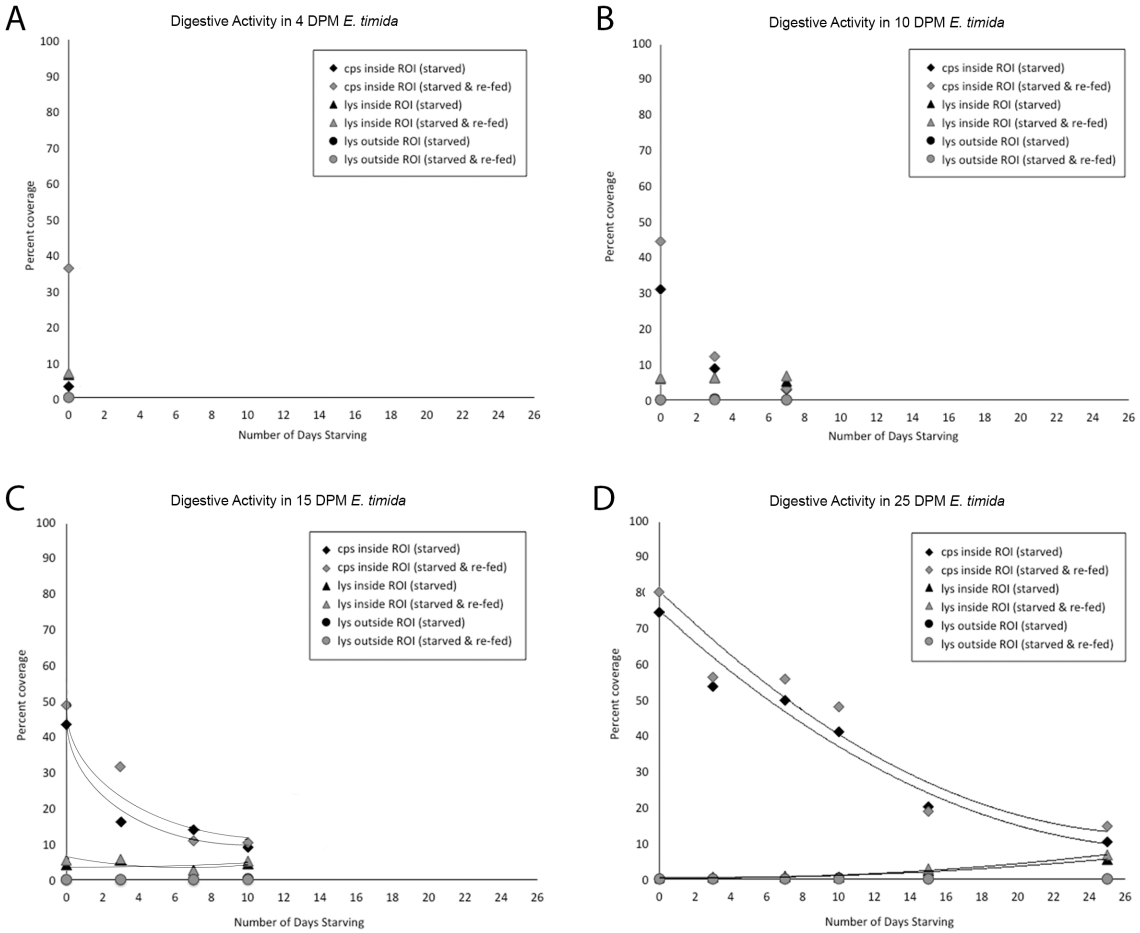
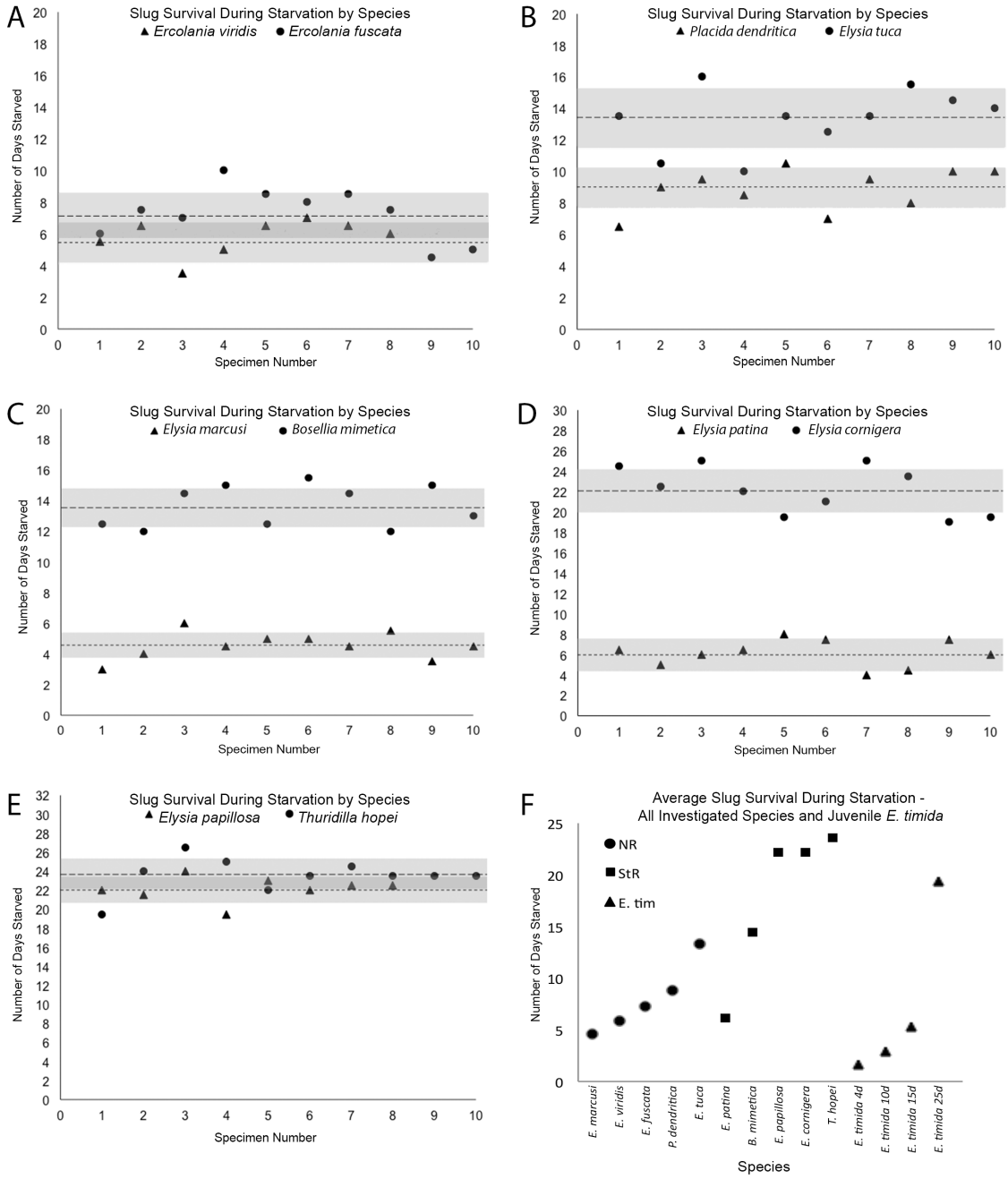


Figure 6.5 Digestive activity as indicated by chloroplast and lysosome abundance in specimens of 4, 10, 15 and 25 Days Post-Metamorphosis (DPM) *E. timida.* Black shapes indicate starved animals and grey shapes indicate starved-then-fed animals. Diamonds show chloroplast abundance inside the digestive gland tubule, here referred to as the region of interest (ROI), triangles denote lysosome abundance inside the ROI and circles depict lysosome abundance outside the ROI. All values are given in percent coverage, the percentage of tissue either inside or outside the ROI where fluorescent signal was measured as compared to the total area covered by the tissue. A: digestive activity in 4 DPM juveniles. B: 10 DPM juveniles. C: 15 DPM juveniles. D: 25 DPM juveniles.

Figure 6.6 (next page) Longevity in starvation by species. A-E: The x-axis shows the individual specimen number and the y-axis records the number of days that specimen survived. The black dotted and dashed lines show the respective averages for each species and the grey bars indicate one standard deviation to each side of the mean. A: *Ercolania viridis* is denoted by triangles and *Ercolania fuscata* by circles. The *E. viridis* average was 5.8 ± 1.1 days (dotted line) and *E. fuscata* averaged 7.25 ± 1.7 (dashed line). B: *Placida dendritica* is represented by triangles and *Elysia tuca* by circles. The *P. dendritica* average was 8.85 ± 1.3 days (dotted line) and *E. tuca* averaged 13.35 ± 1.9 (dashed line). C: *Elysia marcusi* is signified by triangles and *Bosellia mimetica* by circles. The *E. marcusi* average was 4.55 ± 0.89 days (dotted line) and *B. mimetica* averaged 13.65 ± 1.4 (dashed line). D: *Elysia*

***patina* is conveyed by triangles and *Elysia cornigera* by circles. The *E. patina* average was 6.15 ± 1.7 days (dotted line) and *E. cornigera* averaged 22.15 ± 2.3 (dashed line). E: *Elysia papillosa* is designated by triangles and *Thuridilla hopei* by circles. The *E. papillosa* average was 22.1 ± 1.3 days (dotted line) and *T. hopei* averaged 23.55 ± 1.8 (dashed line). F: All of the short- and non-retaining species average longevities in starvation and the *E. timida* juveniles average longevities are depicted, showing the clear difference in juvenile longevities and grouping within either the short-term retention slugs and non-retaining slugs. Circles depict Non-Retaining slug species (NR); squares indicate Short-term Retaining (StR) species and triangles show the different *E. timida* juvenile age groups.**



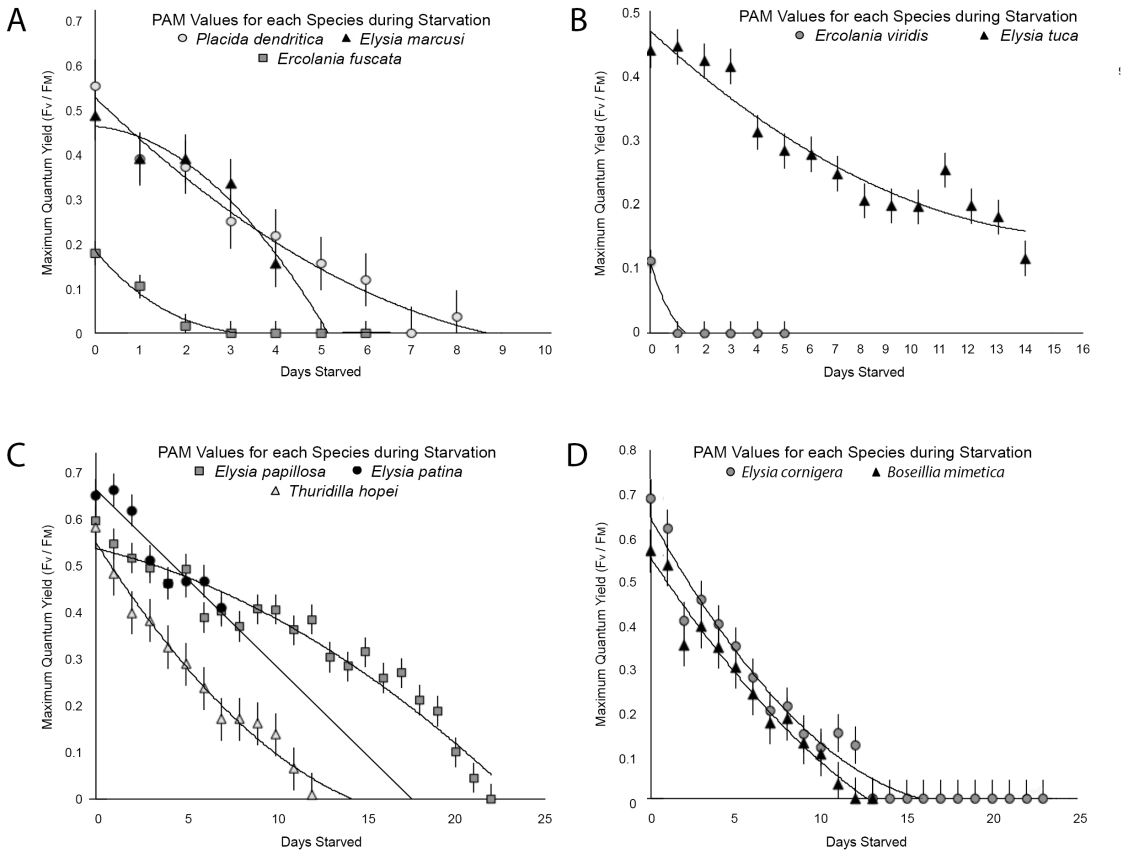


Figure 6.7 Pulse Amplitude Modulated (PAM) fluorometry values for a range of short-term retention and non-retaining species during starvation. A: The PAM values for *Placida dendritica* are indicated by light grey circles, *Elysia marcusi* by black triangles and *Ercolania fuscata* by dark grey squares. *P. dendritica* is best modeled by the function $y = 4.22x^2 - 103.95x + 610.26$ ($R^2 = 0.97$), *E. marcusi* by $y = -15.17x^2 + 21.83x + 441$ ($R^2 = 0.92$) and *E. fuscata* by $y = -1.59x^3 + 28.87x^2 - 169.65x + 320.76$ ($R^2 = 0.98$). B: The PAM values for *Ercolania viridis* are indicated by grey circles and *Elysia tuca* by black triangles. *E. viridis* is best modeled by the function $y = -5.14x^3 + 63.87x^2 - 248.13x + 296$ ($R^2 = 0.95$) and *E. tuca* by $y = 1.14x^2 - 40.43x + 507.49$ ($R^2 = 0.91$). C: The PAM values for *Elysia papillosa* are indicated by dark grey squares, *Elysia patina* by black circles and *Thuridilla hopei* by light grey triangles. *E. papillosa* is best modeled by the function $y = -0.55x^2 - 7.54x + 514.6$ ($R^2 = 0.94$), *E. patina* by $y = -35.59x + 660.95$ ($R^2 = 0.89$) and *T. hopei* by $y = 1.48x^2 - 60.38x + 577.04$ ($R^2 = 0.98$). D: The PAM values for *Elysia cornigera* are indicated by grey circles and *Bosellia mimetica* by black triangles. *E. cornigera* is best modeled by the function $y = 1.91x^2 - 74.94x + 718.98$ ($R^2 = 0.97$) and *B. mimetica* by $y = 1.26x^2 - 62.16x + 615.03$ ($R^2 = 0.97$). The error bars show the standard error.

6.4 Discussion

Elysia timida is increasingly used as a model organism in the search to understand the development and evolution of functional kleptoplasty since it is commonly found and only feeds on one algal species, which limits experimental uncertainty due to multiple incorporated kleptoplast species [9,11,15,16,35,46]. *El. timida* juveniles are incapable of retaining functional chloroplasts, showing that this ability must be developed as the young slug develops. Although Marín and Ros (1993) state that functional kleptoplasty is established after 12 days, they do not provide evidence supporting this claim. The longevity experiments conducted here dispute this claim, instead showing functional kleptoplasty is likely developed more than 15 days after metamorphosis and shell loss – maybe even after 25 days, see below, when algae is continuously available for feeding. This discrepancy may be due to population differences, temperature or a host of other abiotic and biotic factors.

6.4.1 Juvenile handling with and without $MgCl_2$ and cold (Experiments 1 & 2)

The exposure to cold and $MgCl_2$ did not affect juvenile longevity and in most cases, did not seem to affect the animal's locomotion. Both of these techniques are frequently used to immobilize living animals but they both had little effect on juvenile *El.* timida despite functioning in adults [51–53]. They also did not affect the longevity of the animal, as the average longevities for the treated animals showed no significant difference to animals that were untreated.

6.4.2 The development of functional kleptoplasty in juveniles

Only one other sacoglossan species has been investigated regarding larval development and the acquisition of functional kleptoplasty, the LtR, heterokontophyte feeding species *Elysia chlorotica.* Pelletreau et al. [36,42] reported post-metamorphic juveniles requiring 7 days of feeding before they gained the ability to retain functional kleptoplasts and defined the term transient kleptoplasty in reference to the time period when kleptoplasty is being established

but does not function as observed in adults. This conclusion is based on laboratory cultured *El. chlorotica* that were photographed until 10 DPM. Our study shows that *El. timida* needs more than 15 days, suggesting each LtR species gains this ability at a different time and may even gain it through a different process although this will take further investigation to confirm. As previously shown for *E. chlorotica* [42], the data presented here indicates a transient kleptoplasty phase in *E., timida*, after the 15 DPM time point.

This argument is based on two of the experiments presented here. The longevity experiments show juveniles that are 4, 10 or 15 DPM as having very short longevities (a maximum 8 days) in starvation. While these animals survive shorter time periods than seen in adult non-retention forms, the starvation time for 25 DPM *El. timida* juveniles is well above all of the non-retaining slugs (NR), and is well within the range of the short-term retention (StR) slugs observed in this investigation, at a maximum 22 days. This is still only around one fourth of the total starvation survival duration accomplished by adult *E. timida*, (89 days) [9,46]. It is also important to note that the NR and StR forms surveyed here were adult slugs, and had significantly more body mass than the *El. timida* juveniles in our experiments here.

Some previous reports on longevity in starvation are similar to the results presented here (*El. patina, El. papillosa* [48]), while others present discrepancies suggesting a variability in starvation longevity that may be related to factors such as time of the year, light intensity, temperature, size of the investigated specimens or the number of individuals surveyed. Christa et al. [48] reported *El. tuca* capable of surviving 20 days in starvation although they only achieved 13.3 days here. *El. marcusi* survived 9 days in that study (referred to as "*Bosellia marcusi"*) but 4.5 days on average here and *El. cornigera* which was reported starving 12 days in their study and 22.1 days in this investigation. *P. dendritica* also starved more than 11 days in a previous report [47] but only 8.6 days here. Starvation longevity has not been previously assessed for the species, *Er. fuscata*, *Er. viridis, T. hopei* and *B. mimetica,* so this study constitutes the first report. Although this study appears to show a correlation between the duration functional chloroplasts remain in the slug's digestive gland and the time it can withstand starvation (short-term

retaining slugs outlive non-retaining species), the discrepancies described above and the lack of additional reporting on some species suggest that this conclusion should not be made without further and clarifying evidence. This sentiment aligns with previous work that has discussed this topic in detail [11,54].

6.4.3 Functional kleptoplast abundance in juvenile *El. timida*

Although 25 DPM juveniles can withstand starvation longer than all of the NR slug species, many still died with functional chloroplasts in their digestive systems. Adult *E. timida* have been reported as either having or lacking functional kleptoplasts in their bodies after dying of starvation [16,35]. The number of kleptoplasts was drastically reduced compared to the number each individual starts starvation with, however the presence of functional kleptoplasts upon death indicates that not all kleptoplasts are digested and used to meet their energetic needs, even when they are about to die. This suggests the following options, that juvenile slugs are unable to digest all chloroplasts, that the energy required to digest these kleptoplasts is more than the juvenile can spare or that they take up more chloroplasts than they can use. Despite this, the decline in functional kleptoplasts indicates that *El. timida* juveniles of all ages (including the 25DPM specimens) are capable of digesting kleptoplasts. This agrees with transmission electron micrograph studies, where broken down kleptoplasts are seen within juvenile *El. timida* digestive tubules [9]. Other investigations have reported functional kleptoplasts in slugs that have died of starvation [16].

Two distinct groupings are seen within the 4 DPM juveniles. Four of these juveniles had very low amounts of chloroplasts in their bodies, indicating that they had fed less than the other juveniles in their age group. This difference may have affected their longevity, as they all died within the first 48 hours, however some of the juveniles with far higher chloroplast levels also died within this time span, so a direct comparison between longevity and chloroplast abundance cannot be drawn. The same can be said for each of the other age groups, since in each of the populations, there is no obvious advantage to having more chloroplasts in terms of days survived. This may indicate that these chloroplasts are not directly or fully contributing to meeting the slug's nutritional demands throughout the starvation

period, however possible contributions made by the accumulated photosynthates should not be ignored and require further investigation [35,43]. One 25 DPM specimen (Specimen #7, Fig 6.4D) showed a slight increase in chloroplast abundance at 11 days in starvation and this data point lacks a clear explanation since there are no reports of chloroplast division once incorporated. This is likely a random error and may be due to sample movement during scanning.

The overall percent coverage of chloroplasts in juvenile *El. timida* also suggests that transient kleptoplasty is established after 15 days of feeding, but before 25 days. The low percent coverage seen in 4, 10 and 15 DPM juveniles differs significantly from the high abundance seen in adults [46]. Only the 25 DPM juveniles reach the 35-70% coverage seen in the adults although not every 25 DPM juvenile fell within that range (19-43%). The overlap in some juveniles and some adult *El. timida's* ranges here, (35-43%) indicates that at least some of these juveniles are filled with kleptoplasts to the degree with which adults are. This again aligns 25 DPM juveniles more with adults than with the other juvenile populations, despite their still immature morphology (the female gonad had not developed in any of the 25 DPM juveniles examined). This aligns with Schmitt et al. (2014) who stated that it takes 106 days for *El. timida* to develop a fully mature internal morphology and reproductive capabilities.

The variability of juvenile longevity in starvation suggests a range of individual fitness levels, consistent with an *r*-selection-species pattern, where many offspring are produced and very few actually survive. As *El. timida* egg clutches contain many offspring (19-249 according to Schmitt (2014)) and we observed many slugs hatching but not developing through metamorphosis, this finding is consistent with the predicted fitness strategy. The only fitness tests examined in these experiments screened out animals that did not hatch and those that couldn't complete metamorphosis. Therefore, the animals used here were already amongst the fitter specimens, but following a successful metamorphosis, they were not additionally screened for size, weight, ingested kleptoplast content or other factors, meaning a variety of fitness levels should still be present. Despite this, no significant advantage was observed in any of the experiments, where one specimen drastically outlived the others in its age group. The low standard

deviations surrounding the average longevities also suggest that while individual variability and fitness should be considered, it was not overly apparent that some individuals were better suited to survival in starvation.

6.4.4 Digestive activity in *E. timida* juveniles

Lysosomal activity in 4, 10 and 15 DPM animals was relatively stable, with around 5% coverage throughout the starvation periods. As an indicator of digestion in these tissues, this pattern of lysosomal activity suggests a constant digestive process within these animals. This trend does not align with the trend seen in *El. timida* adults [46], where the lysosomal activity is almost non-existent at the beginning of the starvation period and rises exponentially midway through the starvation period. The 25 DPM juveniles also show this pattern, starting at 0% and increasing to 8% at the end. While this is not the sharp increase seen in the adult populations, the trend is the same, starting at 0%, which indicates suppressed digestion and later rising. This further suggests that 25 DPM juveniles behave more like adults than younger juveniles concerning digestion during a starvation period. There was no discernable trend that correlated the number of functional kleptoplasts within an individual with the rate of lysosomal activity in that individual.

Juveniles that were re-introduced to food and allowed to feed for 2 hours before their lysosomal activity and kleptoplast abundance were measured showed the increased kleptoplast abundance within their digestive glands. This was not observed in adult specimens, likely because the number of kleptoplasts gained by feeding is a very small percentage compared to the number they already possess [46]. In each juvenile age group however, the increase in kleptoplast abundance is visible, accounting for a 1-15% increase in the coverage area. Also unlike the adults, every population of juveniles was observed feeding throughout the starvation period, whenever food was supplied again, suggesting they do not lose the ability to feed. The amount of kleptoplasts gained was not always significant, however. A change in lysosomal activity was never significant amongst these populations, so the digestive trends reported here are not affected by a starving slug's recent feeding, at least within the short period between feeding and analysis.

6.4.5 Comparison to short-term and non-retaining species

To collaborate and compare *El. timida* juvenile longevities to various long-, short- and non-retention forms, 10 other sacoglossan species, with varying abilities to perform functional kleptoplasty were starved to uncover the maximum starvation time under laboratory conditions. The non-retention forms *Placida dendritica, Elysia marcusi, Elysia tuca,* were starved, showing various longevities within the expected ranges for these particular species [48]. *Ercolania fuscata* and *Ercolania viridis* were not previously investigated in regards to maximum starvation but their starvation longevity and photosynthetic yield values allow their assignment to the Non-Retaining (NR) group, as defined by Händeler et al. [5]. Short-term retention species *Bosellia mimetica, Thuridilla hopei, Elysia papillosa, El. patina, El. cornigera* also fell within previously published ranges [4,48,55]. When compared to *El. timida* juveniles, these other species provide a basis for the assertion that juvenile *El. timida* under 15-days-old function like non-retention forms, directly digesting chloroplasts and having short lifespans in starvation.

Slugs older than 15 days begin to function like adults, having developed kleptoplast retention and a somewhat extended starvation capacity, despite lacking adult morphology and sexual maturity. A transitional stage as described by Pelletreau et al. [42] would have to begin between 15 and 25 DPM, and the intervals sampled here could not accurately define its beginning. Despite this transitional stage not appearing clearly in the data presented here, the 25 DPM juveniles are likely in this stage. They can only survive up to 22 days of starvation, a mere fifth of the time an adult *El. timida.* This coupled with the fact that some of these juveniles died with kleptoplasts still in their digestive glands and this has not been observed in starving adults (EMJL unpublished results), also suggests that these juveniles have developed many adult-like traits, but are not completely adults regarding the development of functional kleptoplasty. “Why” and “how” these animals transition from non-kleptoplast retaining individuals to long-term retaining adults remains to be resolved.

6.4.6 Elysia timida ecology

Contrasting Marín and Ros (1992), we found adult *El. timida* throughout the summer months at a variety of sampling locations in Italy and Spain, however only those animals collected during May produced egg masses on a regular basis. *A. acetabulum* was found forming caps in May 2015 with full caps present by July 2015. This was observed again in July 2016, suggesting that the yearly cycle *A. acetabulum* follows is less rigid calendar than Ros and Marín (1992, 1993) reported and likely depends on locality and/or specific environmental cues that fluctuate in the natural environment. By August 2015 and 2016, no living *A. acetabulum* was found, only the calcified stalks from the previous season, indicating that the caps had already broken open and released their planktonic cysts. Since only the adult animals we collected in May produced egg masses, the larvae that hatched had Type 2 development [38] and they hatched when *A. acetabulum* is abundantly found and not completely calcified, we can confirm Marín and Ros' (1992) observations regarding these questions.

Our experiment clearly show that functional kleptoplasty is not developed in *El. timida* until they are at least 15 days post-metamorphosis, and even 25 DPM individuals do not survive starvation to the degree with which adults do, but rather exhibit transient kleptoplasty. Future experiments should consider these observations when trying to understand how this remarkable ability functions and has evolved.

6.5 Acknowledgements

We would like to express our thanks to James-Robert Jerschabek Laetz for help collecting the animals, Claudia Müller for her help with animal husbandry, cultivating the algae used in these experiments and confocal microscopy, Diane Laetz for help with the statistics, as well as the entire staff at the Keys Marine Lab, Gregor Christa and Michael Middlebrooks for collection advice. Animal collection was permitted by a Special Activities License granted to Heike Wägele and Elise Laetz (SAL-16-1353C-SR).

Chapter 7

Unsuccessful Experiments and Ongoing Inquiries

Almost all of the experiments presented here involve methods I modified and/or developed. They only succeeded after numerous setbacks and unsuccessful trials. This chapter briefly explains some of the ineffective experiments, their intended purposes as well as their various outcomes. Some of these lines of inquiry led to the work presented in Chapters 2-6, others are still in progress at the present time and others have been discontinued for the time being.

7.1 Fluorescent Staining to Examine Digestive Activity

Investigating intracellular digestion *in situ* was always one of the main objectives in this project. Just prior to this investigation, the acidification of unripe cnidocysts was observed in the digestive gland tubules of *Aeolidiella stephanieae* (Obermann et al. 2012). While this likely has nothing to do with digestion, I was intrigued by the use of fluorescent staining (Ageladine A) to observe pH environments in living slugs. Knowing that intracellular digestion involves lysosomes binding and acidifying food particles enclosed in endosomes and hypothesizing that long-term- and short-term retaining slugs react to incorporated chloroplasts differently, I decided to attempt pH dependent staining in sacoglossan slugs.

Working with Dr. Ulf Bickmeyer (Alfred-Wegener Institute in Bremerhaven, Germany), who discovered Agaladine A and developed the staining methods used in Obermann et al. (2012), I tried to stain various sacoglossans directly after feeding and later in the starvation period. Each attempt failed to show localized signal from Ageladine A in the digestive gland tubules, even when we varied the

staining time, concentration, tissue sample and species. We could not get Ageladine A to penetrate into the living tissue beyond a few cells thick, most of it aggregating in sub-epidermal gland cells. No discernable signal was observed in the vicinity of the newly incorporated chloroplasts, so no results could be reported (Figure 7.1 A-B).

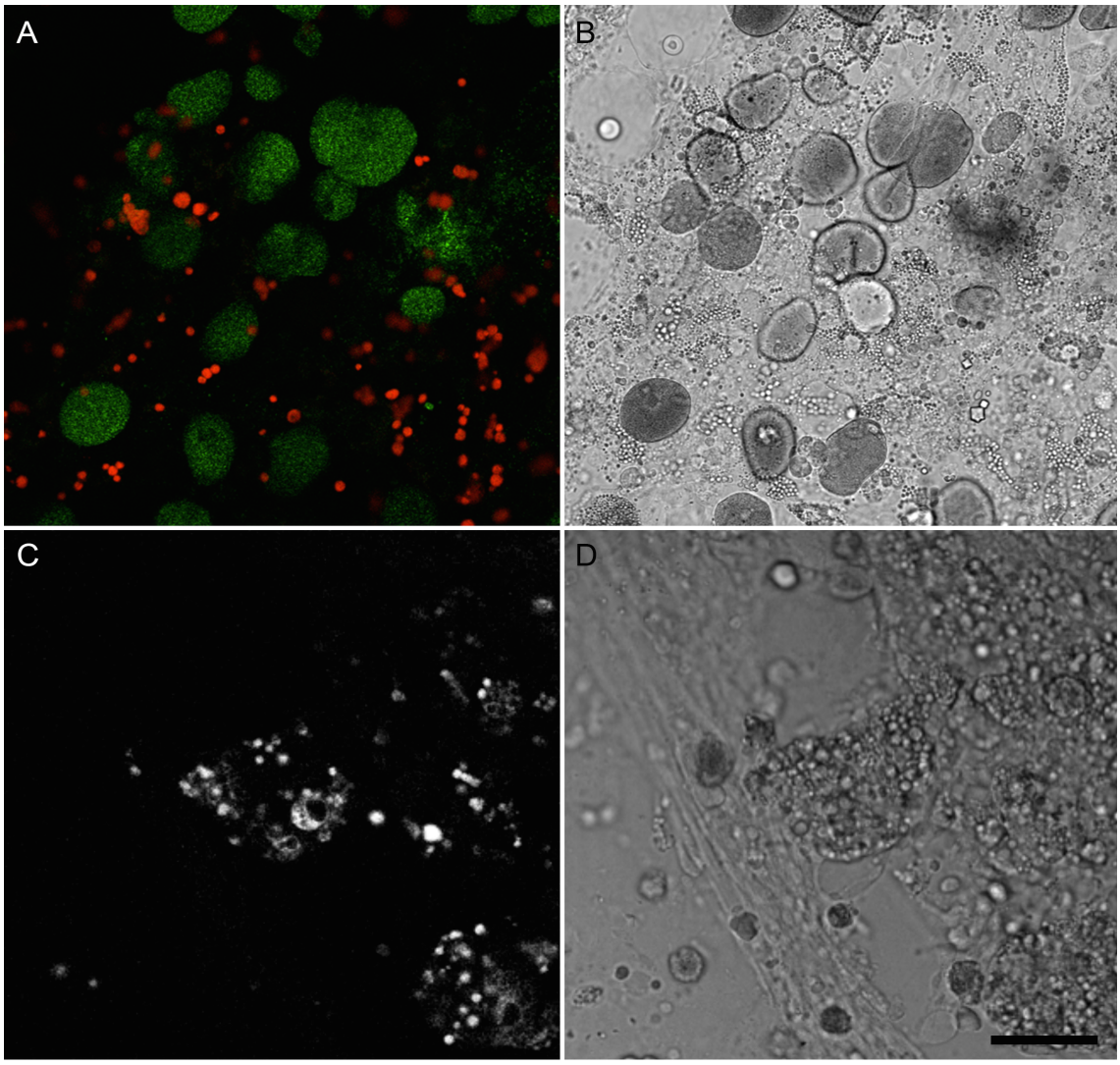


Figure 7.1 Unspecific staining in *Elysia timida* digestive gland cells. A: Ageladine A (falsely colored green) is concentrated in sub-epidermal gland cells located amongst the digestive gland tubule cells containing incorporated plastids (chloroplast autofluorescence falsely colored red), but does not penetrate into the digestive gland cells themselves. B: The same tissue observed using the transmission channel. C: Digestive gland tubule in 21 days starved *E. timida* after 24 hours staining. Lysoglow 84 seems to stain endosomes and lysosomes in the digestive system (as predicted), but the stain is not found throughout the digestive gland making the images un-interpretable. D: Transmission channel image of the same tissue. Scale bar: 50µm.

We also attempted stainings with Lysoglow 84, an Ageladine A derivative that shows higher sensitivity. Lysoglow84 did penetrate into the slug tissues, and could be detected in the digestive gland, however it did not penetrate evenly, even with staining periods exceeding 24 hours. Since this stain measures pH based on the signal intensity – how much this stain aggregates in place is relative to how acidic the location is – and it did not penetrate all tissues, the results could not be interpreted since lower signal could mean either a more alkaline environment or a lack of stain penetration (Figure 7.1 C-D).

The lack of interpretable data from these experiments led me to try another pH dependent stain, acridine orange which proved successful and resulted in multiple publications (Chapters 2, 4, 6).

7.2 Plastid Staining

A large obstacle when examining functional kleptoplasty is the inability to distinguish newly ingested chloroplasts from those that were already in the slug's digestive gland tubules. The absence of healthy adult slugs lacking plastids makes it difficult to follow a single plastid from the algae into the slug and observe what happens to that plastid during starvation. This means that every intracellular experiment occurring after a feeding is not only surveying newly incorporated plastids, but also chloroplasts that are still retained from previous feedings.

In order to distinguish newly incorporated chloroplasts from those already present from previous feedings, I tried a variety of staining techniques aimed at visually altering either the new or old plastids and therefore indicating the newly introduced ones. I attempted to stain the chloroplast nucleoid and stroma in the algae, and proceeding to feed the slug the altered algae. This worked with mixed success. I also varied numerous factors such as staining time and stain concentration to get a positive result, with no success. Since none of the experimental stainings detailed above worked, I suspended work on this matter for the time being and will continue in the coming months.

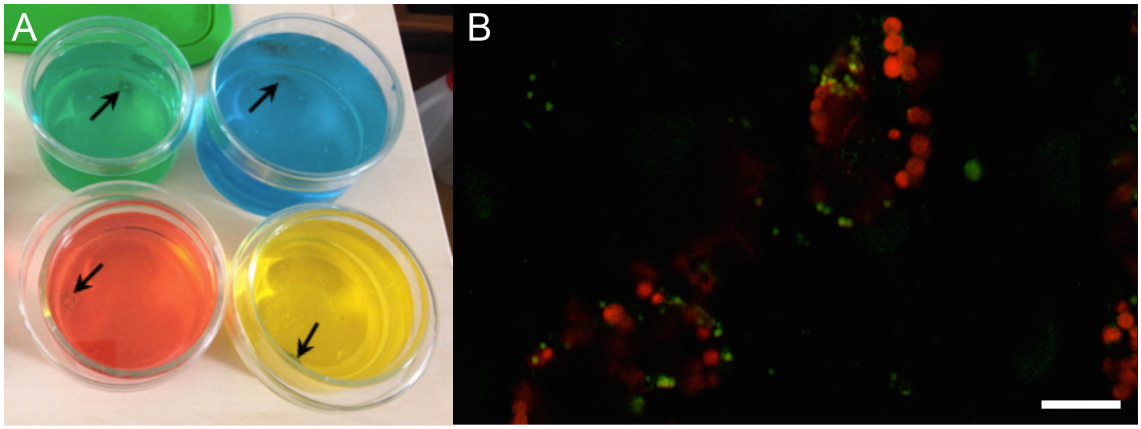


Figure 7.2 Unsuccessful staining of chloroplasts before slug feeding. A: FD&C stains on *Acetabularia acetabulum* caps (indicated by arrows). B: Hoeschst 33342 staining (falsely colored green) in *A. acetabulum.* This experiment failed to stain the chloroplast (red) nucleoids as indicated by a lack of green color inside the chloroplasts. Green staining outside the chloroplasts shows unspecific and unintended staining, likely mitochondria, since they are the only other structure containing DNA in the *A. acetabulum* cap.

7.3 TIC/TOC Immunohistochemistry

Using chlorophyll autofluorescence in living slug tissue is a secure method of measuring functional chloroplast abundance, however the actual number of chloroplasts may be far higher. When imaged on the CLSM, the chloroplasts observed are only chloroplasts with structurally intact chlorophyll a because the signal detected is the autofluorescence emitted by chlorophyll a. This does not reflect the actual chloroplast abundance, which is theoretically higher, the difference stemming from any chloroplasts that are present but no longer contain functional chlorophyll.

To assess the actual chloroplast abundance, I applied immunohistochemical staining to sacoglossan tissues. Since chloroplasts contain proteins not found in metazoans, choosing a marker unrelated to chlorophyll a should reveal the actual chloroplast abundance. Antibodies for the translocon for the inner membrane (TIC) and translocon for the outer membrane (TOC) of the plastid were recommended by my supervisors, because they are unrelated to chlorophyll a and should be unrelated to any chlorophyll a breakdown that occurs before the plastid

itself is digested and because TIC/TOC are plastid specific protein channels and should not stain in the slug's tissue. After numerous trials, I succeeded in staining TIC and TOC in the host algae, however the staining was either incomplete or unspecific and therefore dismissible in slug tissues. TOC was more successful than TIC, however the images I took were still un-interpretable (Figure 7.3). When a more aggressive detergent was applied, the chlorophyll washed out of the cells, so the functional chloroplasts could no longer be identified. Multiple detergents, antibody concentrations, staining times, blocking times and other conditions were attempted, none of which resulted in stainings that were reproducible or interpretable.

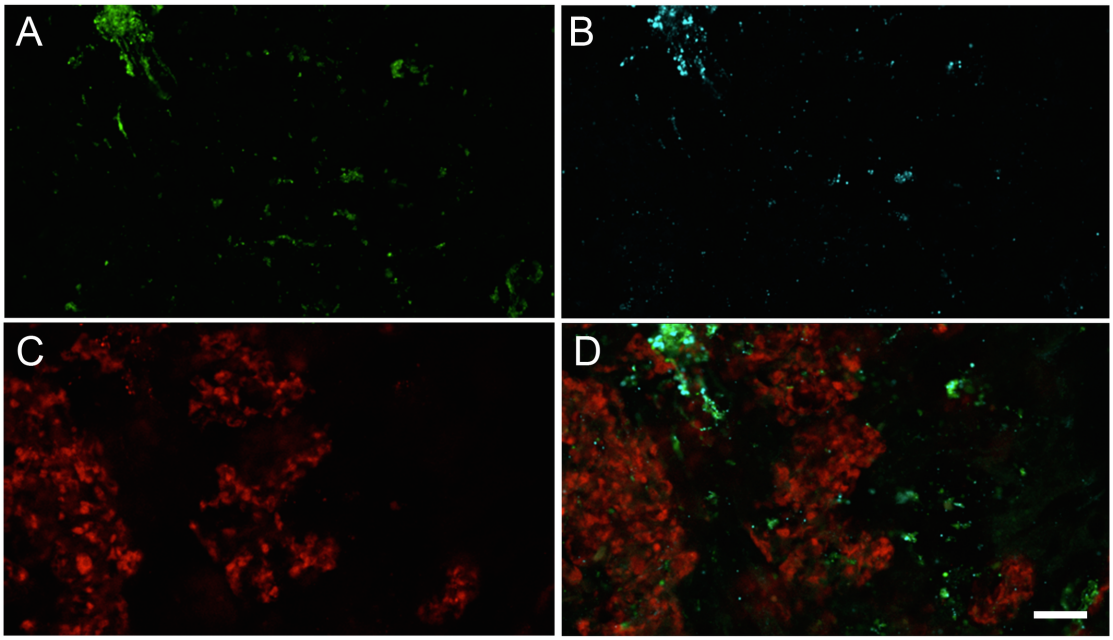


Figure 7.3 Unsuccessful TIC-TOC staining in *E. timida.* A: TOC staining, falsely colored green. B: TIC staining, falsely colored blue. C: Chloroplast autofluorescence (red). The chlorophyll observed here is no longer compactly found in the chloroplasts, showing this method also released the chlorophyll from the plastid. D: Composite of all three images showing little co-localization of plastids and the TIC-TOC markers. Scale bar: 20µm.

7.4 Chloroplast isolation

Throughout each of the cytological experiments presented here, it has become clear that tissue penetration is a huge obstacle. To alleviate this issue I have attempted to isolate chloroplasts from slug tissues. After numerous trials, this has resulted in reproducible success and I will use these isolates in future inquiries.

I intend to section these chloroplasts to observe the number of membranes surrounding each one (an ongoing debate described in Chapter 1.7). Observing only two membranes would neither confirm nor reject the presence of an endosome membrane, since it could have been lost in the extraction process and/or might not have been there. TIC/TOC staining using immunogold labeling could confirm that the two membranes observed here are of algal origin though. If three membranes are observed throughout a partial or complete starvation period, the presence of an endosome membrane would be supported.

Chapter 8

General Discussion

The purpose of this dissertation was to explore many of the unresolved questions regarding functional kleptoplasty in sacoglossan slugs. Functional kleptoplasty is a complex process involving multiple steps (detailed in Chapter 1.7). To understand the mechanisms behind functional kleptoplasty and postulate how it evolved, it is necessary to examine individual factors related to each step. Previous investigations have thoroughly detailed steps 1-3, plastid ingestion and incorporation by: describing adaptive and unique anatomy that allows the ingestion of algal cytoplasm (Chapter 1.1), examining sacoglossan diets (Chapter 1.2), and revealing which of slug/algae species combinations facilitate functional kleptoplasty (Chapter 1.4). Most aspects of plastid retention and eventual breakdown, however, were either unresolved or uninvestigated before this examination. In this dissertation, numerous factors are investigated to elucidate how chloroplasts remain in a plastid-retaining slug's digestive gland, uncover how they benefit a starving slug, reveal differences between long- and short-term plastid retention, examine the lack of plastids later in the starvation period and explore the development of this ability in juvenile slugs. These factors are detailed in the following chapters and the conclusions they present reshape our understanding of how functional kleptoplasty occurs in these species and allows us to hypothesize how it evolved throughout the Sacoglossa.

8.1 Photosynthetic Efficiency

Photosynthetic activity in sacoglossan slugs has been investigated since the late 1960's, using a variety of different methods. The most common method used in recent years is Pulse Amplitude Modulated (PAM) fluorometry, a non-invasive way of measuring chloroplast efficiency in living tissues (detailed in Chapters 1.5,

2.1) (Bhattacharya et al. 2013; Casalduero and Muniain 2008; Christa, Händeler, Kück, et al. 2014; Evertsen et al. 2007; Evertsen and Johnsen 2009; Katharina Händeler et al. 2009; Jesus et al. 2010; Middlebrooks et al. 2011; Pelletreau et al. 2011; Schmitt et al. 2014; Ventura et al. 2013; Vieira et al. 2009; Wägele et al. 2010, 2011; Wägele and Johnsen 2001; Wägele and Martin 2014). PAM Fluorometry has aided the understanding of functional kleptoplasty in sacoglossan slugs by revealing photosynthetic activity in their tissues and by providing a metric for monitoring the decrease in activity during starvation periods.

To date, roughly 85 species have been analyzed displaying a range of photosynthetic abilities and survival durations in starvation. At this point, no oxynoacean taxa has demonstrated photosynthetic activity nor an ability to withstand extended starvation - more than a few weeks (Christa, Händeler, Kück, et al. 2014). Of the investigated limapontioidean genera, only *Costasiella* species that show evidence of photosynthetic activity and extended starvation survival (Christa 2014; Evertsen and Johnsen 2009), although relatively few species have been examined. This study examined two previously uninvestigated species, *Ercolania viridis* and *Ercolania fuscata*, neither of which displayed photosynthetic activity after 1 or 2 days respectively. Although more limapontioidean species need to be investigated for confirmation, this supports the hypothesis that most if not all limapontioidean species other than *Costasiella,* are incapable of retaining functional kleptoplasts (Christa, Gould, et al. 2014). Contrary to this, many plakobranchoideans are capable of retaining chloroplasts for extended time periods and all of the long-term plastid retaining species except *Costasiella ocellifera* are located within this clade.

While this investigation added a few species that were previously uninvestigated furthering our understanding of photosynthetic efficiency within the Sacoglossa, most of these experiments replicated previous findings and were conducted as a metric to assess whether these populations were performing as observed in previous studies. The benefits of having a metric became apparent

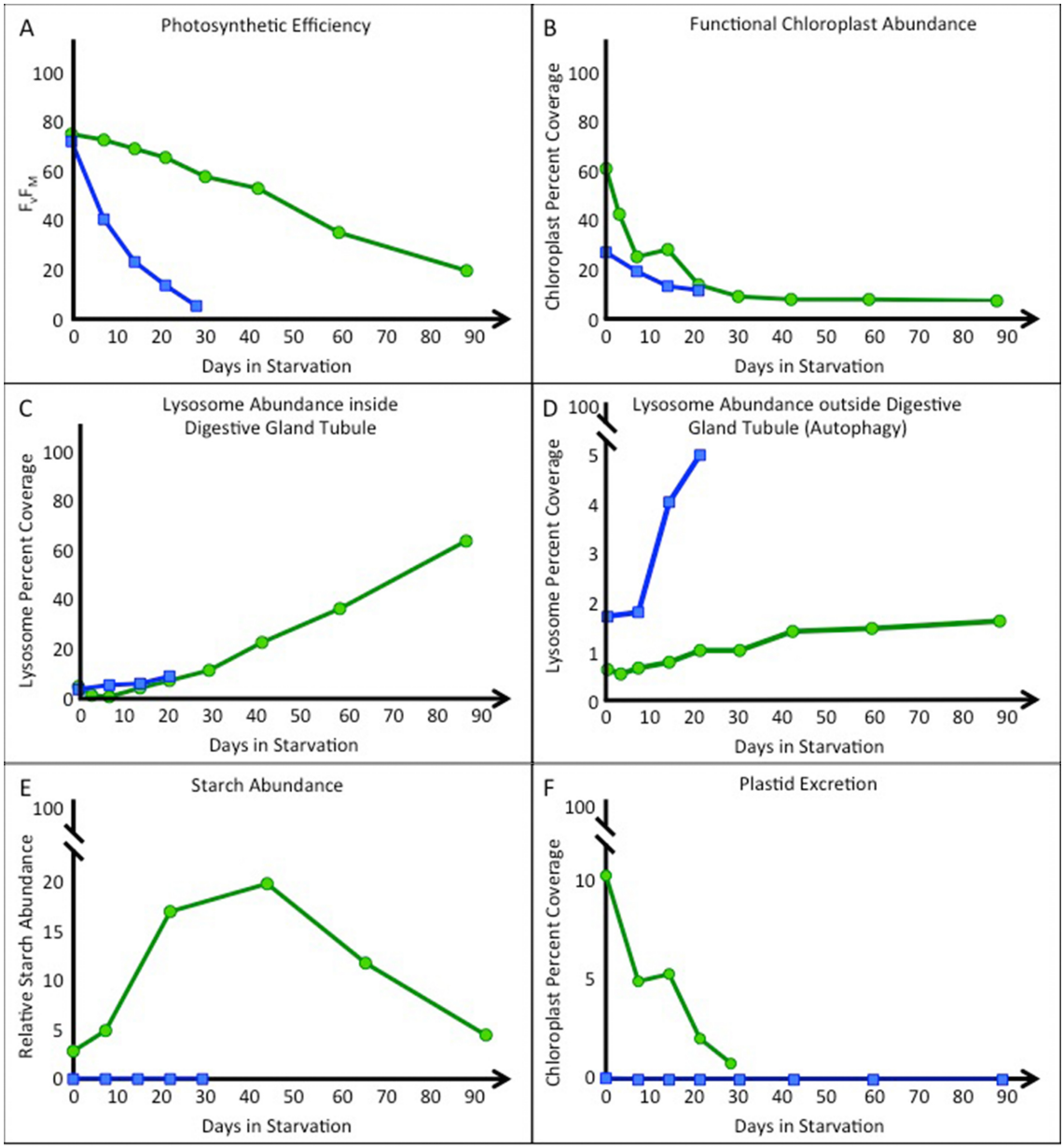


Figure 8.1 Each of the factors examined in this dissertation throughout starvation periods, comparing *E. timida* and *E. cornigera.* Green circles indicate *E. timida,* and blue squares represent *E. cornigera.* A: Photosynthetic efficiency based on PAM Fluorometry results (compiled from Chapters 2,4). PAM results from *E. timida* at 18°C are depicted here, since this represents all of the naturally occurring populations examined (each other temperature treatment was artificially induced). B: The relative abundance of functional chloroplasts within the digestive gland tubule cells (Chapters 2,4). C: The relative abundance of lysosomes within the digestive gland tubule cells (Chapters 2,4). D: The relative abundance of lysosomes outside the digestive gland tubule cells, indicating autophagy (Chapters 2,4). E: The relative amount of starch inside the digestive gland tubule (Chapters 3,5). F: The relative abundance of functional chloroplasts in excrement samples throughout starvation periods (Chapter 4).

after the investigations examining digestive activity (Chapter 2) and starch accumulation (Chapter 3) revealed significant differences in two populations. Only one population's PAM values (the 18°C, fall populations) matched the majority of previous investigations (Figure 8.1A) (Christa, Zimorski, et al. 2013a; Katharina Händeler et al. 2009; Krug et al. 2012; Schmitt et al. 2014). These differences in PAM values and starvation longevity led to further examination and the identification of temperature as a factor that influences photosynthetic efficiency in functional kleptoplasts (Chapter 4).

8.2 Functional Kleptoplast Abundance

A large obstacle in understanding functional kleptoplasty is the lack of a reliable method for quantifying the number of functional chloroplasts incorporated in a slug's digestive gland at any point during feeding or starving periods. Although PAM fluorometry requires functional chlorophyll to be present in order to record a measurement, PAM values themselves are relative and do not provide quantitative measurements (Wägele and Johnsen 2001). By examining living sacoglossan tissues with a confocal laser microscope, this obstacle has largely been mitigated due to the exact same principle used by the PAM Fluorometer: chlorophyll autofluorescence. Confocal microscopy adds a spatial dimension to the data being collected. In this case the area of the digestive gland tubule is measured, which, when coupled with the visualization of chloroplasts as objects inside this space, allows the accurate quantification of functional chloroplasts (Chapters 2,4 and 5).

The advent of this method has enriched our understanding of functional kleptoplasty by revealing significant differences amongst different sacoglossan species as well as different patterns of chloroplast decline in these tissues. In unstarved animals, the long-term plastid retaining species *E. timida* contains a significantly higher plastid abundance than the short-term retaining species *E. cornigera, T. hopei* and short-/long-term retaining *E. viridis* (depending on the study (Baumgartner et al. 2015; Katharina Händeler et al. 2009)) (Chapters 2,4).

Interestingly, *E. timida* acclimatized to higher water temperatures (21°C, 25°C) had significantly lower functional chloroplast abundances than those cultured at 18°C suggesting that temperature may influence *E. timida's* ability to sequester chloroplasts (Chapter 4). Throughout starvation, *E. timida* at all investigated temperatures had higher functional plastid abundances than short-term retention forms, which may indicate that the amount of functional plastids sequestered influences a slug's starvation longevity.

In each of the species where functional chloroplast abundance was recorded, the decrease in plastid abundance did not reflect the decrease in their PAM values. Whereas PAM value decrease was always best-modeled by a linear function, plastid abundance was always best-modeled by a negative power or exponential function, having a sharp decrease in abundance followed by a gradual decrease. This demonstrates the difference in results acquired by each method and confirms that photosynthetic efficiency measurements do not reflect the functional chloroplast abundance. Due to this discrepancy and the significant difference in plastid abundance amongst long- and short-term retaining species, the actual kleptoplast abundance should be assessed in future investigations into functional kleptoplasty (Figure 8.1B).

8.3 Digestive Activity and Plastid Excretion

One of the largest unresolved queries regarding functional kleptoplasty asks why chloroplasts are not digested when they are endocytosed into the digestive gland tubule cell and if/when they are later digested. Most of the experiments presented in this dissertation address this question by examining digestive activity in sacoglossan slugs. They was accomplished after numerous attempts, by measuring lysosomal abundance – an indicator of intracellular digestion - using the fluorescent stain acridine orange, which aggregates in extremely acidic cellular compartments, namely lysosomes (Chapter 2). By uncovering different trends in lysosomal activity for different slug species, ages and temperature treatments, numerous hypotheses regarding functional kleptoplasty now have empirical support.

Long-term plastid retaining species *E. timida* displayed significantly different lysosomal activity within the digestive gland during starvation when compared to short-term retaining species *E. cornigera* and *T. hopei* as well as the ambiguous long/short-term species *E. viridis* (depending on the investigation (Baumgartner et al. 2015; Katharina Händeler et al. 2009)). Each of the short-term retention species display a slow, linear rise in lysosomal activity inside the digestive gland (related to digestion), which occurs steadily throughout the starvation period. Contrastingly, each of the *E. timida* populations surveyed had larger increases in the first half of the starvation period followed by a significantly steeper increase in the second half (Figure 8.1C) (Chapters 2,4). The significant difference in lysosomal activity during the second half of the starvation period indicates a change in the digestive gland cellular environment that spurs digestive activity. The detection was made possible through these experiments, however the nature of this change remains unresolved.

Lysosomal activity outside the digestive gland indicates autophagy and while some autophagy is expected regardless of whether the slug is feeding normally or in starvation, the average rate of autophagy in unstarved specimens increases when the slug enters starvation. This was true for all of the slug species surveyed and indicates an increase in starvation related autophagy. This increase was significant but marginal (0.25%) in each of the *E. timida* and *E. viridis* populations investigated and did not correlate to temperature. *E. cornigera* and *E. timida* displayed significantly different autophagal responses in starvation. While *E. timida* barely revealed signs of autophagy, even late in the starvation period, *E. cornigera* had exponential increases in non-digestive gland lysosomal activity, suggesting *E. cornigera* directly employs autophagy as means to meet its metabolic demands. On the contrary, *E. timida* does not directly engage in autophagal processes, instead subduing this cellular machinery until later in the starvation period when it is moderately performed (Figure 8.1D).

Examining lysosomal activity in sacoglossan slugs furthers our understanding of the mechanisms behind functional kleptoplasty by revealing that short- and long-term plastid retaining species have differences in the digestive gland tubule cellular environment that facilitate this ability. Direct evidence of

plastid digestion and the ability to regulate digestive activity were shown for *E. timida* throughout the starvation period. These results build on conclusions from previous examinations. TEM investigations prior to those presented here, suggested plastids are digested since they reveal fragmented plastids. This cannot be confirmed since these kleptoplasts could also be degrading due to reasons unrelated to slug digestion, i.e. a lack of repair proteins inside the slug that causes their deterioration (Martin et al. 2013; Schmitt and Waegele 2011). The strong correlation between lysosomal abundance and plastid abundance/degradation does not imply causation, but does support the theory that chloroplasts can be digested in *E. timida* (Chapter 2). This is further supported by two experiments presented here: 1) the lack of functional plastids in their excrement (detailed in Chapter 4.3.5), which implies functional chloroplasts do not leave the digestive gland once incorporated and 2) the TEM investigations conducted here, which detail plastid breakdown throughout the starvation period (Chapter 5).

Short-term plastid retaining species *E. cornigera*, *E. viridis* and *T. hopei* do not reveal the same results, instead displaying a slow increase in digestive activity throughout their shorter starvation periods (Chapters 2 and 4). This, coupled with an abundance of functional chloroplasts in *E. cornigera* excrement throughout the starvation period reveal that *E. cornigera* do not digest all of the chloroplasts they ingest (Figure 8.1F) (Chapter 4). The ability to digest chloroplasts, may be precisely what allows *E. timida* to survive extended starvation and prevents the short-term species from extended viability when food is not available. While the actual mechanisms and reasons for the differences in their cytological responses are still unresolved, the investigations presented here prove for the first time, that these species have different physiological reactions within their digestive gland cells, to starvation.

8.4 Photosynthate Production

Numerous investigations have examined the ways in which kleptoplasts help a starving slug withstand starvation. These investigations have revealed algal-

derived carbon in multiple slug-produced compounds indicating a transfer of fixed carbon from the chloroplast to the slug. This is confirmed as photosynthetically fixed carbon by dark experiments, in which photosynthesis cannot function. The carbon that is fixed by slugs in darkness can be attributed to heterotrophic carbon fixation that, when subtracted from the total light fixed carbon, reveals the amount of carbon that is photosynthetically fixed. The presence of photosynthetically derived carbon in slug-produced compounds confirms the hypothesis that slugs receive energetic benefits from incorporating chloroplasts, which may be allowing them to survive starvation.

Despite this conclusion, two hypotheses remain for when the slug actually receives these benefits: 1) ongoing photosynthesis produces photosynthates that are immediately transported to the slug, or 2) the plastids produce photosynthates which accumulate in the plastid until it's eventual breakdown, when they are then made available to the slug (detailed in Chapter 3.1, 5.1). The investigations into photosynthate production presented in this dissertation do not reveal starch production in *E. cornigera,* which does not fit either of these hypotheses. These investigations do support the larder hypothesis for *E. timida* though. Chapter 3 details the accumulation and subsequent loss of starch, within the kleptoplasts sequestered by the long-term starvation-surviving species *E. timida* (Figure 8.1E). This accumulation cannot be due to slug metabolism since metazoans do not utilize starch as an energy storage molecule, meaning the increase in starch observed throughout the first half of the starvation period comes from photosynthesis. The subsequent decrease in starch concentration within these tissues during the second half of the starvation period correlates to the increased lysosomal abundance and therefore increase in digestive activity observed in Chapter 2. This correlation suggests that *E. timida* only gain access to starch in the second half of the starvation period when the chloroplasts themselves are digested (Chapter 8.2E). The delayed access to these photosynthates is likely what sustains them throughout the end of the starvation period when photosynthesis itself is no longer occurring. This conclusion is substantiated by transcriptomic data that shows a downregulation in genes related to *E. timida* metabolism at the beginning of the starvation period, followed by upregulation of metabolic genes later in

starvation (de Vries et al. 2015). The conclusions presented here do not, however, negate the first hypothesis, that photosynthates may also be continuously made available to the slug. Further research is needed to determine the quantities of photosynthates that are directly made available to the slug, versus those that are accumulated as starch and only digested later.

8.5 Juvenile Development

Another aspect of functional kleptoplasty that was explored in this dissertation concerns the development of functional kleptoplasty by each generation, within the long-term plastid retaining species *E. timida.* Previous investigations confirmed that juvenile *E. timida* are incapable of withstanding starvation, even after ingesting algal cell contents and that there is no vertical transmission of chloroplasts from parent to offspring (Rahat 1976; Schmitt et al. 2014; Robert K Trench 1969; Robert K Trench et al. 1969). Despite this, nothing was known about how *E. timida* juveniles develop the ability to sequester plastids and withstand starvation prior to the investigations detailed in Chapter 6. By examining juveniles at different ages regarding chloroplast abundance/digestion, lysosomal activity and starvation longevity, some aspects of the transition from direct digestion to functional kleptoplasty in *E. timida* juveniles are now resolved. Although still morphologically immature, juveniles between 15 and 25 days post-metamorphosis gain the ability to sequester chloroplasts. They are able to maintain chloroplasts inside their digestive gland tubules for almost a month, outlasting even some short-term retention species. Juvenile digestive activity trends change from uniform lysosomal abundance to the same pattern observed in adult specimens, an exponential increase in lysosomal abundance throughout the starvation period. Although the genetic mechanisms behind these developmental changes remain unresolved, the experiments conducted in Chapter 6 identify cytological differences between adult and juvenile animals that may form the basis for future inquiries into the gene regulation involved in the development of functional kleptoplasty.

8.6 Conclusions

The studies presented here examine numerous aspects of functional kleptoplasty, primarily in the model-species *Elysia timida* and *E. cornigera* and the results presented redefine our understanding of how this phenomenon occurs within these species. Previous investigations regarding *E. timida* have examined factors involved in functional kleptoplasty that do not differ between these species, such as its algal food preference, body plan and feeding anatomy as well as factors that distinguish these species from one another and may support long-term kleptoplasty in *E. timida* (Krug et al. 2012). Distinguishing characters include: the ability to withstand starvation for up to a month in *E. cornigera* and almost four months in *E. timida*, different metabolic gene transcription patterns during early starvation, an accumulation of reactive oxygen species in starving *E. cornigera* digestive gland tubules that are lacking in starving *E. timida*, differing decreases in the rates of photosynthetic efficiency during starvation, and pigment concentration (de Vries et al. 2015; Katharina Händeler et al. 2009; Schmitt et al. 2014).

The investigations presented in this dissertation examine numerous other factors, focusing on differences in the digestive gland tubule cellular environment, which impact each species' ability to perform functional kleptoplasty. By discovering starch accumulation within sequestered kleptoplasts in *E. timida*, a significant benefit to plastid retention has been confirmed and the lack of this benefit in *E. cornigera* reveals a possible explanation for the difference in their starvation longevities. The quantification of functional chloroplasts reveals over twice as many functional plastids in *E. timida,* indicating that any benefits received from kleptoplasts are doubled for this species. Monitoring digestive activity outside the digestive system uncovers high rates of autophagy in *E. cornigera* and very low autophagal activity in *E. timida.* Inside the digestive gland tubule, digestive activity also differs greatly with *E. cornigera* displaying only a slight increase in lysosomal activity, that when coupled with the abundance of functional plastids found in its excrement, suggest *E. cornigera* do not digest chloroplasts at all, while *E. timida* do. These factors in combination reveal that *E. timida* and *E.*

cornigera are less similar than previously thought and the understanding of functional kleptoplasty lies within their differences.

8.7 Outlook

Finding factors that unify long-term plastid retaining species and could therefore explain extended plastid retention is difficult since these taxa are different in almost every way they have been examined. Long-term species have been recorded in every temperate and tropical sea across a range of ecosystems, each of which has completely different abiotic factors. They can be stenophagous or polyphagous, cerata-bearing or parapodia-bearing. Many of the experiments previously conducted to assess these aspects have reached differing conclusions. None of the confirmed long-term retention species are closely related, so this ability likely evolved multiple times, each time potentially employing novel mechanisms to facilitate this ability. Furthermore, the algal species ingested can determine the plastid retention capacity within even one slug species. It is due to this complexity that model-systems become of utmost importance.

Despite the numerous advances in understanding functional kleptoplasty presented by this dissertation, many aspects of this phenomenon remain unclear for the *E. timida / E. cornigera* model system. In Chapter 4, examining functional kleptoplast abundance revealed twice as many chloroplasts in *E. timida* as in *E. cornigera* but how *E. timida* incorporates so many more chloroplasts remains unclear. Why *E. cornigera* excretes functional plastids while *E. timida* retains them, when fed the same algal culture, is also unresolved. Chapter 5 details starch accumulation in *E. timida* kleptoplasts although plastids from the same algal culture do not produce starch in *E. cornigera* and no obvious reason supports this finding. Differences in metabolic gene regulation, autophagy and digestive activity also lack clear explanation, serving to distinguish these species but not explain why they differ. These unresolved queries demonstrate that although progress has been made in understanding functional kleptoplasty for this model-system by identifying individual components that differ amongst these species, the larger

picture of how functional kleptoplasty functions in these species has yet to be uncovered.

While examining functional kleptoplasty in *E. timida* and *E. cornigera* does not address the diversity or complexity of this ability throughout the Sacoglossa, it does provide a starting point. The studies presented in this dissertation reveal new methods that can now be applied to species outside this model-system including the limapontioidean species, *Costasiella ocellifera* (long-term plastid retaining) and *Costasiella nonatoi* (non-plastid retaining), which inhabit the same environment and feed on the same algae. These methods can also be applied to polyphagous species such as *E. viridis* and *P. ocellatus*, whose photosynthetic efficiency and longevity in starvation depend on the algal species ingested and have not been examined regarding digestive activity. Expanding investigations of functional kleptoplast abundance, activity, and excretion as well as digestive activity throughout the Sacoglossa may serve to help understand the "big picture" behind functional kleptoplasty, revealing how it likely evolved and how it functions inside these solar-powered animals.

Chapter 9

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