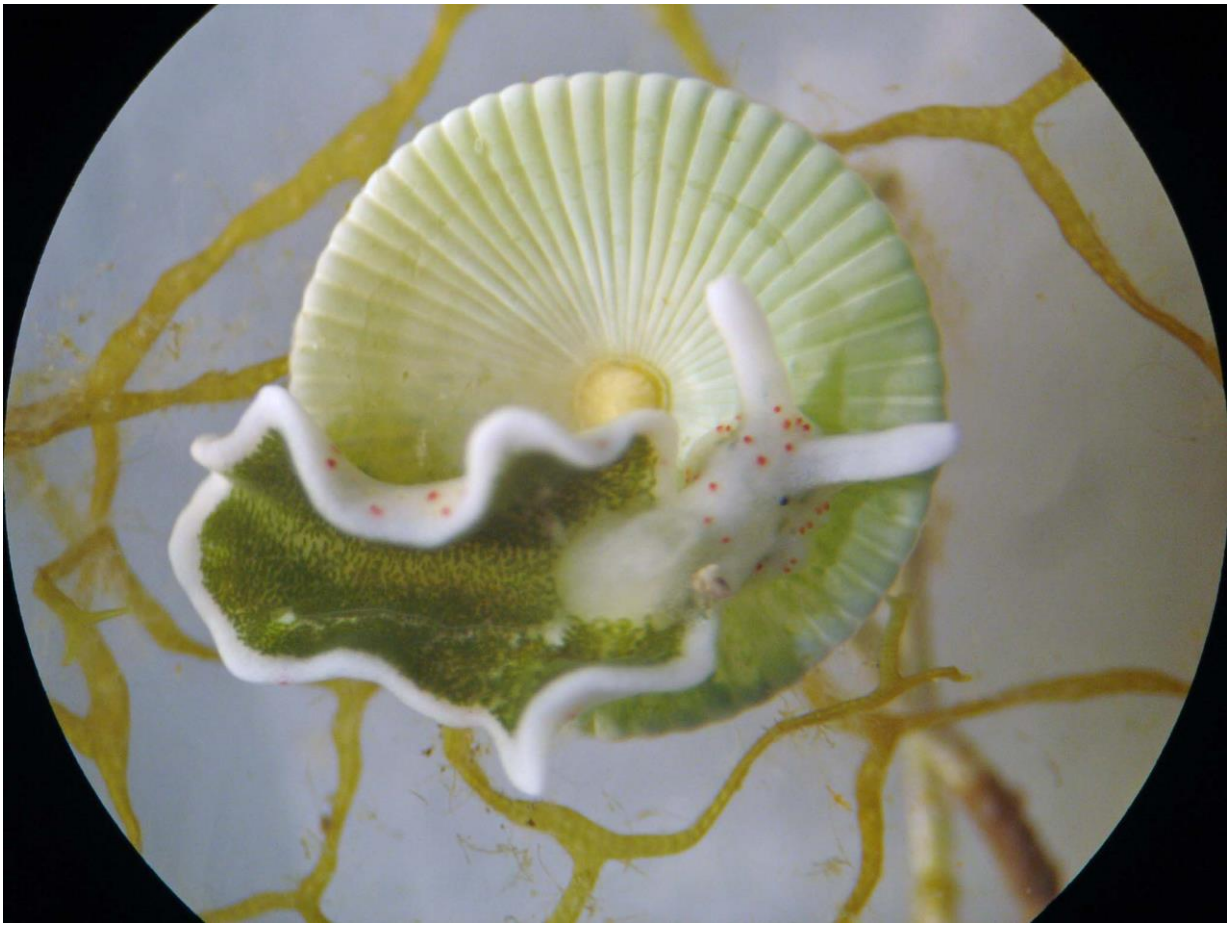
The Phenomenon of Functional Retention of Incorporated Chloroplasts in Sea Slugs (Sacoglossa, Heterobranchia, Mollusca) and Evolutionary Adaptations

Aspects of Photobiology, Cell Biology, Ecology and Behavior



Valérie Schmitt

The Phenomenon of Functional Retention of Incorporated Chloroplasts in Sea Slugs (Sacoglossa, Heterobranchia, Mollusca) and Evolutionary Adaptations

Aspects of Photobiology, Cell Biology, Ecology and Behavior

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Valérie Schmitt

aus

Münster

Bonn Juli 2019

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Gutachter: Prof. Dr. Heike Wägele

2. Gutachter: Prof. Dr. Thomas Bartolomaeus

Tag der Promotion: 05.12.2019

Erscheinungsjahr: 2020

Contents

Summary

The aim of this Ph.D. thesis was to investigate the phenomenon of chloroplast incorporation or 'kleptoplasty' in sacoglossan sea slugs (Sacoglossa, Heterobranchia, Mollusca) and related adaptations – concerning aspects of photobiology, cell biology, ecology and behavior.
The phenomenon of retention of functional chloroplasts from their food algae in sacoglossan sea slugs is a challenge for research, still leaving many questions unsolved, e. g. concerning differences between various sacoglossan species in their capacities of retention of functional chloroplasts. To investigate these differences and potential influencing factors in more detail, this thesis combined photobiological investigations with cell biological, ecological and behavioral analyses. Photosynthetic activity, i. e. the ongoing functioning of incorporated chloroplasts within the slug cells, was analyzed with a Pulse Amplitude Modulated Fluorometer (PAM), an established method to investigate photosynthetic activity in sea slugs. The analyses included several chloroplast-incorporating sea slug species, especially most of the few which are known as "top-performers" of long-term functional retention of chloroplasts: *Elysia timida, Elysia crispata* (*mangrove type* and *reef type*)*, Elysia viridis* and *Plakobranchus ocellatus*. As further comparative species, *Bosellia mimetica, Thuridilla hopei* and *Placida dendritica* were included.
Overall, relevant differences between species-specific spectra of photosynthetic capacities in various sacoglossan sea slug species could be confirmed and also considerable variation within. For *P. ocellatus*, PAM measurements of functional chloroplast retention for over seven months were reported, the longest time period documented up to now. Also, variations in capacities of long-term kleptoplast retention between the two eco-morphotypes *E. crispata mangrove type* and *reef type* could be found. Capacities of kleptoplast retention in *E. viridis* were shorter than expected in relation to former reports and varied strongly in different populations with varying habitats and substrates. Feeding experiments indicated that in addition to its known food algae *Codium fragile*, *E. viridis* also fed on and could incorporate chloroplasts from *Flabellia petiolata*, with even partly better capacities of chloroplast retention than with *C. fragile/vermilara.* Furthermore, several algal chloroplast donors could be confirmed for several sacoglossan species, and be documented with regard to the connected photosynthetic activity and capacity of long-term retention. For *B. mimetica*, better long-term kleptoplast retention capabilities than expected could be documented.
Also within the frame of species-specific spectra, the photosynthetic capacities of integrated chloroplasts displayed variations, which in free-living sea slugs can potentially be influenced

by various factors as season, temperature, food availability, light conditions and further environmental parameters as well as age, size and overall condition of the individuals. Photobehavioral analyses, testing a former hypothesis that chloroplast-retention potentially implies stronger phototactic behavior in sea slugs, in which the non-sacoglossan sea slug species *Cratena peregrina* and *Flabellina affinis* were additionally included as comparison without incorporation of chloroplasts, indicated a different coherence of photobehavior, as some sea slug species without chloroplasts or with rather fast digestion of chloroplasts reacted stronger positively phototactic than species with long-term kleptoplast retention. For *E. timida*, nevertheless a positive phototactic behavior could be observed, which might be connected to special adaptations in this species. With PAM-measurements could be demonstrated e. g. the efficiency to regulate fluorescence F emission of incorporated chloroplasts by varying opening and closing positions of the parapodial lobes in *E. timida*. Furthermore, effects of temperature on capacities of long-term photosynthetic activity were indicated by experimental trials under controlled laboratory conditions. The advantages of the laboratory culture system with *E. timida* as a model organism that could be successfully established could be revealed. In trials within the laboratory culture system, the capacity of *E. timida* to acquire kleptoplasts from another chloroplast donor – the alga *Acetabularia peniculus* – with similar retention capacities compared to their food alga *Acetabularia acetabulum*, could be demonstrated. On a cell biological level, indices for factors concerning special adaptations in relation to incorporation of chloroplasts were elucidated with transmission electron microscopy (TEM). The very first uptake of chloroplasts from the food alga *A. acetabulum* in juvenile *E. timida* could be illustrated with TEM. Furthermore, ecological parameters in the natural environment, especially concerning light conditions, could be demonstrated to affect photosynthetic activity of incorporated chloroplasts, which constituted the first demonstration of this kind. Two sea slug species, *E. timida* and *E. crispata mangrove type*, were investigated underwater with a Diving PAM Fluorometer in their natural habitat in France and in Florida, respectively, concerning kleptoplast photosynthetic activity and combined environmental and behavioral parameters. Distinct differences between the two sea slug species were found concerning habitat, environmental parameters and photosynthetic activities. Photosynthetic activities in both sea slug species and in the food algae of *E. timida, A. acetabulum,* varied in relation to natural light conditions in the sea. These represent to the current knowledge the first photosynthetic measurements of incorporated chloroplasts in sacoglossan sea slugs in their natural environment published so far.

Preface

"The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land. "

T. H. Huxley 1887 (cited from Carl Sagan's book "Cosmos" (1980), First Ballantine Books edition 1985, p.1 (Sagan 1980))

The quest of this Ph.D. thesis was to contribute to the knowledge about the fascinating, yet still enigmatic, phenomenon of functional chloroplast incorporation in 'photosynthetic sea slugs'.

Ever since I first encountered sea slugs I was fascinated by these wonderful living beings and the same accounts to the fascination about endosymbiosis since I first learned about it, and which grew especially with the getting to know organisms in which 'animal' and 'plant' characters are combined, like e. g. *Euglena*. As I experienced that in sacoglossan sea slugs, these fascinating subjects merge together, I could combine these interests in this Ph.D. thesis, and I would have liked to go more deeply into the research of these fascinating subjects, but limitations were posed mainly by the constraints due to most of the time working in parallel to earn my living.

As this Ph.D. thesis was in large part carried out in parallel to working to earn my living, this meant a permanent double challenge during many years. But I am grateful for all that this Ph.D. thesis and the support and grants I received temporarily have given me – so many fascinating impressions and experiences during my work, while diving in the sea and meeting all those fascinating sea creatures, while looking with a stereomicroscope at these splendid sea slugs, observing their different states during their life circles from eggs to whirling veliger larvae to juveniles and adults, fascinating insights into their cells with incorporated chloroplasts, and along with it the people I had the pleasure to meet in connection with my work, in international science projects in Banyuls-sur-Mer, France, and Florida Keys, USA, and of course also in Germany – all these were precious and wonderful experiences for me that I am deeply grateful for, and to many of these people and institutions I am still connected in gratefulness. (Please see also Acknowledgements.)

One thing I regret is the death of every little sea slug dying in the course of the experiments – though they have in general short natural life spans which might in some cases even be shorter in nature as in the laboratory. As far as I could regulate it, I tried to take care to keep the losses as low as possible, e. g. always collect only a small part of individuals of a species that I spotted at a collection place. When I came back to those sites later I always was happy seeing still the same sea slug species crawling around.

Sea slugs are to my mind among the most fascinating living beings and research objects on this planet. These "butterflies of the sea" or "leaves that crawl" seem to be magic, like little elves of the ocean while at the same time representing scientific phenomena.

For me they symbolize in small the beauty, magic and vulnerability of all nature. Thus I hope that contributing to exploring these fascinating creatures and gaining more understanding of their complex and astonishing nature may also enhance the understanding of nature as a whole and the consciousness of the preciousness of our wonderful planet and the need to preserve it.

1 Introduction

"We are symbionts on a symbiotic planet, and if we care to, we can find symbiosis everywhere."

Lynn Margulis in "Symbiotic Planet – A new look at evolution" (1998), Science Masters Series, Basic Books, Sciencewriters, Amherst, Massachusetts, p. 5 (Margulis 1998)

1.1 The phenomenon of chloroplast incorporation in sea slugs and the aims of this Ph.D. thesis

Among the splendid diversity of various forms and colors developed in sea slugs, a special enigmatic phenomenon has evolved in the group of Sacoglossa (Heterobranchia, Mollusca). This phenomenon of 'chloroplast endosymbiosis' or 'kleptoplasty' is the retention of intact and functional chloroplasts from food algae with ongoing photosynthesis inside the slugs' cells and is among metazoans uniquely known from sacoglossan sea slugs (while otherwise known in some marine protists) (Wägele and Johnsen 2001, Händeler, Grzymbowski et al. 2009, Pelletreau, Bhattacharya et al. 2011, Wägele and Martin 2013, Christa, Händeler et al. 2014, Wade and Sherwood 2018). As Rumpho et al. summarize it accurately, some other metazoans in the phyla Mollusca (giant clams, nudibranchs), Porifera (sponges), Cnidaria (corals, anemones and hydra), Acoelomorpha (flatworms) and Chordata (ascidians) are known to have evolved photosynthetic endosymbiosis, but they form symbiotic associations with whole intact unicellular algae or cyanobacteria (Rumpho, Pelletreau et al. 2011) (and references therein). In contrast to this, sacoglossan sea slugs preserve instead of whole organisms only the single organelles, the chloroplasts, out of the algal content that they ingest by their name-giving, characteristic, sucking way of feeding, and incorporate these 'selected' chloroplasts intact and functional into their body and cells (Wägele and Johnsen 2001, Händeler, Grzymbowski et al. 2009, Pelletreau, Bhattacharya et al. 2011, Rumpho, Pelletreau et al. 2011, Wägele and Martin 2013, Christa, Händeler et al. 2014, Christa, Händeler et al. 2015, Laetz, Moris et al. 2017, Laetz and Wägele 2018a). Thus, it is not exactly a real symbiosis after the classic traditional definition of symbiosis, e. g. as "system in which members of different species live in physical contact" or "the living together of differently named organisms" like Lynn Margulis cites it in her book 'Symbiotic Planet' (the second after the definition by Anton deBary who she describes to have coined the term 'symbiosis' in

1873) (Margulis 1998). But it could potentially be regarded as having parallels to the original endosymbiotic process of chloroplasts described also by her. Viera et al. refer to the nomenclature of the term 'kleptoplasty' by Clark et al. that denotes specifically the ability of sacoglossans to ‘‘borrow’’ or ‘‘steal’’ chloroplasts (Vieira, Calado et al. 2009) (reference therein: (Clark, Jensen et al. 1990)). This phenomenon of incorporation of functional chloroplasts in sacoglossan sea slugs is a fascinating and challenging research topic with many questions still unsolved – especially due to the evolution of this specific phenomenon, underlying functioning mechanisms and differences between various sea slugs species in their capabilities for chloroplast retention (Wägele and Martin 2013, Cruz, Cartaxana et al. 2015, Chan, Vaysberg et al. 2018, Melo Clavijo, Donath et al. 2018).

The capability for chloroplast retention varies enormously between different species within the sacoglossan sea slugs, from fast digestion over retention for weeks up to several months, and only a few species are known to retain functional chloroplasts over longer periods (Evertsen, Burghardt et al. 2007, Händeler, Grzymbowski et al. 2009, Pelletreau, Bhattacharya et al. 2011, Rumpho, Pelletreau et al. 2011, Wägele and Martin 2013, Christa, Händeler et al. 2015). With the aim to investigate these differences and potential influencing factors in more detail, this Ph.D. thesis explores several aspects concerning photobiology, cell biology, ecology and behavior, with a focus on sacoglossan species with high potential of functional chloroplast retention. Among the few sacoglossan species known to be capable of extended periods of functional chloroplast retention, one species with the longest known durations of kleptoplast retention is *Elysia chlorotica* Gould, 1870 (Gould 1870), which has already been intensively studied by Rumpho, Pelletreau et al., also in a laboratory culture system (Rumpho, Pelletreau et al. 2011, Pelletreau, Worful et al. 2012). *Plakobranchus ocellatus* van Hasselt, 1824 (Hasselt 1824) is another sacoglossan species that was found to be able to store chloroplasts functional for extended durations and thus was estimated in the comprehensive categorization by Händeler et al. as a long-term retention form (Händeler, Grzymbowski et al. 2009). Reports on *P. ocellatus* were limited, however, thus it was included in several investigations in this thesis as one example for a sacoglossan species with longest durations of functional chloroplast retention (Figure 1.1.1), with the aim to explore and document its full retention potential and as comparison to species with lower capacities of kleptoplasty. In the same categorization, out of several sacoglossan species (including members of Oxynoacea and Plakobranchacea), only two other species were classified as long-term retention species, *Elysia timida* Risso 1818 (Risso 1818) (Figure 1.1.2) and *Elysia*

crispata, Mörch 1963 (MolluscaBase 2019c) (Figure 1.1.3), both also from the same group as *P. ocellatus*, the Plakobranchoidea (Händeler, Grzymbowski et al. 2009).

Figure 1.1.1: Sacoglossan sea slug species with one of the longest durations of long-term retention of kleptoplasts: *Plakobranchus ocellatus*. Inside the wing-like parapodial lobes folded together over the body, green lamella are visible, indicating with their green color the storing of chloroplasts.

Figure 1.1.2: Sacoglossan sea slug species with long-term retention of kleptoplasts: *Elysia timida*. This individual is touching the round rim of its food alga *Acetabularia acetabulum* (left) with the mouth part of its head to suck in the algal sap. Its wing-like parapodia are spread out like a leaf, exposing the incorporated chloroplasts.

Even if *E. timida* had been in the focus of research already since many years, as e. g. revised for three decades of research by Giménez-Casalduero et al. (Giménez-Casalduero, Muniain et al. 2011), it remained still an interesting research object due to many open questions concerning potential adaptations in relation to long-term kleptoplasty, e. g. concerning photobehavior and several further aspects, due to which it was included in several investigations in this thesis as explained in more detail below. Furthermore, it is a common Mediterranean species and was described to have a specific feeding preference for its food alga *Acetabularia acetabulum* Silva 1952 (Silva 1952) by Marin and Ros (Marín and Ros 1992, Marín and Ros 1993). *E. crispata*, on the contrary, was described to acquire kleptoplasts from several algal species (Curtis, Massey et al. 2005). Thus, with its inclusion, polyphagous feeding habits could be compared to a more narrow food spectrum in this thesis. Two differentiated morphotypes of *E. crispata* – a *mangrove type* and a *reef type* (Figure 1.1.3) corresponding to the description by Krug et al. (Krug, Vendetti et al. 2016) – were included in these investigations to examine potential differences.

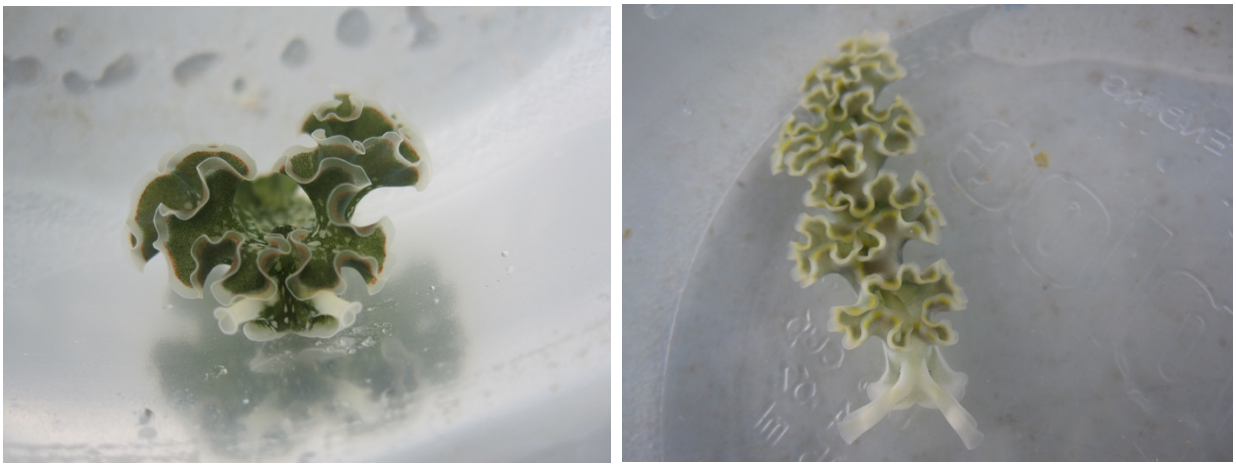


Figure 1.1.3: *Elysia crispata mangrove type* (left) and *reef type* (right). The darker morphotype on the left stemmed from shallow sand/mud grounds near mangroves with low currents, the brighter morphotype on the right stemmed from offshore reefs.

In contrast to the classification by Händeler et al. of *Elysia viridis* Montagu 1804 (updated from (MolluscaBase 2019e), reference therein as description of *Laplysia viridis*: (Montagu 1804)) as a short-term retention species (Händeler, Grzymbowski et al. 2009), Evertsen and Johnsen reported that in their observations, *E. viridis* was capable of long-term retention of chloroplasts from *Codium fragile* (Suringar) Hariot, 1889 (Hariot 1889) while *Placida dendritica* Alder und Hancock 1843 (Alder and Hancock 1843) digested those chloroplasts

rapidly (Evertsen and Johnsen 2009), thus the two species were also included in comparative analyses in this thesis (Figure 1.1.4).

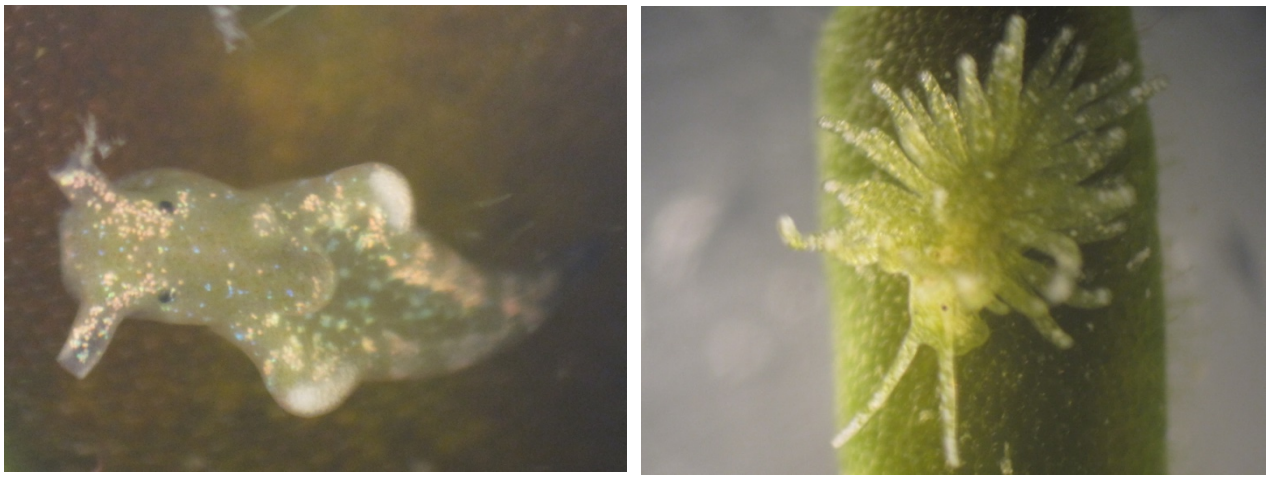


Figure 1.1.4: *Elysia viridis* (left) and *Placida dendritica* (right). Despite found on and feeding on the same food algae, *E. viridis* has longer chloroplast retention capability in contrast to *P. dendritica* with fast chloroplast digestion.

Also included were two further species which were classified as short-term retention forms by Händeler et al. (Händeler, Grzymbowski et al. 2009): *Boselllia mimetica* Trinchese 1890 ((MolluscaBase 2019a), reference therein: (Trinchese 1890)) and *Thuridilla hopei* Vérany 1853 ((MolluscaBase 2019g), reference therein: (Vérany 1853)) (Figures 1.1.5 and 1.1.6).

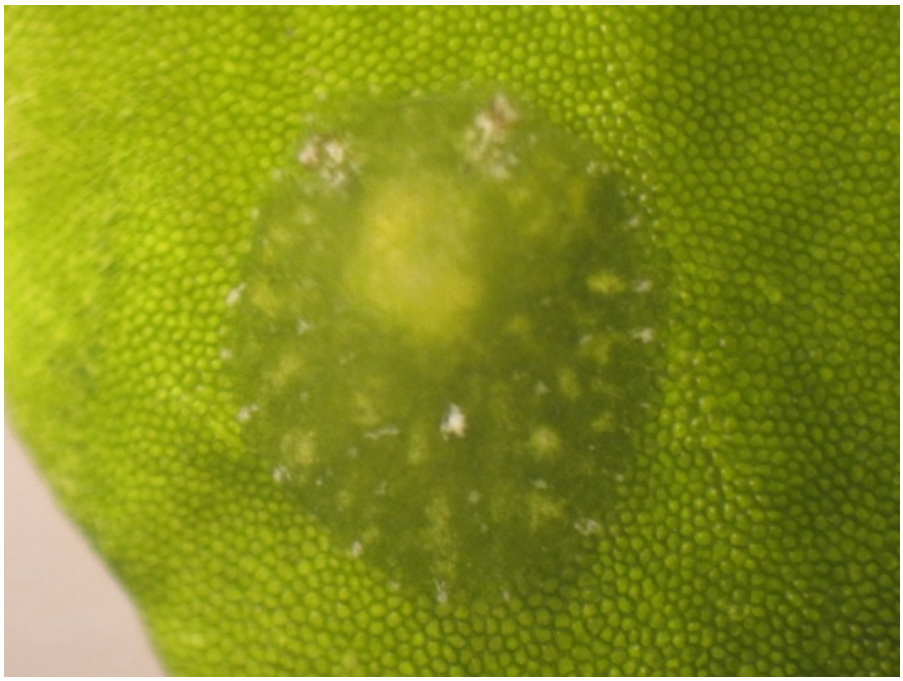


Figure 1.1.5: *Bosellia mimetica* (center) on its food algae *Halimeda tuna*. The round and flat body of the sea slug is hardly distinguishable from its algal underground, only by its different pattern emerging from the regular algal structure – representing clearly the perfectly camouflaging effect by the incorporation of green chloroplasts on green algal ground.

These two species were also included as comparison in the investigations of this thesis, concerning *B. mimetica* with the hypothesis that this species could reveal potentially longer chloroplast retention than expected (chapters 3.3 and 3.4) and concerning *T. hopei* as an exemplary species for fast digestion or short-term retention of chloroplasts (chapters 3.1, 3.3 and 3.4).

Figure 1.1.6: *Thuridilla hopei*. In the case of this species, the differing coloration mirrors the rather fast digestion of incorporated chloroplasts in contrast to long-term retention species.

Furthermore, an additional species which was found together with *E. viridis* in a small habitat of a tidal pool, *Ercolania viridis* (A. Costa, 1866) ((MolluscaBase 2019f), reference therein: (Costa 1866-1869)), was investigated concerning its potential of chloroplast incorporation in this thesis in a comparison of the two species living sympatrically (chapter 3.4) (Figure 1.1.7). As for *Er. viridis* the algal nutrition preferences were not known, this species was examined in feeding experiments, like also other included species, in combination with photobiological investigations and cell biological investigations with the aim to analyze various algal chloroplast donors and potential differences concerning incorporation of chloroplasts and connected photosynthetic capacities (chapter 3.4).

Figure 1.1.7: *Ercolania viridis*. Individuals of this small species were analyzed concerning their potential algal food source and comparatively analyzed with *Elysia viridis* living sympatrically in the same tidal pool.

Despite general extensive investigations of the phenomenon of incorporation of chloroplasts in sacoglossan seas slugs since its discovery (reviewed e. g. in (Wägele and Martin 2013)), many questions remain open, e. g. on a cell biological level, about the exact incorporation of chloroplasts into the slugs' body and cells and potential recognizable differences between various sea slugs species with different capacities of functional chloroplast retention. To address these questions, several investigations with transmission electron microscopy (TEM) were carried out in this thesis – among other investigations one aim was to document the very first uptake and incorporation of chloroplasts from its food algae *A. acetabulum* in juvenile *E. timida* (Schmitt, Händeler et al. 2014) (chapter 3.2 and further results 3.4).
For photobiological investigations in this thesis, analyses of photosynthetic activity of incorporated chloroplasts in the various investigated sea slug and algae species were performed with a Pulse Amplitude Modulated (PAM) Fluorometer. PAM-measurements of chlorophyll a fluorescence of photosystem II represent an established non-invasive method to measure in vivo photosynthetic activities of short- and long-term functionality of incorporated chloroplasts in sea slugs (Wägele and Johnsen 2001, Evertsen, Burghardt et al. 2007, Evertsen and Johnsen 2009, Händeler, Grzymbowski et al. 2009, Vieira, Calado et al. 2009, Jesus,

Ventura et al. 2010). Thus, analyses of photobiology were performed on short-term and long-term scales and combined in this thesis with cell biological analyses as well as behavioral and ecological analyses as explained in more detail in the following.

1.2 Photobehavior / behavioral adaptations

The behavior in sea slugs seems to be not as simple as could possibly be assumed – former own studies (diploma thesis) revealed an astonishingly complex behavior of *E. timida* in relation to mating (Schmitt, Anthes et al. 2007). The assumption seems probable, that in connection with the evolution of kleptoplasty also special behavioral adaptations are developed.

Specialized photobehavior in sea slugs was first reported by Fraenkel with a description of photomenotaxis in *E. viridis* (Fraenkel 1927). He described that he chose *E. viridis* out of many tested opisthobranch species to investigate photomenotaxis due to its fastest and clearest reaction, but did not name the other compared species.

Nearly 50 years later, during which research and knowledge about the phenomenon of incorporated chloroplasts in sacoglossan sea slugs had considerably increased, yet not concerning behavioral adaptations, Weaver and Clark compared five sacoglossan species with or without 'endosymbiotic' chloroplasts (Weaver and Clark 1981). Three 'chloroplast symbiotic' species with incorporated chloroplasts (*Elysia tuca* Marcus and Marcus 1967 (=*Elysia velutinus* Pruvot-Fol, 1947 after (MolluscaBase 2019d), reference therein: (Marcus and Marcus 1967)), *Costasiella lilianae (=Costasiella ocellifera* after Clark (Clark 1984)), and (*Tridachia/)E. crispata*) were compared versus the two 'aposymbiotic' species without chloroplasts *Oxynoe antillarum* Mörch and *Berthelinia carribbea* Edmunds with regard to photobehavior (Weaver and Clark 1981). Weaver and Clark reported orientation to the light source in the 'chloroplast symbiotic' species opposed to light avoidance of the 'aposymbiotic' species (Weaver and Clark 1981). From this, the assumption could be inferred that phototaxis would be potentially enhanced by the possession of incorporated chloroplasts.

To investigate this hypothesis more profoundly and also with taking the factor of longevity of incorporated chloroplasts into account, further analyses of phototactic behavior were performed in this Ph.D. thesis. In a first study, two sacoglossan species, *E. timida* with long-term retention of incorporated chloroplasts and *T. hopei* with only short-term retention or fast

digestion of chloroplasts, were compared concerning phototaxis and other potential behavioral adaptations (Schmitt and Wägele 2011) (chapter 3.1).
The results of that study seemed first to point into the same direction of the assumption indicated by the former studies (Weaver and Clark 1981), but up to that time point, only few species had been observed, and a broad overview over different species was still generally lacking. Thus, to gain an overview over a wider range of species, the investigations on phototaxis were extended with the inclusion of a broader spectrum of species comprising different forms of chloroplast retention. This included the above already described species with very long lasting chloroplast retention (*P. ocellatus*) over long-term or mediate chloroplast retention (*E. timida, E. crispata (mangrove type and a reef type), E. viridis, B. mimetica*) to short-term retention or fast digestion of chloroplasts (*T. hopei, P. dendritica*) plus two non-sacoglossan sea slug species, carnivore nudibranchs feeding on hydrozoans and correspondingly without sequestration of chloroplasts (*Cratena peregrina* and *Flabellina affinis*) (chapter 3.3).
Furthermore, interesting specialized behavioral adaptations are reported for *E. timida* in form of modifying the position of its parapodial lobes from closed to spread, opened leaf-like in relation to different light conditions, first described by Rahat and Monselise (Rahat and Monselise 1979, Monselise and Rahat 1980). Only few reports about this specialized behavior were available so far however. Thus, to investigate this behavior in more detail, behavioral observations of *E. timida* were performed in a semi-natural laboratory setting. The special photobehavior of *E. timida* was analyzed in relation to its capability for long-term retention of chloroplasts in comparison to *T. hopei*, as both are common Mediterranean species that live sympatrically, with a similar body size and structure with movable parapodia, and as the latter is a species with short-term retention or fast digestion of chloroplasts (Marin and Ros 1989, Händeler, Grzymbowski et al. 2009). The two species were comparatively analyzed concerning the behavior in form of the varying positions of the parapodia in relation to light conditions and photosynthetic activity, including e. g. fluorescence (Schmitt and Wägele 2011) (chapter 3.1). *E. timida* was additionally observed concerning its specialized behavior of regulating the exposure of incorporated chloroplasts by varying opening of the parapodia in its natural environment in the sea along with other investigations described in more detail below under ‘Investigations in near-natural and natural settings’ (chapter 3.3).

1.3 Laboratory culture system investigations – *Elysia timida* as a model organism

To investigate *E. timida* also under specialized controlled conditions, a laboratory culture system was established as described in chapter 3.2 (Schmitt, Händeler et al. 2014).
By the time we built up the laboratory culturing system of *E. timida*, most previous studies of sacoglossan sea slugs had been performed on individuals collected from the sea, implying an unknown history of the individuals before collection, concerning e. g. their age, the spectrum of algae they have fed or light conditions they have been exposed to (Schmitt, Händeler et al. 2014). One successful laboratory culture system had been established by Rumpho and coworkers, in which they kept *E. chlorotica* – another of the sacoglossan species with the longest durations of functional kleptoplasty as listed already above – and analyzed the complete life cycle of about ten months in detail (Rumpho, Pelletreau et al. 2011). By the investigations within their laboratory culture, they were also able to document an obligatory primary phase in juvenile *E. chlorotica* to feed on the food algae *Vaucheria litorea* for at least seven days to establish kleptoplasty (Pelletreau, Worful et al. 2012). In another study, Curtis and coworkers kept slugs hatched from egg masses laid in the laboratory by sea slugs of the formerly called sacoglossan species *Elysia clarki* Pierce, Curtis, Massey, Bass, Karl & Finney, 2006 (=*Elysia crispata* Mörch 1863 after (MolluscaBase 2019b), reference therein: (Pierce, Curtis et al. 2006)) (which should probably correspond to *E. crispata mangrove type* in this thesis as explained above), but only for some observations, not to establish a culture system (Curtis, Pierce et al. 2007).
We tested the possibility to establish a laboratory culture system with the sacoglossan species *E. timida*, which is especially suitable as a model organism for investigations of kleptoplasty due to several reasons. As a common Mediterranean species, it was described by Marin and Ros to live in a close relation to its food alga *A. acetabulum* from which it gains its kleptoplasts (Marín and Ros 1992, Marín and Ros 1993). As explained above, it is also one of the few species capable of long-term retention of chloroplasts with up to approximately three months of retaining kleptoplasts functional during starvation (Evertsen, Burghardt et al. 2007, Giménez Casalduero and Muniain 2008, Händeler, Grzymbowski et al. 2009). Furthermore, as described already above concerning photobehavior, *E. timida* reveals several potential specific adaptations to long-term functional kleptoplasty as positive phototaxis and the specialized regulation of the opening degree of the parapodia resulting in more or less exposure of the incorporated chloroplasts (Rahat and Monselise 1979, Monselise and Rahat

1980, Jesus, Ventura et al. 2010, Schmitt and Wägele 2011). An additional combination of the behavioral photo-regulation mechanism of opening or closing the parapodia with a physiological photo-regulation mechanism (xanthophyll cycle) is described to enhance maintenance of photosynthetic capacity for longer durations in *E. timida* (Jesus, Ventura et al. 2010). In all, in several regards *E. timida* provided an interesting potential for investigations as a laboratory model to examine long-term kleptoplasty. Furthermore, former own studies had given first preliminary hints of several characteristics of *E. timida* which could possibly be advantageous for culturing (Schmitt, Anthes et al. 2007). Thus, based on those former studies on the reproduction in *E. timida* (Schmitt, Anthes et al. 2007) and preliminary investigations, we built up a laboratory culture system with *E. timida* as a model organism for investigations concerning long-term kleptoplast retention and related parameters throughout the slugs' entire life cycle (Schmitt, Händeler et al. 2014) (chapter 3.2).

1.4 Investigations in near-natural and natural settings

In addition to investigations of sea slugs in the laboratory or in specialized laboratory cultures like described above, investigations on site in the sea and under near-natural conditions are essential, however, to gain knowledge about conditions of the sea slugs in their natural environment and ecological relations. Thus, this was in the focus of other investigations in this thesis that aimed especially at exploring real life parameters of photosynthetic sea slugs in their natural environment and under near-natural conditions. The intention was to gain data on the chloroplast retention from individuals directly after being collected from the sea, held in near-natural or semi-natural conditions, for longest durations possible, and combine those with behavioral (described above) and ecological investigations. As a part of these investigations, also *P. ocellatus* with one of the longest chloroplast retention potentials as described above was investigated. Even if the high potential for long-term chloroplast retention in *P. ocellatus* had been discovered before, actual observations and PAM-measurements had only been performed for restricted time periods and the full potential was only estimated (Evertsen, Burghardt et al. 2007, Händeler, Grzymbowski et al. 2009, Yamamoto, Hirano et al. 2013, Wade and Sherwood 2017). Thus, the investigations in the frame of this thesis aimed to present the longest time period of PAM-measurements of the photosynthetic activity of incorporated chloroplasts in *P. ocellatus* possible, potentially the whole retention duration or life span (without food supply) (chapter 3.3). Furthermore, as

depicted above, individuals of the species *E. crispata* were included in these investigations corresponding to the two differentiated morphotypes *mangrove type* and *reef type* described by Krug et al. (Krug, Vendetti et al. 2016). Individuals of the two types were compared concerning potential differences in photosynthetic activity of incorporated chloroplasts and other parameters, partly described also in the section with further, unpublished results (chapter 3.4). In all, a spectrum of sea slug species with different degrees of chloroplast retention from fast digestion to long-term retention were included in these investigations, among them most up to now described species with longest durations of long-term chloroplast retention as depicted above. The aim of these investigations was to gain an overview analysis concerning potential differences between different sea slug species with various capacities of chloroplast retention, including several parameters (chapters 3.3 and 3.4). As different algal diets and corresponding possible differences in chloroplast robustness could be affecting capacities of functional maintenance of incorporated chloroplasts in sea slugs, feeding experiments were carried out to achieve more fundamental information concerning feeding in the investigated species, for which some information was available but some still lacking or unclear (chapter 3.4). For *E. viridis* and *E. crispata*, different chloroplast donors as well as different natural habitats were analyzed comparatively.

Though photobiological analyses of sacoglossan sea slugs have been carried out extensively in laboratories as described above, observations in their natural environment are scarce. Monselise and Rahat reported observations concerning the photobiology – especially photobehavior – of *E. timida* in the sea (Monselise and Rahat 1980), but did not document photosynthetic activity of kleptoplasts by PAM, and in general photobiological observations on sacoglossan sea slugs in their natural environment are lacking. Thus, two sea slug species, *E. timida* and *E. crispata (mangrove type),* were investigated concerning photobiology, including ecological and behavioral parameters, underwater directly in the natural setting in their habitats in France and Florida, respectively, by diving with a Diving PAM Fluorometer. These investigations were performed with the aim to provide the first PAM-measurements of photosynthetic sea slugs in their natural environment (results chapter 3.3).

1.5 Aim and structure of this Ph.D. thesis in summary

The aim of this thesis was to investigate special aspects of adaptations in relation to the phenomenon of long-term chloroplast retention or kleptoplasty in sacoglossan sea slugs (Sacoglossa, Heterobranchia, Mollusca) – concerning aspects of photobiology, cell biology, ecology and behavior – especially with regard to differences between various sacoglossan species in capabilities of functional chloroplast retention and factors potentially influencing this capability. The introductive sections above explained the respective focus of the different investigations that are presented in form of two already published studies (chapters 3.1 and 3.2) (Schmitt and Wägele 2011, Schmitt, Händeler et al. 2014) and one further yet unpublished publication manuscript draft (chapter 3.3), all presented in the results sections and all performed as first author. The section "further results" (chapter 3.4) presents several further, until now unpublished, own investigations, including in the first part feeding experiments and long-term photobiological investigations by PAM-monitoring to analyze different single algal chloroplast donors and related photosynthetic activities in various sacoglossan sea slug species, and in the second part analyses with TEM that were performed in combination with the photobiological investigations. Two further publications as co-author, with participation by contribution of photobiological and cell biological investigations conducted in the frame of this Ph.D. thesis, are presented only as citations here (Wägele, Deusch et al. 2011, Martin, Hazkani-Covo et al. 2012); the Ph.D. thesis focusses exclusively on own investigations as first author.

2 Methods

2.1 General methods

Taxonomy nomenclature of sea slugs and algae species that were included in the analyses was checked with "WORMS – World Register Of Marine Species" (www.marinespecies.org) and the connected "MolluscaBase", e. g. concerning species acceptance status, taxonomic citation and original description references, as well as with literature and partly depicted with correction remarks, respectively. Sacoglossan sea slugs species included in the analyses are depicted with author and year of description for taxonomic reference.

The sea slugs investigated in this Ph.D. thesis were in large part collected by the author during research stays at the Observatoire Océanologique at Banyuls-sur-Mer (OOB), France, and the Marine Mote Lab (MML), field station Summerland Key, Florida, USA, while diving or from algae samples in the laboratory. Furthermore, individuals of the *Elysia crispata reef type* were kindly provided by a professional collector from a reef offshore Key West, Florida, USA. Individuals of *Plakobranchus ocellatus* were provided by an aquarium specialist (Frank Richter Meerwasseraquaristik, Chemnitz, Germany) from collections near Cebu, Philippines, and then kept at the Institute for Molecular Evolution (IME), Heinrich-Heine-University of Düsseldorf, Germany, where also the laboratory culture of *Elysia timida* was established.

Photobiological investigations were performed after the methods established for sea slugs by Wägele and Johnsen (Wägele and Johnsen 2001) with a Pulse Amplitude Modulated Fluorometer (Diving PAM Fluorometer or Photosynthesis Yield Analyzer Mini PAM, version 2.0, both WALZ, Germany) (Heinz Walz GmbH 1998) in the laboratory and in the sea (Figure 2.1.1).

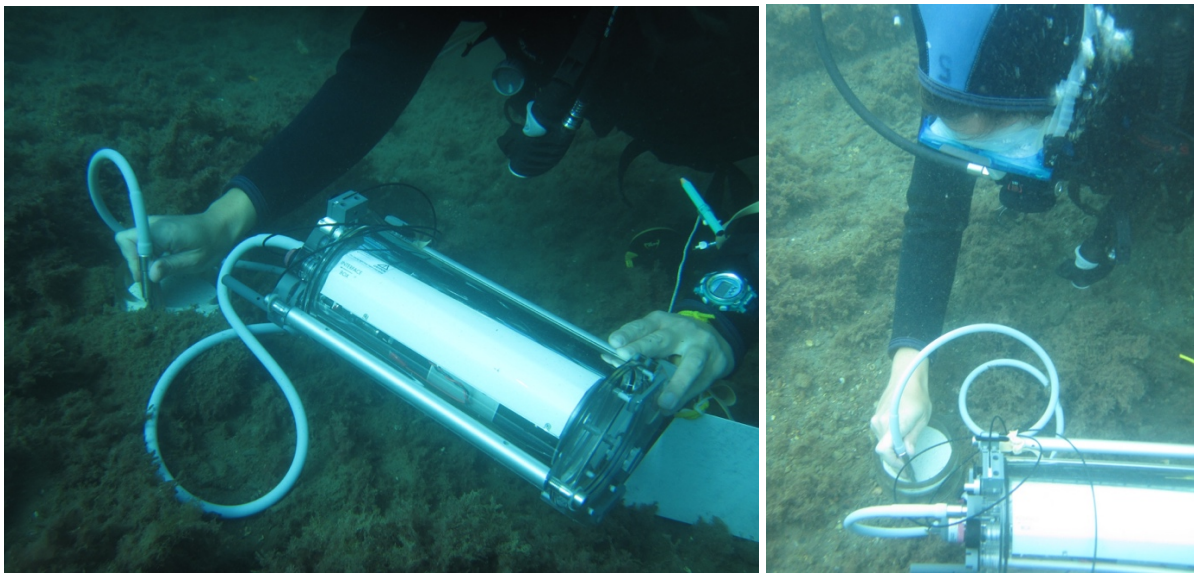


Figure 2.1.1: Underwater investigations of sea slugs in their natural environment with a Diving PAM.

These included measurements of light conditions with an integrated light sensor of the Diving PAM Fluorometer or with a light sensor connected to the Mini-PAM (US-SQS/L, Walz, Germany), measured in PAR (quantum flux density of photosynthetically active radiation, [µmol quanta m^{-2} s^{-1}]) (Heinz Walz GmbH 1998).

Investigations with transmission electron microscopy (TEM) were performed in the first part in collaboration with Rainer Martin at the University of Ulm and in the following at the OOB in the frame of the ASSEMBLE program in collaboration with the technical assistant Marie-Line Escande.

Statistical background to 'choose and use statistics' was based on Dytham's guide (Dytham 2003).

For details, see the methods section in the respective publication / manuscript; in the following, additional methodical notes concerning the further results section are depicted.

2.2 Additional method notes concerning the further results section

2.2.1 Collections and maintenance of sea slugs

Additional method notes on chapter 3.4 are depicted here, as concerning the yet unpublished results presented in this doctoral thesis, a part is presented in form of a publication manuscript with an included method description in chapter 3.3 and another part is presented separately as further unpublished results in chapter 3.4. The chapters 3.3 and 3.4 present both selections of investigations performed during several years, thus the methods are in big part overlapping. The Mediterranean sea slug species included in the investigations in chapter 3.4, *Elysia timida, Elysia viridis, Ercolania viridis, Placida dendritica, Bosellia mimetica* and *Thuridilla hopei,* were investigated at the OOB at Banyuls-sur-Mer, France, in April-September 2010, June-September 2011 and August-October 2012, in major part in the frame of the ASSEMBLE program. Collections were performed either directly by diving or indirectly from collected predefined algae species in the laboratory, in part kindly provided by the divers of the laboratory. Single individuals of *E. viridis* and *Er. viridis* were additionally collected from the special small habitat of a tidal pool in the rocks close to the OOB in April/May 2010 for a

comparative analysis. The individuals of the different species and habitats which were included in the various analyses during the several research stays are displayed in the results in chapter 3.4, respectively, and the inclusion criteria are described in the method section of the publication manuscript draft in chapter 3.3 and below in the data analyses section. Collected algae (mainly *Codium fragile/vermilara, Flabellia petiolata* and *Halimeda tuna*) were separated according to algae species and collection event in flat basins in the laboratory, where they were thoroughly examined for the presence of sea slugs. As *C. fragile* and *C. vermilara* can potentially be growing together and are difficult to distinguish (Bergbauer and Humberg 1999, Hofrichter 2003), they are considered together in the analyses. Similar as in investigations in chapter 3.3, the sea slugs were kept in near-natural conditions in the laboratory, in basins of about 160 cm x 60 cm with running seawater from the laboratory circulation system which draws water from the nearby sea in shallow depths (corresponding sea temperature in 5 m depths, kindly provided by the Réserve Naturelle Cerbère-Banyuls: 2010: April: 12-16 °C, May: 14-18 °C, June: 16-20 °C, July: 17-24 °C, August: 18-23 °C, September: 18-22 °C; 2011: June: 18-21 °C, July: 18-22 °C, August: 19-24 °C, September: 21-23 °C; 2012: August: 17-25 °C, September: 17-23 °C, October: 17-20 °C), or individually in petri dishes with regular water exchange from the laboratory system with highest measured water temperature in petri dishes reaching up to a maximum of 26 °C (measured 28th August 2012). Likewise, the sea slugs were exposed to natural (but not direct sun-) light through a window (orientated to the west) with light intensities between 4-5 µmol quanta m^{-2} s^{-1} up to around 123 µmol quanta m^{-2} s^{-1} during the day (highest values measured end of August 2012 in patches of sunlight falling in with distance to the sea slugs up to 345 µmol quanta m^{-2} s^{-1}). The investigations in chapter 3.4.1 included also 47 individuals of *Elysia crispata mangrove type* after Krug et al. (Krug, Vendetti et al. 2016) which were collected in February 2012 at the MML, field station Summerland Key, Florida, USA, directly under a dock in depths of about 0.40-2 m in calm water on shady sandy/muddy ground with several algae, including the genera *Halimeda, Caulerpa,* and *Penicillus*. These individuals were kept in tanks (40.5 x 92.5 inches) or individually in smaller basins or petri dishes in the outdoor laboratory facilities at the MML, under a roof of a transparent black net to reproduce a slight shady effect imitating natural sunlight transmission in the sea, and an additional material upon the roof to protect from the strong midday solar irradiation, and with supply of running seawater through the laboratory circulation system from the nearby canal in which temperatures measured during observations in the sea ranged around 23-24 °C in March and April 2012. Light conditions measured in proximity to the sea slugs ranged overall between 4-6 µmol quanta m^{-2} s^{-1} in very

shady points up to around 110 µmol quanta m^{-2} s^{-1} in light patches (highest values measured above the tanks in light patches ranged to 575 µmol quanta m^{-2} s^{-1}).

2.2.2 Feeding experiments, PAM-measurements and TEM-investigations

Of 47 *Elysia crispata mangrove type* individuals collected at the MML, Florida, seven individuals were measured as representatives for the first curve of starvation after being collected until values of photosynthetic yield started to drop down. The further 40 individuals were then included into the continuing measurements until all photosynthetic yield values approached zero, respectively, to ensure that approximately all intact embedded chloroplasts had been digested or degraded before starting to feed the individuals with the tested algae in feeding experiments, thus measured photosynthetic activity would stem from newly incorporated chloroplasts of the test algae. For the feeding experiment with *E. crispata mangrove type*, fresh algae of the abundant species *Caulerpa verticillata, Penicillus capitatus, Penicillus lamourouxii, Halimeda incrassata* and *Halimeda monile* from the environment of the slugs were collected every few days from the same collection site where the slugs had been collected, and provided to five starved slug individuals, respectively. For *P. capitatus*, an additional individual was fed as some individuals showed rising of photosynthetic yield and fluorescence values while others did not. The individuals were provided with regularly renewed algae of the respective tested species during the experimental feeding period. If they were in good condition then with raised photosynthetic yield and fluorescence values, algae supply was stopped and PAM-measurements were continued during a second starving period. Feeding trials in the Mediterranean species were performed in an analogous way, with a preceding phase without food supply until photosynthetic yields fell to approach zero, indicating depletion of incorporated chloroplasts, then renewed feeding with chosen algae species collected in the natural environment of the individuals and observation of photosynthetic performances of newly incorporated chloroplasts.
Concerning starving phases in the examined species in general, PAM fluorescence and yield values decreased during the course of starving periods, thus the sensitivity of the PAM was accordingly adapted by adjusting the parameter ‘outgain’ from level 2 (default) to higher levels, up to level 8. Despite of sensitivity adaptation, in single cases false high yield values can potentially be displayed in combination with very low fluorescence values when

fluorescence values are falling to approach zero, which was in single cases respectively left out or replaced by zero for further analysis.
Like in other PAM-investigations in this thesis, PAM-measurements were performed in series of 1-3 measurements with individuals being dark-adapted for 10 minutes before each measurement and with the fibre optic held above the individual with a distance of 0,5-1 cm in the central region of the body part with the parapodia or other body appendixes, depending on the examined species.
The TEM-investigations presented in the further results section 3.4.2 were carried out along with the other investigations at the OOB in the frame of the ASSEMBLE program in collaboration with the technical assistant Marie-Line Escande with methods correspondingly as published in Schmitt et al. (Schmitt, Händeler et al. 2014) (chapter 3.2).

2.2.3 Data analysis

Data analyses presented in chapter 3.4 were performed along with the analyses described in chapter 3.3, with Excel and with data presentation mostly in the form of mean, standard deviation and range. Corresponding with chapter 3.3, the analyses of the PAM-measurements included 1-3 consecutive measurements per individual and measurement day, and the parameter 'days' was partly adapted, e. g. to merge different measurement series together. Also similar as in chapter 3.3, for analyses of long-term PAM-series, single individuals, that were separated from the collection population and fixed for separate transmission electron microscopy investigations after only one PAM-measurement, were excluded. As described also in chapter 3.3, other single individuals which were additionally included in other investigations but at a later time point when photosynthetic yield values had already fallen were included in the analyses. Also corresponding with chapter 3.3, individuals were excluded in rare cases from the analyses, as they were displaying already yield values approaching 0 or very pale color (not green anymore) right from the beginning after collection or if measured too shortly for inclusion into long-term analyses for other reasons.
Concerning data selection for special analyses, for *E. viridis*, a population collected from its known food algae *C. fragile* (Evertsen and Johnsen 2009) – here considered together with *C. vermilara* as explained above – was chosen for the long-term retention analysis displayed in chapter 3.3 and individuals collected from other substrates were analyzed comparatively in separate analyses as described in the further results section.

3 Results

3.1 Publication: Behavioral adaptations in relation to long-term retention of endosymbiotic chloroplasts in the sea slug *Elysia timida* (Opisthobranchia, Sacoglossa)

This chapter provides a publication (Schmitt and Wägele 2011) which has been published as: Valérie Schmitt & Heike Wägele (2011). "Behavioral adaptations in relation to long-term retention of endosymbiotic chloroplasts in the sea slug *Elysia timida* (Opisthobranchia, Sacoglossa)." Thalassas 27(2): 225–238.

Introductive and summarizing information on the publication: The publication presents behavioral and photobiological investigations comparing the two sacoglossan species *Elysia timida* with long-term retention and *Thuridilla hopei* with short-term retention of chloroplasts. There had been few first reports on specialized photobehavior in sacoglossan sea slugs with assumptions of possible connections between behavior and retention of chloroplasts (as described already in the general introduction of this thesis). Thus, the present investigations aimed at analyzing these two sacoglossan species in more detail with special regard to photobehavior and taking the character of long- or short-term retention of chloroplasts into account. One major finding was that both sacoglossan species showed phototactic behavior, with an overall more prominent phototactic behavior in *E. timida* than in *T. hopei*. Also, phototactic behavior could be observed in juvenile *E. timida* before the first incorporation of chloroplasts, indicating phototaxis as a more basic evolution, not a direct influence of chloroplasts. Moreover, a formerly described specialized photobehavior with light-adapted opening and closing of the parapodial lobes could be confirmed in *E. timida* – in contrast to *T. hopei*. Furthermore, the efficiency of this behavior could be demonstrated with a significant relation between parapodia positioning and fluorescence of incorporated chloroplasts, measured with a Pulse Amplitude Modulated Fluorometer. In summary, this publication indicates specialized behavioral adaptations in relation to long-term kleptoplasty in *E. timida*. The project was mainly performed at the Observatoire Océanologique Banyuls-sur-Mer, France, supported by the European Community with an ASSEMBLE grant agreement no. 227799 to Valérie Schmitt and partly by funding of the German Science Foundation (Wa618/12) to Heike Wägele.

THALASSAS

Thalassas, 27 (2): 225-238
An International Journal of Marine Sciences

BEHAVIORAL ADAPTATIONS IN RELATION TO LONG-TERM RETENTION OF ENDOSYMBIOTIC CHLOROPLASTS IN THE SEA SLUG *Elysia timida* (OPISTHOBRANCHIA, SACOGLOSSA)

VALÉRIE SCHMITT [1, 2] & HEIKE WÄGELE [1]

Key words: Sacoglossa, endosymbiosis, chloroplasts, retention, phototaxis, photobehavior.

ABSTRACT

A comparative study was performed to analyze differences in evolutionary adaptations in two sea slug species, *Elysia timida* with long-term retention of endosymbiotic chloroplasts and *Thuridilla hopei* with short-term retention of endosymbiotic chloroplasts. Both sacoglossan species stem from the same habitat and show similar body sizes and structures with parapodial lobes whose position can be actively varied by the slugs. Ethological analyses were carried out concerning the positioning of parapodia and other photobehavioral parameters like phototaxis. In parallel, photosynthetic activity was measured with a Pulse Amplitude Modulated Fluorometer (PAM). In total, 252 *E. timida* individuals and 63 *T. hopei* individuals were included in the analysis. Slugs were collected diving in shallow depths up to 5 m in Banyuls sur mer, France, and kept in the laboratory in basins with running seawater and natural light through a glass window. Behavioral observations and PAM-measurements were performed in 4 time intervals in the course of an observation day in daylight and dark-adapted conditions. Phototactic behavior was found to be present in both compared species, although the phototactic reaction was more pronounced in *E. timida*. Phototaxis was also observed in juvenile *E. timida* before sequestration of first *Acetabularia*-chloroplasts, which indicates no direct current influence of the endosymbiotic chloroplasts. Other parameters, however, like the positioning of the parapodia, were observed to be significantly different between the long-term and short-term storing species. While an adapted changing of the parapodia's position in reaction to light conditions was not observed in *T. hopei*, the typical specialized photobehavior of *E. timida* with active variation of parapodial positions including exposure and protection of integrated chloroplasts could be confirmed and analyzed in this study. Positioning of the parapodia in *E. timida* showed a significant relation to fluorescence values from PAM-measurements demonstrating the efficiency of exposure and protection of embedded chloroplasts.

(1) Zoologisches Forschungsmuseum Alexander Koenig
Adenauerallee 160. 53113 Bonn. Germany
Tel: +49 (0)228 9122 241
Fax: +49 (0)228 9122 202
Email: hwaegele@evolution.uni-bonn.de
(2) Observatoire Océanologique, 66651 Banyuls sur mer, France
Corresponding author: Valerie Schmitt

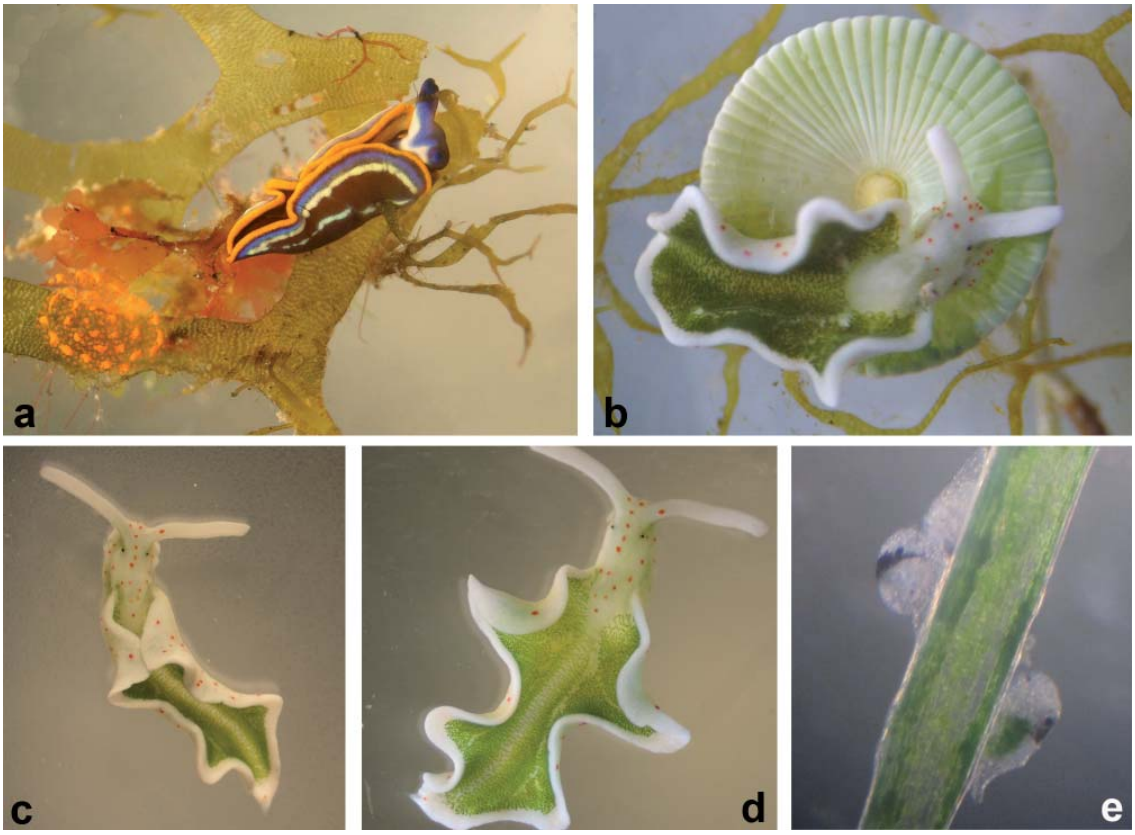


Figure 1:
a Thuridilla hopei *on Dictyota (not a food organism of this species). b* Elysia timida *on its natural food alga* Acetabularia acetabulum. *c* Elysia timida*, parapodial opening level 2. d* Elysia timida*, parapodial opening level 3. e Three juveniles attached to a young* Acetabularia*: on the left two specimens before feeding, on the right one specimen after feeding*

The specific photobehavior of *E. timida* with controlled exposure of parapodial lobes represents a highly specialized evolutionary adaptation in relation to long-term integration of chloroplasts and - state of the art - is only recorded for this species.

INTRODUCTION

Our knowledge on biology and evolution of functional kleptoplasty in various sacoglossan sea slugs has increased lately to a considerable extent (see e.g., Giménez Casalduero and Muniain, 2008; Händeler et al., 2009; Jesus et al., 2010 and literature herein). But when it comes to behavior, our knowledge is still limited. Sacoglossans reveal a variety of evolutionary adaptations when it comes to retain endosymbiotic chloroplasts – especially with regard to behavior. First descriptions of specialized photobehavior in sea slugs were done by Fraenkel (1927) when he examined photomenotaxis in *Elysia viridis.* In a later study comparing five sacoglossan species, the focus was laid on the presence or absence of endosymbiotic chloroplasts in the sea slugs. Three symbiotic species with integrated chloroplasts (*Elysia tuca, Costasiella lilianae* (= *Costasiella ocellifera* after Clark (1984)), and *Elysia crispata*) and two aposymbiotic species (*Oxynoe antillarum* and *Berthelinia carribea*) were analyzed concerning their

photobehavior (Weaver and Clark, 1981). As one result, the symbiotic species oriented towards light while the aposymbiotic species avoided light which points to a possible relationship between symbiotic chloroplasts and phototaxis.

The chloroplast-hosting sacoglossan *Elysia timida* has a specially notable photobehavior, changing the position of its parapodial lobes from a contracted, closed posture to a spread, opened leaf-like posture (Rahat and Monselise, 1979). As *E. timida* varies the position of the parapodia as a reaction to light conditions, a possible nearby conclusion is that this photobehavior could have evolved in relation to the chloroplast-endosymbiosis. *E. timida* is a common Mediterranean species that lives in a close relationship to its food alga *Acetabularia acetabulum* from which it retains its endosymbiotic chloroplasts (Marin and Ros, 1992; Marin and Ros, 1993). With an extensive duration of approximately three months of retaining the endosymbiotic chloroplasts functional during starvation, *E. timida* belongs to the few species with the most extended capability of long-term retention of chloroplasts (Evertsen et al., 2007; Giménez Casalduero and Muniain, 2008; Händeler et al., 2009; Wägele et al., 2010). Recent literature defines long-term retention as lasting functionality of chloroplasts of more than a month opposed to short-term retention lasting about one week (Händeler et al., 2009).

As the special photobehavior of *E. timida* should be analyzed in more detail in this study with regard to its relation to the long-term integration of endosymbiotic chloroplasts, it was compared to a similar Mediterranean species with short-term retention of chloroplasts. The sacoglossan *Thuridilla hopei* is a species with short-term chloroplast endosymbiosis (Marin and Ros, 1989; Händeler et al., 2009) and was chosen as the most suitable comparative species, as both *E. timida* and *T. hopei* are common Mediterranean species that live sympatrically and have about the same body size and structure with parapodial lobes that can be actively closed and opened by the slugs - the basis for the comparison of this behavior. During our studies we analyzed these varying positions in relation to irradiance and tested both species for the presence of phototaxis.

MATERIAL AND METHODS

In total, 252 *Elysia timida* and 63 *Thuridilla hopei* (Fig. 1a and b) were collected in the same habitat in Banyuls sur mer, France, by diving in shallow depths down to about 5 m, in July 2009 and September 2010. Individuals were kept in the laboratory (Observatoire Océanologique, Banyuls sur mer, France) in basins of about 160 cm x 60 cm with running seawater from the laboratory circulation system (21.2 ± 1.0 °C in July 2009 and 19.6 ± 0.9 °C in September 2010). It was attempted to provide the animals semi-natural conditions with exposure to natural (but not direct sun-) light through a window (orientated to the west) with a light intensity of up to 47 and 37 µmol quanta m^{-2} s^{-1} (PAR: photosynthetic active radiation, highest single values measured in July 2009 and September 2010, respectively). Free access to an assortment of various algae from their natural environment, including the preferred food algae *Acetabularia acetabulum (E. timida)* and *Cladophora cf. vagabunda (T. hopei)* (Marin and Ros, 1989) collected from the same collection sites as the animals, was provided. For the various photobehavioral experiments, algae were removed from the basins and running sea water supply was stopped in order to exclude any additional influencing factors. Clutches laid by *E. timida* individuals in the laboratory were kept in petri dishes with artificial sea water and regular water exchange until hatching. Until experiments started, the juveniles were kept in artificial seawater with no food provided. In this state, juveniles are transparent (Fig. 1 e).

First phototaxis study: Elysia timida

The first observations on phototactic behavior included two groups of 50 individuals each in two separate basins. The two basins were both orientated parallel to the window side and for the trial were covered each half with black board. As a result, each

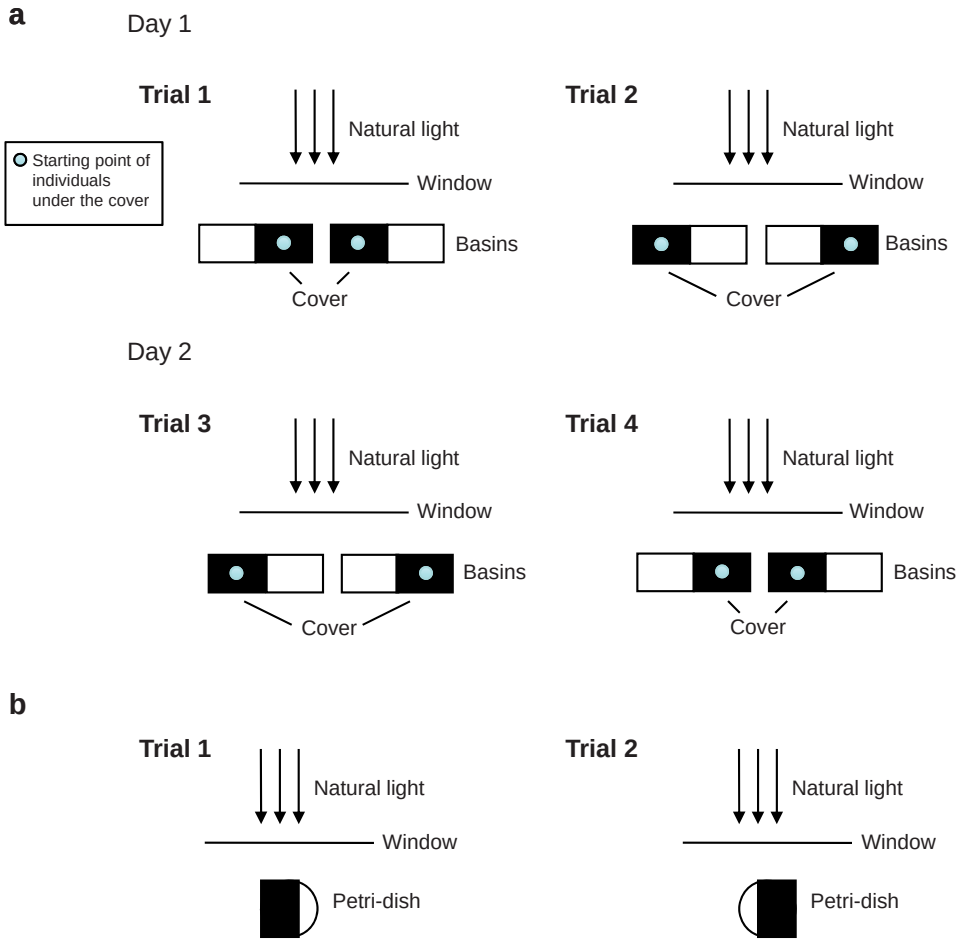


Figure 2:
Schemata of phototaxis experiments. a First and second phototaxis study.
The first phototaxis study with 100 Elysia timida was started by covering the inner sides of the basins and putting 50 individuals each in the middle of the dark covered side of the respective basin (indicated with a grey dot). Cover was changed after 3.5 h to the other side of the basin for the second trial. The next day the experiment was repeated with reversed sides. For the second phototaxis experiment with E. timida and Thuridilla hopei, the procedure of the first day of the first experiment was performed again in the same way.
b Phototaxis experiment with juvenile E. timida.
20 juvenile E. timida were put into one half of a petri-dish which was covered with black paper leaving only a gap of about 1cm for light incidence of natural light through a glass window. The cover was first put on the one side for the first trial, and then changed to the other side for the second trial.

half of the basins was shaded while the other half was illuminated by natural light through the window in the same angle. The first trial was started with covering the right half of the left basin and the left half of the right basin (Fig. 2 a). After 3.5 hours the cover was changed to the respective other side of the basin and observations were continued for another 3.5 hours. On the second day, the same procedure was performed in the reversed way starting with covering the outer sides of the basins first, then changing after 3.5 hours. Thus, in total four trials were performed in two days. This experimental design was chosen in order to equalize any influence from different angles of light incidence or potential other influences from position conditions. The basins were covered at 11 a.m. at each observation day. Before starting the

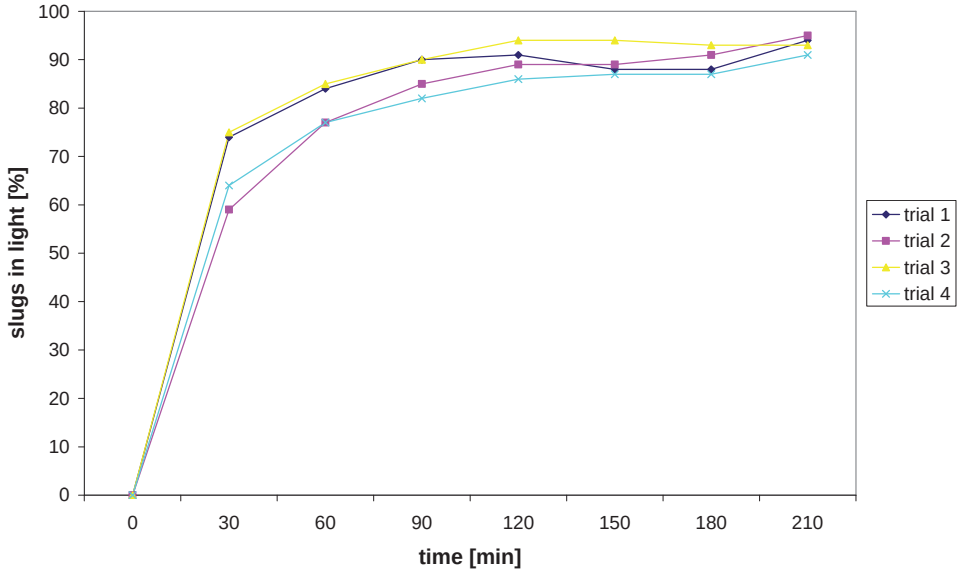


Figure 3:
Phototaxis in Elysia timida.
Two 160 x 60 cm basins were each covered half with black board and 50 Elysia timida individuals were placed under the cover on the dark side of each basin. Every 30 min. until 210 min. locations of specimens were recorded (trial 1). Then the cover was put to the other half of the basin and location recorded until after 210 min. (trial 2). Trial 3 and 4 were performed in the same way.

experiment, the 50 individuals were placed each in the middle of the shaded half of the basin. Starting with 30 minutes after the basins were covered, individuals that had crawled into the illuminated side were counted. The census was repeated every 30 minutes for 3.5 hours for each trial – in total 7 counts.

Second phototaxis study: Elysia timida – Thuridilla hopei

The same trial was performed another day to compare the phototactic behavior in *E. timida* and *T. hopei*. For this, 77 *E. timida* and 48 *T. hopei* were allocated into a group of 40 *E. timida* and 15 *T. hopei* in one basin and a group of 37 *E. timida* and 33 *T. hopei* in the other basin. The basins were covered with black board in the same way as in the first phototaxis trial and the trial was also started at 11 a.m. Again individuals were placed in the middle of the shaded half of the basin. Observation intervals were shortened to 15 minutes and the cover was changed to the other side already after 90 minutes in adaptation to the results of the first phototaxis trial, which had shown that the examination of the phototactic reaction is possible in a short observation period.

Third phototaxis study: juvenile Elysia timida

Six days after hatching of veliger larvae had started in the clutch, 20 juveniles which had turned into the crawling juvenile state were put into a small petri-dish and observed through a stereomicroscope. The petri-dish was covered on the sides and from upside with black paper so that only a small gap of approximately 1 cm was left open to natural light through a glass window (Fig. 2 b). In correspondence with the former phototaxis studies, the juveniles were put under the cover on the dark side and after 30 minutes it was counted how many individuals had moved to the light-exposed area. The cover was then changed to the other side without moving the petri-dish to repeat the trial in the reverse way. Again, the number of individuals which had moved into the light after 30 minutes was evaluated.

Studies on specialized photobehavior

Two trials were performed to analyze the correlation of the parapodial opening and the ground fluorescence: the first contained 25 adult specimens of *E. timida* together with 15 adult specimens of *T. hopei*, the second trial was performed with 50 individuals

of *E. timida* by measuring with a higher sensitivity of the PAM (see below). For the trials, individuals were kept in the basins separated individually in conform containers made out of transparent plastic bottles. Wholes were pierced equally into three rims of each bottle in distances of about 1 cm, permitting exchange of water from the running seawater (mean temperature during the hours of observation 21.5 ± 0.4 °C in July 2009 and 19.8 ± 0.1 °C in September 2010). Each container was stabilized with a stone, which also provided an opportunity for the slugs to hide underneath. Behavioral observations were performed along with PAM-measurements 4 times during an observation day during the time spans 9 a.m. – 12, 12 – 3 p.m., 3 p.m. – 6 p.m. and 6 p.m. – 9 p.m.. Opening level of the parapodial lobes was defined in the following 6 levels and documented in correlation of light intensity (measured in µmol quanta m^{-2} s^{-1}):

0 – parapodia completely closed, the inside of the parapodia is totally covered, slug may be contracted
1 – parapodia are mainly closed with rims of both parapodia coming together over the body for the most part, but opened only a small part so that a little area of the dorsal body can be seen (Fig. 1a)
2 – parapodia are mainly opened, but still the rims of the opposing parapodia touch at least at one, often at two areas, the usual position while crawling (Fig. 1c)
3 – parapodia are opened, the rims of the opposing parapodia do not touch, but still the angle of the parapodia is more upward than sideward (<45°), hence the insides of the parapodia are only partly exposed (Fig. 1d)
4 – parapodia are fully opened, the angle of the parapodia is more sideward than upward (>45°), the rims of the parapodia are still either a little upward or undulated (in contrast to 5)
5 – parapodia are fully opened and absolutely outstretched and flat, angle is totally sideward (90°), the rims of the parapodia are smooth and fully expanded, sometimes even pointing downwards (>90°)

In parallel, fluorescence was measured with the help of a PAM to examine the relation between opening level of parapodia and efficiency of exposure of the chloroplasts.

PAM-measurements

The maximum quantum yield of fluorescence for Photosytem II and ground fluorescence was measured with a Pulse Amplitude Modulated Fluorometer (Diving PAM, WALZ, Germany) during the experiments for the observation of specialized photobehavior. Measurements were performed 4 times per observation day (during the 4 time spans 9 a.m. – 12, 12 – 3 p.m., 3 p.m. – 6 p.m. and 6 p.m – 9 p.m.). Animals were not dark acclimated before measurements in order to obtain the actual fluorescence with regard to actual light intensity and parapodia positions. The maximum quantum yield of fluorescence for PSII in ambient light can be defined as $(F_m' - F_0')/F_m'$ (Wägele and Johnsen, 2001; Jesus et al., 2010) and shows the photosynthetic activity in the actual light regime as a relative value. During measurement, the maximum fluorescence (F_m) is induced by a saturation light pulse triggered by the PAM. The ground fluorescence (F_0) measured directly before the saturation pulse reflects the actual fluorescence under the given light regime. Both values depend on quality and quantity of chloroplasts. But it has to be kept in mind that accurate estimations of fluorescence values may be difficult to obtain and are influenced by other factors (see Wägele and Johnson, 2001). Only two measurements after 6 p.m. in the second study were performed dark-acclimated for comparison.

The fibre optic was held above the animal with a distance of 1 cm in the region of the body part with the parapodia. Since the size of the measured animals was around 10 mm and the head has not to be included in the measurements, the sensor with a cross section of 5 mm covered the body area with the parapodia well.

The second study on the relation of parapodial opening was performed with increased sensitivity

of the PAM by putting the parameters 'outgain' and 'measure-int' from level 2 (default) to level 8 during the whole study.

Ambient light conditions were measured with the light sensor of the PAM.

Statistical analysis

Statistical analysis was performed using Excel and SPSS.

RESULTS

Phototaxis

The first four observational trials to investigate phototaxis in 100 *E. timida* individuals revealed a very distinct and fast phototactic reaction for *E. timida* (Fig. 3). In the first census, 30 minutes after the slugs had been put under the cover in the basin, the majority of individuals (ranging from 59-75% in the four trials) had already moved from the dark covered side of the basin into the light.

The slugs then stayed in the light-exposed areas while the remaining individuals from the dark followed subsequently. When the cover was changed to the other side of the basin, the same fast movement into the light was observed again. Repeating the trial with reversed sides in trial 3 and 4, the reaction was identical. After 3.5 hours of observation in each of the four trials, nearly all of the individuals (ranging from 91-95% in the four trials) were positioned in the light-exposed area of the basin. Only a small percentage did not enter the light side or moved back under the cover. Those individuals were found to be in the border area directly under the rim of the cover where a small amount of light was falling in.

As in this first phototaxis study it became obvious that the phototactic reaction is performed fast and can be examined in a short observation period, the time spans of the second phototaxis study were adapted and shortened to observation intervals of 15 minutes and an overall duration of 90 minutes per trial. In this second phototaxis study with the aim to compare phototactic reactions in *E. timida* and *T. hopei*, phototactic behavior was also seen in *T. hopei* although it was obviously more pronounced in *E. timida* (Fig. 4 a and b).

While after 30 minutes the phototactic reaction of *E. timida* was similar as in the first phototaxis study (mean value of 63% in the two trials compared to 68% in the four trials of the first study), it was slightly lower in *T. hopei* with 50% of individuals counted on the light-exposed side. After 90 minutes, *E. timida* revealed again a comparable result to that in the first study with 81% of the individuals located on average in the light area compared to 86% in the first four trials. In *T. hopei*, however, the phototactic reaction was clearly less pronounced with only 59% of individuals positioned on the light side. Similar as in the first study with exclusively *E. timida*, also in this experiment remaining individuals of *E. timida* and *T. hopei* were found to be in the partly illuminated border area directly under the rim of the cover. Thus *T. hopei* showed a stronger tendency to prefer this border area with only a small amount of light falling in while *E. timida* showed a stronger tendency to prefer the area which was fully illuminated with moderate natural light.

Juvenile *E. timida,* which had reached a crawling state, but had no possibility yet to feed on *Acetabularia acetabulum*, also revealed a distinct phototactic behavior. In both trials with changing the cover from one side to the other like in the studies before, 90% and 95% (respectively) of the 20 juveniles had moved into light after 30 minutes which reflects a very fast and distinct phototactic reaction.

Specialized photobehavior

The individuals of *E. timida* varied their parapodial positions from a nearly closed condition to fully spread leaf-like positions ranging from parapodial opening level 1-5 (Fig. 5 a and b). A complete closure (level 0) was not observed during the trials, but during night and extreme light exposure

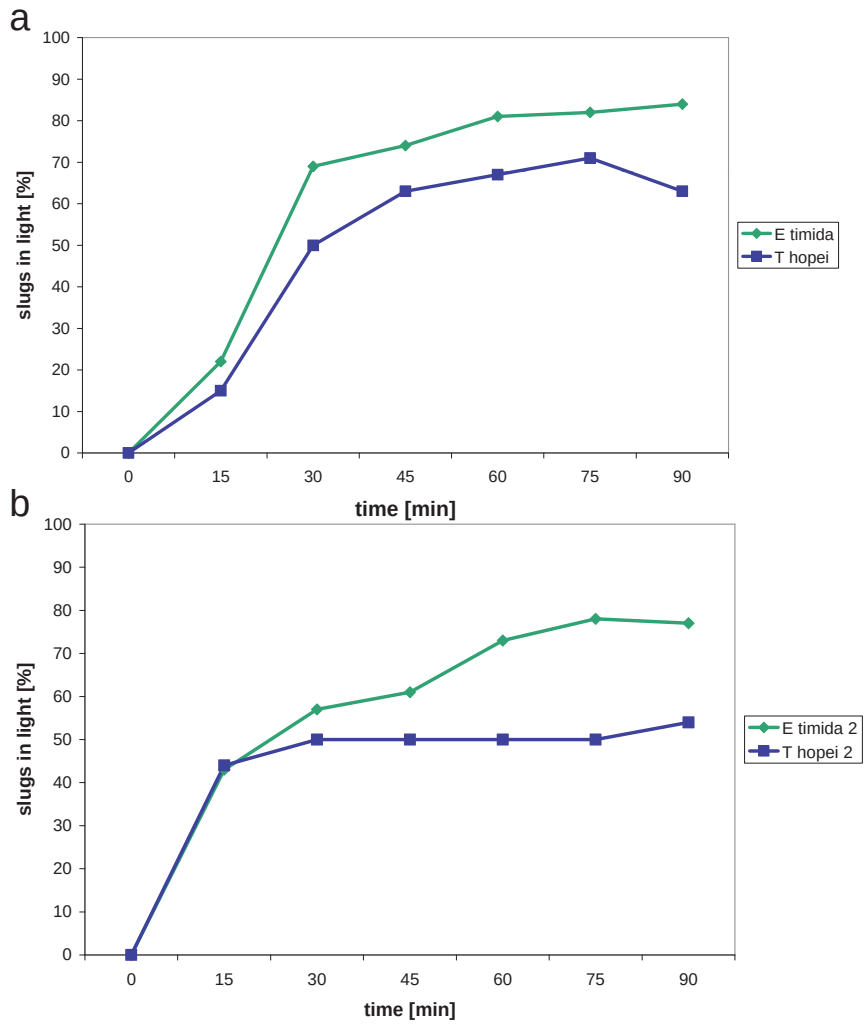


Figure 4:
***Phototactic reaction in* Elysia timida *and* Thuridilla hopei.**
The experiments were performed with 77 Elysia timida *and 48* Thuridilla hopei *in two basins. a First trial with cover on the inner side. b Change of cover to the outer side (after 90 min). Observation intervals were shortened to 15 minutes and duration of one series was limited to 90 minutes.*

(not figured here). *T. hopei*, however, did not show a higher parapodial opening level than 1 (Fig. 1a) during the observations irrespective of irradiance (Fig. 5 a). In the majority of cases (112 out of 120 observational cases), the parapodia were closed (level 0). To examine the ability to open the parapodia, *T. hopei* was also observed in dark conditions, where the slugs sometimes showed an opening level of 3 to 4. Additionally, opening was observed as a reaction to a tactile stimulus by carefully touching the slug's body.

E. timida revealed a tendency of broader exposure of the chloroplasts (parapodia opening levels 3-5) with higher light irradiances, but in the frame of the moderate lux values of the natural light spectrum (and in accordance the reduced photosynthetic active radiation PAR) through a window in the laboratory and the short momentous recordings of behavior, a clear significant correlation between current light intensity measurements and parapodial position in *E. timida* could not be inferred.

The momentary fluorescence values in the PAM-measurements (F_0'), however, increased in strong correspondence with increasing parapodial opening level of *E. timida* individuals which constituted a significant correlation ($p<0.01$ in both of the studies, Spearman rank-order correlation test) (Fig. 6a and b). While with a low parapodial opening level of 1, the momentary fluorescence measured in *E. timida* was similar to that in *T. hopei*, the fluorescence values rose with every higher level of parapodial opening in *E. timida*, reflecting the higher exposure of the imbedded chloroplasts. In contrast, corresponding yield values, which represent relative values, stayed constant irrespective of parapodia position (Fig. 7a and b). This can probably be explained by the increasing measurable maximum fluorescence (F_m) when parapodia show a higher level of opening. No remarkable variances in the ground fluorescence were observed in the measured *T. hopei* individuals (Fig. 6a) and yield values were lower than in *E. timida* (Fig. 7a).

DISCUSSION

In our analyses of phototaxis we observed phototactic behavior in *E. timida* with long-term integration of functional chloroplasts as well as in *T. hopei* with short-term chloroplast integration. In the first phototaxis study with 100 individuals of *E. timida*, approximately all individuals had moved from the dark into the light-exposed area at the end of each of the four trials. The remaining individuals were located in the border area under the rim of the cover where some light was falling in. Thus it can be concluded that *E. timida* in general has an automatic strong and direct phototactic behavior. The second phototaxis study revealed phototactic behavior also in *T. hopei*, but the reaction was less pronounced than in *E. timida*. In comparison, individuals of *T. hopei* showed a stronger tendency to stay in the border area under the rim of the cover with only a slight light incidence or crawl back into this area while individuals of *E. timida* showed a stronger preference of the light-exposed area. With still the majority of slugs choosing the light-exposed area and most remaining individuals staying in the border area with some light incidence, we consider *T. hopei* as a phototactic species, but with a gradual difference of stronger tendency to more shaded areas in contrast to *E. timida*. This corresponds to observations of localities in the sea when collecting the animals. While *E. timida* was found mainly on horizontal, light-exposed rocks, *T. hopei* was found mainly on vertical, half-shaded rocks, often even in little holes in the rock surface. Future experiments with regard to phototaxis may help to elucidate the distinct behavior concerning sensitivity in various light regimes.

Fraenkel (1927) wrote that he chose *Elysia viridis* for his observations on photomenotaxis out of many tested opisthobranch species as *E. viridis* showed the fastest and clearest reaction. Unfortunately he did not describe which other species exactly he compared and in which way. Weaver and Clark (1981) compared the three sacoglossan species *Elysia tuca, Elysia crispata* and *Costasiella lilianae* (= *Costasiella ocellifera* after Clark (1984)) with endosymbiotic chloroplasts and the two sacoglassan species *Oxynoe antillarum* and *Berthelinia carribea* without endosymbiotic chloroplasts concerning their photobehavior. They found that the symbiotic species oriented towards light while the aposymbiotic species avoided light. This indicates a possible correlation of chloroplasts' sequestration and phototaxis. The results of our phototaxis analyses correspond in so far that both investigated species are symbiotic and both show phototactic behavior. As furthermore the phototactic behavior was stronger in *E. timida* with long-term chloroplast retention as in *T. hopei* with short-term retention, the question arises, if species with long-term functional chloroplast retention reveal stronger evolutionary adaptations in relation to endosymbiotic chloroplasts. The phototactic behavior is more probably to be regarded as such an evolutionary adaptation, not as an immediate, direct influence of the chloroplasts on their host. The finding of our study that juvenile *E. timida* already revealed strong phototaxis before the first integration of chloroplasts from *A. acetabulum* supports this assumption.

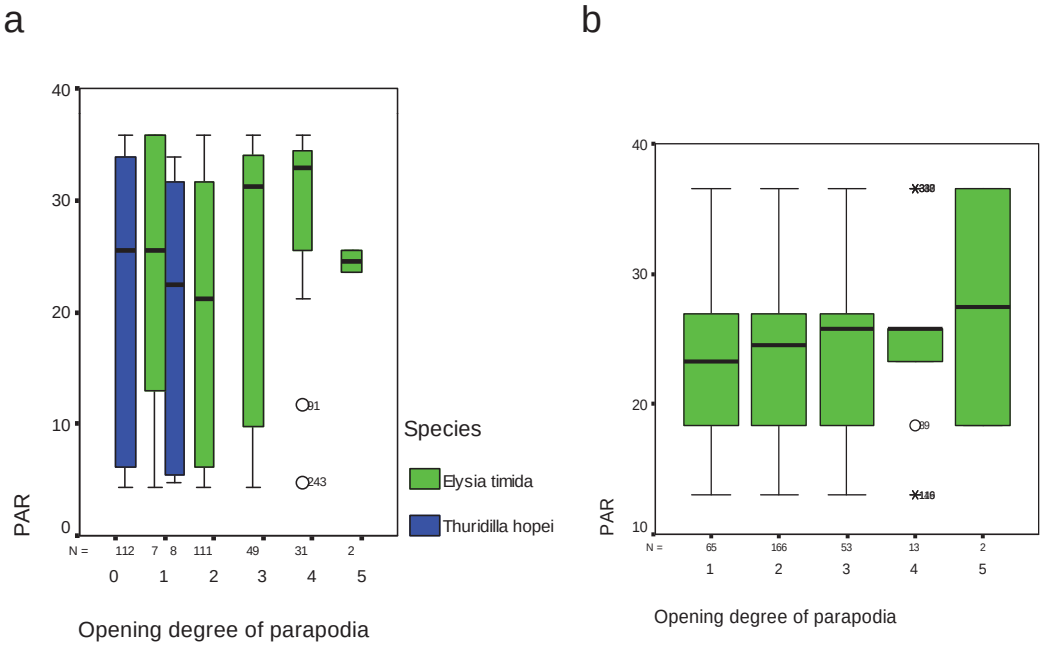


Figure 5:
Current irradiance [PAR: µmol quanta m^{-2} s^{-1}] in relation to opening level of parapodial lobes.
a First trial with 25 Elysia timida *and 15* Thuridilla hopei *measured 4 times on 2 days respectively in July 2009. b Second trial with 50* E. timida*, measured 4 times on 2 days, respectively, in September 2010. Due to seasonal effects, light incidence in the laboratory reached higher values in the measurements in July than in September.* T. hopei *was not observed to open the parapodia more than level 1 (only if touched) and therefore not included in the second analysis. N displays the number of incidences this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.*

Importance of photosynthesis of the endosymbiotic chloroplasts as source of nutrients for *E. timida* was shown in experiments, in which *E. timida* was kept in the dark and thus deprived of the photosynthetic products of their chloroplast. These individuals had lower survival rates and stronger size decreases opposed to those kept in light (Giménez Casalduero and Muniain, 2008). The need of exposure to light for the function of the photosynthetic endosymbionts stands in conflict with potential dangers connected to exposure, e.g. bigger vulnerability through greater exposure to predators, waves and currents and especially damage of photosynthetic endosymbionts through exposure to irradiances higher than a well tolerated maximum (Monselise and Rahat, 1980). The predator problem can be reduced by mechanisms like producing toxic or irritating secretions and cryptic colorations in sacoglossan sea slugs (Cimino and Ghiselin, 1998; Marin and Ros, 2004), even if not fully eliminated. The potential damage of photosynthetic functions through extreme light intensities still poses a difficult problem (Jesus et al., 2010). It seems evident that *E. timida* has evolved an efficient protection mechanism against this photodamage problem with the specialized photobehavior. By closing the parapodia, *E. timida* can react directly to threatening light intensities and form a natural protection shield for the embedded chloroplast in the inside of the parapodia. This mechanism enables *E. timida* to be located permanently in shallow light-exposed areas and adapt to current light irradiances. Opening of the parapodia exposes the chloroplasts to higher irradiation, whereas the closure reduces light penetration. This specialized photobehavior of

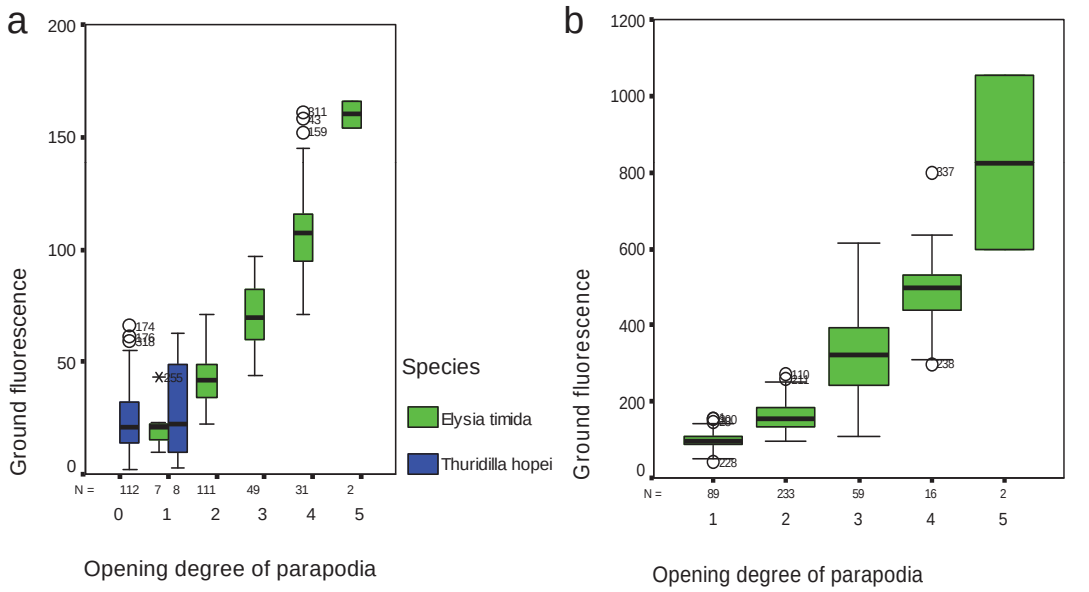


Figure 6:
Ground fluorescence (F_0') in relation to opening level of parapodial lobes.
a First trial with 25 Elysia timida *and 15* Thuridilla hopei *measured 4 times on 2 days respectively. b Second trial with 50* E. timida*, measured 4 times on 2 days, respectively; PAM-settings were increased to high sensitivity (consequently values of momentary fluorescence are higher). N displays the number of times this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.*

E. timida first described by Rahat and Monselise (1979) could be confirmed as a general mechanism by our observations and analyzed in more detail. In our experiments, we used the emission of the fluorescence through the parapodia as a factor to indirectly measure the exposure of the chloroplasts. The closure of the parapodia unambiguously shows that less light penetrates the parapodia and therefore protects the underlying chloroplasts of higher irradiances. With increasing parapodial opening level the momentary ground fluorescence values (F_0') in individuals of *E. timida* increase in strong correspondence, which constituted a significant correlation in our measurements. This reflects the efficiency of the behavior to expose the inlaying chloroplasts to light by opening the parapodia and thus enhancing photosynthetic activity in the integrated chloroplasts. We assume that the maximum fluorescence (F_m') rises also with higher parapodial opening levels, which equalizes the higher values of ground fluorescence. As the overall effective yield value of photosynthetic activity is calculated from (F_m' – F_0')/F_m', the effective yield therefore stayed relatively constant with the varying parapodial opening levels.

Concerning the specialized photobehavior of *E. timida* with light-adapted changing of the position of the parapodial lobes, the examined behavioral reactions were very different in the two compared species. The light-adapted gradual opening of the parapodia as in *E. timida* is apparently not present in *T. hopei*. Although *T. hopei* individuals were observed to actively open their parapodia in reaction to touch or sometimes in darkness, they did not open them wider

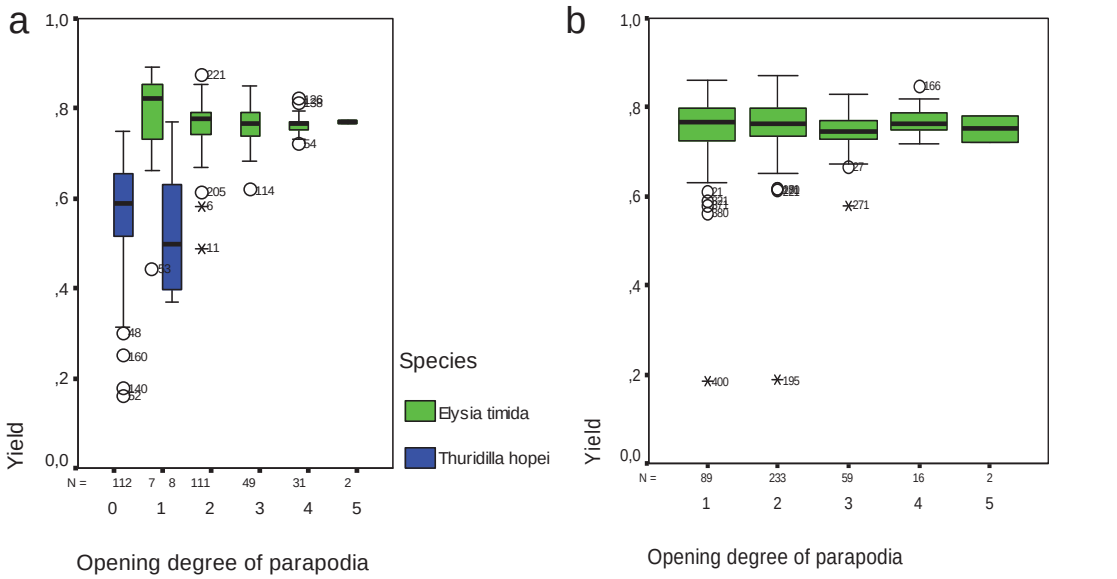


Figure 7:
Yield (F_m' – F_0'/F_m') in relation to opening level of parapodial lobes.
a) First trial with 25 Elysia timida *and 15* Thuridilla hopei *measured 4 times on 2 days respectively. b) Second trial with 50* E. timida*, measured 4 times on 2 days, respectively; PAM-settings were increased to high sensitivity. N displays the number of times this parapodial opening level was counted in the behavioral observations. Boxes represent interquartile ranges divided at median values. Lines are drawn from the top of the box to the largest value within 1.5 interquartile ranges of the top and the same from the bottom. Symbols display outliers outside this range.*

than level 1 in the moderate natural light conditions in the laboratory. The special photobehavior of *E. timida* is also related to the characteristic structure of integrating the chloroplasts into the body. In *E. timida*, the embedded chloroplasts can well be seen as a green area covering the inside of the parapodia while the outsides of the parapodia and the rest of the body are full of white pigment with only another small green stripe on the lower sides of the slug. In contrast *T. hopei*, which exhibits a similar arrangement of branched digestive gland and incorporated chloroplasts, seems to prevent photosynthesis of chloroplasts by shading them permanently with the help of the parapodia. Additionally, the rather dark body coloration may enhance this shielding of sunlight.

E. timida revealed a tendency of increasing exposure of the chloroplasts with higher light irradiances, but in the frame of the moderate lux values of natural light through a window in the laboratory and the short momentous recordings of behavior, a clear significant correlation between current light intensity measurements and parapodial position in *E. timida* could not be inferred. The parapodial position is always connected to the current active state of the individual. Individuals usually start to open their parapodia to higher parapodial opening levels only while sitting in one position for a while. The opening level 2, which was observed in the majority of cases in both experiments, is the characteristic position while crawling. Thus more observations are necessary for detailed results on the relation between light conditions and behavior.

It is not explained so far how exactly the specialized photobehavior of *E. timida* functions. In general, the slug's behavior is in discrepancy anyway: When it exposes itself to higher irradiances, then chloroplasts

suffer from photodamage and can not be repaired, due to lack of genomic equipment (Wägele et al., 2010). When it hides from sunlight, photosynthesis is reduced and contribution to live maintenance is probably minor. Jesus et al. (2010) described that *E. timida* is capable of combining the behavioral photo-regulation mechanism (opening/closing the parapodia) with a functional physiological photo-regulation mechanism (xanthophyll cycle) increasing their photo-regulation capacity as a mechanism to keep their maximum photosynthetic capacity for longer periods. The exact mechanisms of the specialized photobehavior in *E. timida*, however, remain unclear. According to our observations until now, this specialized photobehavior is rather specific for *E. timida*. It represents a highly specialized evolutionary adaptation in relation to long-term retention of chloroplasts with efficient exposure of endosymbiotic chloroplast for high photosynthetic benefit as well as efficient protection of endosymbiotic chloroplasts from photo-damage, enabling functionality of chloroplast endosymbiosis in *E. timida* for one of the most extended durations known so far.

ACKNOWLEDGEMENTS

The project was partly supported by the European Community with an ASSEMBLE grant agreement no. 227799 to VS and partly by the German Science Foundation (Wa618/12) to HW. Furthermore, we thank the Observatoire Océanologique Banyuls sur mer for providing laboratory facilities and assistance and also Susanne Gunkel for assistance in breeding juvenile *E. timida*.

REFERENCES

Evertsen J, Burghardt I, Johnsen G, Wägele H (2007). Retention of functional chloroplasts in
some sacoglossans from the Indo-Pacific and Mediterranean. Marine Biology, 151: 2159-2166.

Cimino G, Ghiselin MT (1998). Chemical defence and evolution in Sacoglossa (Mollusca: Gastropoda: Opisthobranchia). Chemoecology, 8: 51-60.

Clark KB (1984). New records and synonymies of Bermuda Opisthobranchs (Gastropoda). The Nautilus, 98: 85-97.

Fraenkel G (1927). Über Photomenotaxis bei *Elysia viridis* Mont.. Zeitschrift für vergleichende Physiologie, 6: 385-401.

Giménez Casalduero F, Muniain C (2008). The role of kleptoplasts in the survival rates of *Elysia timida* (Risso, 1818): (Sacoglossa: Opisthobranchia) during periods of food shortage. Journal of Experimental Marine Biology and Ecology, 357: 181-187.

Händeler K, Grzymbowski Y, Krug PJ, Wägele H (2009). Functional chloroplasts in metazoan cells – a unique evolutionary strategy in animal life. Frontiers in Zoology, 6: 28.

Jesus B, Ventura P, Calado G (2010). Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanism in the solar-powered sea slug *Elysia timida* (Risso 1818). Journal of Experimental Marine Biology and Ecology, 395: 98-105.

Marin A, Ros J (1989). The chloroplast-animal association in four Iberian sacoglossan opisthobranchs: *Elysia timida*, *Elysia translucens*, *Thuridilla hopei* and *Bosellia mimetica*. Scientia Marina, 53: 429-440.

Marin A, Ros J (1992). Dynamics of a plant-herbivore relationship: the photosynthetic ascoglossan *Elysia timida* and the chlorophycean *Acetabularia acetabulum*. Marine Biology, 112: 667-682.

Marin A, Ros J (1993). Ultrastructural and ecological aspects of the development of chloroplast retention in the sacoglossan gastropod *Elysia timida*. Journal of Molluscan Studies, 59: 95-104.

Marin A, Ros J (2004). Chemical defenses in Sacoglossan Opisthobranchs: Taxonomic trends and evolutive implications. Scienta Marina, 68: 227-241.

Monselise EBI, Rahat M (1980). Photobiology of *Elysia timida* (Mollusca: Opisthobranchia): observations in the sea. Israel Journal of Zoology, 29: 125–128.

Rahat M, Monselise EBI (1979). Photobiology of the chloroplast-hosting mollusc Elysia timida (Opisthobranchia). Journal of Experimental Biology, 79: 225-233.

Wägele H, Deutsch O, Händeler K, Martin R, Schmitt V, Christa G, Pinzger B, Gould SB, Dagan T, Klussmann-Kolb A, Martin W (2010). Transcriptomic evidence that

longevity of aquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobranchus ocellatus* does not entail lateral transfer of algal nuclear genes. Molecular Biology and Evolution, 28: 699-706.

Wägele H, Johnsen G (2001). Observations on the histology and photosynthetic performance of "solar-powered" opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. Organisms Diversity and Evolution, 1: 193-210.

Weaver S, Clark KB (1981). Light intensity and color preferences in five ascoglossan (=sacoglossan) molluscs (Gastropoda, Opisthobranchia): A comparison of chloroplast symbiotic and aposymbiotic species. Marine and Freshwater Behavior and Physiology, 7: 297-306.

3.2 Publication: Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of *Elysia timida* (Sacoglossa, Heterobranchia)

This chapter provides a further publication (Schmitt, Händeler et al. 2014) which has been published as:
Valerie Schmitt, Katharina Händeler, Susanne Gunkel, Marie-Line Escande, Diedrik Menzel, Sven B. Gould, William F. Martin and Heike Wägele (2014). "Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of *Elysia timida* (Sacoglossa, Heterobranchia)." Frontiers in Zoology 11(1): 5.

Introductive and summarizing information on the publication: The publication comprises a description of the laboratory culture system established with the aim to investigate the sacoglossan sea slug *Elysia timida* as a model organism for long-term retention of functional chloroplasts, plus reports of investigations performed within the system. Laboratory culture systems can potentially provide advantages for investigating sea slugs, as e. g. specialized controlled conditions, known life history of individuals and continuous observations of life cycles. The present publication demonstrates that several characteristics of *E. timida* proved to be advantageous for culturing. As one central finding, juvenile *E. timida* fed directly on their adult diet *Acetabularia acetabulum* – in contrast to the previous notion that they first require *Cladophora dalmatica*. The very first intake of chloroplasts from *A. acetabulum* by juvenile *E. timida* could be documented by transmission electron microscopy. Consequently, one algal food source was sufficient for the culture system. In trials within the system, *E. timida* was able to incorporate chloroplasts from *Acetabularia peniculus* – though not abundant in its natural environment – with comparable retention capabilities to chloroplasts from *A. acetabulum*. Furthermore, indications for potential influences of temperature on kleptoplasts' photosynthetic activity could be found in trials with *E. timida* within the controlled system. Also, retention with chloroplasts of defined origin and for longer periods than before could be documented. The project was performed at the Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf, Germany, and at the Observatoire Océanologique Banyuls-sur-Mer, France, supported by the European Community with an ASSEMBLE grant agreement no. 227799 to Valérie Schmitt and partly by funding of the German Science Foundation (Wa618/12) to Heike Wägele and an ERC advanced grant no. 232975 to William F. Martin.

FRONTIERS IN ZOOLOGY

Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of *Elysia timida* (Sacoglossa, Heterobranchia)

Schmitt *et al.*

BioMed Central

Schmitt *et al. Frontiers in Zoology* 2014, **11**:5
http://www.frontiersinzoology.com/content/11/1/5

FRONTIERS IN ZOOLOGY

RESEARCH Open Access

Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of *Elysia timida* (Sacoglossa, Heterobranchia)

Valerie Schmitt[1,2], Katharina Händeler[2], Susanne Gunkel[2], Marie-Line Escande[3], Diedrik Menzel[4], Sven B Gould[2], William F Martin[2] and Heike Wägele[1*]

Abstract

Introduction: The Mediterranean sacoglossan *Elysia timida* is one of the few sea slug species with the ability to sequester chloroplasts from its food algae and to subsequently store them in a functional state in the digestive gland cells for more than a month, during which time the plastids retain high photosynthetic activity (= long-term retention). Adult *E. timida* have been described to feed on the unicellular alga *Acetabularia acetabulum* in their natural environment. The suitability of *E. timida* as a laboratory model culture system including its food source was studied.

Results: In contrast to the literature reporting that juvenile *E. timida* feed on *Cladophora dalmatica* first, and later on switch to the adult diet *A. acetabulum*, the juveniles in this study fed directly on *A. acetabulum* (young, non-calcified stalks); they did not feed on the various *Cladophora spp.* (collected from the sea or laboratory culture) offered. This could possibly hint to cryptic speciation with no clear morphological differences, but incipient ecological differentiation. Transmission electron microscopy of chloroplasts from *A. acetabulum* after initial intake by juvenile *E. timida* showed different states of degradation — in conglomerations or singularly — and fragments of phagosome membranes, but differed from kleptoplast images of *C. dalmatica* in juvenile *E. timida* from the literature. Based on the finding that the whole life cycle of *E. timida* can be completed with *A. acetabulum* as the sole food source, a laboratory culture system was established. An experiment with PAM-fluorometry showed that cultured *E. timida* are also able to store chloroplasts in long-term retention from *Acetabularia peniculus*, which stems from the Indo-Pacific and is not abundant in the natural environment of *E. timida*. Variations between three experiment groups indicated potential influences of temperature on photosynthetic capacities.

Conclusions: *E. timida* is a viable laboratory model system to study photosynthesis in incorporated chloroplasts (kleptoplasts). Capacities of chloroplast incorporation in *E. timida* were investigated in a closed laboratory culture system with two different chloroplast donors and over extended time periods about threefold longer than previously reported.

Keywords: Endosymbiosis, Chloroplasts, Kleptoplasty, Photosynthetic sea slug, Solar powered sea slug, Sacoglossa, *Elysia*, Model organism

* Correspondence: h.waegele@zfmk.de
[1]Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany
Full list of author information is available at the end of the article

Introduction

The phenomenon of "long-term retention" of functional chloroplasts from food algae with ongoing photosynthesis inside the slugs' cells over longer time periods than a month only occurs in very few sea slug species [1-5]. Among animals, such kleptoplasty is only known for sacoglossan sea slugs [4-6]. Thus, these plastid bearing marine slugs are interesting in their own right, but they can further act as model organisms to study a special kind of "symbiosis". With few exceptions that we discuss below, most sacoglossan studies were performed on individuals collected from the sea, which implies that the history of the animals before collection, that is for example their age, repertoire of algae they have fed on or light conditions they have experienced is unknown. Transmission electron microscopy (TEM) and, more recently, molecular analyses provided insights into their food spectrum [4,7-15], but overall data is still sparse. Rumpho and coworkers kept the long-term retention species *Elysia chlorotica* Gould, 1870 [16] successfully in a laboratory culture system and characterized the entire life span of approximately ten months for the first time [3]. In a new study they report lab-reared cultures of regularly fed individuals they kept for more than two years, and without observing 'annual mortality' that earlier reports had documented [17]. Within their laboratory culture system they found that juveniles needed to feed on *Vaucheria litorea* (Agardh, 1823) [18] for at least seven days to establish kleptoplasty [17]. Curtis and coworkers [12] raised slugs hatched from egg masses laid in the laboratory by adult sea slugs, which were originally collected from the sea and fed the offspring after metamorphosis, but only for a limited period of time for subsequent experiments.

The laboratory culture of sea slugs permits long-term studies under controlled conditions und with animals of known individual history. It allows developmental investigations and reduces the burden on natural sea slug populations [3,17]. Laboratory culture can foster research progress on kleptoplast maintenance in slugs, which is still poorly understood [1-4,19-27]. Several interesting evolutionary adaptations in relation to long-term retention of chloroplasts are described for *Elysia timida* (Risso, 1818) [28], including positive phototaxis and closing or opening their parapodial lobes to modulate light flux [29-32]. Further, a physiological photo-regulation mechanism in form of the xanthophyll cycle to increase the maintenance of its photosynthetic capacities was also postulated [30].

Here we report the captive breeding and culturing of *E. timida,* which provides the opportunity to use this slug as a novel model organism to study feeding behavior, chloroplast sequestration, long-term kleptoplast retention and kleptoplast photosynthesis throughout the slugs' entire life cycle.

Results

Elysia timida individuals collected from the sea mated frequently under laboratory conditions and produced considerable amounts of egg masses (Table 1). The stability and orange coloration proved to be advantageous for culturing, as clutches could be recognized easily in the aquaria, removed and handled separately in petri dishes. Within short development periods of up to three weeks, the offspring developed in their egg capsules and hatched as free-swimming veliger or as crawling juveniles, with the larval shell still attached (Table 2). Final metamorphosis into shell-less, crawling juveniles took place within three to four days and without the presence of any algae. The whole development of freshly laid clutches into metamorphosed juveniles was completed within 24.8 ± 3.0 days on average in the example group of observed clutches in the marine laboratory Observatoire Océanologique Banyuls-sur-mer, and 20.0 ± 2.6 days in the example group of observed clutches later in the laboratory culturing system in Düsseldorf (Table 2). More than 100 eggs on average per single clutch were counted in an example group of 45 clutches in the laboratory culturing system (Table 3). In an exemplary clutch containing 215 eggs, the entire life cycle (Figure 1) was observed. After 106 days 122 individuals were still alive, translating into a survival rate of 57%. At this time point two new clutches were found in the glass bowl, indicating sexual maturity of the reared offspring.

Juvenile *E. timida* fed directly on the diet usually consumed by adult slugs, *A. acetabulum*, when young non-calcified stalks collected from the sea in the environment of adult slug populations were presented. In contrast to this, none of the hatched juveniles from more than 20 observed clutches fed on any *Cladophora spp.* sampled at the collection sites. This was confirmed through laboratory feeding trials with juveniles, which fed exclusively on *A. acetabulum* and not *C. dalmatica* or any of the other algae offered (Table 4). Based on these findings a closed laboratory culture system of *E. timida*, and which included simultaneous cultivation of *A. acetabulum*, was established (Figure 1). As plastids are not inherited by the offspring

Table 1 Reproduction under laboratory conditions

Tank group	1	2
N° individuals	92	87
Captivity period [days]	44	28
N° clutches	218	207
Average of clutches per day	4.95	7.39
Average of clutches per individual	2.37	2.38

Number of clutches in two different groups of adult *Elysia timida* kept at the marine laboratory OOB (Observatoire Océanologique Banyuls-sur-mer, France). The sea slugs were collected freshly from the sea and kept in two 5 1 tanks with aerated seawater and constant supply of *Acetabularia acetabulum* food algae.

Table 2 Development under laboratory conditions

	Marine laboratory (OOB) n = 37, 16–22°C		Laboratory culturing system (IME) n = 17, 20–23°C	
Metamorphosis state	**Mean**	**SD**	**Mean**	**SD**
Period since clutch deposition until hatching as veliger or juvenile with shell [days]	20.9	2.4	16.7	2.7
Metamorphosis from hatching into shell-less crawling juvenile [days]	3.8	1.0	3.3	1.9
Total development time from clutch deposition to shell-less crawling juvenile [days]	24.8	3.0	20.0	2.6

Time periods from egg laying to development of shell-less crawling juveniles of *Elysia timida* at the OOB (Oberservatoire Océanologique Banyuls-sur-mer, France) and IME (Institute for Molecular Evolution, Düsseldorf, Germany). SD standard deviation.

from the adult slugs, juveniles are transparent after hatching. Upon initial feeding on *A. acetabulum*, juveniles take up the green chloroplasts into the digestive gland revealing its bilateral structure (Figure 2; Additional file 1). The uptake of chloroplasts into the digestive glandular cells was documented by TEM (Figure 3) at two different time points: (1) juveniles were fixed 2–3 hours after feeding on *A. acetabulum* had commenced, and (2) a second group was preserved two days after permanent supply of *A. acetabulum*. In both cases chloroplasts in various states were found, in single and as aggregates. Juveniles that had been fixed 2–3 hours after their first chloroplasts meal and had been continuously feeding before fixation showed more single chloroplasts that still appeared intact than in a juvenile fixed two days after the beginning of feeding and constant food supply, in which more pronounced degradation was observed. Aggregates of chloroplasts surrounded by a phagosome membrane and with first signs of degradation were also visible in individuals fixed 2–3 hours after initiation of feeding (Figure 3C). While intact chloroplasts appeared to be embedded directly in the cytoplasm, chloroplasts in the process of degradation showed pronounced gaps between them and their surroundings and partially fragmented phagosome membranes. These gaps were more pronounced around aggregates of several chloroplasts (Figure 3A-F).

In order to examine if adult *E. timida* feed on other algae species and incorporate their chloroplasts, we designed a three-phased experiment (see material and methods for details). In total, 50 individuals from the laboratory culture kept on *A. acetabulum* were first starved until the photosynthetic yield values of Pulse Amplitude Modulated fluorometry (PAM)-measurements approached F_v/F_m values of zero (Phase 1, Figure 4A). Three different groups with different temperature background were used (for details see below). Subsequently, a set of different algae was offered in individual feeding trials lasting one month (Phase 2). These individuals were then again subjected to starvation and photosynthetic activity documented through PAM measurements (Phase 3). For the total group of 50 individuals, yield values remained on a high level of photosynthetic activity of about 0.8 during more than a month of starvation, reflecting high levels of intact chloroplasts (Figure 4A). PAM values then slowly decreased indicating gradual degradation of more and more chloroplasts, until the green coloration of the slugs bleached after about 90 days (88,56 ± 22,64 days; range: 42–135 days; n = 46 surviving until bleached and approaching zero). F_v/F_m values at this point approached zero on average.

The 50 individuals were investigated in three subsequent series during phase 1, with the first starting in winter, the second in spring and the third in summer (end of January, middle of March 2012 and end of June, respectively; Figure 4B). The first group of *E. timida* individuals (winter group; blue) was exposed to the overall coldest temperatures and revealed the best photosynthetic performance with yield values staying at high levels the longest. The spring series (Figure 4B; green) had the same maximum ambient temperature of 21.6°C measured as the first group, but was exposed on average to a slightly higher temperature. This group showed a slightly lower long-term photosynthetic activity than the winter group. The summer group (Figure 4B; red), which was exposed to the overall highest temperatures – especially during the first phase of the experiment – with highest measured values reaching 24.0°C, showed a faster decrease of photosynthetic activity (Figure 4B).

In total, 39 of the 50 specimens survived the first starvation period (Phase 1) and thus could be included into phase 2 of the experiment. Of these, nine specimens were fed on *A. acetabulum* and nine on *A. peniculus,* which led to an increase of photosynthetic yield values measured (Table 5). During the re-feeding phase (phase 2), individuals fed with *A. acetabulum* showed overall only slightly higher photosynthetic yield values of 0.76 ± 0.11 (range: 0.31-0.86), than those fed with *A. peniculus* (0.72 ± 0.09; range: 0.44-0.82). Like juveniles, the adults (15 individuals tested) did neither feed on *C. dalmatica* nor on other potential

Table 3 Reproductive output under laboratory conditions

	N° eggs per clutch
Mean	108.36
SD	52.75
Minimum	19
Maximum	249

Average, minimum and maximum numbers of eggs in spawn of *Elysia timida* (counted in an example group of 45 clutches).

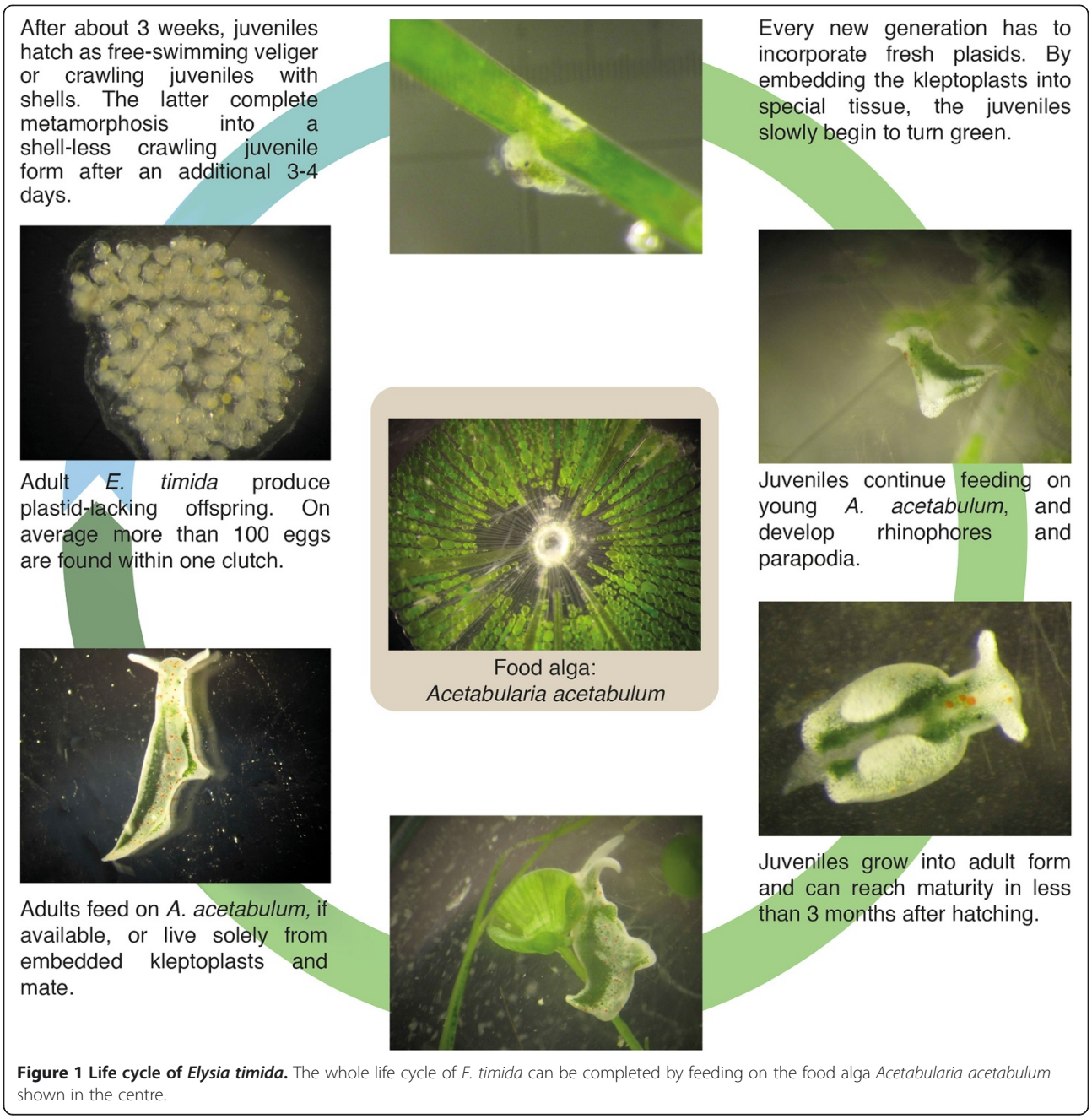


Figure 1 Life cycle of *Elysia timida*. The whole life cycle of *E. timida* can be completed by feeding on the food alga *Acetabularia acetabulum* shown in the centre.

Table 4 Algae consumption in juvenile *Elysia timida*

	Consumed by juveniles	N° positive/total in two trial series
Acetabularia acetabulum	yes	6/25 (n = 10 + 15)
Acetabularia peniculus	no	0/25 (n = 10 + 15)
Cladophora dalmatica	no	0/25 (n = 10 + 15)
Cladophora rupestris	no	0/25 (n = 10 + 15)

Feeding acceptance of juvenile *E. timida* in the laboratory culture system was tested. Two series with juveniles from two different clutches (40 individuals from the first clutch and 60 individuals from the second clutch) were performed and observed during 3 weeks. Feeding was determined by the green coloration through uptake of algal sap into the transparent juveniles.

plastid donors (*V. litorea* and *C. verticillata)*, the food of other long-term retention sea slugs species (*E. chlorotica* and *E. clarki*, respectively; Table 5). These individuals did not show a photosynthetic yield recovery and often quickly died despite of being supplied with the test algae.

All surviving individuals from phase 2 with a feeding phase of one month were subjected to a second starvation period. Out of the nine individuals from each alga, five (on *A. acetabulum*) and four (on *A. peniculus*) completed this second starvation phase (phase 3) until yield values again approached zero. Also the course of the

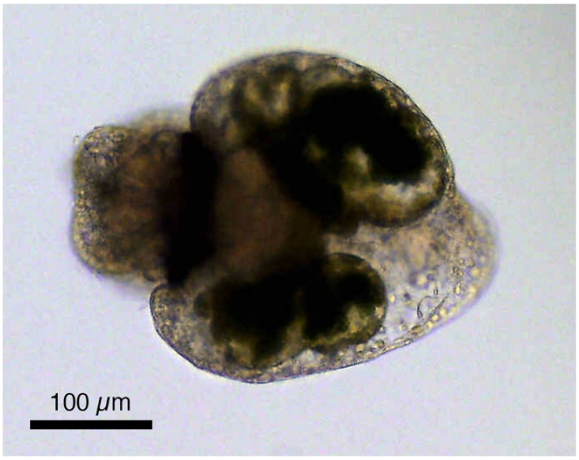


Figure 2 Juvenile *Elysia timida* after the first uptake of *Acetabularia acetabulum* cytosol. When transparent juveniles feed for the very first time on *A. acetabulum*, the cytosol of the algae including the plastids, fill up the digestive gland, thereby revealing its bilobed structure. The individual was fixed 2–3 hours after the first *A. acetabulum* meal, and about 5–6 days after hatching. The individual is oriented with its head to the left side, the digestive gland can be seen in the middle with the two balloon-like protrusions that are filled up and colored greenish by the plastids.

photosynthetic capacities during the subsequent starvation phase (phase 3) revealed similar retention characteristics. Yield values started to decrease slightly sooner in chloroplasts from *A. peniculus* on average, but individuals from both algal donor groups revealed high photosynthetic activity throughout approximately one month and subsequent parallel degradation (Figure 5A).

One individual lived for so long that after a first starving phase (phase 1) and new feeding of *A. peniculus* (phase 2 and subsequent phase 3), it could go through an additional feeding phase with *A. acetabulum* (Figure 5B). As it survived more than 8 months in the experiment — and had also grown up for some time previously in the laboratory system — it had reached an age of approximately ten months and was thus the individual of *E. timida* with the longest recorded life span in the laboratory so far. Comparing the two curves of photosynthetic activity during and after re-feeding with each algal plastid donor, demonstrates that photosynthetic capacities were very similar for chloroplasts of the two different algae species within the same individual (Figure 5B). PAM Yield values during the feeding phase were slightly higher after the second re-feeding with *A. acetabulum* with 0.78 ± 0.04 compared to 0.70 ± 0.07 after the first re-feeding with *A. peniculus*. The duration of chloroplast retention after the stop of feeding was very similar for the two different algal donors in this particular individual: Yield values approached zero after 47 days and 49 days of starvation after being fed with *A. peniculus* and *A. acetabulum,* respectively.

Discussion

Elysia timida individuals kept together in basins were observed to mate in their species-specific mating habit as previously described [33]. Furthermore, the typical orange coloration that egg masses of *E. timida* display due to extra-capsular yolk [15], as well as the size and relative stability of egg masses, is advantageous for culturing. Egg masses can be recognized easily in the basins and deposited and handled in separate containers, such as glass bowls. Marín and Ros [15] reported two seasonally varying development types for *E. timida* with intracapsular development into crawling juveniles during a short winter period, and lecithotrophic development with a short veliger phase of 3–4 days between hatching and metamorphosis in spring and autumn (with June to August missing) from Mazarrón Bay, Spain. Our observations from the first collections in Banyuls-sur-mer, France, in May/June 2010 also showed lecithotropic development with hatching of free-swimming veliger and crawling juveniles with a shell, which is cast off within 3–4 days. Single juveniles without a shell were rarely observed.

The development period from clutch deposition until hatching was longer in Banyuls-sur-mer (21 days) than in laboratory culture (17 days), which corresponded to the 16–18 days described by Marín and Ros [15]. This might have been influenced by temperature differences during clutch maturation, which was lower in Banyuls-sur-mer (16–22°C) than in the laboratory (20–23°C), again corresponding to the 20–24°C from Marín and Ros [15]. The lecithotrophic or intracapsular development with a metamorphosis induced without an external (algal) trigger is advantageous for culturing, since planktotrophic development imposes many more problems. It complicates water exchange due to free-swimming larvae and planktonic algae have to be provided, which implies additional effort and further possible difficulties. This was shown by Trowbridge [34] for *E. viridis*, and by Rumpho and coworkers [3,17] for *E. chlorotica*. In the latter species metamorphosis depends on the presence of filaments of the food algae for adult *E. chlorotica*, the heterokontophyte *Vaucheria litorea*.

The number of eggs in individual clutches in the laboratory culture system showed a slightly wider range (minimum of 19 to a maximum of 249) than reported by Marín & Ros [15] with 34–168 eggs per clutch, but ranges around a similar level. They counted 140 eggs per clutch on average, versus 108 in our laboratory system.

The close relationship between adult *E. timida* and its food algae *A. acetabulum* (Linnaeus) Silva, 1952 [35] was described recently [36]. Consistently, *E. timida* in laboratory culture accepted *A. acetabulum* and in our hands, juvenile *E. timida* were not observed to feed on *C. dalmatica* and *C. rupestris* purchased from a commercial supplier or other *Cladophora spp.* collected from the

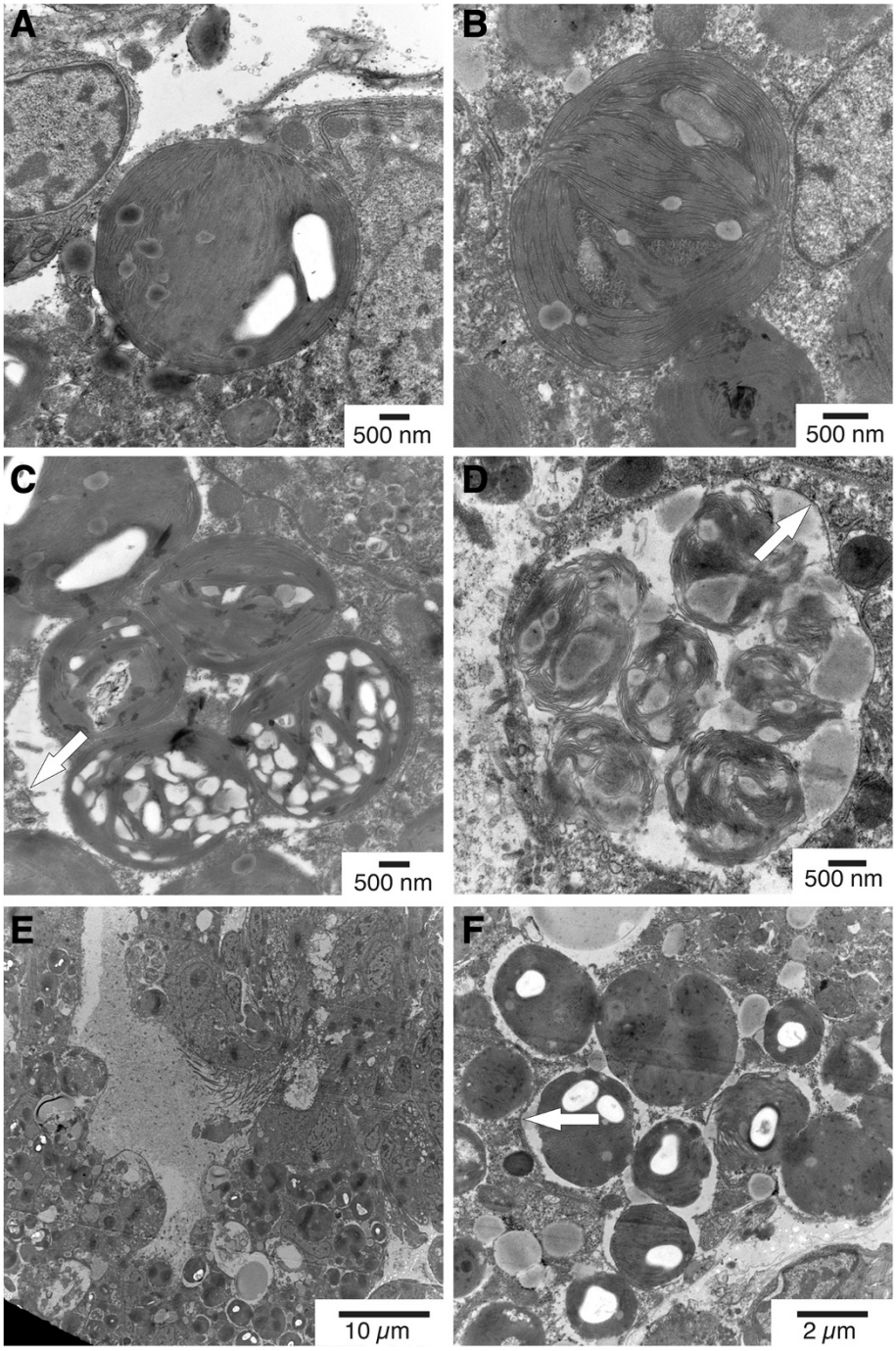


Figure 3 Ultrastructural investigations. Chloroplasts in digestive glandular cells in two different juvenile *Elysia timida* specimens fixed 2–3 h after the beginning of the very first feeding on *Acetabularia acetabulum* **(A,B,C)**. Chloroplasts in different states of degradation in juvenile *E. timida* fixed 2 days after the beginning of the very first feeding on *A. acetabulum* and free food supply until fixation **(D,E,F)**. Around some degrading chloroplasts gaps are evident and some are enclosed in conglomerations. Fragments of phagosome membranes are highlighted by arrows.

slugs' natural habitat. Instead, juvenile *E. timida* fed directly on *A. acetabulum*, if young non-calcified stalks of the algae were provided. By contrast, Marín and Ros reported that juvenile *E. timida* fed on *C. dalmatica* before switching to *A. acetabulum* as an adult diet [15]. Giménez-Casalduero et al. [37] reported several cases of variations in *E. timida*, differing in morphological, reproductive or other features, including genetic differentiations. Molecular markers will be needed to analyze whether feeding differences suggest incipient speciation in *E. timida* or not.

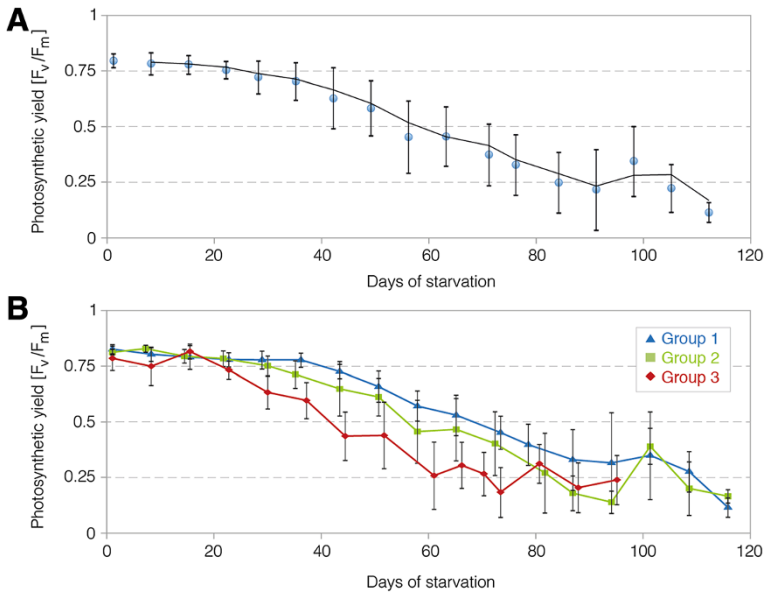


Figure 4 Photosynthetic long-term performance of *Acetabularia acetabulum* plastids in *Elysia timida* during starvation. **A)** Photosynthetic yields of PAM-measurements of all the 50 *E. timida* individuals included in the experiment. **B)** Photosynthetic yields of PAM-measurements, divided into the three different serial trial groups of *E. timida* individuals [Group1: start January 31st 2012, n = 24, temperature range: 19(or lower)-22°C; group 2: start March 13th 2012, n = 16, temperature range 20-22°C; group 3: start June 25th 2012, n = 10, temperature range: 20-24°C]. Regular interval temperature measurements started end of March to avoid overheating due to rising temperatures in spring and summer.

As can be seen in the video of juvenile *E. timida* feeding on young stalks of *A. acetabulum* (Additional file 1), the juveniles are first transparent and only become green upon incorporation of the first chloroplasts from *A. acetabulum*. The chloroplasts integrated into the digestive gland cells differed clearly from those of *Cladophora dalmatica* (Kützing 1843) [38] chloroplasts documented in the literature for juvenile *E. timida*, which had a distinct pyrenoid [15]. Only 2–3 hours after the first initiation of feeding, plastid degradation had already commenced as shown by our tissue fixed 2-3 hours after feeding start. This shows that chloroplasts ingested intact, are then quickly digested; most likely, as juveniles need a large and rapid nutrient supply for their growth and development. Marín and Ros [15] reported that "host membranes of the phagocytic vacuole" (p. 98) surrounded chloroplasts from *C. dalmatica* in juvenile *E. timida*. A distinct, complete phagocytic membrane around chloroplasts from *A. acetabulum* could not be clearly defined in our electron micrographs of juvenile *E. timida*, but fragments resembling phagosome membranes were recognizable. In some cases, chloroplasts seem to lie freely in the cytoplasm with direct contact to the cytosol, in others, however, a distant gap between chloroplast and cytoplasm was observed, resembling the gap around chloroplasts of *C. dalmatica* in juvenile *E. timida* displayed by Marín and Ros [15]. Around those gaps, and especially around aggregations of several chloroplasts, parts of an enclosing phagocytic membrane can be seen in our electron micrographs, pointing to a possible correlation between degradation (digestion) and the presence of a phagocytic membrane – which however has to be backed up by more investigations.

Table 5 Algae consumption in adult *Elysia timida*

	Fed on by adult *E. timida*	N° trials n = 39
Acetabularia acetabulum	yes	Positive: 9
		Negative: 2
		Unsure:1
Acetabularia peniculus	yes	Positive: 9
		Negative: 2
		Unsure:1
Cladophora dalmatica	no	3 (all negative)
Vaucheria litorea	no	8 (all negative)
Caulerpa verticillata	no	4 (all negative)

Feeding acceptance of *E. timida* for different alga as potential chloroplast donors was tested. Feeding success was determined by the increase of photosynthetic yield in PAM-measurements.

Evertsen et al. [11] described phagosome membranes around chloroplasts in the sea slug *Placida dendritica* (Alder und Hancock, 1843) [39], which underwent degradation, while intact chloroplasts in *Elysia viridis* (Montagu, 1804) [40] lie directly in the cytoplasm. This corresponds to the report from Marín and Ros [15] of phagocytic membranes around the chloroplasts that were probably about to be degraded in juvenile *E. timida*, in

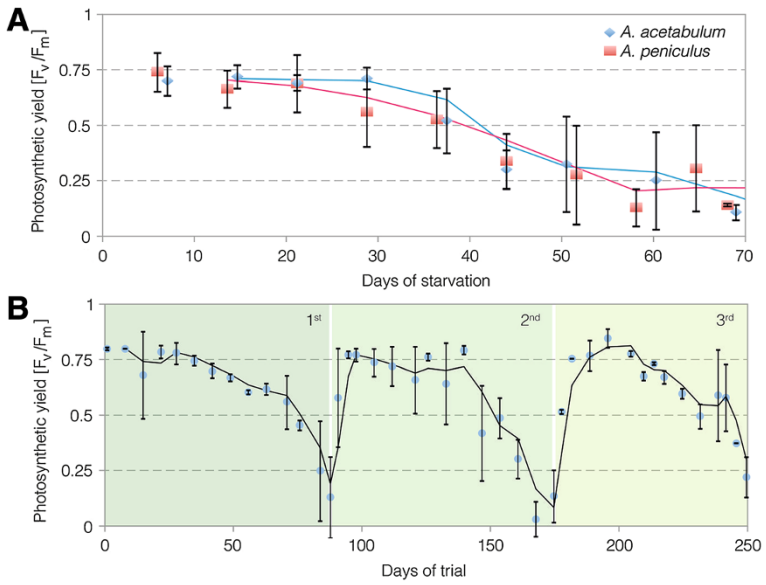


Figure 5 Photosynthetic long-term capacities of *Acetabularia acetabulum* and *A. peniculus* chloroplasts in *Elysia timida* during starvation. A) Long-term retention of chloroplasts from *A. acetabulum* and *A. peniculus* in *E. timida* during the second starvation phase. *E. timida* individuals had gone through a first starvation phase until depletion of former chloroplasts, than fed again for a month with either *A. acetabulum* or *A. peniculus* and consecutively measured for long-term retention during a second starvation phase with PAM fluorometry. Measurement days have been grouped. **B)** Long-term retention of chloroplasts from *A. acetabulum* and *A. peniculus* in an *E. timida* individual, which completed three starvation phases. First starvation reflects yields of chloroplasts from *A. acetabulum*, the second starvation phase those from *A. peniculus* and the third starvation phase again those from plastids of *A. acetabulum*. The curves of the second and third phase include the respective re-feeding phase of a month and the consecutive starvation phase. Means and standard deviations are calculated for three consecutive measurements per measurement day.

contrast to intact chloroplasts without an additional layer of phagocytic membranes in adult *E. timida*. Our assumption that chloroplasts of *A. acetabulum* in juvenile *E. timida* are first digested after their initial intake is also in accordance with new findings of Pelletrau et al. [17]: chloroplasts in juvenile *E. chlorotica* are first degraded and an initial feeding phase of a week was needed until degradation decreased and chloroplast incorporation was established. Juvenile *E. timida* in our laboratory culture system died within 2–3 weeks after metamorphosis, when no feeding occurred. As kleptoplasts in photosynthetic sea slugs are not inherited through the eggs, a new repertoire of kleptoplasts needs to be established by every generation [3,17].

PAM fluorometry is an established method to measure photosynthetic capacities of long-term functionality of incorporated chloroplasts in sea slugs [4-6,11,30,41-43]. The data presented here indicate that *E. timida* maintains chloroplasts from *A. acetabulum* as well as from *A. peniculus* (R. Brown ex Turner) Solms-Laubach 1895 [44] in a functional state under laboratory conditions. Giménez-Casalduero and coworkers reported that *E. timida* fed on other algal species during laboratory trials, but unfortunately they did not specify on which [37].

Measurements of photosynthetic activity during the first phase of laboratory culture correspond well to earlier data from *E. timida* specimens that were measured for three weeks after collection from the natural habitat [4]. However, our data indicate better long-term capacities when compared to other published PAM-data of *E. timida* [5,30]. Jesus and coworkers [30] showed data for complete starvation phases until approaching F_v/F_m values of zero for *E. timida* from Mar Menor and Mazarrón (Spain), but these individuals approached F_v/F_m values of zero after about 40 days of starvation in contrast to the roughly 90 days on average in our experiments. They kept the slugs under lower light conditions of 40 μmol quanta m^{-2} s^{-1} in 10 hours light per day in contrast to our 86 μmol quanta m^{-2} s^{-1} light for 12 hours a day in the experimental setting.

The three serial trial groups showed differences in photosynthetic capacities indicating a potential influence of the different temperature conditions the individuals were exposed to, and which reflected seasonal influences. The instantaneous temperature optimum for carbon fixation in *E. timida* from Mar Menor, Spain, is described to be 25°C [45]. However, differences in the temperature optimum can be attributed to geographic variation. Clark et al. [46] also reported a temperature optimum of 25°C for carbon fixation in the chloroplasts of the sea slug *Costasiella ocellifera* (Simroth, 1895) [47]. This species is exposed to higher temperatures in its natural habitat in Florida than *E. timida* from the coldest part of the

Mediterranean, namely Southern France. In contrast, Stirts and Clark stated an optimum of 15°C for maximum photosynthesis-based carbon fixation for *Elysia tuca* (Marcus and Marcus, 1967) [48], another chloroplast-equipped sea slug from Florida [49]. Further experiments focusing on the effect of temperature differences on long-term photosynthetic activity are however required to better understand the influence.

We were able to compare photosynthetic capacities of chloroplasts from two different algal donors in one and the same sea slug species. Long-term retention capacities with chloroplasts from both algal donors were very similar, showing that *E. timida* is able to store chloroplasts from an algal species that is – to our knowledge – not present in its natural habitat. How kleptoplasts stay photosynthetically active and are maintained in sea slugs over prolonged periods of time is still unresolved and remains the most intriguing aspect of the slug-kleptoplast association. Characteristics of the plastids alone cannot be the determining factor, as chloroplasts of the same food alga show different fates in different sea slug species. Such is the case for the alga *Codium fragile* (Hariot, 1889) [50], which serves as a food source for *Placida dendritica* that digests directly, as well as for *Elysia viridis* with short- to long-term retention of the kleptoplasts [11]. Christa et al. [7] showed that in the long-term retention sea slug *Plakobranchus ocellatus* van Hasselt, 1824 [51] only chloroplasts from one algal species (out of the originally 6 present) were likely contributing to photosynthesis after 64 days of starvation. This indicates differences in plastid characteristics across different algae. Retention of functional kleptoplasts of both algal donors in our study was shorter in the phases after renewed feeding than in the first starving phase after being taken from the culture system with constant feeding, possibly due to the advanced age of these animals and/or due to exhaustion from a complete depletion of plastids in the first phase of our experiments.

Conclusions

We have been able to maintain populations of *Elysia timida* in continuous culture since June 2010. The finding that juvenile *E. timida* fed directly on the adult diet *A. acetabulum* is eminent in the light of future analyses, since only a single food source system has to be provided during complete life cycles. We also demonstrated for the first time that *E. timida* is able to perform long-term retention in culture with an alternative algal chloroplast donor. Transmission electron microscopy on juvenile *E. timida* showed that chloroplasts from *A. acetabulum* are first taken up intact while feeding for the first time, but degradation processes already commence 2–3 hours after initial uptake of algal material. This is clear evidence that juveniles need to feed and digest, before long term incorporation is possible. Intact chloroplasts appeared to reside directly in the cytoplasm, whereas gaps and membranous fragments, maybe of phagosomal origin, were observed around single or conglomerations of chloroplasts in various states of degradation. In conclusion, *Elysia timida* proved to be a tractable laboratory culture model system, which opens up new possibilities to investigate long-term plastid retention.

Materials and methods

Preliminary investigations for the suitability of the model organism

Initial investigations were performed at the marine biological institute Observatoire Océanologique at Banyuls-sur-mer (OOB), France, from April to June 2010. The first generation of *E. timida* individuals (n = 179) for the laboratory culture was collected in depths of up to 2 m in proximity to the OOB, along with different algae from their natural environment, including *A. acetabulum*. Slugs and algae were kept in 5 1 tanks with supply of seawater from the institutional circulation system of the OOB. The aquaria were regularly checked for freshly laid clutches, which were carefully removed and transferred into petri dishes. As soon as veliger larvae had metamorphosed into crawling juveniles, feeding trials were performed including young non-calcified stalks of *A. acetabularia* as well as *Cladophora spp.* collected in the direct environment of the slugs, as *C. dalmatica* was formerly reported as a food source for juvenile *E. timida* [15].

Laboratory culture system

At the Institute for Molecular Evolution (IME), Heinrich-Heine-University of Düsseldorf, Germany, adult *E. timida* individuals were kept in 12 1 tanks in aerated artificial seawater (37–38 g/l, hw_Marinemix professional, hw-Wiegandt GmbH) in a climate chamber at 14–16°C. The water was partly renewed once a week, and in the meantime, evaporating water was replaced with demineralized freshwater from the laboratory system in order to keep a constant salinity level. Each tank was equipped with an aquarium pump to have a consistent water circulation and aeration. The influx area of the pumps was covered with filter cartridges to prevent slugs from streaming in and net barriers were installed to avoid getting slugs close to the pump. To provide free access to food algae, *A. acetabulum* was offered in the tanks in glass bowls covered by nets through which *E. timida* individuals could easily enter and exit but algae were kept in and prevented from floating. *A. acetabulum* was renewed when sucked out or looking old. The slugs were held under a light regime of 12 h to 12 h light/dark photoperiod in relatively low light intensities (tanks half-shaded with paper) of about 20–50 μmol quanta m^{-2} s^{-1} (PAR: photosynthetic active radiation, measured in water above bottom of tanks

where individuals were located) (neon tubes Osram L 58 W/840 LumiLux cool white). This resembles holding conditions recently reported by Pelletreau et al. [17] for their cultivation of *E. chlorotica* at 12:12 L:D cycle at 40 μmol quanta m^{-2} s^{-1} (measured at light-water interface). As they stated for *E. chlorotica* – and as is as well accurate for *E. timida* – optimal light intensities for maintenance of specimens have not yet been experimentally verified. The applied light regime for *E. timida* in our culture system ranged around the value of 31.33 μmol quanta m^{-2} s^{-1} reported by Giménez-Casalduero and Muniain [52] for rapid saturation of the photosynthetic apparatus of *E. timida* from the Mar Menor lagoon. In our laboratory conditions, *E. timida* individuals appeared to be in good condition and did not reveal signs of light stress.

Freshly laid clutches were carefully removed and transferred into glass bowls and kept either in the climate chamber (14–16°C) or at room temperature (~19-24°C according to season) and under a light intensity of about 20–30 μmol quanta m^{-2} s^{-1} (PAR, measured above the containers). The artificial seawater for the cultivation of the clutches was filtered with a sterile-filtering-apparatus (142 mm Edelstahl-Druckfiltrationsgerät, Sartorius Stedim Biotech GmbH, Germany). The water in the glass bowls was regularly exchanged in part until veliger larvae or juveniles hatched. When hatchlings had reached a crawling juvenile state, they were provided with young stalks of *A. acetabulum* and kept in the small containers until they had grown to an adult state and could be placed into a 12 1 tank.

The algae were also cultivated in the climate chamber (14–16°C) or in the laboratory room at room temperature (~19-24°C according to season) and additionally in climate boxes at 21°C, all with 12 h to 12 h day/light regime (neon cultivation tubes, approximately 130–200 quanta m^{-2} s^{-1}). For the medium of the algae, the artificial seawater (37–38 g/l, Tropic Marine Pro Reef, Zoo Zajac, Duisburg, or equivalent) was first filtered with a sterile-filtering-apparatus (142 mm Edelstahl-Druckfiltrationsgerät, Sartorius Stedim Biotech GmbH, Germany) and than enriched with f/2 medium (Guillards F/2 Marine Water, Sigma, 20 ml/l). Stock cultures of *A. acetabulum* (Mediterranean) and *A. peniculus* (Indopacific) were maintained according to Berger & Kaever [53].

Feeding experiments with juvenile *E. timida*

In preliminary feeding experiments with juvenile *E. timida* at the marine laboratory OOB, the selected algae were added to petri dishes with clutches from which juveniles were hatching and the direct reaction of the juveniles was observed through a stereomicroscope ($n \geq 20$ clutches). *A. acetabulum* and different *Cladophora spp.* that had been freshly collected from the sea in the surrounding area of *E. timida* populations were tested.

The feeding experiment with juvenile *E. timida* in the laboratory culture at the IME was performed in two series with offspring of two different clutches from the culture. Juveniles that had completed metamorphosis into the shell-less crawling juvenile form were carefully pipetted with a glass pipette (sterilized in boiling water) into four separate small glass bowls – 10 individuals per bowl from the first trial clutch, 15 individuals per bowl from the second trial clutch, respectively. In each of the four glass bowls one test algal species was added: *A. acetabulum*, *A. peniculus*, *C. dalmatica* or *C. rupestris*. The immediate reaction of the juveniles to the offered algae was observed through a stereomicroscope for more than 30 minutes per bowl. At this transparent state of the juveniles, feeding can clearly be determined by the intake of the green algal sap. Potential feeding progress was subsequently controlled every 2–3 days by recording green-colored versus transparent individuals for a period of 3 weeks. The feeding response to the laboratory-cultured *A. acetabulum* and *A. peniculus* was tested, and furthermore to *C. dalmatica,* which is described as a food source for juvenile *E. timida* in the literature [15]. *C. rupestris* was included as an additional *Cladophora*-species.

Long-term retention PAM fluorometry experiment

The experiment to observe capacities of long-term retention of chloroplasts from different algae in *E. timida* was performed in the laboratory culture system at the Institute for Molecular Evolution, from January 2012 to October 2012. In total, 50 individuals of *E. timida* from the laboratory culture were included (one additional individual had been excluded directly from the analysis as it died after only 7 days of observation). The overall 50 individuals were divided into three serial groups, the first starting January 31th 2012 ($n = 24$, temperature range: 19(or lower)-22°C), the second starting March 13th 2012 ($n = 16$, temperature range: 20–22°C) and the third starting June 25th 2012 ($n = 10$, temperature range: 19–24°C). Regular interval temperature measurements started end of March to avoid overheating due to rising temperatures in spring and summer.

During the experiment, the slugs were kept individually in petri dishes under a 12 h to 12 h dark/light regime with light intensities of 86 μmol quanta m^{-2} s^{-1} (PAR, measured in air above petri dishes) provided by full spectrum lamps (Androv Medicals, Germany). Photosynthetic activity was measured as maximum quantum yield of fluorescence for photosystem II with a Pulse Amplitude Modulated Fluorometer (Photosynthesis Yield Analyzer Mini PAM, version 2.0, WALZ, Germany) following the methods after Wägele and Johnsen [43]. For the measurement, the fibre optic was held above the slug with a distance of 0,5 – 1 cm covering the body region with the parapodia well with the sensor of a cross section of 5 mm. Three consecutive

measurements with the possibility to acclimate again in between were taken of each individual. As F_v/F_m values decreased considerably during the course of starving periods, the sensitivity of the Mini-PAM was individually adapted by putting the parameter 'outgain' from level 2 (default) to higher levels, up to level 8. Ambient light conditions were measured with a light sensor connected to the Mini-PAM (US-SQS/L, Walz, Germany).

The experiment was performed in the following 3 phases:

Phase 1: Individuals grown on *A. acetabulum* were held separately without any further food supply until yield values approached 0, assuming that incorporated chloroplasts were degraded to a non-functional state. Phase 2: Individuals were than allowed to feed on the different newly provided test algae for a month in order to assure that they recovered completely from the starving period and could fully replenish with new chloroplasts to a state of storing. *A. acetabulum* and *A. peniculus* from the culture system were provided to compare two related species of which one is not the natural food due to separate geographic distribution. *Cladophora dalmatica*, described as a food source for juvenile *E. timida* [15], was also comparatively provided. Furthermore, *Vaucheria litorea* was offered as a comparative food alga (*V. litorea* K-0379, SCCAP (Scandinavian Culture Collection of Algae and Protozoa)), as it is described as chloroplast donor of *E. chlorotica* with extensive durations of long-term retention of chloroplasts [3,25-27]. Furthermore, *Caulerpa verticillata* (collected at the Mote Laboratory, Florida Keys, USA) was included because it was observed to be a potential chloroplast donor for long-term retention in *Elysia clarki* (unpublished results VS and HW). Feeding on the various algae was supervised by measuring photosynthetic activity. Phase 3: After the feeding phase of one month, the food algae were removed and the long-term retention photosynthetic performance of the individuals with the newly incorporated chloroplasts was surveyed by regular PAM fluorometry measurements. For the evaluation of PAM fluorometry data, means and standard deviations were calculated first for the three consecutive measurements per individual per day and then grouped for the respective group analyses.

Transmission electron microscopy

For electron microscopic examinations of the very first incorporation of chloroplasts, juvenile *E. timida* were fixed in a mix of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M Cacodylate buffer after observed feeding on *A. acetabulum* in two time series: the first after 2–3 hours since the beginning of feeding and continued feeding until fixation; the second after 2 days since the beginning of feeding and continued free access to the food alga. The samples were post-fixed with 1% OsO_4 and dehydrated in an ethanol series and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed at 80KV in the transmission electron microscope (Hitachi H7500) at the OOB.

Availability of supporting data

The data sets supporting the results of this article are available by the responsible author (HW).

Additional file

Additional file 1: Video 1. Juvenile *Elysia timida* feeding. Metamorphosed into the shell-less juvenile state, young transparent *E. timida* feed for the first time – on young stalks of *Acetabularia acetabulum* – and turn green through the incorporated chloroplasts.

Abbreviations

IME: Institute for molecular evolution; OOB: Observatoire Océanologique Banyuls-sur-mer; PAM: Pulse amplitude modulated; TEM: Transmission electron microscopy.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

VS performed collections and preliminary investigations to start the laboratory culturing system, maintenance of the culture system, feeding experiments, video recordings and TEM investigations of juveniles, the long-term retention PAM-experiment, data processing and drafted the manuscript. KH participated in the design of the study, established protocols for the laboratory culturing system and partly supervised the establishing of the laboratory culture system by SG. SG performed collections and preliminary investigations to start the laboratory culture system, interpretation of data and established the culture methods at the laboratory. MLE performed TEM investigations of juveniles with VS and assisted in interpreting the results. DM established the culture of *A. acetabulum* and *A. peniculus* and provided specimens and protocols for culturing. WM and SBG initiated, designed and supervised the laboratory culturing system for the slugs and revised data interpretation and final analysis. HW initiated the study, collected material, designed experiments and helped in data interpretation and drafting the manuscript. All authors have revised the manuscript critically for important intellectual content and approved the final manuscript.

Authors' information

VS is PhD student under the supervision of HW and worked in a collaboration project with the culture system at the department of WM. KH was postdoctoral student at the Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf. SG was diploma student at the Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf. MLE is technical assistant for microscopy at the Observatoire Océanologique, Banyuls-sur-mer. DM is professor at the Institut für Zelluläre und Molekulare Botanik, Rheinische Friedrich-Wilhelms-Universität Bonn. SBG is postdoc in the group of WM, who is professor at the Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf. HW is professor at the Zoologisches Forschungsmuseum Alexander Koenig, Bonn.

Acknowledgements

The project was supported by the European Community with an ASSEMBLE grant agreement no. 227799 to VS, a grant of the German Science Foundation to HW (Wa618/12), and an ERC advanced grant no. 232975 to WM. Furthermore, we sincerely thank the Observatoire Océanologique Banyuls-sur-mer for providing laboratory facilities. We also thank Daniel Niederau for assistance with the laboratory culture. We thank Margarete Stracke (Düsseldorf) for excellent technical work on slug cultivation and Rainer Martin for his initial help in TEM preparations.

Author details

[1]Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany. [2]Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf,

Germany. [3]Observatoire Océanologique, 66651 Banyuls-sur-mer, France. [4]Institut für Zelluläre und Molekulare Botanik, Zellbiologie der Pflanzen, Rheinische Friedrich-Wilhelms-Universität Bonn, Kirschallee 1, 53115 Bonn, Germany.

Received: 24 October 2013 Accepted: 10 January 2014
Published: 16 January 2014

References

1. Wägele H, Martin W: **Endosymbioses in sacoglossan seaslugs: Photosynthetic animals that keep stolen plastids without borrowing genes.** In *Endosymbiosis.* Edited by Löffelhardt W. Heidelberg: Springer. in press.
2. Pelletreau KN, Bhattacharya D, Price DC, Worful JM, Moustafa A, Rumpho ME: **Sea slug kleptoplasty and plastid maintenance in a metazoan.** *Plant Physiol* 2011, **155:**1561–1565.
3. Rumpho ME, Pelletreau KN, Moustafa A, Bhattacharya D: **The making of a photosynthetic animal.** *J Exp Biol* 2011, **21:**303–311.
4. Wägele H, Deutsch O, Händeler K, Martin R, Schmitt V, Christa G, Pinzger B, Gould SB, Dagan T, Klussmann-Kolb A, Martin W: **Transcriptomic evidence that longevity of acquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobranchus ocellatus* does not entail lateral transfer of algal nuclear genes.** *Mol Biol Evol* 2011, **28:**699–706.
5. Händeler K, Grzymbowski Y, Krug PJ, Wägele H: **Functional chloroplasts in metazoan cells – a unique evolutionary strategy in animal life.** *Front Zool* 2009, **6:**28.
6. Evertsen J, Burghardt I, Johnsen G, Wägele H: **Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean.** *Mar Biol* 2007, **151:**2159–2166.
7. Christa G, Wescott L, Schäberle T, König GM, Wägele H: **What remains after 2 months of starvation of sequestered algae in a photosynthetic slug, *Plakobranchus ocellatus* (Sacoglossa, Opisthobranchia), by barcoding.** *Planta* 2013, **237:**559–572.
8. Maeda T, Hirose E, Chikaraishi Y, Kawato M, Takishita K, Yoshida T, Verbruggen H, Tanaka J, Shimamura S, Takaki Y, Tsuchiya M, Iwai K, Maruyama T: **Algivore or phototroph? *Plakobranchus ocellatus* (Gastropoda) continuously acquires kleptoplasts and nutrition from multiple algal species in nature.** *PLoS One* 2012, **7**(7):e42024. doi:10.1371/journal.pone.0042024.
9. Händeler K, Wägele H, Wahrmund U, Rüdinger M, Knoop V: **Slugs' last meals: molecular identification of sequestered chloroplasts from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda).** *Mol Ecol Resour* 2010, **10:**968–978.
10. Curtis NE, Schwartz JA, Pierce SK: **Ultrastructure of sequestered chloroplasts in sacoglossan gastropods with differing abilities for plastid uptake and maintenance.** *Invert Biol* 2010, **129**(4):297–308.
11. Eversten J, Johnsen G: **In vivo and in vitro differences in chloroplast functionality in the two north Atlantic sacoglossans (Gastropoda, Opisthobranchia) *Placida dendritica* and *Elysia viridis*.** *Mar Biol* 2009, **156:**847–859.
12. Curtis NE, Pierce SK, Massey SE, Schwartz JA, Maugel TK: **Newly metamorphosed *Elysia clarki* juveniles feed on and sequester chloroplasts from algal species different from those utilized by adult slugs.** *Mar Biol* 2007, **150:**797–806.
13. Curtis NE, Massey SE, Pierce SK: **The symbiotic chloroplasts in the sacoglossan *Elysia clarki* are from several algal species.** *Invert Biol* 2006, **125**(4):336–345.
14. Curtis NE, Massey SE, Schwartz JA, Maugel TK, Pierce SK: **The intracellular, functional chloroplasts in adult sea slugs (*Elysia crispata*) come from several algal species, and are also different from those in juvenile slugs.** *Microsc Microanal* 2005, **11**(Suppl2):1194–1195.
15. Marín A, Ros J: **Ultrastructural and ecological aspects of the development of chloroplast retention in the sacoglossan gastropod *Elysia timida*.** *J Mollus Stud* 1993, **59:**95–104.
16. Gould AA: *Report on the Invertebrata of Massachusetts. Second Edition, comprising the Mollusca.* Boston: Wright and Potter; 1870.
17. Pelletreau KN, Worful JM, Sarver KE, Rumpho ME: **Laboratory culturing of *Elysia chlorotica* reveals a shift from transient to permanent kleptoplasty.** *Symbiosis* 2012, **58:**221–232.
18. Agardh CA: *Species Algarum Rite Cognitae, cum Synonymis, Differentiis Specificis et Descriptionibus Succinctis. Volumen Primum Pars Posterior.* Lundea [Lund]: Ex officina Berlingiana; 1823:399–531.
19. Pierce SK, Fang X, Schwartz JA, Jian X, Zhao W, Curtis NE, Kocot K, Yang B, Wang J: **Transcriptomic evidence for the expression of horizontally transferred algal nuclear genes in the photosynthetic sea slug *Elysia chlorotica*.** *Mol Biol Evol* 2012, **29:**1545–1556.
20. Schwartz JA, Curtis NE, Pierce SK: **Using algal transcriptome sequences to identify transferred genes in the sea slug, *Elysia chlorotica*.** *Evol Biol* 2010, **37:**29–37.
21. Pierce SK, Curtis NE, Schwartz JA: **Chlorophyll a synthesis by an animal using transferred algal nuclear genes.** *Symbiosis* 2009, **49:**121–131.
22. Rumpho ME, Worful JM, Lee J, Kannan K, Tylor MS, Bhattacharya D, Moustafa A, Manhart JR: **Horizontal gene transfer of the algal nuclear gene psbO to the photosynthetic sea slug *Elysia chlorotica*.** *Proc Natl Acad Sci* 2008, **105:**17867–17871.
23. Pierce SK, Curtis NE, Hanten JJ, Boerner SL, Schwartz JA: **Transfer, integration and expression of functional nuclear genes between multicellular species.** *Symbiosis* 2007, **43:**57–64.
24. Rumpho ME, Summer EJ, Green BJ, Fox TC, Manhart JR: **Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to function for months in the cytosol of a sea slug in the absence of an algal nucleus?** *Zoology* 2001, **104:**303–312.
25. Rumpho ME, Summer EJ, Manhart JR: **Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis.** *Plant Physiol* 2000, **123:**29–38.
26. Pierce SK, Biron RW, Rumpho ME: **Endosymbiotic chloroplasts in molluscan cells contain proteins synthesized after plastid capture.** *J Exp Biol* 1996, **199:**2323–2330.
27. Mujer CV, Andrews DL, Manhart JR, Pierce SK, Rumpho ME: **Chloroplast genes are expressed during intracellular symbiotic association of *Vaucheria litorea* plastids with the sea slug *Elysia chlorotica*.** *Proc Natl Acad Sci* 1996, **93:**12333–12338.
28. Risso A: **Mémoire sur quelques gastéropodes nouveaux, nudibranches et tectibranches observés dans la Mer de Nice.** *J Phys Chim Hist Nat Arts* 1818, **87:**368–377.
29. Schmitt V, Wägele H: **Behavioural adaptation in relation to long-term retention of endosymbiotic chloroplasts in the sea slug *Elysia timida* (Opisthobranchia, Sacoglossa).** *Thalassas* 2011, **27**(2):226–238.
30. Jesus B, Ventura P, Calado G: **Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanism in the solar-powered sea slug *Elysia timida* (Risso, 1818).** *J Exp Mar Bio Ecol* 2010, **395:**98–105.
31. Monselise EBI, Rahat M: **Photobiology of *Elysia timida* (Mollusca: Opisthobranchia): observations in the sea.** *Isr J Zool* 1980, **29:**125–128.
32. Rahat M, Monselise EBI: **Photobiology of the chloroplast-hosting mollusc *Elysia timida* (Opisthobranchia).** *J Exp Biol* 1979, **79:**225–233.
33. Schmitt V, Anthes N, Michiels NK: **Mating behavior in the sea slug *E. timida* (Opisthobranchia, Sacoglossa): hypodermic injection, sperm transfer and balanced reciprocity.** *Front Zool* 2007, **4:**17. doi:10.1186/1742-9994-4-17.
34. Trowbridge CD: **The missing links: larval and post-larval development of the ascoglossan opisthobranch *Elysia viridis*.** *J Mar Biol Ass UK* 2000, **80:**1087–1094.
35. Silva PC: **A review of nomenclatural conservation in the algae from the point of view of the type method.** *Univ Calif Publ Botany* 1952, **25:**241–323.
36. Marín A, Ros J: **Dynamics of a plant-herbivore relationship: the photosynthetic ascoglossan *Elysia timida* and the chlorophycean *Acetabularia acetabulum*.** *Mar Biol* 1992, **112:**667–682.
37. Giménez-Casalduero F, Muniain C, González-Wangüemert, Garrote-Moreno A: ***Elysia timida* (Risso, 1818) three decades of research.** *Anim Biodivers Conserv* 2011, **34**(1):217–227.
38. Kützing FT: *Phycologia Generalis Oder Anatomie, Physiologie und Systemkunde der Tange… Mit 80 Farbig Gedruckten Tafeln, Gezeichnet und Graviert vom Verfasser. pp. [Part 1]: [i]-Xxxii, [1]-142, [Part 2]: 143–458, 1, err.], pls 1–80.* Leipzig: F.A. Brockhaus; 1843.
39. Alder J, Hancock A: **Notice on a British species of *Calliopaea* d'Orbigny, and on four new species of *Eolis* with observations on the development and structure of the nudibranchiate Mollusca.** *Ann Mag Nat Hist* 1843, **12:**233–238.
40. Montagu G: **An account of some new and rare British shells and animals.** *Trans Linn Soc London* 1815, **11:**179–204.
41. Middlebrooks ML, Pierce SK, Bell SS: **Foraging behavior under starvation conditions is altered via photosynthesis by the marine gastropod, *Elysia clarki*.** *PLoS One* 2011, **6:**e22162. doi:10.1371/journal.pone.0022162.
42. Vieira S, Calado G, Coelho H, Serôdio J: **Effects of light exposure on the retention of kleptoplastic photosynthetic activity in the sacoglossan mollusc *Elysia viridis*.** *Mar Biol* 2009, **156:**1007–1020.

43. Wägele H, Johnsen G: **Observations on the histology and photosynthetic performance of "solar-powered" opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae.** *Org Divers Evol* 2001, **1:**193–210.
44. Solms-Laubach H: **Monograph of the Acetabularieae.** *Transactions of the Linnean Society of London, Second Series, Botany* 1895, **5:**1–39. pls 1–4.
45. Marín A, Ros J: **The chloroplast-animal association in four Iberian Sacoglossan Opisthobranchs: *Elysia timida, Elysia translucens, Thuridilla hopei* and *Bosellia mimetica*.** *Scient Mar* 1989, **53:**429–440.
46. Clark KB, Jensen KR, Stirts HM, Fermin C: **Chloroplast symbiosis in a non-elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa): Effects of temperature, light intensity and starvation on carbon fixation rate.** *Biol Bull* 1981, **160:**43–45.
47. Simroth H: **Neuere Arbeiten über Opisthobranchien.** *Zool Centralbl* 1895, **2:**513–515.
48. Marcus E, Marcus E: **American opisthobranch molluscs Part I, Tropical American Opisthobranchs.** *Stud Trop Oceanogr Miami* 1967, **6:**1–256.
49. Stirts HM, Clark KB: **Effects of temperature on products of symbiotic chloroplasts in *Elysia tuca* Marcus (opisthobranchia: ascoglossa).** *J Exp Mar Bio Ecol* 1980, **43:**39–47.
50. Hariot P: *Mission Scientifique du Cap Horn. 1882–1883. Botanique I. Algues.* Paris: Gauthier-Villars et fils; 1889.
51. Hasselt JC, v In: Férussac A: **Extrait d'une lettre du Dr. J.C. van Hasselt au Prof. van Swinderen sur mollusques de Java (traduit de l'Allgem. konst en letterbode, 1824, nos. 2,3,4) Tjuringe (ile Java) le 25 Mai 1823 (1).** *Bull Sci Nat Géol* 1824, **3:**237–248.
52. Giménez-Casalduero F, Muniain C: **Photosynthetic activity of the solar-powered lagoon mollusk *Elysia timida* (Risso, 1818) (Opisthobranchia: Sacoglossa).** *Symbiosis* 2006, **41:**151–158.
53. Berger S, Kaever J: *Dascycladades: An Illustrated monograph of A Fascinating Algal Order.* Stuttgart, New York: Georg Thieme Verlag; 1992:213–218.

doi:10.1186/1742-9994-11-5
Cite this article as: Schmitt *et al.*: Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of *Elysia timida* (Sacoglossa, Heterobranchia). *Frontiers in Zoology* 2014 **11**:5.

3.3 Unpublished results: Kleptoplast photosynthetic activity and photobehavior in different sacoglossan sea slugs (Heterobranchia, Mollusca) in near-natural and natural settings (manuscript draft)

This chapter presents yet unpublished results in form of a manuscript draft as first author with the following major contributions: Valérie Schmitt performed all investigations and analyses and drafted the manuscript as first author in the frame of the Ph.D. thesis. Heike Wägele is supervisor of the Ph.D. thesis and supported investigations, analyses and manuscript drafting and made comments on the manuscript. One part of the investigations was performed at the Mote Marine Laboratory, field station Summerland Key, Florida, USA, where Gregor Christa participated in a part of the measurements and afterwards with comments on the manuscript. A part of the investigations was performed in the frame of a collaboration project under additional supervision of William F. Martin at the Institut für Molekulare Evolution, Heinrich-Heine-Universität Düsseldorf, Germany. There, Margarete Stracke participated in a part of the measurements and is acknowledged for excellent co-work as a technical assistant. The major part of investigations was carried out in the frame of the ASSEMBLE program (grant agreement no. 227799 to Valérie Schmitt) at the Observatoire Océanologique at Banyuls-sur-Mer, France. (For complete acknowledgements, see the general entire acknowledgements of this thesis in chapter 6.)

The manuscript comprises an extract of results of investigations during several years, focusing on investigations in natural and near-natural or semi-natural settings. Several further selected results connected to these investigations are presented in the further result section in chapter 3.4.

Abstract

Retention of functional chloroplasts from their food algae in sacoglossan sea slugs – which is also named kleptoplasty – is still a challenge for research, with many questions unsolved. One question concerns differences between sacoglossan species in their capacities of retention of functional chloroplasts. This study presents data on the ongoing functioning of incorporated chloroplasts within the slug cells, in relation to behavioral and ecological aspects in several chloroplast-incorporating sea slugs, including species which are known as the "top-performers" of long-term functional retention of chloroplasts: *Elysia timida, Elysia crispata* (*mangrove type* and *reef type*)*, Elysia viridis* and *Plakobranchus ocellatus*. For comparison, *Bosellia mimetica, Thuridilla hopei* and *Placida dendritica* were included. For *P. ocellatus*, we report measurements (PAM, Pulse Amplitude Modulated Fluorometer) of functional chloroplast retention for over seven months, the longest time period documented up to now. We can confirm extreme differences between the various sea slug species with regard to capacities of retention of functional chloroplasts, including variations between the two eco-morphotypes *E. crispata mangrove type* and *reef type*. Capacities of kleptoplast retention in *E. viridis* were shorter than expected from former reports. We conducted an analysis testing a former hypothesis that chloroplast-retention implies stronger phototactic behavior in sea slugs, in which the non-sacoglossan sea slug species *Cratena peregrina* and *Flabellina affinis* were additionally included as comparison without incorporation of chloroplasts. As one result, some sea slug species without chloroplasts or with rather fast digestion of chloroplasts reacted more positively phototactic than some species with long-term kleptoplast retention. *E. timida* and *E. crispata mangrove type* were investigated underwater with a Diving PAM Fluorometer in their natural habitat in France and in Florida, respectively. We found distinct differences between the two sea slug species concerning environmental parameters and photosynthetic activities. Photosynthetic activities of chloroplasts in both sea slug species and in the food algae of *E. timida, Acetabularia acetabulum,* varied depending on natural light conditions in the sea. These represent to our knowledge the first photosynthetic measurements of sea slugs in their natural environment published so far.

Introduction

Retention of functional chloroplasts, also called 'kleptoplasty', is within metazoans only known from sacoglossan sea slugs (Wägele and Johnsen 2001, Händeler, Grzymbowski et al. 2009, Pelletreau, Bhattacharya et al. 2011, Wägele and Martin 2013, Christa, Händeler et al. 2014). Despite an increase of investigations on this phenomenon, many questions are still unsolved – especially with regard to underlying mechanisms as well as differences in the ability to maintain functional chloroplasts (Wägele and Martin 2013, Cruz, Cartaxana et al. 2015, Chan, Vaysberg et al. 2018, Melo Clavijo, Donath et al. 2018). These differences range between different sacoglossan species from fast digestion of chloroplasts over retention for weeks up to several months or possibly a life span (Evertsen, Burghardt et al. 2007, Händeler, Grzymbowski et al. 2009, Pelletreau, Bhattacharya et al. 2011, Wägele, Deusch et al. 2011, Wägele and Martin 2013, Christa, Händeler et al. 2015). To examine these differences and potential influencing factors, we performed comparative investigations on photosynthetic activity, behavior and ecology of species with different capacities of chloroplast retention, including most of the few species which are known as "top-performers" of long-term functional retention of chloroplasts: *Elysia timida, Elysia crispata, Elysia viridis* and *Plakobranchus ocellatus*. Of *E. crispata,* we compared two different types – a *mangrove type* and a *reef type,* according to the identification by Krug et al. of *E. crispata* as one species with various differentiated morphotypes (Krug, Vendetti et al. 2016). To compare photosynthetic activities between the various species, we conducted analyses with a Pulse Amplitude Modulated Fluorometer (PAM), an established method to explore photosynthetic activity in sea slugs (Wägele and Johnsen 2001, Evertsen, Burghardt et al. 2007, Evertsen and Johnsen 2009, Händeler, Grzymbowski et al. 2009, Vieira, Calado et al. 2009, Jesus, Ventura et al. 2010).

In general, intensive investigations of sea slugs have already been carried out in the laboratory or even in specialized laboratory cultures with the advantages of controlled conditions (Rumpho, Pelletreau et al. 2011, Pelletreau, Worful et al. 2012, Bhattacharya, Pelletreau et al. 2013, Pelletreau, Weber et al. 2014, Schmitt, Händeler et al. 2014, Laetz and Wägele 2017, Chan, Vaysberg et al. 2018). To gain knowledge about conditions of the sea slugs in their natural environment and ecological relations, however, investigations on site and under near-natural conditions are essential. Thus, investigations of real life parameters of photosynthetic sea slugs in their natural environment and under near-natural conditions are in a special focus in this present study. As one factor, behavioral adaptations of the sea slugs are assumed to

have developed and play a potential role for the functionality of chloroplasts, e. g. in the species *E. timida* with a modulation of opening posture of the parapodial lobes as a potential regulation mechanism toward light (Rahat and Monselise 1979, Monselise and Rahat 1980, Jesus, Ventura et al. 2010, Schmitt and Wägele 2011) or also including more species and further photobehavioral parameters, e. g. phototaxis (Weaver and Clark 1981, Schmitt and Wägele 2011). A recent study described aspects of photobehavior in *E. viridis* and *Placida dendritica*, including acclimation to different light regimes (Cartaxana, Morelli et al. 2018). A broad overview of several sea slugs species regarding phototaxis is lacking up to now, however. To test the assumption that chloroplast-retention possibly implies stronger phototactic behavior in sea slugs, we performed a photobehavior trial in parallel to the analyses of photosynthetic activity profiles with the same spectrum of sea slug species in focus. Additionally, the non-sacoglossan sea slug species *Cratena peregrina* and *Flabellina affinis*, carnivore nudibranchs feeding on hydrozoans, were included in this experiment as comparison without sequestration of chloroplasts.
Observations concerning the photobiology – especially photobehavior – of *E. timida* in the sea are described by Monselise and Rahat (Monselise and Rahat 1980), but otherwise reports on photobiological observations on sacoglossan sea slugs in their natural environment are scarce. Consequently, we performed underwater investigations of two sea slug species, *E. timida* and *E. crispata (mangrove type),* concerning photobiology including ecological and behavioral parameters directly in their natural habitats by diving with a Diving PAM Fluorometer, and report to our knowledge the first PAM-measurements of sea slugs in their natural environment published so far.

Methods

Collections and maintenance of sea slugs

Seven sacoglossan species with different capacities of functional chloroplast retention were investigated in this study, including the two different types *mangrove type* and *reef type* of *Elysia crispata,* after Krug et al. (Krug, Vendetti et al. 2016) and individuals of the species complex *Plakobranchus ocellatus* after Krug et al. (Krug, Vendetti et al. 2013) (Figure 3.3.1).

Figure 3.3.1: The sacoglossan sea slug species in the focus of this study (from up to down, left to right): *Plakobranchus ocellatus* (Cebu, Philippines)*, Elysia crispata mangrove type* and *Elysia crispata reef type* (Florida Keys, USA), and *Elysia timida, Elysia viridis, Bosellia mimetica, Placida dendritica* and *Thuridilla hopei* (Banyuls-sur-mer, France).

The two nudibranch species *Cratena peregrina* and *Flabellina affinis* without incorporation of chloroplasts were additionally included as a comparison in the photobehavior analyses (see below).

The 32 examined individuals of *P. ocellatus* were provided by an aquarium specialist (Frank Richter Meerwasseraquaristik, Chemnitz, Germany) from collections near Cebu, Philippines, end of November 2011. Specimens were kept at the Institute for Molecular Evolution (IME), Heinrich-Heine-University of Düsseldorf, Germany, in 25 1 tanks in aerated artificial seawater under a light regime of 12 h / 12 h light / dark photoperiod in relatively low light intensities (tanks half-shaded with paper) of about 20–50 µmol quanta m^{-2} s^{-1} (PAR: photosynthetic active radiation) (neon tubes Osram L 58 W/840 LumiLux cool white) in a climate chamber at temperatures ranging around 14–22 °C in the course of the period of several months until June 2012, attempting to keep temperature mostly constant above 20 °C with installations of aquarium heaters set at 22 °C, thus attempting to adapt temperature to the origin environment, as well as salinity (around 35 g/l). This is approximately similar to conditions described by Pelletreau et al. (Pelletreau, Worful et al. 2012) for culturing *Elysia chlorotica* and also those applied for *P. ocellatus* in other investigations (Christa, Wescott et al. 2013) – with our temperature spectrum ranging between the lower and higher temperatures applied in these investigations, respectively. For further details on conditions in the laboratory system at the IME see Schmitt et al. (Schmitt, Händeler et al. 2014). For phototactic trials and PAM-measurements, *P. ocellatus* individuals were temporarily taken out of the basins and transferred into an observatory basin or individually in petri-dishes, respectively, at room temperature (range: ~19-22 °C) and replaced back into the holding basins thereafter.

Elysia timida, Elysia viridis, Placida dendritica, Bosellia mimetica, Thuridilla hopei, Cratena peregrina and *Flabellina affinis* were investigated at the Oberservatoire Océanologique at Banyuls-sur-mer (OOB), France, in April-September 2010, June-September 2011 and August-October 2012. They were collected either by diving or from collected predefined algae species in the laboratory, partly provided by the divers of the laboratory. The numbers of individuals included in the analyses are displayed in the results, respectively, and the inclusion criteria are described below in the data analyses section.

The sea slugs were kept – depending on the respective experimental design – either in basins of about 160 cm x 60 cm with running seawater from the laboratory circulation system or individually in petri dishes with regular water exchange from the laboratory system. The system draws water from the nearby sea in shallow depths (corresponding sea temperature in 5 m depths, kindly provided by the Réserve Naturelle Cerbère-Banyuls: 2010: April: 12-

16 °C, May: 14-18 °C, June: 16-20 °C, July: 17-24 °C, August: 18-23 °C, September: 18-22 °C; 2011: June: 18-21 °C, July: 18-22 °C, August: 19-24 °C, September: 21-23 °C; 2012: August: 17-25 °C, September: 17-23 °C, October: 17-20 °C). Temperatures measured in shallow depths above 5 m during the own underwater investigations in the nearby sea in September and October 2012 varied correspondingly between 19-21 °C. In the laboratory, water temperature in petri dishes reached up to a maximum of 26 °C (measured 28th August 2012). Light conditions in the laboratory were near-natural with exposure to natural but not direct sunlight through a window (orientated to the west), light intensities ranging between 4-5 µmol quanta m^{-2} s^{-1} up to around 123 µmol quanta m^{-2} s^{-1} during the day (highest values measured end of August 2012 in patches of sunlight falling in with distance to the sea slugs up to 345 µmol quanta m^{-2} s^{-1}).

Collected *E. crispata* (Florida, USA) corresponded in habitat and morphology to two morphotypes described by Krug et al. (Krug, Vendetti et al. 2016): 47 individuals of *E. crispata mangrove type* were collected in February 2012 at the Marine Mote Lab (MML), field station Summerland Key, Florida, USA, directly under a dock in depths of about 0.40-2 m in calm water on shady sandy/muddy ground with several algae, including the genera *Halimeda, Caulerpa,* and *Penicillus*, while 30 individuals of the *E. crispata reef type* were kindly provided by a professional collector, collected from a reef overgrown with fine algae offshore Key West in March 2012. All *E. crispata* individuals were kept in tanks (40.5 x 92.5 inches) or individually in smaller basins or petri dishes in the tanks of the outdoor laboratory facilities at the MML, under a transparent black net reproducing a slight shady effect imitating natural sunlight transmission in the sea, and an additional material upon the roof to protect from the strong midday solar irradiation. The tanks received running seawater from the laboratory circulation system with supply from the nearby canal in which temperatures measured during observations in the sea ranged around 23-24 °C in March and April 2012. Measured light intensities where the sea slugs were positioned ranged overall between 4-6 µmol quanta m^{-2} s^{-1} in very shady points up to around 110 µmol quanta m^{-2} s^{-1} in light patches (highest values measured above the tanks in light patches ranged to 575 µmol quanta m^{-2} s^{-1}).

Phototaxis trials

Phototaxis trials were performed with methods after Schmitt and Wägele (Schmitt and Wägele 2011), from which also data of *E. timida* and *T. hopei* were included for comparison.

For each trial, one half of the basin was covered with a black cover to darken this half while the other was left uncovered. Individuals were placed in the middle of the darkened side and observed for 90 minutes for each trial. The trials were performed two to four times with changing the cover from one side to the other and starting with different sides to focus on the phototactic effect only and exclude other factors as far as possible under the given field/laboratory conditions. The new trials with *E. viridis, P. dendritica*, *B. mimetica, C. peregrina* and *F. affinis* were performed in the same laboratory room at the OOB as in Schmitt and Wägele (Schmitt and Wägele 2011), with light intensities varying between 11-19 µmol quanta m^{-2} s^{-1}. The trials with *E. crispata* were performed at the MML in one of the outdoor tanks as described above with black plastic bag film as cover and with light intensities varying between 6-26 µmol quanta m^{-2} s^{-1} (*reef type*) and 10-112 µmol quanta m^{-2} s^{-1} (highest values in single light patches) (*mangrove type*). The trial with *P. ocellatus* was performed in a plastic tank-box in a laboratory room at the IME under full-spectrum lamps (Androv, Germany) under a light intensity of 27 µmol quanta m^{-2} s^{-1} and with the same shadowing procedures as in the other trials, using black paper for covering. For all investigated species, light conditions were overall attempted to be kept low to moderate, in order to exclude photo-damage of chloroplasts and potential light-avoidance due to high light intensities, as well as approximately similar in the different trials as far as possible under the given field/laboratory conditions, under which variations could not be completely excluded. Phototaxis trials were performed in general with individuals soon after collection from the sea or been fed in the laboratory, thus the individuals should be in a relatively saturated state with incorporated chloroplasts depending on the specific capacity for chloroplast retention.

PAM measurements in the laboratory and in the sea

The photosynthetic activity of incorporated chloroplasts was monitored with a Pulse Amplitude Modulated Fluorometer (Diving PAM Fluorometer or Photosynthesis Yield Analyzer Mini PAM, version 2.0, both WALZ, Germany). The measured maximum quantum yield of fluorescence for PSII is defined as $(F_m - F_0)/F_m = F_v/F_m$ (with a dark-adapted sample) which is measured routinely with PAM in sea slugs (Wägele and Johnsen 2001, Evertsen, Burghardt et al. 2007, Evertsen and Johnsen 2009, Händeler, Grzymbowski et al. 2009, Vieira, Calado et al. 2009, Jesus, Ventura et al. 2010, Schmitt and Wägele 2011, Schmitt, Händeler et al. 2014). The individual sea slugs were investigated in series of 1-3 consecutive

measurements per measurement day for various periods depending on the respective experiment and the capacity of the analyzed sea slug species for long-term retention of chloroplasts. For measurement series in the laboratory (OOB, IME and MML), individuals were dark-adapted for 10 minutes before each measurement. For each single measurement, the fibre optic was held above the individual with a distance of 0,5-1 cm in the central region of the body part with the parapodia or other body appendixes, depending on the examined species. Since fluorescence and yield values decreased considerably during the course of starving periods, the sensitivity of the PAM was accordingly adapted by putting the parameter 'outgain' from level 2 (default) to higher levels, up to level 8. Even with adaptation of the sensitivity of measurements, in single cases false high yield values can potentially be displayed in combination with very low fluorescence values when fluorescence values are falling to approach zero, which was in single cases respectively left out or replaced by zero for further analysis.

Ambient light conditions in the laboratory and field were measured with an integrated light sensor of the Diving PAM Fluorometer or with a light sensor connected to the Mini-PAM (US-SQS/L, Walz, Germany) in PAR (quantum flux density of photosynthetically active radiation, [µmol quanta m^{-2} s^{-1}]) (Heinz Walz GmbH 1998).

To determine the photosynthetic yield under natural conditions, two species, *E. timida* and *E. crispata mangrove type*, were measured with the Diving PAM Fluorometer in their respective underwater environment. Underwater measurements of *E. timida* at Banyuls-sur-Mer, France, were performed in two different depth levels, one between the water surface and a maximum depth of around 1.5 m and one around 5 m depth. For the investigations of *E. crispata mangrove type*, a population that was found under a shallow dock close to the MML, field station Summerland Key, Florida, was observed and measured in maximum around 1.5 m depth. PAM-measurements were performed at the place where the respective individual was spotted, in the natural light conditions (effective quantum yield ($\Delta F/F_m' = (F_m' - F_0')/F_m'$) (' meaning light-acclimated here) under ambient light conditions, equivalent to (or counterpart of) the maximum quantum yield under dark-adaptation described above). Relative rate of electron transport (ETR) as measured with the Diving-PAM results from calculation by the formula: ETR=E=YIELD x PAR x 0.5 x ETR-factor, as depicted by the Diving-PAM operational handbook (Heinz Walz GmbH 1998).

As individuals had to be measured on a neutral background to ensure precise measurement of their photosynthetic performance and exclude adulterations from algae etc., a glass bowl with a gray-white cement ground was used as background for the measurement and for background

calibration of the Diving-PAM Fluorometer. For the single measurement, the glass bowl was put beneath the spotted individual and the individual was carefully put in it. Measurements were performed directly in the natural light conditions without dark-adaptation and in some cases additionally in a second measurement with preceding dark-adaptation for comparison, for which a black plastic film bag was used to cover the glass bowl with the respective individual inside. For the analysis of the photobehavior in the natural habitat, the parapodia opening degree was analyzed for the first moment in which the respective individual was spotted. The parapodia opening degree was determined after the categories of opening levels defined in Schmitt and Wägele from zero – completely closed to ascending increasing opening degrees (Schmitt and Wägele 2011).

Data analysis

Data were analyzed with Excel and presented mostly with mean, standard deviation and range. For the analyses of the PAM-measurements, 1-3 consecutive measurements per individual and measurement day were included. For the analysis and presentation, the parameter 'days' was partly adapted, e. g. to merge different measurement series together. To determine one representative population per species for the analysis of long-term photosynthetic performance (in Figure 3.3.2), inclusion criteria were: one exemplary group per species was chosen, which included the highest number of individuals for ideally one collection or alternatively from collections within the same timeframe of only 1-3 days distance and from the same region/habitat or substrate/algae with being in the laboratory either without food supply after collection or on the algae for no more than 1-3 days and with exclusion of feeding experiments in the analyzed period to display long-term conditions without food supply. Only for *P. dendritica* a further individual was included from another collection of the same algae with a bigger time distance of 18 days between collections and being on the algae for 5 days previous to the experiment in order to obtain a population of at least 5 exemplary individuals for the analysis. For *E. viridis*, individuals collected from its known food algae *Codium fragile* (Evertsen and Johnsen 2009) – which is considered together with *Codium vermilara* here as the two species can potentially grow together and be difficult to distinguish – were chosen (in contrast to *E. viridis* found on other substrates). For all species, single individuals, that were separated from the collection population and fixed for separate transmission electron microscopy investigations after only one PAM-measurement,

were excluded from the long-term analyses. Other single individuals which were additionally included in other investigations but at a later time point when photosynthetic yield values had already fallen were included in the long-term analyses. In rare cases, individuals were excluded from the long-term analyses, as they were displaying already yield values approaching 0 or very pale color (not green anymore) right from the beginning after collection or if measured too shortly for inclusion into long-term analyses for other reasons.

Results

Photosynthetic activity of incorporated chloroplasts in different sacoglossan species

The photosynthetic activity of incorporated chloroplasts differed extremely in the various sacoglossan sea slug species during periods without food supply (Figure 3.3.2). By far the longest duration of high kleptoplast photosynthetic activity was observed in individuals of the *P. ocellatus* species complex with maximum quantum yield F_v/F_m remaining on a high level during several months with only a minimal decline. In the course of this long period, individuals of *P. ocellatus* died even if kleptoplasts still showed residual photosynthetic activity in the last measurements. On day 148, 17 of the 32 individuals were still alive, on day 203 still six individuals and two on day 210.
With high photosynthetic activity for around three weeks, followed by a slow decline during the course of a further month, *E. timida* revealed distinctively shorter chloroplast retention capacities than *P. ocellatus* but still longer lasting high yields than the other observed species (Figure 3.3.2). In *E. viridis, E. crispata mangrove type* and *reef type* as well as *B. mimetica*, a spectrum of intermediate photosynthetic capacities was observed (Figure 3.3.2).
E. crispata mangrove type and *reef type* revealed different photosynthetic activities with the decline of activity being more rapid in the *mangrove type* than the *reef type* (Figure 3.3.2). In *T. hopei*, photosynthetic values were at a high level when collected freshly from the sea, but decreased very fast within a few days (Figure 3.3.2). In *P. dendritica*, photosynthetic values were low right from the beginning (Figure 3.3.2). Yield measurements of the non-sacoglossan hydrozoan-feeding nudibranchs *C. peregrina* (n=10) and *F. affinis* (n=3) always displayed zero when measured with the PAM-Fluorometer directly after collections. These individuals were consequently not included in PAM-measurement series, but only in the phototaxis experiment as comparison species without incorporation of chloroplasts.

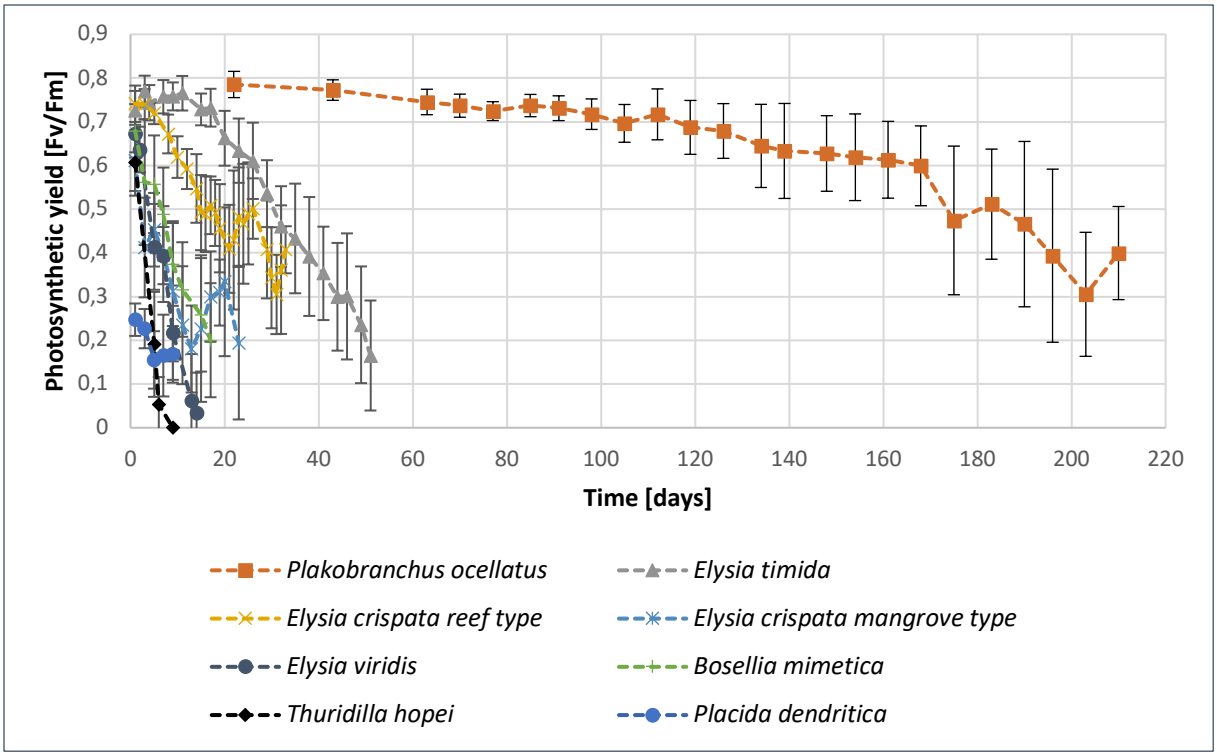


Figure 3.3.2: Photosynthetic maximum quantum yield F_v/F_m (dark-adapted) during periods without food supply in different sacoglossan sea slug species with various capacities of retention of incorporated chloroplasts after collection from the sea.
Plakobranchus ocellatus (Cebu, Philippines, n=32, start (of measurements) 20th December 2011, IME, temperature range: ca. 14-22 °C)
Elysia timida (Banyuls-sur-Mer, France, n=22, start April 13th 2010, OOB, ca. 13-18 °C)
Elysia crispata reef type (Florida Keys, USA, n=10, start 26th March 2012, MML, ca. 23-24 °C)
Elysia crispata mangrove type (Florida Keys, USA, n=1+6 start 7/10th February 2012, MML, ca. 23-24 °C)
Elysia viridis (Banyuls-sur-Mer, France, n=8, start 30th June 2011, OOB, ca. 19-22 °C)
Bosellia mimetica (Banyuls-sur-Mer, France, n=5+3+2, start 7/9/11th August 2012, OOB, ca. 17-25 °C)
Thuridilla hopei (Banyuls-sur-Mer, France, n=10, start 8th July 2011, OOB, ca. 18-22 °C)
Placida dendritica (Banyuls-sur-Mer, France, n=1+4, start 13th/31st August 2012, OOB, ca. 17-25 /ca. 19-23 °C)
IME – Institute for Molecular Evolution, Heinrich-Heine-University, Düsseldorf, Germany; MML – Marine Mote Lab, field station Summerland Key, Florida, USA; OOB – Oberservatoire Océanologique Banyuls- sur-mer, France

Phototactic behavior

The same sacoglossan species investigated concerning photosynthetic capacities of incorporated chloroplasts were correspondingly investigated concerning phototactic behavior (Figure 3.3.3) to investigate potential correlations between long-term chloroplast retention capacities and behavior.

The nudibranchs *C. peregrina* and *F. affinis,* which were included as comparative sea slug species without incorporated chloroplasts as described above, as well as *P. dendritica* with fast digestion of chloroplasts, showed the most positive phototactic reactions in this trials.

E. timida individuals revealed slightly less prominent but also overall mostly positively phototactic behavior. In contrast, the long-term retention species *P. ocellatus* showed more rather avoiding of direct light than exposure. All other species showed intermediate phototactic behavior (Figure 3.3.3).

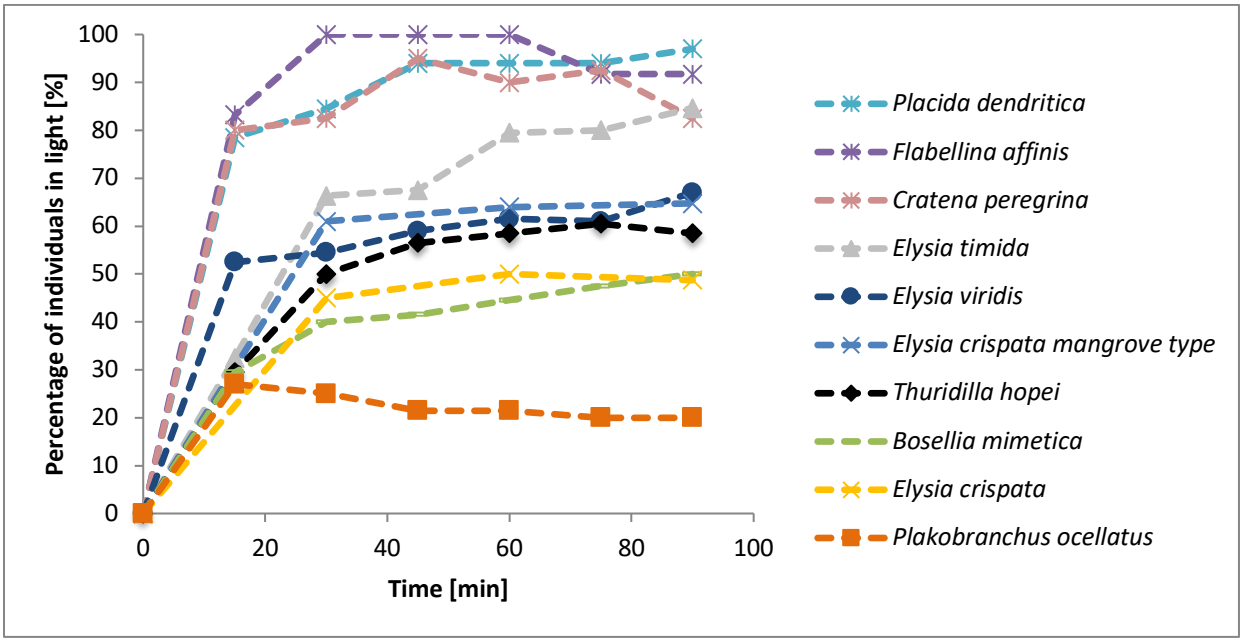


Figure 3.3.3: Phototactic reaction in various sea slug species with and without incorporated chloroplasts and with different degrees of chloroplast retention (for comparison see Figure 3.3.2). Data represent the percentage of sea slug individuals that had moved into the light after being placed on the dark side of a basin under a cover at the beginning of the respective trial. Each data row displays a summary of two to four trials in which the side of the dark cover was changed to exclude influences from other factors than light. All experiments were performed in a laboratory room at the OOB with natural sunlight after the methods applied already in former trials from which data of *E. timida* and *T. hopei* were included, except the trials with *E. crispata* which were performed under shaded natural sunlight in outside basins at the MML and the trials with *P. ocellatus* which were performed with artificial sunlight at the IME (see methods). Species with long-term to intermediate retention of chloroplasts: *Plakobranchus ocellatus* (n=30), *Elysia timida* (n=177), *Elysia viridis* (n=50), *Elysia crispata reef type* (n=20), *Elysia crispata mangrove type* (n=40), *Bosellia mimetica* (n=50). Species with fast decrease of chloroplasts: *Thuridilla hopei* (n=48), *Placida dendritica* (n=8). Species without chloroplasts: *Cratena peregrina* (n=10), *Flabellina affinis* (n=3).

Underwater studies

The underwater investigations of the two species *E. crispata mangrove type* and *E. timida* in their natural habitats in Florida and France, respectively, revealed remarkable differences concerning light conditions and kleptoplast photosynthetic activity. The site where *E. crispata mangrove type* was observed was a shady, shallow (~ 1 m depth), sandy area under a dock, where stronger sunlight was only entering for a short time period in the late afternoon (Figure 3.3.4A).

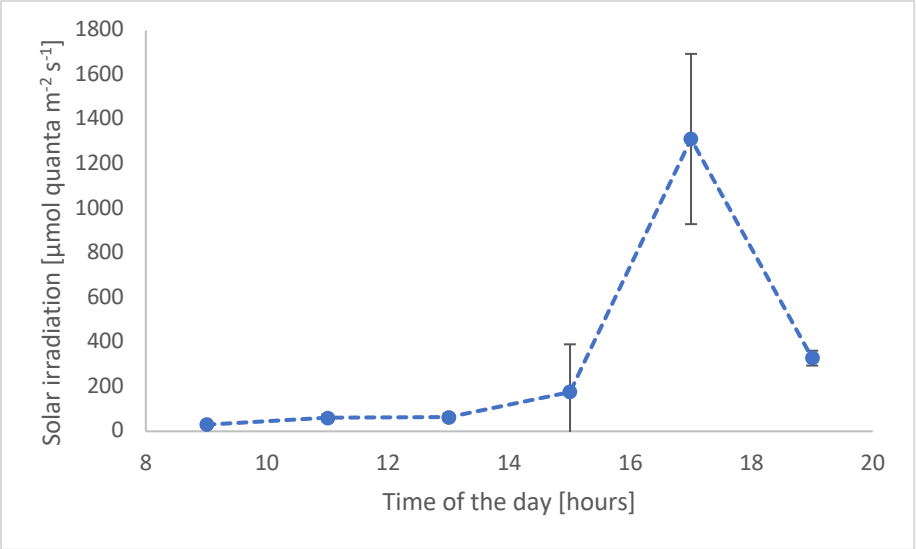


Figure 3.3.4A: Full day curve of light conditions in the course of an exemplary day (24th April 2012) at the investigated site of *Elysia crispata mangrove type* in their natural habitat in a shallow (< 1 m depth) sandy area under a dock (Florida Keys, USA). Each measurement point represents the mean of a series of measurements in the investigated habitat of individuals of *E. crispata mangrove type* during diving at the respective timepoint of the day. The curve mirrors the special light conditions in this habitat under a dock with shading until the afternoon when sunlight fell under the dock provoking an irradiation peak. (Summerland Key, Florida, USA, 24th April 2012, ca. 23-24 °C).

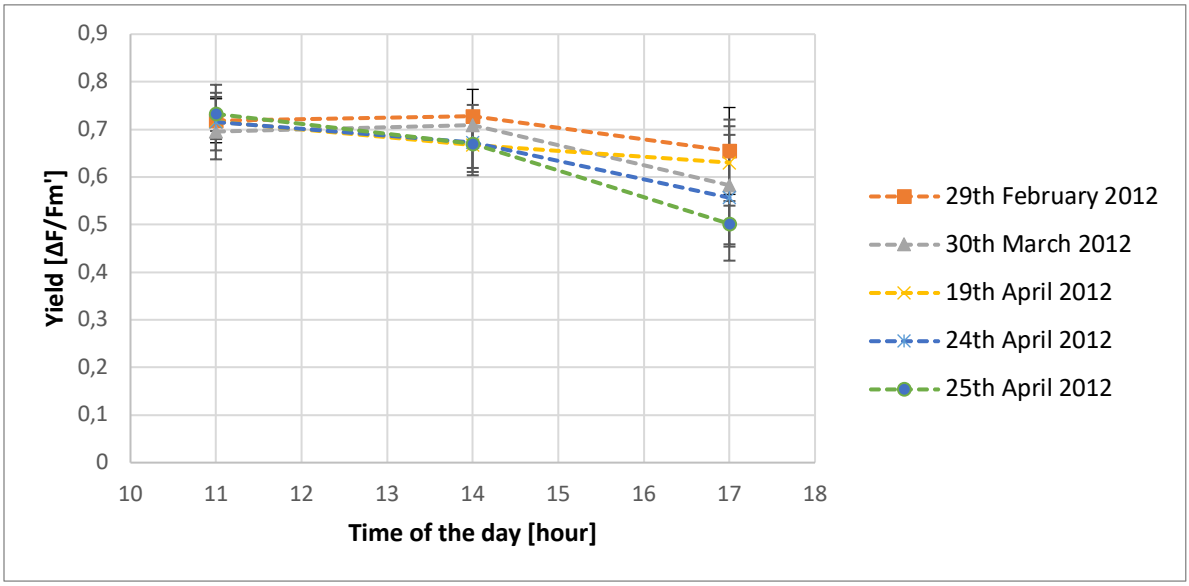


Figure 3.3.4B: Effective quantum yield of photosystem II (ΔF/Fm') measured in individuals of *Elysia crispata mangrove type* in the course of the day in their natural habitat in a shallow (< 1 m depth) sandy area under a dock (Florida Keys, USA) during 3 dives at around 11 o'clock, 14 o'clock and 17 o'clock on 5 days. Each measurement point represents a mean of consecutive measurements in 5-9 different individuals of *Elysia crispata mangrove type* that could be found and measured during diving at the respective timepoint of the respective day. *Elysia crispata mangrove type* (Summerland Key, Florida, USA, n=7+9+7, 29th February 2012, n=5+5+5, 30th March 2012, n=5+5+5, 19th April 2012, n=5+5+5, 24th April 2012, n=5+5+5, 25th April 2012, ca. 23-24 °C).

Corresponding to the special light conditions with a solar irradiation peak in the afternoon, the kleptoplast effective quantum yields $\Delta F/F_m$' in the examined individuals of *E. crispata mangrove type* were always higher during the time of the day with low solar irradiation and fell remarkably with the stronger solar irradiation in the afternoon (Figure 3.3.4B). The difference was less prominent on the 19th April with a rather cloudy, rainy weather in the course of the day which diminished the solar irradiation peak in the afternoon. Combining the results of all five investigation days, the difference was statistically significant with the overall mean effective quantum yield $\Delta F/F_m$' of 0.585 ± 0.061 (range: 0.502 – 0.655) at 17 o'clock being significantly lower compared to both the overall mean of 0.689 ± 0.027 (range: 0.668 – 0.728) at 14 o'clock and 0.716 ± 0.013 (range: 0.696 – 0.732) at 11 o'clock (paired t-test: p=0.01, respectively).

In the habitat of the investigated individuals of *E. timida*, on the contrary, the shallow open Mediterranean bays were exposed to sunlight already in the morning, reaching normally a peak in the midday sun and falling in the course of the afternoon with lower sun stand and less solar irradiation reaching the bay ground, all naturally varying with weather conditions (Figure 3.3.4C). During morning and midday, the solar irradiation varied strongly depending on the degree of clouds, as e. g. on 17th September 2012, when it was more cloudy around 14 o'clock than in the measurements before, and punctual measurements of solar irradiation during midday outside the water varied already between around 1420 µmol quanta m^{-2} s^{-1} with more sunlight and 723 µmol quanta m^{-2} s^{-1} with more clouds. Inside the water, an additional potentially influencing factor was shadow from rocks, which probably was the major reason for the lower solar irradiation on the sites where the measurements were taken in the morning on 7th September 2012, when the measurement of solar irradiation outside the water before diving (1015 µmol quanta m^{-2} s^{-1}) was only slightly lower than on the other days (1122 µmol quanta m^{-2} s^{-1}, 1135 µmol quanta m^{-2} s^{-1}, 1076 µmol quanta m^{-2} s^{-1}, 1120 µmol quanta m^{-2} s^{-1}, respectively). The same correlation described for *E. crispata mangrove type* above also applied for the observations in *E. timida* in reversed sequence during the day (Figure 3.3.4D). While there was no significant difference between the lower mean effective quantum yields $\Delta F/F_m$' of 0.459 ± 0.068 (range: 0.371 – 0.562) measured during diving at 11 o'clock and 0.505 ± 0.083 (range: 0.427 – 0.608) at 14 o'clock in stronger solar irradiation, the overall mean effective quantum yield $\Delta F/F_m$' of 0.654 ± 0.017 (range: 0.627 – 0.672) at 17 o'clock with lower solar irradiation was significantly higher compared to both (paired t-test: p=0.002 and p=0.01, respectively) (overall means for all samples taken together).

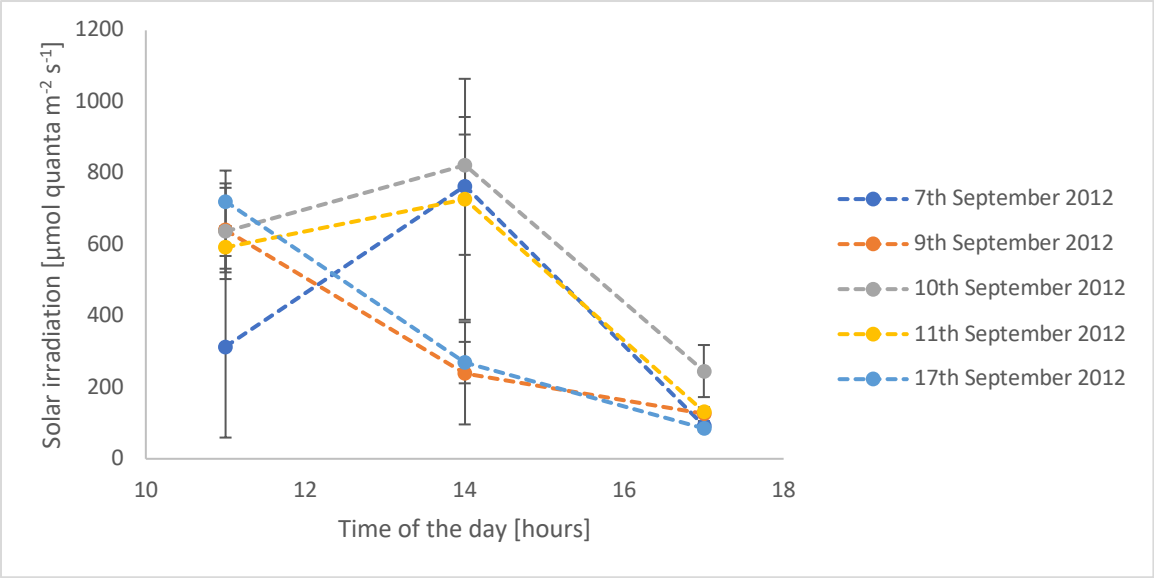


Figure 3.3.4C: Natural light conditions measured along with the photosynthetic yield of *Elysia timida* in the course of a day in depths of < 1 m in their natural habitat in a Mediterranean bay (Banyuls-sur-Mer, France) during 3 dives at around 11 o'clock, 14 o'clock and 17 o'clock on 5 days. Each measurement point represents the mean of a series of measurements in the investigated habitat of individuals of *Elysia timida* during diving at the respective timepoint of the respective day. Higher standard deviations mirror stronger variations due to weather and shadows from rocks underwater during morning and midday. (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, n=5+5+5, 9th September 2012, n=5+5+5, 10th September 2012, n=5+5+4, 11th September 2012, n=5+5+5, 17th September 2012, ca. 20-21 °C).

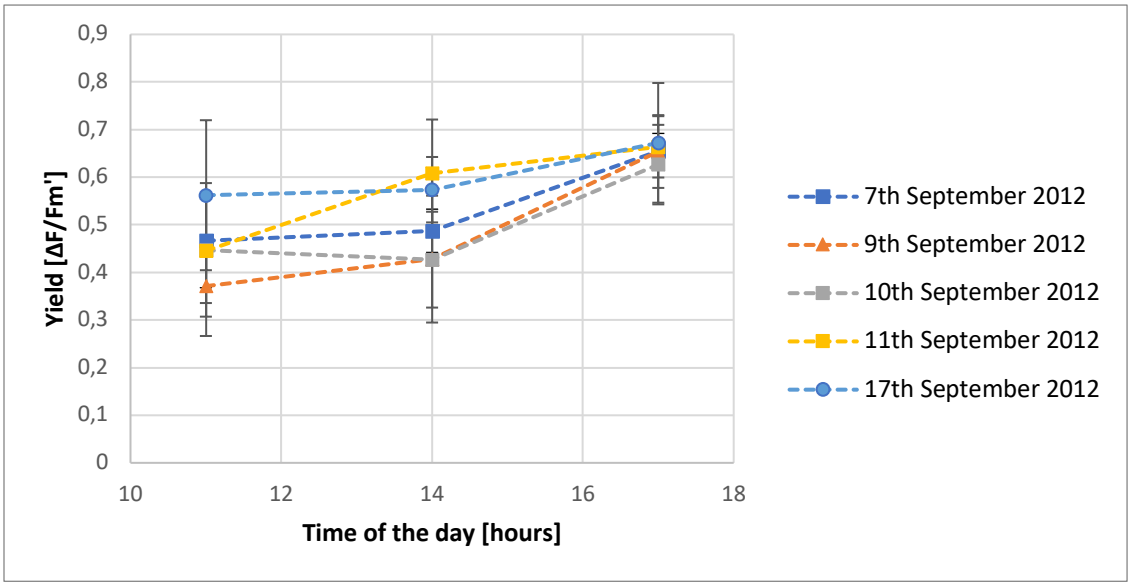


Figure 3.3.4D: Effective quantum yield of photosystem II (ΔF/Fm') measured in individuals of *Elysia timida* in the course of the day in their natural habitat in shallow depths (< 1 m) in a Mediterranean bay (Banyuls-sur-Mer, France) during 3 dives at around 11 o'clock, 14 o'clock and 17 o'clock on 5 days. Each measurement point represents a mean of consecutive measurements in 4-5 different individuals of *E. timida* that could be found and measured during diving at the respective timepoint of the respective day. *E. timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, n=5+5+5, 9th September 2012, n=5+5+5, 10th September 2012, n=5+5+4, 11th September 2012, n=5+5+5, 17th September 2012, ca. 20-21 °C).

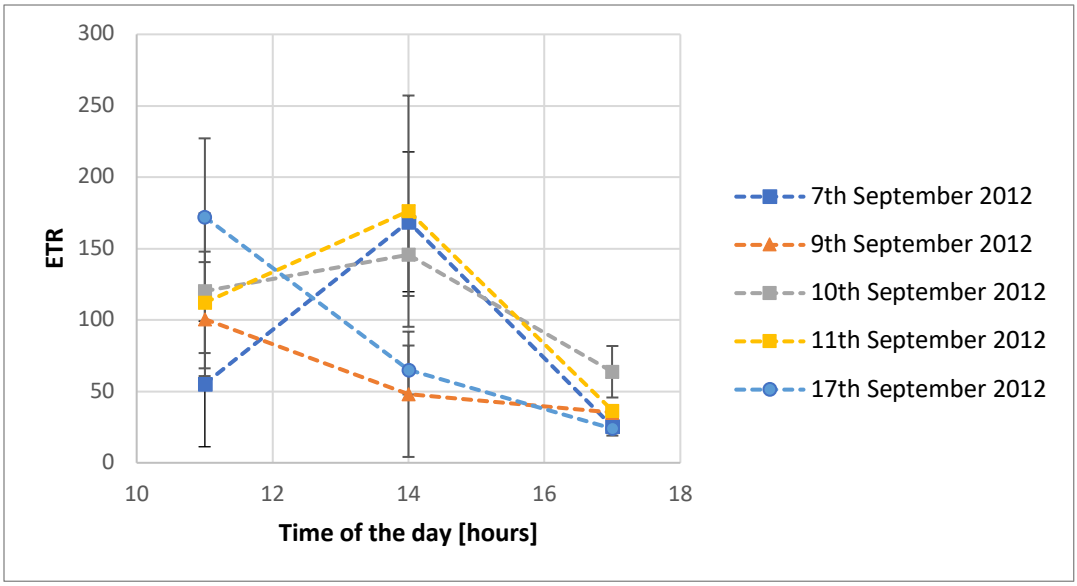


Figure 3.3.4E: Relative rate of electron transport (ETR) measured in individuals of *Elysia timida* in the course of the day in their natural habitat in shallow depths (< 1 m) in a Mediterranean bay (Banyuls-sur-Mer, France) during 3 dives at around 11 o'clock, 14 o'clock and 17 o'clock on 5 days. Each measurement point represents a mean of consecutive measurements in 4-5 different individuals of *E. timida* that could be found and measured during diving at the respective timepoint of the respective day. *E. timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, n=5+5+5, 9th September 2012, n=5+5+5, 10th September 2012, n=5+5+4, 11th September 2012, n=5+5+5, 17th September 2012, ca. 20-21 °C).

Photosynthetic relative rate of electron transport (ETR) (Figure 3.3.4E) measured in *E. timida* with the Diving-PAM as calculated with a formula including a multiplication of the yield and PAR-parameter of the ambient light conditions (see methods) corresponded coherently as means per dive with the respective irradiation conditions displayed above. On the three days with the typical irradiation curves with a midday peak (7th, 10th and 11th September), the ETR showed the same tendency in the course of the day with higher ETR during high midday irradiation. On the other two days, when the respective irradiation around midday was lower, correspondingly also the ETR was lower.

In addition to the investigations in shallow depths, individuals of *E. timida* were also examined in deeper dives around 5 m depth at around 12 o'clock and 15 o'clock on 3 different days. Light intensities in depths of around 5 m were overall lower than observed in the shallower depths and showed less variation (Figure 3.3.4F). In accordance with that and with the correlation of lower solar irradiation and higher effective quantum yields described above, the overall mean effective quantum yield $\Delta F/F_m'$ in individuals of *E. timida* in 5 m depths was with 0.634 ± 0.016 (range: 0.623 – 0.652) at 12 o'clock and 0.625 ± 0.063 (range: 0.575 – 0.695) at 15 o'clock comparatively high (Figure 3.3.4G) and only slightly lower than in shallower depths at 17 o'clock described above (overall means for all samples taken together).

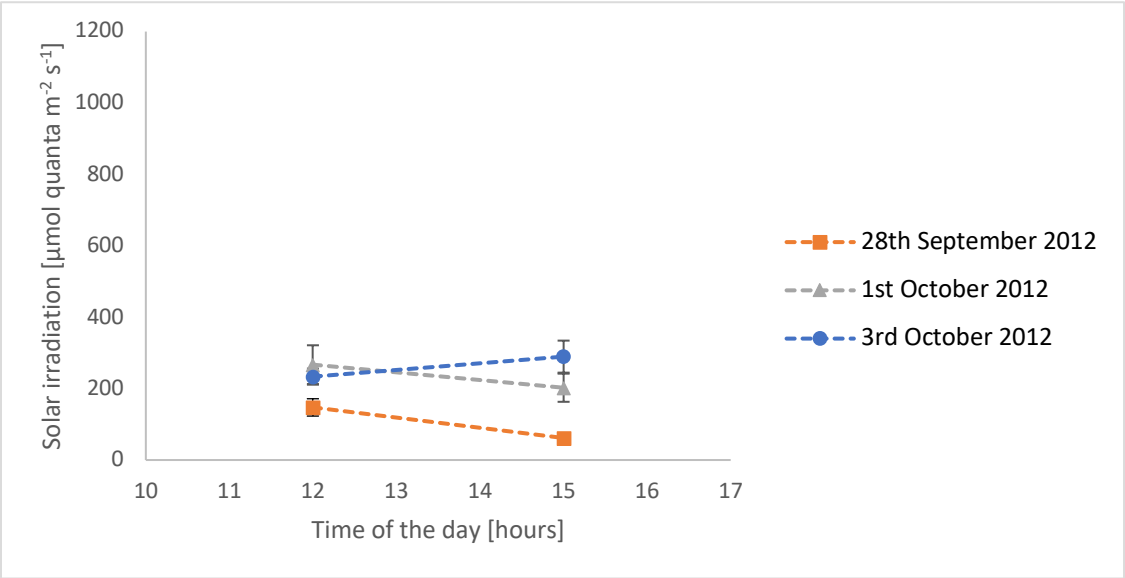


Figure 3.3.4F: Natural light conditions measured along with the yield for *Elysia timida* in depths of around 5 m in their natural habitat in a Mediterranean bay (Banyuls-sur-Mer, France) during 2 dives at around 12 o'clock and 15 o'clock on 3 days. Each measurement point represents the mean of a series of measurements in the investigated habitat of individuals of *Elysia timida* during diving at the respective timepoint of the respective day. Y-axis adapted for comparison with figure 4C of light intensities in shallower depths. (Banyuls-sur-Mer, France, n=10+14, 28th September 2012, n=12+17, 1st October 2012, n=12+14, 3rd October 2012, ca. 19 °C).

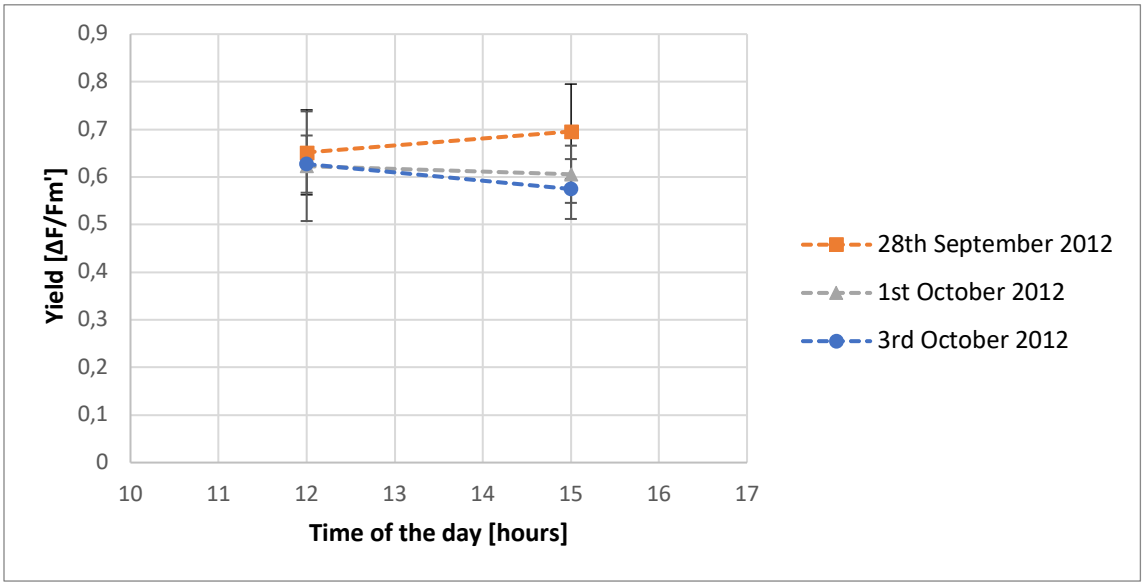


Figure 3.3.4G: Effective quantum yield of photosystem II (ΔF/Fm') measured in individuals of *Elysia timida* in the course of the day in their natural habitat in depths of around 5 m in a Mediterranean bay (Banyuls-sur-Mer, France) during 2 dives at around 12 o'clock and 15 o'clock on 3 days. Each measurement point represents a mean of consecutive measurements in different individuals of *E. timida* that could be found and measured during diving at the respective timepoint of the respective day. *Elysia timida* (Banyuls-sur-Mer, France, n=10+14, 28th September 2012, n=12+17, 1st October 2012, n=12+14, 3rd October 2012, ca. 19 °C).

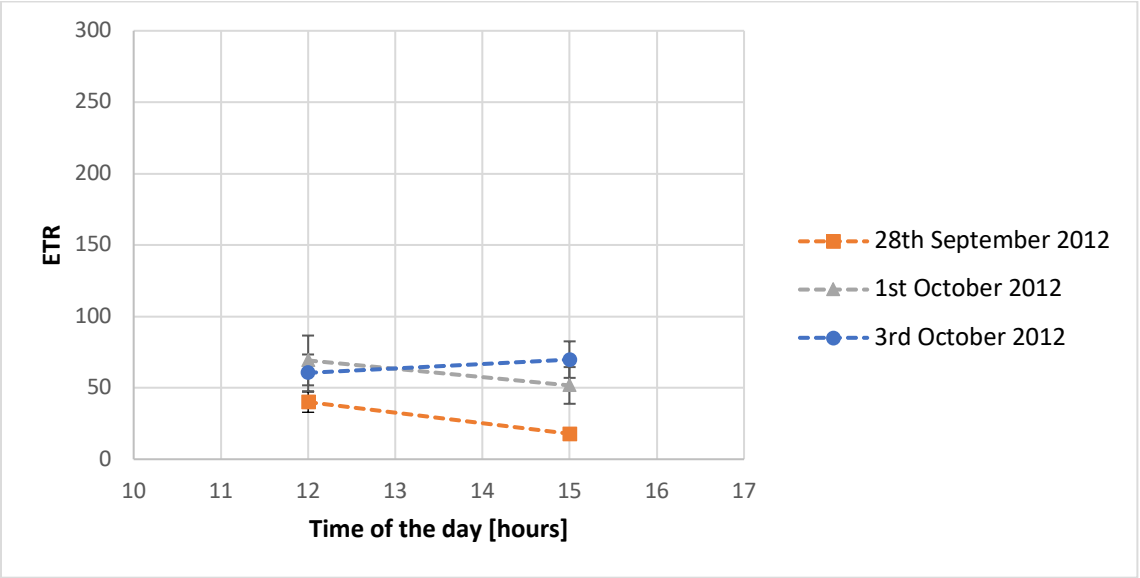


Figure 3.3.4H: Relative rate of electron transport (ETR) measured in individuals of *Elysia timida* in the course of the day in their natural habitat in depths of around 5 m in a Mediterranean bay (Banyuls-sur-Mer, France) during 2 dives at around 12 o'clock and 15 o'clock on 3 days. Each measurement point represents a mean of consecutive measurements in different individuals of *E. timida* that could be found and measured during diving at the respective timepoint of the respective day. Y-axis adapted for comparison with figure 4E of ETR in shallower depths. *Elysia timida* (Banyuls-sur-Mer, France, n=10+14, 28th September 2012, n=12+17, 1st October 2012, n=6+14, 3rd October 2012, ca. 19 °C).

As in the lower depths described above, also in *E. timida* in deeper depths around 5 m the relative rate of electron transport (ETR) (Figure 3.3.4H) measured with the Diving-PAM corresponded coherently as means per respective dive in relation to the respective irradiation conditions displayed above. Overall, the ETR in *E. timida* in these deeper depths were distinctively lower compared to the ETR in shallower depths.

Individuals of *A. acetabulum,* close to where individuals of *E. timida* were spotted, emitted higher fluorescence F (about double) and slightly lower or higher photosynthetic yield $\Delta F/F_m$' values than the individuals of *E. timida* measured during the same dive with the respective similar light conditions, revealing overall the same correlation of lower effective quantum yield $\Delta F/F_m$' with higher solar irradiation as well as higher effective quantum yield $\Delta F/F_m$' with lower solar irradiation (Table 3.3.1).

Date/ Timepoint Dive	*Elysia timida*				*Acetabularia acetabulum*			
	Nr. individuals	Fluorescence	Effective quantum yield	Solar irradiation [µmol quanta m^{-2} s^{-1}]	Nr. individuals	Fluorescence	Effective quantum yield	Solar irradiation [µmol quanta m^{-2} s^{-1}]
7th September 2012 11 o'clock	N=4	229 ± 111 (range: 166 – 394)	0.467 ± 0.099 (range: 0.338 – 0.578)	314 ± 254 (range: 91 – 578)	N=4	639 ± 104 (range: 549 – 774)	0.511 ± 0.134 (range: 0.368 – 0.636)	459 ± 184 (range: 178 – 579)
11th September 2012 14 o'clock	N=5	234 ± 70 (range: 156– 343)	0.608 ± 0.113 (range: 0.453 – 0.755)	727 ± 338 (range: 129– 926)	N=2	554 ± 0 (range: 554 – 554)	0.334 ± 0.064 (range: 0.453 – 0.755)	686 ± 211 (range: 537– 835)
1st October 2012 12 o'clock	N=12	474 ± 127 (range: 283– 741)	0.623 ± 0.115 (range: 0.425 – 0.771)	267 ± 54 (range: 151– 329)	N=4	1119 ± 521 (range: 790– 1896)	0.599 ± 0.089 (range: 0.522 – 0.728)	263 ± 8 (range: 256– 275)
1st October 2012 15 o'clock	N=17	588 ± 113 (range: 156– 343)	0.606 ± 0.060 (range: 0.499 – 0.710)	202 ± 39 (range: 137– 274)	N=3	955 ± 207 (range: 744– 1157)	0.713 ± 0.087 (range: 0.653 – 0.812)	203 ± 21 (range: 187– 226)

Table 3.3.1: Fluorescence, effective quantum yield of photosystem II (ΔF/Fm') and natural light conditions measured in individuals of *Elysia timida* and their food algae *Acetabularia acetabulum* in their natural habitat in shallow depths of < 1 m and 5 m in Mediterranean bays (Banyuls-sur-Mer, France). Each measurement point represents a mean of consecutive measurements in the respective indicated number of individuals of *E. timida* and *A. acetabulum* found and measured during diving per each respective dive. (Banyuls-sur-Mer, France, 7th September 2012, 11th September 2012, 1st October 2012, ca. 19-21 °C).

When aligning all mean effective quantum yield $\Delta F/F_m$' values per dive with the respecting mean solar irradiation of the respective dive, for shallow and deep dives for *E. timida* and *A. acetabulum*, respectively, the described correlation of higher yields with lower irradiation and vice versa was more prominent in *A. acetabulum* than in *E. timida* (Standard pearson product-moment correlation: *A. acetabulum* r=-0,982, *E. timida* r=-0,698) (Figure 3.3.5A). Relative rate of electron transport (ETR) (Figure 3.3.5B) measured in *E. timida* plotted in the same way, revealed the corresponding correlation of higher ETR with higher solar irradiation, which in this case was more pronounced in *E. timida*.

As the ETR is in strong correlation with the respective irradiance which can show fluctuations between local, punctual measurements, the single values of ETR were also analyzed with their respectively corresponding single irradiance values, illustrating clearly the strong correlation of ETR in *E. timida* and *A. acetabulum* with the current ambient solar irradiation (Figure 5C). Concerning behavioral observations, a tendency was observed that individuals of *E. timida* held their parapodia more closed in higher light intensities (Standard pearson product-moment correlation: parapodia *E. timida* r=-0,682 (Figure 3.3.6).

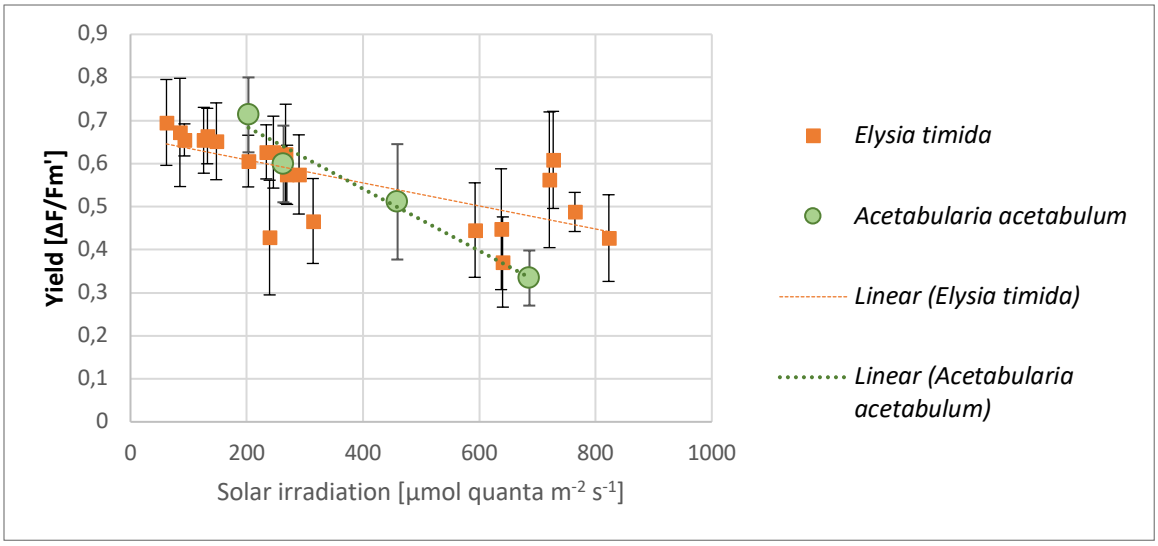


Figure 3.3.5A: Effective quantum yield of photosystem II (ΔF/Fm') and natural light conditions for *Elysia timida* and their food algae *Acetabularia acetabulum* in depths of < 1 m and 5 m in their natural habitat (Banyuls-sur-Mer, France). Each point represents a mean of measurements in different individuals of *E. timida* and *A. acetabulum* which could be found and measured per each respective dive. *E. timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, 11/14/17 o'clock dive, n=5+5+5, 9th September 2012, 11/14/17 o'clock dive, n=5+5+5, 10th September 2012, 11/14/17 o'clock dive, n=5+5+4, 11th September 2012, 11/14/17 o'clock dive, n=5+5+5, 17th September 2012, 11/14/17 o'clock dive, n=10+14, 28th September 2012, 12 and 15 o'clock dive, n=12+17, 1st October 2012, 12 and 15 o'clock dive, n=12+14, 3rd October 2012, 12 and 15 o'clock dive, ca. 19-21 °C), *A. acetabulum* (Banyuls-sur-Mer, France, n=4, 7th September 2012, 11 o'clock dive, n=2, 11th September 2012, 14 o'clock dive, n=4+3, 1st October 2012, 12 and 15 o'clock dive, ca. 19-21 °C).

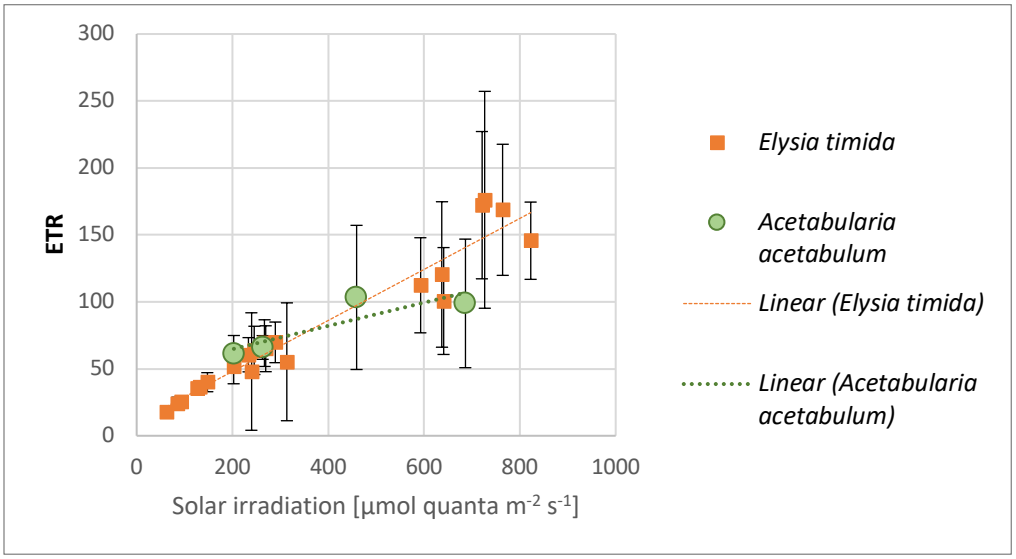


Figure 3.3.5B: Relative rate of electron transport (ETR) and natural light conditions for *Elysia timida* and their food algae *Acetabularia acetabulum* in depths of < 1 m and 5 m in their natural habitat (Banyuls-sur-Mer, France). Each point represents a mean of measurements in different individuals of *E. timida* and *A. acetabulum* which could be found and measured per each respective dive. *E. timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, 11/14/17 o'clock dive, n=5+5+5, 9th September 2012, 11/14/17 o'clock dive, n=5+5+5, 10th September 2012, 11/14/17 o'clock dive, n=5+5+4, 11th September 2012, 11/14/17 o'clock dive, n=5+5+5, 17th September 2012, 11/14/17 o'clock dive, n=10+14, 28th September 2012, 12 and 15 o'clock dive, n=12+17, 1st October 2012, 12 and 15 o'clock dive, n=6+14, 3rd October 2012, 12 and 15 o'clock dive, ca. 19-21 °C), *A. acetabulum* (Banyuls-sur-Mer, France, n=4, 7th September 2012, 11 o'clock dive, n=2, 11th September 2012, 14 o'clock dive, n=4+3, 1st October 2012, 12 and 15 o'clock dive, ca. 19-21 °C).

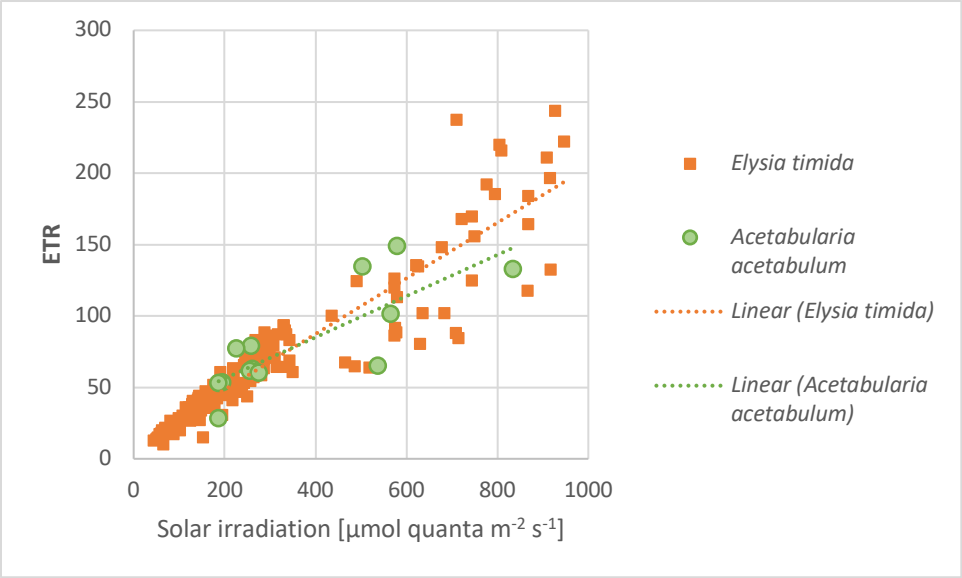


Figure 3.3.5C: Relative rate of electron transport (ETR) in relation to the respective punctual natural light irradiation for *Elysia timida* and their food algae *Acetabularia acetabulum* in depths of < 1 m and 5 m in their natural habitat (Banyuls-sur-Mer, France). Each point represents a single PAM-measurement including ETR and PAR in different individuals of *E. timida* and *A. acetabulum*. *E. timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, 11/14/17 o'clock dive, n=5+5+5, 9th September 2012, 11/14/17 o'clock dive, n=5+5+5, 10th September 2012, 11/14/17 o'clock dive, n=5+5+4, 11th September 2012, 11/14/17 o'clock dive, n=5+5+5, 17th September 2012, 11/14/17 o'clock dive, n=10+14, 28th September 2012, 12 and 15 o'clock dive, n=12+17, 1st October 2012, 12 and 15 o'clock dive, n=6+14, 3rd October 2012, 12 and 15 o'clock dive, ca. 19-21 °C), *A. acetabulum* (Banyuls-sur-Mer, France, n=4, 7th September 2012, 11 o'clock dive, n=2, 11th September 2012, 14 o'clock dive, n=4+3, 1st October 2012, 12 and 15 o'clock dive, ca. 19-21 °C).

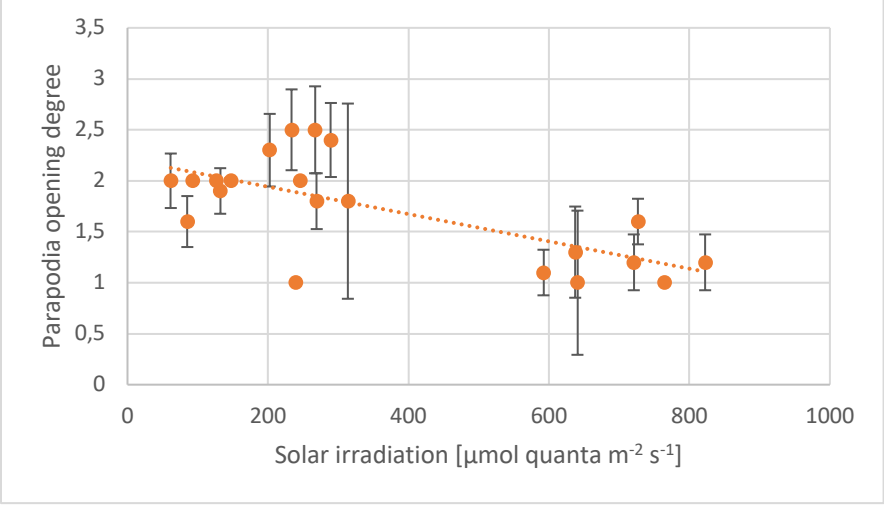


Figure 3.3.6: Parapodia opening degree and natural light conditions measured in individuals of *Elysia timida* in shallow depths of < 1 m and 5 m in their natural habitat (Banyuls-sur-mer, France). Each measurement point represents a mean of observations in individuals of *E. timida* which could be found and measured per each respective dive. Documented was the parapodia opening degree observed in the moment the respective individual was spotted. Parapodia opening degree from 0 – totally closed, over 1 – parapodia mainly closed, to further ascending degrees of opening (see methods). *Elysia timida* (Banyuls-sur-Mer, France, n=4+4+4, 7th September 2012, 11/14/17 o'clock dive, n=5+5+5, 9th September 2012, 11/14/17 o'clock dive, n=5+5+5, 10th September 2012, 11/14/17 o'clock dive, n=5+5+4, 11th September 2012, 11/14/17 o'clock dive, n=5+5+5, 17th September 2012, 11/14/17 o'clock dive, n=10+14, 28th September 2012, 12 and 15 o'clock dive, n=12+17, 1st October 2012, 12 and 15 o'clock dive, n=12+14, 3rd October 2012, 12 and 15 o'clock dive, ca. 19-21 °C).

Discussion

Photosynthetic activity of incorporated chloroplasts in different sacoglossan species

Concerning differences in photosynthetic capacities between and within different sea slug species, various factors can have possible influences. The potential effect of temperature on photosynthetic performance in *E. timida* was indicated in previous studies (Schmitt, Händeler et al. 2014, Laetz and Wägele 2018b). Also, different food algae and with that different characteristics of chloroplasts from various donors as potential cause for variations in retention of chloroplasts have been reported, e. g. in *E. viridis* (Baumgartner, Pavia et al. 2015, Rauch, Tielens et al. 2018) and also in a broad overview comprising investigations of 26 sacoglossan species (Christa, Händeler et al. 2014). In our results, individuals of *E. viridis* collected from *C. fragile/vermilara* showed with only about 14 days a much shorter retention duration than expected, as e. g. Evertsen and Johnsen reported long high photosynthetic capacities of functional chloroplasts in *E. viridis* from the Trondheim fjord in Norway, which after 73 days of observed starvation they estimated to last photosynthetic for about 5-9 months (Evertsen and Johnsen 2009). In contrast to this, Viera et al. reported retention of functional chloroplasts in individuals of *E. viridis* of the west coast of Portugal, held in low light conditions of 30 µmol photons m^{-2} s^{-1}, to last for 15 up to 57 days (Vieira, Calado et al. 2009) which resembled more to our results in the present study but was also overall longer. The results in our present study of only about two weeks of retention of functional chloroplasts in *E. viridis* also applied to further collection populations throughout different research stays with some variations, but could also differ considerably with different habitats and substrates (own observations). *E. viridis* seems to be an example for a species with strong intraspecific variations in chloroplast retention in different habitats and geographical regions and also potentially in relation to different algal food sources, which might indicate a high degree of adaptation or even differentiation into a species complex with various ecological types (Evertsen and Johnsen 2009, Rauch, Tielens et al. 2018).
Concerning the fast degradation of chloroplasts from *Codium* algae in *P. dendritica* in contrast to *E. viridis*, we could confirm the former results of Evertsen and Johnsen (Evertsen and Johnsen 2009) as overall low photosynthetic yield values measured in *P. dendritica* even with saturate food algae supply directly after collection clearly reflected, that despite uptake of chloroplasts the degradation of chloroplasts was so strong, that photosynthetic yield was far

below species with retention of chloroplasts, e. g. *E. viridis,* collected from the same food algae.

We also found differences in kleptoplast photosynthetic performance between the populations of *E. crispata reef type* and *mangrove type*, which confirms the ecological differentiation, probably with different diets and with that different chloroplast donors, as described by Krug et al. (Krug, Vendetti et al. 2016).

With our PAM-measurements of over seven months, *P. ocellatus* is the species with the longest measured duration of chloroplast retention so far. Until now, only distinctively shorter periods were measured for this species and the total duration of long-term-retention of chloroplasts was only estimated, e. g. with a coefficient of 11 month calculated by Evertsen at al. (Evertsen, Burghardt et al. 2007). Händeler et al. presented measurements for 75 days with still relatively prominent photosynthetic activity (Händeler, Grzymbowski et al. 2009). Some studies present distinctively shorter durations (Christa, Wescott et al. 2013, Yamamoto, Hirano et al. 2013, Wade and Sherwood 2017). Intraspecific differences in photosynthetic capacities in *P. ocellatus* might be caused by widely diverse food spectra and ecological differentiation. In a recent study for *Plakobranchus cf. ianthobapsus* from 10 sites across the Main Hawaiian Islands during winter and summer seasons, Wade and Sherwood reported an extremely diverse food spectrum with sequestration of chloroplasts from 23 algal species from across the siphonous green algal order *Bryopsidales* (Wade and Sherwood 2018). A former study from specific Hawaian individuals of *P. ocellatus* reported sequestration of chloroplasts from up to 11 bryopsidalean algal species (Wade and Sherwood 2017). Maeda et al. reported also a broad spectrum of green algal species as food and chloroplast sources for *P. ocellatus* from Japan (Maeda, Hirose et al. 2012). High diversity occurring in *P. ocellatus* lead to the current definition as a species complex with probably 10 species varying in distribution and nutrition preferences (Krug, Vendetti et al. 2013, Wade and Sherwood 2017). For *P. ocellatus* from the Philippines, like the individuals in our present study, a former study described also a broad food spectrum including members of the genera *Halimeda, Caulerpa, Udotea, Acetabularia* and further unidentified algae, with an emphasis on *H. macroloba* (Christa, Wescott et al. 2013).

In a further study, the algal species that seem to be essential for long-term-functional retention in general were defined to belong to the taxa *Halimeda, Caulerpa, Penicillus, Avrainvillea, Acetabularia* and *Vaucheria*, while none of these were found in *Thuridilla*, the only plakobranchoidean genus without long-term retention forms (Christa, Händeler et al. 2014). Ventura et al. investigated especially kleptoplasts and short-term retention in *T. hopei*

(Ventura, Calado et al. 2013). Our results confirmed formerly reported short-term retention in *T. hopei* (also e. g. (Händeler, Grzymbowski et al. 2009), with interestingly potential high photosynthetic yields directly after collection which resemble those of species with long-term retention of chloroplasts, but consecutively fast complete degradation of chloroplasts within about a week.

Concerning the various sacoglossan species analyzed in the present study, we could confirm distinct differences between species-specific spectra of photosynthetic capacities, which in free-living sea slugs can potentially be influenced by various factors as season, temperature, food availability, light conditions and further environmental parameters as well as age, size and overall condition of the individuals. Viera et al. reported e. g. influence of light on photosynthetic activity in *E. viridis* (Vieira, Calado et al. 2009) and Cruz et al. reported photoprotection in sequestered plastids of sea slugs and respective algal sources (Cruz, Cartaxana et al. 2015). Underlying mechanisms and factors for longevity of incorporated chloroplasts are still unclear and potentially diverse (Cruz, Cartaxana et al. 2015, Chan, Vaysberg et al. 2018). Pelletreau et al. published a study indicating that *Elysia chlorotica* can profit from the incorporated chloroplasts to gain potentially its life-time-energy (Pelletreau, Worful et al. 2012). With the results for *P. ocellatus* of this study with a similar extremely long potential to live without food supply while retaining high levels of photosynthetic yields $\Delta F/F_m$ we can partly confirm this with the restriction that the observed individuals showed falling yield values during the course of observations accumulating before dying, indicating degradation of chloroplasts. In all, research remains highly interesting concerning this enigmatic phenomenon of functional chloroplast incorporation which different Sacoglossa species have evolved in varying perfection and which can potentially be further influenced by various ecological and behavioral parameters – like light conditions in the natural environment.

Photobehavior

Behavioral adaptations of the sea slugs in relation to functional kleptoplasty are assumed to have developed and play a potential role for the functionality of chloroplasts, e. g. in the species *E. timida* with a special modulation of more or less opening posture of the parapodial lobes resulting in more or less light reaching the incorporated chloroplasts (Rahat and Monselise 1979, Monselise and Rahat 1980, Jesus, Ventura et al. 2010, Schmitt and Wägele

2011). In a study comparing three sacoglossan species with incorporated chloroplasts (*Elysia tuca, Costasiella lilianae (= Costasiella ocellifera* after Clark (Clark 1984)), and *Elysia crispata*) versus two without chloroplast retention (*Oxynoe antillarum* and *Berthelinia carribea*), Weaver and Clark reported that the 'chloroplast-symbiotic' species oriented towards light while the 'aposymbiotic' species avoided light and supposed a possible relationship between incorporated chloroplasts and phototaxis (Weaver and Clark 1981). Results from our previous study, comparing *E. timida* with long-term retention versus *T. hopei* with fast degradation of chloroplasts, seemed partly to confirm the hypothesis that chloroplast-retention might imply stronger phototactic behavior, as *E. timida* revealed stronger positive phototaxis movement than *T. hopei* (Schmitt and Wägele 2011). The phototaxis trial in the present study was performed in parallel to the analyses of photosynthetic profiles with the same sea slug species in focus to test the hypothesis in more detail, that chloroplast-retention possibly implies stronger phototactic behavior in sea slugs. With an overview including more species in the present study, however, the hypothesis cannot be confirmed in this simple way. In our present study, the non-sacoglossan species *C. peregrina* and *F. affinis* without chloroplasts and the sacoglossan *P. dendritica* with fast digestion of chloroplasts showed a highly positive phototactic reaction while several species with either long chloroplast retention as *P. ocellatus* or mediate retention profiles rather reacted with caution or avoidance versus direct light exposure, even in these comparatively low light intensities. The rather light-shy reaction revealed by *P. ocellatus* can consequently also not really be explained by different light-conditions in the laboratory as the light intensity of 27 µmol quanta m^{-2} s^{-1} was relatively low and in a similar intensity spectrum to the light conditions in the other trials. Furthermore, as a side observation, individuals of *P. ocellatus* that were exposed to the sunlight-lamp in the laboratory in boxes with sand, started to burry themselves partly in the sand. As a species with long-term retention of chloroplasts, *Elysia timida* revealed nevertheless increasing movement into direct light exposure in our trials. This might potentially be connected to the adaptations that are described for this species, e. g. as the modulation of the wing-like parapodial lobes to close and cover the inner lying chloroplasts (Rahat and Monselise 1979, Monselise and Rahat 1980, Jesus, Ventura et al. 2010, Schmitt and Wägele 2011). However, interpretation of photobehavior in sacoglossans still requires caution, with potential photodamage of chloroplasts by high irradiation taken carefully into account. Viera et al. investigated the effect of light exposure on the photosynthetic activity of kleptoplasts in *E. viridis* by comparing two different experimental treatments of keeping the individuals either in 'high' light conditions of 140 µmol photons

m^{-2} s^{-1} or low light conditions of 30 µmol photons m^{-2} s^{-1}, and reported a distinctly more rapid decrease of photosynthetic activity with high light conditions of retention, lasting only 6 to 15 days, opposed to the much slower decrease in low light conditions with retention lasting from 15 to 57 days (Vieira, Calado et al. 2009). In their recent study investigating aspects of photobehavior in *E. viridis* and *Placida dendritica*, Cartaxana et al. included the factor of acclimation to different light regimes, which they inferred to have modulating influences on photobehavior (Cartaxana, Morelli et al. 2018). Overall, various potential regulation mechanisms and additional further factors could possibly influence photobehavior, a complex phenomenon, with positive phototaxis being probably a more basic evolution, as sea slug species without chloroplasts and also juvenile *E. timida* before integration of chloroplasts showed positive phototactic behavior (Schmitt and Wägele 2011).

Underwater studies

Despite extensive investigations of photosynthetic sea slugs under laboratory conditions, reports of investigations of photosynthetic activities in their natural environment are not known to us. The present study is the first that performed PAM-investigations of the photosynthetic yield $\Delta F/F_m$' of kleptoplasts of two sacoglossan species underwater in their natural habitat in relation to environmental light conditions. We found variations in kleptoplast photosynthetic activity during the course of the day in the species *E. timida* and *E. crispata mangrove type* with a correlation to light conditions in the natural environment which are varying depending on habitat, weather and other environmental conditions. In both species, there was overall the same correlation of lower effective quantum yield $\Delta F/F_m$' with higher solar irradiation as well as higher effective quantum yield $\Delta F/F_m$' with lower solar irradiation, which was even more prominent in the food algae *A. acetabulum* of *E. timida*. For the interpretation of photosynthetic activity in natural environments in general, several potential influencing factors have to be considered, as e. g. specific photosynthetic optima or other intrinsic (e. g. state of the chloroplasts, state of the sea slug) and extrinsic (e. g. light conditions, temperature) parameters. Concerning the correlation between lower irradiances with higher effective quantum yield $\Delta F/F_m$', the special character of the PAM-fluorometry in relation to the complex photosynthetic mechanisms has to be considered: in contrast to the maximum quantum yield of PS II which is measured after dark acclimation with 'maximal number of open reaction (oxidized) centers in photosystem II available to process photons'

(Wägele and Johnsen 2001), here, in the natural environment conditions, we measure the effective quantum yield $\Delta F/F_m$' under ambient light conditions, which decreased with increasing irradiation. As a measure of photosynthetic rate, the relative rate of electron transport (ETR), however, which includes the quantum flux density of photosynthetically active radiation of the ambient light as a multiplication factor, revealed the corresponding positive correlation of higher ETR with higher solar irradiation, which in this case was more pronounced in *E. timida* than in *A. acetabulum*. In coherence with the ambient light conditions, the ETR was overall distinctively higher in the *E. timida* individuals in the shallower depths close to the surface than in the deeper depths of around 5 m. Fluorescence F in individuals of *E. timida* measured underwater was always distinctively lower (about half as much) than in their food algae *A. acetabulum*. This might be a result of higher density of chloroplasts in the algae and/or partly influenced by the shading effect of the parapodia, also diminishing emission of fluorescence, as described before (Schmitt and Wägele 2011). Observations revealed a tendency that parapodia of individuals of *E. timida* were more closed in stronger light intensities pointing to the parapodial covering effect, corresponding to former descriptions (Rahat and Monselise 1979, Monselise and Rahat 1980, Jesus, Ventura et al. 2010, Schmitt and Wägele 2011). Casalduero and Muniain supposed that opening and closing of parapodia seems to modulate excess light in connection with a rapid saturation of the photosynthetic apparatus which they inferred from estimates and models in an analysis of photosynthetic activity in *E. timida* (Casalduero and Muniain 2006). During observations of *E. crispata mangrove type*, we saw indices for a movement of parapodia with light modulation but it was not directly recognizable in a clear way, probably also due to the more undulated structure of the parapodia and different distribution of chloroplasts throughout the body, which impeded a clear distinction of parapodial opening levels similar to those in *E. timida*. A recent study reported observations of parapodia modulation in relation to irradiance in *E. viridis*, with increasing light levels leading to a closure of parapodia (Cartaxana, Morelli et al. 2018). Parapodia modulation and photobehavior overall remain interesting research topics with potential clarification with further investigations.

References manuscript draft

1. Baumgartner, F. A., et al. (2015). "Acquired Phototrophy through Retention of Functional Chloroplasts Increases Growth Efficiency of the Sea Slug Elysia viridis." PLoS One **10**(4): e0120874.
2. Bhattacharya, D., et al. (2013). "Genome analysis of Elysia chlorotica Egg DNA provides no evidence for horizontal gene transfer into the germ line of this Kleptoplastic Mollusc." Mol Biol Evol **30**(8): 1843-1852.
3. Cartaxana, P., et al. (2018). "Kleptoplast photoacclimation state modulates the photobehaviour of the solar-powered sea slug Elysia viridis." J Exp Biol **221**(Pt 12).
4. Casalduero, F. G. and C. Muniain (2006). "Photosynthetic activity of the solar-powered lagoon mollusc Elysia timida (Risso, 1818) (Opisthobranchia: Sacoglossa)." Symbiosis **41**: 151–158.
5. Chan, C. X., et al. (2018). "Active Host Response to Algal Symbionts in the Sea Slug Elysia chlorotica." Mol Biol Evol **35**(7): 1706-1711.
6. Christa, G., et al. (2015). "Phylogenetic evidence for multiple independent origins of functional kleptoplasty in Sacoglossa (Heterobranchia, Gastropoda)." Org Divers Evol **15**: 23.
7. Christa, G., et al. (2014). "Identification of sequestered chloroplasts in photosynthetic and non-photosynthetic sacoglossan sea slugs (Mollusca, Gastropoda)." Front Zool **11**(1): 15.
8. Christa, G., et al. (2013). "What remains after 2 months of starvation? Analysis of sequestered algae in a photosynthetic slug, Plakobranchus ocellatus (Sacoglossa, Opisthobranchia), by barcoding." Planta **237**(2): 559-572.
9. Clark, K. B. (1984). "New records and synonymies of Bermuda Opisthobranchs (Gastropoda)." The Nautilus **98**: 85-97.
10. Cruz, S., et al. (2015). "Photoprotection in sequestered plastids of sea slugs and respective algal sources." Sci Rep **5**: 7904.
11. Evertsen, J., et al. (2007). "Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean." Marine Biology **151**: 2159-2166.
12. Evertsen, J. and G. Johnsen (2009). "In vivo and in vitro differences in chloroplast functionality in the two north Atlantic sacoglossans (Gastropoda, Opisthobranchia) Placida dendritica and Elysia viridis." Marine Biology **156**: 847-859.
13. Händeler, K., et al. (2009). "Functional chloroplasts in metazoan cells - a unique evolutionary strategy in animal life." Front Zool **6**: 28.
14. Heinz Walz GmbH (1998). Underwater Fluorometer Diving-PAM - Submersible Photosynthesis Yield Analyzer Handbook of Operation.
15. Jesus, B., et al. (2010). "Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanisms in the solar-powered sea slug Elysia timida (Risso, 1818)." Journal of Experimental Marine Biology and Ecology **395**: 98–105.
16. Krug, P. J., et al. (2013). "Integrative species delimitation in photosynthetic sea slugs reveals twenty candidate species in three nominal taxa studied for drug discovery, plastid symbiosis or biological control." Mol Phylogenet Evol **69**(3): 1101-1119.
17. Krug, P. J., et al. (2016). "Molecular and morphological systematics of Elysia Risso, 1818 (Heterobranchia: Sacoglossa) from the Caribbean region." Zootaxa **4148**(1): 1-137.
18. Laetz, E. M. J. and H. Wägele (2017). "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." PLoS One **12**(10): e0182910.
19. Laetz, E. M. J. and H. Wägele (2018). "How does temperature affect functional kleptoplasty? Comparing populations of the solar-powered sister-species Elysia timida Risso, 1818 and Elysia cornigera Nuttall, 1989 (Gastropoda: Sacoglossa)." Front Zool **15**: 17.
20. Maeda, T., et al. (2012). "Algivore or phototroph? Plakobranchus ocellatus (Gastropoda) continuously acquires kleptoplasts and nutrition from multiple algal species in nature." PLoS One **7**(7): e42024.
21. Melo Clavijo, J., et al. (2018). "Polymorphic adaptations in metazoans to establish and maintain photosymbioses." Biol Rev Camb Philos Soc.
22. Monselise, E. B. I. and M. Rahat (1980). "Photobiology of Elysia timida (Mollusca: Opisthobranchia): observations in the sea." Isr J Zool **29**: 125–128.

23. Pelletreau, K. N., et al. (2011). "Sea slug kleptoplasty and plastid maintenance in a metazoan." Plant Physiol **155**(4): 1561-1565.
24. Pelletreau, K. N., et al. (2014). "Lipid accumulation during the establishment of kleptoplasty in Elysia chlorotica." PLoS One **9**(5): e97477.
25. Pelletreau, K. N., et al. (2012). "Laboratory culturing of Elysia chlorotica reveals a shift from transient to permanent kleptoplasty." Symbiosis **58**(1-3): 221-232.
26. Rahat, M. and E. B. I. Monselise (1979). "Photobiology of the chloroplast-hosting mollusc Elysia timida (Opisthobranchia)." J Exp Biol **79**: 225–233.
27. Rauch, C., et al. (2018). "The ability to incorporate functional plastids by the sea slug Elysia viridis is governed by its food source." Marine Biology **165**(82).
28. Rumpho, M. E., et al. (2011). "The making of a photosynthetic animal." J Exp Biol **214**(Pt 2): 303-311.
29. Schmitt, V., et al. (2014). "Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of Elysia timida (Sacoglossa, Heterobranchia)." Front Zool **11**(1): 5.
30. Schmitt, V. and H. Wägele (2011). "Behavioural adaptation in relation to long-term retention of endosymbiotic chloroplasts in the sea slug Elysia timida (Opisthobranchia, Sacoglossa)." Thalassas **27**(2): 226–238.
31. Ventura, P., et al. (2013). "Photosynthetic efficiency and kleptoplast pigment diversity in the sea slug Thuridilla hopei (Vérany, 1853)." Journal of Experimental Marine Biology and Ecology **441**: 105-109.
32. Vieira, S., et al. (2009). "Effects of light exposure on the retention of kleptoplastic photosynthetic activity in the sacoglossan mollusc Elysia viridis." Marine Biology **156**: 1007-1020.
33. Wade, R. M. and A. R. Sherwood (2017). "Molecular determination of kleptoplast origins from the sea slug Plakobranchus ocellatus (Sacoglossa, Gastropoda) reveals cryptic bryopsidalean (Chlorophyta) diversity in the Hawaiian Islands." J Phycol **53**(3): 467-475.
34. Wade, R. M. and A. R. Sherwood (2018). "Updating Plakobranchus cf. ianthobapsus (Gastropoda, Sacoglossa) host use: Diverse algal-animal interactions revealed by NGS with implications for invasive species management." Mol Phylogenet Evol. **128**:172-181.
35. Wägele, H., et al. (2011). "Transcriptomic evidence that longevity of acquired plastids in the photosynthetic slugs Elysia timida and Plakobranchus ocellatus does not entail lateral transfer of algal nuclear genes." Mol Biol Evol **28**(1): 699-706.
36. Wägele, H. and G. Johnsen (2001). "Observations on the histology and photosynthetic performance of “solar-powered” opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae." Org Divers Evol **1**: 193–210.
37. Wägele, H. and R. Martin (2013). Endosymbioses in sacoglossan seaslugs: plastid-bearing animals that keep photosynthetic organelles without borrowing genes. Endosymbiosis. W. Löffelhardt. Vienna, Springer Vienna**:** 291–324.
38. Weaver, S. and K. B. Clark (1981). "Light intensity and color preferences in five ascoglossan (=sacoglossan) molluscs (Gastropoda, Opisthobranchia): A comparison of chloroplast symbiotic and aposymbiotic species." Marine and Freshwater Behavior and Physiology **7**: 297-306.
39. Yamamoto, S., et al. (2013). "Effects of photosynthesis on the survival and weight retention of two kleptoplastic sacoglossan opisthobranchs." Journal of the Marine Biological Association of the United Kingdom **93**(1): 209-215.

3.4 Further unpublished results

3.4.1 Feeding experiments and inter- and intraspecific differences within species-specific spectra of photosynthetic capacities in various sacoglossan sea slug species

This section depicts further yet unpublished results in the frame of the investigations in near-natural or semi-natural settings presented in chapter 3.3. As described in the introduction, feeding experiments were carried out to gain more fundamental information about algal chloroplast donors and their potential implications on various capabilities of chloroplast retention in the different investigated sea slug species. These investigations were combined with TEM-investigations which are described below in chapter 3.4.2. in more detail. As the investigations presented here in chapter 3.4 were performed along with those in chapter 3.3, the methods overlap in major part as explained and completed with additional method notes in the overall method section (chapter 2). Here, the further results including feeding experiments and PAM-investigations are reported, with an overview of different investigation years.

In the feeding experiments, specific algae could be confirmed as chloroplast donors by using PAM-measurements, resulting in an overview of investigated sacoglossan species and their algal chloroplast donor with respective PAM-values (Table 3.4.1). The different sacoglossan species were analyzed concerning their species-specific photosynthetic yield (F_v/F_m, maximal yield after dark adaptation) after collection and thus in a natural, normally saturated and chloroplast-replenished state (column 1). This was compared to the photosynthetic yield F_v/F_m of the associated algae in a freshly collected, natural state. Furthermore, individuals of the sacoglossan species were kept without food supply and monitored with PAM-measurements until photosynthetic yields fell down and approached zero, indicating depletion of incorporated chloroplasts (column 3). These individuals were then supplied anew with the respective algae species in focus and observed with following PAM-measurement-series. Rising values of measured photosynthetic yield F_v/F_m in sea slugs after renewed feeding with the respective algae species were considered as evidence for the incorporation of the algal chloroplasts by the sea slugs and documented (column 4). Similar to individuals of *Bosellia mimetica* and *Elysia viridis*, an individual of *Elysia timida* revealed the highest photosynthetic yield after renewed feeding and could apparently well replenish with chloroplasts from *Acetabularia acetabulum* already within three to four hours of feeding.

Overall, photosynthetic yields measured in sea slugs after renewed feeding corresponded well to those after collection and those of the respective algae, except for *Placida dendritica* and *Ercolania viridis* with distinctively lower photosynthetic yields than those of the respective food algae (Table 3.4.1).

Sea Slug species with exemplary photosynthetic yield [F_v/F_m] after collection	**Algae species with exemplary photosynthetic yield [F_v/F_m]**	**Last photosynthetic yield [F_v/F_m] in sea slug before renewed feeding**	**Best relevant photosynthetic yield [F_v/F_m] in sea slug after renewed feeding with respective algae °**
Bosellia mimetica, (n=10), 0.679 ± 0.061 (0.568-0.774) (Figure 3.3.2, chapter 3.3)	*Halimeda tuna*, (n=12), 0.695 ± 0.049 (0.589-0.762) **§**	0.186 ± 0.136 (0.029 to 0.272) (n=3)	0.703 ± 0.043 (0.658 to 0.743) (n=3) (2d n=2 #, 4d n=1)
Placida dendritica (n=5), 0.247 ± 0.037 (0.190-0.278) (Figure 3.3.2)	*Codium fragile/vermilara* (n=8), 0.768 ± 0.032 (0.727-0.806) **§**	0.146 ± 0.090 (0.042 to 0.203) (n=1)	0.257 ± 0.042 (0.215 to 0.300) (n=1) (3d) *
Ercolania viridis (n=5), 0.126 ± 0.078 (0.019 to 0.198) (Figure 3.4.4)	*Chaetomorpha aerea/linum,* (n=2), 0.659 ± 0.038 (0.632-0.685) **§**	0.000 ± 0.000 (0.000 to 0.000) (n=2)	0.224 ± 0.048 (0.019 to 0.258) (n=2) (2d)
Elysia viridis (n=8), 0.673 ± 0.043 (0.601-0.732) (Figure 3.3.2)	*Codium fragile/vermilara* (n=8), 0.768 ± 0.032 (0.727-0.806) **§** *Flabellia petiolata (originally on Codium fragile/vermilara)* (n=4), 0.725 ± 0.036 (0.682-0.761) **§** *Flabellia petiolata (originally on Flabellia petiolata)* (n=4), 0.725 ± 0.036 (0.682-0.761) **§**	0.373 ± 0.018 (0.360 to 0.385) (n=1) * 0.201 ± 0.189 (0.000 to 0.376) 0.000 ± 0.000 (0.000 to 0.000)	0.669 ± 0.046 (0.618 to 0.706) (n=1) (5d) * 0.690 ± 0.115 (0.559 to 0.777) (n=3) 0.641 ± 0.019 (0.627 to 0.654) (n=2) (5d+13d)
Elysia timida (n=22), 0.725 ± 0.045 (0.610-0.816) (Figure 3.3.2)	*Acetabularia acetabulum* (n=11), 0.748 ± 0.042 (0.675-0.805) **§**	0.000 ± 0.000 (0.000 to 0.000) (n=1) *	0.703 ± 0.025 (0.675 to 0.723) (n=1#) (0d, 3-4 hrs) *
Elysia crispata mangrove type (n=7), 0.612 ± 0.081 (0.523-0.774) (Figure 3.3.2)	*Caulerpa verticillata* (n=6), 0.662 ± 0.066 (0.575-0.738) **$** *Penicillus capitatus* (n=4), 0.645 ± 0.180 (0.392-0.788) **$**	0.000 ± 0.000 (0.000 to 0.000) (n=3) 0.000 ± 0.000 (0.000 to 0.000) (n=1) *	0.666 ± 0.015 (0.653 to 0.687) (n=3) (7d n=2, 8d n=1) 0.667 ± 0.066 (0.601 to 0.733) (n=1) *

Table 3.4.1: Photosynthetic yield F_v/F_m measured with PAM in algae and sacoglossan species in feeding experiments. Data are displayed with n= number of individuals, mean ± SD (range). All samples dark adapted before PAM-measurement of maximum yield [F_v/F_m], except the two algal species measured in the sea as indicated. ° Best relevant photosynthetic yield reached during phase of renewed feeding (duration of renewed feeding until measurement time point [days]); * When n=1 mean calculated of consecutive measurements of one measurement time point in this individual; # Individual(s) fixed for TEM, values could have risen higher with longer feeding; § after collection; $ in the sea

Thuridilla hopei

In *T. hopei*, collected in July 2011, photosynthetic yield values were at a relatively high level when collected freshly from the sea, but decreased very fast within a few days (Figure 3.3.2 in chapter 3.3). This corresponded to another collection population of *T. hopei* found during another summer season (n=4, August 2012) revealing relatively similar photosynthetic yields in the course of the measurements: directly after collection, the mean photosynthetic yield F_v/F_m was 0.566 ± 0.234 (range: 0.222 to 0.744) and declined fast until day 5 to 0.222 ± 0.262 (range: 0 to 0.512) and approached zero already on day 9 in all investigated individuals of *T. hopei* of both collection populations with comparable seasonal and temperature conditions.

Placida dendritica

In *P. dendritica*, collected from *C. fragile/vermilara* in August 2012, photosynthetic values were low right from the beginning, F_v/F_m ranging from only 0.190 to 0.278 (Figure 3.3.2 in chapter 3.3). Another collection population (n=6, June 2011) displayed even lower yield values directly after collection from *C. fragile/vermilara* with a mean F_v/F_m of 0.048 ± 0.029 (range: 0.02 to 0.103), hardly above zero, and thus was excluded from the analysis. Overall, low photosynthetic yield values measured in *P. dendritica* even directly after collection clearly reflected that despite uptake of chloroplasts the level of degradation of chloroplasts is so high that photosynthetic yield is far below species with retention of chloroplasts, e. g. *E. viridis,* collected from the same food algae (Figure 3.3.2 in chapter 3.3).

Bosellia mimetica

Individuals of *B. mimetica* were always collected from the chlorophyte *Halimeda tuna*, but nevertheless displayed slight differences between different collection populations. Compared to the exemplary population of August 2012 displayed in Figure 3.3.2 (Figure 3.3.2 in chapter 3.3), other collection populations of July and August 2010 (n=5 and n=5) showed lower yield values right after collection with mean F_v/F_m 0.612 ± 0,066 (range: 0.503 to 0.665) and 0.542 ± 0.104 (range: 0.378 to 0.626), respectively, and approaching zero in about 20 days. In summer 2011, however, some individuals showed overall slightly higher starting values with

e. g. 0.699 ± 0.033 (range: 0.643 to 0.733) in June/July 2011 (n=8) and 0.686 ± 0.007 (range: 0.681 to 0.691) in August 2011 (n=2) and longer lasting photosynthetic performance up to even still a mean yield of 0.195 ± 0.090 (range: 0.131 to 0.258) after 34 days without food supply in September 2011 (n=2). One single individual in July 2011, which was collected from *H. tuna* four days after collection from the sea, even displayed a starting yield of 0.764, survived a period of more than 30 days without food and could successfully be fed anew with supply of *H. tuna* (Figure 3.4.1, Table 3.4.1).

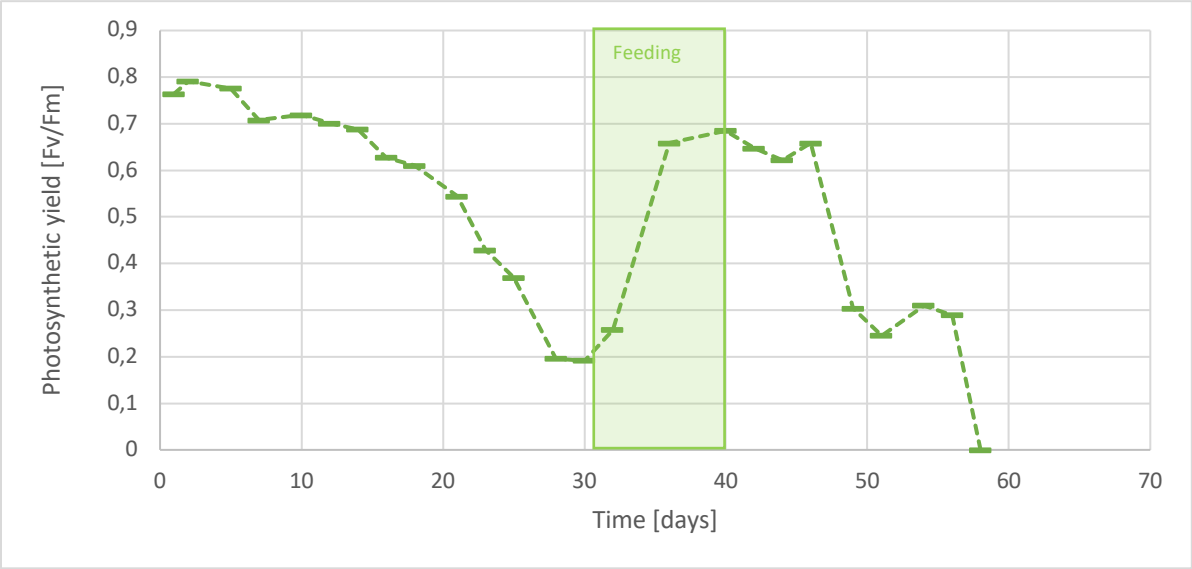


Figure 3.4.1: Photosynthetic yield and successful renewed feeding of an individuum of *Bosellia mimetica* with the algae *Halimeda tuna* after more than 30 days without food supply. On day 32 of the first period without food supply after collecting the sea slug from *H. tuna*, the feeding phase of eight days was started until day 40. Then the food supply was stopped again and a second period without food supply observed. (Last value replaced with 0 as correction for false high value.) *Bosellia mimetica* (Banyuls-sur-Mer, France, n=1, start 12th July 2011, OOB, ca. 18-24 °C).

After eight days of renewed feeding, it had retained its green color and high yield values and could be observed during a second staving period, during which high yield values lasted for about a week (Figure 3.4.1). The three described individuals with longer retention durations (one July 2011, two September 2011) were also comparatively large with sizes of 7, 8 and 11 millimeters (mm), in contrast to e. g. an overall mean size of 4.5 ± 1 mm (range: 3.5 to 7 mm) in the exemplary collection population of August 2012 displayed in Figure 3.3.2 in chapter 3.3. On the basis of the available data, an overall clear correlation between size of the individual slugs and retention duration was not evident, however.

Elysia viridis

Feeding experiments showed that *E. viridis* – in addition to its known food alga *C. fragile* – also fed on another alga, *Flabellia petiolata,* with subsequent chloroplast retention. In a comparative feeding trial, eight individuals of *E. viridis* collected from *C. fragile/vermilara* were at first held without food supply until photosynthetic yield values had fallen, indicating depletion of incorporated chloroplasts, and then supplied with *C. fragile/vermilara* (n=4) or *F. petiolata* (n=4) during 10 days. Interestingly, three of the four individuals supplied with *F. petiolata* showed distinct rising of photosynthetic yield F_v/F_m, indicating that they had fed on the algae and incorporated chloroplasts, with even higher photosynthetic yields and better chloroplast retention than before when collected from *C. fragile/vermilara* (Figure 3.4.2, Table 3.4.1).

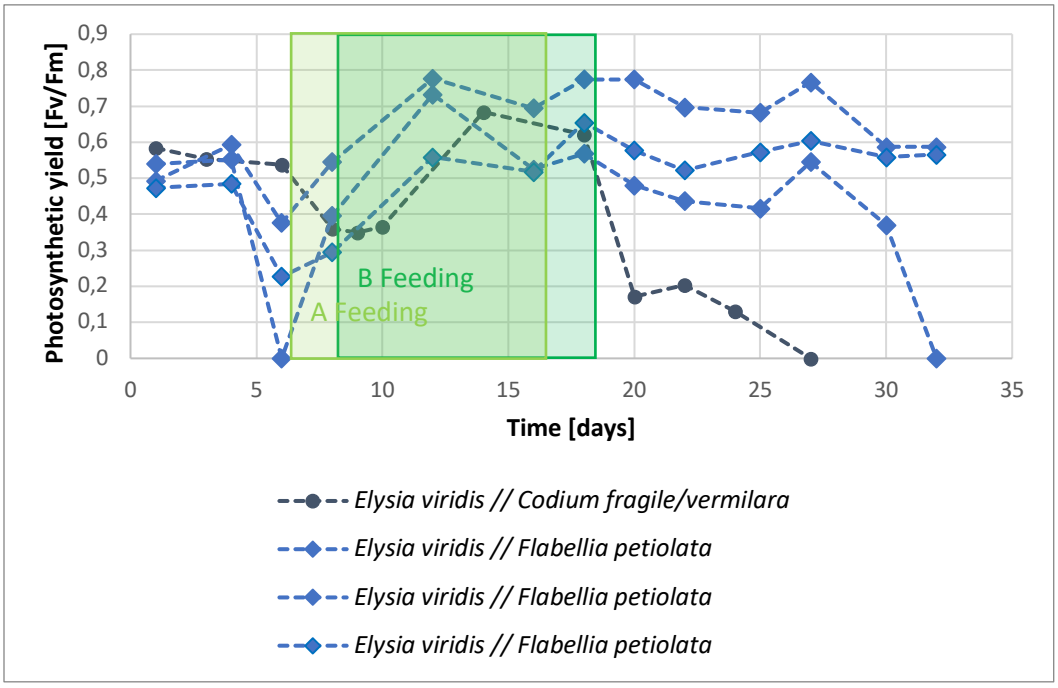


Figure 3.4.2: Photosynthetic yield F_v/F_m and successful renewed feeding of individuals of *Elysia viridis* which had originally been collected from *Codium fragile/vermilara* and after a first period without food supply fed with either *Flabellia petiolata* (group A) or *Codium fragile/vermilara* (group B). After collection from *C. fragile/vermilara,* individuals of *E. viridis* were first held without food supply until photosynthetic yield was approaching zero on day 6 in group A (n=4) and day 8 in group B (n=4) and both groups were then fed on the following day with *F. petiolata* (group A) or *C. fragile/vermilara* (group B), respectively. Three of four individuals in group A and one of four individuals in group B could be successfully fed mirrored by distinct rising of photosynthetic yield F_v/F_m. Supply of the respective algae was stopped in both groups after 10 days on day 16 (group A) and day 18 (group B) and photosynthetic yield was observed in the following period without food supply. *Elysia viridis* (Banyuls-sur-Mer, France, group A n=3(/4), start 5th August 2011, OOB, group B n=1(/4), start 3rd August 2011, ca. 19-24 °C), both groups from the same collection of *Codium fragile/vermilara* (2nd August 2011).

These individuals supplied with *F. petiolata* visibly retained their green color and held relatively high levels of photosynthetic yield during two further weeks without algae supply. Only one of the four regained just little green color and lower photosynthetic yields, leaving the proof of chloroplast uptake in this individual unclear. Of the four individuals supplied with *C. fragile/vermilara*, three showed only low, unclear rising of photosynthetic yield and died despite of algae supply. Only one survived longer than the renewed feeding period and could apparently well replenish with chloroplasts from *C. fragile/vermilara,* retaining a saturated green color and high photosynthetic yields but those fell within a week to approach zero (Figure 3.4.2, Table 3.4.1).

In further reversed trials, individuals of *E. viridis* found originally on *F. petiolata* were after a starving period attempted to be fed anew with *C. fragile/vermilara* (n=4) or *F. petiolata* (n=4). While these individuals did not show clear proofs of chloroplast uptake and retention with *C. fragile/vermilara*, two of the four individuals newly fed with *F. petiolata* showed clear rising of photosynthetic yields, indicating incorporation and functional retention of chloroplasts to approach zero after about two further weeks without food supply (Figure 3.4.2B, Table 3.4.1).

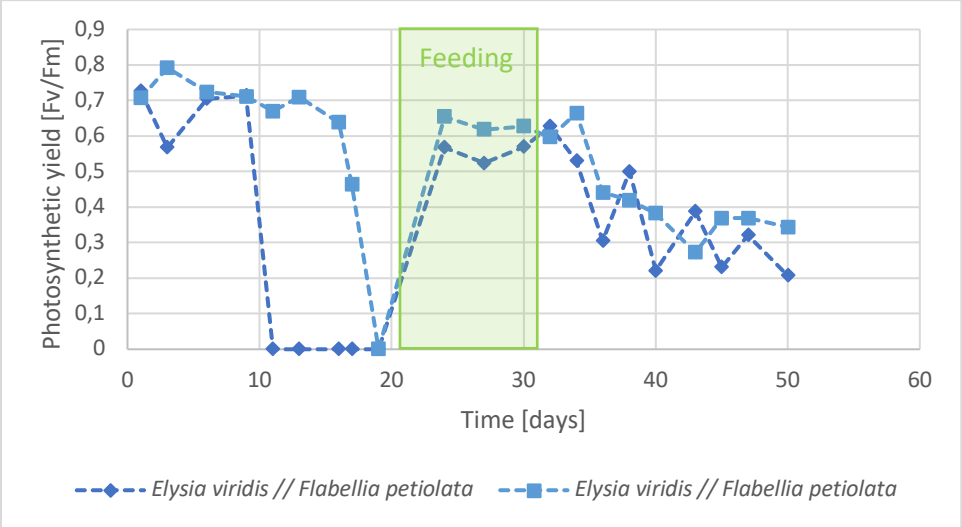


Figure 3.4.2B: Photosynthetic yield F_v/F_m and successful renewed feeding of individuals of *Elysia viridis* which had originally been collected from *Flabellia petiolata* and after a first period without food supply fed successfully with *F. petiolata*. After collection from *F. petiolata,* individuals of *E. viridis* had been first held without food supply until photosynthetic yield was approaching zero on day 11 in one individual and day 19 in another. Both were then fed successfully with *F. petiolata* (day 21) mirrored by distinct rising of photosynthetic yields. Supply of the algae was stopped after 10 days on day 31 and photosynthetic yield observed in the following period without food supply. *Elysia viridis* (Mediterranean, Banyuls-sur-Mer, France, start 13th August 2012, OOB, n=2, ca. 17-25 °C,), both from the same collection of *F. petiolata* (13th August 2012).

In an overview of long-term retention of chloroplasts in assembled different collections populations of *E. viridis* from either *F. petiolata* or *C. fragile/vermilara*, respectively, the individuals from *F. petiolata* showed overall higher mean photosynthetic yield F_v/F_m after collection with 0.710 ± 0.059 (range: 0.627 to 0.845) compared to those from *C. fragile/vermilara* with 0.555 ± 0.120 (range: 0.333 to 0.732) (Figure 3.4.3).

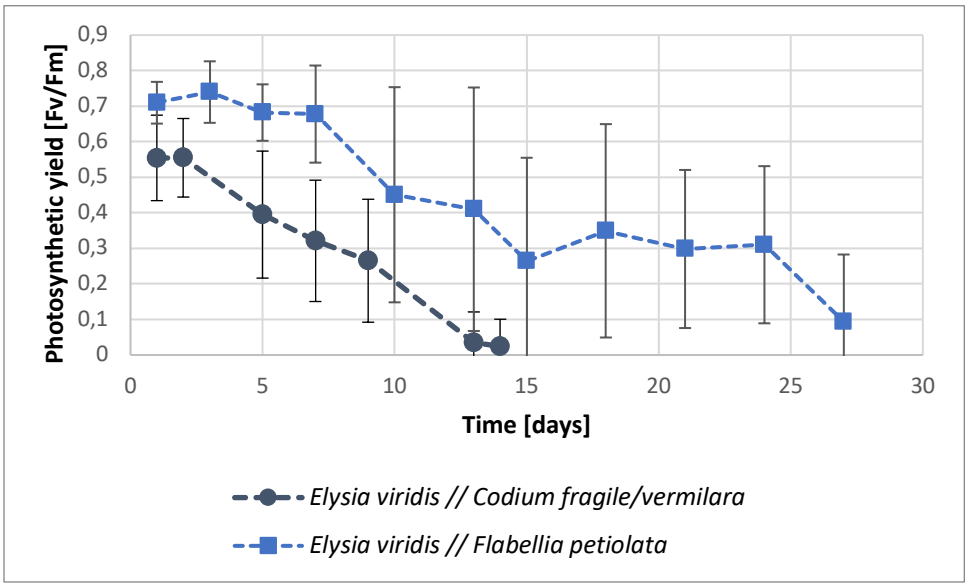


Figure 3.4.3: Photosynthetic yield F_v/F_m without algae supply after collection of *Elysia viridis* from either *Flabellia petiolata* or *Codium fragile/vermilara* in several collection populations assembled together. *Elysia viridis* from *Flabellia periolata* (Banyuls-sur-Mer, France, n=4+1, start 9th/11th August 2012, OOB, ca. 17-25 °C, n=7 start 13th August 2012, ca. 17-25 °C). *Elysia viridis* from *Codium fragile/vermilara* (Banyuls-sur-Mer, France, n=8, start 30th June 2011, OOB, ca. 19-22 °C, n=4+4, start 3rd/5th August 2011, ca. 19-22 °C, n=7 start 31st August 2011, ca. 21-23 °C, n= 5, start 9th August 2012, ca. 17-25 °C).

Within the *E. viridis* populations from *F. petiolata,* there was a tendency for longer chloroplast retention duration with bigger size as most individuals with longer retention were bigger with 8, 8 ,9 and 10 mm as the average 6.6 ± 1.9 mm (range: 4 to 10 mm) of these assembled populations, but one other individual with the second longest chloroplast retention measured only 4 mm. Also, the mean size of the assembled populations from *C. fragile/vermilara* was slightly bigger with 7.2 ± 1.3 mm (range: 5 to 9 mm). Thus, again a clear correlation of body size with chloroplast retention duration was not evident and the difference in photosynthetic capacities between individuals from *F. petiolata* or *C. fragile/vermilara* could not be explained by size.

In another trial as control, in which individuals which had been found on *F. petiolata* (n=2) or *C. fragile/vermilara* (n=4) were provided with the same respective algae during a period of at least two to three weeks after collection, the photosynthetic yield of the individuals stayed on similar high levels during the whole time with supply of both either *F. petiolata* or

C. fragile/vermilara and also stayed on relatively high levels for about further two weeks without food supply (Figure 3.4.3B).

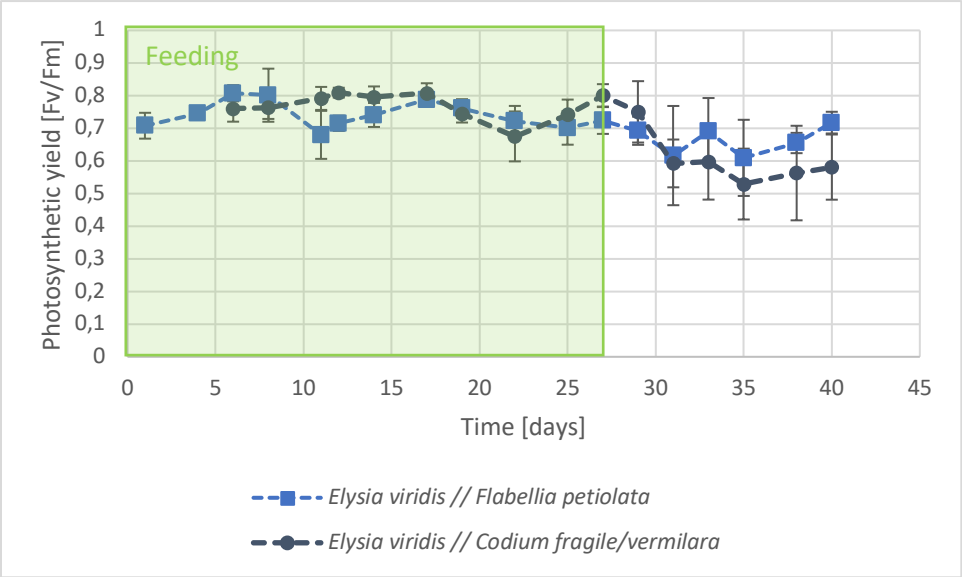


Figure 3.4.3B: Photosynthetic yield F_v/F_m during a phase with algae supply after collection of *Elysia viridis* collected from and provided with either *Flabellia petiolata* or *Codium fragile/vermilara* and a phase without algae supply in the following. Individuals of *E. viridis* were collected from either *F. petiolata* or *C. fragile/vermilara* and provided with the same respective algae for two to three weeks until day 27. On day 27, algae supply was stopped in both groups and the following two weeks without algae supply observed (day 28-40). *Elysia viridis* from *Flabellia petiolata* (Banyuls-sur-Mer, France, n=2, start 18th August 2012, OOB, ca. 17-25 °C). *Elysia viridis* from *Codium fragile/vermilara* (Banyuls-sur-Mer, France, n=2+1+1, start 23rd/25th/29th August 2012, OOB, ca. 17-25 °C).

Overall, *F. petiolata*, could be confirmed as a potential food algae and chloroplast donor for *E. viridis* in addition to *C. fragile/vermilara,* with the relevant proving photosynthetic yield F_v/F_m after successful renewed feeding being quite similar with both food algae (Table 3.4.1) and with even a tendency for better photosynthetic performance with longer retention of chloroplasts with *F. petiolata* in the investigated individuals.

In a comparison of different populations of *E. viridis* from different habitats, a population from a special habitat of a small seasonal tidal pool in the rocks, filled with a mix of algae including *Acetabularia acetabulum, Cladophora sp., Chaetomorpha sp.,* and *Enteromorpha sp*., was compared to a collection population from offshore. In the protected, calm environment of the temporarily constant tidal pool, found individuals had big sizes of 12, 13, 14 and even to 18 mm, appeared to be in very good condition with strong green coloring and high photosynthetic mean yield F_v/F_m of 0.747 ± 0.021 (range: 0.720 to 0.767) after

collection, lasting on high levels without food supply for about two weeks than declining slowly to approach zero after more than a month in total (Figure 3.4.4).

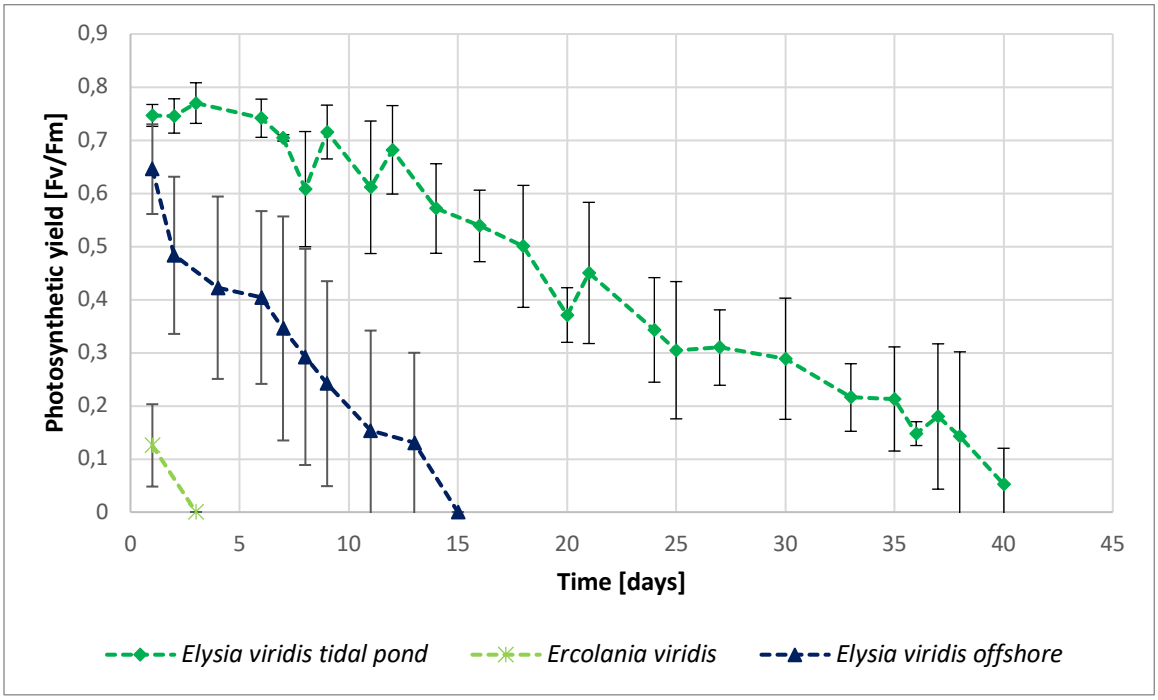


Figure 3.4.4: Photosynthetic yield F_v/F_m during periods without food supply after collection of *Elysia viridis* and *Ercolania viridis* from the small habitat of a tidal pool and *Elysia viridis* from offshore collections during the same time period. Tidal pool: *Ercolania viridis* (Banyuls-sur-Mer, France, n=3+2, start 21st April and 21st May 2010, OOB, ca. 13-15 °C and 15-16 °C), *Elysia viridis* (Banyuls-sur-Mer, France, n=4, start 21st April 2010, OOB, ca. 13-18 °C). Offshore boat collection: *Elysia viridis* (Banyuls-sur-Mer, France, n=2(1+1)+3, start 15th/20st April and 20st May 2010, OOB, ca. 13-16 °C and 15-18 °C).

Individuals of *E. viridis* obtained from offshore collections from mixed substrates (including e. g. *Posidonia* sp. and *Halimeda tuna)* during the same time period, were visibly smaller in size with 9.4 ± 2.2 mm (range: 6 to 12 mm) and revealed overall lower photosynthetic yields with 0.646 ± 0.084 (range: 0.507 to 0.712) after collection, falling distinctly faster to approach zero (Figure 3.4.4). The latter corresponded in photosynthetic yield and retention duration to the exemplary collection of Figure 3.3.2 in chapter 3.3, obtained from *C. fragile/vermilara*, which was even smaller in size with 7.6 ± 1.1 mm (range: 6 to 9 mm) and showed only slightly higher photosynthetic yield F_v/F_m with 0.673 ± 0.043 (range: 0.601 to 0.732), also falling to approach zero in about two weeks. Similar as described in the results above, again there are hints to a tendential influence of size on photosynthetic yield, but no evident correlation on the base of this data.

Elysia viridis and *Ercolania viridis* in the same habitat

Individuals of a population of *Ercolania viridis* – a small species of only some millimeters size with cerata like *Placida dendritica* – living in the same small habitat of the tidal pool as the *E. viridis* individuals described above, showed very low photosynthetic yield values directly after collection with a mean of 0.126 ± 0.078 (range: 0.019 to 0.198) which approached zero already after 2 days without food supply (Figure 3.4.4), resembling results of *P. dendritica* depicted above. Of the algae *Acetabularia acetabulum, Cladophora sp., Chaetomorpha sp., and Enteromorpha sp.* found in the habitat and offered to the individuals of *Er. viridis*, only *Chaetomorpha linum/aerea* could be confirmed as food algae (Table 3.4.1, also confirmed in video recordings). The photosynthetic yield values measured in two individuals who could be successfully fed anew with these algae were similar to those measured directly after collection and similarly had approached zero already on day 3 without food supply (Table 3.4.1).With that, *Er. viridis* represents another example of a cerata-bearing sacoglossan species that shows fast digestion of chloroplasts in contrast to the parapodia-bearing *E. viridis*, in the same habitat with the same presence of food algae in the environment – similar to the cerata-bearing *P. dendritica* which feeds on *C. fragile/vermilara* alike as *E. viridis* (Table 3.4.1, Figure 3.3.2 in chapter 3.3).

Elysia crispata mangrove type and *reef type* – two differentiated morphotypes from different habitats

Of *Elysia crispata,* a comparison of two different populations of *Elysia crispata* corresponding to *Elysia crispata mangrove type* and *reef type* with regard to their habitat origin was performed. As depicted in chapter 3.3, the two eco-morphotypes *Elysia crispata mangrove type* and *reef type,* revealed different photosynthetic performances in these two collection populations that stemmed from a flat, calm, sandy-muddy mangrove-near environment and an off-shore reef, with a more rapid decline of photosynthetic activity in the *mangrove type* than the *reef type* (Figure 3.3.2 in chapter 3.3).
For *E. crispata mangrove type*, two algal chloroplast donors could be confirmed (Table 3.4.1). Of the five provided algae species which were found to be abundant in the direct environment of the collection site where *E. crispata mangrove type* individuals were collected and that had been provided to the starved individuals, only *Caulerpa verticillata* and *Penicillus capitatus*

were found to evoke a distinct rising of photosynthetic yield in the examined individuals (Table 3.4.1) (negative feeding trials (see discussion): *Penicillus lamourouxii, n=5, Halimeda incrassata n=5, Halimeda monile n=5)*. In individuals that survived a second starving period, the course of photosynthetic yields with replenished depots from the two different chloroplast donor algae was relatively similar with slightly better photosynthetic performance with chloroplasts from *C. verticillata* (Figure 3.4.5).

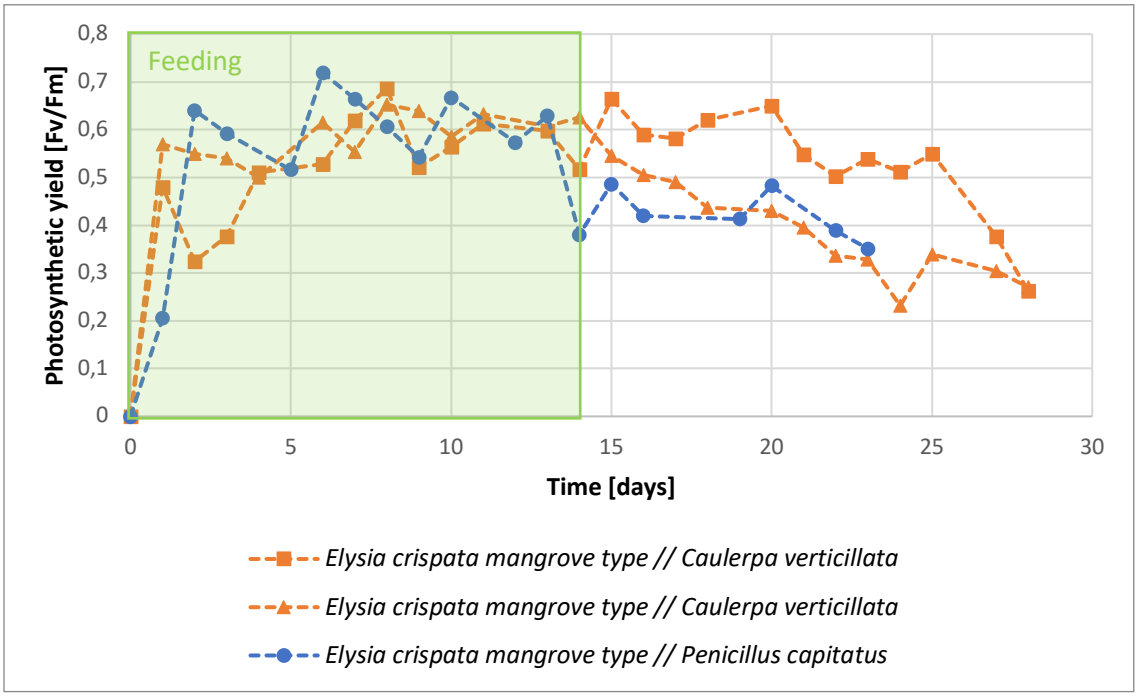


Figure 3.4.5: Photosynthetic yield F_v/F_m of incorporated chloroplasts from two different algal donors in renewed feeding of individuals of *Elysia crispata mangrove type* which after a first period without food supply and chloroplast depletion were fed with either *Caulerpa verticillata* (group A) or *Penicillus capitatus* (group B). After collection, individuals of *E. crispata mangrove type* had first been held without food supply until photosynthetic yield had approached zero after 40 days (group A) and 54 days in (group B) and both groups were then fed on the following day with either *Caulerpa verticillata* (group A) or *Penicillus capitatus* (group B), respectively. Supply of the respective algae was stopped after 14 days and photosynthetic yield was observed in the following period without food supply. *Elysia crispata mangrove type* (Florida Keys, USA, n=2+1, start 20th March / 4th April 2012, MML, ca. 23-24 °C).

3.4.2 Transmission electron microscopy investigations

Investigations with TEM were carried out to examine the incorporation of chloroplasts in several sacoglossan species with different capabilities of chloroplast retention as explained in the introduction and methods (chapters 1 and 2). The very first incorporation of chloroplasts of *A. acetabulum* in juvenile *E. timida* could be documented and published as displayed in chapter 3.2. This section here presents yet unpublished results from TEM-investigations performed during research stays in parallel to photobiological, behavioral and ecological analyses in the frame of the ASSEMBLE program with the technical assistance of Marie-Line Escande at the Observatoire Océanologique in Banyuls-sur-Mer, France, and along with the investigations described in chapters 3.2, 3.3 and 3.4.1. These TEM-investigations included *E. timida* as an exemplary species for long-term retention of chloroplasts, two exemplary species for intermediate, potential short- or long-term retention, *E. viridis* and *B. mimetica,* and two species with short-term retention or fast digestion, *T. hopei* and *P. dendritica*. In addition, the respective food algae were analyzed comparatively, as far as known and available. These comprised the food algae *A. acetabulum* (of *E. timida), H. tuna* (of *B. mimetica),* and *C. fragile/vermilara* (of *P. dendritica* and *E. viridis*). In coherence with the detection of *F. petiolata* as food algae and chloroplast donor for *E. viridis* (chapter 3.4.1), this algae was additionally analyzed. Of several TEM-investigations, a selection of results is presented here.

Sea slug species with intermediate to long-term chloroplast retention

Bosellia mimetica

B. mimetica collected from *H. tuna*, were fixed with the algae for TEM on the same day (28th June 2011) as representatives for the natural state. In that natural state, some chloroplasts lie apparently intact in the cytoplasm while others appear to be in degradation states (Fig. 3.4.6). In some cases, several chloroplasts seem to be enclosed and digested together in a digestive conglomerate vessel/vacuole, where in this case, also a starch grain seems to be included (Fig. 3.4.6). This individual was fixed for TEM directly after collection from *H. tuna* collected that day and PAM-measurements (F_v/F_m: 0.714, 0.675, 0.728), representing the natural state. Also in another individual representing the natural state, chloroplasts in different states were observed, some with dark conglomerates/droplets and some with bright conglomerates, apparently starch grains/granules, some with both (PAM: F_v/F_m: 0.693, 0.706, 0.649).

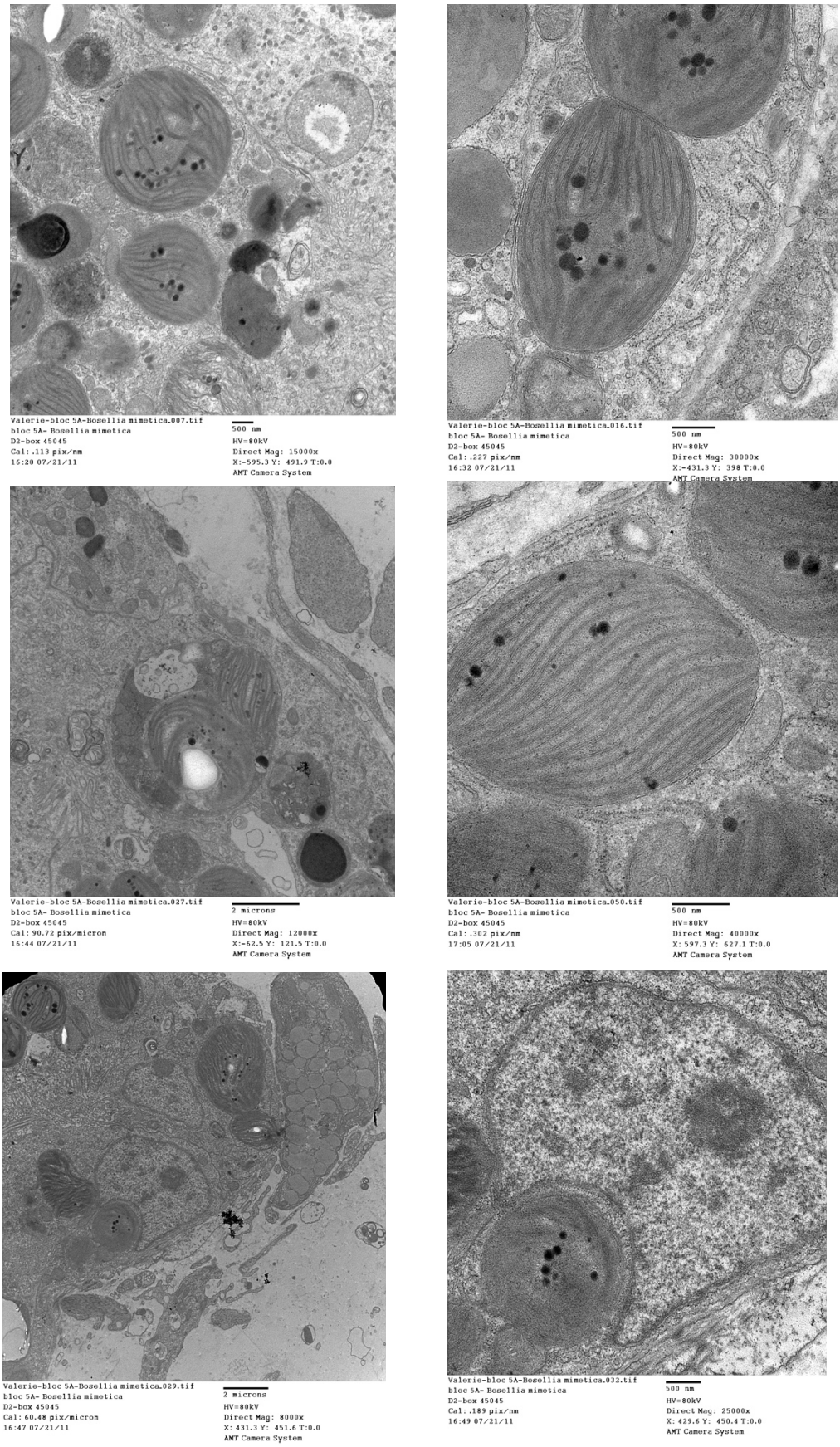


Figure 3.4.6: TEM micrographs of chloroplasts in *B. mimetica*, collected on 28th June 2011 from *H. tuna* and fixed on the same day, thus presenting a natural state in summer. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

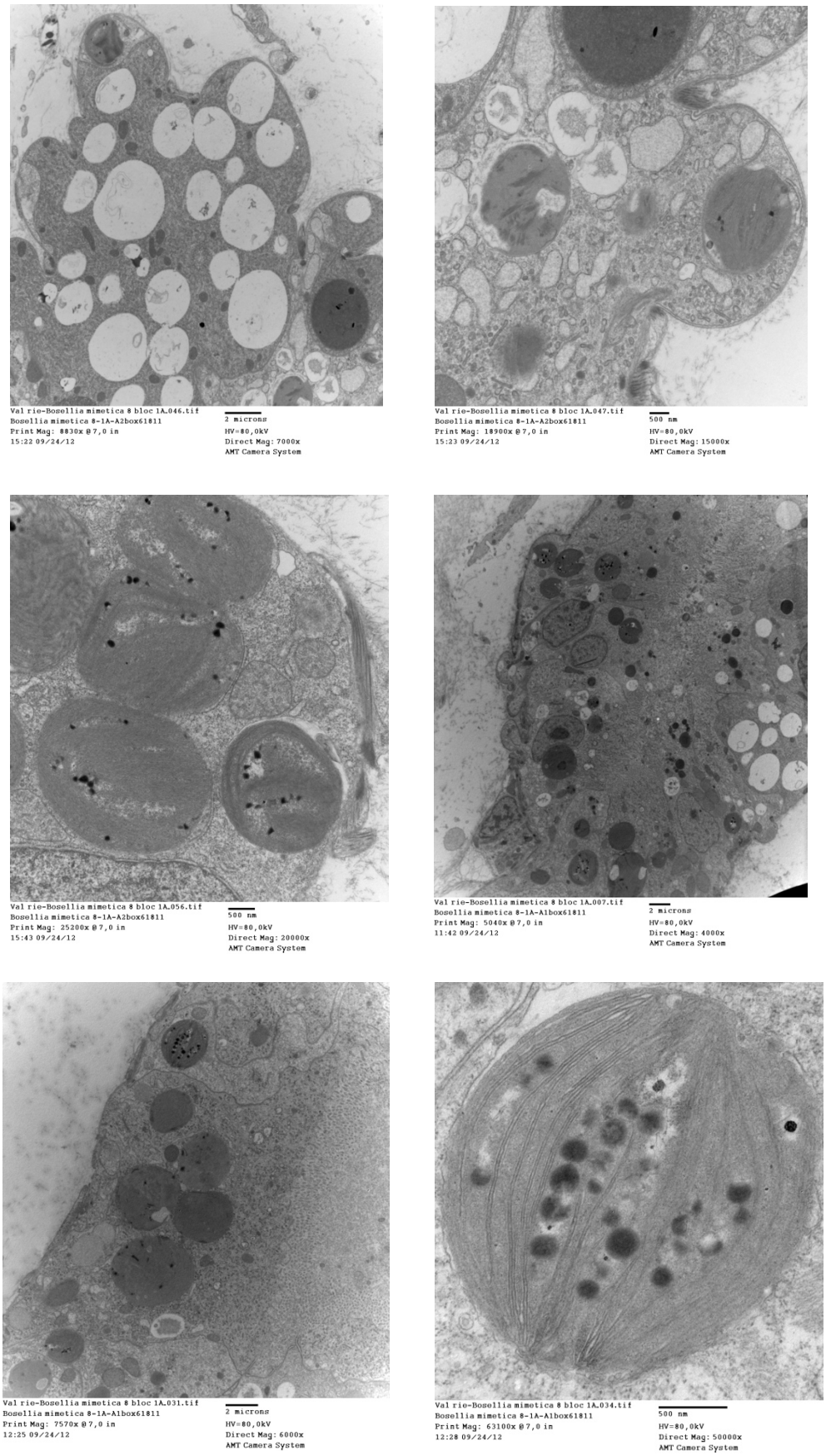


Figure 3.4.7: TEM micrographs of chloroplasts in *B. mimetica*, collected on 8th/9th August 2012 from *H. tuna* and starved for 11/12 days until fixation on 21st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In *B. mimetica*, the difference between the natural and the control state of a fed individual in contrast to the condition starved for 11 days was visible with more recognizable degradation of chloroplasts in the starved state (Fig. 3.4.7) (see above). Still, some single chloroplasts still appeared quite intact (Fig. 3.4.7).

In individuals of *B. mimetica* that were first starved for nine or 11 days, respectively, and then provided with *H. tuna* for two days or two hours, respectively, chloroplasts in different states, from apparently intact to remnants could be observed as expected (Fig. 3.4.8 and 3.4.9).

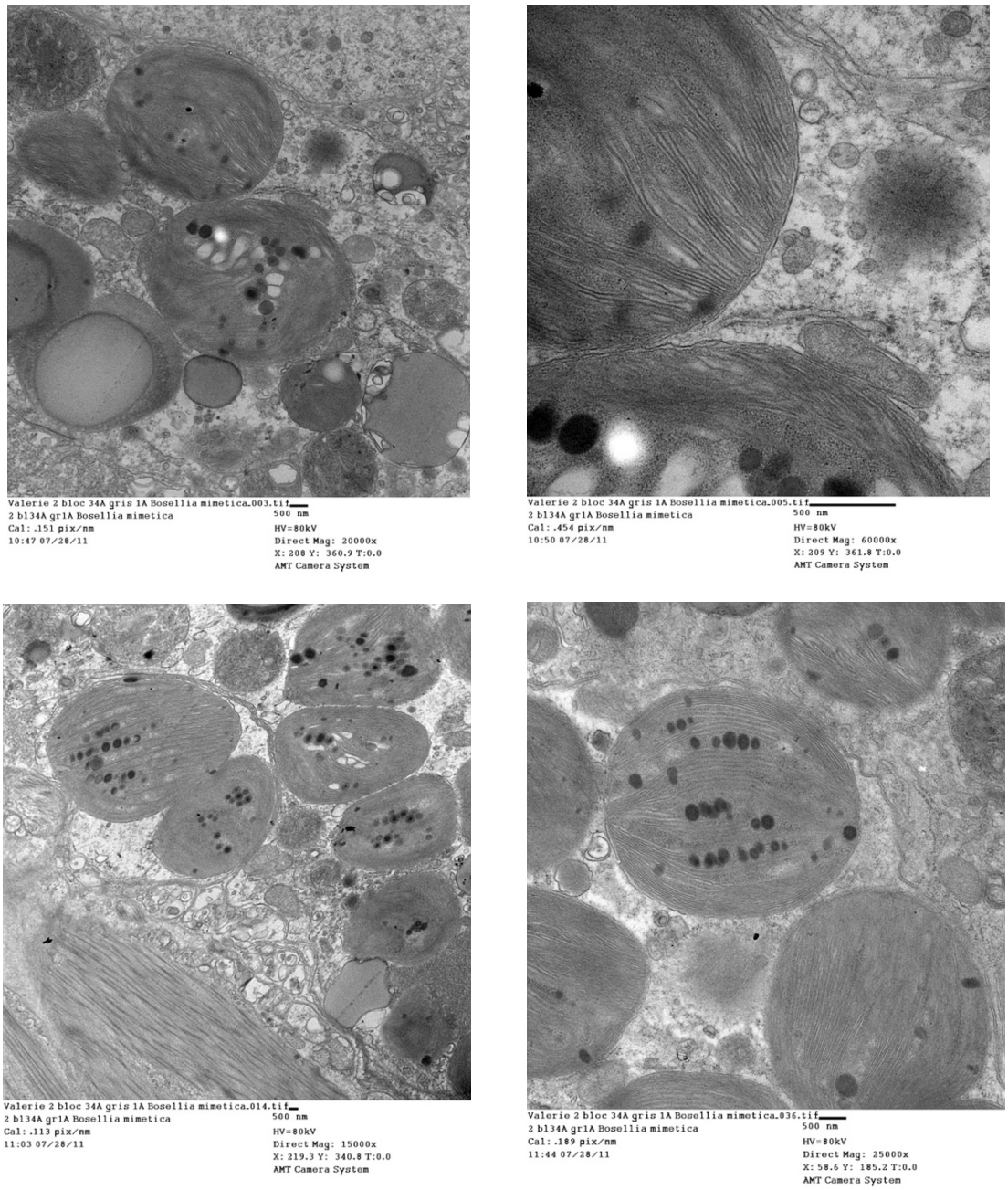


Figure 3.4.8: TEM micrographs of chloroplasts in *B. mimetica*, taken on 8th July 2011 from *H. tuna* and starved for nine days with accompanying PAM-measurements until an observed feeding experiment with *H. tuna* on 17th July and continuous supply of *H. tuna* for two days until fixation on 19th July 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

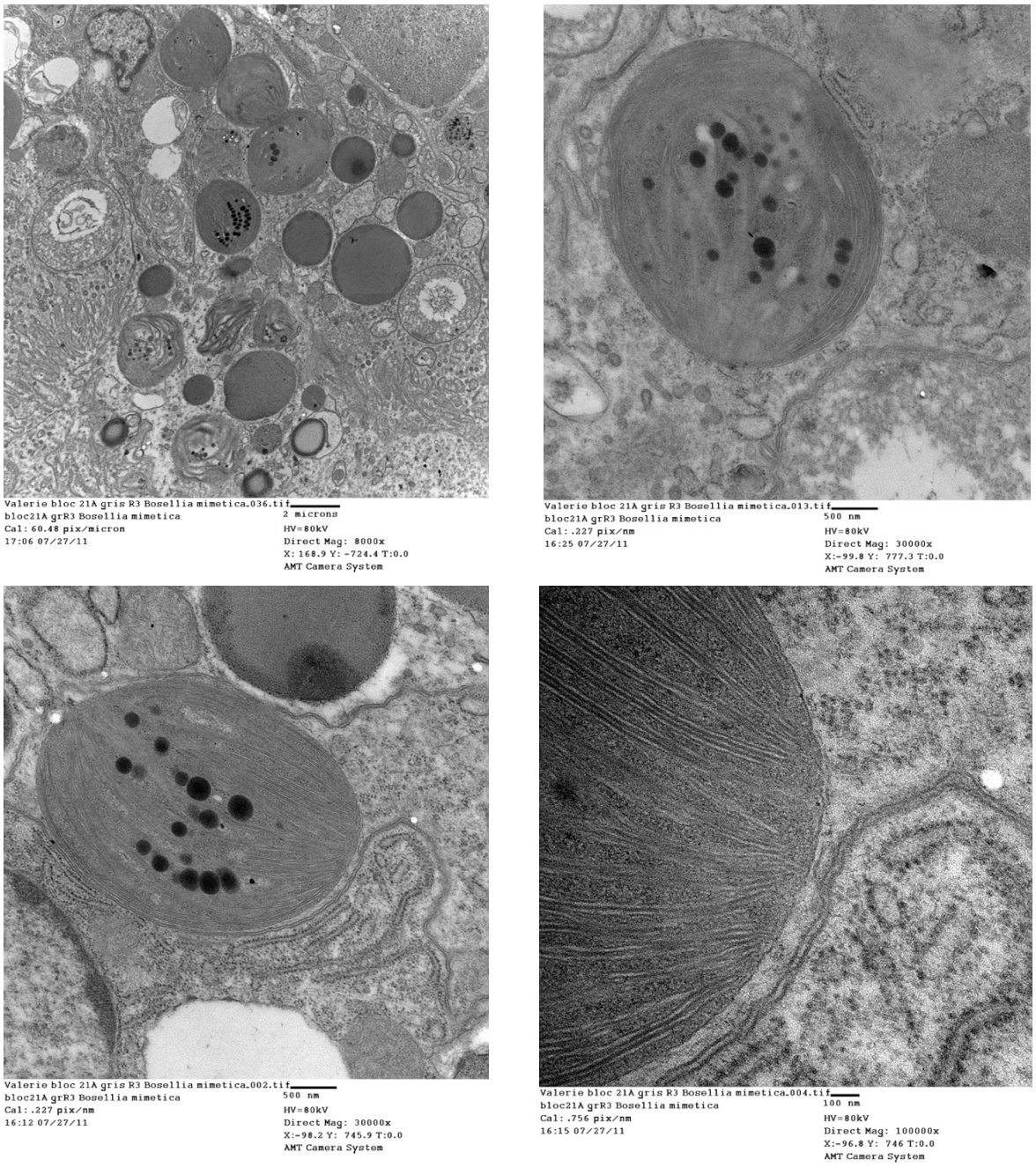


Figure 3.4.9: TEM micrographs of chloroplasts in *B. mimetica*, taken on 8th July 2011 from *H. tuna* and starved for 11 days with accompanying PAM-measurements until an observed feeding experiment with *H. tuna* on 19th July and continuous supply of *H. tuna* for about two hours until fixation on 19th July 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In all states observed in individuals of *B. mimetica*, chloroplasts often contained many dark droplets (probably e. g. plastoglobuli/lipids) sometimes appearing in rows in thylakoids, similar as observed also in *H. tuna,* though chloroplasts appear in general rounder when incorporated in the sea slugs (Fig. 3.4.8 and 3.4.9).

A control individual of *B. mimetica*, which was collected on 16th August 2012 and kept in the laboratory for 13 days with supply of *H. tuna* before fixation, however, showed also indices of degradation of chloroplasts (Fig. 3.4.10), but as far as could be observed, much less than the starved individuals and similar to the natural state as depicted above.

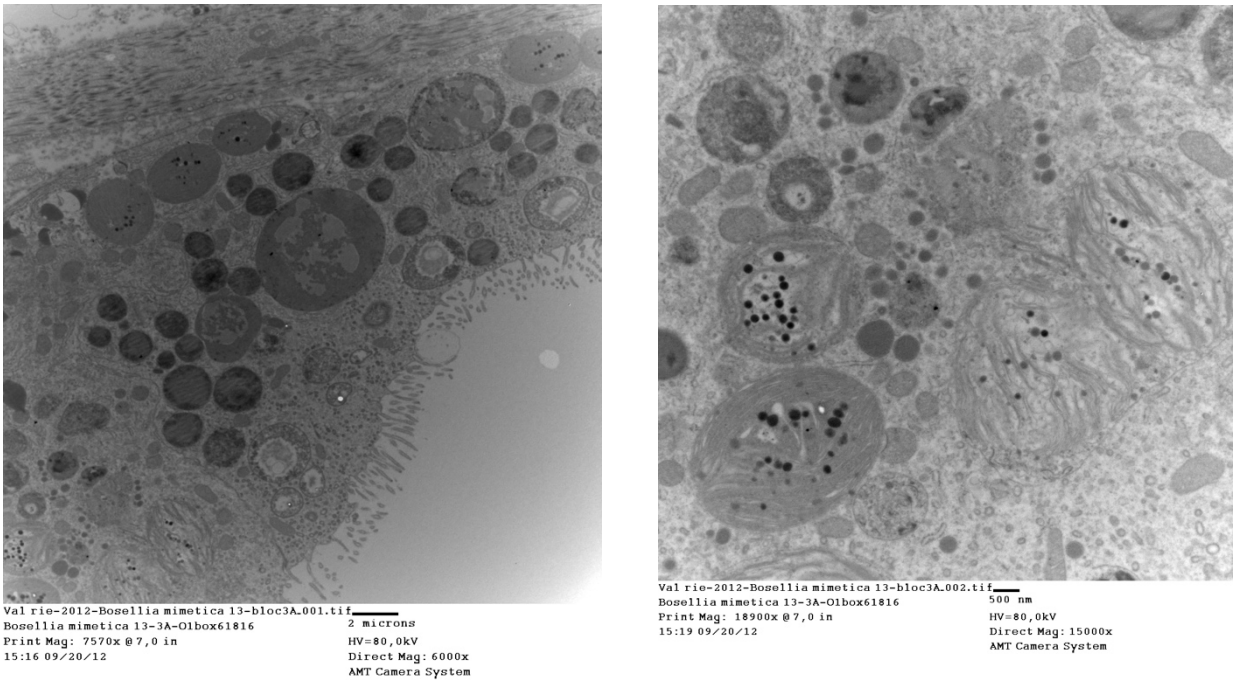


Figure 3.4.10: TEM micrographs of chloroplasts in *B. mimetica*, control individual of *B. mimetica*, which was collected on 16th August 2012 and kept in the laboratory for 13 days with supply of *H. tuna* before fixation. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Food algae of *Bosellia mimetica: Halimeda tuna*

In *H. tuna*, collected on 28th June 2011 and fixed on the same day as a representative for the natural state, round to very elongated chloroplasts were seen, some of them with bright (elongated) grains, also dark droplets in rows between thylakoids (Fig. 3.4.11). The bright conglomerates or grains, that are depicted already for chloroplasts in *B. mimetica* above and in further algae and sacoglossan species in the following are probably starch grains/granules and thus considered in the following as such. The extremely elongated forms of chloroplasts were only observed in algae, chloroplasts incorporated in sacoglossan individuals were only observed with in general rounder forms as depicted above.

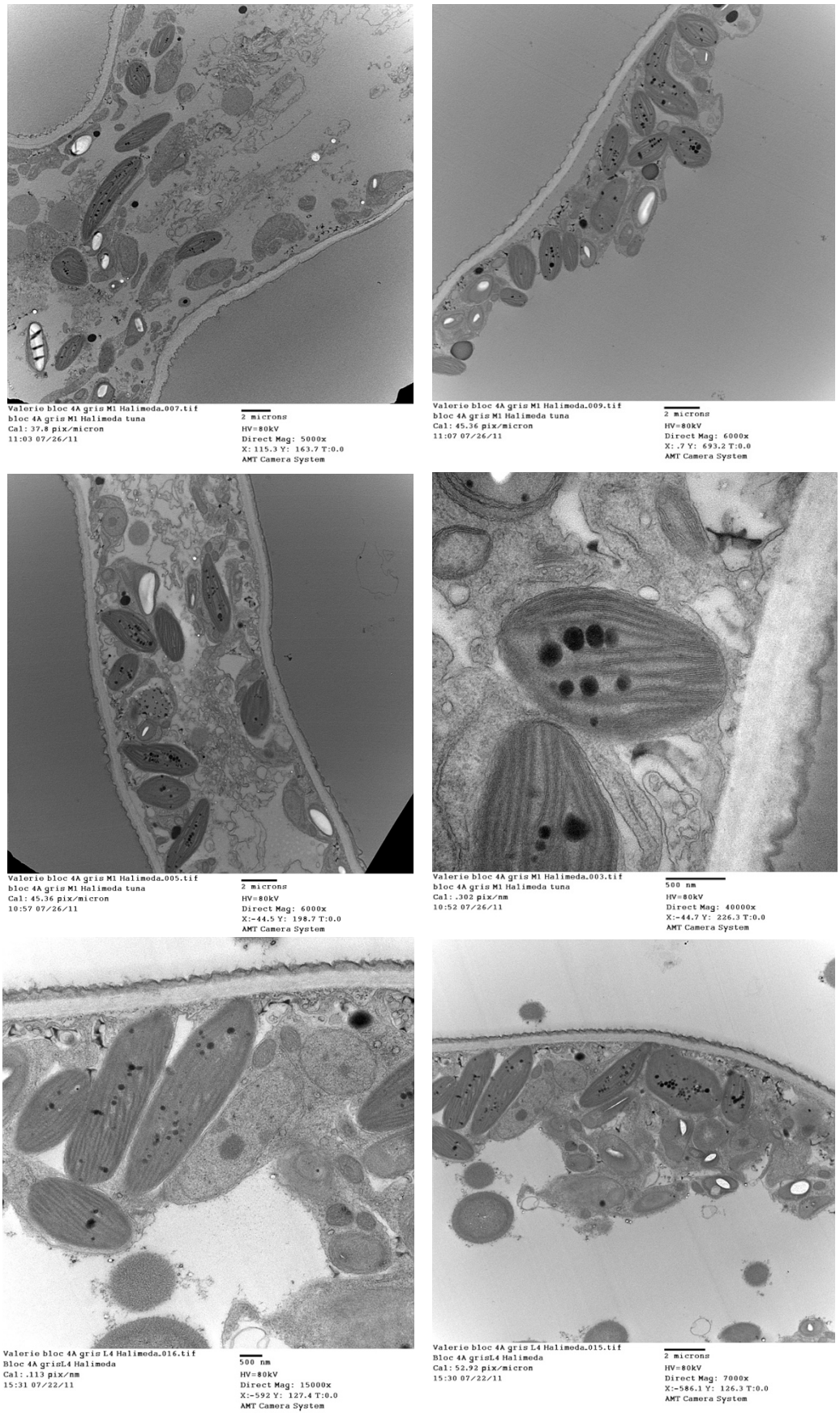


Figure 3.4.11: TEM micrographs of chloroplasts in *H. tuna*, collected in late summer on 28th June 2011 and fixed on the same day, thus presenting a natural state in summer. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Elysia timida

In a control individual of long-term retention species *E. timida*, that was supplied with its food algae *A. acetabulum* for about two weeks after collection, the cytoplasm of some cells was apparently filled with round and slightly differently patterned appearing chloroplasts (Fig. 3.4.12). The same was observed in another individual kept as a control or example for the saturated state two weeks after collection in the laboratory with supply of *A. acetabulum*. Some single chloroplasts were also observed to contain starch granules (Fig. 3.4.13).

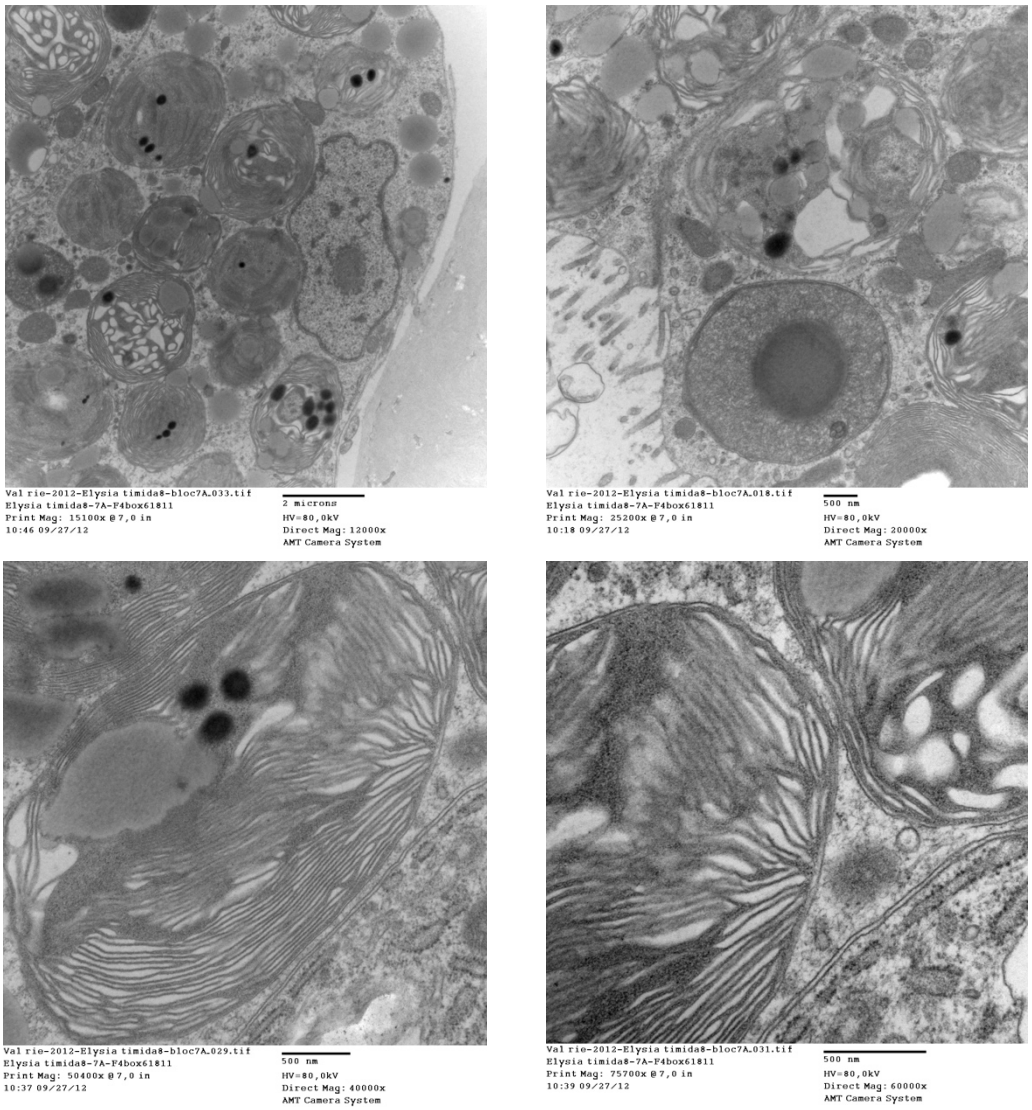


Figure 3.4.12: TEM micrographs of chloroplasts in a control individual of *E. timida*, which was collected on 16th August 2012 and kept in the laboratory for 13 days with supply of *A. acetabulum* before fixation on 29th August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

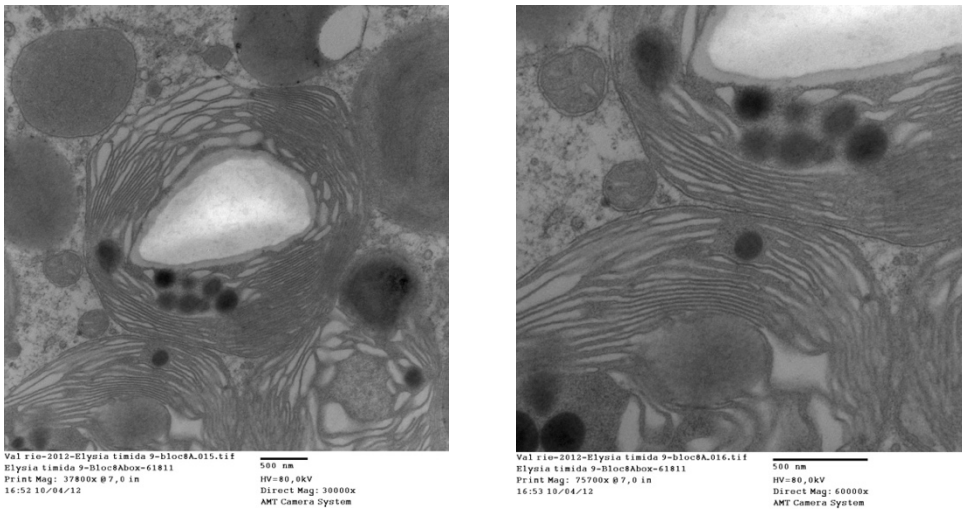


Figure 3.4.13: TEM micrographs of chloroplasts in another control individual of *E. timida*, which was collected on 16th August 2012 and kept in the laboratory for 13 days with supply of *A. acetabulum* before fixation on 29th August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

An individual of *E. timida* collected and investigated in the year ago, directly fixed after collection on the same day, representing the natural state, also showed incorporated chloroplasts in different states, some apparently containing starch granules (Fig. 3.4.14).

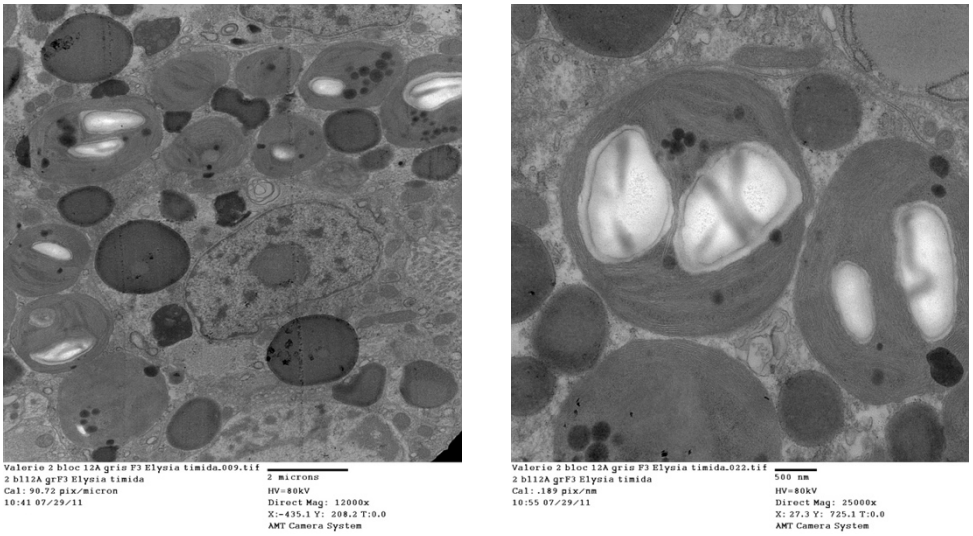


Figure 3.4.14: TEM micrographs of chloroplasts in an individual of *E. timida*, which was collected on 28th June 2011 and fixed directly on the same day for TEM after PAM measurements as a representative for the natural state. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In an *E. timida* individual starved for 12 days, among different states of chloroplasts some apparently still intact chloroplasts were seen, some few chloroplasts seemed to contain starch granules (Fig. 3.4.15). Also another *E. timida* individual starved for 12 days showed

chloroplasts in different states and differently looking patterns, some appearing close to nuclei (Fig. 3.4.16).

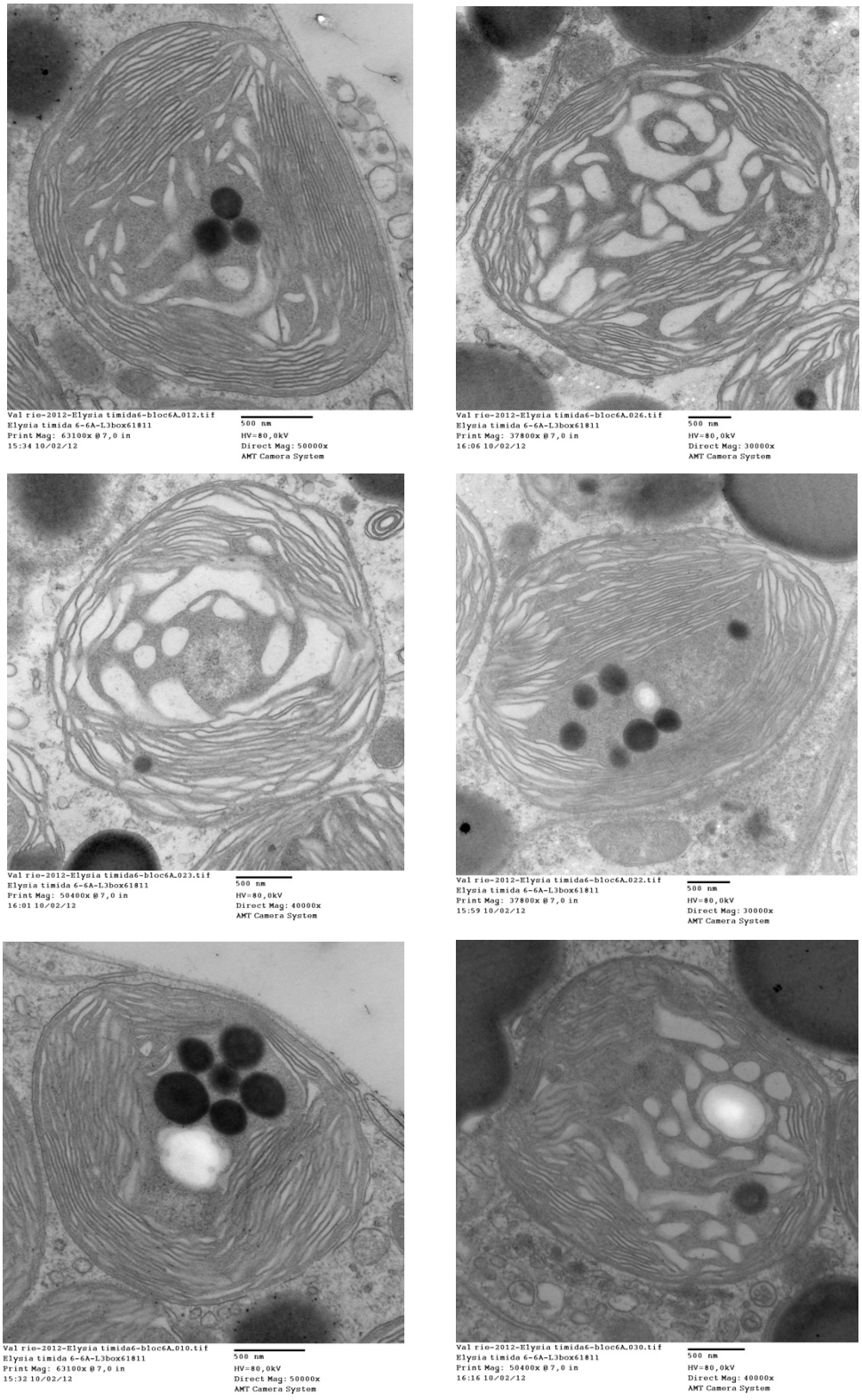


Figure 3.4.15: TEM micrographs of chloroplasts in an individual of *E. timida*, which was collected on 9th August 2012 and kept in the laboratory for 12 days without food supply until fixation on 21st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

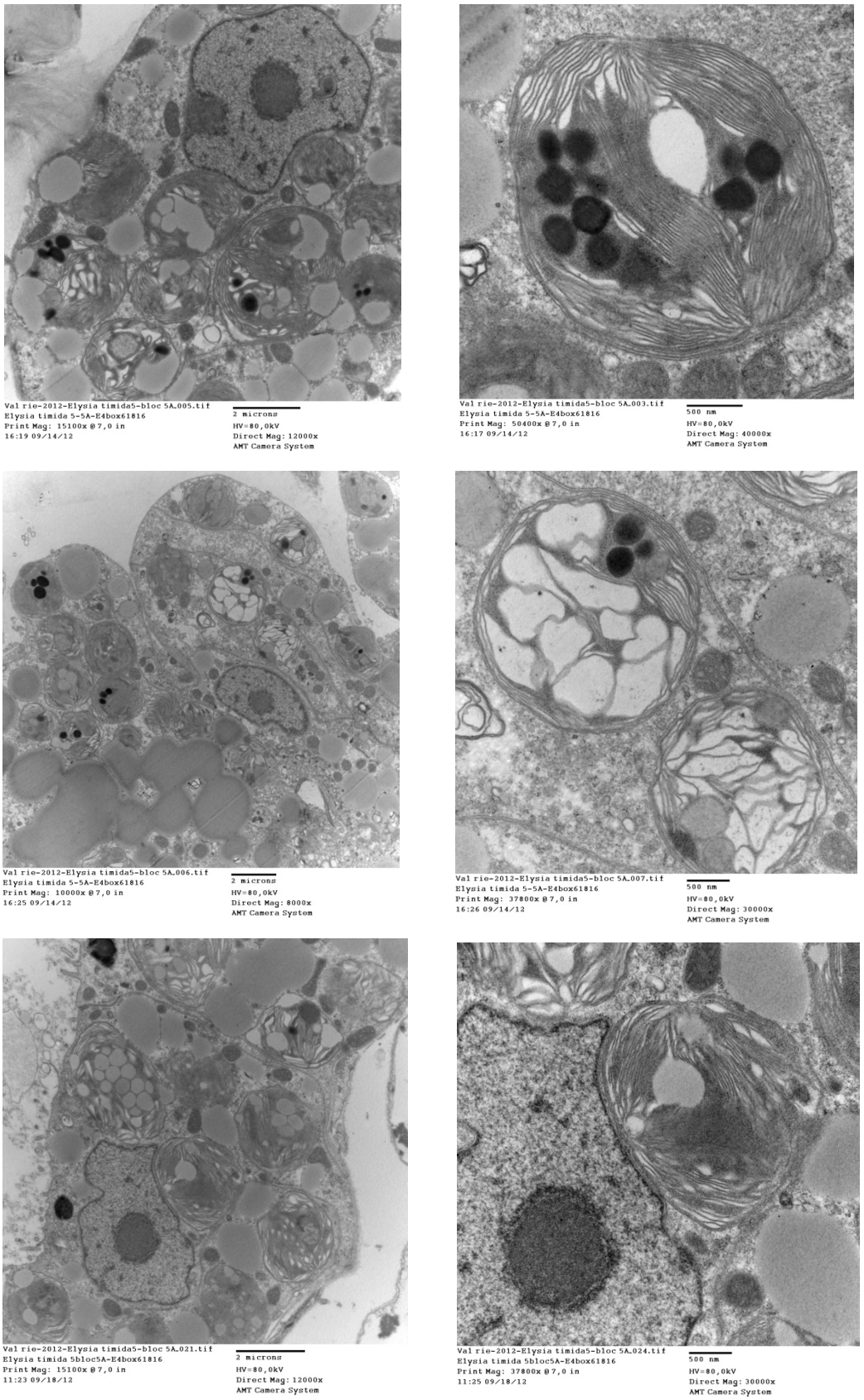


Figure 3.4.16: TEM micrographs of chloroplasts in another individual of *E. timida* which was collected on 9th August 2012 and kept in the laboratory for 12 days without food supply until fixation on 21st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

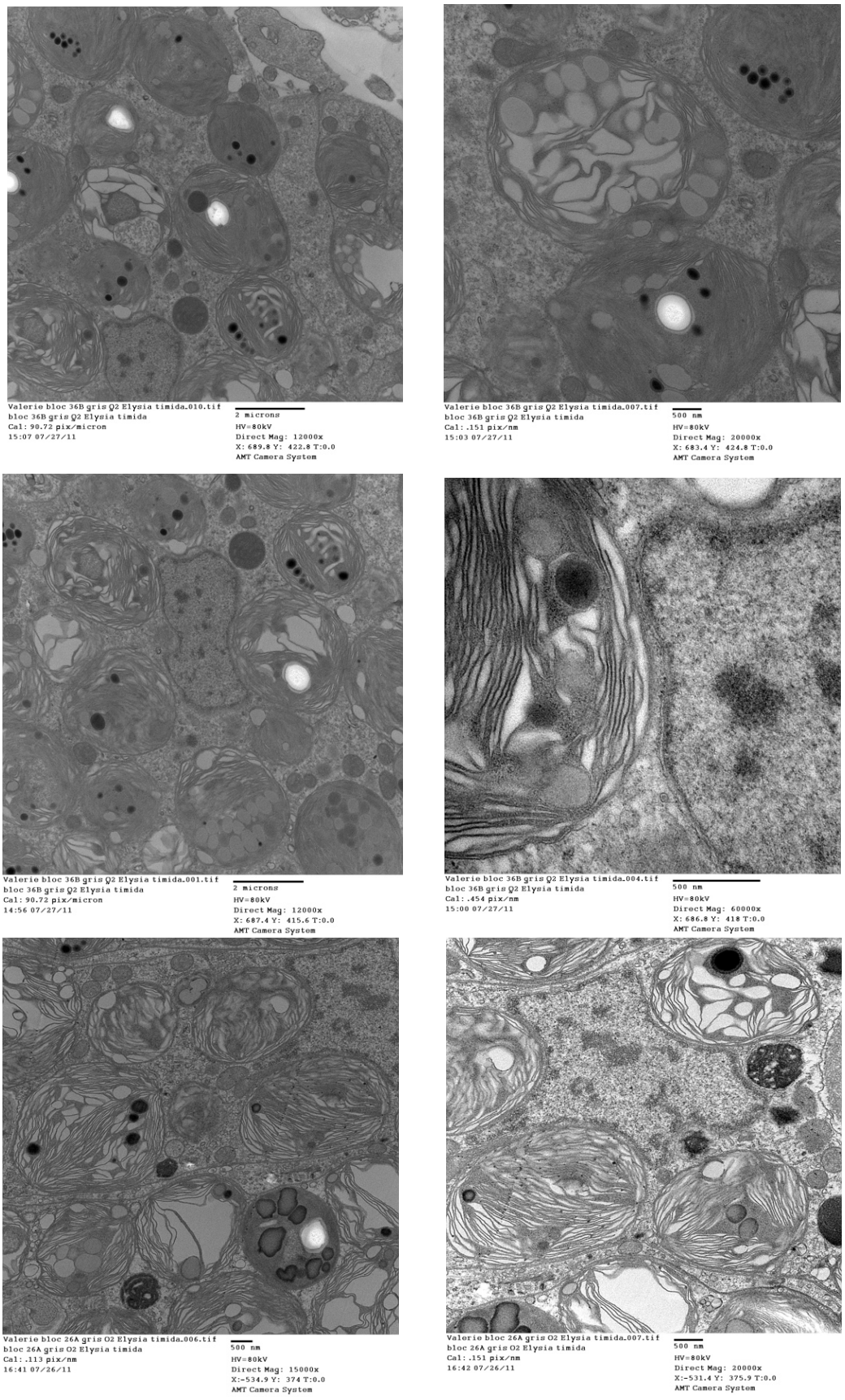


Figure 3.4.17: TEM micrographs of chloroplasts in two individuals of *E. timida*, which were kept in the laboratory without food supply for 18 or 20 days, respectively, from 30th June 2011 to 17th and 19th July 2011 until being supplied with *A. acetabulum* in a feeding experiment for two days (individual figures 1-4) or about two hours (individual figures 5-6), respectively, until fixation on 19th July 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In two *E. timida* individuals that were kept without food supply for 18 or 20 days, respectively, and then supplied with *A. acetabulum* for two days or two hours, respectively, in a feeding experiment to investigate incorporation of chloroplasts, different states of chloroplasts were observed, sometimes in proximity to nuclei (Fig. 3.4.17), similar as observed also in other individuals and displayed already above.

Food algae of *Elysia timida: Acetabularia acetabulum*

TEM-analyses of *A. acetabulum* displayed round to elongated shaped chloroplasts of which some were observed to contain starch granules and dark conglomerates or droplets (probably e. g. plastoglobuli/lipids), in some cases both (Fig. 3.4.18 and 3.4.19).

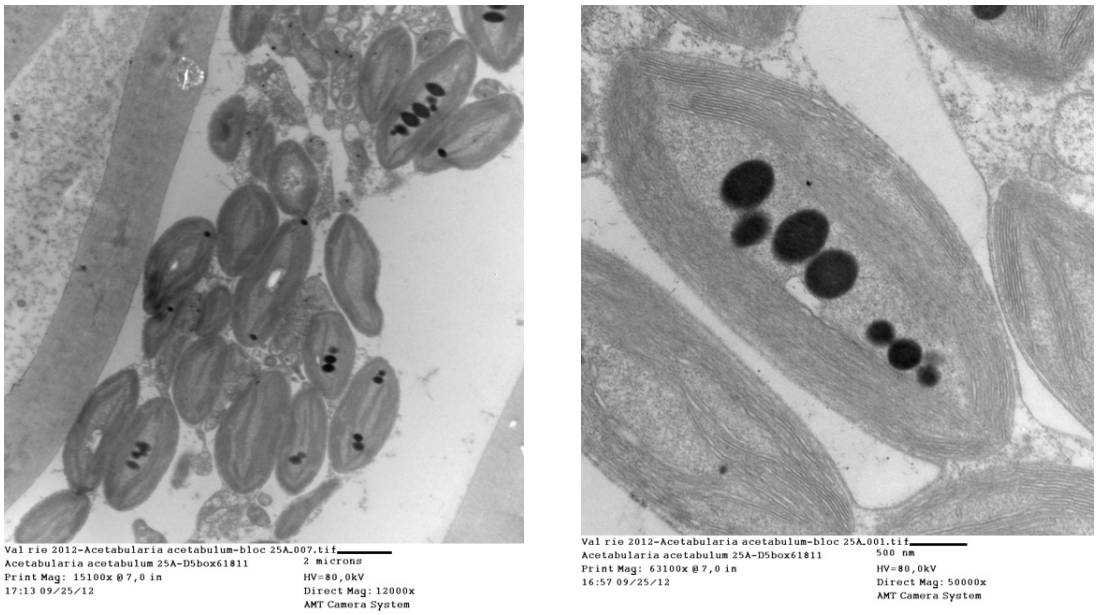


Figure 3.4.18: TEM micrographs of chloroplasts in *A. acetabulum*, collected in late summer on 28th August 2012 on a stone, on which they continued to grow in intact condition for three days in the laboratory where they were separated from their natural underground just before fixation, thus presenting a natural state in late summer of the annual cycle of *A. acetabulum*. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

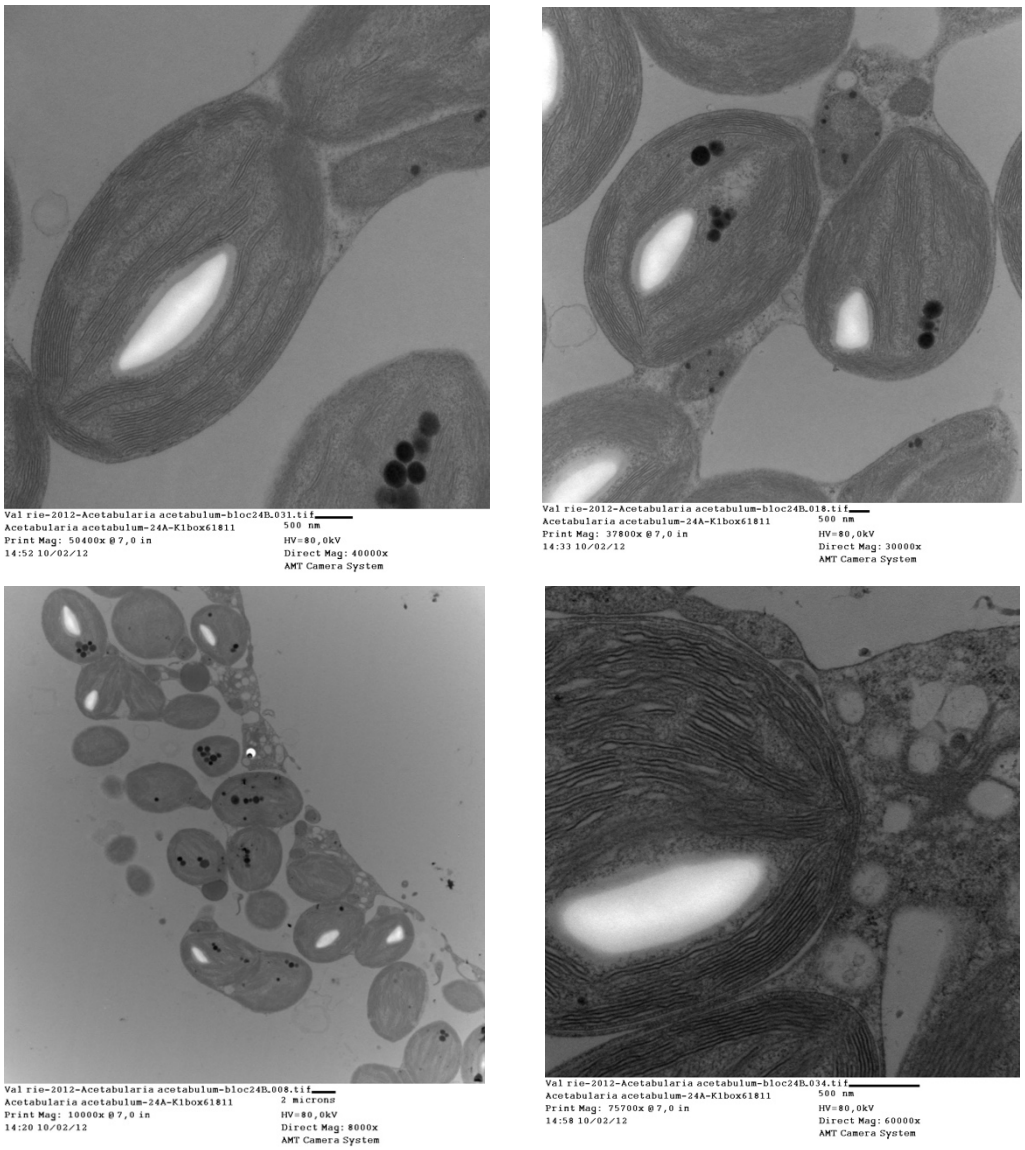


Figure 3.4.19: A further sample of TEM micrographs of chloroplasts in *A. acetabulum*, collected in late summer on 28th August 2012 on a stone, continued to grow in intact condition for three days in the laboratory and separated from their natural underground just before fixation, representing a natural state in late summer of the annual cycle of *A. acetabulum*. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

These samples of *A. acetabulum* had been collected in late summer on 28th August 2012 on a stone, on which they continued to grow in intact condition for three days in the laboratory where they were separated from their natural underground just before fixation, thus presenting a natural state in late summer of the annual cycle of *A. acetabulum*. In another individual investigated in the end of June the year before (28th June 2011), freshly collected and directly fixed, a similar picture was observed with chloroplasts looking very similar, some containing starch granules or dark droplets or both.

Elysia viridis

In an individual of *E. viridis* investigated in 2011, fixed directly after collection from *C. fragile/vermilara* on the same day, thus representing the natural state, chloroplasts in different states of degradation were observed and some apparently containing starch granules (Fig. 3.4.20).

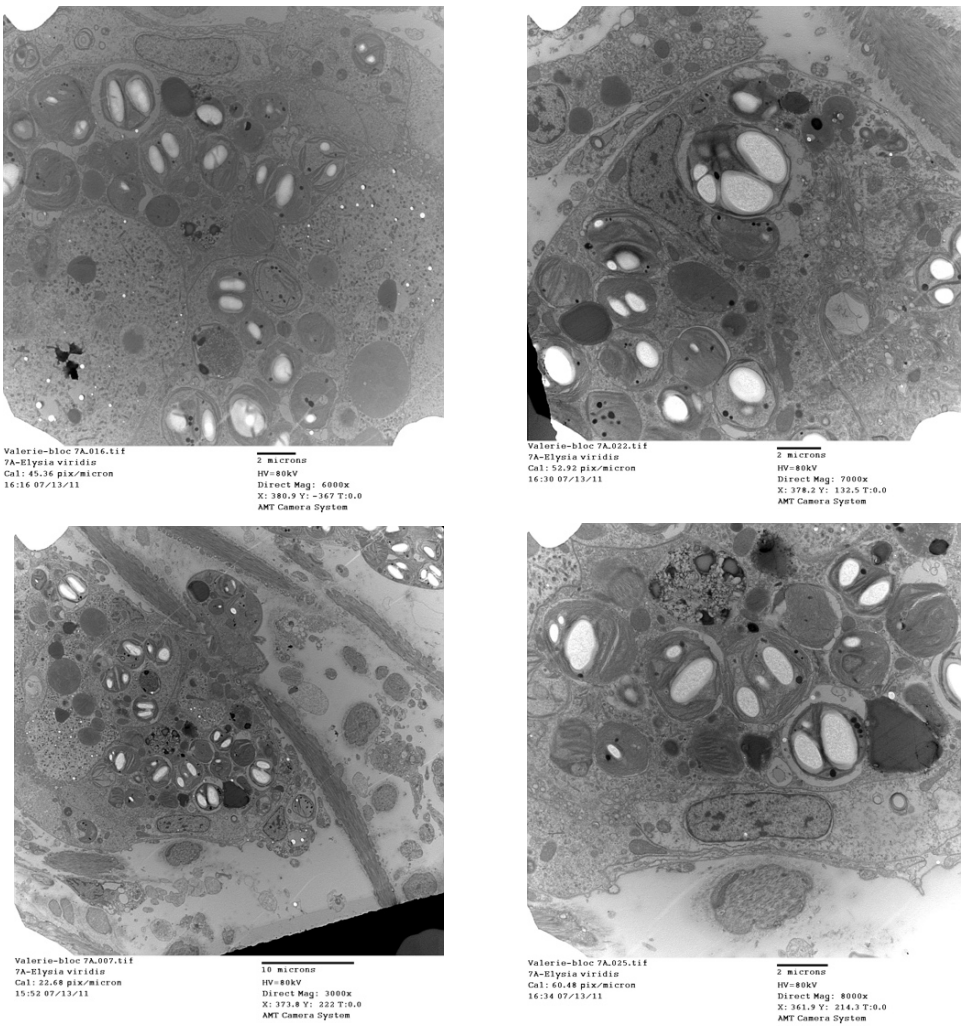


Figure 3.4.20: TEM micrographs of chloroplasts in an individual of *E. viridis*, which was collected on 28th June 2011 from *C. fragile/vermilara* and fixed directly on the same day for TEM after PAM measurements as a representative for the natural state. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In a control individual of *E. viridis*, collected from *C. fragile/vermilara* and kept in the laboratory for 12 days with supply of *C. fragile/vermilara*, also chloroplasts in different states could be observed, often appearing in proximity to nuclei, though partly appearing enclosed in membranes (Fig. 3.4.21).

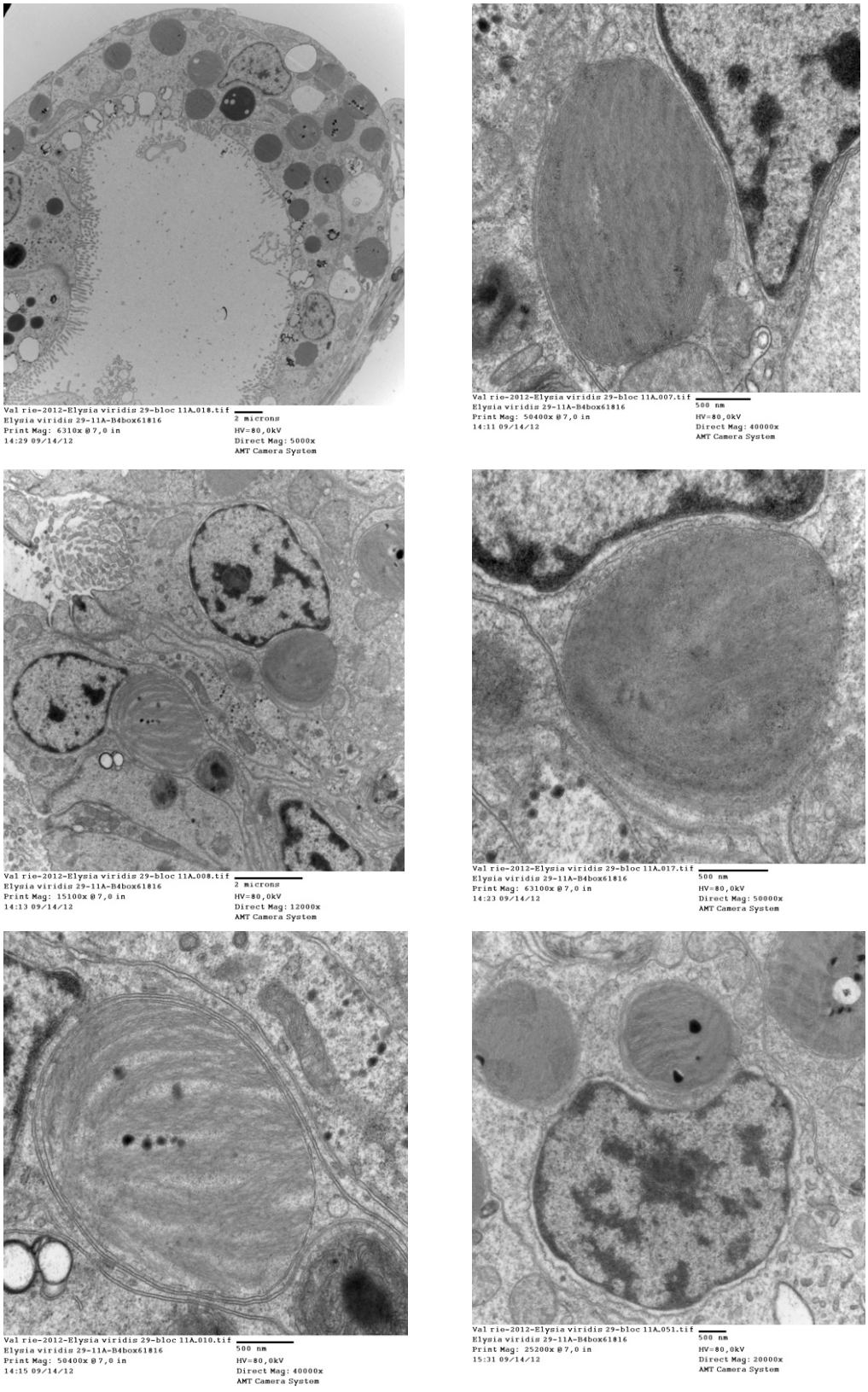


Figure 3.4.21: TEM micrographs of chloroplasts in an individual of *E. viridis*, which was collected on 19th August 2012 from *C. fragile/vermilara* and was kept in the laboratory with further supply of *C. fragile/vermilara* during 12 days until fixation on 31st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In *E. viridis* originally collected from *C. fragile/vermilara* and then kept in the laboratory the first three days on the algae and then seven days without food supply, some chloroplasts appeared still quite intact and different states of degradation were recognizable (Fig. 3.4.22).

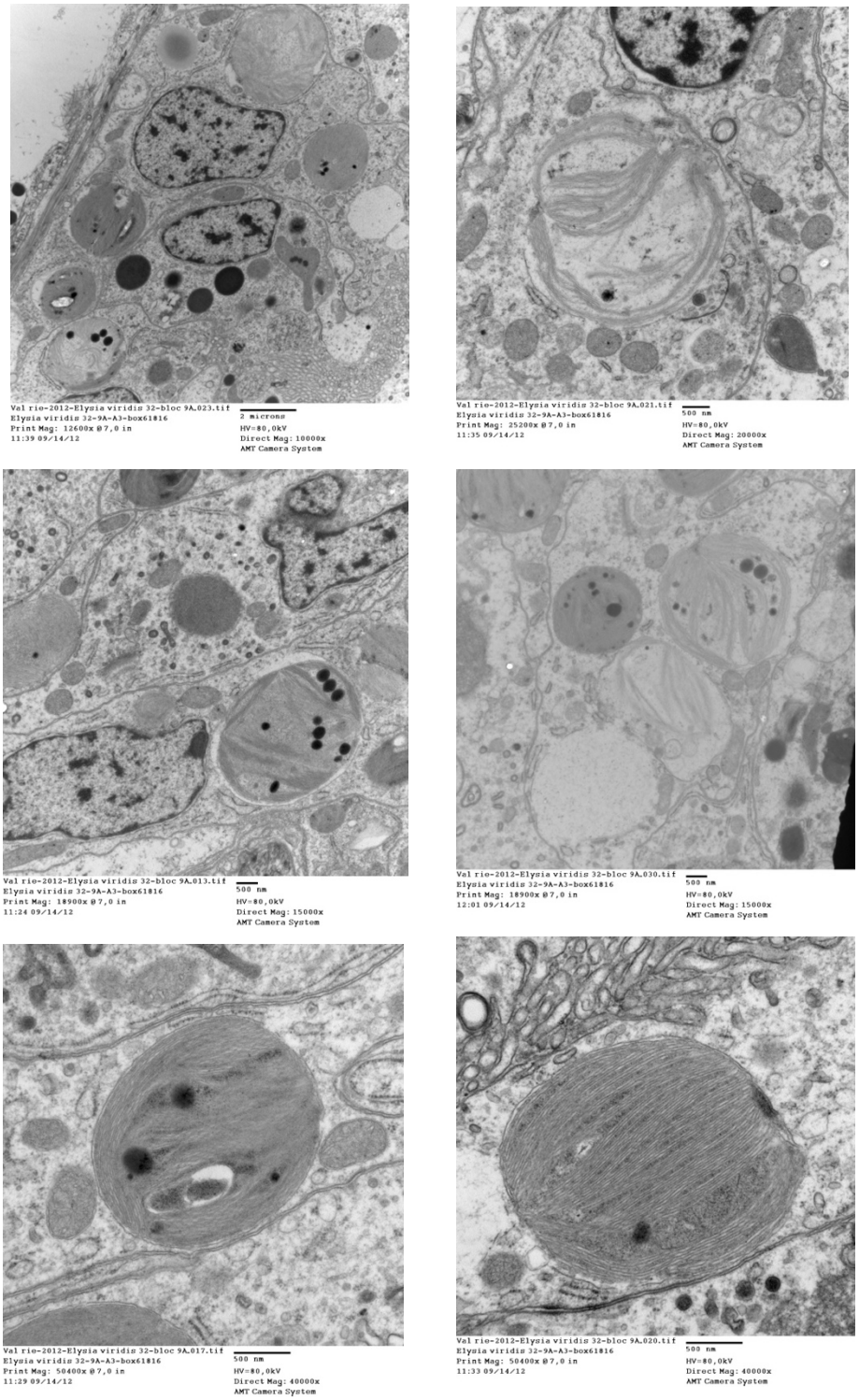


Figure 3.4.22: TEM micrographs of chloroplasts in an individual of *E. viridis*, which stemmed from *C. fragile/vermilara* collected on 22nd August 2012 and was kept in the laboratory first for three days on *C. fragile/vermilara* until 25th August 2012, then about a week without food supply until fixation on 31st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In *E. viridis* collected from *F. petiolata* and kept for further two weeks in a petri dish with supply of *F. petiolata* as control representative for a saturated state, round and intact appearing chloroplasts were observed among different states (Fig. 3.4.23).

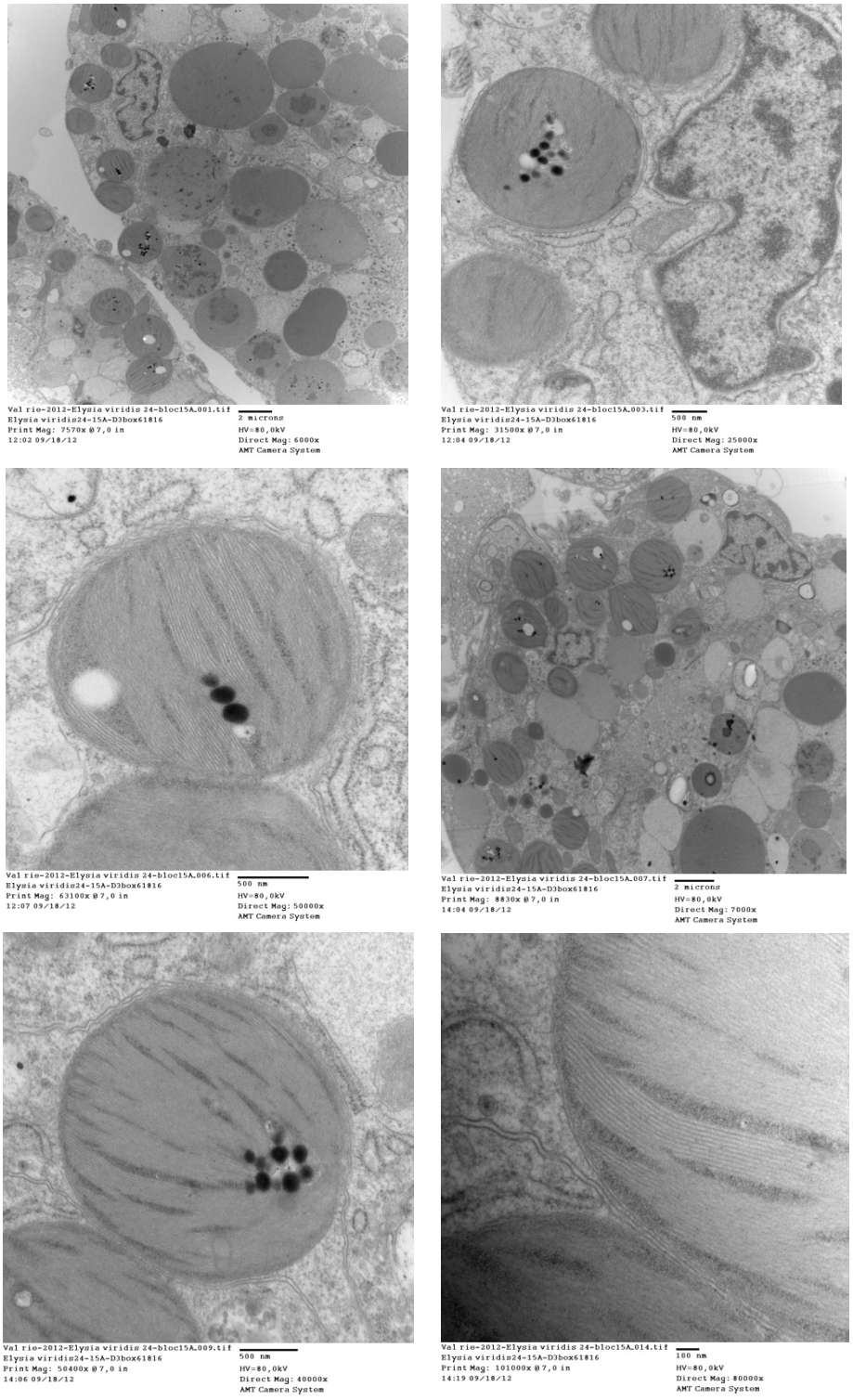


Figure 3.4.23: TEM micrographs of chloroplasts in an individual of *E. viridis*, which was collected on 17th August 2012 from *F. petiolata* (collected on 13th August 2012) and was kept in the laboratory with further supply of *F. petiolata* until fixation on 29th August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In *E. viridis* collected from *F. petiolata* and starved for 12 days, still many chloroplasts seemed to lie intact in the cytoplasm and some contained starch granules (Fig. 3.4.24).

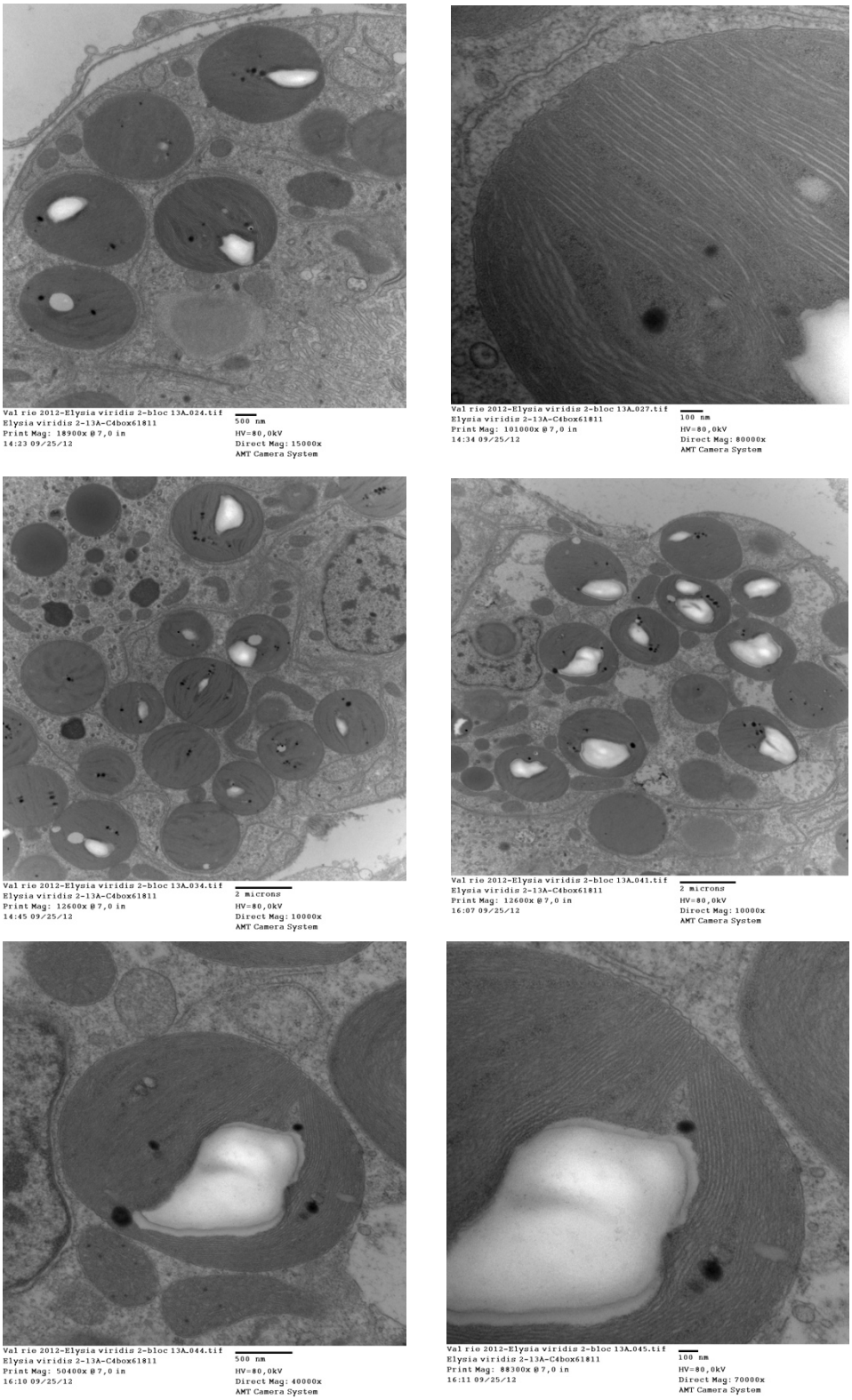


Figure 3.4.24: TEM micrographs of chloroplasts in an individual of *E. viridis*, which was collected on 9th August 2012 from *F. petiolata* (collected on 8th August 2012) and was kept in the laboratory without food supply until fixation on 21st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In a parallel feeding trial to compare the incorporation of chloroplasts from *C. fragile/vermilara* and *F. petiolata*, individuals of *E. viridis* that had been either collected from *C. fragile/vermilara* or *F. petiolata* were kept without food supply for two days in the laboratory and then provided with the same algae species they had been collected from, respectively, for about two hours of observed feeding. In the *E. viridis* individuals from *C. fragile/vermilara*, chloroplasts in different states were observed, several appearing to contain starch granules (Fig. 3.4.25). In the *E. viridis* individuals from *F. petiolata*, also different states of chloroplasts were seen, many appearing intact and with the characteristic pattern already detected in other individuals from *F. petiolata* as depicted above (Fig. 3.4.26).

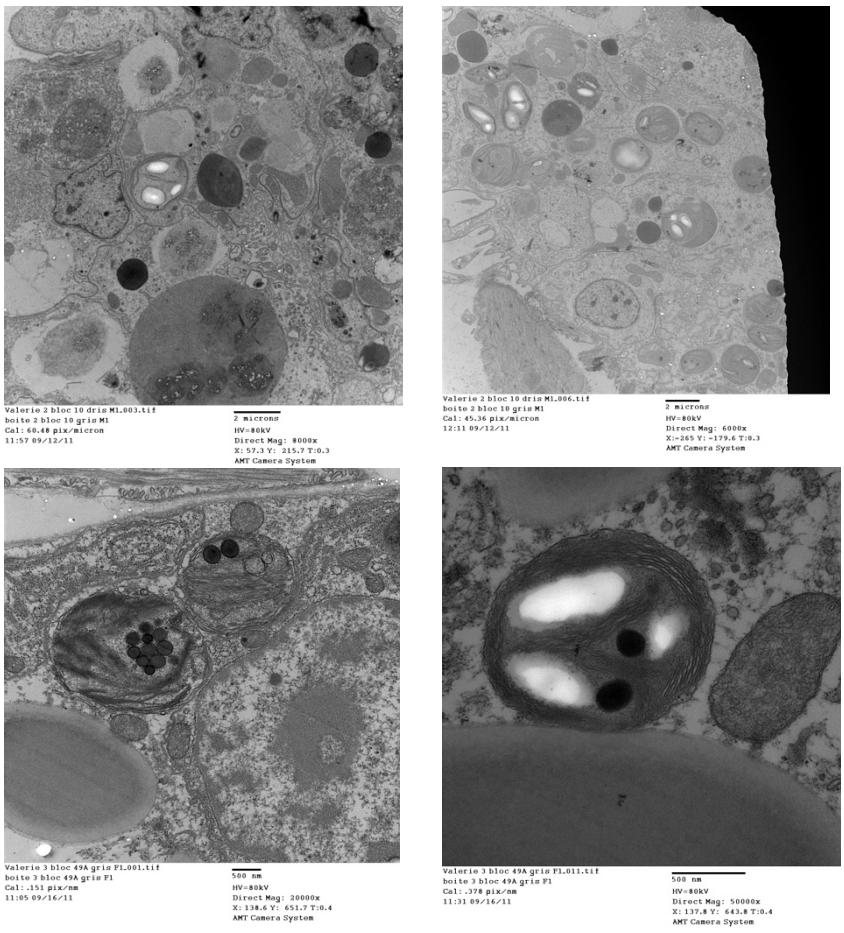


Figure 3.4.25: TEM micrographs of chloroplasts in two individuals of *E. viridis*: one (picture 1-2) was collected on 28th June 2011 from *C. fragile/vermilara* and was kept in the laboratory two days without food supply, then supplied with *C. fragile/vermilara* and observed to feed on *C. fragile/vermilara* for about an hour until fixation on 1st July 2011; the second (picture 3-4) was taken from *C. fragile/vermilara* (collected on 31st August 2011) on 5th September 2011 and was kept in the laboratory two days without food supply, then supplied with *C. fragile/vermilara* and observed to feed on *C. fragile/vermilara* for about two hours until fixation on 7th September 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

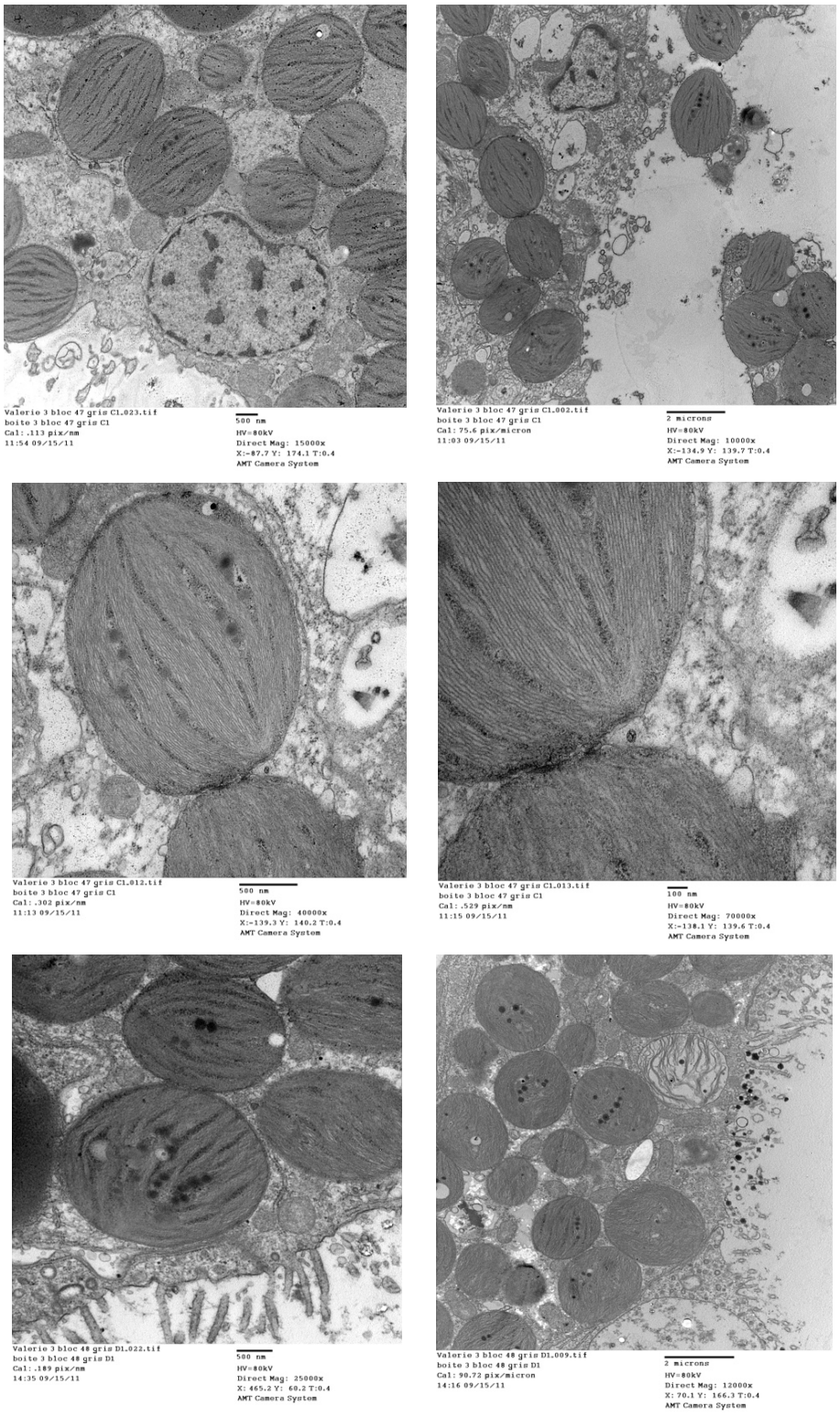


Figure 3.4.26: TEM micrographs of chloroplasts in two individuals of *E. viridis* that were both collected from *F. petiolata* (collected on 31st August 2011) on 5th September 2011, then kept in the laboratory two days without food supply, then supplied with *F. petiolata* and observed to feed on *F. petiolata* for about two hours until fixation on 7th September 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Food algae of *Elysia viridis: Flabellia petiolata*

In *F. petiolata*, round to partly extremely elongated chloroplasts densely packed with thylakoids and indices for starch granules were observed (Fig. 3.4.27 and 3.4.28).

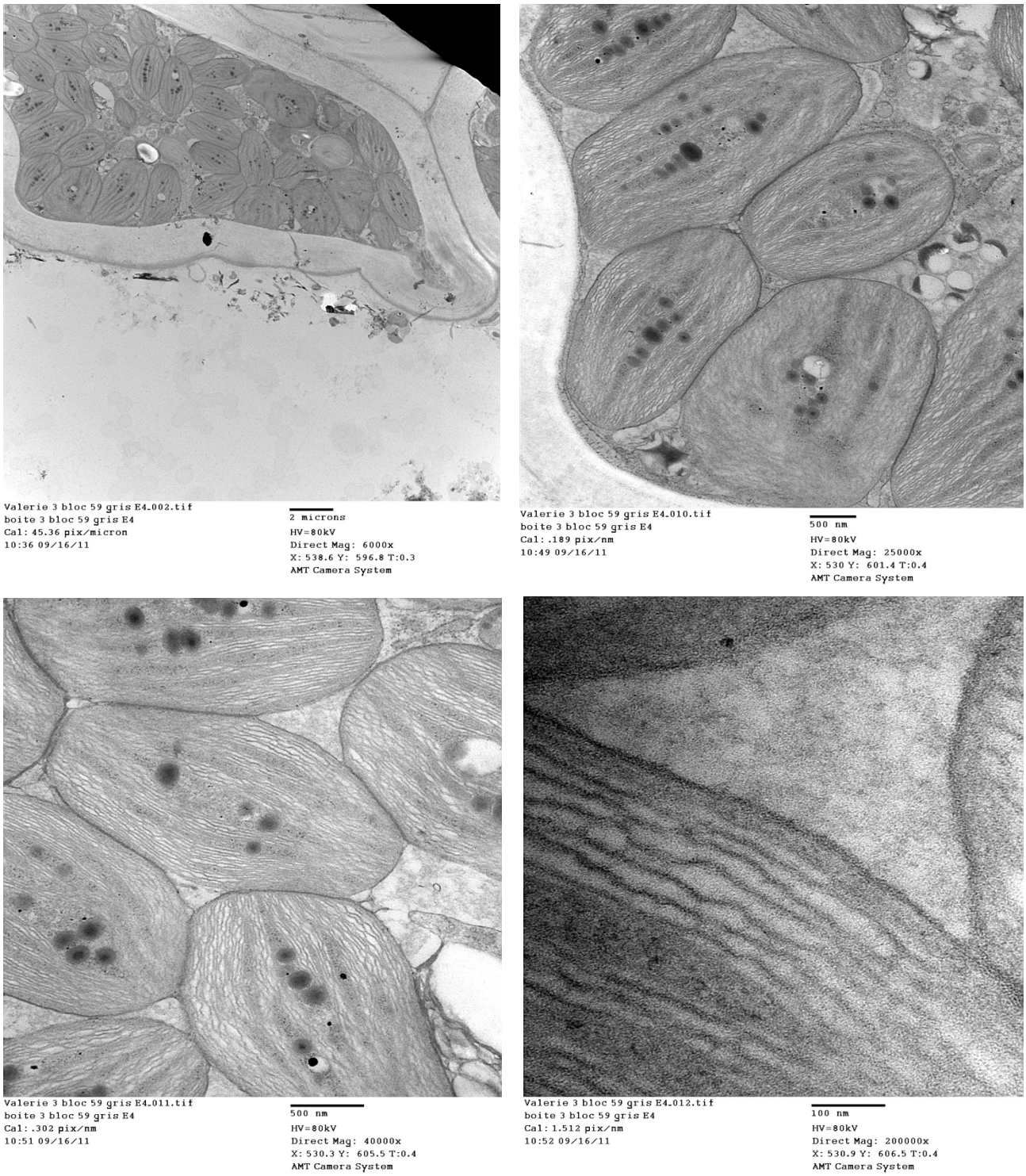


Figure 3.4.27: TEM micrographs of chloroplasts in *F. petiolata*, collected on 31st August 2011 and kept several days in fluent sea water in the laboratory until a piece being separated/cut for fixation on 7th September 2011. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

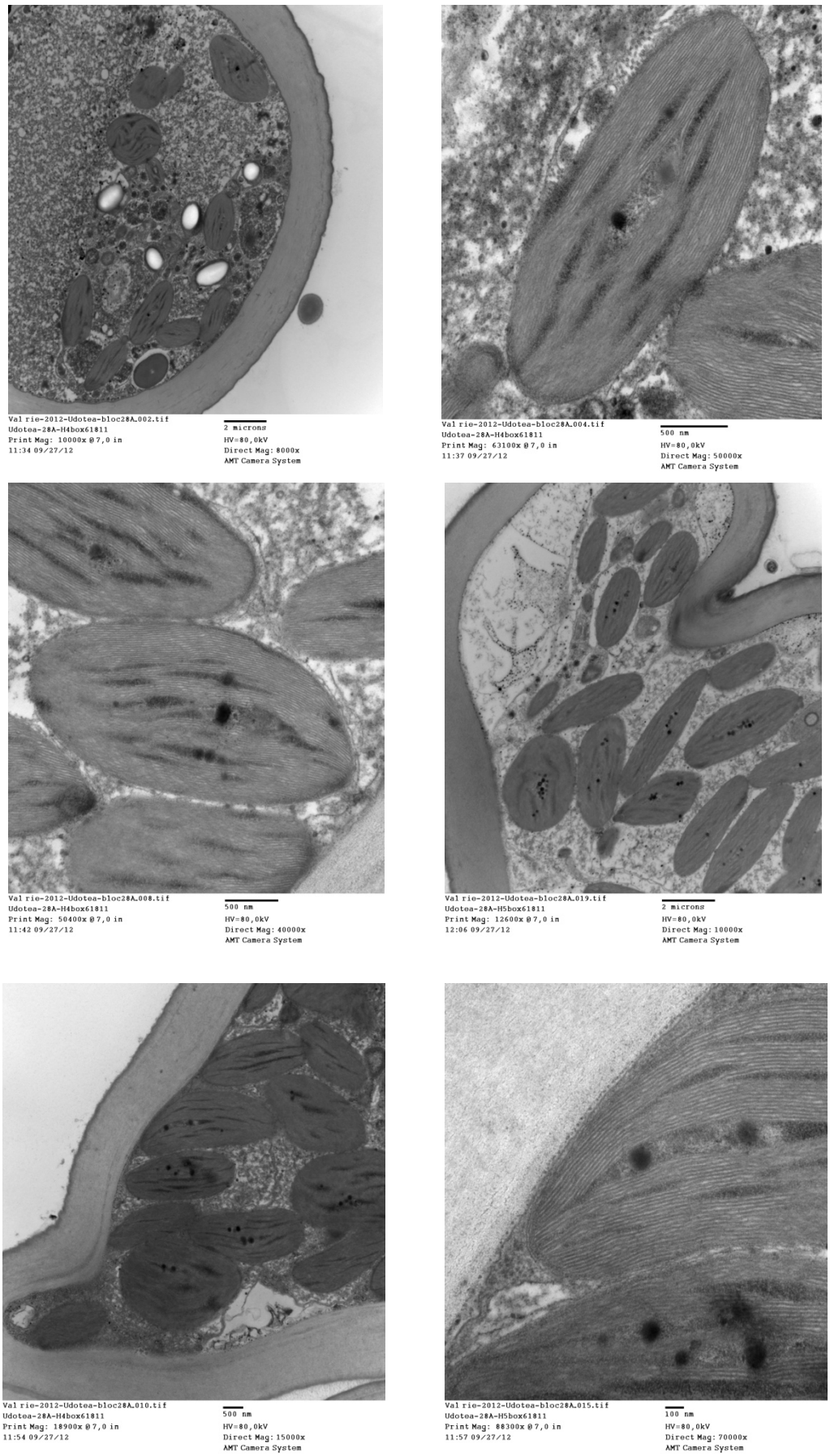


Figure 3.4.28: TEM micrographs of chloroplasts in *F. petiolata*, collected on 24th August 2012 and kept several days in fluent sea water in the laboratory until a piece being separated/cut for fixation on 31st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Food algae of *Elysia viridis* and *Placida dendritica: Codium fragile/vermilara*

In TEM-micrographs of *C. fragile/vermilara*, chloroplasts appeared also as round to elongated, often narrowing to the two opposite ends in tips. Many prominent bright conglomerates as starch granules were recognizable. In this sample, collected on 28th August 2012 and kept three days in fluent sea water in the laboratory until fixation, thylakoids often appear to be relatively 'loosely packed'. (Fig. 3.4.29). Also a sample investigated in the year before, freshly collected and fixed on the same day, 28th June 2011, revealed elongated chloroplasts with similar forms and both starch granules and dark droplets.

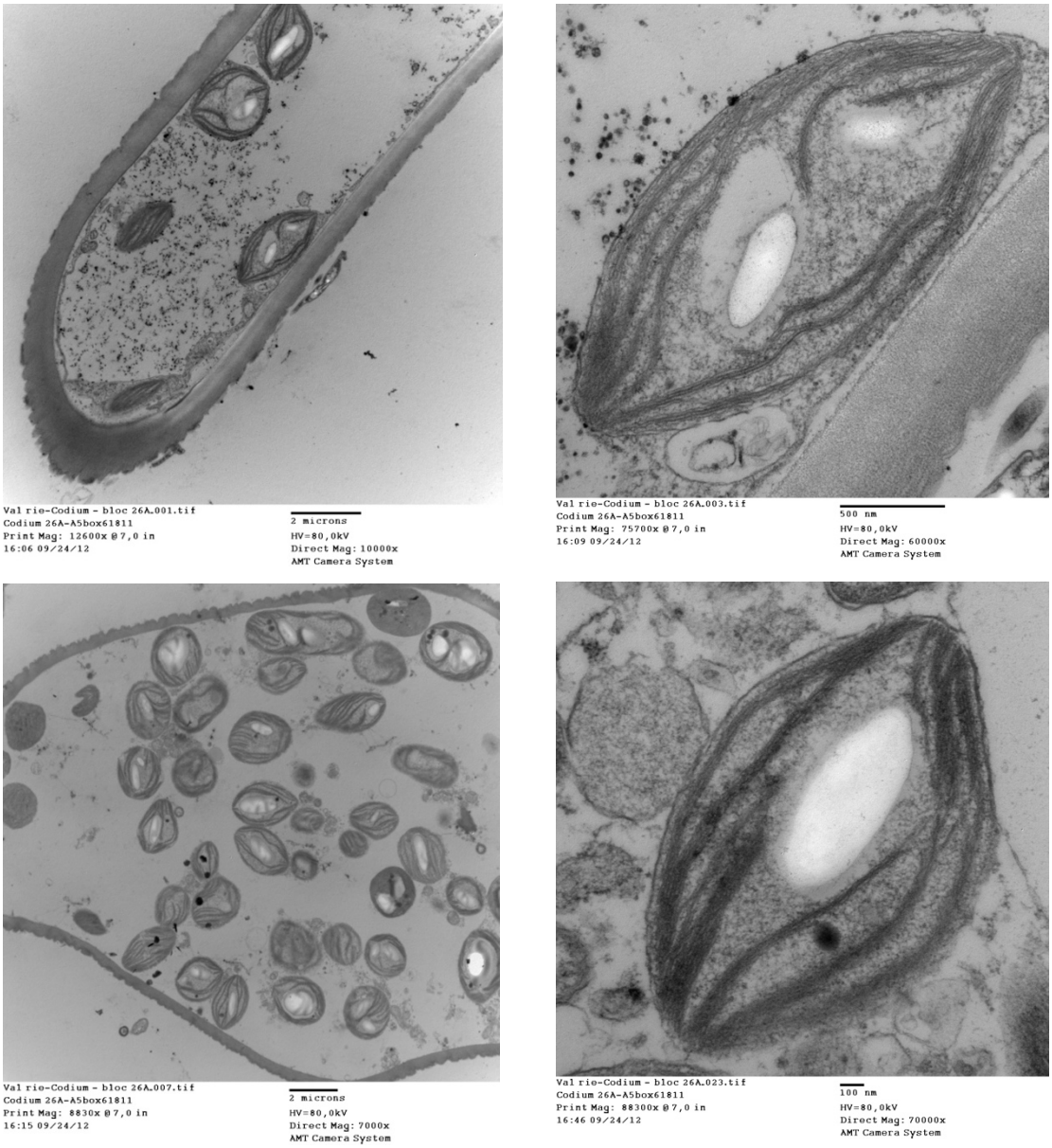


Figure 3.4.29: TEM micrographs of chloroplasts in *C. fragile/vermilara*, collected in late summer on 28th August 2012 and kept three days in fluent sea water in the laboratory until fixation. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Sea slug species with short-term retention or fast digestion of chloroplasts

Placida dendritica

In *P. dendritica*, different fixation states could be observed, all after collection from *C. fragile/vermilara*: Taken from *C. fragile/vermilara* and kept without food supply for 3 days (natural state and starved 3 days) and for 11 days (long starvation), collected and kept with supply of *C. fragile/vermilara* for 3 days (fresh/natural state and fed), and control individual held for two weeks in the laboratory with supply of *C. fragile/vermilara* (control, saturated state). In the individuals with supply of *C. fragile/vermilara*, digestive gland cells were apparently filled with chloroplasts and especially with numerous prominent starch granules as observed also in the chloroplasts in *C. fragile/vermilara* described above. Also, chloroplasts in different degradation states and without starch granules could be seen, as well as appearing in proximity to nuclei (Fig. 3.4.30 and 3.4.31).

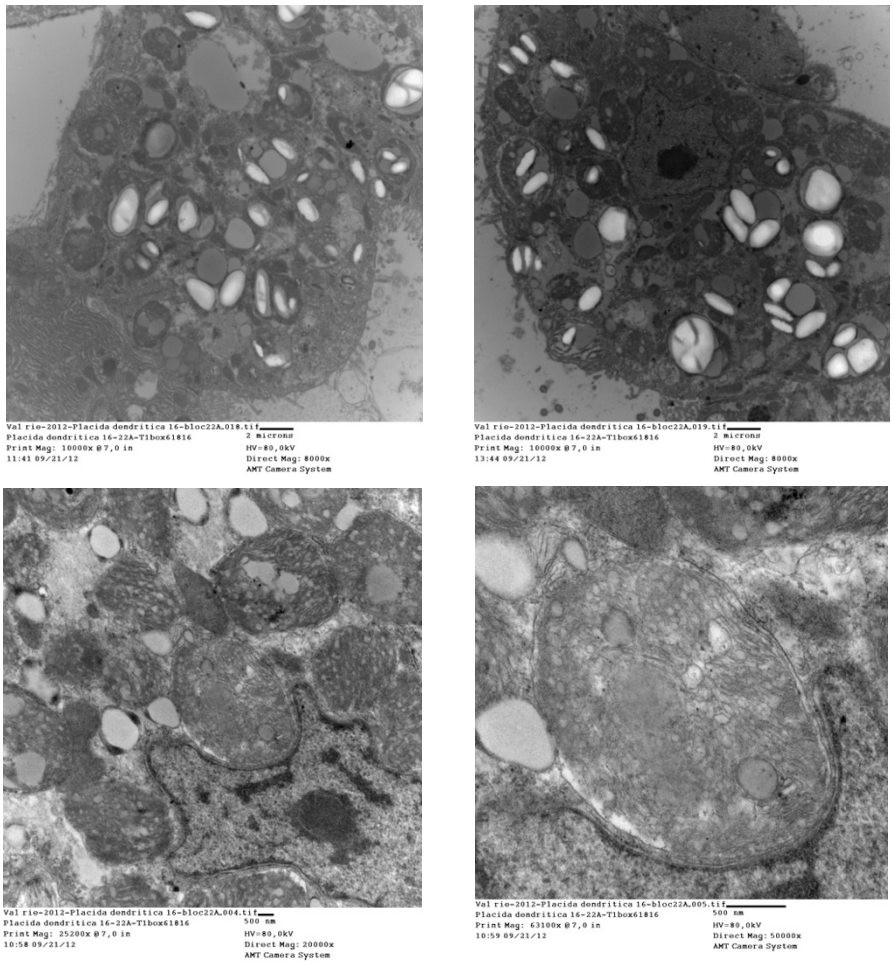


Figure 3.4.30: TEM micrographs of chloroplasts in an individual of *P. dendritica*, which was collected on 28th August 2012 from *C. fragile/vermilara* and was kept in the laboratory with further supply of *C. fragile/vermilara* for three days until fixation on 31st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

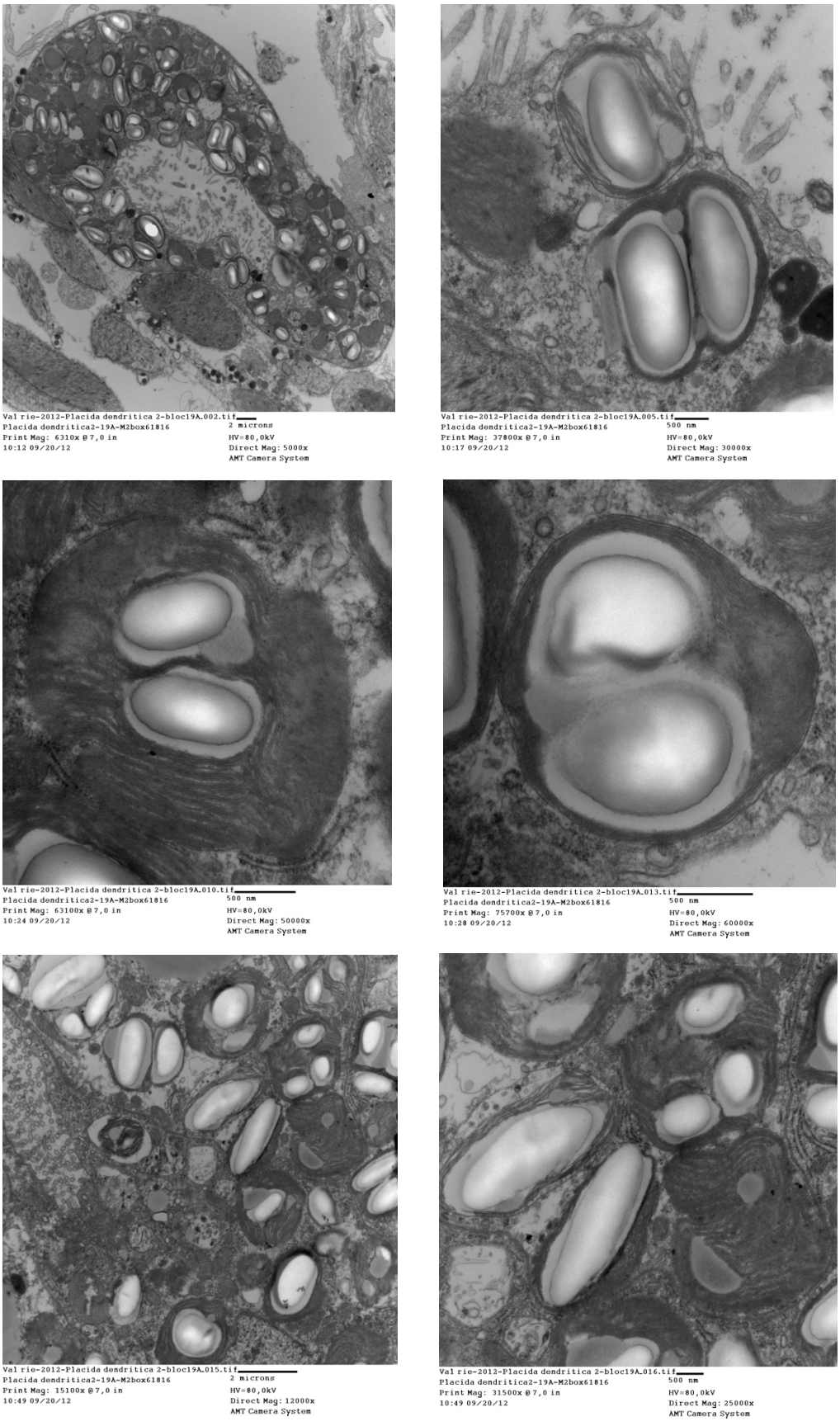


Figure 3.4.31: TEM micrographs of chloroplasts in a control individual of *P. dendritica*, which was collected on 17th August 2012 from *C. fragile/vermilara* (collected on 16th August 2012) and was kept in the laboratory with further supply of *C. fragile/vermilara* for 13 days until fixation on 29th August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In an individual fixed in the year ago, as representing the natural state directly after fresh collection of *C. fragile/vermilara* on that day, also many chloroplasts appeared to contain starch grain inclusions (Fig. 3.4.32).

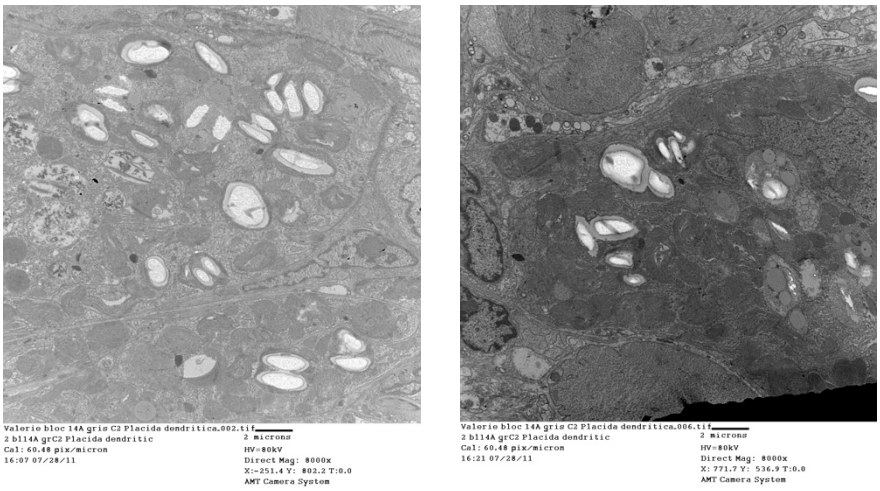


Figure 3.4.32: TEM micrographs of chloroplasts in an individual of *P. dendritica*, which was collected on 28th June 2011 with and from *C. fragile/vermilara* and was directly fixed on the same day as representative for the natural state. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

In the starved individuals, only degradation states or remnants of chloroplasts were seen (and no starch anymore) (Fig. 3.4.33 and 3.4.34).

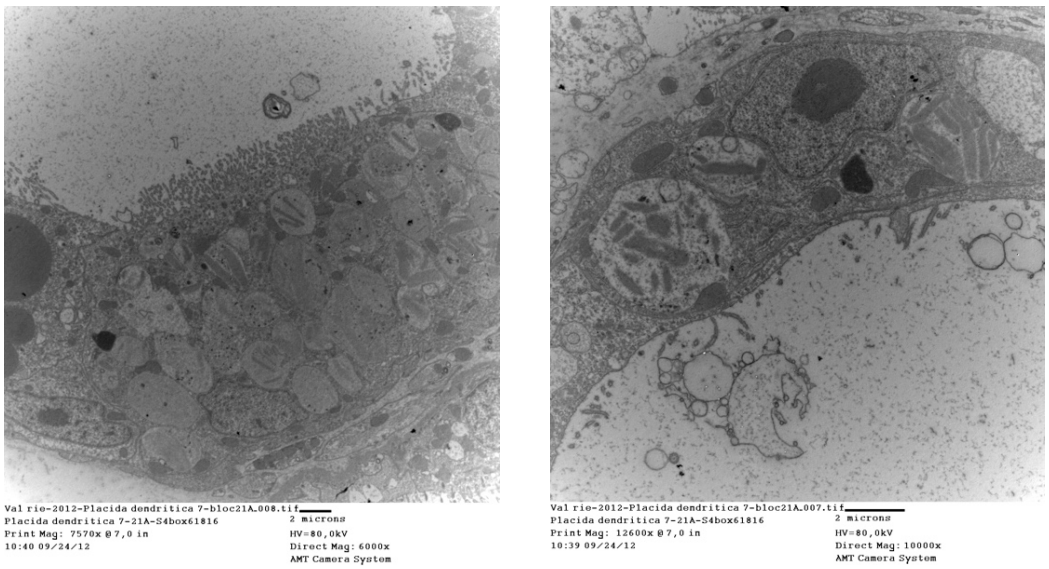


Figure 3.4.33: TEM micrographs of degradation of chloroplasts in an individual of *P. dendritica*, which was collected on 28th August 2012 from *C. fragile/vermilara* (collected on 22nd August 2012) and then was kept for three days without food supply until fixation on 31st August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

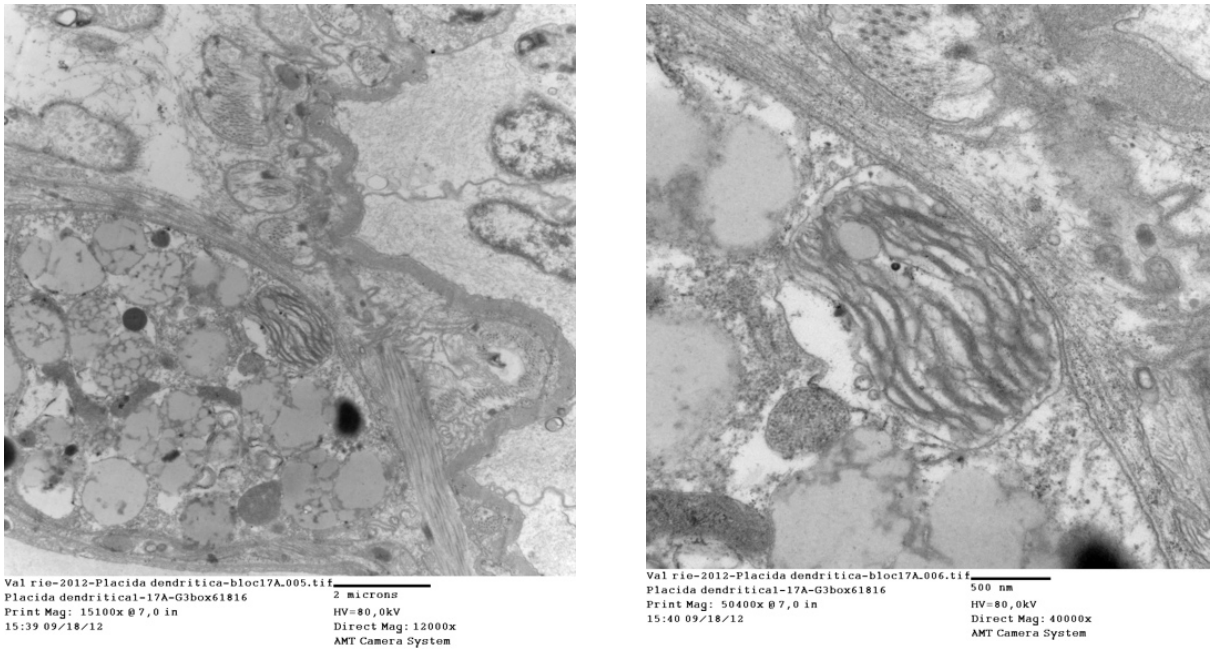


Figure 3.4.34: TEM micrographs of degradation of chloroplasts in an individual of *P. dendritica*, which was collected on 13th August 2012 from *C. fragile/vermilara* (collected on 8th August 2012) and then was kept without food supply for 11 days until fixation on 24th August 2012. Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

Thuridilla hopei

In individuals of *T. hopei* freshly collected and directly fixed on the same day, thus representing the natural state, chloroplasts in different states of degradation were observed, also some chloroplasts with starch granules (Fig. 3.4.35). Yield values in PAM-measurements before fixation were relatively high with F_v/F_m 0.614, 0.515, 0.605 (Th11-1) and 0.442, 0.510, 0.603 (Th11-2), respectively.

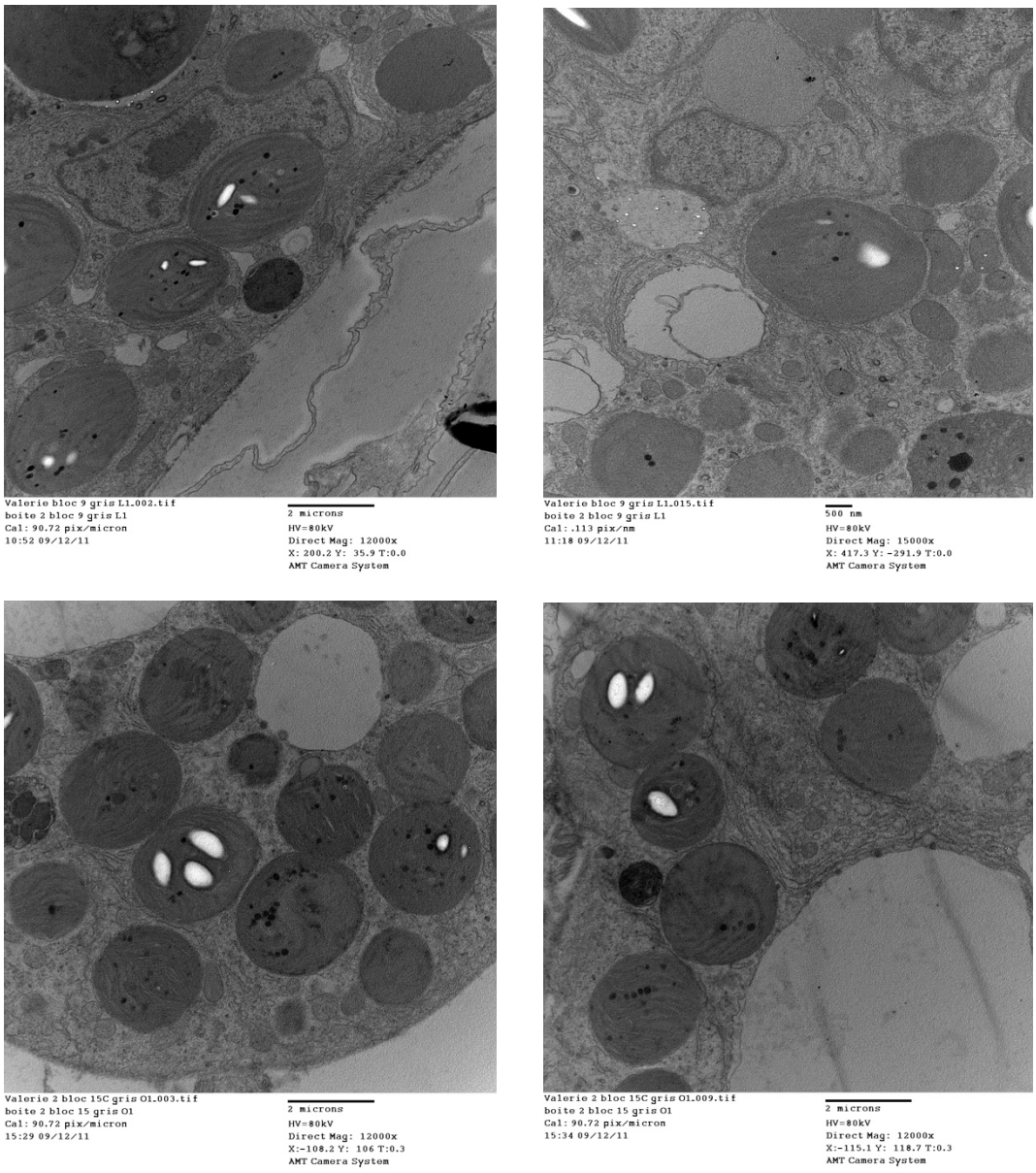


Figure 3.4.35: TEM micrographs of chloroplasts in two individuals of *T. hopei*, which were collected on 28th June 2011 and fixed directly on the same day as representative for the natural state (picture 1-2: Th11-1, PAM F_v/F_m 0.614, 0.515, 0.605; picture 3-4: Th11-2, PAM F_v/F_m 0.442, 0.510, 0.603). Bright conglomerates – probably starch granules. Dark conglomerates or droplets – probably plastoglobuli/lipids.

4 Discussion

4.1 Photobehavior / behavioral analyses

The results of the first study examining phototaxis in chapter 3.1 corresponded in part to the former results by Weaver and Clark who reported that the three 'chloroplast symbiotic' sacoglossan species *Elysia tuca* Marcus and Marcus 1967 (=*Elysia velutinus* Pruvot-Fol, 1947 after (MolluscaBase 2019d), reference therein: (Marcus and Marcus 1967)), *Elysia crispata* and *Costasiella lilianae (=Costasiella ocellifera* after Clark (Clark 1984)) oriented towards light while the two 'aposymbiotic' sacoglossan species *Oxynoe antillarum* and *Berthelinia carribea* without chloroplasts avoided light, which could point to a possible correlation of chloroplasts retention and phototaxis (Weaver and Clark 1981). Investigating the two Mediterranean sacoglossan species *Elysia timida* and *Thuridilla hopei*, the results in chapter 3.1 correspond in so far to the former hypothesis of Weaver and Clark depicted above, that both investigated species incorporate chloroplasts and both showed phototactic behavior to a certain degree (Schmitt and Wägele 2011). As an additional parameter, the longevity of functional retention of chloroplasts was taken into account in the analysis, leading to the result that the phototactic behavior was stronger in *Elysia timida* with long-term chloroplast retention than in *Thuridilla hopei* with short-term retention. Thus, the question arose, if species with long-term functional chloroplast retention reveal stronger evolutionary adaptations in relation to kleptoplasty concerning their phototactic behavior (Schmitt and Wägele 2011). It was inferred that the phototactic behavior is more probably to be regarded as an evolutionary adaptation, not as an immediate, direct influence of the chloroplasts on their host, which was supported by the finding of the study that juvenile *Elysia timida* already revealed strong phototaxis before the first uptake of chloroplasts (Schmitt and Wägele 2011) (chapter 3.1).

Thus, further analyses on phototaxis were performed including more species with different capacities of chloroplast retention and correspondingly taking the factor of longevity of functional chloroplast retention more into account (chapter 3.3). Included were several species with different capacities of chloroplast retention which were in parallel examined with long-term photobiological analyses. These included most of the few sacoglossan species which are known as the "top-performers" of long-term functional retention of chloroplasts: *Elysia timida, Elysia crispata* (*mangrove type* and *reef type*), *Elysia viridis* and *Plakobranchus ocellatus.* Furthermore *Bosellia mimetica, Thuridilla hopei* and *Placida*

dendritica were included as further comparative sacoglossan species, and additionally the non-sacoglossan nudibranchs *Cratena peregrina* and *Flabellina affinis* as comparison without incorporation of chloroplasts. The more extended analyses in chapter 3.3 with an overview including more species revealed that the background of photobehavior turns out to be more complex than formerly assumed and the former hypothesis cannot be confirmed in this simple way. On the contrary, in this study, the non-sacoglossan species *C. peregrina* and *F. affinis* without chloroplasts and the sacoglossan *P. dendritica* with fast digestion of chloroplasts revealed a highly positive phototactic reaction while several species with either long chloroplast retention capacities as *P. ocellatus* or mediate retention profiles rather reacted with caution or avoidance versus direct light exposure, even in the comparatively low light intensities (chapter 3.3).

Concerning phototactic behavior in chloroplast-carrying sacoglossan sea slugs there is a potential conflict between exposure of chloroplasts to light for functioning of photosynthesis on one hand, and on the other hand the danger of photodamage of chloroplasts due to high solar irradiance. Giménez Casalduero and Muniain reported a benefit of exposure of the chloroplasts for nutritional profits from photosynthesis in experiments, in which *E. timida* kept in the dark and thus deprived of the photosynthetic products of their chloroplasts had lower survival rates and stronger size decreases opposed to those kept in light (Giménez Casalduero and Muniain 2008). Recently, also Cartaxana et al. report experiments with the conclusion that photosynthesis of its kleptoplasts is nutritionally relevant in *E. viridis* (Cartaxana, Trampe et al. 2017). The exposure to light for the photosynthetic function of the kleptoplasts might imply also exposure to predators and currents, however, and irradiances higher than a well-tolerated maximum might damage the kleptoplasts (Monselise and Rahat 1980). While the threat of predators might be potentially decreased by self-defense with toxic or irritating secretions and/or camouflaging colorations in sacoglossans (Cimino and Ghiselin 1998, Marin and Ros 2004), the impairment of kleptoplasts through too high irradiation remains a danger (Jesus, Ventura et al. 2010). Also Viera et al. reported a negative effect of strong light exposure on the photosynthetic activity of kleptoplasts in *E. viridis* as individuals kept in "high" light conditions of 140 µmol photons m^{-2} s^{-1} showed a distinctly more rapid decrease of photosynthetic activity with retention lasting only 6 to 15 days opposed to the much slower decrease of photosynthetic activity in low light conditions of 30 µmol photons m^{-2} s^{-1} with retention lasting from 15 to 57 days (Vieira, Calado et al. 2009). Thus, the potentially balancing behavior concerning phototaxis observed in the various sacoglossan species in chapter 3.3 could possibly make sense as evolutionary adaptations in relation to

functional chloroplast retention. A recent study assumes also specific adaptations with reports that kleptoplast photoacclimation state to different light conditions modulated the photobehavior of the sea slug *E. viridis* (Cartaxana, Morelli et al. 2018).

For *E. timida*, further specialized protection mechanism against the photodamage problem are described and were analyzed in more detail in this Ph.D. thesis. Opening or closing the parapodia to different degrees in *E. timida* in different surrounding light intensities automatically either forms a natural protection shield for the embedded chloroplasts in the inside of the parapodia or exposes them like a leaf. First described by Rahat and Monselise (Rahat and Monselise 1979, Monselise and Rahat 1980), this specialized photobehavior of *E. timida* could be confirmed by the results in this thesis and analyzed in more detail (Schmitt and Wägele 2011) (chapters 3.1 and 3. 3). In the analyses in chapter 3.1, the emission of the fluorescence through the parapodia in individuals of *E. timida* was used as a factor to indirectly measure the exposure of the chloroplasts. The momentary ground fluorescence values (F0') increased in strong correspondence with increasing parapodial opening level, resulting in a significant correlation. This clearly reflected the efficiency of the behavior to either opening of the parapodia and exposing the incorporated chloroplasts to light or closing the parapodia leading to less light entering the parapodia and therefore protection of the underlying chloroplasts (Schmitt and Wägele 2011). This constitutes a highly specific adaptation enabling *E. timida* to reside in shallow light-exposed areas with potential flexible adaptation to current light conditions as could also be confirmed by the observations of *E. timida* in the natural environment in chapter 3.3.

Jesus et al. reported furthermore a capability in *E. timida* of combining the behavioral photo-regulation mechanism (opening/closing the parapodia) with a functional physiological photo-regulation mechanism (xanthophyll cycle) for longer conservation of maximal photosynthetic capacity (Jesus, Ventura et al. 2010). Probably further photoprotection mechanisms exist, also in other species. Cartaxana et al. described in their recent publication modulation of parapodia in reaction to light irradiance in *E. viridis* (Cartaxana, Morelli et al. 2018). In investigations of this thesis, individuals of the *E. crispata mangrove type* were also observed with hints pointing to that behavior, but the opening and closure of parapodia could not (yet) be distinguished so clear. Within all the observations in the frame of this thesis, *E. timida* was the only species observed to reveal this behavior in this special distinct form, which might possibly be also connected to its characteristic natural body structure, with a distinct division of certain locations of chloroplast incorporation mainly in the inside of the parapodia, and white parapodial outsides as potential protection shields. These specific body structures and

behavior in *E. timida* could potentially represent specialized adaptations in relation to long-term retention of functional chloroplasts with a flexible potential of exposure of kleptoplasts for photosynthetic benefit as well as protection from damage through too high irradiations, enabling functionality of incorporated chloroplasts for among the most extended durations known so far (Schmitt and Wägele 2011) (chapters 3.1 and 3.3).

4.2 Laboratory culture system investigations – *Elysia timida* as a model organism

The laboratory culture system with *E. timida* could successfully be established and several investigations with the advantages of the controlled conditions could be performed (Schmitt, Händeler et al. 2014) (chapter 3.2). Several characteristics of the species *E. timida* were shown to be advantageous for laboratory culturing. For example, specific traits concerning reproduction turned out to be beneficial, like mating habits, as well as size, stability and coloration of egg masses and a mainly intracapsular development with only a potential short free-swimming veliger phase of three days, as in parts previously described (Marín and Ros 1993, Schmitt, Anthes et al. 2007, Schmitt, Händeler et al. 2014). Furthermore, in contrast to former reports of juvenile *E. timida* feeding first on *Cladophora dalmatica* Kützing 1843 (Kützing 1843) before starting to feed on *Acetabularia acetabulum* as preferred food algae in adults observed by Marin and Ros (Marín and Ros 1993), our juvenile *E. timida* individuals fed directly on young, non-calcified, stalks of *A. acetabulum* and rejected all samples of different *Cladophora* species offered in our investigations (Schmitt, Händeler et al. 2014). This could possibly indicate incipient speciation – several cases of variations in *E. timida*, differing in morphological, reproductive or other features, including genetic differentiations were reported (Giménez-Casalduero, Muniain et al. 2011), but further analyses would be needed to clarify this. For the culturing, our finding that juveniles fed directly on *A. acetabulum* was advantageous, as the laboratory culture system could be established with only one food algae for the whole life cycle of *E. timida* (Schmitt, Händeler et al. 2014).
In the frame of the laboratory culture system, several trials could be performed with respective controlled conditions and the focus of the analyses on specific factors. Data on long-term photosynthetic activity of incorporated chloroplasts in *E. timida* individuals in the laboratory culture during periods without food supply corresponded to those or were longer lasting than in reports in the literature (Händeler, Grzymbowski et al. 2009, Jesus, Ventura et

al. 2010, Wägele, Deusch et al. 2011). A three-phase analysis of the long-term photosynthetic activity of incorporated chloroplasts in *E. timida* individuals in the laboratory culture with three trial groups with different starting points (winter, spring, summer) indicated a potential influence of the different temperature conditions (reflecting seasonal influences) the individuals were exposed to, on durability and effectivity of long-term photosynthetic activity of incorporated chloroplasts (Schmitt, Händeler et al. 2014). Based on this, further investigations by Laetz et al. also indicated an influence of temperature on photosynthetic activity in kleptoplasts in *E. timida* (Laetz and Wägele 2018b). In another study, Laetz et al. also supposed temperature as a potential influencing factor on photosynthetic starch production in kleptoplasts in *E. timida* (Laetz, Moris et al. 2017). An influence of temperature of the photosynthetic activity of incorporated chloroplasts seems likely. In this context, several different optimal temperatures for carbon fixation are described for the kleptoplasts in some sacoglossan sea slugs, e. g. 25 °C for *E. timida* from Mar Menor, Spain (Marin and Ros 1989) and astoundingly two very different temperatures from two species from Florida, *Costasiella ocellifera* and *Elysia tuca* Marcus and Marcus 1967 (=*Elysia velutinus* Pruvot-Fol, 1947 after (MolluscaBase 2019d), reference therein: (Marcus and Marcus 1967)) with also 25 °C (Clark, Jensen et al. 1981) and 15 °C (Stirts and Clark 1980), respectively. Within the controlled laboratory culture conditions, we could also demonstrate for the first time the capability of *E. timida* to incorporate chloroplasts from another algal donor, namely *Acetabularia peniculus* (R. Brown ex Turner) Solms-Laubach 1895 (Solms-Laubach 1895) – which is not abundant in its natural environment (as far as stand of knowledge) – and to perform long-term retention with it (Schmitt, Händeler et al. 2014). Previously, the stand of reporting was a rather specific food preference of adult *E. timida* for *A. acetabulum* (Marín and Ros 1992), plus the primary juvenile diet on *C. dalmatica* (Marín and Ros 1993). One publication stated feeding of *E. timida* on other algae in laboratory trials, but did not specify the respective algal species (Giménez-Casalduero, Muniain et al. 2011). Comparing the photosynthetic activity of chloroplasts from the two different algal donors in *E. timida* individuals, a long-term experiment in the laboratory culture system revealed a very similar outcome concerning durability and effectivity of photosynthetic capacities of kleptoplasts from *A. acetabulum* and *A. peniculus* – showing that chloroplasts from two different (even if related) algal donors can result in similar photosynthetic long-term capacities in one sea slug species (Schmitt, Händeler et al. 2014). In all, the experiments performed in the laboratory culture system could profit from the controlled defined conditions and the advantage of known history of individuals, e. g. the clear definition that measured photosynthetic activities

stemmed only from clearly determined chloroplasts of *A. acetabulum* and *A. peniculus.* Thus, advantages of laboratory systems to study kleptoplasty in sea slugs could be confirmed. Analyses of sea slugs in controlled laboratory settings or even in specialized laboratory cultures provide advantages as e. g. permitting long-term studies under controlled conditions and with individuals of known individual history, as well as the opportunity of developmental investigations (Rumpho, Pelletreau et al. 2011, Pelletreau, Worful et al. 2012, Bhattacharya, Pelletreau et al. 2013, Pelletreau, Weber et al. 2014, Schmitt, Händeler et al. 2014, Laetz and Wägele 2017, Chan, Vaysberg et al. 2018).

4.3 Cell biological investigations by TEM

In the ultrastructural investigations of evolutionary adaptations in relation to kleptoplasty, juvenile *E. timida* could be analyzed with TEM concerning the very first intake of chloroplasts in their life feeding the first time on *A. acetabulum* (Schmitt, Händeler et al. 2014) (chapter 3.2). These TEM micrographs revealed clear differences of the chloroplasts from *A. acetabulum* integrated into the juveniles' digestive gland cells compared to those of *C. dalmatica* documented in the literature for juvenile *E. timida* (Marín and Ros 1993). Also, the process of first uptake and degradation of chloroplasts could be documented in our electron micrographs; degradation had already begun only two to three hours after the first initiation of feeding as shown by tissues fixed two to three hours after feeding start (Schmitt, Händeler et al. 2014). Most probably, the first chloroplasts are ingested intact, but then rapidly digested for the high initial nutrient need of juveniles for their growth and development. In their reports of chloroplasts from *C. dalmatica* in juvenile *E. timida*, Marín and Ros describe that those chloroplasts were surrounded by "host membranes of the phagocytic vacuole" (p. 98) (Marín and Ros 1993). In our electron micrographs of juvenile *E. timida,* some chloroplasts seem to be embedded intact in the cytoplasm with direct contact to the cytosol, while around others a distinct gap between chloroplast and cytoplasm was observed, similar to the gap around chloroplasts of *C. dalmatica* in juvenile *Elysia timida* shown by Marín and Ros (Marín and Ros 1993). Around those gaps, and especially around aggregations of several chloroplasts, fragments resembling an enclosing phagocytic membrane were recognizable in our electron micrographs (Schmitt, Händeler et al. 2014). The results point to a possible correlation between degradation (digestion) and the presence of a phagocytic membrane – which however would have to be examined in more detail by

further investigations. In accordance to this assumed relation would be the description of Marín and Ros of phagocytic membranes around the chloroplasts in juvenile *E. timida* probably being about to be degraded in contrast to intact chloroplasts without an additional layer of phagocytic membranes in adult *E. timida* (Marín and Ros 1993). Also corresponding to this assumed relation, Evertsen et al. described phagosome membranes around chloroplasts in *P. dendritica* in relation to fast degradation in contrast to intact chloroplasts lying directly in the cytoplasm in *E. viridis* with long-term functional retention (Evertsen and Johnsen 2009). Martin et al. depicted observations of differences in the way chloroplasts are enveloped in different sacoglossan species with various capacities of chloroplast retention that point into the same direction that in sea slugs' cells, chloroplasts surrounded by membranes or 'envelopes' are digested more rapidly, and chloroplasts without those 'envelopes' can be maintained longer (Martin, Walther et al. 2015).

The assumption that chloroplasts of *A. acetabulum* in juvenile *E. timida* are primarily digested in the first time after their initial intake is also in accordance with findings of Laetz et al. that functional kleptoplasty was not established until after at least 15 days (of feeding) post metamorphosis in juvenile *E. timida* (Laetz and Wägele 2017). For juvenile *Elysia chlorotica*, Pelletrau et al. also reported that chloroplasts are degraded in the first time and an initial feeding phase of a week was needed until degradation decreased and kleptoplasty was established (Pelletreau, Worful et al. 2012).

Further TEM-investigations presented with a selection of results in the further results section, provided a comparative overview over several sea slug species with different chloroplast retention and their respective food algae. In general, chloroplasts appeared more round when incorporated in the sea slugs than in the algae where they displayed more elongated shapes, which indicates a special incorporation in the sea slugs. Corresponding to the feeding experiments in the further results section, indicating good long-term retention capacities for chloroplasts from *F. petiolata* in *E. viridis,* many chloroplasts were observed in *E. viridis* from *F. petiolata* with round and intact appearance and a characteristic pattern, also after a certain time of starving. Incorporated chloroplasts in *E. timida* revealed a very special diversity of patterns which confirmed former own TEM results (Wägele, Deusch et al. 2011, Schmitt, Händeler et al. 2014). These special diverse patterns could point to a specialized way of chloroplast incorporation in *E. timida* in connection with its good capabilities for long-term chloroplast retention, which however would have to be clarified by further investigations.

Different states of chloroplast degradation were observed also in freshly collected and control individuals and have to be interpreted carefully due to a limited knowledge concerning history

of collected individuals and factors in the process of retention and digestion. The same accounts to the appearance or presence of starch granules which should be interpreted carefully due to the general complex, flowing, nature of the physiological processes and the general difficulties of clear determination with TEM. The TEM overview of insights into incorporation of chloroplasts in various sea slugs in this thesis provided a general spectrum picture of different states of chloroplast incorporation from apparently intact, containing photosynthesis products as e. g. starch grains, and different states of degradation – and represents basis findings for further investigations. Laetz et al. described quantified starch accumulation by kleptoplast photosynthesis during starving periods in *E. timida* – serving as potential nutrient reserves being digested gradually as needed, as subsequent degradation was observed in the later course of starving (Laetz, Moris et al. 2017). In a comparative study, Laetz and Wägele did not find the same starch accumulation in *Elysia cornigera*, however, it has to be taken into account, that *E. cornigera* was fed with *A. acetabulum* for comparison equality with *E. timida*, but this is another *Acetabularia*-species than *E. cornigera* feds on naturally (Laetz and Wägele 2018a). Laetz and Wägele found a certain amount of starch grains also in *A. acetabulum* and in unstarved individuals of *E. timida* and *E. cornigera* (Laetz and Wägele 2018a), which could of course also be a product of photosynthesis while chloroplasts were still in the algae. With these results, the findings in this present thesis are thus overall in coherence, as here starch grains were observed to a certain degree in different states in the various species, also in natural, presumably saturated states of sea slugs and in algae. With investigations on retention of functional kleptoplasts and digestive activity in *E. timida, E. viridis* and *T. hopei*, Laetz et al. found furthermore that in digestive processes the number of chloroplasts and lysosomes was indirectly proportional, with decreasing plastid density when starvation begins (Laetz, Rühr et al. 2016). This described progressive degradation of chloroplasts, as also illustrated by Laetz and Wägele (Laetz and Wägele 2018a) with vacuoles containing fluid and/or fragments of chloroplasts, is also in coherence with the TEM-investigations in this thesis.

4.4 Investigations in near-natural and natural settings

With the underwater investigations in form of PAM-measurements of photosynthetic activity in *E. timida* and *E. crispata mangrove type* in combination with environmental factors on site in their natural habitats, presented in chapter 3.3, this Ph.D. thesis provides the first data of

the photosynthetic yield ΔF/Fm' of kleptoplasts of two sacoglossan species underwater in their natural habitat in relation to environmental light conditions. Photosynthetic sea slugs have been extensively investigated under laboratory conditions as described above, but reports of investigations in their natural environment are lacking up to now.

The underwater studies showed distinct differences between the two sacoglossan species *E. timida* and *E. crispata mangrove type* concerning their habitats and environmental conditions and photosynthetic activities in relation to environmental conditions. Kleptoplast photosynthetic activity revealed variations during the course of the day in both species *E. timida* and *E. crispata mangrove type* in correlation to light conditions in the natural environment which are varying depending on habitat, weather, etc. This resulted overall in the same correlation of lower photosynthetic yield ΔF/Fm' with higher solar irradiation as well as higher photosynthetic yield ΔF/Fm' with lower solar irradiation, which was even more prominent in the food algae *A. acetabulum* of *E. timida*. As discussed in more detail in chapter 3.3, concerning this correlation, the special character of the PAM-measurements in relation to the complex photosynthetic mechanisms has to be taken into consideration for the interpretation of the results, as in contrast to the maximum quantum yield of PS II, the effective quantum yield $\Delta F/F_m'$ under ambient light conditions is measured here, which decreased with increasing irradiation. The relative rate of electron transport (ETR), however, including the quantum flux density of photosynthetically active radiation of the ambient light as a multiplication factor, provided measurements of photosynthetic rate here, revealing the corresponding positive correlation of higher ETR with higher solar irradiation (chapter 3.3). Interestingly, fluorescence F in individuals of *E. timida* measured underwater in their natural habitat was always distinctively lower (about half as much) than in their food algae *A. acetabulum*, which might be caused by the shading effect of the parapodia, diminishing emission of fluorescence, that was investigated in this Ph.D. thesis also under semi-natural laboratory conditions in the first study (Schmitt and Wägele 2011) (chapter 3.1).

The results of investigations of long-term capacities of retention of functional chloroplasts in the various sacoglossan species in chapter 3.3 could partly confirm former results but also showed new results as described in chapter 3.3. The profiles of capacities of functional chloroplast retention differed extremely in the various investigated species with *P. ocellatus* revealing outstanding capacities of long-term chloroplast retention compared to the other species. Thus, the results of observations of long-term kleptoplast retention in *P. ocellatus* over seven months could confirm *P. ocellatus* as one of the few species with extremely long durations of chloroplast retention and provided the longest period measured so far (chapter

3.3). As depicted in more detail in the discussion in chapter 3.3, reports about chloroplast retention in *P. ocellatus* were until now only restricted to shorter periods of actual observation and partly subsequent estimations of the total potential duration (Evertsen, Burghardt et al. 2007, Händeler, Grzymbowski et al. 2009, Christa, Wescott et al. 2013, Yamamoto, Hirano et al. 2013, Wade and Sherwood 2017). An explanation for observed differences in kleptoplast retention capacities in *P. ocellatus* could be ecological differentiation and varying chloroplast donors, as broad and diverse food spectra have been described for *P. ocellatus* from different regions (Maeda, Hirose et al. 2012, Christa, Wescott et al. 2013, Wade and Sherwood 2017). Due to its high intraspecific diversity, *P. ocellatus* is also defined as species complex of possibly ten species with diverse distribution and nutrition profiles (Krug, Vendetti et al. 2013, Wade and Sherwood 2017). The individuals of *P. ocellatus* investigated in this Ph.D. thesis stemmed from the Philippines, for which origin Christa et al. described a broad food spectrum in *P. ocellatus*, comprising species of the genera *Halimeda, Caulerpa, Udotea, Acetabularia* and further unidentified algae – with emphasizing especially *Halimeda macroloba* with regard to its potential implication for long-term retention (Christa, Wescott et al. 2013), which is discussed also below in context to the overall observed inter- and intraspecific variation in retention capacities in this thesis.

Also, differences in photosynthetic performance were demonstrated in chapter 3.3 between the populations of *E. crispata reef type* and *mangrove type*, which might indicate ecological differentiation and different algal chloroplast donors, as Krug et al. described ecological differentiation of the two different morphotypes of *E. crispata reef type* and *mangrove type* (Krug, Vendetti et al. 2016). As a contrasting result of investigation of species-specific long-term retention profiles in chapter 3.3, individuals of *E. viridis* collected from its known food algae *C. fragile/vermilara* showed shorter retention duration than expected due to former reports of Evertsen and Johnsen of very long high photosynthetic capacities of functional chloroplasts in *E. viridis* (Evertsen and Johnsen 2009). Thus, the results in chapter 3.3 revealed *E. viridis* as a species with rather intermediate than long-lasting chloroplast retention. They corresponded more to shorter retention duration in *E. viridis* reported by e. g. Viera et al. (Vieira, Calado et al. 2009) and thus potentially confirms *E. viridis* as an example for a species with strong intraspecific variations in capacities of chloroplast retention in different habitats and geographical regions and also in relation to different algal food sources, which possibly indicates a high degree of adaptation and differentiation into a species complex with various ecological types (Evertsen and Johnsen 2009, Rauch, Tielens et al. 2018). Baumgartner et al. described distinct differences in photosynthetic capabilities and

resulting parameters like growth efficiency in *E. viridis* due to whether they acquired 'highly functional kleptoplasts' from *C. fragile* or kleptoplasts 'of limited functionality' from *Cladophora rupestris* (Baumgartner, Pavia et al. 2015). Results from feeding experiments presented in the further results section 3.4 also revealed distinct differences between various collection populations of *E. viridis*, e. g. from different habitats and different food sources, and thus pointed to the same direction, as explained in more detail below in section 4.5.

4.5 Feeding experiments and inter- and intra-specific differences

Besides potential influences on photobehavior like described above in *E. timida*, the special parapodia-bearing body form could also have some potential implications for the capacity of chloroplast retention, as the pattern was described that sea slug species known with the highest durations of long-term retention of functional chloroplasts are species with wing-like parapodial lobes (Händeler, Grzymbowski et al. 2009, Rumpho, Pelletreau et al. 2011). As an exception, the cerata-bearing Limapontioidea *Costasiella ocellifera* is described with functional kleptoplasty with durations of several weeks and some other species of the genus with short-term chloroplast retention – thus the origin of functional kleptoplasty is assumed to have evolved earlier than at the base of the *Plakobranchoidea*, but at the base of the more basal *Plakobranchacea* or with potential multiple origins (Christa, Händeler et al. 2015). Further analyses comprising more species and details will potentially show clearer relations. Up to now, the known sea slug species with the longest durations of long-term retention of functional chloroplasts during several months are parapodia-bearing forms. This former general pattern could be confirmed in this study, concerning the distinct differences between the parapodia-bearing *E. viridis* and the cerata-bearing *P. dendritica* collected from the same food algae *Codium fragile/vermilara* which have formerly already been described by Evertsen et al. (Evertsen and Johnsen 2009).
In addition, we found *E. viridis* and the cerata-bearing *Ercolania viridis* living sympatrically in a tidal pool, showing the same difference, with the photosynthetic performance of *Er. viridis* strongly corresponding to those of *P. dendritica*, as described in the further results section 3.4. Interestingly, even in a saturated state, the two cerata-bearing sea slug species *P. dendritica* and *Er. viridis* always had photosynthetic yields far below those of their respective food algae, while *E. viridis* and the other observed sea slug species with longer

retention of chloroplasts showed photosynthetic yields resembling those of their food algae (chapter 3.4, Table 3.4.1).

Individuals of the tidal-pool-population of *E. viridis* were remarkably bigger in size and revealed distinctively higher longer-lasting retention of chloroplasts than the other collection populations from the sea during the three years of investigations. This might have been caused by the different habitat conditions and/or different nutrition sources. As explained above, e. g. Baumgartner et al. described better retention capacities and increased growth rates in *E. viridis* feeding on *C. fragile* compared to *E. viridis* feeding on *C. rupestris* (Baumgartner, Pavia et al. 2015). In addition to the formerly described food algae *C. fragile/vermilara*, the results in chapter 3.4 could detect *F. petiolata* as algal food and chloroplast donor in *E. viridis*, with even a tendency for better retention capacities with chloroplasts from *F. petiolata*. When both algal donors were supplied to *E. viridis* individuals in a further additional trial, photosynthetic yields remained high with supply of the one or the other algae. This could possibly imply that either the incorporated chloroplasts depots are maintained untouched and newly uptaken chloroplasts are digested or that degrading chloroplast depots are refilled by newly uptaken chloroplasts. Evertsen et al. reported in relation to a former study by Gallop et al. that feeding individuals replaced 75% of their chloroplasts during a nine-day period, while starving individuals only lost 15% in the same period (Evertsen and Johnsen 2009).

The reversed trial of trying to feed *E. viridis* individuals originally collected from *F. petiolata* after a starvation period with *C. fragile/vermilara*, did not function, which could possibly hint to individual food preference and adaptation, finally leading to ecological differentiation. If eggs of sea slugs are deposited on or close to colonies of specific potential food algae and juveniles grow up on these colonies, they might adapt to the respective algae.

In our feeding experiment with individuals of *E. crispata mangrove type*, we could identify out of an assortment of algae abundant in the environment the two algal species *Caulerpa verticillata* and *Penicillus capitatus* as chloroplast donors, which resulted in only slightly different photosynthetic profiles of chloroplast retention (chapter 3.4). Curtis et al. described *E. clarki* – which probably corresponds to the *E. crispata mangrove type* as explained in the introduction – to feed on different algae, with nutrition preferences of juveniles differing from those of adults (Curtis, Pierce et al. 2007).

For some sacoglossan species, very specialized food preferences are described, e. g. *E. timida* with a close relationship to *A. acetabulum* despite of other algae species present in the environment which serve other sacoglossan species as food algae and chloroplast donor, e. g.

C. fragile/vermilara (Marín and Ros 1992). Our previous laboratory trials, however, revealed that *E. timida* also accepted *A. peniculus*, which is not abundant in its natural environment, as a food algae and chloroplast donor resulting in similar photosynthetic capacities (Schmitt, Händeler et al. 2014) (chapter 3.2). Also in the present study, *E. viridis* individuals originally stemming from *C. fragile/vermilara* could successfully replenish with chloroplasts from *F. petiolata* and reach even better photosynthetic capacities than before (chapter 3.4). In conclusion, there are specialized food preferences developed in sacoglossan sea slugs, but apparently also a certain potential flexibility for adaptation to other algal sources. In an overview about various sacoglossan species with their food sources analyzed by barcoding and in relation to chloroplast retention, Christa et al. report that long-term retention forms can as well be specialized as *E. timida* feeding on *A. acetabulum,* but also polyphagous as *P. ocellatus* feeding on several algae species, and all in all determined the algal species apparently essential for long-term chloroplast retention to belong to the genera *Halimeda, Caulerpa, Penicillus, Avrainvillea, Acetabulari*a and *Vaucheria* (Christa, Händeler et al. 2014). In a former study, as cited also above, Christa et al. already reported barcoding analyses identifying a broad food spectrum in *P. ocellatus*, comprising species of the genera *Halimeda, Caulerpa, Udotea, Acetabularia* and further unidentified algae – with an emphasis on *Halimeda macroloba,* especially concerning contribution to long-term retention (Christa, Wescott et al. 2013).

Simple observational feeding trials as in the present investigations can in general only serve in limited way as positive evidence, as negative reactions in feeding trials can be caused by multiple factors, e. g. individuals not being in good condition anymore due to preceding starving, local or temporary feeding preferences or avoidances due to various factors etc. For example, feeding trials with *Halimeda incrassata* (n=5) in *E. crispata mangrove type* individuals in chapter 3.4 were negative, though *H. incrassata* was reported as a potential food source by barcoding analyses in formerly named "*E. clarki*" (Christa, Händeler et al. 2015) which should correspond to the *E. crispata mangrove type.* Christa et al. criticized that their analyses applying the marker rbcL might not have been able to detect *Caulerpa* species as potential food sources as they could identify several *Caulerpa* species analyzing *Elysia tomentosa, Volvatella viridis* and *P. ocellatus* with the application of *tufA* as barcoding marker (Christa, Händeler et al. 2015). In the present feeding observations in chapter 3.4, *C. verticillata* could be confirmed as potential chloroplast donor in individuals of the *E. crispata mangrove type* with following chloroplast retention of at least about a week without food supply in individuals that had been starved before and then supplied newly with

C. verticillata (chapter 3.4). The advantage of a methodical procedure like this, is that chloroplasts from special single algal donors can be analyzed. However, this does not represent the natural state in which sea slugs have potential access to various algal species and has further methodical limitations, e. g. clear differentiation of the algal species. Nevertheless, the present feeding analyses in chapter 3.4 can provide useful fundamental results which can also provide a basis for further analyses.

The parameter of size of the investigated individuals should also be taken carefully into account in future analyses of long-term photosynthetic capacities of integrated chloroplasts. The results here pointed to e. g. a tendency in individuals of *E. viridis* and *B. mimetica* of better or longer retention of functional chloroplasts with bigger size. It is obvious that within a sea slug species, individuals of bigger size have most probably better or more capacities to store chloroplasts and with more chloroplast deposit capacities also longer-lasting residue to degrade time-after-time. Even more as smaller individuals if still in the process of growing will probably digest more chloroplasts to obtain energy for their growth, as e. g. Laetz and Wägele described that juveniles primarily digest first meals of chloroplasts and that functional kleptoplasty is not developed in *E. timida* until after at least 15 days post-metamorphosis, with individuals even after 25 days post-metamorphosis not surviving starvation to a similar extent as adults, but rather in a still uncomplete, transient form of kleptoplasty (Laetz and Wägele 2017). On the other hand, the possible advantages from bigger size and with that bigger chloroplast deposit capacity are probably limited by various factors as e. g. age, condition and death of the individuals and a mixture of other factors which play a role in the life of the free-living individuals whose life-histories before collection are unknown.

Concerning the different sea slug species, the species with higher durations of long-term retention of chloroplasts are mostly also species which are slightly bigger in sizes, more in a centimeter-scale than in a millimeter scale, however, more has to be known to have a clearer overview over these potential relations and factors. Only several sacoglossan species have been investigated while others not yet and even for the species under investigations still a lot of questions remain open up to now in the exploration of this fascinating, yet still enigmatic phenomenon.

5 Conclusions

In conclusion, the following summarized major findings could be achieved:
On a cell biological level, a comparative overview of different species could be provided and indices for special adaptations in relation to incorporation of chloroplasts were found. As one major finding, the very first uptake of chloroplasts from their food alga *Acetabularia acetabulum* could be revealed by transmission electron microscopy in juvenile *Elysia timida*. Based on this finding, that the whole life cycle of *Elysia timida* can be completed with only one food alga, a laboratory culture system could be successfully established. The advantages of the laboratory culture system with *E. timida* as a model organism could be demonstrated. In a trial within the culture system, it could be revealed that *E. timida* can use also chloroplasts from another chloroplast donor – *Acetabularia peniculus* – to establish kleptoplasty with similar retention capabilities as with *Acetabularia acetabulum*. Furthermore, effects of temperature on capacities of long-term photosynthetic activity were indicated by experimental trials under the controlled laboratory conditions.
With the measurements of over seven months of long-term retention in *Plakobranchus ocellatus*, the longest period so far could be documented. The results of the feeding experiments presented in the further results section provide basic findings; several algal chloroplast donors could be confirmed and it could be revealed that *Elysia viridis* also fed on and incorporated chloroplasts from *Flabellia petiolata*, with even partly better capacities of chloroplast retention than with *Codium fragile/vermilara*. Overall, differences between species-specific spectra of photosynthetic capacities in various sacoglossan seas slug species could be confirmed and also considerable variation within. Variations within the frame of the species-specific spectra of the photosynthetic capacities of integrated chloroplasts can in free-living sea slugs potentially be influenced by various factors as e. g. season, temperature, food availability, light conditions and further environmental parameters, as well as age, size and overall condition of the individuals. Ecological parameters in the natural environment, especially concerning light conditions, could be demonstrated to affect photosynthetic activity of kleptoplasts, which constituted the first demonstration of this kind. Photobehavior was found to be more complex than assumed in former hypotheses and could be confirmed as forming specific adaptations in relation to incorporation of chloroplasts. In *E. timida*, the efficiency of the specialized photobehavior of parapodia modulation could be demonstrated by the effect of the emission of fluorescence F and the specialized photobehavior could also be confirmed in underwater investigations. All in all, the complete explanations of the

specialized phenomenon of chloroplast incorporation in sacoglossan sea slugs rest enigmatic – this thesis contributed with some aspects to the knowledge of this fascinating phenomenon.

6 Acknowledgements

First, I thank my supervisor Professor Heike Wägele for the opportunity to perform this Ph.D. thesis and the support and patience she gave me.
Furthermore, I thank Professor William F. Martin for two temporary posts as scientific coworker in a collaboration project at the Institute for Molecular Evolution, Heinrich-Heine-University of Düsseldorf, Germany.
Thanks are also due to Professor Thomas Bartolomaeus for being the second referee for this thesis as well as two further referees.
Also, I thank Professor Rainer Martin, University of Ulm, for introducing me to the techniques of transmission electron microscopy and collaboration with it. For further transmission electron microscopy investigations, I especially thank Marie-Line Escande for technical assistance and collaboration at the Observatoire Océanologique Banyuls-sur-Mer, France, in the frame of the ASSEMBLE program.
I sincerely thank that the project was in several parts supported by the European Community in the frame of the ASSEMBLE program with a grant agreement no. 227799 to Valérie Schmitt and partly by a grant of the German Science Foundation (Wa618/12) to Heike Wägele and an ERC advanced grant no. 232975 to William F. Martin. Furthermore, I thank the Observatoire Océanologique Banyuls-sur-Mer, France, and the Mote laboratory, Florida, USA, for providing laboratory facilities as well as very good and friendly co-work during research stays. I address special thanks to Gregor Christa for cooperation, participating in a part of the measurements in Florida and some comments on some parts on the manuscript.
At the Institute for Molecular Evolution, Heinrich-Heine-University of Düsseldorf, Germany, I especially thank Margarete Stracke for excellent work as technical assistant, which I always could fully rely on, even when I was on a research stay and had to survey the running experiments through our communication from abroad. Also, I thank Susanne Gunkel for assistance in breeding juvenile *E. timida* and establishment of the culture system, as well as Daniel Niederau for assistance with the laboratory culture. And I sincerely thank the secretaries Doris Matthée and Ariane Baab for all their support. I thank all my colleagues,

especially to name Katharina Händeler, Re Young Wu and Houda, for collaboration and nice times together.
Concerning research stays in Banyuls, I also thank the secretaries of the OOB, Francois for talks about photosynthesis and life, Michele Nishiguti for diving together and often sharing the laboratory room, Pascal Romans for helpful assistance with laboratory / aquarium issues, Pascal from diving school for being my diving and security guide when I was taking underwater measurements with PAM while scuba diving and thus needed a security guide in contrast to underwater measurements while I was snorkeling alone. Furthermore I thank Géraldine Criquet, Daniela, Maria Jesus Herrero, Claire and after naming just those who helped with special issues as e. g. accommodation during research stays before I received the grants for the research stays and with that helped a lot, also in general all the nice people who supported me in any way, especially accompanying me sometimes when I was going to collect sea slugs so I did not have to go every time alone, which sometimes was not easy, especially with a lot of equipment to carry, and thus their companionships were a big help and joy, furthermore as they were always astonishingly willing to listen to all my sea slug stories.

Many of the above and some who are not named here, but all went with me through that times, are still connected to me in friendship, even if sometimes separated by distance and time, and I thank all these people that they have been at my side and walked a piece of this way with me. I am so grateful to all the friends and friendly people who gave me courage.

In all, I am deeply grateful for all the support I received from all sides (and please if anyone misses being named above or not enough, feel included in this whole entity).

7 References

1. Alder, J. and A. Hancock (1843). "Notice on a British species of Calliopaea d'Orbigny, and on four new species of Eolis with observations on the development and structure of the nudibranchiate Mollusca." Ann Mag Nat Hist **12**: 233–238.
2. Baumgartner, F. A., et al. (2015). "Acquired Phototrophy through Retention of Functional Chloroplasts Increases Growth Efficiency of the Sea Slug Elysia viridis." PLoS One **10**(4): e0120874.
3. Bergbauer, M. and B. Humberg (1999). Was lebt im Mittelmeer? Ein Bestimmungsbuch für Taucher und Schnorchler. Stuttgart, Kosmos.
4. Bhattacharya, D., et al. (2013). "Genome analysis of Elysia chlorotica Egg DNA provides no evidence for horizontal gene transfer into the germ line of this Kleptoplastic Mollusc." Mol Biol Evol **30**(8): 1843-1852.
5. Cartaxana, P., et al. (2018). "Kleptoplast photoacclimation state modulates the photobehaviour of the solar-powered sea slug Elysia viridis." J Exp Biol **221**(Pt 12).
6. Cartaxana, P., et al. (2017). "Kleptoplast photosynthesis is nutritionally relevant in the sea slug Elysia viridis." Nature Scientific Reports **7**(7714): 1-10.
7. Casalduero, F. G. and C. Muniain (2006). "Photosynthetic activity of the solar-powered lagoon mollusc Elysia timida (Risso, 1818) (Opisthobranchia: Sacoglossa)." Symbiosis **41**: 151–158.
8. Chan, C. X., et al. (2018). "Active Host Response to Algal Symbionts in the Sea Slug Elysia chlorotica." Mol Biol Evol **35**(7): 1706-1711.
9. Christa, G., et al. (2015). "Phylogenetic evidence for multiple independent origins of functional kleptoplasty in Sacoglossa (Heterobranchia, Gastropoda)." Org Divers Evol **15**: 23.
10. Christa, G., et al. (2014). "Identification of sequestered chloroplasts in photosynthetic and non-photosynthetic sacoglossan sea slugs (Mollusca, Gastropoda)." Front Zool **11**(1): 15.
11. Christa, G., et al. (2013). "What remains after 2 months of starvation? Analysis of sequestered algae in a photosynthetic slug, Plakobranchus ocellatus (Sacoglossa, Opisthobranchia), by barcoding." Planta **237**(2): 559-572.
12. Cimino, G. and M. T. Ghiselin (1998). "Chemical defence and evolution in Sacoglossa (Mollusca: Gastropoda: Opisthobranchia)." Chemoecology **8**: 51-60.
13. Clark, K. B. (1984). "New records and synonymies of Bermuda Opisthobranchs (Gastropoda)." The Nautilus **98**: 85-97.
14. Clark, K. B., et al. (1990). "Survey for functional kleptoplasty among West Atlantic Ascoglossa (=Sacoglossa) (Mollusca: Opisthobrachia)." Veliger **33**: 339–345.
15. Clark, K. B., et al. (1981). "Chloroplast symbiosis in a non-elysiid mollusc, Costasiella lilianae Marcus (Hermaeidae: Ascoglossa (=Sacoglossa): Effects of temperature, light intensity and starvation on carbon fixation rate." Biol Bull **160**: 43–45.
16. Costa, A. (1866-1869). "Saggio sui molluschi eolididei del Golfo di Napoli. Annuario del Museo Zoologico della Reale Università di Napoli, (1863) 3: 59-90, pl. 1-3 [1866]; (1864) 4: 26-37, pl. 1-2 [1867]; (1865) 5: 46-53, pl. 2 [1869], available online at http://biodiversitylibrary.org/page/11331073."
17. Cruz, S., et al. (2015). "Photoprotection in sequestered plastids of sea slugs and respective algal sources." Sci Rep **5**: 7904.
18. Curtis, N. E., et al. (2005). "The intracellular, functional chloroplasts in adult sea slugs (Elysia crispata) come from several algal species, and are also different from those in juvenile slugs." Microsc Microanal **11(Suppl 2)**.

19. Curtis, N. E., et al. (2007). "Newly metamorphosed Elysia clarki juveniles feed on and sequester chloroplasts from algal species different from those utilized by adult slugs." Marine Biology **150**: 797–806.
20. Dytham, C. (2003). Choosing and Using Statistics - A Biologist's Guide. Second Edition., Blackwell Science.
21. Evertsen, J., et al. (2007). "Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean." Marine Biology **151**: 2159-2166.
22. Evertsen, J. and G. Johnsen (2009). "In vivo and in vitro differences in chloroplast functionality in the two north Atlantic sacoglossans (Gastropoda, Opisthobranchia) Placida dendritica and Elysia viridis." Marine Biology **156**: 847-859.
23. Fraenkel, G. (1927). "Über Photomenotaxis bei Elysia viridis Mont.." Zeitschrift für vergleichende Physiologie **6**: 385-401.
24. Giménez Casalduero, F. and C. Muniain (2008). "The role of kleptoplasts in the survival rates of Elysia timida (Risso, 1818): (Sacoglossa: Opisthobranchia) during periods of food shortage." Journal of Experimental Marine Biology and Ecology **357**: 181-187.
25. Giménez-Casalduero, F., et al. (2011). "Elysia timida (Risso, 1818) three decades of research." Anim Biodivers Conserv **34**(1): 217–227.
26. Gould, A. A. (1870). Report on the Invertebrata of Massachusetts. Second Edition, comprising the Mollusca. Boston: Wright and Potter.
27. Händeler, K., et al. (2009). "Functional chloroplasts in metazoan cells - a unique evolutionary strategy in animal life." Front Zool **6**: 28.
28. Hariot, P. (1889). "Mission Scientifique du Cap Horn. 1882–1883." Botanique I. Algues. Paris: Gauthier-Villars et fils.
29. Hasselt, J. C. v. (1824). "In: Férussac A: Extrait d'une lettre du Dr. J.C. van Hasselt au Prof. van Swinderen sur mollusques de Java (traduit de l'Allgem. konst en letterbode, 1824, nos. 2,3,4) Tjuringe (ile Java) le 25 Mai 1823 (1)." Bull Sci Nat Géol **3**: 237–248.
30. Heinz Walz GmbH (1998). Underwater Fluorometer Diving-PAM - Submersible Photosynthesis Yield Analyzer Handbook of Operation.
31. Hofrichter, R. (2003). Das Mittelmeer - Fauna, Flora, Ökologie. Band II/1. Heidelberg, Berlin., Spektrum Akademischer Verlag.
32. Jesus, B., et al. (2010). "Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanisms in the solar-powered sea slug Elysia timida (Risso 1818)." Journal of Experimental Marine Biology and Ecology **395**: 98-105.
33. Krug, P. J., et al. (2013). "Integrative species delimitation in photosynthetic sea slugs reveals twenty candidate species in three nominal taxa studied for drug discovery, plastid symbiosis or biological control." Mol Phylogenet Evol **69**(3): 1101-1119.
34. Krug, P. J., et al. (2016). "Molecular and morphological systematics of Elysia Risso, 1818 (Heterobranchia: Sacoglossa) from the Caribbean region." Zootaxa **4148**(1): 1-137.
35. Kützing, F. T. (1843). "Phycologia Generalis Oder Anatomie, Physiologie und Systemkunde der Tange. Mit 80 Farbig Gedruckten Tafeln, Gezeichnet und Graviert vom Verfasser. pp. [Part 1]: [i]-Xxxii, [1]-142, [Part 2]: 143–458, 1, err.], pls 1–80. Leipzig: F.A. Brockhaus."
36. Laetz, E. M. J., et al. (2017). "Photosynthate accumulation in solar- powered sea slugs - starving slugs survive due to accumulated starch reserves." Frontiers in Zoology **14**(4): 1-9.
37. Laetz, E. M. J., et al. (2016). "Examining the retention of functional kleptoplasts and digestive activity in sacoglossan sea slugs. ." Org Divers Evol **17**: 87–99.

38. Laetz, E. M. J. and H. Wägele (2017). "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." PLoS One **12**(10): e0182910.
39. Laetz, E. M. J. and H. Wägele (2018a). "Comparing amylose production in two solar-powered sea slugs: the sister taxa Elysia timida and E. cornigera (Heterobranchia: Sacoglossa)." Journal of Molluscan Studies: 1-6.
40. Laetz, E. M. J. and H. Wägele (2018b). "How does temperature affect functional kleptoplasty? Comparing populations of the solar-powered sister-species Elysia timida Risso, 1818 and Elysia cornigera Nuttall, 1989 (Gastropoda: Sacoglossa)." Front Zool **15**: 17.
41. Maeda, T., et al. (2012). "Algivore or phototroph? Plakobranchus ocellatus (Gastropoda) continuously acquires kleptoplasts and nutrition from multiple algal species in nature." PLoS One **7**(7): e42024.
42. Marcus, E. and E. Marcus (1967). "American opisthobranch mollusks Part I, Tropical American Opisthobranchs, Part II, Opisthobranchs from the Gulf of California." Studies in Tropical Oceanography **6**: 1–256, pl251. page(s):229-231.
43. Margulis, L. (1998). Symbiotic Planet - A New Look At Evolution. Sciencewriters, Amherst, Massachusetts, Basic Books.
44. Marin, A. and J. Ros (1989). "The chloroplast-animal association in four Iberian sacoglossan opisthobranchs: Elysia timida, Elysia translucens, Thuridilla hopei and Bosellia mimetica." Scientia Marina **53**: 429-440.
45. Marin, A. and J. Ros (2004). "Chemical defenses in Sacoglossan Opisthobranchs: Taxonomic trends and evolutive implications." Scienta Marina **68**: 227-241.
46. Marín, A. and J. Ros (1992). "Dynamics of a plant-herbivore relationship: the photosynthetic ascoglossan Elysia timida and the chlorophycean Acetabularia acetabulum." Marine Biology **112**: 667–682.
47. Marín, A. and J. Ros (1993). "Ultrastructural and ecological aspects of the development of chloroplast retention in the sacoglossan gastropod Elysia timida." J Mollus Stud **59**: 95–104.
48. Martin, R., et al. (2015). "Variable retention of kleptoplast membranes in cells of sacoglossan sea slugs: plastids with extended, shortened and non-retained durations." Zoomorphology **134**(4): 523-529.
49. Martin, W., et al. (2012). "Gene transfers from organelles to the nucleus: How much, what happens, and why none in Elysia?" Journal of Endocytobiosis and Cell Research **23**: 16-20.
50. Melo Clavijo, J., et al. (2018). "Polymorphic adaptations in metazoans to establish and maintain photosymbioses." Biol Rev Camb Philos Soc.
51. MolluscaBase (2019a). "MolluscaBase. Bosellia mimetica Trinchese, 1891. Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=138851 on 2019-06-29."
52. MolluscaBase (2019b). "MolluscaBase. Elysia clarki Pierce, Curtis, Massey, Bass, Karl & Finney, 2006. Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=390340 on 2019-06-29."
53. MolluscaBase (2019c). "MolluscaBase. Elysia crispata Mörch, 1863. Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=420572 on 2019-06-29."
54. MolluscaBase (2019d). "MolluscaBase. Elysia tuca Ev. Marcus & Er. Marcus, 1967. Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=420575 on 2019-06-29."

55. MolluscaBase (2019e). "MolluscaBase. Elysia viridis (Montagu, 1804). Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=139686 on 2019-06-29."
56. MolluscaBase (2019f). "MolluscaBase. Ercolania viridis (A. Costa, 1866). Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=141563 on 2019-06-29."
57. MolluscaBase (2019g). "MolluscaBase. Thuridilla hopei (Vérany, 1853). Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=139687 on 2019-06-29."
58. Monselise, E. B. I. and M. Rahat (1980). "Photobiology of Elysia timida (Mollusca: Opisthobranchia): observations in the sea." Isr J Zool **29**: 125–128.
59. Montagu, G. (1804). "Description of several marine animals found on the south coast of Devonshire. Transactions of the Linnean Society, London. 7: 61-85, pl. 6-7., available online at https://doi.org/10.1111/j.1096-3642.1804.tb00282.x ".
60. Pelletreau, K. N., et al. (2011). "Sea slug kleptoplasty and plastid maintenance in a metazoan." Plant Physiol **155**(4): 1561-1565.
61. Pelletreau, K. N., et al. (2014). "Lipid accumulation during the establishment of kleptoplasty in Elysia chlorotica." PLoS One **9**(5): e97477.
62. Pelletreau, K. N., et al. (2012). "Laboratory culturing of Elysia chlorotica reveals a shift from transient to permanent kleptoplasty." Symbiosis **58**(1-3): 221-232.
63. Pierce, S. K., et al. (2006). "A morphological and molecular comparison between Elysia crispata and a new species of kleptoplastic sacoglossan sea slug (Gastropoda: Opisthobranchia) from the Florida Keys, USA. ." Molluscan Research **26**(1): 23-38.
64. Rahat, M. and E. B. I. Monselise (1979). "Photobiology of the chloroplast-hosting mollusc Elysia timida (Opisthobranchia)." J Exp Biol **79**: 225–233.
65. Rauch, C., et al. (2018). "The ability to incorporate functional plastids by the sea slug Elysia viridis is governed by its food source." Marine Biology **165**(82).
66. Risso, A. (1818). "Mémoire sur quelques gastéropodes nouveaux, nudibranches et tectibranches observés dans la Mer de Nice." J Phys Chim Hist Nat Arts **87**: 368–377.
67. Rumpho, M. E., et al. (2011). "The making of a photosynthetic animal." J Exp Biol **214**(Pt 2): 303-311.
68. Sagan, C. (1980). Cosmos. New York, Toronto, A Ballantine Book Published by The Random House Publishing Group.
69. Schmitt, V., et al. (2007). "Mating behaviour in the sea slug Elysia timida (Opisthobranchia, Sacoglossa): hypodermic injection, sperm transfer and balanced reciprocity." Front Zool **4**: 17.
70. Schmitt, V., et al. (2014). "Chloroplast incorporation and long-term photosynthetic performance through the life cycle in laboratory cultures of Elysia timida (Sacoglossa, Heterobranchia)." Front Zool **11**(1): 5.
71. Schmitt, V. and H. Wägele (2011). "Behavioral adaptation in relation to long-term retention of endosymbiotic chloroplasts in the sea slug Elysia timida (Opisthobranchia, Sacoglossa)." Thalassas **27**(2): 226–238.
72. Silva, P. C. (1952). "A review of nomenclatural conservation in the algae from the point of view of the type method." Univ Calif Publ Botany **25**: 241–323.
73. Solms-Laubach, H. (1895). "Monograph of the Acetabularieae." Transactions of the Linnean Society of London, Second Series, Botany **5**: 1–39. pls 31–34.

74. Stirts, H. M. and K. B. Clark (1980). "Effects of temperature on products of symbiotic chloroplasts in Elysia tuca Marcus (opisthobranchia: ascoglossa)." J Exp Mar Bio Ecol **43**: 39–47.
75. Trinchese, S. (1890). "Descrizione del nuovo genere Bosellia." Memorie della Regia Accademia delle Scienze dell'Istituto di Bologna **5**: 773-778.
76. Ventura, P., et al. (2013). "Photosynthetic efficiency and kleptoplast pigment diversity in the sea slug Thuridilla hopei (Vérany, 1853)." Journal of Experimental Marine Biology and Ecology **441**: 105-109.
77. Vérany, G. B. (1853). "Catalogue des Mollusques Céphalopodes, Ptéropodes, Gastropodes, Nudibranches etc. des environs de Nice." Journal de Conchyliologie **4**: 375-392.
78. Vieira, S., et al. (2009). "Effects of light exposure on the retention of kleptoplastic photosynthetic activity in the sacoglossan mollusc Elysia viridis." Marine Biology **156**: 1007-1020.
79. Wade, R. M. and A. R. Sherwood (2017). "Molecular determination of kleptoplast origins from the sea slug Plakobranchus ocellatus (Sacoglossa, Gastropoda) reveals cryptic bryopsidalean (Chlorophyta) diversity in the Hawaiian Islands." J Phycol **53**(3): 467-475.
80. Wade, R. M. and A. R. Sherwood (2018). "Updating Plakobranchus cf. ianthobapsus (Gastropoda, Sacoglossa) host use: Diverse algal-animal interactions revealed by NGS with implications for invasive species management." Mol Phylogenet Evol. **128**: 172-181.
81. Wägele, H., et al. (2011). "Transcriptomic evidence that longevity of acquired plastids in the photosynthetic slugs Elysia timida and Plakobranchus ocellatus does not entail lateral transfer of algal nuclear genes." Mol Biol Evol **28**(1): 699-706.
82. Wägele, H. and G. Johnsen (2001). "Observations on the histology and photosynthetic performance of “solar-powered” opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae." Org Divers Evol **1**: 193–210.
83. Wägele, H. and R. Martin (2013). Endosymbioses in sacoglossan seaslugs: plastid-bearing animals that keep photosynthetic organelles without borrowing genes. Endosymbiosis. W. Löffelhardt. Vienna, Springer Vienna**:** 291–324.
84. Weaver, S. and K. B. Clark (1981). "Light intensity and color preferences in five ascoglossan (=sacoglossan) molluscs (Gastropoda, Opisthobranchia): A comparison of chloroplast symbiotic and aposymbiotic species." Marine and Freshwater Behavior and Physiology **7**: 297-306.
85. Yamamoto, S., et al. (2013). "Effects of photosynthesis on the survival and weight retention of two kleptoplastic sacoglossan opisthobranchs." Journal of the Marine Biological Association of the United Kingdom **93**(1): 209-215.