# Molecular mechanisms underpinning photosymbiosis onset and maintenance in marine slugs

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## Summary

Photosymbiosis is the association between heterotrophs and autotrophs and is the main driver for ecological success in oligotrophic aquatic ecosystems. Ciliates, Radiolaria, foraminifers, sponges, cnidarians, acoelomorpha and rhabdocoelan worms, mollusks, ascidians, and some amphibians are examples of heterotroph hosts. They harbor mainly intracellular photobionts like cyanobacteria, dinoflagellates, unicellular green algae, and in special cases, even "stolen" chloroplasts. In mollusks like sea slugs three photosymbiotic strategies occur: 1) cladobranchs from the superfamilies Fionoidea, Arminoidea, and Aeolidioidea associate with Symbiodiniaceae obtained from their photosymbiotic cnidarian prey, 2) cladobranch species belonging to Dendronotoidea that acquire their photobiont directly from their environment, and 3) sacoglossan sea slugs that feed on macroalgae and sequester the photosynthetically active chloroplasts, known as kleptoplasts.

The study of photosymbiosis breakdown has increased in the past twenty years given the imminent threat of climate change to the survival of marine ecosystems, in particular coral reefs. To understand how and why the photosymbiosis is disrupted, it is mandatory to study how it begins and how it is maintained. Many studies have addressed these questions at the molecular and cellular level using the Cnidaria-Symbiodiniaceae model, given its ecological role as main reef-builders. In this line, the host innate immune system came into focus, given its important role in distinguishing phototrophic partners from harmful pathogens. However, the mechanisms behind the onset and maintenance of the photosymbiosis in non-calcifying animals like sea slugs remain largely unknown. The aim of the present study was to investigate the onset and maintenance of the photosymbiosis in marine slugs.

First, knowledge of photosymbiosis in cladobranchs and unanswered questions about this association were compiled into a review, which proposed cladobranchia as a model for studying photosymbiosis evolution. Second, the host innate immune receptors involved in the process of photobiont recognition were identified in the sacoglossans *Elysia cornigera, Elysia timida,* and *Elysia chlorotica,* and the cladobranch *Berghia stephanieae*. Based on the gene expression profile of these species, scavenger receptors (SRs) from the class B and E, C-type lectins, and thrombospondin-type-1 repeat proteins (TSRs) were identified as potential candidates for plastid and Symbiodiniaceae recognition. Particularly for the latter, fibrinogen-like proteins are promising candidates in cladobranchs. Further, the diverse SRs and TSRs repertoire in sacoglossan sea slugs and cladobranchs is similar to the one present in other photosymbiotic animals like the cnidarians.

The maintenance of the photosymbiosis was also a focus in this study. The downstream signaling cascades and immune pathways that are activated or suppressed to promote the host tolerance to the symbiont, and thus its persistence within the host, were analyzed in *B. stephanieae* and *E. chlorotica*. Based on the gene expression, the transforming growth factor β (TGF- β) pathway is not involved in the immunotolerance to the photobiont in sea slugs, contrary to what is known from cnidarians. As other mollusks, *B. stephanieae* and *E. chlorotica* lack key elements for the activation of the pathway such as the TGF- β sensu stricto ligand. Moreover, the digestion and symbiont maintenance occur simultaneously in *B. stephanieae* during feeding on symbiotic anemones. A transcriptional signal for symbiont expulsion via vomocytosis is also activated in this slug. In contrast, the general immune response seems to be suppressed in *E. chlorotica*, and no vomocytosis activation was detected while feeding, likely contributing to kleptoplast maintenance.
The antioxidant defense against toxic by-products like reactive oxygen species (ROS) was activated since early stages in *E. chlorotica* and in adult *B. stephanieae* in the presence of the photobiont. The antioxidant activity was further analyzed in *B. stephanieae* during feeding and starvation under different light intensities. Starvation alone triggers autophagy and may cause symptoms of oxidative stress regardless of the light condition. This effect is heightened in light deprived conditions and was also evidenced in *E. viridis*. Starvation in darkness can generate an energy metabolic change and an increase of lysosomal abundance in the digestive gland of the slug. Kleptoplasts nutritional support is not enough to prevent length loss during starvation and they function more as food storage that can be accesed via autophagy.

New genomic tools like mitochondrial genomes and transcriptomes are also presented here. The mitogenome of *B. stephanieae* and *E. viridis* were sequenced using a combination of Illumina short-reads and Oxford Nanopore long-reads. The mitogenomes are similar to other heterobranch species containing 13 protein-encoding genes, 21 tRNAs and 2 rRNAs. Particular to *B. stephanieae* is the presence of a duplicated tRNA- Ser 1, whereas in *E. viridis* a tRNA-S2 gene is missing.

The results presented here are pioneer work in the photosymbiosis in marine slugs and contribute to understanding how the photosymbiosis begins and how it is maintained. The process of photobiont recognition in marine slugs shares similarities to the cnidarian system, which is expected considering how conserved and ancient the innate immune system in metazoans is. Comparative studies with non-photosymbiotic congeners and stable symbiotic species, as well as from the photobiont perspective and the other members of the microbiome, are needed to fully understand the photosymbiosis in sea slugs.

# Chapter 1

## General Introduction

*Photosynthetic symbiosis*

Symbiosis is the relationship between organisms that live together (De Bary, 1879) found in various unicellular and multicellular species. It ranges from parasitic to mutualistic relationships and is an imperative driver of evolution (Sapp et al., 1994). Some heterotrophic and phototrophic organisms form a particular type of symbiosis known as photosymbiosis or photosynthetic symbiosis (Cowen, 1988) (Figure 1). This mixotrophy includes endosymbiosis and organelle retention that can be facultative or obligate interactions (Johnson, 2011). Often, the phototrophic partner (photobiont) resides intracellularly in the heterotrophic partner (host), but there are also extracellular photosymbioses. The heterotroph provides the compounds needed for photosynthesis to the photobionts, such as ammonium and carbon dioxide. In turn, the phototroph translocates photosynthetic products, mainly glucose, to the host (Buchner, 1921; Yonge, 1934; Melo Clavijo et al., 2018) (Figure 1). This nutrient exchange is essential for long-term photosymbioses and forms the basis for the stability of many aquatic food webs (Chapman, 2013).

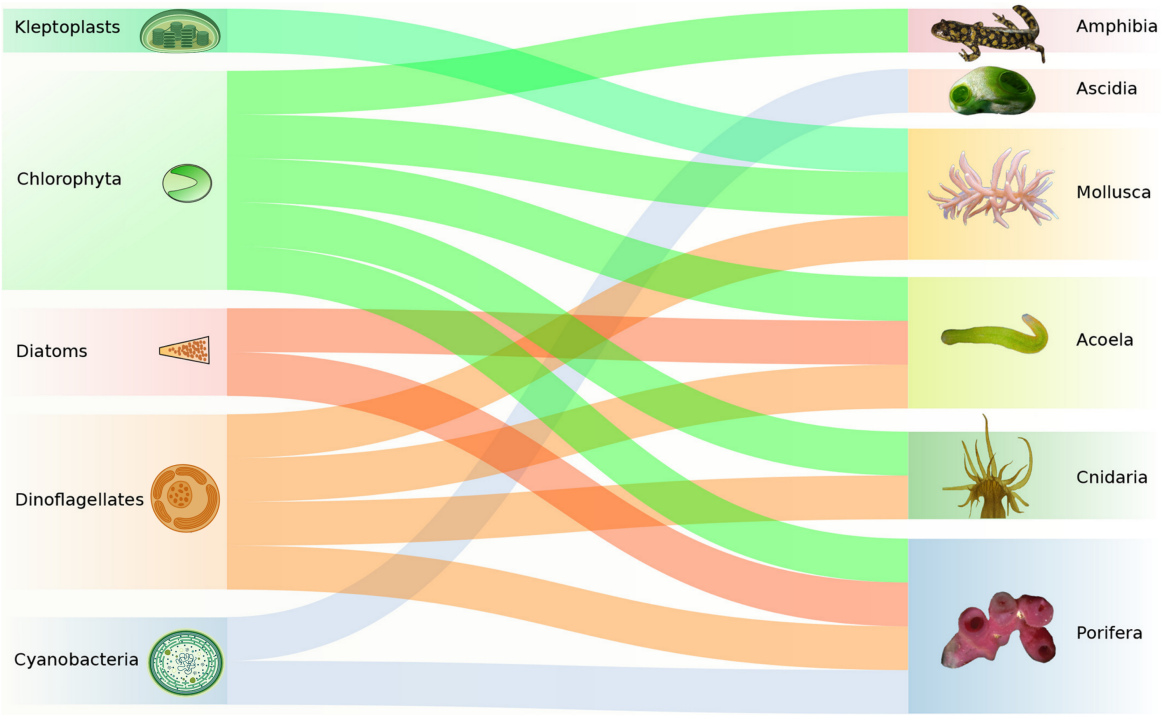


**Figure 1.** Photosymbiosis in Metazoa (modified from Melo Clavijo et al., (2018)). Photosynthetic symbionts on the left side, animal hosts on the right side. Lines connect symbionts with hosts. Modified with BioRender.com

However, the metabolic integration between the partners and the degree of dependency varies (Johnson, 2011), and so does the stability of the symbiosis. The contribution from the photobiont to the host's nutrition ranges from 70 % (Muscatine et al., 1981; Trench et al., 1981; Hawkins & Klumpp, 1995; Tanaka et al., 2006; Tremblay et al., 2012) to up to 170 % in some cases (Muscatine et al., 1984; Thomas et al., 2023). If the photobionts cannot fully support the hosts, the animals forage to obtain nitrogen, phosphorus, and other essential compounds (Davies, 1984; Muscatine et al., 1984). Thus, photosymbiosis provides a considerable advantage to animals, especially in low-nutrient environments (Johnson, 2011; Stanley & Lipps, 2011; Grube et al., 2017). The nutritional support enables a higher and faster growth rate of the heterotrophs (Fitt et al., 1986; Starzak et al., 2014; Gabay et al., 2018) and, in some cases, allows higher calcification rates that form the basis of the oldest and most productive marine ecosystem, the coral reefs (Stanley & van de Schootbrugge, 2009; Stanley & Lipps, 2011; Drake et al., 2020).

The ability to associate with a phototroph partner and establish a photosymbiosis is present in a few Metazoa phyla (Melo Clavijo et al., 2018). It involves animal hosts such as sponges, cnidarians, acoelomorph and rhabdocoelan worms, mollusks, ascidians, and even some amphibians, and also different photobionts like cyanobacteria, dinoflagellates, diatoms, unicellular green algae, and in a special case, stolen chloroplasts (Venn et al., 2008; Melo Clavijo et al., 2018; Maruyama & Kim, 2020). Most animal hosts are marine and inhabit coral reef ecosystems; just a few cases are found in freshwater habitats (Melo Clavijo et al., 2018). Common to almost all animal hosts are some adaptations that allow them to harbor photobionts and promote their photosynthetic activity by increasing the light uptake (Venn et al., 2008). For instance, large surface areas are provided by the corresponding volume ratio in the body plan. Also, morphological adaptations such as an extended mantle and highly branched digestive systems in some mollusks facilitate exposure of photobionts to light (Norton et al., 1992; Farmer et al., 2001; Burghardt et al., 2008a,b; Hernawan, 2008; Moore & Gosliner, 2011). Tentacle specialization occurs in some anemones, where one type harbors only symbionts and is extended during the day, while the other type is long and filled with nematocysts, well adapted to feeding and defense (Day, 1994; Venn et al., 2008). With increased and prolonged UV-light exposure, the risk of thermal and light stress increases for both the host and the photobiont. To protect itself and the photobiont's photosystem against potential light and/or thermal stress, the animal host has developed a range of protection mechanisms. For example, as a photoprotective mechanism, some animal hosts produce fluorescent proteins and accumulate mycosporine-like amino acids (MAAs) that act as natural sun blockers (Banaszak et al., 2000; Shick

& Dunlap, 2002; Roth, 2014). Some hosts also modify their behavior according to the day-night cycle by retracting and extending the tissues harboring the photobionts (Venn et al., 2008; Sorek et al., 2014). By doing so, the hosts relocate the photobionts to shallow or deeper parts of the tissue (Fang et al., 2016). The animal host also migrates vertically and horizontally in the water column to access light during the day or more nutrients at night (Djeghri et al., 2019).

The adaptations to a photosymbiotic lifestyle are not only present in the heterotroph partner, but also in the photobiont. The most common photobiont in various animal hosts belongs to the family Symbiodiniaceae, Dinoflagellata (Taylor, 1974; Stat et al., 2006; LaJeunesse et al., 2018). Members of this family can be host-specific like clade I, which is only found in foraminiferans, or associate with hosts that harbor multiple genera/species of Symbiodiniaceae, or have an exclusive free-living lifestyle like *Effrenium voratum* (H.J.Jeong, S.Y.Lee, N.S.Kang & LaJeunesse) LaJeunesse & H.J.Jeong 2018 (LaJeunesse et al., 2018; Maruyama & Kim, 2020). Within Symbiodiniaceae, several distinct genotypes with different morphological, physiological, and ecological traits exist. For instance, genera in Symbiodiniaceae differ in cell size (LaJeunesse, 2001), inorganic carbon acquisition (Brading et al., 2013), photosynthetic carbon fixation (Leal et al., 2015), growth rates, chlorophyll level (Hennige et al., 2009), MAAs synthesis (Banaszak et al., 2000), enzymatic antioxidant repertoire (Krueger et al., 2015), to name a few. These distinct attributes can impact how Symbiodiniaceae and its host -the holobiont- respond to environmental changes (Tchernov et al., 2004; Goulet et al., 2005, 2008, 2019). To establish a photosymbiosis, the free-living Symbiodiniaceae have to transform from a motile stage (with flagella) to a non-motile stage (a coccoid form) (Koike et al., 2004; Mohamed et al., 2020). While in the coccoid stage, the cell wall structure is thinner (Palincsar et al., 1988; Wakefield et al., 2000; Pasaribu et al., 2015). In symbiosis, the cell cycle of Symbiodiniaceae also changes. The G1 phase, where the cell grows and prepares for mitosis, is extended dramatically (Stambler, 2011). The progression from G1 phase to S is constrained when Symbiodiniaceae is *in hospite* (Smith & Muscatine, 1999). At the molecular level, symbiotic Symbiodiniaceae exhibit an expansion of bicarbonate, glucose, and ammonium transporters compared to other dinoflagellates (Aranda et al., 2016). Symbiotic species also show massive genome divergence (more transposable elements, genetic duplication, structural rearrangements, and pseudogenisation) compared to free-living ones within Symbiodiniaceae (González-Pech et al., 2021).

The adaptations mentioned above contribute to the successful association between heterotrophs and autotrophs, the holobiont. At a deeper level, molecular adaptations enable heterotroph hosts to recognize specific endosymbionts and maintain them in a vacuole free from digestion (Johnson, 2011). These adaptations of the holobiont are still being uncovered and explored with the help of new genomic, transcriptomic, proteomic, and metabolomic tools (e.g., Gordon & Leggat, 2010; Shinzato et al., 2011; Meyer & Weis, 2012; Barshis et al., 2013; Pernice & Levy, 2014; Baumgarten et al., 2015; Aranda et al., 2016; Shoguchi et al., 2018; Maor-Landaw et al., 2020; González-Pech et al., 2021). A number of studies have also explored the molecular and cellular events involved in the photosymbiosis (e.g., Rosset et al., 2021; Tortorelli et al., 2022), the adaptations required by both partners to establish the symbiosis (Allemand & Furla, 2018; González-Pech et al., 2021), and the effects of stress on the symbiosis (e.g., Weis, 2008; Baird et al., 2009; Cziesielski et al., 2019; Maire et al., 2022; van Woesik et al., 2022).

*Innate immunity and photosymbiosis*

As with other symbiotic relationships, addressing fundamental questions such as how the photosymbiosis is initiated and how it is maintained requires an examination of the immune system and its role in the onset of the photosynthetic association (Kitano & Oda, 2006; McFall-Ngai et al., 2010; Chu & Mazmanian, 2013; Cao et al., 2017). In all organisms, the immune system is the first line of defense against microbial invaders. In addition to detecting and eliminating potential pathogens, this system also recognizes and manages potential symbionts (Parkin & Cohen, 2001). Two immune systems are present in animals: the innate immune system and the adaptive immune system (Pancer & Cooper, 2006; Boehm & Swann, 2014; Buchmann, 2014). Generally, the innate immune system acts immediately after any microbe invasion and distinguishes between own cells and pathogens (Janeway & Medzhitov, 2002). In a more precise way, the adaptive immune system recognizes specific microbial invasive proteins (antigens) by specific host proteins (antibodies) (Pancer & Cooper, 2006; Boehm & Swann, 2014). Additionally, the adaptive immune system provides immunological memory of infection, which enables it to only respond when a particular pathogen is present (Janeway & Medzhitov, 2002).

In invertebrates only the innate immune system is present, while in vertebrates both the innate and the adaptive immune system are working together (Pancer & Cooper, 2006; Boehm & Swann, 2014; Buchmann, 2014). The fact that invertebrates only rely on their innate immunity does not

imply that it is less complex. For instance, innate immunity memory has evolved in animal lineages lacking an adaptive immune response as a result of functional epigenetic reprogramming. This promotes the rise, storage, and recall of new immunological capacities in innate immunity cells (Gourbal et al., 2018). Furthermore, invertebrates exhibit many gene expansions of different immunological components that do not occur in vertebrates (Adema, 2015; Zhang et al., 2015; Neubauer et al., 2016; Kamm et al., 2019; Emery et al., 2021). These components are highly diverse and contribute to the invertebrate innate immune response to a huge variety of microbe invaders.

Microbe detection or recognition is the first action when a microbe invader is present (Janeway & Medzhitov, 2002; Nyholm & McFall-Ngai, 2004; Davy et al., 2012; Nyholm & Graf, 2012). During the initial contact between the host and the microbe invader, a molecular signaling process initiates the recognition process. In both the animal host and the microbe, cellular receptors (secreted or transmembrane) participate in this process (Nyholm & McFall-Ngai, 2004; Davy et al., 2012; Nyholm & Graf, 2012). In the host these receptors are called pattern recognition receptors (PRRs), while in the microbe they are known as microbe associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs) (Nyholm & Graf, 2012) (Figure 2).

The interaction between PRRs-MAMPs/PAMPs triggers different signaling cascades that result in the elimination of the pathogen or the uptake of the potential symbiont (Davy et al., 2012). In the context of photosymbiosis, PRRs like scavenger receptors (SRs) and C-type lectins have been proposed as key players in the onset of this association (Jimbo et al., 2000, 2005; Koike et al., 2004; Rodriguez-Lanetty et al., 2006; Wood-Charlson et al., 2006; Kvennefors et al., 2008, 2010; Lehnert et al., 2014; Neubauer et al., 2016).

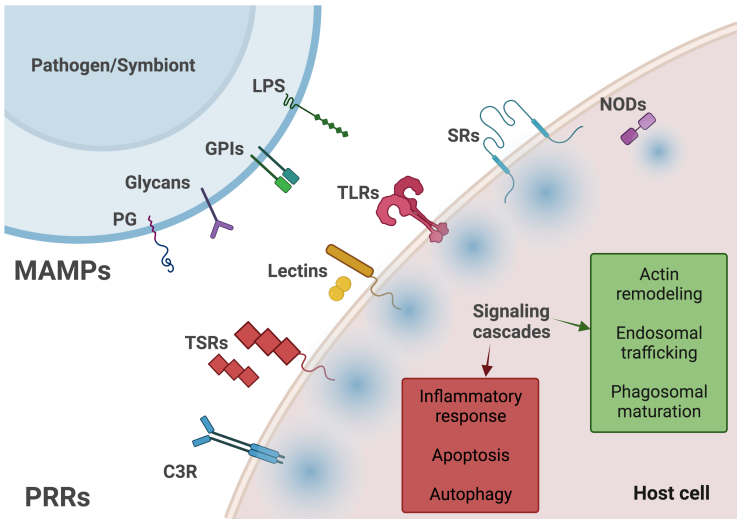


**Figure 2.** MAMPs and PRRs in photosymbiosis. C3R, TSRs, Lectins, TLRs, SRs, and NODs have been proposed as PRRs involved in the symbiont recognition in photosymbioses. PG, glycans, GPIs, and LPS are some of the MAMPs that can be present on the cellular surface of microbes. MAMPs-PRRs interaction can trigger signaling cascades that promote the elimination of the pathogen (red) or the uptake of the symbiont (green). Model based on Davy et al., (2012) and created with BioRender.com.

In the same way, other elements of the innate immunity like the complement system (Poole et al., 2016) and the thrombospondin-type-1 repeat (TSRs) domain-containing

proteins (Neubauer et al., 2017) have been hypothesized to act indirectly in the recognition process by binding to PRRs or other secondary proteins to amplify the host response (reviewed in Davy et al., 2012; Fransolet et al., 2012; Mansfield & Gilmore, 2019). Toll-like receptors (TLRs) and NOD-like receptors (NDRs) have been identified in photosymbiotic animals (e.g. Emery et al., 2021) and their role in the symbiont recognition has been suggested, based on their involvement in pathogen recognition and self/altered-self/non-self-recognition (Mohamed et al., 2020). Nevertheless, functional studies are still required to establish their role in the symbiosis establishment.

Once the potential symbiont is recognized and engulfed, several host immune pathways are set off that will promote the symbiont permanence within the host cell. There is a general immunosuppression in the host, where the complement system (Poole et al., 2016), the transforming growth factor β (TGF-β) (Detournay et al., 2012; Berthelier et al., 2017; Fuess et al., 2020), and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) immune signaling pathways (DeSalvo et al., 2010; Wolfowicz et al., 2016; Mansfield et al., 2017) play key roles in the symbiont maintenance (reviewed in Mansfield & Gilmore, 2019). The general down-regulation of the host immune response prevents the elimination of the symbiont either via apoptosis, selected autophagy (Davy et al., 2012), expulsion (vomocytosis) (Jacobovitz et al., 2021), and/or digestion (symbiophagy) by inhibiting the phagosome maturation (Hill & Hill, 2012; Mohamed et al., 2016, 2020).

If the phagosome does not mature and the symbiont avoids elimination, a functional symbiosome will be established in intracellular symbioses. This allows the communication between the host and the symbiont, including nutrient exchange (Davy et al., 2012; Hill & Hill, 2012; Rosset et al., 2021). However, in some photosymbioses, such as the one between clams from the family Cardiidae and Symbiodiniaceae, the symbiont is located extracellularly in the digestive tubular system (Norton et al., 1992; Farmer et al., 2001; Hernawan, 2008). In the acoel *Symsagittifera roscoffensis* (Graff, 1891) the symbionts (the green algae *Tetraselmis convolutae* (Parke & Manton) R.E.Norris, Hori & Chihara, 1980) even reside in the extracellular spaces of the parenchyma (Bailly et al., 2014). In both examples no proper individual symbiosome is established, but the metabolic exchange between partners still occurs resulting in a stable symbiosis (Yellowlees et al., 2008; Leggat et al., 2002; Bailly et al., 2014). Thus, the membrane surrounding the extracellular spaces where the symbionts are located might act as a symbiosome-like environment involved in signaling, acidification, and molecule trafficking (Bailly et al., 2014).

In photosymbioses the photosynthetic activity of the symbionts is the main feature. The symbionts need particularly carbon dioxide to perform this light-dependent reaction. The dissolved inorganic carbon can be obtained by three different pathways. i) The animal host takes up $CO_2$ from the surrounding seawater as bicarbonate, and transports it to the symbiont through the symbiosome via bicarbonate transporters. In the symbiosome, bicarbonate is converted into $CO_2$ by carbonic anhydrases, and is then available for photosynthesis. ii) The symbionts can take up carbon dioxide directly from the seawater. iii) The symbionts receive $CO_2$ as a result of the host metabolism (reviewed in Furla et al., 2000a,b; Leggat et al., 2002; Yellowlees et al., 2008; Davy et al., 2012). Through photosynthesis, the acquired carbon is fixed and several organic compounds like amino acids are synthesized by the symbiont. The photosynthetically fixed carbon and a portion of the other photosynthates are translocated to the host supporting respiration, growth, and reproduction (Muscatine et al., 1984; Davy et al., 2012; Tremblay et al., 2014). Waste products of the host catabolic processes like ammonium are assimilated and recycled by the symbiont, and relocated to the host as free amino acids or glycoconjugates (glycans or carbohydrates linked to a protein, peptide or lipid) (Sutton & Hoegh-Guldberg, 1990; Markell & Trench, 1993; Wang & Douglas, 1997; Markell & Wood-Charlson, 2010). Dissolved inorganic nitrate is taken up from the water column by the animal host, but only the photosynthetic symbiont can assimilate it, converting it to ammonium and subsequently transforming it to amino acids (Tanaka et al., 2006; Leggat et al., 2007; Yellowlees et al., 2008; Davy et al., 2012).

*Symbiosis breakdown*

Environmental changes like high temperatures, ocean acidification, high sedimentation, changes in salinity, increased nutrients or pollutants (heavy metals), and excessive light exposure are stress factors that can disrupt the symbiosis (reviewed in Weis, 2008; Lesser, 2011; Maor-Landaw & Levy, 2016; Cziesielski et al., 2019; van Woesik et al., 2022). As a result of drastic changes in abiotic factors, photosynthesis may be disrupted, chloroplasts may be damaged, and the photobiont may produce excessive amounts of reactive oxygen species (ROS) such as $H_2O_2$ and singlet oxygen (Lesser, 1997, 2006; Weis, 2008). The excessive production of ROS exceeds the ability of the antioxidant system of the photobiont to quench ROS (Lesser, 1997, 2006; Weis, 2008). Consequently, ROS accumulate in the photobiont and also diffuse into the host cells. Yet, it is unclear how ROS from the symbionts leak into the animal cells while *in hospite*. In addition, elevated temperature and light can also directly damage the host mitochondria leading to increased

ROS levels (Nii & Muscatine, 1997; Weis, 2008; Lesser, 2011). Generally, high levels of ROS can cause oxidative damage to cells via lipid peroxidation, protein oxidation, and DNA degradation, leading to a general metabolic dysfunction (Lesser, 2006). This then leads to a stress response in the animal host to counteract the ROS stress, inducing the disruption of the symbiosis, the so-called bleaching (Lesser, 1997, 2011; Weis, 2008). Yet, bleaching also occurs independent of symbiont-derived ROS (Tolleter et al., 2013; Rädecker et al., 2021; Dungan et al., 2022). Upsetting the balance of nutrient exchange between both partners can also jeopardize the stability of the photosymbiotic association.

Currently, most studies have used the model Cnidaria-Symbiodiniaceae to study photosymbiosis, because this association is crucial in the health and maintenance of coral reef ecosystems, especially in relation to climate change (reviewed in Weis, 2008; Lesser, 2011; Maor-Landaw & Levy, 2016; Cziesielski et al., 2019; van Woesik et al., 2022). There have been multiple bleaching events in reef ecosystems over the past few years, and some experts predict even more in the near future (van Woesik & Kratochwill, 2022). Questions about the photosymbiosis onset and maintenance, particularly at the molecular and cellular level, have become more relevant when trying to understand what happens when the symbiosis is disrupted. In addition to contributing to the development of strategies that will help coral reef ecosystems recover and maintain, this research also paves the way for the study of other photosymbiotic systems, their evolution, and their response to global warming. For instance, in sea slugs.

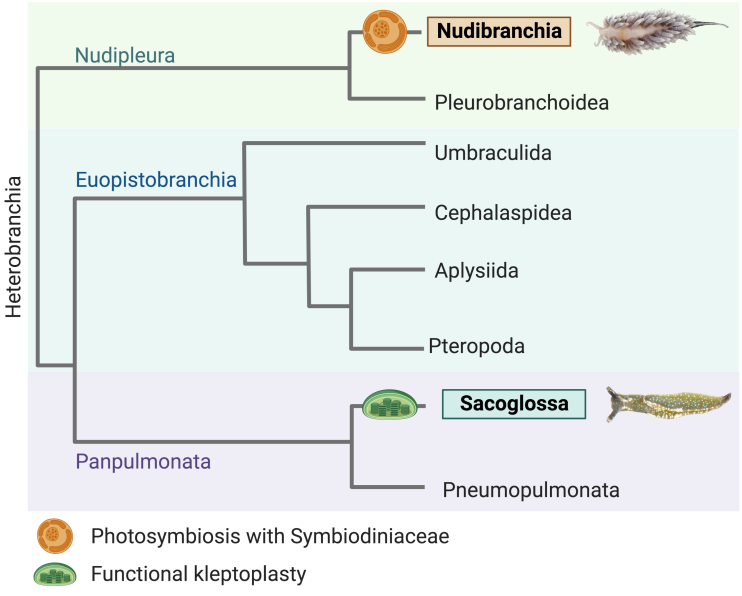


**Figure 3.** Photosymbiosis in Heterobranchia. Schematic dendogram showing the main taxa in Heterobranchia based on Zapata et al. (2014) and Krug et al. (2022). Dinoflagellate and chloroplast indicate the presence of photosynthetic associations within Nudibranchia and Sacoglossa, respectively. Created with BioRender.com.

*Photosymbiosis in marine slugs*

There are five types of photosymbiosis among mollusks: i) bivalves from the family Cardiidae associate with dinoflagellates from the family Symbiodiniaceae (Li et

al., 2020); ii) the caeonogastropod species *Aliger gigas* (Linnaeus, 1758) hosts Symbiodiniaceae and maintains an apparent mutualistic symbiosis in larval stages that becomes parasitic in adulthood (García Ramos & Banaszak, 2014); this association is debatable and needs more evidence; iii) cladobranch gastropods (Nudibranchia) from the family Dendronotoidea acquire Symbiodiniaceae from the water column (Burghardt & Wägele, 2014); iv) cladobranch gastropods from the families Arminoidea, Fionoidea, and Aeolidioidea obtain Symbiodiniaceae by feeding on photosymbiotic cnidarians (Rola et al., 2022); v) and a special case in gastropods belonging to the superorder Sacoglossa, where the symbiont is not an entire organism, but a sequestered organelle obtained from the algal food source- the so-called functional kleptoplasty (Wägele & Martin, 2014). A particular focus will be placed here on the photosymbiosis of marine slugs.

Photosymbiotic cladobranchs are usually carnivorous that prey on sea anemones (Actinaria), hydrozoans (Hydrozoa), soft corals (Alcyonacea), and stony corals (Scleractinia) (Rola et al., 2022). In contrast, Sacoglossa are herbivorous that feed on algae usually belonging to the Ulvophyceae or Xantophyceae (Wägele & Martin, 2014). In these sea slugs, unique and rare forms of photosymbioses occur (Figure 3). Most cladobranchs become a secondary host, but members of the genus *Melibe* Rang, 1829 are the primary host of Symbiodiniaceae (Rola et al., 2022). One of the most unusual forms is found in sacoglossan sea slugs that steal photosynthetically active chloroplasts (functional kleptoplasty) and integrate them intracellularly (Wägele et al., 2010). Whether functional kleptoplasty can be classified as a mutualistic symbiosis is still debatable. While Symbiodiniaceae contribute greatly to the nutritional requirements of their host and can divide *in hospite* (e.g. Muscatine et al., 1984; Stambler, 2011), the contribution of kleptoplasts is reduced, and kleptoplasts cannot divide in the slug's cytosol (de Vries et al., 2014a; Wägele & Martin, 2014; Rauch et al., 2017a).

Despite the differences between these two systems, for example, the nature of the endosymbiont (a whole organism vs. a sequestered organelle), some similarities can be observed when these distantly related taxa are compared. In both superorders, Cladobranchia and Sacoglossa, different levels of the photosynthetic association can be identified. Depending on the ability of the slug to retain the symbiont, the morphology and the branching pattern of the digestive gland system, six levels were proposed by Kempf (1991) for cladobranchs: (1) non-photosymbiotic species that instantly digest the dinoflagellates, or (2) that expel them in a healthy state without incorporation; (3) species that can incorporate the dinoflagellates but keep them up to 6 days and then defecate them undigested,

or (4) species that can keep the symbionts for short time and digest them; (5-6) species with a highly branched and specialized digestive system that can harbor large populations of healthy symbionts and can maintain them for months during starvation periods. This classification might be summarized in three different levels according to the stability of the symbiosis: non-symbiotic species (levels 1 and 2 from Kempf's classification), species with an unstable symbiosis (levels 3 and 4), and stable photosymbiotic species (levels 5-6) (Rola et al., 2022). Similarly, the classification proposed by Clark et al., (1990) for Sacoglossa included six levels of kleptoplast retention. Level one included species that cannot retain the kleptoplasts and immediately digest them. Level six grouped species with long-term retention function and kleptoplasts' photosynthetic activity longer than one week. Nowadays, only three levels of kleptoplast retention are used for classifying the sacoglossan slugs: non-retention form (NR), where the chloroplasts are incorporated but immediately digested; short-retention form (StR), where the kleptoplasts are kept intracellularly for a week; and long retention-form, where the retention extends over several months (Evertsen et al., 2007; Händeler et al., 2009).

The evolution of the photosynthetic partner acquisition (organism or organelle) in sea slugs seems to be convergent, but it is not yet entirely clear. In cladobranchs, it seems that the ability to establish a symbiotic relationship with Symbiodiniaceae evolved independently in Dendronotoidea, Arminoidea, Fionoidea, and Aeolidioidea. Yet, it is still unexplored whether unstable photosymbiosis is a precursor to stable photosymbiosis, or if it evolved separately (Rola et al., 2022). Similarly, functional kleptoplasty evolved multiple times in Sacoglossans: twice as short-term retention in *Costasiella* Pruvot-Fol, 1951 and Plakobranchoidea, and six times independently as long-term retention (Christa et al., 2015; Hirokane et al., 2022).

Most studies on cladobranch photosymbiosis have focused on the diversity and systematics of species harboring dinoflagellates (e.g. Moore & Gosliner, 2011; Carmona et al., 2013; Moore & Gosliner, 2014). The cladobranch symbionts were originally called zooxanthellae (e.g. Rousseau, 1934, 1935; Rudman, 1981a,b), but with advances in dinoflagellate systematics, the symbiont was re-named *Symbiodinium* Freudenthal, 1962 (e.g. Burghardt et al., 2005, 2008; Burghardt & Wägele, 2014). Phylogenetically distinct clades within *Symbiodinium* were later described (Pochon et al., 2006), and several studies have classified the cladobranch symbiont according to this classification (Loh et al., 2006; FitzPatrick et al., 2012; Wecker et al., 2015; Yorifuji et al., 2015; Ziegler et al., 2014). Only a few recent studies have identified the symbiont genotype present in the cladobranch

host (Monteiro et al., 2019; Melo Clavijo et al., 2022) based on the most recent classification of the Symbiodiniaceae (LaJeunesse et al., 2018). The photophysiological aspects of the symbiosis have been addressed by focusing on the photosynthetic activity of the symbiont *in hospite* (Wägele & Johnsen, 2001; Burghardt et al., 2005; Burghardt et al., 2008a,b; Burghardt & Wägele, 2004, 2014), particularly during starvation. Yet, many questions remain regarding how the photosymbiosis is initiated, the nature of this association, whether and how nutrients are exchanged between the partners, and the consequences of the breakdown of this association.

Earlier research on functional kleptoplasty in Sacoglossa focused on morphological factors such as the location of the kleptoplasts within the cytosol of the sacoglossan slug and whether host membranes surrounded the stolen organelles (reviewed in Wägele & Martin, 2014). Numerous studies have addressed the most intriguing question regarding functional kleptoplasty: how does the kleptoplast remain photosynthetically active inside an animal cell without the proteins and enzymes encoded by the nucleus? Different hypotheses have been proposed including a later dismissed horizontal gene transfer (Pierce et al., 1996, 2012; Rumpho et al., 2008; Wägele et al., 2011; de Vries et al., 2014b; Wägele & Martin, 2014; Rauch et al., 2015), dual targeting of core proteins (Rumpho et al., 2000, 2011), kleptoplast intrinsic properties such as longevity and robustness (Giles & Sarafis, 1972; Trench & Ohlhorst, 1976; Green et al., 2005; de Vries et al., 2013, 2014a; Serôdio et al., 2014; Cruz et al., 2015), and the compatibility between kleptoplasts with specific properties and slugs that can incorporate and maintain them (de Vries et al., 2014b, 2015; Wägele & Martin, 2014; Rauch et al., 2018). Still a big question is how well the kleptoplasts support the slug's nutritional requirements, and whether or not the slug exchanges nutrients with the kleptoplasts at all (de Vries et al., 2014a,b; Cartaxana et al., 2017; Laetz et al., 2017a; Rauch et al., 2017a; Cruz et al., 2020; Frankenbach et al., 2021). However, similar to cladobranchs, the cellular and molecular events behind the selective incorporation of kleptoplasts and their maintenance within the slug's cytosol remain unexplained.

**Aims and scope of the present dissertation**

Most of the knowledge we know about photosymbiosis comes from calcifying animals like the scleractinian corals-Symbiodiniaceae model. In contrast, little is known about the initiation and maintenance of the photosymbiosis, the nutrient exchange between partners, and the resilience of photosymbiosis in non-calcifying animals, such as sea slugs. The present study aims to fill this

knowledge gap and gain a deeper understanding of the biology of photosymbiosis in marine slugs, its evolution, physiology, and the cellular and molecular mechanisms that lead to the photosynthetic association. In particular, new genomic tools are offered to study photosymbiosis in non-calcifying marine slugs (Chapter 2.3 Melo Clavijo et al., 2022; Chapter 2.4 Frankenbach et al., 2023; Chapter 2.5 Melo Clavijo et al., 2021). The study seeks to find answers to the following questions:

I. Are cladobranchs a suitable model for studying the molecular mechanisms and the evolution of photosymbiosis? (Chapter 2.1: Rola et al., 2022).
II. How does the sea slug host recognize the potential symbiont? (Chapter 2.2: Melo Clavijo et al., 2020; Chapter 2.3: Melo Clavijo et al., 2022).
III. What cellular mechanisms are necessary to establish a stable photosymbiosis and kleptoplasty in sea slugs? (Chapter 2.3: Melo Clavijo et al., 2022; Chapter 2.6).
IV. What is the effect of starvation in the animal host? (Chapter 2.3: Melo Clavijo et al., 2022; Chapter 2.4: Frankenbach et al., 2023).
V. How is the response of the slug host to abiotic factors like light and continuous darkness combined with starvation stress? (Chapter 2.4: Frankenbach et al., 2023; Chapter 2.6).

## Results

The results of this dissertation cover some of the main processes in the initiation and maintenance of the photosymbiosis in sea slugs, as well as the physiology and biology of photosymbiotic sea slugs. These are presented as individual chapters that correspond to scientific publications. Unpublished data are presented in Chapter 2.6.

### Chapter 2.1

Rola, M., Frankenbach, S., Bleidißel, S., Sickinger, C., Donath, A., Frommlet, J. C., Greve, C., Serôdio, J., Preisfeld, A., **Melo Clavijo, J.** & Christa, G., (2022). Cladobranchia (Gastropoda, Nudibranchia) as a promising model to understand the molecular evolution of photosymbiosis in animals. *Frontiers in Marine Science, 8,* 1920. DOI:10.3389/fmars.2021.745644

This review gathers the main aspects of the photosymbiosis between sea slugs belonging to the suborder Cladobranchia and dinoflagellates from the family Symbiodiniaceae. Here we propose the taxon Cladobranchia as model for studying the evolution of photosymbiosis based on the presence of different levels of the symbiosis and different modes of photobiont acquisition within the taxon. My contribution to this paper was 20 % and included literature revision, drafting, and revision of the manuscript.

### Chapter 2.2

**Melo Clavijo, J.**, Frankenbach, S., Fidalgo, C., Serôdio, J., Donath, A., Preisfeld, A. & Christa, G., (2020). Identification of scavenger receptors and thrombospondin-type-1 repeat proteins potentially relevant for plastid recognition in Sacoglossa. *Ecology and Evolution, 10*(21), 12348-12363. DOI:10.1002/ece3.6865.

In this publication we found a shared and a species-specific gene expression of immune receptors potentially relevant for plastid recognition by analyzing the gene expression of three sacoglossan sea slugs that are able to incorporate plastids of different algal sources. The core set of expressed genes is similar than the one known from photosymbiotic cnidarians, suggesting convergently

evolved mechanisms for symbiont recognition in taxonomically diverse animal hosts. My contribution to this paper was 60 % and included data collecting, analyses, drafting, and revision of the manuscript.

**Chapter 2.3**

**Melo Clavijo, J.,** Sickinger, C., Bleidißel, S., Gasparoni, G., Tierling, S., Preisfeld, A., & Christa, G. (2022). The nudibranch *Berghia stephanieae* (Valdés, 2005) is not able to initiate a functional symbiosome to maintain *Breviolum minutum* (J.E.Parkinson & LaJeunesse, 2018). *Frontiers in Marine Science*. DOI: 10.3389/fmars.2022.934307.

In this publication we present the first gene expression assessment in a dinoflagellate-bearing nudibranch that compares symbiotic and aposymbiotic animals feeding, starving, and re-feeding after a starvation period. The recognition machinery in nudibranchs is similar to the ones present in sacoglossan sea slugs and cnidarians. Further, we show that in this unstable symbiosis the slug can recognize, engulf the dinoflagellates, and activate antioxidant mechanisms in their presence, but is not able to suppress the immune response against the dinoflagellates nor inhibit the phagosome maturation, leading to their digestion or expulsion. My contribution to this paper was 60 % and included data collecting, experimental design, analyses, drafting, and revision of the manuscript.

**Chapter 2.4**

Frankenbach, S., **Melo Clavijo, J.,** Brück, M., Bleidißel, S., Simon, M., Gasparoni, G., Lo Porto, C., Laetz, E.M., Greve, C., Donath, A., Pütz, L., Sickinger, C., Serôdio, J., & Christa, G. (2023). Shedding light on starvation in darkness in the plastid-bearing sea slug *Elysia viridis* (Montagu, 1804). *Marine Biology*, *170*(89). DOI: 10.1007/s00227-023-04225-0

In this publication we address the effect of darkness in starved plastid-bearing sea slugs beyond the inhibition of photosynthesis. The gene expression analysis showed that starvation induces stress, and as response autophagy might be triggered. Starvation in darkness not only inhibits photosynthesis of the kleptoplasts, but also induces a major metabolic depression and a shift to alternative energy generation mechanisms. My contribution to this paper was 40 % and included data collecting, analyses, drafting, and revision of the manuscript.

## Chapter 2.5

**Melo Clavijo, J.**, Drews, F., Pirritano, M., Simon, M., Salhab, A., Donath, A., Frankenbach, S., Serôdio, J., Bleidißel, S., Preisfeld, A. & Christa, G., 2021. The complete mitochondrial genome of the photosymbiotic sea slug *Berghia stephanieae* (Valdés, 2005) (Gastropoda, Nudibranchia). *Mitochondrial DNA Part B,* 6(8), 2281-2284. DOI: 10.1080/23802359.2021.1914211

In this publication we present the mitochondrial genome of *Berghia stephanieae* obtained with hybrid assembly of short and long reads. The mitogenome is similar to other nudibranchs in general, with the exception of a duplicated *tRNA-Ser 1*. My contribution to this paper was 60 % and included data collecting, analyses, drafting, and revision of the manuscript.

## Chapter 2.6

### Unpublished data

**Melo Clavijo, J.**, Frankenbach, S., Serôdio, J., Preisfeld, A., & Christa, G. Reduction of the innate immune response might promote the establishment of functional kleptoplasty in juveniles of the slug *Elysia chlorotica* Gould, 1870. My contribution to this paper was 60 % and included data collecting, analyses, drafting, and revision of the manuscript

**Melo Clavijo, J.**, Baltaci, O., Preisfeld, A., & Christa, G. Starvation coupled with excessive light or darkness causes oxidative stress in *Berghia stephanieae* (Valdés, 2005). My contribution to this paper was 65 % and included data collecting, analyses, drafting, and revision of the manuscript

## Chapter 2.1

# Cladobranchia (Gastropoda, Nudibranchia) as a promising model to understand the molecular evolution of photosymbiosis in animals

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# Cladobranchia (Gastropoda, Nudibranchia) as a Promising Model to Understand the Molecular Evolution of Photosymbiosis in Animals

*Marcellina Rola[1†], Silja Frankenbach[2†], Sabrina Bleidissel[1], Corinna Sickinger[1], Alexander Donath[3], Jörg C. Frommlet[2], Carola Greve[4], João Serôdio[2], Angelika Preisfeld[1], Jenny Melo Clavijo[1*] and Gregor Christa[1*]*

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Symbiosis with photoautotrophic organisms has evolved in various species and even whole animal lineages, which allowed them to directly benefit from photosynthesis. This so-called *photosymbiosis* is best studied in cnidarians, which primarily establish symbioses with dinoflagellates from the family Symbiodiniaceae. In most other animals the mechanisms of establishing photosymbiosis, the physiological basis, and the evolution of a photosymbiotic life history remain poorly understood. Sea slugs belonging to the Cladobranchia (Gastropoda, Nudibranchia) are no exception, and are a rather neglected animal lineage in the research field of photosymbiosis. Yet, studying these sea slugs holds great potential to establish a unique photosymbiosis model, as they are the only known taxon that has evolved two different strategies to acquire their symbiont: either from cnidarian prey (thus becoming a secondary host) or directly out of the water column. The mechanisms for photobiont uptake and maintenance are unknown for these sea slugs, but might be similar to those of cnidarians. However, in terms of the evolution of photosymbiosis, Cladobranchia seem to share many commonalities with more closely related sea slugs belonging to the Sacoglossa, which only maintain the chloroplasts of the algae they feed on. Hence, Cladobranchia have the potential to shed light on the evolution of photosymbiosis in taxonomically divergent animals that also harbor photobionts of different evolutionary lineages.

**Keywords: Nudibranchia, photosynthetic symbiosis, sea slugs, Symbiodiniaceae, symbiont recognition**

## INTRODUCTION

Symbiotic relationships shape genomic and morphological plasticity, which is a driving force of evolution within prokaryotes and eukaryotes (Margulis, 1981; Burki et al., 2020). Most common are symbioses between heterotrophic organisms, but some protists (Decelle, 2013; Decelle et al., 2015; Foster and Zehr, 2019) and a few animal lineages (Melo Clavijo et al., 2018) engage in symbioses

with photoautotrophic organisms (photobionts) – the so-called *photosymbiosis* (Cowen, 1988; Stanley, 2006). Among animals, stony corals (Scleractinia) are probably the best-known example of a successful photosymbiosis. Corals primarily form a mutualistic symbiosis with dinoflagellates of the family Symbiodiniaceae (Fensome, 1993), that are referred to zooxanthellae when *in hospite*. Yet, more recently the mutualism of the photosymbiosis has been questioned and some authors refer to a host-controlled parasitism of the photobiont (Wooldridge, 2010; Blackstone and Golladay, 2018). Independent on the exact nature of the symbiosis, the photosymbiosis of corals and Symbiodiniaceae is based on an interdependent nutrient exchange cycle between the host and the photobiont (Stanley and Helmle, 2010; Tornabene et al., 2017). It has been shown that the coral host obtains nutritional support by the photobiont in the form of sugars, amino acids and some other essential nutrients, sometimes even superseding its nutritional requirements, allowing the coral host to thrive in oligotrophic waters (Muscatine and Porter, 1977; Falkowski et al., 1984; Lin et al., 2015) and enhances their ability to form skeletons and build up the coral reef structure (Stanley and Lipps, 2011; Tambutté et al., 2011; Roth, 2014). In return, the coral host provides carbon dioxide and ammonium, which are key limiting compounds for the photobiont (e.g., Yellowlees et al., 2008). The coordination of this nutrient exchange cycle is complex. Its disruption, for instance caused by heat stress, can lead to the breakdown of the symbiosis and can result in the expulsion of the photobionts. This process is known as bleaching and often eventually results in the coral's death leading in extreme cases to mass bleaching events, as seen in recent years, that are endangering entire reef communities across the globe (Suggett and Smith, 2020).

Aside from Scleractinia, photosymbiosis in metazoans is not well understood (Melo Clavijo et al., 2018). Considering its potential benefit for the heterotrophic host, it remains unclear why the symbiosis with a photobiont has evolved only in a few metazoan lineages. It is likely that photosymbiotic animals share components of a common genetic tool kit, which are essential for the initiation and maintenance of a photosymbiosis. This set of genes probably includes a large fraction related to the innate immune system (Gross et al., 2009; Davy et al., 2012; Mansfield and Gilmore, 2019), which is also highly relevant for other animal-microbe symbioses (e.g., McFall-Ngai et al., 2012; Schmittmann et al., 2021). Genomic data could help to understand these molecular mechanisms, but are still scarce for most photosymbiotic animal lineages (Melo Clavijo et al., 2018). Fortunately, genome-sequencing initiatives such as the recently launched Aquatic Symbiosis Genomics Project by the Welcome Sanger Institute and the Gordon and Betty Moore Foundation are addressing this lack of genomic data. Among others, this initative focuses on key photosymbiotic species in different lineages, which will provide a wealth of data and hence enable us to boost our understanding of photosymbioses. However, the acquisition of genomes of non-photosymbiotic congeners will be eminent to identify relevant genomic adaptations promoting photosymbiosis. Analyzing and comparing photosymbiotic and non-photosymbiotic animals within and between different lineages will have the potential to unravel their common genomic adaptations for photobiont recognition and maintenance. Such comparisons also hold the key to clarify at which point in the evolutionary history the animal host acquired distinct adaptations needed for photosymbiosis and if these adaptations evolved convergently or homologously.

We propose that a specific group of sea slugs, the Cladobranchia that belong to the Nudibranchia, should be studied in more detail. This lineage could considerably contribute to our understanding of photosymbiotic processes and the evolution of photosymbiosis in distantly related animals.

## "Butterflies of the Sea"

Nudibranchia are a morphologically diverse and colorful group of non-shelled sea slugs, belonging to the Heterobranchia (Burmeister, 1837) and consisting of the suborder Doridina and the suborder Cladobranchia. Over 4,000 nudibranch species have been described and, due to their colorful appearance, they fascinate scientists and non-scientists alike and are often called "butterflies of the sea" (Anderson, 1995). Current research on Nudibranchia focuses on assessing their biodiversity (e.g., Eisenbarth et al., 2018; Fritts-Penniman et al., 2020; Korshunova et al., 2021), their developmental biology and life cycle (Page, 1993; Kristof and Klussmann-Kolb, 2010; Ahmadian et al., 2016; Togawa et al., 2019), and phylogenetic relationships within the different groups (e.g., Carmona et al., 2013; Goodheart et al., 2015a,b; Karmeinski et al., 2021 Korshunova et al., 2021). Furthermore, because most Nudibranchia lost their protective shell, alternative defense strategies, such as mimicry of food sources (Gosliner and Behrens, 1990), calcareous needles (Cattaneo-Vietti et al., 1995), the synthesis of toxic metabolites (Bogdanov et al., 2017), and storing and using cnidocysts "stolen" from their cnidarian food source (Obermann et al., 2012; Goodheart et al., 2018) are investigated. Especially the potential pharmaceutical relevance of their secondary metabolites (reviewed by Cimino and Gavagnin, 2006; Putz et al., 2010; Fisch et al., 2017) makes them an interesting group for researchers. Photosymbiosis is only found in the Cladobranchia that comprise approximately 1,000 species and that can be identified by the lack of gills and their large dorsal appendices, the cerata, that also function as respiratory organ. However, photosymbiosis in Cladobranchia is not well understood.

## Cladobranchia Evolved Different Strategies of Photobiont Acquisition

Like most photosymbiotic animals, Cladobranchia acquire their photobionts anew in each generation (i.e., horizontally, instead of vertically from their parents), which is the most common mechanism of photobiont acquisition in animals (reviewed in Melo Clavijo et al., 2018). However, only Cladobranchia evolved two different modes of horizontal photobiont acquisition – out of the water column or from photosymbiotic cnidarians (**Figure 1**). Within the Cladobranchia, members of the Dendronotoidea (Allmann, 1845), such as *Melibe engeli* Risbec, 1937 (**Figure 1**), experienced morphological modifications that resulted in a fan-like mouth opening (Gosliner, 1987), allowing them to effectively ingest the photobionts out of the water column

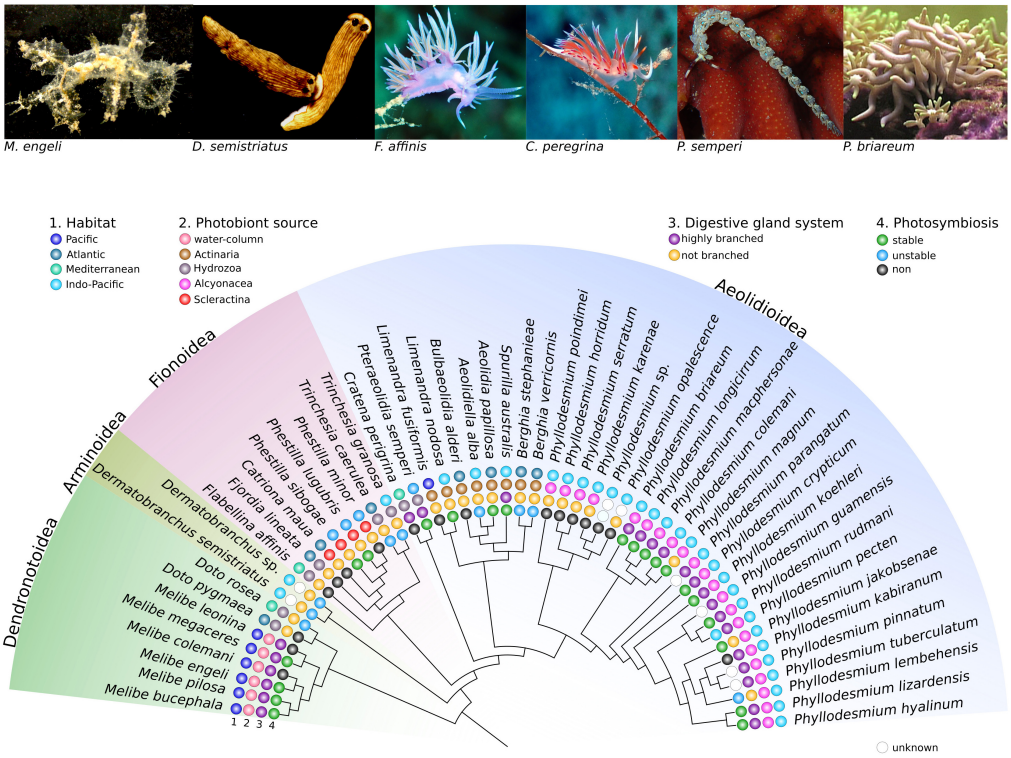


**FIGURE 1 |** Cladogram of selected Cladobranchia species showing different levels of photosymbiosis. Members of Cladobranchia are widespread distributed (1. Habitat) and acquire their photobiont horizontally either from the water column or by feeding on photosymbiotic cnidarians (2. Photobiont source). Different degree of ramification of the digestive gland system is present in Cladobranchia (3. Digestive gland system) that might be correlated to the stability of the photosymbiosis (4. Photosymbiosis). The cladogramm and the respective information are based on published data (Wägele and Willan, 2000; Johnson and Gosliner, 2012; Moore and Gosliner, 2014; Goodheart et al., 2018; Karmeinski et al., 2021). The images were taken by Heike Waegele (*Melibe engeli, Dermatobranchus semistriatus)*, Daniela Kupschus (*Flabellina affinis*, *Cratena peregrina*, *Pteraeolidia ianthina*), and Sabrina Bleidissel (*Phyllodesmium briareum*).

(Bleidissel, 2010; Burghardt and Wägele, 2014). To the best of our knowledge, adults of the genus *Melibe* are the only sea slugs to obtain their photobionts this way. The vast majority of Cladobranchia, however, acquire the photobionts by feeding on cnidarian prey and "stealing" the cnidarians' photobionts (Rudman, 1981; Kempf, 1984; Wägele, 2004). This is a unique photobiont acquisition strategy in animals that evolved in Cladobranchia probably multiple times in the superfamilies Arminoidea, Iredale and O'Donoghue (1923), Fionoidea Gray (1827), and Aeolidioidea Gray (1827) (**Figure 1**).

## Not All Cladobranchia Can Maintain Photobionts

Subsequent to the uptake, Cladobranchia selectively phagocytize the photobionts into epithelial cells of their digestive gland system (DGS). The DGS branches throughout the entire body and, particularly, into the cerata (**Figures 2A,B**). Once the algae are phagocytized in the epithelial cells, they remain in their coccoid state and are surrounded by the phagosomal membrane (**Figures 2C,D**; Wägele and Johnsen, 2001; Wakefield and Kempf, 2001). In most cladobranchs, like *Flabellina affinis* (Gmelin, 1791) or *Cratena peregrina* (Gmelin, 1791) (**Figure 1**), the algae are then rapidly digested, while some species, like *Berghia stephanieae* (Valdés, 2005) (**Figure 2A**) are capable to maintain the algae photosynthetically active for a few days (Monteiro et al., 2019). Regarding the organismic interaction that we describe in this review we refer to the general symbiotic terminology. Within that terminology, the existing subcategories of photosymbiosis are defined based on the beneficial aspects and the time-span of interaction, following the definitions by Kempf (1991). Based on this, maintaining the photobiont for a short term is here referred to an unstable photosymbiosis, because the algae reside intracellularly, but the slugs, like *B. stephanieae*, tend to digest the algae within a couple of days, or expel them from the cells and secrete them in a viable state in the feces. Species that have evolved an unstable photosymbiosis do not appear to benefit from the photosynthesis performed by the acquired photobionts. They are neither able to maintain their symbionts nor their biomass when solely relying on the photobiont as

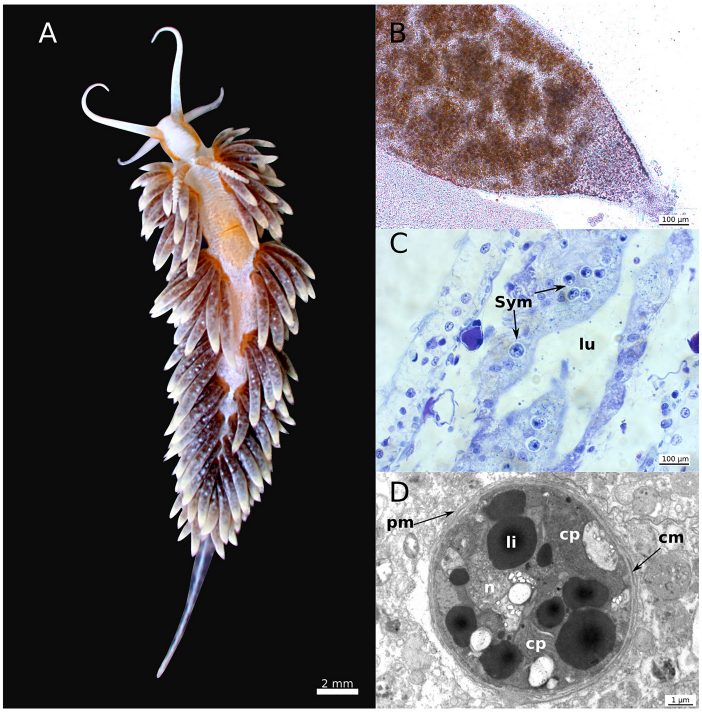


**FIGURE 2 |** Morphology and microscopic details of *Berghia stephanieae*. Morphology of *B. stephanieae* **(A)**. Detail of a cerata of *B. stephanieae* bearing the photobionts **(B)**, histological cross-section through a cerata showing the host cells of the cerata bearing the photobiont **(C)**, and detail of the coccoid (non-motile) stage of intracellular Symbiodiniaceae **(D)**. cm: Symbiodiniaceae cell membrane, cp: chloroplast, li: lipid droplets, lu: lumen of the host digestive gland, n: nucleus, pm: host phagosomal membrane, Sym: Symbiodiniaceae. The images were taken by Gregor Christa **(A,B)**, Jenny Melo **(C)** and Elise Laetz **(D)**.

source of nutrition (Kempf, 1991; McFarland and Muller-Parker, 1993; Bleidissel, 2010; Monteiro et al., 2019). Yet, some taxa have evolved the ability to establish a stable photosymbiosis, maintaining the photobionts for months. For a couple of these photosymbiotic species, like *Melibe engeli* and *Phyllodesmium briareum* (Bergh, 1896), it has been shown that the photobiont can fully support the host, enhancing growth and the ability of long-term reproduction without a reduction in the quantity and quality of egg-masses under regular light conditions (Kempf, 1984; Burghardt and Wägele, 2004, 2006, 2014; Burghardt et al., 2005, 2008a,b; Burghardt and Gosliner, 2006). These observations support the idea that at least these Cladobranchia species and Symbiodiniaceae are involved in a mutualistic symbiosis, which is unique in gastropods. More closely related Sacoglossa sea slugs, that are in a sort of photosymbiosis with chloroplasts of their algal prey, are not able to grow, or even maintain their biomass, if they are exclusively dependent on their ingested chloroplasts for more than a couple of weeks (Pelletreau et al., 2012; Christa et al., 2014). Further, they are not able to maintain the quantity and quality of egg-masses during periods of food depletion (Cartaxana et al., 2019). Hence, Cladobranchia provide a unique opportunity to understand which genomic adaptations are needed to evolve a mutualistic photosymbiosis in sea slugs. Comparative analyses of photosymbiotic and non-photosymbiotic Cladobranchia with Sacoglossa and Cnidaria might uncover if these genomic adaptations are based on convergent evolution or if, for instance, epigenetic modifications are involved in activating specific genes in photosymbiotic slugs.

## Mechanisms for Photobiont Recognition and Maintenance Are Unknown in Cladobranchia

The selective incorporation of photobionts in Cladobranchia is a complex process. It remains unknown how the slugs are able to distinguish between photobionts and plankton and further digest all plankton (e.g., in *Melibe*) or tissues of the cnidarian prey while maintaining the photobionts intact. The mechanisms of photobiont recognition by the epithelial cells of the DGS have not yet been addressed, while in cnidarians the photobiont recognition is based on a set of animal host pattern recognition receptors (PRRs). Specific microbe associated molecular patterns (MAMPs) of the photobiont (Neubauer et al., 2016, 2017; Mansfield and Gilmore, 2019) bind to the PRRs, which triggers downstream signaling cascades to maintain the photobiont (Davy et al., 2012). The PRRs of Cladobranchia might be similar to

those of their cnidarian host, as they incorporate the same photobiont and hence need to recognize the same MAMPs. However, Cladobranchia could also just use different PRRs to recognize other MAMPs to identify the equivalent photobiont. The incorporation process might even be more similar to the selective uptake of chloroplasts by more closely related members of sea slugs belonging to the Sacoglossa (Chan et al., 2018; Melo Clavijo et al., 2020). Using phylogenetic and domain specific analyses of the respective receptors will help to understand if the relationship of the respective PRRs is matching the taxonomy of the host. In combination with subsequent functional analyses, for instance by gene expression and manipulation, a list of candidate genes can be generated that could be involved in photobiont recognition. Therefore, Cladobranchia are the only animals that could permit a direct comparison of photobiont recognition mechanisms, in particular PRRs, between a primary (Cnidaria) and secondary (Cladobranchia) host of the same photobiont. At the same time, Cladobranchia allow comparisons with mechanisms of chloroplast recognition in the more closely related Sacoglossa. These analyses may provide insights into the relevance of PRRs in photosymbiosis across taxonomically divergent animal taxa. Furthermore, they may elucidate how conserved the signaling pathways for initiating photosymbiosis are – independent from the taxonomic lineage of the photobiont.

## Evolution of Stable Photosymbiosis in Cladobranchia

The evolution of a stable photosymbiosis in Cladobranchia is still not well understood. It appears that photosymbiosis evolved several times independently in different Cladobranchia superfamilies (**Figure 1**). For instance, it might have evolved independently in the Dendronotoidea and a monophyletic group including Arminoidea, Fionoidea, and Aeolidioidea. This would explain the evolution of the two different acquisition modes. Alternatively, photosymbiosis could also have evolved in each of the superfamilies independently. It is furthermore unknown whether unstable and stable photosymbiosis evolved separately, or if stable photosymbiosis evolved from unstable photosymbiosis.

Commonly, photosymbiotic Cladobranchia have a highly branched digestive gland system and specialized digestive structures, such as large circular chambers, fine tubules and cisternae that are located at the tips and harbor the photobionts (Burghardt and Wägele, 2014). These structures are considered to enlarge colonizable space and optimize light attenuation, increasing photobiont density and photosynthesis, respectively (Rudman, 1991; Burghardt et al., 2008a,b; Moore and Gosliner, 2011). Yet, despite of having a highly branched digestive gland system, species of *Phestilla* or *Limenandra* (**Figure 1**), and species like *Melibe leonina* (Gould, 1852) or *Phyllodesmium kabiranum* Baba, 1991, are non-photosymbiotic (**Figure 1**). Aside of this exception the genus *Phyllodesmium* shows a correlation between photosymbiosis and a highly branched digestive gland system (Rudman, 1991; Burghardt et al., 2008a,b; Moore and Gosliner, 2011). A highly branched DGS might not be a prerequisite to evolve a stable photosymbiosis, but it appears to be advantageous to harbor larger numbers of photobionts and to optimize photosynthesis.

The cnidarian prey is considered important for the stability of the photosymbiosis in Cladobranchia (Wägele et al., 2010). For instance, members of the genus *Phyllodesmium*, in which most species have a stable photosymbiosis, feed exclusively on xeneid cnidarians belonging to the Alcyonacea. However, *Pteraeolidia semperi* (Bergh, 1970), also in a stable photosymbiosis, obtains the photobionts from Hydrozoa, so that the food source is not strictly connected to photosymbiosis (**Figure 1**). Instead, it seems to be based on obtaining specific secondary metabolites for defense purposes (Bogdanov et al., 2017) and the feeding preference is rather taxon-specific. Independent of the source of the photobiont, the efficiency of the photosymbiosis may be further influenced by the specific algal taxon. For instance, in other stable photosymbiotic systems, such as Cnidaria-Symbiodiniaceae and Bivalvia-Symbiodiniaceae associations, the animal hosts could benefit from more physiologically resilient photobionts during increased ambient temperature (Hume et al., 2016; Cziesielski et al., 2018; Mies, 2019; Cunning and Baker, 2020). When corals are in symbiosis with multiple symbiodiniacean genera, the active removal of less resilient strains results in an adaptive bleaching, which might increase the animal's fitness and improve the stability of the symbiosis considerably (Ziegler et al., 2014; Bayliss et al., 2019; Chen et al., 2019). So far, only a few studies have investigated the diversity and composition of Symbiodiniaceae in Cladobranchia (Loh et al., 2006; FitzPatrick et al., 2012; Ziegler et al., 2014; Wecker et al., 2015; Yorifuji et al., 2015). Nevertheless, these studies have not uncovered any correlation between specific Symbiodiniaceae taxa and the ability to establish a photosymbiosis with Nudibranchia. It rather seems that symbionts are taken up from the cnidarian prey indiscriminately. Future analyses of photobiont abundance in cladobranchs in comparison to their cnidarian prey will help to understand if the slugs are able to distinguish between Symbiodiniaceae taxa and selectively expel less beneficial photobionts. Comparative metabarcoding analyses, as available for some cnidarians and their Symbiodiniaceae composition (Fujise et al., 2021), is still lacking for sea slugs, but are needed to reveal the relevance for a stable photosymbiosis in Cladobranchia. It might be further worth to investigate, if the slugs play a role in genotype dispersion of symbiodiniaceans in marine habitats. The fact that symbionts are transferred from a sessile to a motile host could potentially enhance the dispersion of symbiodiniaceans and could change the composition of clades and strains in environmental populations (Parker, 1984). This could be beneficial for other sessile photosymbiotic animals and their symbiont uptake, facing environmental changes with different adapted symbionts (Umeki et al., 2020).

Regardless of the stability of the photosymbiosis, little is known on how nutrients are exchanged between the slugs and the algae. The phagosomal membrane surrounding photobionts in cnidarians is known as symbiosome (Hill and Hill, 2012). The symbiosome plays a crucial role in the successful establishment of the symbiosis. Transporters relevant for nutrient exchange, i.e., sugars from the algae to the animal and dissolved inorganic compounds from the animal to the algae, are

situated in the symbiosomal membrane (Sproles et al., 2018). In cladobranchs it is unknown if and which transporters are present on the phagosomal membrane and how comparable it is to the symbiosome in terms of functionality. Future immuno-histochemical studies could give valuable insights into the localization of photosymbiosis-relevant receptors and their role in the nutritional exchange between the two partners, while metabolomics could provide important information on the interdependent nutrient exchange between both partners. Further, it remains to be shown whether in species with a stable photosymbiosis the symbiosis is beneficial for the host and whether the symbiosis is mutualistic or even some sort of parasitism as proposed for other photosymbiotic animals by some authors (Lesser et al., 2013; Blackstone and Golladay, 2018; Androuin et al., 2020).

## CLADOBRANCHIA CAN SHED LIGHT ON PHOTOSYMBIOSIS

Cladobranchia are a promising model to deepen the knowledge on fundamental processes that lead towards the evolution of photosymbiosis in animals as they resemble a connecting link between the well studied photosymbiosis in cnidarians and the less understood animal lineages such as sea slugs. Future research combining genomics, metabolomics, physiological, and immuno-histochemical studies, as well as phylogenetic analyzes of key receptors or proteins involved in photosymbiosis, will highlight if photosymbiosis evolved convergently or homologously in the different animal lineages.

## AUTHOR CONTRIBUTIONS

MR, JM, and GC designed the figures. All authors searched and analyzed the literature, wrote the draft, and reviewed and accepted the final version of the manuscript.

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## Chapter 2.2

# Identification of scavenger receptors and thrombospondin-type-1 repeat proteins potentially relevant for plastid recognition in Sacoglossa.

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# Identification of scavenger receptors and thrombospondin-type-1 repeat proteins potentially relevant for plastid recognition in Sacoglossa

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## Abstract

Functional kleptoplasty is a photosymbiotic relationship, in which photosynthetically active chloroplasts serve as an intracellular symbiont for a heterotrophic host. Among Metazoa, functional kleptoplasty is only found in marine sea slugs belonging to the Sacoglossa and recently described in Rhabdocoela worms. Although functional kleptoplasty has been intensively studied in Sacoglossa, the fundamentals of the specific recognition of the chloroplasts and their subsequent incorporation are unknown. The key to ensure the initiation of any symbiosis is the ability to specifically recognize the symbiont and to differentiate a symbiont from a pathogen. For instance, in photosymbiotic cnidarians, several studies have shown that the host innate immune system, in particular scavenger receptors (SRs) and thrombospondin-type-1 repeat (TSR) protein superfamily, is playing a major role in the process of recognizing and differentiating symbionts from pathogens. In the present study, SRs and TSRs of three Sacoglossa sea slugs, *Elysia cornigera*, *Elysia timida*, and *Elysia chlorotica*, were identified by translating available transcriptomes into potential proteins and searching for receptor specific protein and/or transmembrane domains. Both receptors classes are highly diverse in the slugs, and many new domain arrangements for each receptor class were found. The analyses of the gene expression of these three species provided a set of species-specific candidate genes, that is, SR-Bs, SR-Es, C-type lectins, and TSRs, that are potentially relevant for the recognition of kleptoplasts. The results set the base for future experimental studies to understand if and how these candidate receptors are indeed involved in chloroplast recognition.

KEYWORDS

*Elysia*, Kleptoplasty, photosymbiosis, sacoglossa, scavenger receptors, thrombospondin

## 1 | INTRODUCTION

Animals of many metazoan phyla establish a mutualistic symbiotic relationship with photosynthetic partners (Melo Clavijo et al., 2018). This so-called photosymbiosis allows the respective host to passively gain access to the benefits of photosynthesis, while the symbionts are protected against biotic and abiotic factors and are supplied with compounds relevant for the photosynthesis, such as $CO_2$ (Davy et al., 2012; Dean et al., 2016; Muscatine & Porter, 1977). Photosymbiotic processes, like the initiation of the symbiosis, mechanisms of symbiosis disruption, and the physiological benefits of both partners, are probably best understood in cnidarians (Davy et al., 2012; Fransolet et al., 2012; Koike et al., 2004; Lehnert et al., 2014; Neubauer, Poole, Detournay, Weis, & Davy, 2017; Neubauer, Poole, Neubauer, et al., 2017; Schwarz et al., 2008; van der Burg et al., 2016; Wood-Charlson et al., 2006). However, in other photosymbiotic systems, for example, in sacoglossan sea slugs, these mechanisms are less understood. Sacoglossa sea slugs suck out the cell content of their prey, mainly macroalgae, and some species then exclusively incorporate the chloroplasts into their own cytosol (de Vries, Christa, & Gould, 2014). These "stolen plastids" (kleptoplasts) retain their photosynthetic activity even for weeks or months in the absence of any nuclear support from their original host (Händeler et al., 2009; Rauch et al., 2017; Wägele et al., 2011). This photosymbiotic system involving an animal host and photosynthetically active kleptoplasts is called "functional kleptoplasty" (Gilyarov, 1983; Waugh & Clark, 1986) and in metazoans it was only further described for two rhabdocoelan species (van Steenkiste et al., 2019).

Most Sacoglossa species are not able to retain functional kleptoplasts and even in the species that do, the stability of the association varies (see, e.g., Christa et al., 2015, 2017; Cruz et al., 2014; de Vries et al., 2015). For instance, the shelled Oxynooidea and most of the shell-less "Limapontioidea" are not able to incorporate functional kleptoplasts (non-retention, NR; Figure 1). However, some members of the Costasiellidae and most members of the Plakobranchoidea retain the chloroplasts for a few days up to a couple of weeks (short-term retention, StR; Figure 1) (Christa et al., 2014; Händeler et al., 2009). Only five species are known in which the kleptoplasts are photosynthetically active for more than three months (long-term retention, LtR) (Christa et al., 2015). Among functional plastid-bearing Sacoglossa, the LtR species *Elysia chlorotica* Gould, 1870 and *Elysia timida* Risso, 1818, as well as the StR species *Elysia cornigera* Nuttall, 1989 are the most intensively investigated species (see, e.g., de Vries et al., 2014; Gimenez-Casalduero et al., 2011; Rumpho et al., 2008). It has been hypothesized that in *E. chlorotica* (LtR) functional kleptoplasty takes at least seven to 10 days postmetamorphosis to become stable. Generally, the process toward a stable functional kleptoplasty can be split into an initial phase, in which the chloroplasts are primarily recognized, a transient phase, in which the kleptoplasts are incorporated but still digested, and a stable phase, in which the kleptoplasts support the slugs during development by to a small degree (Pelletreau et al., 2012).

The reasons for the different abilities to maintain functional kleptoplasty are still unknown. Based on observations that the food source alone is not sufficient (Christa et al., 2015), it is assumed that the right combination of abiotic factors, such as temperature (Laetz & Wägele, 2018), slug intrinsic factors (i.e., genomic adaptations to support the kleptoplasts), and algae chloroplast intrinsic factors (i.e., longevity of proteins relevant for photosynthesis), leads to long-term functional kleptoplasty (de Vries et al., 2014).

Nevertheless, the food source might be important for the initiation of the symbiosis. While *E. timida* (LtR) and *E. cornigera* (StR) feed on the ulvophyte *Acetabularia acetabulum* P.C Silva, 1952 (Christa et al., 2013; de Vries et al., 2014), *E. chlorotica* (LtR) feeds on the heterokontophyte *Vaucheria litorea* C. Agardh, 1823 (Rumpho et al., 2000) (Figure 1). However, the evolutionary origin of the

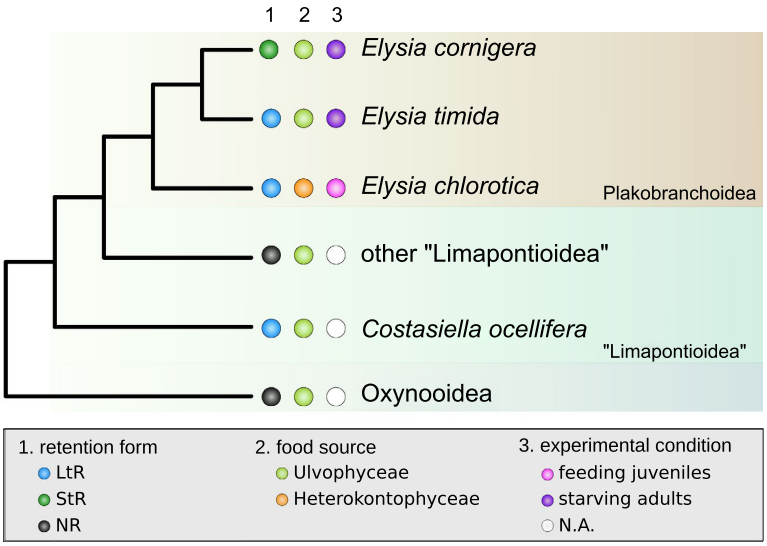


**FIGURE 1** Simplified phylogenetic relationship based on Christa et al. (2015) of *Elysia timida*, *Elysia cornigera*, and *Elysia chlorotica* as well as information on the retention form and food algae, and the experimental condition the three slugs were exposed to. The shelled Oxynooidea are the most basal Sacoglossa. *Costasiella ocellifera* is the only known member of the paraphyletic "Limapontioidea" in which long-term functional kleptoplasty is known (Christa et al., 2015)

chloroplasts of these two algae is quite different. Chloroplasts of *A. acetabulum* evolved from a primary endosymbiosis in the chlorophyte lineage, while those of *V. litorea* evolved from a secondary endosymbiotic event in the rhodophyte lineage (Gould et al., 2008). The different evolutionary origin of the chloroplasts, and with this potential differences in the composition of glycans or lipopolysaccharides of the inner and outer chloroplast membranes, might thus have implications on their recognition by the host. However, it remains to be understood how the slugs specifically recognize the chloroplasts and if the recognition differs for kleptoplasts originating from different algal lineages.

The innate immune system probably plays a major role in the initiation of the photosymbiosis process (van der Burg et al., 2016; Davy et al., 2012; Fransolet et al., 2012; Koike et al., 2004; Lehnert et al., 2014; Mansfield & Gilmore, 2019; Neubauer, Poole, Detournay, et al., 2017; Neubauer, Poole, Neubauer, et al., 2017; Poole et al., 2016; Schwarz et al., 2008; Wood-Charlson et al., 2006). Particularly, interactions between pattern recognition receptors (PRRs) of the host cell and microbe-associated molecular patterns (MAMPs) of the microbe/symbiont cell trigger different signaling cascades, which are essential to discriminate a pathogen from a symbiont (Davy et al., 2012; Fransolet et al., 2012; Wood-Charlson et al., 2006). Among PRRs, innate immune receptors, such as scavenger receptors (SRs), extracellular matrix proteins, like the thrombospondin-type-1 repeat (TSR) domain-containing proteins, and cnidarian ficolin-like receptors (CniFLs), have been shown to be involved in symbiont recognition (Baumgarten et al., 2015; Davy et al., 2012; Mansfield & Gilmore, 2019; Neubauer, Poole, Detournay, et al., 2017; Neubauer, Poole, Neubauer, et al., 2017; Rodriguez-Lanetty et al., 2006; van der Burg et al., 2016; Wood-Charlson et al., 2006). Especially, receptors of the SR-B and SR-E class play a major role in symbiont recognition (reviewed in Davy et al., 2012; Mansfield & Gilmore, 2019) and SR-B receptors are further thought to interact with proteins containing TSR domains. This interaction might trigger the immunosuppressive transforming growth factor β (TGF-β) pathway (Detournay et al., 2012; Li et al., 2006; Masli et al., 2006; Yehualaeshet et al., 1999), which seems to prevent a host immune response and to promote symbiont colonization and establishment (Detournay et al., 2012).

In sacoglossan sea slugs, a detailed examination of PRRs is missing and was so far only briefly investigated in the LtR species *Elysia chlorotica* (Chan et al., 2018). Here, we describe the abundance of SRs and TSRs in the StR species *E. cornigera*, and the LtR species *E. timida*, and *E. chlorotica*. To this end, we analyzed the available differential gene expression data with regard to the specific expression of both PRR groups, either during the different stages toward a stable functional kleptoplasty (*E. chlorotica*), or in freshly fed animals compared to different starvation periods (*E. cornigera* and *E. timida*). Our results revealed that Sacoglossa have a diverse SR and TSR repertoire, similar to photosymbiotic cnidarians. The expression profiles of the two PRR classes provided a set of species-specific candidate genes that might be involved in chloroplast recognition in Sacoglossa.

## 2 | METHODS

### 2.1 | Analyzed species

Publicly available RNA datasets of three Sacoglossa species were used for the analyses of the abundance and expression of SRs and TSRs: *Elysia chlorotica* (LtR; NCBI SRA sample accession SRS3101883) (Chan et al., 2018), *Elysia timida* (LtR; SRS706683), and *Elysia cornigera* (StR; SRS706681) (de Vries et al., 2015). The retrieved datasets were generated under different experimental conditions: From *Elysia chlorotica*, total RNA was extracted by pooling > 20 individuals each from unfed juveniles (aposymbiotic) and from juveniles feeding for five, seven, and 10 days postmetamorphosis (Chan et al., 2018), always in triplicates. From *Elysia timida* and *Elysia cornigera*, total RNA was extracted by pooling > seven individuals from freshly fed adults (fed) and from adults starved for four and seven days, and additionally from adults starved for 30 days for *E. timida*(de Vries et al., 2015).

### 2.2 | Annotation of transcriptomic data

For all species, the available assembled transcriptomes (*Elysia cornigera*: NCBI TSA version GBRW00000000.1; *E. timida*: TSA version GBRM00000000.1; *E. chlorotica:* http://cyanophora.rutgers.edu/Elysia-expression/) were first clustered using CD-HIT v4.6.8 with default parameters (Fu et al., 2012; Li & Godzik, 2006). For *Elysia cornigera*, we obtained 458,434 transcript clusters, for *E.* timida 274,479, and for *E. chlorotica* 129,716. The clustered transcriptomes were translated into the longest open reading frame to retrieve potential proteins using TransDecoder v3.0.1 (Haas & Papanicolaou, 2015) with default settings. The datasets were then subjected to a BLASTP search against the UniProt database version 11/13/19 (The UniProt Consortium, 2019) setting the E-value to $1e^{-10}$. Taxonomic assignment for each protein sequence was performed using the UniProt taxonomic database and sequences were subsequently filtered for Metazoa annotations (Appendix S1). Using this approach, we obtained 29,444 annotated proteins for *E. cornigera*, 20,445 for *E. timida*, and 13,389 for *E. chlorotica*.

### 2.3 | Identification of scavenger receptors and thrombospondin-type-1 repeat proteins

The domain architecture of the filtered protein sequences was characterized by using HMMER v.3.1b2 (Eddy, Wheeler, & the HMMER Development Team, 2015) with default settings against the protein database PfamA 31.0 (Finn et al., 2016). Transmembrane regions (TM) were identified using the TMHMM server v.2.0 (Krogh et al., 2001; Sonnhammer et al., 1998). Sequences were then filtered for the different receptor class specific domains, as defined in PrabhuDas et al. (2014, 2017). For example, protein sequences having an N-terminal cytoplasmic tail, a transmembrane domain,

spacer region, α-helical coiled coil domain, collagen domain, and a C-terminal scavenger receptor cysteine-rich (SRCR) domain were annotated as a member of the SR-A class; protein sequences containing a CD36 domain in the form of an extracellular loop flanked by two transmembrane regions were annotated as a member of the SR-B class; protein sequences having a transmembrane region with a single C-type lectin domain were annotated as a member of the SR-E-like class, because sequence homology is not sufficient to include them in a SR-E group. To be classified as SR-E, a scavenger activity must be experimentally demonstrated (PrabhuDas et al., 2014, 2017). Protein sequences containing a transmembrane region with multiple SRCR domains were annotated as a member of the SR-I class. All proteins that contained C-type lectin domains, at least one transmembrane domain, and which could not be assigned to SR-Es were classified as C-type lectins. All proteins containing SRCR domains and that would not be assigned to SRs were classified as SRCR members.

Protein sequences were characterized as a member of the TSR superfamily if they contained a thrombospondin-type-1 (TSP1) domain, a disintegrin and metalloproteinase with thrombospondin motifs spacer 1 domain (ADAMTS Spacer 1), or a Sema domain. A further classification of the various TSR family members followed the definition given by Adams and Tucker (2000), Tucker (2004), and Adams and Lawler (2011). For instance, thrombospondins (TSPs) have an invariant carboxy-terminal region consisting of repeats of epidermal growth factor (EGF)-like domains, 13 calcium-binding type 3 repeats, a homologous L-type lectin domain in the C-terminal region, and N-terminal region that varies in domain composition (Adams & Lawler, 2011). Repeats of the TSP1 domain are named as TSR. The TSR domain in cnidarians is similar to that in vertebrates (Adams & Tucker, 2000; Silverstein, 2002; Tan et al., 2002). It includes six cysteine residues, a protein and glycosaminoglycan (GAG) binding site formed by the motif WXXWXXW, a RXRXRX motif consisting of polar residues (such as arginine, lysine, and glutamine). Further, it contains binding regions for SR-B proteins formed by the motifs CSVTCG and GVQTRXR (Neubauer, Poole, Neubauer, et al., 2017). Members of the ADAMTS group have a signal peptide, a prodomain, a metalloproteinase catalytic domain, a disintegrin-like domain, a central TSP1-like domain repeat, a cysteine-rich domain, a spacer region with variable length, and C-terminus with a variable number of TSP1 domains (Porter et al., 2005). Semaphorins, a group of secreted and transmembrane proteins, were identified by the presence of the Sema domain (Raper, 2000). Out of the eight classes of semaphorins (1 to 7 plus class V for viruses), class 5 is also classified as TSR, due to the presence of the TSP1 domain (Adams & Tucker, 2000; Tucker, 2004). Properdin, a further member of the TSR superfamily, is characterized only by the presence of six consecutive TSP1 domains (Nolan et al., 1991, 1992; Sun et al., 2004). In the present study, sequences similar to properdin were defined as TSR-TM (without transmembrane regions). Sequences containing only TSP1 domains with a transmembrane region were grouped as TSR + TM. Proteins were further filtered for a minimum length of 150 amino acids and an independent E-value of $1e^{-5}$ as recommended in the manual of HMMER v.3.1b2 (Eddy et al., 2015). A sequence logo of the TSP1 domains of those TSR sequences that were differentially expressed (see below) was created using the weblogo server (http://weblogo.berkeley.edu/logo.cgi) and compared to the general Pfam TSP1 domain motif downloaded from https://pfam.xfam.org/.

### 2.4 | Gene expression analyses

The datasets used in this study were previously analyzed using different tools. In order to avoid any method-based difference, we de novo analyzed the gene expression. For this, the respective short reads were downloaded for each species from the short read archive deposited in GenBank (see above) analyses. Reads were then mapped using Bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) onto the clustered transcriptomes. Transcript abundance of sequences with a raw read count of at least 100 raw counts in any two samples tested was estimated using RSEM (Li & Dewey, 2011) implemented in Trinity v.2.9.0 (Grabherr et al., 2011). Differential gene expression analyses were performed using edgeR v3.30.3 (Robinson et al., 2010). For feeding juveniles of *E. chlorotica*, we compared specimens fed for five days to the aposymbiotic state (initial); specimens fed for seven days with specimens fed for five days (transient); and specimens fed for 10 days with specimens fed for seven days (stable), to investigate whether the expression of the receptors changed during the different hypothesized stages to establish a stable functional kleptoplasty (Pelletreau et al., 2012). For *E. timida* and *E. cornigera*, we compared the freshly fed animals to the different starvation periods, in order to identify genes that might be relevant, while the slugs are feeding. We then focused on genes that were highly expressed in feeding animals compared to all starvation periods. Only genes with a $\log_2$ fold change (L2FC) >1 or <−1 were considered as significantly differentially expressed, because we assumed the expression of a gene to be relevant when it changes twofold. Further, because for *E. cornigera* and *E. timida* no biological replicates are available, we used a L2FC threshold of < −1 or >1 as a way to infer meaningful expression changes.

## 3 | RESULTS

### 3.1 | Abundance of scavenger receptors in *Elysias*

No putative SR-A receptor proteins could be identified in any of the investigated *Elysia* species. A total of eight potential SR-B proteins, a varying number of SR-E-like proteins, with the highest number identified in *E. timida* (15), and two to four SR-I proteins were found in all sea slugs (Figure 2). Additionally, numerous protein sequences containing one or multiple CTLD, often combined with various other domains, were found. Especially in *E. cornigera*, a high diversity (41) of C-type lectin proteins was found. Additionally, proteins containing

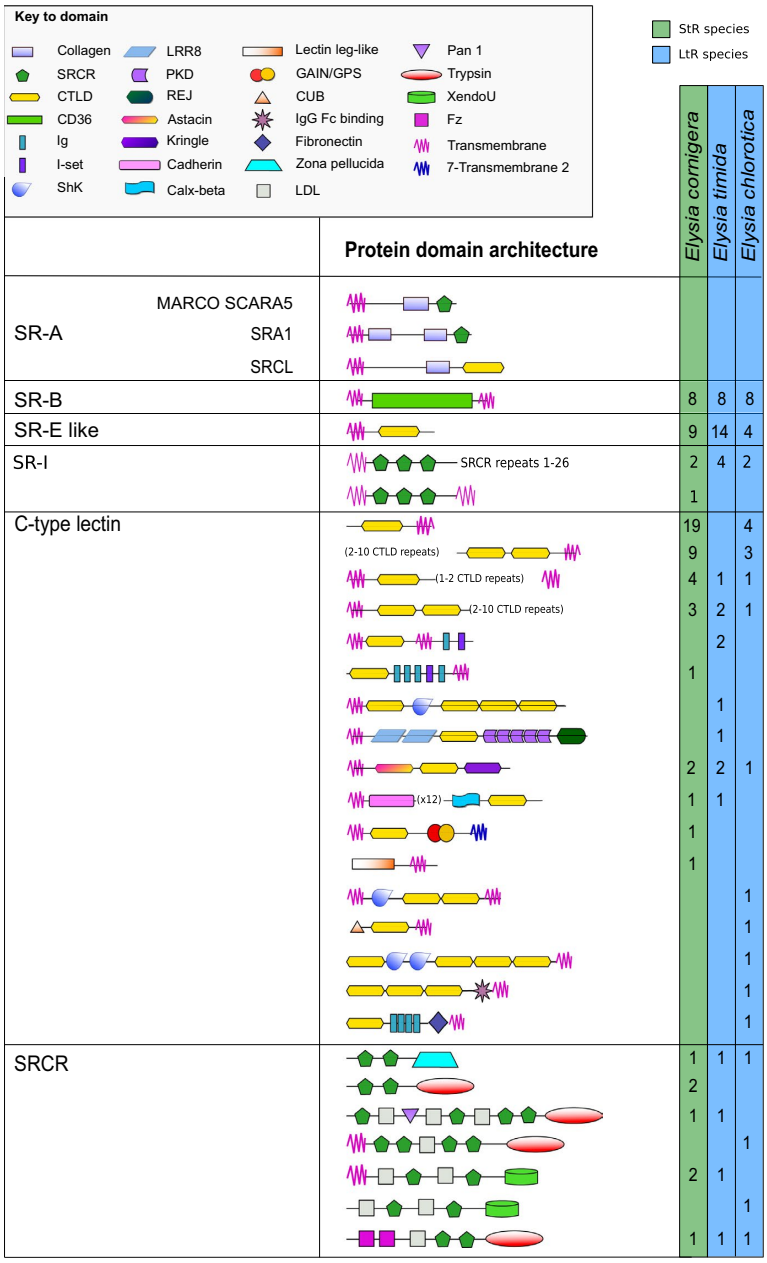


**FIGURE 2** Overview about the diversity of scavenger receptor proteins in *Elysia timida*, *Elysia cornigera*, and *Elysia chlorotica*. As no putative SR-A proteins were identified in any of the slugs, the general domain architecture of SR-A proteins in humans is shown as a reference. Astacin, peptidase family M12A; CD36, cluster of differentiation 36; CTLD, C-type lectin domain; GAIN/GPS, G protein-coupled receptors autoproteolysis inducing domain; Ig, immunoglobulin domain; I-set, immunoglobulin I-set domain; LDL, low-density lipoprotein domain; LRR8, leucine-rich repeat 8 domain; PKD, polycystic kidney disease domain; REJ, receptor of egg jelly domain; ShK, *Stichodactyla helianthus* $K^+$ channel toxin domain; SRCR, scavenger receptor cysteine-rich domain

SRCR domains combined with other domains were found in all three slugs (Figure 2).

### 3.2 | Abundance of TSRs in *Elysias*

In all analyzed *Elysia* species, a potential thrombospondin type 5/cartilage oligomeric matrix protein (COMP) homologue was identified (Figure 3). Additionally, several different putative ADAMTS-like, semaphorin, and plexin homologues were found. The vast majority of TSR sequences only contained TSP1 sequences (Figure 3). Furthermore, for each species an Astacin homologue and several homologues containing either of von Willebrand factor A (VWA) domains or immunoglobulin-like domains, or UNC-5 domains, as well as putative homologues of RPE-spondin and spondin (Figure 4). The highest diversity of TSR receptors (15 different arrangements) was found for proteins that contained one or several TSP1 domains combined with a variety of different receptors (Figure 4).

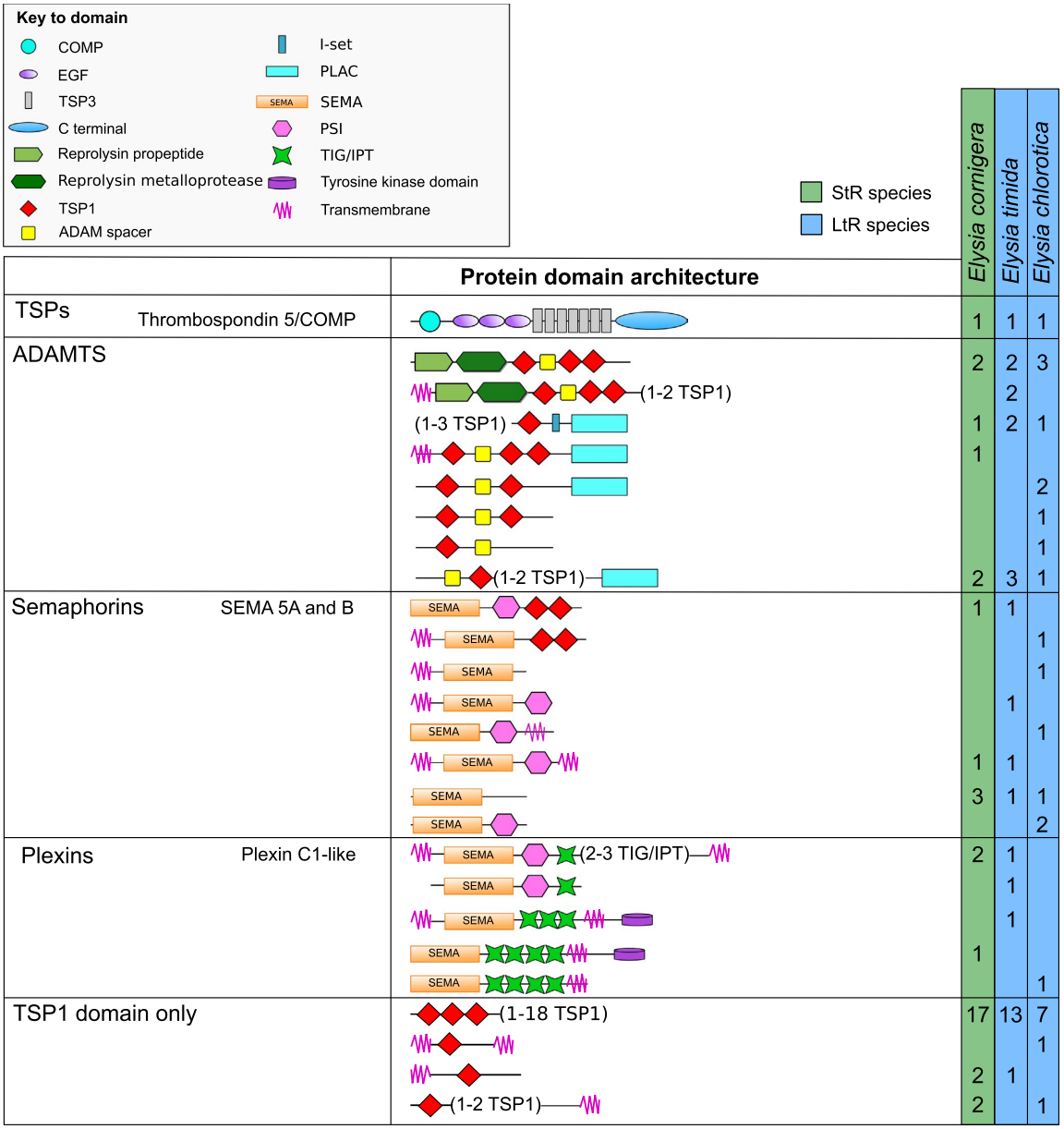


**FIGURE 3** Overview about the diversity of thrombospondin-type-1 repeat (TSR) proteins in *Elysia timida*, *Elysia cornigera*, and *Elysia chlorotica*. COMP, cartilage oligomeric matrix protein; EGF, epidermal growth factor domain; I-set, immunoglobulin I-set domain; PLAC, protease and lacunin domain; PSI, plexin repeat; TIG/IPT, immunoglobulin-like, plexins, transcription factor domain; TSP1, thrombospondin-type-1 domain; TSP3, thrombospondin-type-3 domain

### 3.3 | Expression of PRRs in adults of the StR *E. cornigera* and the LtR *E. timida*

In the StR species *E. cornigera*, six out of 68 genes belonging to the SRs class were significantly upregulated in the freshly fed animal, compared to both starvation periods (Table 1, Figure 5). One of these genes is a putative SR-B homologue (GBRW01136834.1), a putative Perlucin homologue (GBRW01100272.1) belonging to the SR-E receptors, and four C-type lectins. Among the C-type lectins are two putative C-type mannose receptor 2 sequence homologues (GBRW01106608.1 and GBRW01163094.1), and one putative Versican core protein homologue (GBRW01163094.1) that all contained two C-type lectin domains (CTLD) and one transmembrane (TM) region. Further, we found one gene to be significantly

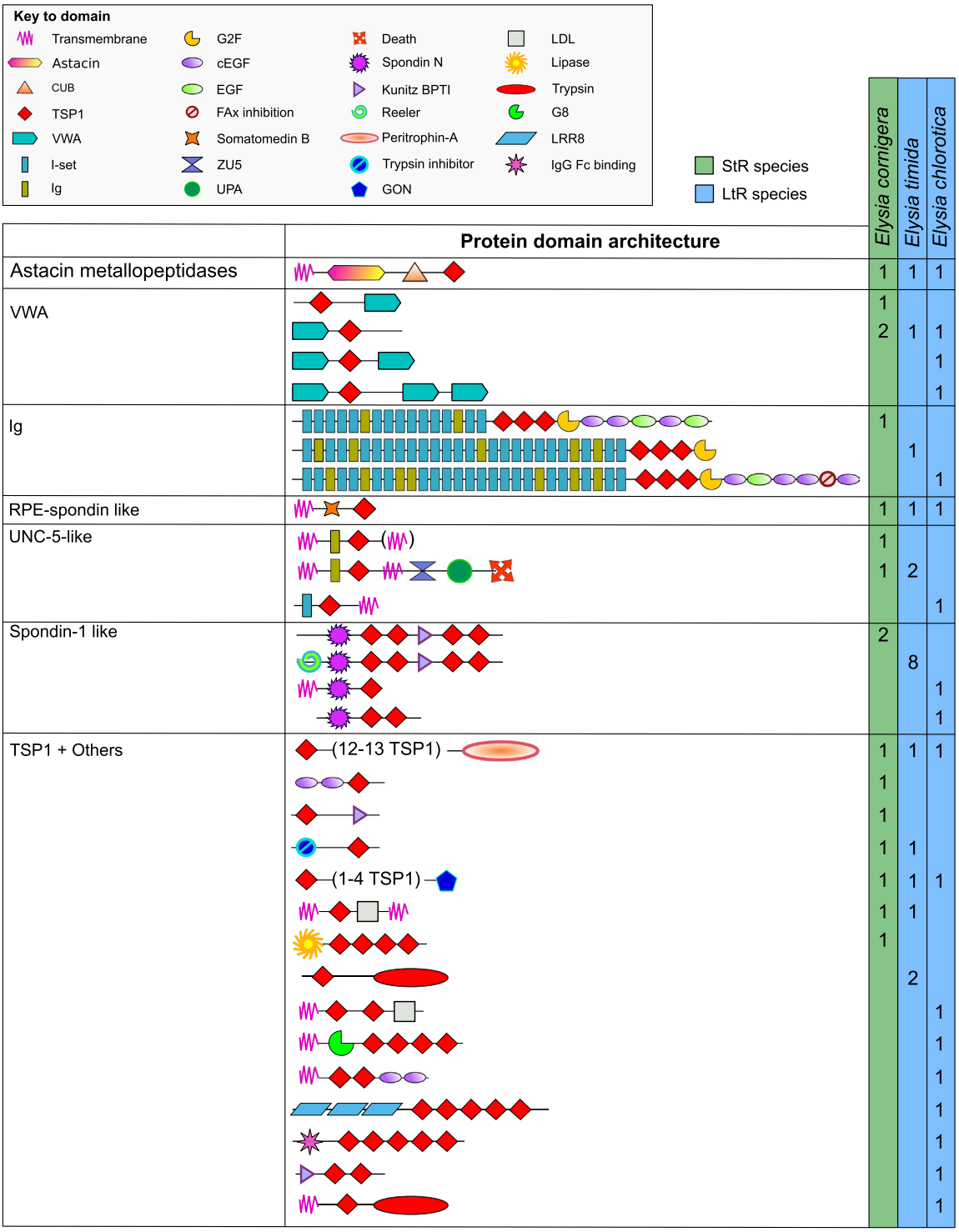


**FIGURE 4** Overview about the diversity of new domain arrangements of thrombospondin–type-1 repeat (TSR) proteins in *Elysia timida*, *Elysia cornigera*, and *Elysia chlorotica*. CUB, complement C1r/C1s, Uegf, Bmp1 domain; EGF, epidermal growth factor domain; Ig, immunoglobulin domain; I-set, immunoglobulin I-set domain; Kunitz BPTI, Kunitz bovine pancreatic trypsin inhibitor domain; LDL, low-density lipoprotein domain; LRR8, leucine-rich repeat 8 domain; TSP1, thrombospondin-type-1 domain; VWA, von Willebrand factor type A domain

**TABLE 1** Scavenger receptors and thrombospondin-type 1 receptors of the StR species *Elysia cornigera* and the LtR species *Elysia timida* that were significantly upregulated in freshly fed animals

| Species | Receptor | Domain arrangement | Gene_ID | UniProtKB annotation | $log_2$ fold change | | |
|---|---|---|---|---|---|---|---|
| | | | | | Day 4 | Day 7 | Day 30 |
| *Elysia cornigera* (StR) | SR-B | TM + CD36 + TM | GBRW01136834.1 | Scavenger receptor class B member 1 | 2.41 | 1.43 | – |
| | SR-E like | TM + CTLD | GBRW01100272.1 | Perlucin | 3.04 | 1.32 | – |
| | C-type lectins | CTLD + CTLD +TM | GBRW01106608.1 | C-type mannose receptor 2 | 2.53 | 8.50 | – |
| | | | GBRW01166191.1 | C-type mannose receptor 2 | 2.11 | 1.15 | – |
| | | | GBRW01163094.1 | Versican core protein | 1.22 | 2.28 | – |
| | | TM + CTLD +CTLD + TM | GBRW01019759.1 | Snaclec agglucetin subunit beta-1 | 1.69 | 1.17 | – |
| | TSP1 | TSP1 | GBRW01123401.1 | Hemicentin-1 | 3.24 | 2.69 | – |
| *Elysia timida* (LtR) | SR-E like | TM + CTLD | GBRM01064602.1 | Collectin-10 | 2.66 | 3.54 | 2.08 |
| | | | GBRM01009636.1 | C-type lectin 37 Da | 2.80 | 2.54 | 6.14 |
| | | | GBRM01066486.1 | C-type lectin 37 Da | 3.87 | 3.66 | 6.09 |
| | | | GBRM01066478.1 | Perlucin | 3.69 | 3.92 | 4.63 |
| | | | GBRM01039872.1 | C-type mannose receptor 2 | 2.90 | 2.61 | 2.36 |
| | C-type lectins | TM + CTLD +TM | GBRM01017414.1 | Collectin-12 | 3.58 | 1.73 | 2.72 |
| | ADAMTS | TSP1 + TSP1 + I-set + Plac | GBRM01039431.1 | Protein madd-4 | 8.92 | 9.47 | 10.06 |

*Note:* The $log_2$ fold change shows the expression in freshly fed animals compared to the respective starvation period.
Abbreviations: CD36, cluster of differentiation 36; CTLD, C-type lectin domain; I-set, immunoglobulin I-set domain; Plac, protease and lacunin domain; TM, transmembrane region; TSP1, thrombospondin-type-1 domain.

upregulated (GBRW01019759.1) which contained two CTLDs flanked at both sides by a TM region and was annotated as putative Snaclec agglucetin subunit beta-1 homologue.

In freshly fed animals of the StR *E. cornigera*, only one out of 53 identified TSRs was significantly expressed compared to both starvation conditions (Table 1, Figure 6). This sequence (GBRW01123401.1) only contained one TSP domain and was annotated as Hemicentin-1.

Regardless of the starvation duration, six out of the 40 genes classified as SRs were significantly upregulated in the freshly fed animal of the LtR species *Elysia timida* (Table 1, Figure 5). Five of those genes belonged to the SR-E like class, all containing one TM and one CTLD. Among those genes were a putative Collectin-10 (GBRM01064602.1), two C-type lectin 37Da homologues (GBRM01009636.1 and GBRM01066486.1), one putative Perlucin homologue (GBRM01066478.1), and one putative C-type mannose receptor 2 homologue (GBRM01039872.1). Additionally, one C-type lectin homologue (GBRM01017414.1), containing one CTLD flanked at both sites by TM regions, was significantly upregulated.

In freshly fed adults of the LtR *E. timida*, only one out of 52 TSR genes was significantly upregulated compared to all three starvation conditions (Table 1, Figure 6). This gene (GBRM01039431.1) was classified as ADAMTS member and contained two TSP domains, one I-set domain, and one PLAC domain and was annotated as Protein madd-4 homologue.

### 3.4 | Expression of PRRs in feeding juveniles of the LtR *E. chlorotica*

In feeding juveniles of the LtR species *Elysia chlorotica*, four of the 33 receptors classified as SRs were expressed during the initial phase of functional kleptoplasty (Table 2, Figure 5). Out of those two SR-B receptor homologues (c104938_g1_i1_1-2480 and c128999_g1_i3_1-4140) and two genes belonging to the C-type lectins, a putative snaclec B1 homologue (c119366_g1_i1_1-1981) and a putative secretory phospholipase A2 receptor homologue (c124460_g1_i2_1-2516) were significantly upregulated during the initial phase of functional kleptoplasty (Figure 5). During the transient phase of kleptoplasty, one of the previous upregulated SR-B homologues (c104938_g1_i1_1-2480) was significantly down-regulated, while the other SR-B homologue and the two C-type lectins were not significantly altered. During the stable phase, the gene expression of SRs changed considerably. Out of 33 genes, 20 were significantly upregulated, while the remaining sequences did not change significantly. Overall, five SR-B, one SR-E like, both SR-I, nine C-type

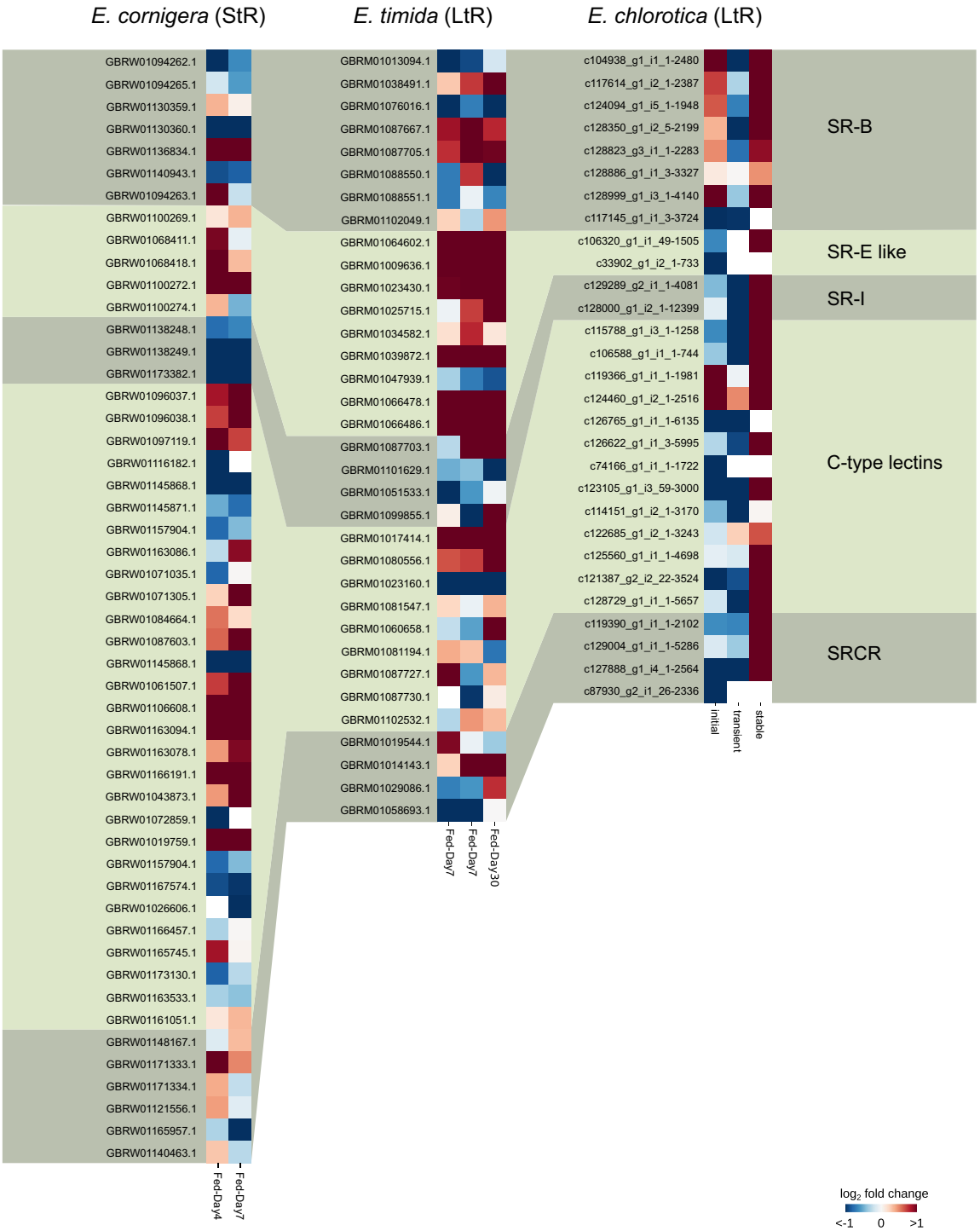


**FIGURE 5** Gene expression profile of scavenger receptors in *Elysia cornigera* (StR), *Elysia timida* (LtR) and *Elysia chlorotica* (LtR). Shown is the $\log_2$ fold change (L2FC) of the gene expression and only genes that were differentially expressed in at least one condition are displayed

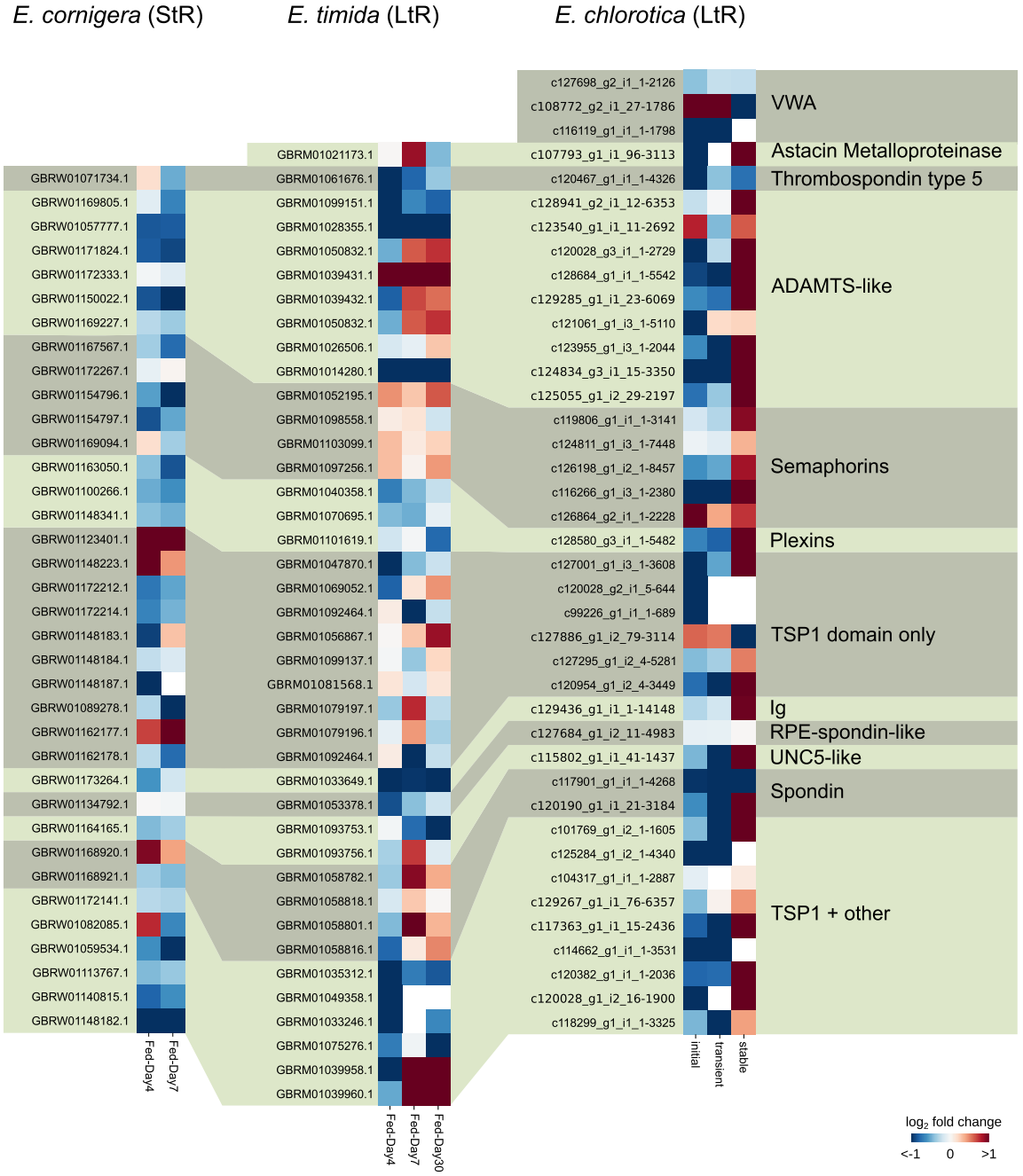


**FIGURE 6** Gene expression profile of thrombospondin-type-1 repeat (TSR) proteins in *Elysia cornigera* (StR), *Elysia timida* (LtR) and *Elysia chlorotica* (LtR). Shown is the $\log_2$ fold change (L2FC) of the gene expression and only genes that were differentially expressed in at least one condition are displayed

lectins, and three SRCR homologues were upregulated during the stable phase.

In feeding juveniles of the LtR species *E. chlorotica*, only two TSR genes, a collagen alpha-5 chain homologue (c126864_g2_i1_1-2228), which contains a VWA and a TSP1 domain, as well as a Plexin-B homologue (c126864_g2_i1_1-2228), which contains a Sema domain (SEMA) and a PSI integrin domain (PSI), were significantly upregulated during the initial phase of functional kleptoplasty (Table 2, Figure 6).

**TABLE 2** Scavenger receptors and thrombospondin-type-1 receptors of the LtR species *Elysia chlorotica*

| Species | Receptor | Domain arrangement | Gene_ID | UniportKB annotation | $log_2$ fold change | | |
|---|---|---|---|---|---|---|---|
| | | | | | Initial | Transient | Stable |
| *Elysia chlorotica* (LtR) | SR-B | TM + CD36 + TM | c104938_g1_i1_1-2480 | Lysosome membrane protein 2 | 2.02 | −2.12 | 1.33 |
| | | | c128999_g1_i3_1-4140 | Scavenger receptor class B member 1 | 2.58 | −0.38 | 1.90 |
| | C-type lectins | CTLD + CTLD +TM | c119366_g1_i1_1-1981 | Snaclec B1 | 1.10 | −0.05 | 3.17 |
| | | | c124460_g1_i2_1-2516 | Secretory phospholipase A2 receptor | 1.68 | 0.48 | 2.95 |
| | VWA | VWA + TSP1 + VWA | c108772_g2_i1_27-1786 | Collagen alpha−5 chain | 1.05 | 2.00 | −1.55 |
| | Semaphorin | SEMA + PSI | c126864_g2_i1_1-2228 | Plexin-B | 1.33 | 0.39 | 0.72 |

*Note:* The $log_2$ fold change shows the expression during the different stages of functional kleptoplasty. The focus was set on genes expressed during the initial phase.

Abbreviations: CD36, cluster of differentiation 36; CTLD, C-type lectin domain; SEMA, Sema domain; TM, transmembrane region;TSP1, thrombospondin-type-1 domain; VWA, von Willebrand factor type A domain.

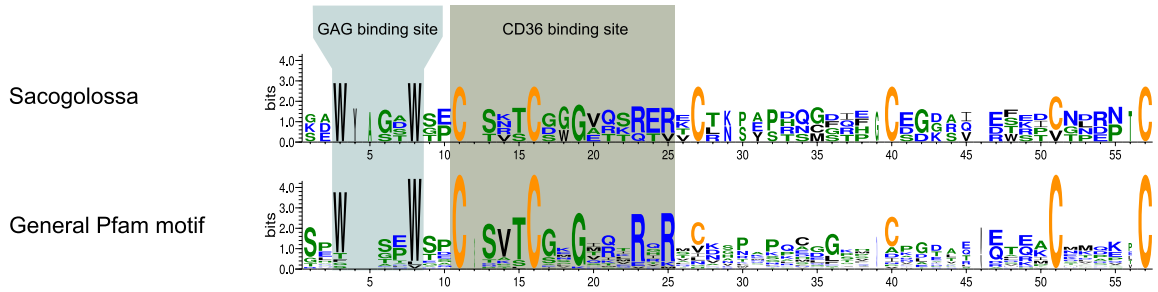


**FIGURE 7** Sequence logo of the TSP1 domains of TSR genes that were differentially expressed throughout the experimental conditions in the three slugs. The profile was compared to the general Pfam profile of TSP1 domains. The glycosaminoglycan (GAG) and CD36 binding sites are highlighted in the boxes, and the six conserved cysteines are shown in orange

This general expression profile does not change during the transient phase of functional kleptoplasty, with the exception that the Plexin-B homologue (L2FC 0.39) was not significantly regulated anymore. During the stable phase of functional kleptoplasty, the expression profile of TSRs changes, similar as of SRs, extensively (Figure 6). Overall, 18 genes were significantly upregulated during the stable phase, three significantly down-regulated, and 19 did not change. Especially, seven ADAMTS domain-containing proteins were significantly upregulated (average L2FC 2.13 ± 0.67). Among genes containing only TSP1 domains, two were significantly upregulated as well; a Hemicentin-1 homologue (c127001_g1_i3_1-3608, L2FC 1.04) containing 14 TSP1 domains and a thrombospondin-type-1 domain-containing protein 7A homologue (c120954_g1_i2_4-3449, L2FC 1.48) containing two TSP1 domains and a transmembrane region.

Generally, the TSP1 domains of the upregulated TSR sequences of the slugs were made out of six conserved cystein residues, containing a WXXW (where X is any amino acid) motif, a motif similar to the CSVTCG motif and a subsequent RXR motif (Figure 7).

## 4 | DISCUSSION

To shed light on the initiation of functional kleptoplasty, we analyzed the abundance and domain architecture of scavenger receptors (SRs) and thrombospondin-type-1 repeat (TSR) protein superfamily in three kleptoplastid-bearing sea slugs *Elysia cornigera* (StR), *E. timida* (LtR), and *E. chlorotica* (LtR). All species possess a similar SRs and TSRs receptor repertoire independent on the ability to either maintain the kleptoplasts in the short or long term. We could only find minor differences in the number of genes and the diversity of some receptors mainly between *E. cornigera*/*E. timida* and *E. chlorotica*. This might, however, be based on the different experimental setups and developmental stage of the used specimens with an according different gene expression profile, rather than genomic differences, for example, gene duplication, diversification, or losses. Nevertheless, the general abundance is also similar to that found in cnidarians (Neubauer, Poole, Detournay, et al., 2017; Neubauer, Poole, Neubauer, et al., 2017).

Independently of the experimental condition, the gene expression profiles of the three species provided a set of species-specific candidate genes, in particular SR-B, SR-E, C-type lectins, and TSR genes, that might be relevant for plastid recognition (Figure 8). Receptors belonging to those classes are likewise upregulated during the onset of a symbiosis in cnidarians (Mohamed et al., 2016; Neubauer, Poole, Neubauer, et al., 2017) and at least for SR-Bs and TSRs their involvement in symbiont recognition was verified by physiological trials (Neubauer, Poole, Detournay, et al., 2017; Neubauer, Poole, Neubauer, et al., 2017). We found TSP1 domain motifs of the TSR proteins (six conserved cystein residues, the protein and glycosaminoglycan binding motif, and a motif similar to the CD36 binding motif) (Zhang & Lawler, 2007), that are needed for potential interactions of the TSRs with SR-B receptors (Detournay et al., 2012; Neubauer, Poole, Neubauer, et al., 2017). The candidate TSR genes identified in the slugs differ among the three species and to those known from cnidarians, where a previous analysis of the gene expression showed an upregulation of semaphorin 5A and a trypsin-like gene (Neubauer, Poole, Neubauer, et al., 2017). Thus, if there is an interaction of TSRs with SR-Bs in the slugs, the exact TSR proteins involved vary with the different slugs and compared to cnidarians. Yet, whether the identified candidate genes in the slugs are indeed involved in symbiont recognition and whether the TSRs are interacting with the SR-Bs (Figure 8), and hence are involved in promoting the symbiont tolerance by the host (Detournay et al., 2012; Mansfield & Gilmore, 2019), remains to be experimentally tested.

CTLD-containing receptors are upregulated in all three slugs, and evidence exists that glycan–lectin interactions are relevant in the symbiont recognition process in cnidarians (reviewed in Davy et al., 2012; Mansfield & Gilmore, 2019). For instance, lectins can bind to conserved glycans in *Symbiodinium* cell walls inducing phagocytic processes, and the activation of the complement pathway (Davy et al., 2012; Fransolet et al., 2012; Koike et al., 2004; Lin et al., 2000; Logan et al., 2010; Poole et al., 2016; Wood-Charlson et al., 2006). Lectins have been found surrounding symbionts in gastrodermal host cells (Jimbo et al., 2000, 2005; Kvennefors et al., 2008, 2010) and they can induce *Symbiodinium* transformation, from the motile stage to a coccoid nonmotile stage, suitable for the symbiosis establishment (Koike et al., 2004).

An involvement of lectins in chloroplast recognition is, however, uncertain. In some plant species, for instance in the pea *Pisum sativum* (Keegstra & Cline, 1982), the outer membrane of the chloroplasts lacks glycoproteins, which would prevent interactions with lectins. Whether the chloroplasts of the Sacoglossan food sources lack glycoproteins too is unknown (Figure 8). The outer envelope of primary chloroplasts, as in *Acetabularia acetabulum*, the food source of *E. cornigera* and *E. timida*, is generally rich in galactolipids (mono- and digalactosyldiacylglycerol), phosphatidylcholine, and low in phospholipids, with a small portion of phosphatidylglycerol (Block et al., 2007; Keegstra & Yousif, 1986), and has the highest lipid to protein ratio among any plant membrane (Block et al., 1983). Secondary chloroplasts, as in *Vaucheria litorea*, the food source of *E. chlorotica*, possess three to four membranes consisting also of mono- and digalactosyldiacylglycerol, although the precise location of these galactolipids in the various plastid membranes is still unclear (Petroutsos et al., 2014). In the case of *V. litorea* plastids, the membranes are also associated with the endoplasmic reticulum in what is called the chloroplast endoplasmic reticulum (Graves et al., 1979; Rumpho et al., 2000). In *E. chlorotica*, the outer two membranes of the kleptoplasts are, however, degraded (Rumpho et al., 2000). What mechanism underpins this degradation and whether it occurs before or after the ingestion is unknown, but might be an, additional, important factor regarding chloroplast recognition. Nevertheless, an involvement of SR-E-like or other C-type lectin domain-containing receptors should not be ruled out (Figure 8).

The recognition process also includes the release of compounds by the symbiont. For instance, glycoconjugates are thought to serve as species-specific signaling molecules, important during recognition and maintenance of the symbiont (Markell & Wood-Charlson, 2010). So far, there is no evidence of the secretion of potential recognition

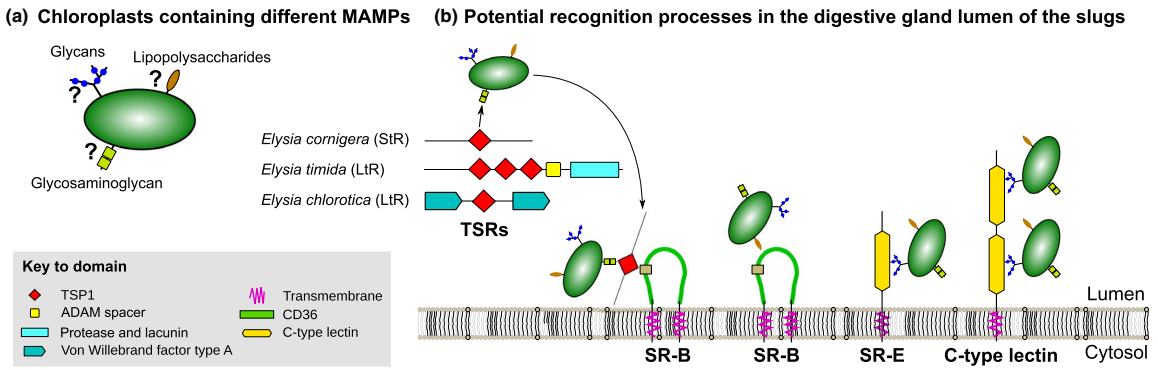


**FIGURE 8** Schematic overview of potential recognition processes in Sacoglossa sea slugs. (a) The exact composition of glycans, lipopolysaccharides, and glycosaminoglycans of the chloroplast is still unknown for *Acetabularia acetabulum* and *Vaucheria litorea*. (b) TSRs are expressed in a species-specific manner and might bind to glycosaminoglycan to enhance binding to SR-B. The chloroplasts can potentially also directly bind to SR-Bs through lipopolysaccharides. Further, SR-E and C-type lectins can bind to glycans

signal molecules from the chloroplast in a kleptoplastic system and at least the lipidome does not undergo any shifts during the onset of functional kleptoplasty (Rey et al., 2017). Thus, if and how the chloroplasts might be actively enhance the recognition process remains elusive.

The present study made a step toward compiling a list of candidate genes potentially involved in chloroplast recognition in Sacoglossa, but the exact mechanisms are still far from being understood. This is in part due to the fact that available transcriptomic data are heterogeneous, making it hard to infer a general pattern. Furthermore, in particular for juveniles of *E. chlorotica* it is nearly impossible to discriminate between gene expression related to development or chloroplast recognition. Based on the expression analyses between the different phases of functional kleptoplasty, it seems that during each transition, the gene expression changes considerably, especially during the stable phase of functional kleptoplasty, which could be more related to development than to functional kleptoplasty (see also Chan et al., 2018). Future research should thus focus on generating homogenous datasets including aposymbiotic animals in order to help understand how Sacoglossa can recognize and subsequently maintain their kleptoplasts. This task is, however, particularly complicated, because only for the StR species *Elysia viridis* aposymbiotic adults could be cultured so far under laboratory conditions (Rauch et al., 2018), but there is no transcriptomic dataset available for this species. Further, a comparative analysis using shelled species, that digest the chloroplast extracellularly, combined with homogenous datasets of StR and LtR species, would have the potential to allow for a better understanding of the mechanisms and the evolution of gene expression related to incorporate chloroplasts in plastid-bearing sea slugs.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTION

**Jenny Melo Clavijo:** Conceptualization (equal); Formal analysis (equal); Investigation (lead); Methodology (equal); Visualization (equal); Writing-original draft (lead). **Silja Frankenbach:** Formal analysis (equal); Resources (equal); Validation (equal); Writing-review & editing (equal). **Cátia Fidalgo:** Formal analysis (equal); Validation (equal); Writing-review & editing (equal). **Joao Serôdio:** Formal analysis (equal); Resources (equal); Writing-review & editing (equal). **Alexander Donath:** Data curation (equal); Formal analysis (equal); Methodology (equal); Validation (equal); Writing-review & editing (equal). **Angelika Preisfeld:** Formal analysis (equal); Resources (equal); Supervision (lead); Writing-review & editing (equal). **Gregor Christa:** Conceptualization (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (equal); Project administration (lead); Resources (equal); Supervision (supporting); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal).

## DATA AVAILABILITY STATEMENT

Raw reads of *Elysia chlorotica* (SRA sample accession SRS3101883), *Elysia timida* (SRS706683), and *Elysia cornigera* (SRS706681) are accessible via download from GenBank. The assembled transcriptomes are accessible via download from GenBank (*E. cornigera*: GBRW00000000.1; *E. timida*: GBRM00000000.1) or elsewhere (*E. chlorotica:* http://cyanophora.rutgers.edu/Elysia-expression/). Annotation tables of each species can be downloaded from DRYAD: https://doi.org/10.5061/dryad.ttdz08kw2.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## Supplementary Material

The supplementary tables can be found online under the section "Supplementary Information" at: https://onlinelibrary.wiley.com/doi/full/10.1002/ece3.6865

## Chapter 2.3

# The nudibranch *Berghia stephanieae* (Valdés, 2005) is not able to initiate a functional symbiosome to maintain *Breviolum minutum* (J.E.Parkinson & LaJeunesse, 2018).

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# The nudibranch *Berghia stephanieae* (Valdés, 2005) is not able to initiate a functional symbiosome-like environment to maintain *Breviolum minutum* (J.E.Parkinson & LaJeunesse 2018)

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Photosymbiosis is found in different animal lineages and is best understood in cnidarians. A successful initiation and maintenance of the symbiosis between the animal hosts and the photosymbiotic partners is based on a recognition by specific host receptors. This triggers signaling cascades that promote the photobiont tolerance by the host, including an interpartner nutrient exchange and the ability of the host to cope with increased levels of reactive oxygen species (ROS) generated by the photobiont. Key to the successful symbiosis is the inhibition of the phagosomal maturation resulting in the formation of the symbiosome. In animals other than cnidarians, little is known about the photosymbiosis initiation and maintenance, for instance in sea slugs belonging to the Nudibranchia. Here, we investigated the gene expression profile of *Berghia stephanieae*, which is able to incorporate *Breviolum minutum* from its cnidarian prey *Exaiptasia diaphana* (Rapp, 1829) but is not able to maintain the algae for more than a couple of days during starvation. We show that the recognition of the algae is based on similar mechanisms present in cnidarians, and we identified some additional candidate genes that might be molluscan specific for photobiont recognition. Downstream, *B. stephanieae* responds to increased levels of ROS but is not able to stop the phagosomal maturation or decrease the immune response against *B. minutum*, which seem to be the key factors missing in *B. stephanieae* that accounts for the unstable symbiosis in this slug. Hence, *B. stephanieae* can be considered a transitional state toward a stable photosymbiosis and can help to elucidate general aspects of the evolutionary processes involved in establishing photosymbioses in animals.

KEYWORDS

nudibranchia, photosynthetic symbiosis, onset of symbiosis, *de novo* transcriptome, symbiont recognition

## Introduction

Several animal lineages evolved the ability to engage in a symbiotic relationship—the so-called *photosymbiosis*—with unicellular algae or Cyanobacteria (Melo Clavijo et al., 2018). This symbiosis is especially beneficial for the animal host in oligotrophic environments (Stanley and Lipps, 2011; Roth, 2014) and is based on a nutritional exchange between the animal host and the photosynthetic symbiont (the photobiont) (Matthews et al., 2017; Rädecker et al., 2021). Most photosymbiotic animals acquire the photobiont from the environment (horizontal transmission), but some marine slugs belonging to the Cladobranchia (Gastropoda, Nudibranchia) evolved a unique strategy. Cladobranchs feed on photosymbiotic cnidarians and specifically incorporate the prey's symbiotic dinoflagellates (Symbiodiniaceae) into epithelial cells of their own digestive gland system (Rudman, 1987; Burghardt and Wägele, 2004; Burghardt et al., 2005; Burghardt and Wägele, 2006; Burghardt et al., 2008; Wägele et al., 2010). This process makes the slugs the only known secondary host of photobionts in the animal kingdom (Rousseau, 1934; Rousseau, 1935; Wägele and Johnsen, 2001). Depending on the Cladobranchia species, the algae are then directly digested intracellularly (non-symbiotic species) or maintained physiologically active for variable times as photobionts, ranging from a couple of weeks (unstable symbiosis) to several months (stable symbiosis) (reviewed in Rola et al., 2022). In species with unstable symbiosis, the photobionts are often found photosynthetically active in the animal's feces, suggesting a symbiont expelling mechanism that might be based on vomocytosis (Jacobovitz et al., 2021); in species with stable symbiosis, the healthy photobionts are not expelled and are even able to divide intracellularly (Kempf, 1991; Burghardt and Wägele, 2004; Burghardt and Wägele, 2004; Burghardt et al., 2005; Burghardt and Wägele, 2006; Burghardt et al., 2008; Wägele et al., 2010; Rola et al., 2022).

The evolution of photosymbiosis in Cladobranchia, and hence the mechanisms underpinning the selective photobiont recognition and the subsequent maintenance for the varying time periods, remains unknown. Regardless of the evolutionary distance between the slugs and their cnidarian prey, photobiont recognition and maintenance mechanisms might indeed be similar (Chan et al., 2018; Melo et al., 2020). The winnowing, a series of complex steps that occur to establish a stable symbiosis (Nyholm and McFall-Ngai, 2004), has been well described in cnidarians (Davy et al., 2012) and can serve as a reference to understand if the same mechanisms are at play in Cladobranchia. For instance, in cnidarians the successful initiation of the photosymbiosis is initiated by the interaction of pattern recognition receptors (PRRs) of the host, such as scavenger receptors and lectins, with membrane-associated molecular patterns (MAMPs) of the photobiont like glycans (reviewed in Davy et al., 2012, and Mansfield and Gilmore, 2019). This is followed by an inhibition of the phagosomal maturation, where Rab proteins, V-ATPases, and lysosomes are highly involved (Davy et al., 2012). The resulting membrane surrounding the photobiont intracellularly is known as symbiosome (Neckelmann and Muscatine, 1983; Hinde and Trautman, 2001; Kazandjian et al., 2008) and is highly relevant for interpartner nutritional exchange (Hill and Hill, 2012; Mohamed et al., 2016). A stable photosymbiosis is then based on tolerating the photobiont, involving processes like the quenching of reactive oxygen species (ROS), a constant nutrient exchange (Rädecker et al., 2021), and the decrease of the innate immune response where the complement system and signaling pathways like the transforming growth factor-beta (TGF-β), the Toll-like receptor (TLR), and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are major players (Mansfield and Gilmore, 2019). Only recently, the initial steps of the photosymbiosis establishment have been addressed in the context of the functional kleptoplasty of Sacoglossa sea slugs, revealing evidence for a recognition machinery that is in part conserved among evolutionary distant related taxa (Chan et al., 2018; Melo Clavijo et al., 2020). Yet, in Cladobranchia, and other photosymbiotic animals, mechanisms involved in the establishment of photosymbiosis have not yet been studied.

In the present study, we address this knowledge gap by using the cladobranch *Berghia stephanieae* (Valdés, 2005). *B. stephanieae* obtains its photobionts by feeding on sea anemones from the genus *Exaiptasia* (Carroll and Kempf, 1990; Valdés, 2005; Dionísio et al., 2013) and is considered a transitional form toward a stable photosymbiosis: even though the photobionts are kept for a few days to 1 week, their maintenance does not seem to provide any physiological advantages to the slug (Kempf, 1991; Bleidißel, 2010; Mies et al., 2017; Monteiro et al., 2019a, b). Moreover, *Exaiptasia diaphana* is used as a model organism to study the photosymbiosis in cnidarians (Dungan et al., 2020), and both animals can be cultivated in symbiotic and aposymbiotic (symbiont-free) states under laboratory conditions. Hence, *B.*

*stephanieae* is an ideal organism for studying the evolution of photosymbiosis in Cladobranchia, because it allows a direct comparison of known mechanisms involved in photobiont recognition and maintenance in taxonomically divergent taxa. Studying the gene expression of *B. stephanieae*, we found that one major step toward the evolution of a stable photosymbiosis in Cladobranchia might be based on the inhibition of the photobiont digestion/expulsion and the immunosuppression response of the host against the photobiont.

## Methods

### *Exaiptasia diaphana* culture

Symbiotic individuals of *Exaiptasia diaphana* were obtained from a local provider (Seepferdchen24 Meeresaquaristik GmbH, Germany) in February 2019 and were maintained in a 55-l tank (60 cm × 30 cm × 30 cm) filled with circulating artificial seawater (ASW) (AB Reef Salt, Aqua Medic, Germany), keeping the salinity at 33 Practical Salinity Units (PSU), the temperature at 25°C, pH at 8, and light intensity at 30 µmol photons $m^{-2}$ $s^{-1}$ provided by RGB top light for reef tanks (Daylight Sunrise 520, Sera), on a 12-h light/12-h dark cycle. To ensure a stable microbiome in the tank (Godoy-Olmos et al., 2019; Xiao et al., 2019), 2 BactoBalls (Fauna Marin GmbH, Germany) were added and replaced every 2 weeks. In parallel, brine shrimp *Artemia* were cultivated using the Artemio Set and 16 g of ArtemioMix eggs + salt (JBL, Germany). The anemones were fed with freshly hatched brine shrimp nauplii *Artemia* two to three times per week.

In order to produce aposymbiotic individuals of *E. diaphana*, 40 animals were placed in a separate 10-l tank with circulating artificial seawater and menthol (20% w/v in ethanol; Carl Roth, Germany) at a final concentration of 0.19 mM as recommended by Matthews et al. (2016). Minor changes to this procedure were done and are described as follows: to induce bleaching without causing mortality, the anemones were placed in the menthol tank for 6 h at light intensity of 30 µmol photons $m^{-2}$ $s^{-1}$ provided by RGB top light for reef tanks and later incubated in another 55-l tank with ASW and 0.21 M monolinuron (Algol, JBL, Germany) for 18 h in total darkness. At the beginning of this 18-h incubation period, the anemones were fed with freshly hatched nauplii of *Artemia* three times per week. Monolinuron acts as a photosynthesis blocker, thus preventing unwanted algal blooms and reducing the possibility of any symbiont recolonization (Arnaud et al., 1994). This process of menthol + monolinuron incubation was done for 4 consecutive days, with a 3-day pause, during which the anemones were kept in the monolinuron tank in total darkness. The bleaching procedure was performed for 1 month at 23°C, with pH 8, and the salinity was kept at 33 PSU, compensating any loss due to evaporation with newly fresh water. The bottom of all tanks was cleaned, and 30% of the water was changed weekly. To confirm the aposymbiotic status, individuals were visually inspected with the stereo microscope (SteREO Discovery V.8, Zeiss, Germany), light microscope (Imager A2, Zeiss, Germany), and fluorescence microscope (Biozero, Keyence, Japan).

### *Berghia stephanieae* culture

Six breeding pairs of *B. stephanieae* were obtained from a local provider (Seepferdchen24 Meeresaquaristik GmbH, Posthausen) in February 2019 and cultivated in the lab at 23° C, at a day/night cycle of 12 h/12 h. Each pair was kept in a 50-ml plastic container with a lid (75 mm, FAUST, Germany) in 35 ml of ASW. Water changes (50%) were made three times per week, with freshly prepared ASW with a salinity of 33 PSU, pH of 8.0, and temperature of 23°C. The slugs were fed with small symbiotic *E. diaphana* anemones (7-mm foot and 4-mm oral disk or 4-mm foot and 3-mm oral disk) three times per week. Once the breeding pairs spawned, the egg masses were collected and placed individually in 25-ml plastic containers with lids (55 mm, FAUST, Germany) with ASW and maintained as stated above.

The embryological development was monitored, and as soon as the animals hatched they were separated into two groups: symbiotic and aposymbiotic. Depending on the group, the animals were given several cut tentacles as food, symbiotic tentacles for the symbiotic group and aposymbiotic tentacles, or small aposymbiotic anemone pieces for the aposymbiotic group, three times a week. When the animals had developed cerata and were around 1 cm in length, they were fed with small symbiotic or aposymbiotic anemones (7-mm foot and 4-mm oral disk or 4-mm foot and 3-mm oral disk), according to their feeding group.

### Symbiodiniaceae identification

In order to identify the Symbiodiniaceae genotype present in *B. stephanieae* and in its anemone prey *E. diaphana*, total DNA was extracted from three anemones and three slugs from our cultures using the E.Z.N.A® Mollusc DNA Kit, Omega (Georgia, USA). Then, in a total volume of 20 µl, 5 µl of the total DNA was used as a PCR template using 10 µl of 2× concentrated GoTaq® Green Master Mix (Promega, USA) and 10 mM of forward and reverse primers for the Internal Transcriber Space 2 (ITS2) gene attached to Illumina adapter sequences (ITS2 forward: TCT TTC CCT ACA CGA CGC TCT TCC GAT CT GTG AAT TGC AGA ACT CCG TG (Pochon et al. (2001)); ITS2 reverse: GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T CCT CCG CTT ACT TAT ATG CTT (Stat et al. (2009)); Illumina adapter sequence underlined). A three-step PCR was done using

a Gradient Mastercycler® (Eppendorf, Germany), with a 15-min initial denaturation at 95°C, followed by 30 cycles of 1-min denaturation at 94°C, 1 min of primer annealing at 54°C, and 1 min of elongation at 70°C, and ending in a 10-min final elongation at 70°C. The PCR products were visualized with an electrophoresis gel and subsequently purified with 0.7 volume of AMPure XP beads (Beckman Coulter, USA). A second PCR was performed with 10 µl of 2× Q5® High-Fidelity Master Mix (New England Biolabs, UK), 10 mM of Illumina sequencing adapters containing TruSeq indexes, and 5 µl of purified PCR products as template in a 20-µl total volume. The amplification protocol included a 5-min initial denaturation at 95°C, 9 cycles of 1 min at 94°C denaturation, a 1-min annealing step at 52°C, and 1 min of elongation at 70°C, ending with a 10-min elongation at 70°C. The PCR products were purified with 0.7 volume of AMPure XP beads, quantified with a Qubit fluorometer (Thermo Fisher, USA), and concentrated equimolarly to 5 nM. The samples were sequenced on a MiSeq platform (Illumina, Germany) at the Saarland University using the MiSeq Reagent Nano Kit v2 (500-cycle) chemistry (Illumina, Germany). The quality of raw reads was inspected using FastQC v0.11.8 Andrews (2010), adapters were removed, and sequences were quality trimmed using cutadapt v1.15 Martin (2011) with -m 10 -O 17 -e 0 -q 20,20 parameters. Subsequently, the sequences were imported into QIIME 2 v2021.4 (Bolyen et al., 2019) and were denoised with a maximum error rate of 2 using the dada2 plugin (Callahan et al., 2016). For Symbiodiniaceae assignment, we first trained an ITS2 classifier using the Symbiodatabacea database published by Fujise et al. (2021) and then followed the qiime2 pipeline to obtain Symbiodiniaceae annotations for our samples. Annotations were only considered valid if at least 150 sequences could be assigned to the according annotation for each sample (Supplementary Table 1, Supplementary Figure 1; BioProject ID: PRJNA812737; BioSample IDs: SAMN29176735-SAMN29176740; GenBank SRA: SRR19736587-SRR19736592).

## Experimental design and sample collection

Nine nudibranchs fed with symbiotic anemones were selected from the culture, and each individual was placed in one 25-ml container with ASW. The animals were separated into three groups, with three animals per group. The first group was fed three times a week with symbiotic anemones (Fed). One hour after feeding, they were snap frozen on dry ice in a -80°C freezer until processing. The second group was fed only once followed by a starvation period of 7 days (Starved). Once the starvation period ended, the animals were snap frozen at -80°C until processing. The third group was fed only once, starved for 7 days, and refed with small symbiotic anemones (Re-fed). One hour after refeeding, they were snap frozen at -80°C until processing.

The same experimental design was applied for other nine nudibranchs fed with aposymbiotic anemones, divided into three groups, with three animals per group. The groups were as follows: Fed with aposymbiotic anemones, Starved, and Re-fed with aposymbiotic anemones after starvation.

During the experiment, the water of all 18 containers was changed (50%) three times a week and they were maintained with the parameters previously described. For each feeding group, samples were collected and stored as stated before.

## RNA isolation and sequencing

For the 18 slug samples corresponding to six feeding conditions described before (BioSample IDs: SAMN26426654-SAMN26426659), total RNA was extracted from whole animals using the my-Budget RNA Mini Kit (Bio-Budget Technologies, Germany) following the manufacturer's instructions. The RNA concentration was quantified with Qubit fluorometric quantification (Thermo Fisher, USA) and NanoDrop™ One/One$^{C}$ (Thermo Fisher, USA). A total amount of 800 µg RNA per sample was used for sequencing with Novogene (UK). Poly(A) mRNA enrichment, library preparation using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, USA), and 150-bp paired-end sequencing using the Illumina NovaSeq 6000 System were done by Novogene (UK). Samples were run in multiple lanes separating symbiotic from aposymbiotic samples, and all samples were multiplexed using double indices. Batch effects based on using different lanes are excluded based on principal component analysis (PCA) showing that the separation of samples is based on symbiont presence/absence or on feeding state.

## Transcriptome assembly and annotation

A total of 1,210,391,662 reads were obtained (Supplementary Table 2; BioProject ID: PRJNA812737; GenBank SRA: SRR18218271-SRR18218254). Given the presence of the prey and the symbiont genes in the raw reads, a filtering step was performed by mapping the raw reads to the nucleotide sequences of the genome scaffolds of *E. diaphana* (Baumgarten et al., 2015), *Cladocopium* sp. *C1$^{acro}$*, *Fugacium kawagutii* (Liu et al., 2018), *S. microadriaticum* (Aranda et al., 2016), and *Breviolum minutum* (Shoguchi et al., 2013) using BBSplit as implemented in BBTools v38.90 with qin=33 as input parameter (Bushnell, sourceforge.net/projects/bbmap/). This filtering step resulted in 1,038,020,410 reads that could not be mapped to either the anemone or the dinoflagellates (Supplementary Table 2). An assembly for these unmapped reads was done using Trinity v2.12.0 (Henschel et al., 2012; Haas et al., 2013), which resulted in 1,554,424 transcripts. The assembled transcriptome was then clustered with CD-HIT-EST v4.8.1 with -c 0.9 -n 7 -B 1 -g 1 -s

0.9 parameters (Li and Godzik, 2006; Fu et al., 2012), which reduced the assembly to 1,274,094 transcript clusters (Supplementary Data 1). TransDecoder v5.5.0 (Haas and Papanicolaou, 2016) was then used to translate the transcript clusters into the longest open reading frame (Supplementary Data 2). To assess sequence completeness, the amino acid sequences were compared against the mollusca_odb10 BUSCO database using BUSCO v5.1.3 (Supplementary Table 3). The amino acid dataset was then annotated by a BLASTP search (part of the BLAST+ package v2.9.0) against the UniProt database version 03/2021 (The UniProt Consortium, 2021), the genomes of *Fugacium kawagutii*, *Cladocopium* sp. *C1$^{acro}$* (Liu et al., 2018), *S. microadriaticum* (Aranda et al., 2016), and *Exaiptasia diaphana* (Baumgarten et al., 2015), setting the E-value to 1e$^{-10}$. Functional annotations of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers were obtained using eggNOG-mapper v2 (Cantalapiedra et al., 2021). Taxonomic assignment for each protein sequence was done using the UniProt taxonomic database. Sequences were subsequently filtered for metazoan annotations excluding annotations with their best hit to *Exaiptasia* and annotations of bacteria, fungi, and plants (Supplementary Data 3). The annotations were then screened for innate immune elements involved in symbiont recognition and maintenance such as scavenger receptors, C-type lectins, Toll-like receptors, complement components, the thrombospondin-type-1 repeat (TSR) domain-containing proteins, components of the TGF-ß signaling pathway, genes associated with immune suppression, apoptosis, phagosome maturation, nutrient transporters, and reactive oxygen species (ROS) quenching. An additional search with InterProScan was done in order to identify the transcripts with a characteristic domain architecture correspondent to the innate immune recognition receptors that were not identified based on the annotation, following the classification done in our previous work (Melo Clavijo et al., 2020). Additionally, KEGG pathways of apoptosis, autophagy, Toll-like receptor, complement, and TGF-ß signaling pathways were reconstructed using the online KEGG Mapper tool (https://www.genome.jp/kegg/tool/map_pathway.html).

## Differential gene expression analysis

The transcript abundance of sequences was estimated with kallisto v0.46.1 (Bray et al., 2016) following the Trinity pipeline v2.12.0 (Henschel et al., 2012; Haas et al., 2013). Differential expression analysis was done with DeSeq2 v1.32.0 (Love et al., 2014) implemented in Trinity. Differentially expressed genes (DEGs) were defined with FDR p-values of 0.005 and 0.05. Genes with expression values of more than three TMM in at least two replicates of one condition were considered. The normalized expression data corresponding to all genes found in the transcriptome, the annotated ones, and the DEGs were analyzed *via* principal components using the prcomp function in R v4.0.2 (R Core Team, 2021). Gene ontology analysis with adaptive clustering were performed using the GOMWU R package (https://github.com/z0on/GO_MWU) to measure whether each GO category was significantly enriched according to the expression value (Wright et al., 2015). EuKaryotic Orthologous Groups (KOG) class enrichment tests were performed using the KOGMWU R Package v1.2 (Dixon et al., 2015) and published gene expression data of animals under different experimental conditions: the coral *Stylophora pistillata* exposed to heat stress (Meyer et al., 2011; Dixon et al., 2015), different fluorescence morphotypes of the larvae of the coral *Acropora millepora* (Strader et al., 2016), symbiotic *vs.* aposymbiotic states of the anemone prey *Exaiptasia diaphana* (Lehnert et al., 2014), and the dauer and diapause dataset of the nematode *Caenorhabditis elegans* (Sinha et al., 2012) and the midge *Sitodiplosis mosellana* (Gong et al., 2013). With the KOGMWU analysis, delta ranks are computed as the difference between the mean rank of genes in a KOG class and the mean rank of all other genes (Dixon et al., 2015; Matz, 2019). These comparisons using KOG classes and delta ranks of different datasets aid to identify a general pattern of expression in our samples, and the effect of the presence of the symbiont and the starvation as a stress factor. Schematic models were created with BioRender.com.

# Results

*E. diaphana* and *B. stephanieae* both harbored *Breviolum minutum* as a photobiont. Only in one anemone did we additionally identify *Cladocopium* and *Symbiodinium*, but in low frequency (248 and 191 sequences, respectively; Supplementary Figure 1, Supplementary Table 1).

## Gene expression plasticity depends on symbiotic state and starvation

Overall, the gene expression pattern of animals feeding and refeeding with symbiotic prey were distinctly separated in a principal component space from each other and from all other experimental conditions (Figure 1). The samples corresponded to animals feeding and refeeding with aposymbiotic prey, and starved symbiotic and aposymbiotic animals clustered together, independently of the group of genes selected for the analysis (only annotated genes, only differentially expressed genes).

For the comparison between symbiotic *vs.* aposymbiotic fed animals and symbiotic *vs.* aposymbiotic refed animals, significantly enriched gene ontology (GO) terms were identified in the Biological Processes (BP), Cellular Components (CC), and Molecular Function (MF) categories

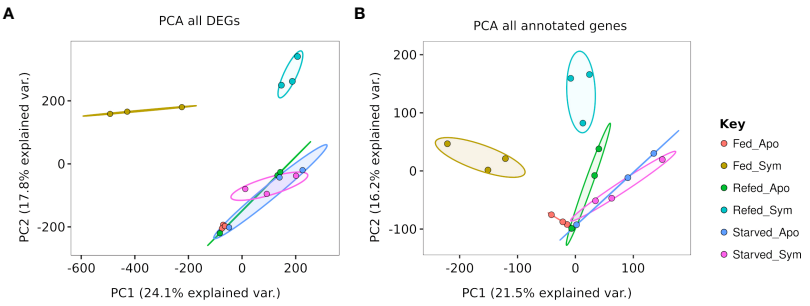


FIGURE 1
Principal component analysis of rlog-transformed counts for **(A)** all differentially expressed genes (DEGs) (n = 184,798) and **(B)** all annotated genes (n = 40,229) identified in the transcriptome of *Berghia stephanieae*.

(Supplementary Figure 2). No significantly enriched GO terms could be found for starved animals.

In symbiotic fed animals, the GO term "antigen processing and presentation of peptide antigen *via* MHC class II" in the BP category was predominantly upregulated. Processes in the BP category related to protein hydrolysis such as "positive regulation of peptidase activity" and "positive regulation of cysteine-type endopeptidase activity," "regulation of cell death," "metabolic and biosynthetic processes," and "gene expression" were predominantly downregulated. Within the CC category, the GO term "plasmodesma," important in cellular communication, was enriched exclusively among the upregulated genes. Regarding the MF category, GO terms associated with protein endohydrolysis were strongly upregulated, while signaling receptor activity was downregulated in fed animals.

In refed animals, cellular components and biological processes depending on the microtubule cytoskeleton and related to cellular division were enriched among the upregulated genes. GO terms related to the extracellular region and the nucleoplasm were downregulated in symbiotic refed animals (Supplementary Figure 2).

We then compared the KOG delta ranks with previously published data on cnidarians that were exposed to heat stress (Meyer et al., 2011; Dixon et al., 2015), on different fluorescence morphotypes of the larvae of the coral *Acropora millepora* (Strader et al., 2016), on the anemone prey *Exaiptasia diaphana*, for which a comparison of the gene expression between symbiotic and aposymbiotic animals is available (Lehnert et al., 2014), and with the dauer and diapause dataset of the nematode *Caenorhabditis elegans* (Sinha et al., 2012) and the midge *Sitodiplosis mosellana* (Gong et al., 2013), respectively (Figure 2). Two main clusters were identified: one cluster included animals under stress conditions, while the other one consisted of unstressed animals. Comparisons of the gene expression between symbiotic *vs.* aposymbiotic fed and refed *B. stephanieae* and its prey *E. diaphana* clustered within the unstressed group. Starvation in *B. stephanieae* resulted in a similar gene expression compared to heat-stressed corals, regardless of the symbiotic state. In the stressed cluster, KOG terms belonging to the category "Metabolism" are predominantly downregulated, while KOG terms belonging to "Cellular processes and signaling" and "Information storage and processing" are mainly upregulated. Only the KOG term "Translation, ribosomal structure and biogenesis" was significantly enriched among upregulated genes in symbiotic *vs.* aposymbiotic fed *B. stephanieae*, while the terms "Transcription" and "Signal transduction mechanisms" were significantly enriched among the downregulated genes. In aposymbiotic starved *vs.* fed animals, the term "Cytoskeleton" was significantly enriched among downregulated genes. The term "Posttranslational modification, protein turnover, chaperones" was significantly enriched among upregulated genes.

## Symbiont recognition in *B. stephanieae*

The symbiont recognition process in *E. diaphana* is a receptor-mediated contact of the host's PRRs and the symbiont's MAMPs. Among the PRRs, the scavenger receptors from class B (SR-B) and E (SR-E), C-type lectins (like collectins), thrombospondin-type-1 repeat domain-containing proteins (TSRs), and the complement system have been previously linked to the symbiont recognition process (Figure 3A) (reviewed in Davy et al., 2012; Mansfield and Gilmore, 2019). The role in the onset of the photosynthetic symbiosis of other PRRs like Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NODs), fibrinogen-related proteins (FREPs), and peptidoglycan recognition proteins (PGRPs) is unknown. We found most of these aforementioned receptors in our gene expression analyses of *Berghia stephanieae* (Table 1).

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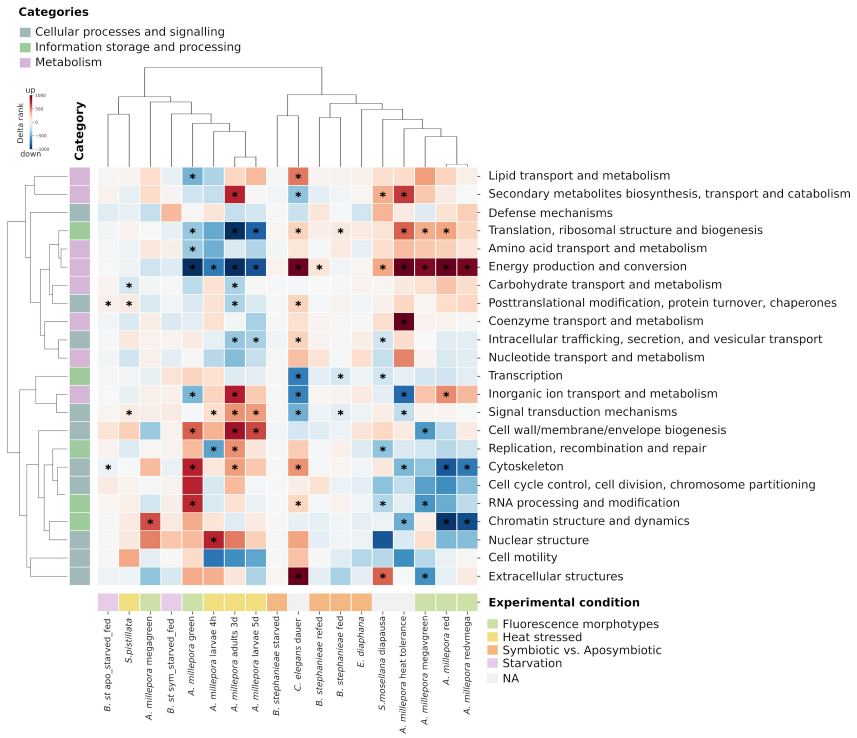


FIGURE 2
Gene expression profile based on KOG classes. Clustered heatmap of enriched KOG classes in *B.* stephanieae compared to publicly available datasets of other organisms exposed to stress factors: different fluorescence morphotypes of the larvae of the coral *Acropora millepora* (Strader et al., 2016), heat-stressed coral *Stylophora pistillata* (Meyer et al., 2011; Dixon et al., 2015), symbiotic *vs.* aposymbiotic *Exaiptasia diaphana* (Lehnert et al., 2014), the dauer state of the nematode *Caenorhabditis elegans* (Sinha et al., 2012), and the diapause state of the midge *Sitodiplosis mosellana* (Gong et al., 2013). KOG classes within three main KOG categories are shown. The regulation of the KOG classes is color-coded, where red indicates upregulation and blue downregulation based on the delta rank score. Asterisks show the KOG classes that were significantly enriched (FDR = 0.1).

Overall, 15 genes were classified as scavenger receptors, from which six belong to class B, three to class E-like, and six to class I. In order to find potential candidates involved in symbiont recognition, we compared the gene expression of these receptors between symbiotic and aposymbiotic refed animals, after a 7-day starvation period. We expected those genes relevant for symbiont recognition to be upregulated in symbiotic refed animals, because our chosen time frame of refeeding of 1 h after a starvation period should induce a photobiont-specific gene expression. In animals regularly fed, this expression pattern might be lost, as observed in *Acropora digitifera* (Mohamed et al., 2016). Out of the six SR-B genes, two were significantly upregulated: a homologue of the lysosome membrane protein 2 (DN954_c6_g1; L2FC 4.53) was upregulated in symbiotic refed animals, while the other SR-B homologue annotated as SRB1 (DN224730_c0_g3; L2FC 5.33) was upregulated in aposymbiotic refed animals (Supplementary Figure 3). From the three identified SR-E-like receptors, a snaclec agkisacutacin subunit A ($p < 0.005$: DN2954_c1_g1, L2FC 3.43) and fibulin-5 ($p < 0.05$: DN4915_c0_g1, L2FC 1.85) were significantly upregulated in symbiotic refed animals (Supplementary Figure 3). None of the SR-I genes were differentially expressed (Supplementary Data 3). Forty-one genes were classified as C-type lectins, and out of them, one C-type mannose receptor was significantly differentially expressed in symbiotic refed animals ($p < 0.005$: DN264_c2_g1, L2FC 1.36). Additionally, collectin 12 and a low-affinity immunoglobulin epsilon Fc receptor were highly expressed in symbiotic refed animals (Supplementary Figure 3 and Table 1). Nine genes were grouped as SRCR, but none of them was differentially expressed (Supplementary Data 3). Among the TSRs, eight genes were classified as ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), 15 as semaphorins, three as plexins, 11 as sequences

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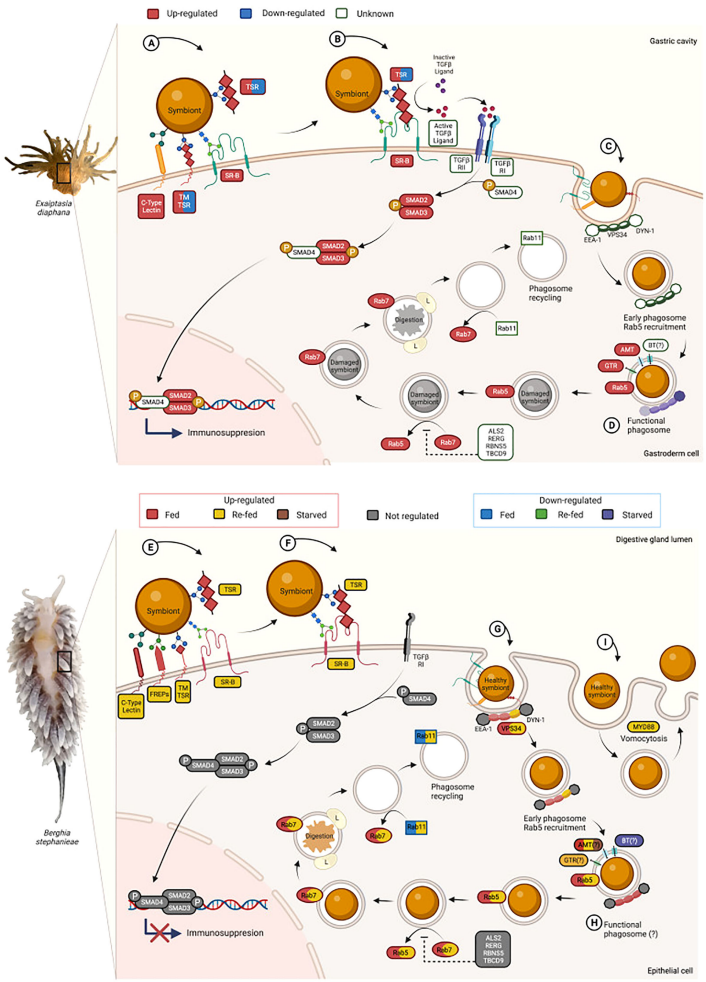


FIGURE 3
Model of the cellular events involved in the symbiont recognition and maintenance in the sea anemone *Exaiptasia diaphana* **(A-D)** based on previously published works (Chen et al., 2003, 2004, 2005, Davy et al., 2012, Detournay et al., 2012, Fransolet et al., 2012, Lehnert et al., 2014, Mohamed et al., 2016, Neubauer et al., 2016, 2017, Ishii et al., 2019) and our results on *Berghia stephanieae* **(E-I)**. In *E. diaphana*, the first step of the winnowing is the recognition of the potential symbiont **(A)** mediated by the host's pattern recognition receptors (PRRs) such as SR-Bs, SR-E-like, C-type Lectins, and TSRs. **(B)** The recognition by TSRs and/or SR-Bs seems to trigger the tolerogenic TGF-β pathway, with a high phosphorylation (P) of the transcription factor SMAD2/3 that promotes an immunosuppression response. **(C)** The phagocytosis of the potential symbiont takes place, where activator proteins like EEA-1, VPS34, and DYN-1 induce the early phagosome formation by the recruitment of Rab5. The phagosomal maturation continues by the replacement of Rab5 with Rab7, which induces the recruitment of lysosome-associated membrane protein (LAMP) and the fusion of the phagosome with lysosomes (L). Once the phagosome content is successfully degraded, the recyclement of the phagosome is triggered by the replacement of Rab7 with Rab11. **(D)** In stable symbiosis, the phagosomal maturation is inhibited and a functional symbiosome is established, where a nutrient exchange takes place *via* bicarbonate, glucose, and ammonium transporters, among others. In B. *stephanieae*, the recognition of the photobiont **(E)** might be mediated by PRRs such as SR-Bs, SR-E-like, C-type Lectins, TSRs, and/or FREPs. **(F)** The tolerogenic TGF-β pathway is not regulated, and core components like TGF-β ligand and TGF-βRII are missing. **(G)** During the early phagosome formation, only the activator protein VPS34 (PIK3C3) is activated and the recruitment of Rab5 is triggered. In contrast with its prey, in B. *stephanieae* there is no selective symbiont elimination, instead, the phagosomal maturation continues for healthy symbionts, marked by the expression of Rab7. Once the photobiont is successfully digested, the phagosome recycling is activated by the replacement of Rab7 with Rab11. **(H)** Since the phagosomal maturation is not inhibited, it seems that no functional symbiosome can be established or maintained, so no nutrient exchange takes place. **(I)** The alternative is that the photobionts cannot escape vomocytosis, given that MyD88 is activated preventing the maintenance of the photobiont in the animal host, and promoting the expulsion of healthy photosynthetically active dinoflagellates.

TABLE 1 Innate immune genes potentially involved in the photosymbiosis initiation in *Berghia stephanieae*.

| Receptor group | Gene ID | UniProtKB annotation | Protein entry | L2FC re-fed Apo *vs.* Sym |
|---|---|---|---|---|
| SR-B | DN954_c6_g1 | Lysosome membrane protein 2 | P27615 | 4.53** |
| SR-B | DN224730_c0_g3 | Scavenger receptor class B member 1 (SRB1) | P16671 | 5.33** |
| SR-E-like | DN2954_c1_g1 | Snaclec agkisacutacin subunit A | Q9IAM1 | 3.43** |
| SR-E-like | DN4915_c0_g1 | Fibulin-5 | Q9WVH9 | 1.85* |
| SR-E-like | DN1503_c0_g3 | Low affinity immunoglobulin epsilon Fc receptor | P20693 | 2.11 |
| C-type lectin | DN264_c2_g1 | C-type mannose receptors | Q9UBG0 | 1.36** |
| C-type lectin | DN79_c0_g1 | Collectin-12 | A6QP79 | 1 |
| C-type lectin | DN52138_c0_g1 | Low affinity immunoglobulin epsilon Fc receptor | P20693 | 0.3 |
| TSR | DN47020_c0_g1 | Coadhesin | B3EWZ3 | 7.15** |
| TSR | DN23670_c0_g4 | ADAMTS adt-1 | G5ECS8 | 7.69** |
| TSR | DN24418_c0_g1 | Hemicentin-1 | D3YXG0 | 7.69** |
| TSR | DN31129_c0_g1 | Hemicentin-1 | Q96RW7 | 7.29** |
| TSR | DN50968_c0_g1 | Hemicentin-1 | D3YXG0 | 6.74** |
| TSR | DN82106_c0_g1 | Hemicentin-1 | Q96RW7 | -2.32** |
| TSR | DN9953_c4_g1 | Hemicentin-1 | D3YXG0 | 1.9** |
| TSR | DN33987_c0_g2 | Spondin-1 | Q8VCC9 | 7.17** |
| Complement | DN16476_c0_g1 | Mannose-binding protein A | P39039 | 0.13 |
| Complement | DN2766_c0_g2 | Mannose-binding protein C | Q66S61 | 0.65 |
| TLR pathway | DN4567_c0_g1 | Myeloid differentiation factor 88 | A5HNF6 | 0.59 |
| FREPs | DN72561_c0_g1 | Fibroleukin | Q29RY7 | 0.66 |
| PGRPs | DN1038_c0_g5 | Peptidoglycan recognition protein 3 | Q96LB9 | 1.81 |

List of candidate proteins for photobiont recognition in *Berghia stephanieae* identified by comparing the gene expression between symbiotic re-fed animals and apo-symbiotic re-fed animals. Asterisks denote the p-value < 0.005 (**) and p-value < 0.05 (*).

containing only the TSP1 (thrombospondin-type-1) domain with transmembrane region (TM), 103 as TSP1 without TM, one astacin metallopeptidase, 2 as F-spondin-like, and 27 as sequences containing TSP1 in combination with other domains (Supplementary Data 3). Out of the 114 TSRs, eight were significantly differentially expressed in symbiotic refed animals ($p < 0.005$). From those eight TSRs, one was annotated as Coadhesin (DN47020_c0_g1, L2FC 7.15), one as ADAMTS adt-1 (DN23670_c0_g4, L2FC 7.69), five as Hemicentin-1 (DN24418_c0_g1, L2FC 7.69; DN31129_c0_g1, L2FC 7.29; DN50968_c0_g1, L2FC 6.74; DN82106_c0_g1, L2FC -2.32, DN9953_c4_g1, L2FC 1.9), and one as Spondin-1 (DN33987_c0_g2, L2FC 7.17) (Supplementary Figure 3).

Furthermore, 35 activator genes of the complement system were identified (Supplementary Data 3), yet none of them were significantly differentially expressed. Only one mannose-binding protein A and one mannose-binding protein C were highly expressed in symbiotic refed animals compared to aposymbiotic refed ones (Supplementary Figure 4; Table 1).

We identified 40 genes involved in the TLR pathway, out of which 30 were classified as Toll-like receptors (TLRs), five as myeloid differentiation primary response protein (MyD88), and five as the nuclear factor NF-kappa-B (NF-kB). Further, 13 genes were grouped as NOD-like receptors (Supplementary Data 3). None of the TLRs, MyD88, NF-kB, and NODs was significantly differentially expressed, except for one MyD88 that was upregulated in symbiotic refed animals (Supplementary Figure 4; Table 1). Sixty-eight genes were classified as fibrinogen-related proteins (FREPs), and three peptidoglycan recognition proteins (PGRPs) were identified, but none of these genes were differentially expressed in symbiotic refed animals (Supplementary Data 3; Supplementary Figure 4). Only one FREP annotated as fibroleukin (Supplementary Figure 4) and one PGRP annotated as peptidoglycan recognition protein 3 were upregulated in symbiotic refed animals compared to aposymbiotic refed ones (Supplementary Figure 4; Table 1).

## TGF-β signaling pathway is not responsible for symbiont tolerance in *B. stephanieae*

Once the symbiont is recognized in cnidarians, signaling cascades like the TGF-β signaling pathway might be triggered, leading to an immunosuppression response (Detournay et al., 2012) (Figure 3B). However, in *B. stephanieae*, core elements like the activation ligand TGF-β *sensu stricto* and TGF-β receptor II (TGFβRII) were not present (Figure 3F), and only one bone morphogenetic protein 1 (BMP1) (DN5506_c0_g1,

L2FC -2.63) was significantly downregulated in symbiotic refed animals. None of the other TGF-β signaling pathway components were expressed above three TMM in any of the feeding conditions (Supplementary Data 3).

## Phagosome maturation: Photobiont digestion and maintenance occur simultaneously

In cnidarians, the potential photobionts are phagocytized, after the successful recognition, into cells of the animal host and are still surrounded by the phagosomal membrane. In order to maintain the photobiont, the phagosomal maturation has to be prevented (Figure 3C) (Hill and Hill, 2012). Usually, newly formed phagosomes fuse with early endosomes and acquire the necessary proteins for maturation including Ras-related protein 5 (Rab5), early endosomal antigen 1 (EEA1), dynamin (DYN-1), and vacuolar sorting protein-34 (VPS34, also known as PIK3C3) (Kinchen and Ravichandran, 2008; Lee et al., 2020). In *E. diaphana*, Rab5 is only present in phagosomes with healthy photobionts (Chen et al., 2004). The early phagosome matures into late phagosome by the replacement of Rab5 with Rab7, promoting the fusion with lysosomes *via* lysosome-associated membrane protein (LAMP1 and LAMP2) recruitment, the accumulation of V-ATPases, and the subsequent acidification of the late phagosome (Lee et al., 2020). Once phagosomal degradation is initiated, the endosome is recycled by the replacement of Rab7 and the recruitment of Rab11. In cnidarian photosymbiosis, proteins like Rab22, amyotrophic lateral sclerosis2 (ALS2), TBC1 domain family member 9 (TBCD9), rabenosyn-5 (RBNS5), and Ras-related and estrogen-regulated growth inhibitor (RERG) might block Rab7 by the constant activation of Rab5, preventing the maturation of the phagosome (Mohamed et al., 2016; Lee et al., 2020). In *B. stephanieae*, Rab5A (DN60084_c0_g1) and Rab7 (DN353992_c0_g1) were significantly upregulated in symbiotic fed and refed animals ($p < 0.005$: Rab5A L2FC 8.25, L2FC 7.39; Rab7 L2FC 7.71, L2FC 8.09, respectively) (Supplementary Figure 5). Rab7A (DN23467_c1_g2, L2FC 1.90) was significantly upregulated only in symbiotic refed animals (Supplementary Figure 5). None of the activators of Rab5 (EEA1, dynamin) was significantly differentially expressed in any feeding condition; only VPS34/PIK3C3 was upregulated in symbiotic fed and refed animals (Supplementary Figure 5). Rab11A and Rab11B were downregulated in symbiotic fed animals, while they were upregulated in symbiotic refed animals. LAMP1 was significantly differentially expressed in symbiotic refed animals (DN5522_c5_g1, L2FC 1.33, $p < 0.05$). V-ATPases were significantly upregulated during feeding ($p < 0.005$: DN35123_c0_g2, L2FC 10.69; $p<0.05$: DN23173_c0_g1, L2FC 1.62; DN4712_c0_g1, L2FC 1.3) and refeeding with symbiotic prey ($p < 0.005$: DN35123_c0_g2, L2FC 8.39; $p < 0.05$: DN10201_c0_g2, L2FC 1.09; DN13065_c0_g2, L2FC 1.06; DN32461_c0_g1, L2FC 2.55; DN4147_c0_g1, L2FC 0.66) (Supplementary Data 3).

## Functional phagosome: Amino acid metabolism and nutrient transporters

In cnidarians, the arrested phagosomes harboring healthy photobionts that evade maturation would communicate with the animal host *via* membrane transporters to obtain the necessary compounds for photosynthesis (dissolved inorganic carbon in the form of bicarbonate or as CO2), translocate produced organic compounds to the host (e.g., glycerol, glucose, amino acids, lipids), and recycle the host's waste products like nitrogen (Figure 3D) (Matthews et al., 2017). The metabolic exchange is key for the stability of the symbiosis. In *B. stephanieae*, we compared symbiotic and aposymbiotic animals for each experimental condition and identified several transporters such as ammonium, bicarbonate, and glucose transporters (Figure 3H; Supplementary Figure 6). Ammonium transporters were significantly upregulated in symbiotic fed animals ($p < 0.05$: DN2986_c0_g2, L2FC 2.01), symbiotic refed animals ($p < 0.005$: DN340748_c0_g1, L2FC 8.29), and starved symbiotic animals ($p < 0.05$: DN8797_c3_g2, L2FC 2.24). Bicarbonate transporters were significantly downregulated in symbiotic starved animals ($p < 0.005$: DN3291_c0_g2, L2FC -3.58; $p < 0.05$: DN3291_c0_g1, L2FC -3.41), but not differentially expressed in any other comparison. Once transported over the phagosomal membrane, bicarbonate must be converted to $CO_2$, to make it available for photosynthesis, by carbonic anhydrases (Weis, 1993). Several carbonic anhydrases (Supplementary Figure 6) were significantly upregulated in symbiotic refed ($p < 0.005$; DN78523_c0_g1, L2FC 1.6; DN72715_c0_g1, L2FC 3.04) and symbiotic starved animals ($p < 0.05$; DN5108_c0_g1, L2FC -2.46; DN78523_c0_g1, L2FC 1.73; DN87999_c0_g1, L2FC 1.48).

For the animal host, the received glucose is a major nutrient and is essential to assimilating waste products, like ammonium, for the synthesis of amino acids. Hence, we expected that glucose transporters were upregulated in all symbiotic animals, in order to maintain the photobionts. Yet, in *B. stephanieae* glucose transporters were only significantly upregulated in symbiotic refed animals ($p < 0.05$: DN1101_c0_g1, L2FC 1.28) (Supplementary Figure 6).

Ammonium is mainly used in the amino acid synthesis through the glutamine synthetase (GS)— glutamine oxoglutarate aminotransferase (GOGAT). In this GS-GOGAT cycle, glutamate dehydrogenases (GDH) convert ammonium into glutamate and GS transforms ammonium into glutamine.

In *B. stephanieae*, one GDH was significantly downregulated in symbiotic refed animals ($p < 0.05$: DN134779_c0_g1, L2FC 2.16) (Supplementary Figure 6). One GS homologue was significantly upregulated in symbiotic refed animals ($p < 0.005$: DN34097_c3_g2, L2FC 2.16) and symbiotic starved animals ($p < 0.05$: DN34097_c3_g2, L2FC 1.99) (Supplementary Figure 6). No GOGAT homologue could be identified.

### Host oxidative stress is promoted by the photobiont presence

The presence of the photobiont may induce additional stress to the animal host by photosynthetic-derived reactive oxygen species (ROS). The maintenance of the photobiont is linked to the ability of the host to cope with this additional stress induced by the photobiont (Richier et al., 2005; 2006; Lesser, 2011). Hence, we expected ROS quenching mechanisms to be active in symbiotic animals. In *B. stephanieae*, catalases were significantly upregulated in symbiotic refed animals ($p < 0.005$: DN2499_c9_g2, L2FC 1.83) but also during feeding and refeeding with aposymbiotic prey ($p < 0.005$: DN5526_c0_g1, L2FC -2.46, -2.63, respectively) (Supplementary Figure 7). Glutathione peroxidases were also significantly upregulated in symbiotic refed animals ($p < 0.005$: DN11199_c1_g1, L2FC 2.42; $p < 0.05$: DN39313_c1_g1, L2FC 1.92) but significantly downregulated in aposymbiotic refed animals (DN39313_c1_g2, L2FC -4.59). Glutathione S-transferases were significantly upregulated in symbiotic refed animals ($p < 0.005$: DN57713_c0_g1, L2FC 2; $p < 0.05$: DN32195_c4_g1, L2FC 0.89). Likewise, peroxiredoxins and peroxiredoxin-like proteins were significantly differentially expressed during feeding and refeeding with symbiotic prey compared to aposymbiotic fed and refed animals ($p < 0.005$: DN1905_c0_g2, L2FC 8.75, 9.10; DN40077_c0_g2, L2FC 11.03, 9.63, respectively) (Supplementary Figure 7).

## Discussion

In the present study, we provide the first differential gene expression analysis of a dinoflagellate-bearing sea slug belonging to Cladobranchia. The comparison of symbiotic and aposymbiotic animals either feeding, refeeding, or starving revealed a unique insight into mechanisms involved in the recognition of Symbiodiniaceae obtained from the slugs' cnidarian prey (Figure 3E). Starvation induced a gene expression profile similar to heat-stressed corals (Meyer et al., 2011; Dixon et al., 2015), including a downregulation of essential metabolic processes (Figure 2). Moreover, in *Berghia stephanieae* mechanisms to cope with reactive oxygen species are induced in the presence of the photobiont (Supplementary Figure 7). Yet, the immune response against the photobiont is not suppressed and the phagosome maturation is not inhibited. Thus, the slug are probably not able to maintain the photobionts in the long term perhaps due to the inability to initiate the formation of a functional and stable symbiosome that supports a nutrient exchange between host and photobiont.

*B. stephanieae* and *E. diaphana* harbored the same symbiodiniaceae genotype (Supplementary Figure 1, Supplementary Table 1), and we found that the recognition of the algae in *B. stephanieae* (Figure 3E) might depend on the same molecular machinery present in its cnidarian prey *E. diaphana*, involving SR-B, SR-E-like, C-type lectins, and TSRs (reviewed in Mansfield and Gilmore, 2019) (Figure 3A). Hence, the photobiont recognition seems to be conserved among taxonomically divergent host taxa and independent on the phylogenetic origin of the phototrophic organism, or organelle (Melo Clavijo et al., 2020). Yet, SR-B has multi-ligand recognition and diverse cellular functions. For instance, SR-Bs recognize MAMPs from pathogens like fungi and bacteria, polyanionic ligands such as high-density and low-density lipoproteins (HDL, LDL), and oxidized phospholipids, apoptotic cells, and amyloid proteins (PrabhuDas et al., 2017). Additionally, SR-B is involved in lipid transport and phagocytosis (Silverstein and Febbraio, 2009; Yu et al., 2021). Furthermore, SR-B homologues are also highly expressed in aposymbiotic *B. stephanieae*. Hence, in the case of SR-B, the determination of a clear involvement in symbiont recognition in the slugs is complicated. This also applies to the other aforementioned receptors, but at least experimental verification has shown the relevance of SR-B, C-type lectins, and TSRs in symbiont recognition in cnidarians (Jimbo et al., 2000; Koike et al., 2004; Neubauer et al., 2016; Neubauer et al., 2017), which is why we consider these receptors as promising candidates for photobiont recognition in *B. stephanieae*.

Further candidates in photobiont recognition and photosymbiosis initiation in cnidarians belong to the TLRs and NODs, although their involvement is debated (Mansfield and Gilmore, 2019). TLRs and NODs can activate the NF-κB transcription factor, which play a central role in the immune response, inflammatory process, and cytokine release and modulation (Doyle and O'Neill, 2006; Dev et al., 2010). In cnidarian symbiosis, NF-κB is downregulated by Symbiodiniaceae (DeSalvo et al., 2010; Wolfowicz et al., 2016; Mansfield et al., 2017; Mansfield and Gilmore, 2019), yet in *B. stephanieae* NF-κB is not regulated, nor are TLRs or NODs (Supplementary Figure 4).

A potential new candidate in symbiont recognition in cladobranchs is fibroleukin, which belongs to a highly diversified group of glycoproteins in mollusks known as FREPs. Members of FREPs contain a fibrinogen domain followed by different domains (Romero et al., 2011), can recognize bacteria and fungi (Kenjo et al., 2001; Middha and Wang, 2008; Wu et al., 2011; Xiang et al., 2014; Huang et al., 2015), and are involved in agglutination, clotting, and phagocytosis during the destruction of pathogens (Huang

et al., 2015; Buchmann, 2014). There is no evidence for an involvement of fibroleukin in other photosymbioses, but more detailed analyses could illuminate a potential function.

Finally, PGRP 3 was highly expressed in symbiotic *B. stephanieae*, which belongs to receptors containing a peptidoglycan-binding type 2 amidase (PGRP) domain that binds to peptidoglycan (PG), lipopolysaccharides (LPS), and lipoteichoic acid (Royet et al., 2011). Their main role is in pathogen defense in invertebrates and in the host-microbiota symbiosis establishment, for instance, between the bioluminescent bacteria *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* (Dierking and Pita, 2020). LPS and PG are mostly present in the outer cell walls of bacteria (Diks et al., 2001), but so far there is no evidence of their presence in dinoflagellates. Yet, in *B. stephanieae* PGRP might be involved in bacterial recognition inducing a shift in the bacteriome related to the presence of a photobiont, as previously shown in its prey *E. diaphana* (Röthig et al., 2016).

After recognizing the algae and a subsequent phagocytosis, *B. stephanieae* is mainly digesting or excreting the algae and is not establishing a stable mutualistic symbiosis. Well-known photobiont maintenance mechanisms in cnidarians such as the TGF-ß pathway (Detournay et al., 2012; Berthelier et al., 2017), the complement system (Poole et al., 2016), the TLR pathway (Jacobovitz et al., 2021), and downstream NF-κB activation (DeSalvo et al., 2010; Wolfowicz et al., 2016; Mansfield et al., 2017) were not present or regulated in *B. stephanieae*, which would be essential for symbiont tolerance (reviewed in Mansfield and Gilmore, 2019).

For instance, the recognition of Symbiodiniaceae, possibly *via* SR-B and/or TSRs, triggers tolerogenic pathways like the TGF-β pathway that leads to the suppression of the host innate immune response in *E. diaphana* (Detournay et al., 2012) (Figure 3B). Yet, in *B. stephanieae*, none of the elements of this pathway were regulated. Moreover, core elements of the TGF-ß pathway were missing (e.g., the ligand TGF-β sensu stricto and the TGF-β receptor II); thereupon, there is no immunosuppression *via* the TGF-β pathway (Figure 3F). Indeed, TGF-β sensu stricto and TGF-β RII are missing in several mollusks (Herpin et al., 2004), which is why alternative pathways could be relevant for immunosuppression, such as the pathways of the complement system.

The complement system is relevant for recognition and promotes phagocytosis (Poole et al., 2016), and its downregulation seems to play a role in the onset and maintenance of the photosymbiosis in cnidarians (Kvennefors et al., 2010; Ganot et al., 2011; Poole et al., 2016; Mansfield and Gilmore, 2019). The activation of the complement system can be achieved through different pathways (Rus et al., 2005; Dunkelberger and Song, 2010; Kemper et al., 2010), resulting in the cleavage of the C3 protein that labels pathogens and recruits the macrophages for phagocytosis (Dunkelberger and Song, 2010). In *B. stephanieae*, we could only identify a potential triggering of the lectin pathway of the complement system through the mannose binding lectin (MBL2) and/or a possible activation *via* the alternative pathway through collectin-12 (Ma et al., 2015). Yet, essential ficolins and downstream components for this pathway were not differentially expressed in *B. stephanieae* (Supplementary Data 3). Thus, the complement pathway might not be relevant for the symbiosis onset in *B. stephanieae* either.

The transcriptional repression of MyD88 and NF-κB may also promote symbiosis establishment (DeSalvo et al., 2010; Wolfowicz et al., 2016; Mansfield et al., 2017; Mansfield and Gilmore, 2019; Jacobovitz et al., 2021). However, MyD88 is activated and NF-κB is not regulated in the symbiotic *B. stephanieae*, pointing to an active immune response against the algae, rather than an immunosuppression. Furthermore, it has been shown that the same symbiodiniaceae genotype performs differently depending on the host (Goulet et al., 2019). Thus, even though *E. diaphana* is in a stable photosymbiosis with *Breviolum minutum* B1/B2, *B. stephanieae* might not be able to establish a stable photosymbiosis with the strain due to photobiont intrinsic properties. Further, *E. diaphana* can be colonized by other less beneficial and productive Symbiodiniaceae such as *Symbiodinium microadriaticum* and *Durusdinium trenchii* (Gabay et al., 2018), or the heterologous *Cladocopium goreaui* (Tortorelli et al., 2020). In previous studies, *B. stephanieae* fed with anemones harboring *S. microadriaticum* A1 was able to incorporate these photobionts but only retained them for 10 days maximum (Mies et al., 2017; Monteiro et al., 2019b). Whether *B. stephanieae* can incorporate and maintain other Symbiodiniaceae genotypes present in its prey remains to be seen. To date, there is no report that identified the native symbiont genotype of this slug in the wild.

Vital for the stability of the symbiosis in intracellular photosymbioses is the inhibition of the phagosome maturation (Hill and Hill, 2012; Mohamed et al., 2016; Mohamed et al., 2020). The high expression of Rab7 in symbiotic *B. stephanieae* in combination with lysosomes recruiting LAMPs and the V-ATPases rather suggest an ongoing phagosome maturation leading to the photobiont digestion, instead of its maintenance and the establishment of the functional symbiosome (Chen et al., 2003; Chen et al., 2004; Fransolet et al., 2012) (Figure 3G). Yet, V-ATPases can also act as carbon concentration mechanisms by acidifying the phagosome (Kinchen and Ravichandran, 2008; Kinchen et al., 2008). This would then lead to a conversion of bicarbonate to $CO_2$ and a subsequent translocation of $CO_2$ to the photobiont in cnidarians and mollusks (Armstrong et al., 2018; Barott et al., 2022), promoting photosynthesis (Barott et al., 2015). For the system to work properly, carbonic anhydrases and bicarbonate transporters would be needed (Weis et al., 1989; Weis and Levine, 1996; Grasso et al., 2008; Ganot et al., 2011; Meyer and Weis, 2012; Ip et al., 2017; Chew et al., 2019), but carbonic anhydrases were highly expressed only in symbiotic *B. stephanieae* (Supplementary Figure 6).

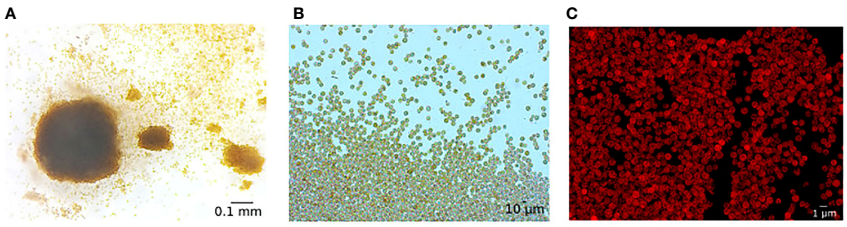


FIGURE 4
Fecal pellet of *B.stephanieae* containing intact, photosynthetically active dinoflagellates. Image taken **(A)** by Corinna Sickinger with a stereo microscope (SteREO Discovery.V8, Zeiss, Germany), 80× magnification, **(B)** by Jenny Melo with a light microscope (Imager A2, Zeiss, Germany), 200×, and **(C)** with a fluorescence microscope (BZ-X800E, Keyence, Japan), using the Texas Red Filter and a 400× magnification.

Other mollusks use different hosting strategies for harboring Symbiodiniaceae and are able to establish stable symbioses. In clams belonging to the family Cardiidae, Symbiodiniaceae are located in extracellular spaces known as the zooxanthellal tubular system (Norton et al., 1992; Hernawan, 2008). In these clams, a symbiosome *per se* is absent. Yet, the ectodermal membrane surrounding the zooxanthellal tubular system may act as some sort of symbiosome involved in signaling, acidification, and molecule transport (Armstrong et al., 2018) to establish a stable symbiosis, similar to other extracellular stable photosymbioses such as the acoelomorph *Symsagittifera roscoffensis* and the green algae *Tetraselmis convolutae* (Bailly et al., 2014).

In addition, some ammonium and glucose transporters were upregulated in symbiotic *B. stephanieae*, but starvation had no effect on the expression of these transporters (Supplementary Figure 6), which would be expected in a stable symbiosis. Particularly, ammonium is important for the algae *in hospite* to ensure a high photosynthetic activity (Taylor, 1978; Koop et al., 2001; Yellowlees et al., 2008), which is actively provided to the algae and is used to control the algal growth (Rädecker et al., 2018; Cui et al., 2019; Xiang et al., 2020). When heat stressed, corals increase the catabolic degradation of amino acids to fuel the GS-GOGAT cycle to generate α-ketoglutarate needed for the tricarboxylic acid (TCA) cycle but simultaneously increase the translocation of ammonium to the algae (Rädecker et al., 2021). As a result, the algae use their photosynthates to grow instead of translocating these to the host (Baker et al., 2018). In the slug's prey *E. diaphana*, the GS-GOGAT cycle is highly upregulated (Cui et al., 2019), while in the slug we could only observe an upregulation of GS in symbiotic animals when feeding, but not during starvation (Supplementary Figure 6). Thus, the GS-GOGAT cycle is probably not connected to a symbiotic relationship in *B. stephanieae* and is likely used to catabolize proteins during starvation to fuel the TCA cycle, similar to heat-stressed corals.

In addition to a nutrient exchange, the swift detoxification of reactive oxygen species (ROS) produced primarily by the photobiont is fundamental in a stable photosymbiosis. In *B. stephanieae*, ROS quenching mechanisms were predominantly activated in the presence of the algae (Supplementary Figure 7), similar to photosymbiotic cnidarians (Ganot et al., 2011; Meyer and Weis, 2012; Matthews et al., 2017; Yuyama et al., 2018). Starvation-induced stress prior to feeding seemed to enhance ROS quenching, particularly inducing the glutathione peroxidase.

In summary, the main challenges for cladobranchs to establish a stable photosymbiosis are thus the inhibition of the phagosomal maturation, which might be based on the successful nutrient exchange with the algae and suppressing immune responses that promote the expulsion of the photosynthetic partner. In combination with previous observations of both digested and intact photobionts in juveniles and in the feces of *B. stephanieae* (Figure 4) (Kempf, 1991; Bleidißel, 2010; Mies et al., 2017; Monteiro et al., 2019b), symbiophagy is probably the dominant process that might be even accompanied by vomocytosis as the TLR pathway is not downregulated (Jacobovitz et al., 2021) (Figure 3I). Given that gene regulation can take place at different steps and levels (posttranscriptional, translational, posttranslational, epigenetic), changes in gene expression are not definitive evidence of activation/inhibition of signaling pathways but provide information of the overall regulation of cellular processes at a certain time (Day & Tuite, 1998; Gibney and Nolan, 2010; Zhao et al., 2017).

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Author contributions

JC and GC sourced, processed the data, performed the data analyses, and edited the figures. GG and ST performed the MiSeq library preparation, sequencing, and data demultiplexing. JC, CS, SB, AP, and GC discussed and interpreted the results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.934307/full#supplementary-material

Additional Supplementary Data can be found at online at: 10.6084/m9.figshare.19690843.

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## Supplementary Material

**Supplementary Table 1.** Symbiodiniaceae annotation.

| Species | Sample-ID | BioSample IDs | SRR Accession | Input | Filtered | % of input passed filter | Denoised | Merged | % of input merged |
|---|---|---|---|---|---|---|---|---|---|
| *Exaiptasia diaphana* | Exaiptasia2 | SAMN29176735 | SRR19736592 | 2219 | 1997 | 90 | 1978 | 1886 | 84.99 |
| *Exaiptasia diaphana* | Exaiptasia3 | SAMN29176736 | SRR19736591 | 2233 | 2066 | 92.52 | 2036 | 1941 | 86.92 |
| *Exaiptasia diaphana* | Exaiptasia4 | SAMN29176737 | SRR19736590 | 2600 | 2443 | 93.96 | 2417 | 2188 | 84.15 |
| *Berghia stephanieae* | Berghia1 | SAMN29176738 | SRR19736589 | 6016 | 5708 | 94.88 | 5686 | 5495 | 91.34 |
| *Berghia stephanieae* | Berghia2 | SAMN29176739 | SRR19736588 | 7887 | 7583 | 96.15 | 7572 | 7283 | 92.34 |
| *Berghia stephanieae* | Berghia3 | SAMN29176740 | SRR19736587 | 6090 | 5790 | 95.07 | 5779 | 5479 | 89.97 |

| Sample-ID | Non-chimeric | % of input non-chimeric | *Breviolum* | *Symbiodinium* | *Cladocopium* | % Annotated |
|---|---|---|---|---|---|---|
| Exaiptasia2 | 1886 | 84.99 | 1447 | 248 | 191 | 100 |
| Exaiptasia3 | 1941 | 86.92 | 1779 | 88 | 74 | 100 |
| Exaiptasia4 | 2188 | 84.15 | 1977 | 88 | 123 | 100 |
| Berghia1 | 4895 | 81.37 | 4886 | 0 | 9 | 100 |
| Berghia2 | 6608 | 83.78 | 6557 | 38 | 13 | 100 |
| Berghia3 | 5479 | 89.97 | 5466 | 0 | 13 | 100 |

**Supplementary Table 2.** Summary of RNA sequencing data information of *Berghia stephanieae*.

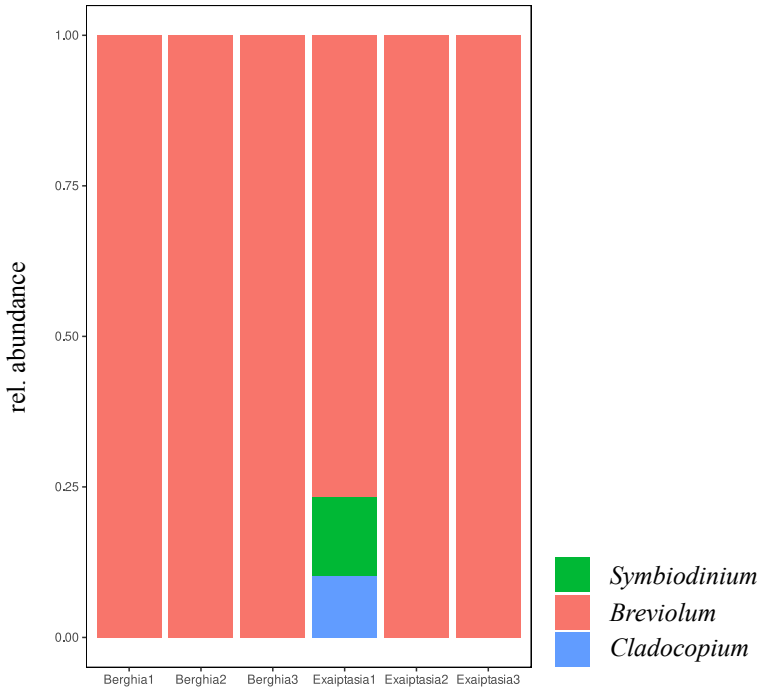
| Condition | | Sample | Raw reads Paired-end | Raw data | Effective(%) | Error(%) | Q20(%) | Q30(%) | GC(%) | Raw reads after BBSplit (paired-end) |
|---|---|---|---|---|---|---|---|---|---|---|
| Aposymbiotic | Starved | A1 | 57536758 | 8.6 | 98.53 | 0.03 | 97.32 | 92.92 | 40.47 | 56122922 |
| | Starved | A2 | 56845036 | 8.5 | 98.06 | 0.03 | 96.82 | 91.8 | 39.62 | 55607248 |
| | Starved | A3 | 55144492 | 8.3 | 97.74 | 0.03 | 96.92 | 91.9 | 41.53 | 52946256 |
| | Refed | A4 | 56416476 | 8.5 | 98.35 | 0.03 | 97.27 | 92.58 | 41.14 | 53381120 |
| | Refed | A5 | 55628646 | 8.3 | 98.14 | 0.03 | 96.86 | 91.85 | 39.84 | 48248998 |
| | Refed | A6 | 69410298 | 10.4 | 98.1 | 0.03 | 96.91 | 91.86 | 42 | 54072846 |
| | Fed | A7 | 74426506 | 11.2 | 99.16 | 0.03 | 97.69 | 93.49 | 46.75 | 60356744 |
| | Fed | A8 | 82084356 | 12.3 | 98.78 | 0.03 | 97.26 | 92.74 | 50.65 | 69681974 |
| | Fed | A9 | 79560100 | 11.9 | 98.82 | 0.03 | 97.38 | 92.85 | 45.07 | 69341948 |
| Symbiotic | Fed | S1 | 78401652 | 11.1 | 98.74 | 0.03 | 97.56 | 93.46 | 40.59 | 55718024 |
| | Fed | S2 | 78858224 | 11.7 | 98.76 | 0.03 | 97.7 | 93.77 | 40.8 | 48075996 |
| | Fed | S3 | 57619200 | 8.1 | 98.39 | 0.03 | 97.36 | 93.19 | 40.09 | 42975048 |
| | Refed | S4 | 72221684 | 10.1 | 98.5 | 0.03 | 97.74 | 93.79 | 40.76 | 60200342 |
| | Refed | S5 | 64023254 | 9.1 | 98.46 | 0.03 | 97.78 | 93.83 | 40.25 | 50726274 |
| | Refed | S6 | 62739464 | 9.3 | 98.74 | 0.03 | 97.71 | 93.72 | 40.44 | 55700052 |
| | Starved | S7 | 67119036 | 9.9 | 98.74 | 0.03 | 97.74 | 93.72 | 38.12 | 65869354 |
| | Starved | S8 | 73935468 | 11 | 98.77 | 0.03 | 97.74 | 93.73 | 40.09 | 72357830 |
| | Starved | S9 | 68421012 | 10.1 | 98.88 | 0.03 | 97.61 | 93.59 | 39.2 | 66637434 |
| **Total** | | | **1210391662** | | | | | | | **1038020410** |

**Supplementary Table 3.** BUSCO analysis results of the transcriptome of *Berghia stephanieae*.

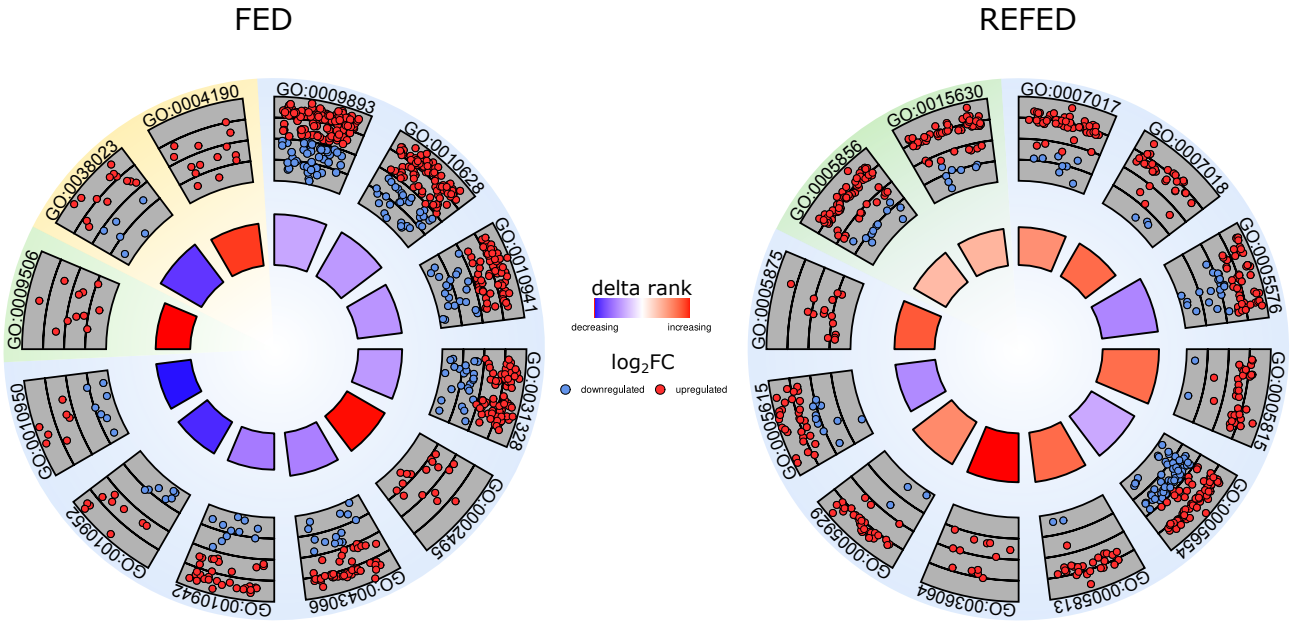
| | | |
|---|---|---|
| Complete BUSCOs (C) | 4591 | 86.7% |
| Complete and single-copy BUSCOs (S) | 1633 | 30.8% |
| Complete and duplicated BUSCOs (D) | 2958 | 55.9% |
| Fragmented BUSCOs (F) | 152 | 2.9% |
| Missing BUSCOs (M) | 552 | 10.4% |
| Total BUSCO groups searched | 5295 | |

For the Supplementary Data 1-3 of this publication, please visit the link: 10.6084/m9.figshare.19690843

All the supplementary material for this publication is available at: https://www.frontiersin.org/articles/10.3389/fmars.2022.934307/full#supplementary-material



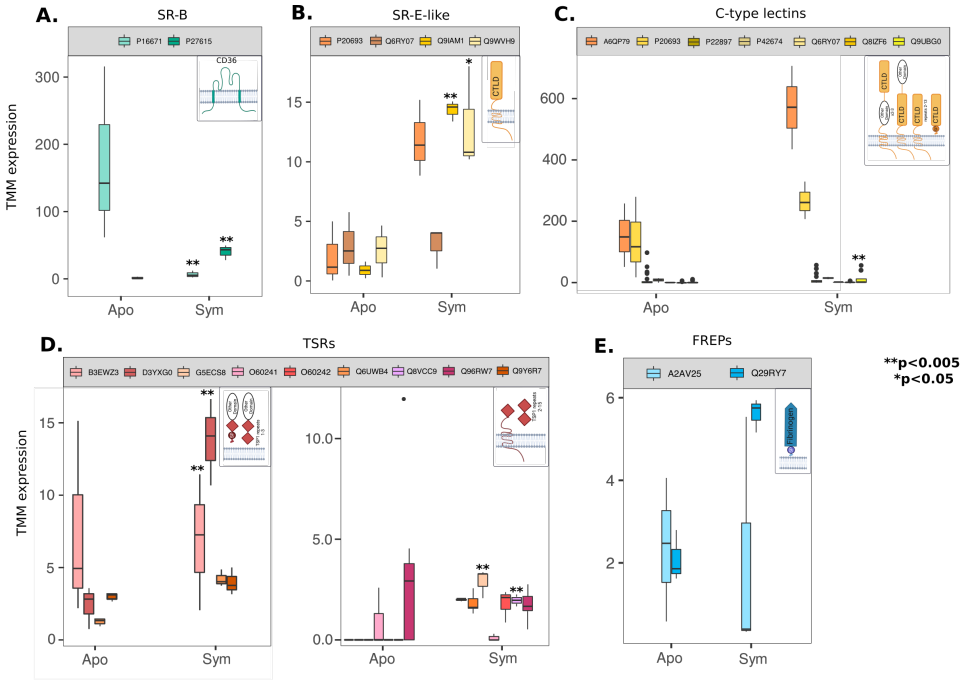

**Supplementary Figure 1.** Relative abundance of Symbiodiniaceae genera present in *B. stephanieae* and *E. diaphana*. Annotations were based on the obtained ITS 2 sequences, Symbiodatabacea database, and qiime2 pipeline. Green for *Symbiodinium*; Red for *Breviolum*; Blue for *Cladocopium*



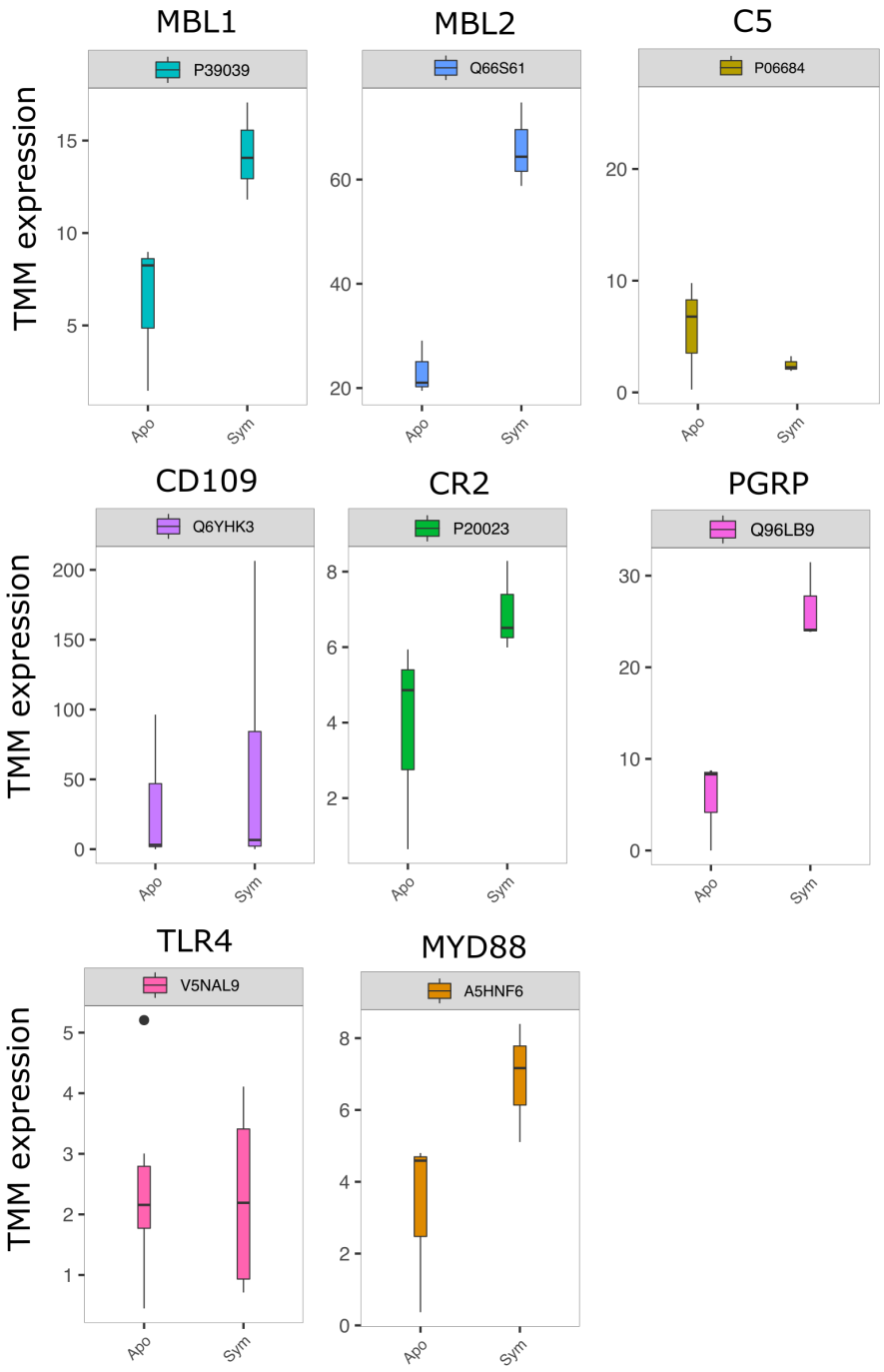

| GO-ID | GO-term | category |
|---|---|---|
| GO:0009893 | positive regulation of metabolic process | BP |
| GO:0010628 | positive regulation of gene expression | BP |
| GO:0010941 | regulation of cell death | BP |
| GO:0031328 | positive regulation of biosynthetic process | BP |
| GO:0002495 | antigen processing and presentation of peptide antigen via MHC class II | BP |
| GO:0043066 | negative regulation of cell death | BP |
| GO:0010942 | positive regulation of cell death | BP |
| GO:0010952 | positive regulation of peptidase activity | BP |
| GO:0010950 | positive regulation of cysteine-type endopeptidase activity | BP |
| GO:0009506 | plasmodesma | CC |
| GO:0038023 | signaling receptor activity | MF |
| GO:0004190 | aspartic-type endopeptidase activity | MF |

| GO-ID | GO-term | category |
|---|---|---|
| GO:0007017 | microtubule-based process | BP |
| GO:0007018 | microtubule-based movement | BP |
| GO:0005576 | extracellular region | BP |
| GO:0005815 | microtubule organizing center | BP |
| GO:0005654 | nucleoplasm | BP |
| GO:0005813 | centrosome | BP |
| GO:0036064 | ciliary basal body | BP |
| GO:0005929 | cilium | BP |
| GO:0005615 | obsolete extracellular region part | BP |
| GO:0005875 | microtubule associated complex | BP |
| GO:0005856 | cytoskeleton | CC |
| GO:0015630 | obsolete cytoskeletal part | CC |

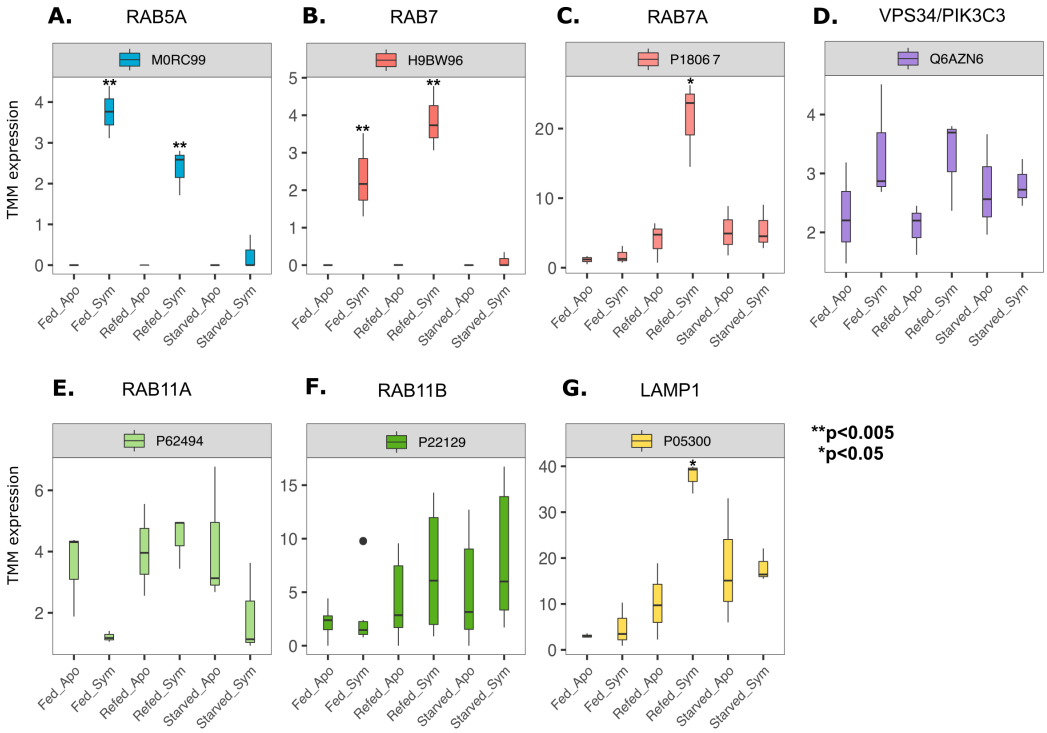
**Supplementary Figure 2.** Rank-based gene ontology enrichment analysis (GOMWU) of Fed and Re-fed symbiotic *B. stephanieae* compared to aposymbiotic state. The outer circle shows a scatter plot for each GO term of the log2FC of the assigned genes. Red small circles display up- regulation and blue ones down- regulation based on their delta-rank scores. GO term categories shown here correspond to Biological Process (BP), Cellular Components (CC), and Molecular Function (MF). The figure was created using GOplot (Walter et al., 2015).



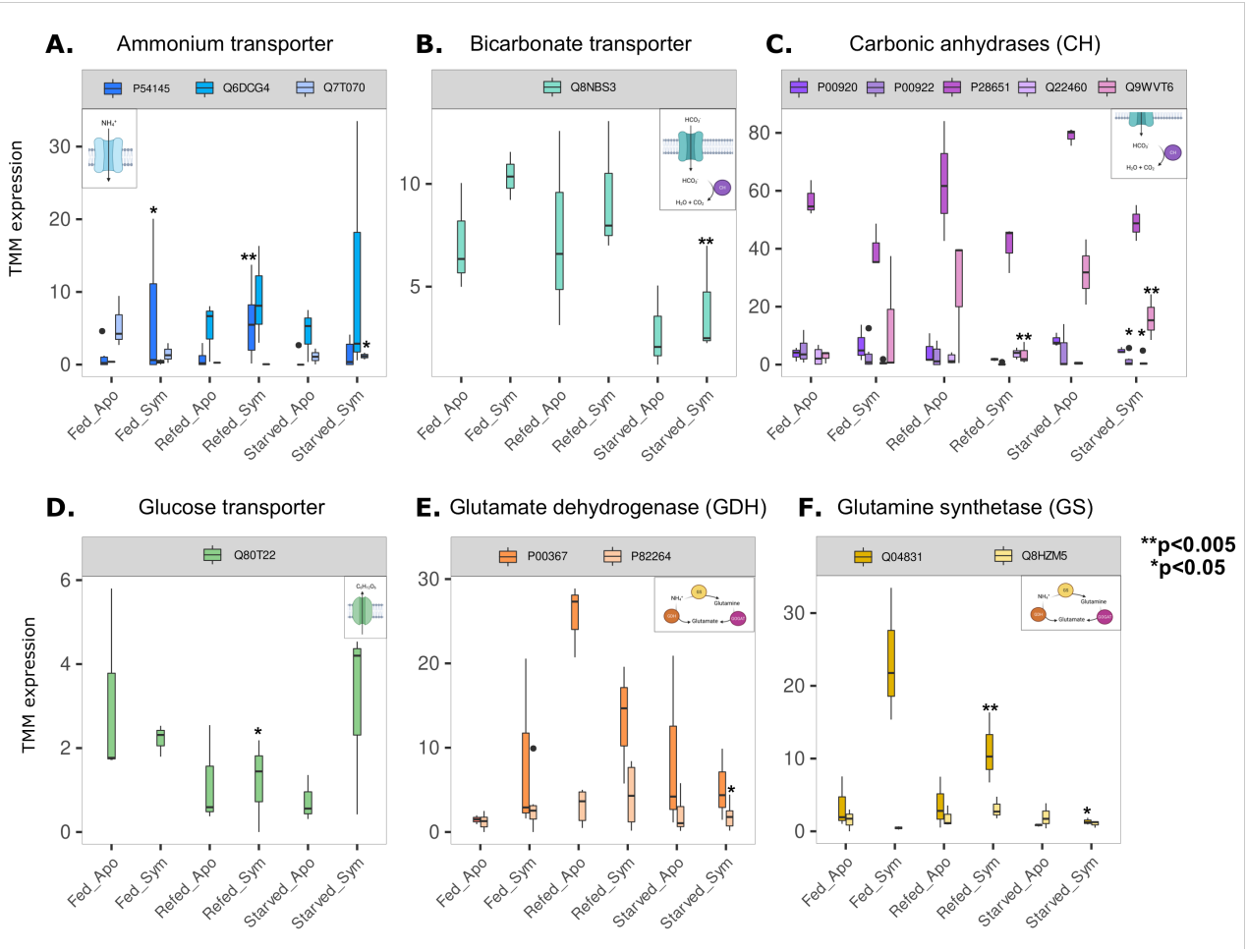

**Supplementary Figure 3.** Gene expression profile of pattern recognition receptors (PRRs) in B. stephanieae. (A) Scavenger receptor class B, (B) Scavenger receptor class E-like, (C) C-type lectins, (D) Thrombospondin-type-1 repeat domain-containing proteins, (E) Fibrinogen-related proteins. Genes were grouped by the UniProt protein identifier. Boxplots show the expression values in TMM, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. The domain architecture for each group of receptors is also shown. UniProt ID: P16671: Scavenger receptor class B member 1; P27615: Lysosome membrane protein 2; P20693: Low affinity immunoglobulin epsilon Fc receptor; Q6RY07: Acidic mammalian chitinase; Q9IAM1: Snaclec agkisacutacin subunit A; Q9WVH9: Fibulin-5; A6QP79: Collectin-12; P22897: Macrophage mannose receptor 1; P42674: Blastula protease 10; Q8IZF6: Adhesion G-protein coupled receptor G4; Q9UBG0: C-type mannose receptor 2; B3EWZ3: Coadhesin; D3YXG0: Hemicentin-1; G5ECS8: ADAMTS adt-1; O60241: Adhesion G protein-coupled receptor B2; O60242: Adhesion G protein-coupled receptor B3; Q6UWB4: Serine protease 55; Q8VCC9: Spondin-1; Q96RW7: Hemicentin-1; Q9Y6R7: IgGFc-binding protein; A2AV25: Fibrinogen C domain-containing protein 1; Q29RY7: Fibroleukin.



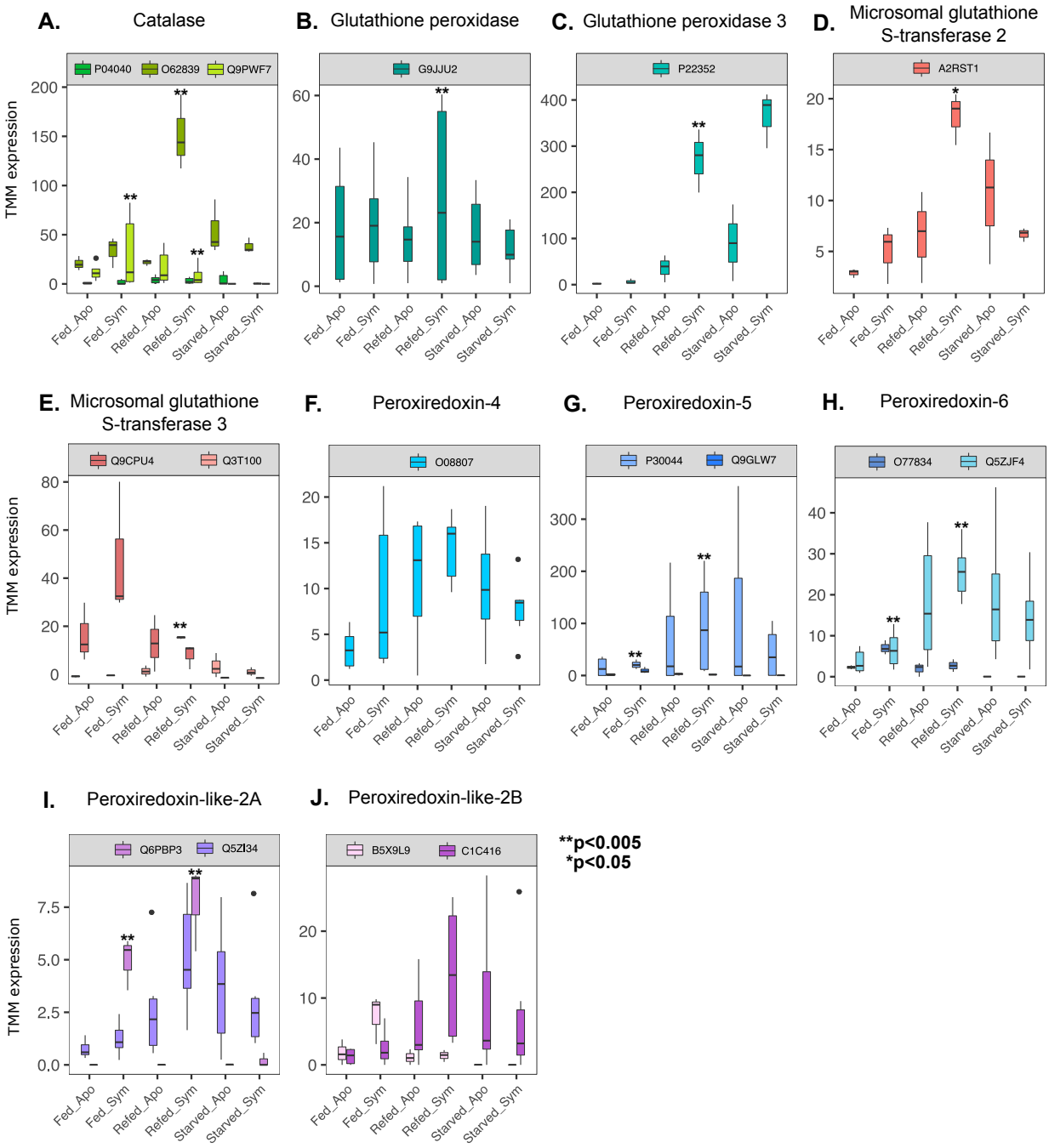

**Supplementary Figure 4.** Gene expression profile of innate immune genes relevant for symbiont recognition in *Berghia stephanieae*. Genes were grouped by the UniProt protein identifier. Boxplots show the expression values in TMM, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. UniProt ID: P39039: Mannose-binding protein A; Q66S61: Mannose-binding protein C (MBL2); P06684: Complement C5; Q6YHK3: CD109 Antigen; P20023: Complement receptor type 2; Q96LB9: Peptidoglycan recognition protein 3; V5NAL9: Toll-like receptor 4; A5HNF6: Myeloid differentiation primary response protein MyD88.




**Supplementary Figure 5.** Expression profile of genes involved in the phagosome maturation events in *B. stephanieae*. (A) Ras-related protein Rab-5A, (B) Ras-related protein Rab7, (C) Ras-related protein Ra-7a, (D) Phosphatidylinositol 3-kinase catalytic subunit type 3, (E) Ras- related protein Rab-11A, (F) Ras-related protein Rab-11B, (G) Lysosome-associated membrane glycoprotein 1. Genes were grouped by the UniProt protein identifier. Boxplots show the expression values in TMM, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. UniProt ID: M0RC99: Ras-related protein Rab-5A; H9BW96: Ras-related protein Rab7; P18067: Ras-related protein Rab-7a; Q6AZN6: Phosphatidylinositol 3-kinase catalytic subunit type 3; P62494: Ras-related protein Rab-11A; P22129: Ras- related protein Rab-11B; P05300: Lysosome-associated membrane glycoprotein 1.




**Supplementary Figure 6.** Gene expression profile of transporters and enzymes involved in the hypothetical nutrient exchange in *B. stephanieae*. (A) Ammonium transporter, (B) Bicarbonate transporter, (C) Carbonic anhydrases, (D) Glucose transporter, (E) Glutamate dehydrogenase, (F) Glutamine synthetase. Genes were grouped by the UniProt protein identifier. Boxplots show the expression values in TMM, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. UniProt ID: P54145: Putative ammonium transporter 1; Q6DGC4: Tetraspanin; Q7T070: Ammonium transporter Rh type B; Q8NBS3: Sodium bicarbonate transporter-like protein 11; P00920: Carbonic anhydrase 2; P00922: Carbonic anhydrase 2; P28651: Carbonic anhydrase-related protein; Q22460: Beta carbonic anhydrase 1; Q9WVT6: Carbonic anhydrase 14; Q80T22: Sodium-dependent glucose transporter 1; P00367: Glutamate dehydrogenase 1, mitochondria; P82264: Glutamate dehydrogenase, mitochondria; Q04831: Glutamine synthetase; Q8HZM5: Glutamine synthetase.




**Supplementary Figure 7.** Gene expression profile of ROS quenching elements in *B. stephanieae*. (A) Catalase, (B-C) Glutathione peroxidase, (D-E) Microsomal glutathione S-transferase, (F-H) Peroxiredoxin, (I-J) Peroxiredoxin-like. All feeding conditions are shown. Genes were grouped by the UniProt protein identifier. Boxplots show the expression values in TMM, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. UniProt ID: P04040: Catalase; O62839: Catalase; Q9PWF7: Catalase; G9JJU2: Glutathione peroxidase; P22352: Glutathione peroxidase 3; A2RST1: Microsomal glutathione S-transferase 2; Q9CPU4: Microsomal glutathione S-transferase 3; Q3T100: Microsomal glutathione S-transferase 3; O08807: Peroxiredoxin-4; P30044: Peroxiredoxin-5; Q9GLW7: Peroxiredoxin-5, mitochondrial; O77834: Peroxiredoxin-6; Q5ZJF4: Peroxiredoxin-6; Q6PBP3: Peroxiredoxin-like 2A; Q5ZI34: Peroxiredoxin-like 2A; B5X9L9: Prostamide/prostaglandin F synthase; C1C416: Prostamide/prostaglandin F synthase.

## Chapter 2.4

# Shedding light on starvation in darkness in the plastid-bearing sea slug *Elysia viridis* (Montagu, 1804)

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**Abstract**

Sacoglossa are known for stealing photosynthetically active chloroplasts from their macroalgal food and incorporating them into their cytosol. The nutritional support these alien organelles (kleptoplasts) provide to the slugs is still debatable. Comparing slugs starved in continuous darkness (non-photosynthetic condition) and light (photosynthetic condition) is often used to understand the contribution of the kleptoplasts to the slugs' metabolism. Here, we examined the slugs' side of starvation in darkness to better understand the effects of darkness on the slugs. We compared the gene expression profile and digestive activity of *Elysia viridis*, starved for one week under ambient light and continuous darkness. Starvation in darkness led to the up-regulation of genes related to glucose deficiency, while genes involved in the development, cellular organization, and reproduction were down-regulated. This specific gene expression may counteract reduced nutrient availability under non-photosynthetic conditions. Under photosynthetic conditions, kleptoplasts may have a higher nutritional value and may be able to support some metabolic processes. It appears that the slugs can only access kleptoplast photosynthates through autophagy during starvation. Nevertheless, autophagy and length reduction in darkness are highly elevated compared to light conditions, suggesting that more slug tissue is needed to satisfy the nutritional demands under non-photosynthetic conditions. Since we did not detect a gene expression related to the export of photosynthates to the slugs, our results support the hypothesis that slugs use kleptoplasts as larders accessible via autophagy. As long as the kleptoplasts are functional, they provide an energetic support, helping the slugs to reduce starvation-induced stress.

## Introduction

The symbiotic relationship between animals and photosynthetic organisms is known as photosymbiosis and is considered especially beneficial for animal hosts (Melo Clavijo et al. 2018). Corals and their symbionts, the unicellular algae Symbiodiniaceae, are one of the best-studied systems of photosymbioses. The nutritional boost provided by symbionts increases the calcification rate of corals, facilitating the formation of large reef structures (Stanley and Lipps 2011). However, there is a special form of photosymbiosis found in many sacoglossan sea slugs (Händeler et al. 2009) and a few microturbellarian flatworms (Van Steenkiste et al. 2019). These two taxa only incorporate the chloroplasts from their algal prey into their cells, a process known as kleptoplasty.

In some Sacoglossa, the "stolen" chloroplasts (kleptoplasts) remain photosynthetically active for weeks or months in the absence of any support from the algal nucleus (Wägele and Martin 2014). Even after decades of research, it is still uncertain what mechanisms are necessary to enable this so-called functional kleptoplasty in Sacoglossa. In the slugs, the expression of genes involved in kleptoplast recognition (Melo Clavijo et al. 2020; Mendoza et al. 2023) and maintenance (de Vries et al. 2015; Chan et al. 2018; Maeda et al. 2021) may mediate the establishment of functional kleptoplasty. Furthermore, the phototactic behavior of the slugs (Weaver and Clark 1981; Schmitt and Wägele 2011; Cartaxana et al. 2018) and chloroplast photoprotection mechanisms (Christa et al. 2018; Cartaxana et al. 2019; Havurinne and Tyystjärvi 2020; Havurinne et al. 2021) are considered to increase the longevity of kleptoplasts in slugs.

Compared to other photosymbiotic systems, the nutritional relevance of bearing functional kleptoplasts is less apparent (Rauch et al. 2017a). For instance, it is estimated that kleptoplasts can provide up to 60 % of the required carbon (Raven et al. 2001; Rauch et al. 2017a), while symbionts in other photosymbioses can fully support the host up to over 170 % carbon (Thomas et al. 2023). To understand the nutritional relevance of kleptoplasts to sacoglossans, starvation experiments under continuous light (photosynthetic condition) and constant darkness (non-photosynthetic condition) are often conducted. Several studies have shown that dark-starved animals lose body size and weight faster than light-starved animals (Cartaxana et al. 2017; Shiroyama et al. 2020). In others, the effects of starvation in darkness were only apparent after several weeks (Casalduero and Muniain 2008; Maeda et al. 2021). The differences between dark-starved and light-starved slugs have been mainly interpreted as a result of blocked photosynthetic activity. However, some species can even overcome several months of starvation without functional kleptoplasts under natural light conditions (Klochkova et al. 2013). Further, alternative explanations are often not considered (Christa et al. 2014b). For instance, continuous darkness is an unnatural condition that can affect the slug's metabolism independently of kleptoplasts' photosynthesis or the slugs' behavior. Thus, some studies have used a photosynthesis blocker (e.g., Monolinuron) to avoid darkness-induced artifacts and showed similar starvation tolerances compared to photosynthetic conditions (Christa et al. 2014a; de Vries et al. 2015; Laetz et al. 2017b).

Regardless of the exact support by the kleptoplasts, there is no doubt that slugs can metabolize the kleptoplasts' photosynthates (Kremer 1976; Hinde 1978; Cruz et al. 2020). However, how they access these photosynthates is unclear. From corals, it is known that the host supports the

photobionts actively with bicarbonate and ammonium, while the photobionts provide sugars to the host (Barott et al. 2015). This exchange of nutrients is crucial for a successful and long-term symbiosis and requires a symbiosome (Mohamed et al. 2016; Matthews et al. 2017). The symbiosome is a membrane that surrounds the symbiont and contains transporters relevant for translocating ammonium and bicarbonate to the symbiont and glucose to the host (Thies et al. 2022). The disruption of this nutrient exchange may lead to the breakdown of the symbiosis (Rädecker et al. 2021). In sacoglossan sea slugs, it is unknown whether there are nutrient transporters in the membrane surrounding the kleptoplasts and how sacoglossan sea slugs can access the photosynthetically derived assimilates. In some species, kleptoplasts accumulate starch (Evertsen and Johnsen 2009; Pelletreau et al. 2014; Laetz et al. 2017b; Cruz et al. 2020), which at least questions an active export of glucose. It even appears that the slugs digest the kleptoplasts while feeding (Maeda et al. 2012; Frankenbach et al. 2021) and during starvation (Laetz et al. 2017a) to access the photosynthates. Hence, the kleptoplasts may instead function as larders and their products as reserves (Pelletreau et al. 2014; Laetz et al. 2017b) accessed by digestion.

In the present study, we analyzed the gene expression profile of the plastid-bearing sea slug *Elysia viridis* (Montagu, 1804) under the presence and absence of light to address the effects of darkness on starvation specifically. We focused on energy metabolism, mitochondrial function, and transporters relevant to the exchange of nutrients between the host and the symbiont to maintain symbiosis. We further combined the gene expression of central components of autophagy with the analyses of kleptoplast and lysosome abundance to better understand how the slugs might access the kleptoplasts' photosynthates.

## Material and Methods

*Sea slug collection and laboratory culturing*

The specimens of *Elysia viridis* used for transcriptome analyses were collected from their food source *Codium tomentosum* Stackhouse, 1797 in September 2020 at the rocky beach of Figueira da Foz, Portugal (40°10'05.0"N, 8°53'20.9"W) and immediately transported to the laboratory. Animals were kept in sets of 10 individuals in 500 ml of natural GF/F (0.7 $\mu$m pore size, Whatman) filtered seawater at 18 ± 1 °C, 25-30 $\mu$mol photons $m^{-2}$ $s^{-1}$ (white fluorescence lamps, Philips, TL-D 36W/54) at a 12h/12h day/night cycle and a salinity of 33 PPT. We provided the macroalgae *Bryopsis hypnoides* J.V.Lamouroux, 1809 as a food source as it can be easily cultured under

laboratory settings and it is a source of chloroplasts that remain functional in *E. viridis* (Rauch et al. 2018). *Bryopsis hypnoides* was cultured in autoclaved seawater enriched with F/2 Medium (Guillard 1975) under constant aeration and the same light regime and temperature as the slugs. Weekly, 80 % of the seawater and 100 % medium were changed. The slugs remained in culture five months before the experiment to ensure they were fully acclimated to laboratory conditions.

*Experimental setup, RNA extraction, and sequencing*

Nine randomly chosen slugs (BioSample IDs: SAMN26303207 - SAMN26303215) were used in a starvation experiment. Each slug was transferred into a 50 ml Falcon tube filled with 35 ml natural GF/F filtered seawater, with 50% of the seawater changed daily. After an acclimation period of three weeks, the slugs were randomly chosen to starve in constant darkness for one week (SD; n=3), to starve for one week under culturing light conditions (SL; n=3) or to serve as the fed control group under culturing light conditions (FED; n=3). All slugs (SD, SL, and FED) were fixed in liquid nitrogen at the end of the experiment. All animals were subsequently freeze-dried at -80 °C for two days. Total RNA from each sample was extracted using the my-Budget RNA Mini Kit (Bio-Budget Technologies, Germany) following the manufacturer's instructions. The RNA concentration was quantified with Qubit fluorometric quantification (ThermoFisher, USA) and NanoDrop™ One/OneC (ThermoFisher, USA). Poly(A) mRNA enrichment, library preparation using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, USA), and 150 bp paired-end sequencing using the Illumina NovaSeq system was done by Novogene (UK).

*Transcriptome assembly and annotation*

The sequencing resulted in 709,603,664 quality filtered (Q30) paired-end reads (on average 78,844,851 per library; Online Resource 1; BioProject ID: PRJNA810987, Accession Nr.: SRR18163429 – SRR18163437). Reads were assembled using Trinity v2.12.0 (Grabherr et al. 2011), and the assembled transcriptome was then clustered with CD-HIT-EST v.4.8.1 with -c 0.9 -n 7 -B 1 -g 1 -s 0.9 parameters (Fu et al. 2012) (Online Resource 2). For each contig, the longest open reading frames were obtained using TransDecoder v5.5.0 (Haas and Papanicolaou 2015). The data set was then annotated by a BLASTP search (part of the BLAST+ package v2.9.0) against the UniProt database version 09/2021 (The UniProt Consortium 2019). Subsequently, all non-annotated genes were subjected to a BLASTP search against a molluscan-specific TrEMBL database. Taxonomic assignment for each protein sequence was obtained from the UniProt and TrEMBL taxonomic database (Online Resource 3). Sequences and annotations were subsequently filtered for

metazoan annotations to exclude annotations of bacteria, fungi, and plants in downstream analyses. Functional annotations of gene ontology (GO) terms, cluster of Eukaryotic Orthologous groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers for the filtered data set were obtained by using eggNOG mapper v.2.1.6 in default mode (Huerta-Cepas et al. 2017) (Online Resource 4).

*Differential gene expression analysis*

The transcript abundance of sequences was estimated with kallisto v0.46.2 (Bray et al. 2016) following the Trinity pipeline v2.12.0 (Online Resource 1) to obtain raw read counts and normalized trimmed mean of M-values (TMM). Differential expression analysis was performed with DeSeq2 v1.32.0 (Love et al. 2014) using the raw reads matrix implemented in Trinity. Differentially expressed genes (DEGs) were defined by a false discovery rate (FDR) < 0.05. Principal Component Analysis (PCA) of annotated DEGs was done using the prcomp() function in the stats package in R v. 4.1.0 (R Core Team 2020) using the TMM values for each gene to visualize the effects of starvation on the general gene expression. Gene ontology analyses with adaptive clustering were performed using the GOMWU R package to measure whether each GO category was significantly enriched according to the expression value (Wright et al. 2015). EuKaryotic Orthologous Groups (KOG) class enrichment tests were performed using the KOGMWU R Package (Dixon et al. 2015). KOGMWU allows the comparison of the gene expression of samples analyzed under different conditions. The correlation is based on the KOG delta ranks across the data sets. The delta rank is the difference between the mean rank of genes in a KOG class and the mean rank of all other genes (Dixon et al. 2015). To get a general idea of how starvation under different light conditions affects *E. viridis*, we compared our data set with published data of developing juveniles of *Elysia chlorotica* Gould, 1870 (Chan et al. 2018), heat-stressed colonies of *Stylophora pistillata* (Esper, 1792) (Rädecker et al. 2021), and symbiotic vs. apo-symbiotic *Exaiptasia diaphana* (Rapp, 1829) (Lehnert et al. 2014).

*DNA extraction, sequencing, and mitochondrial genome assembly and annotation*

High molecular weight DNA was extracted using the approach described in Melo Clavijo et al. (2021) from one specimen of *E. viridis* collected in Aguda, Portugal (41°02'52.6"N, 8°39'16.0"W) in September 2017. The sample was fixed in 70 % EtOH and was transported to the University of Aveiro. Single-end library prep of 500-600 bp read length for Illumina HiSeq2500 instrument (1x 100 nucleotides) was prepared using the tagmentation protocol (Picelli et al. 2014). Overall,

54,437,385 raw single-end Illumina reads with lengths between 50 and 95 nucleotides [nt] (SRR18210511 and SRR18210512) were quality trimmed using BBDuk v.38.91 of the BBMap tool (Bushnell et al. 2017) setting trimq=20. Sequence quality was subsequently verified using fastqc v.0.11.9 (Andrews 2010). We then used a hybrid assembly approach using Spades v.3.11.1 in default mode (Antipov et al. 2016), including 1,159,884 Nanopore long reads (SRR18251374) generated using the LSK109 kit following the protocol in Melo Clavijo et al. (2021). The mitochondrial genome was identified using a BLASTN search of a cytochrome oxidase 1 nucleotide sequence of *Elysia viridis* (GenBank accession: MN223463) against the assembly. The extracted contig had a length of 14,204 base pairs (bp) and was subsequently manually edited to obtain the circular genome. The circular sequence consisted of 14,149 bp and was annotated using the MITOS2 webserver (Donath et al. 2019). The annotation was manually curated using Geneious Prime v.2022.0.2 (https://www.geneious.com), using the published mitochondrial genomes of four other *Elysia* species as reference: *Elysia chlorotica* (NC_010567; (Rumpho et al. 2008)), *Elysia cornigera* Nutall 1989 (NC_035489; (Rauch et al. 2017b)), *E. timida* (Risso, 1818) (NC_035490; (Rauch et al. 2017b)), and *Elysia ornata* (Swainson, 1840) (NC_030537; (Karagozlu et al. 2016)). A circular view of the mitochondrial genome was created using OrganellarGenomeDraw (Lohse et al. 2013).

*Kleptoplast and lysosomal abundance measurements*

Twenty specimens of *E. viridis* used for kleptoplast and lysosomal abundance analyses were collected in May 2017 in Aguda, Portugal (41°02'46.7"N, 8°39'14.6"W), in rocky intertidal pools and transferred to the laboratory at the Zoological Research Museum Alexander Koenig (Bonn, Germany). Specimens were cultured in groups of 10 individuals in 500 mL artificial seawater (ABReef Salt, Aqua Medic, Germany, ASW) in glass jars with a salinity of 33 PPT under a 12h/12h day/night cycle setting the light intensity to 30 $\mu$mol photons $m^{-2}$ $s^{-1}$ provided by daylight sunrise LED X-change tubes (Sera, Germany) at 20 ± 1 °C. The water was changed three times a week. The food source, *Bryopsis hypnoides,* was given to the slugs three times a week for five weeks. *B. hypnoides* was cultured under the conditions mentioned above. For each light treatment (SD, SL, and FED), three randomly chosen slugs were used and cultured under the same conditions in individual 50 mL plastic containers filled with 35 mL of ASW. Slugs were imaged using a DP21 camera mounted on an SZX12 stereo microscope (Olympus, Tokyo, Japan) at the beginning of the experiment and, in the case of the SD and SL group, after one week of starvation, and the length measured as an indicator of fitness. The average length of each slug was calculated from five

images taken during movement to obtain the point of maximum elongation. Chlorophyll *a* fluorescence was subsequently measured in each slug using a Diving Pulse Amplitude Modulated (Diving-PAM) fluorometer (Walz, Effeltrich Germany). For this, samples were dark acclimated for 5 minutes, and then the maximum quantum yield ($F_v/F_m$; $F_v = F_m - F_o$; where $F_o$ and $F_m$ are the minimum and maximum fluorescence emitted by dark-adapted samples, respectively) was determined by applying a saturation pulse (pulse duration 0.8 ms, white light, >5,000 $\mu$mol photons $m^{-2}$ $s^{-1}$) (Serôdio et al. 2010). One measurement per specimen was performed by placing the optic fiber about 3–5 mm above the slug's pericardium to obtain $F_0$ values of around 200–500. Measurements were taken at the beginning of the experiment and immediately after one week of starvation before preparing the samples for confocal microscopy and served as a measure of kleptoplast fitness.

To determine the lysosomal and kleptoplast abundance, SD, SL, and FED animals were examined by confocal laser scanning microscopy using a Leica SPE CLSM (Leica Microsystems AG, Germany). Kleptoplasts were detected via chlorophyll *a* autofluorescence. The lysosome abundance of the slugs was visualized by incubating them for 30 minutes in 5 $\mu$M acridine orange (Sigma-Aldrich, Germany). After incubation, the slugs were vivisected into ~0.5 mm thick transverse sections. The samples were then placed on a microscope slide and imaged using a 488 nm laser to excite both acridine orange and chlorophyll *a*. The wavelength range accepted for acridine orange emission was set to 645 nm to 770 nm to distinguish acridine orange from chlorophyll *a* (acridine orange dimer peak emission is 656 nm) (Laetz et al. 2017a). Chlorophyll *a* autofluorescence was recorded, setting the emission from 600-640 nm (peak 633 nm). Five different sections per slug were imaged. Each section was made up of 8 images 1 µm in depth (z plane) for a total of 8 µm of tissue depth. Thus, for each experimental condition (SD, SL, FED), 15 image stacks were analyzed. The images were processed in ImageJ v. 1.52i (Schindelin et al. 2012) by defining a region of interest and subsequently quantifying the fluorescence signals. All images were processed using the same settings for the fluorescence signals.

## Results

*Starvation, particularly in darkness, induced changes in energy metabolism*

The transcriptome of *Elysia viridis* consisted of 366,188 contigs that translated into 104,180 amino acid sequences based on potential open reading frames. We could annotate 22,996 (22.07 % of all

protein sequences) of these amino acid sequences, most likely belonging to *E. viridis* (Online Resource 5). Additionally, 205 protein sequences (0.20 %) could be assigned to Archaea, 10,599 sequences (10.17 %) were assigned to Bacteria, and 925 sequences (0.89 %) were assigned to Viridiplantae (Online Resource 3). For all subsequent analyses, we only used the genes that most likely belonged to *E. viridis*.

We analyzed the gene expression in *Elysia viridis* and compared freshly fed (FED) animals and those starved for one week under a 12h / 12h day/night light cycle (SL) or in complete darkness (SD). Generally, SD resulted in a different gene expression than SL; both were different from the gene expression of FED specimens. An exception was a replicate of SL animals (SL02) that showed little correlation with the other samples (Fig. 1 A) and showed a higher PC variance compared to the additional replicates (Fig. 1 B). Thus, this sample was excluded from the subsequent differential gene expression analyses. Overall, we identified 1,862 differentially expressed genes (DEGs) when comparing FED and SD samples, 216 DEGs when comparing FED and SL samples, and 879 DEGs when comparing SL and SD samples (Online Resource 5).

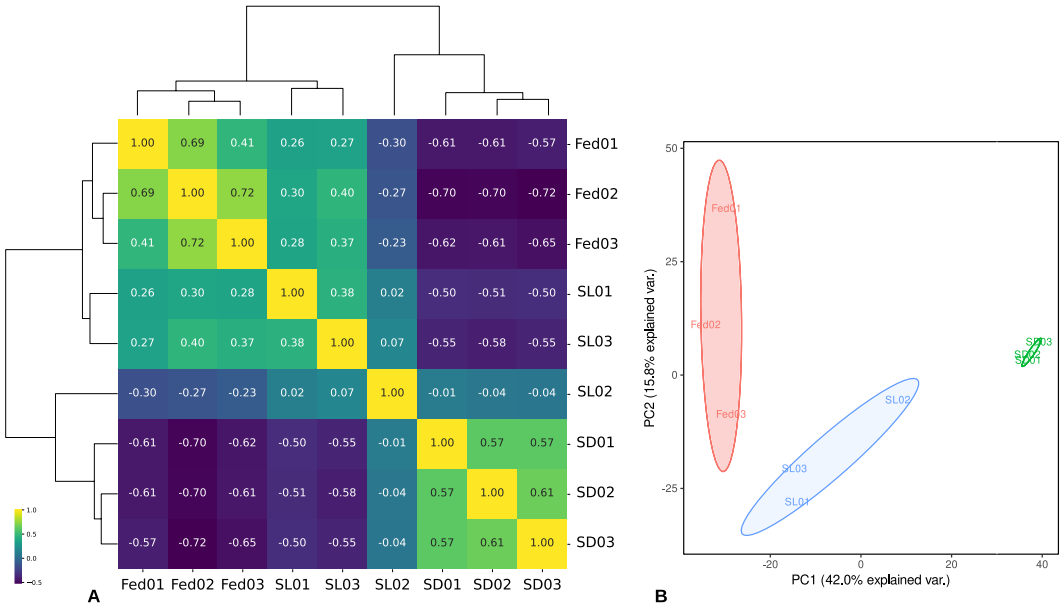


**Fig. 1.** Hierarchical clustering of Pearson correlations **(A)** and Principal Component Analysis **(B)** of differentially expressed genes in specimens of *Elysia viridis* freshly fed (FED), starved in darkness (SD), or starved in light (SL).

We found no significantly enriched Gene Ontology (GO) term among DEGs when using GOMWU to compare FED and SL animals (Online Resource 6). In the Biological Process (BP) division, we found 22 significantly enriched GO-terms (FDR < 0.1) when comparing SD to FED animals and 24 significantly enriched GO-terms (FDR < 0.1) when comparing SD to SL animals (Fig. 2). The

majority of these GO terms were down-regulated in SD animals and could be assigned to the cell cycle and development, independent of the comparison (Fig. 2). Two GO terms related to translation and the GO term "cellular amino acid metabolism" were enriched and up-regulated in SD animals. The GO term "protein folding" was up-regulated when comparing SD to SL animals. GO terms associated with the "cytoskeleton", "microtubule organization center", and "cilium" in the cellular compartment (CC) division were enriched and down-regulated in SD animals, independent of the comparison; The GO term "ribosome" was enriched and up-regulated (Online Resource 6). In the Molecular Function (MF) division, the GO terms "cytoskeletal protein binding" and "transcription factor binding" were enriched and down-regulated in SD compared to FED animals. In contrast, the GO terms "ligase activity", "lyase activity", and "isomerase activity" were enriched and up-regulated (Online Resource 6).

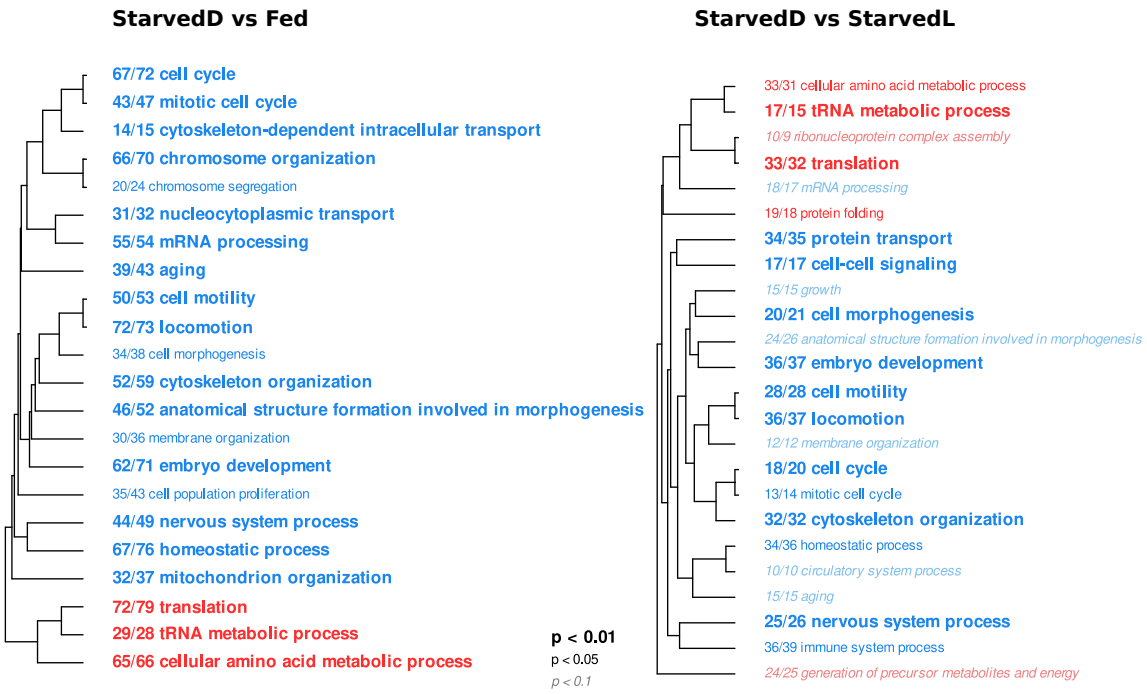


**Fig. 2:** Gene ontology (GO) terms significantly enriched in the Biological Process (BP) category with genes either up- (red) or down-regulated (blue) in specimens of *Elysia viridis* starved in darkness (SD) compared to freshly fed (FED) or light starved (SL) animals. Shown are genes with an FDR < 0.05 relative to the total number of all genes within the BP category.

We analyzed the euKaryotic Orthologous Groups (KOG) using KOGMWU to investigate further the general expression profile (Online Resource 7). We found one main cluster made of fed individuals of *Elysia chlorotica* and one main cluster made of our starved samples of *Elysia viridis* and two selected cnidarians (Fig. 3). The expression of genes assigned to the respective KOG

categories of SD animals, independent of the comparison with FED or SL animals, clustered with individuals of *Exaiptasia diaphana* (comparison of symbiotic vs. apo-symbiotic animals). The gene expression of SL vs. FED individuals of *E. viridis* clustered with heat-stressed individuals of *Stylophora pistillata* (Fig. 3).

Similarly, as in the Gene Ontology analyses, we could not find significantly enriched KOG categories in SL vs. FED individuals of *E. viridis* (Fig. 3). In SD animals, the categories "Energy production and conversion" and "Cell wall/membrane/envelope biogenesis" were enriched with mainly up-regulated genes, regardless of being compared to FED or SL animals. Moreover, in SD vs. SL animals, the KOG categories "Nucleotide transport and metabolism", "Amino acid transport and metabolism", and "Carbohydrate transport and metabolism" were enriched among up-regulated genes in SD animals (Fig. 3).

The KOG categories "RNA processing and modification", "Cytoskeleton", and "Signal transduction mechanisms" were enriched and down-regulated independent of the comparison of SD animals, supporting our GO enrichment analyses. Furthermore, the KOG categories "Intracellular trafficking, secretion, and vesicular transport" and "Posttranslational modification, protein turnover, chaperones" were enriched and down-regulated in the SD vs. FED comparison (Fig. 3).

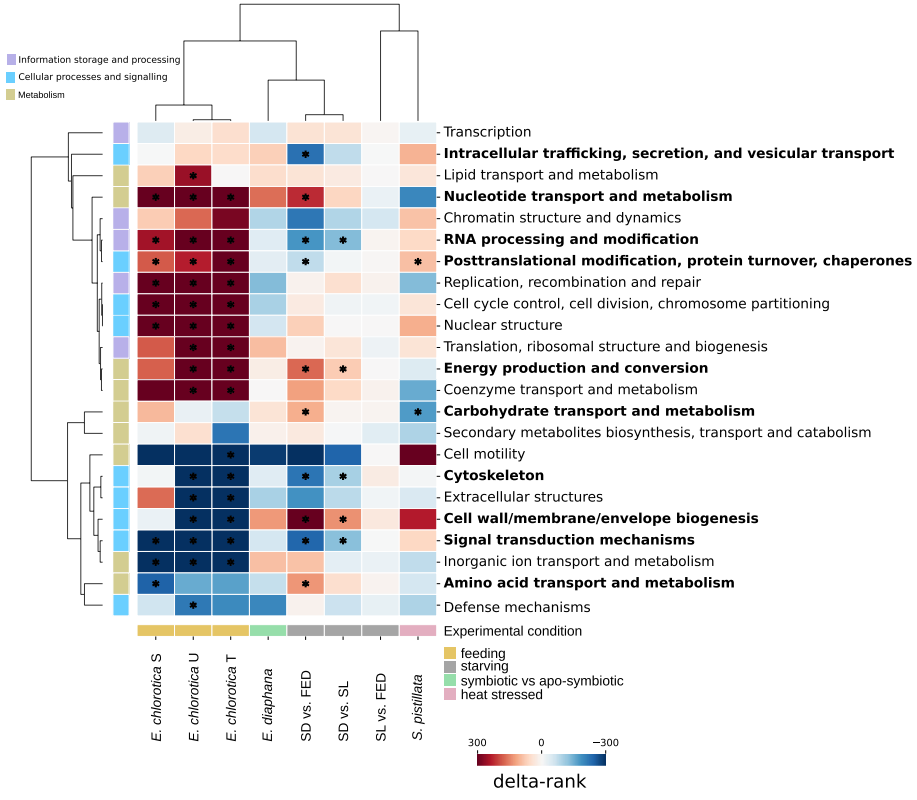


**Fig. 3.** Heat map of enrichment of cluster of Eukaryotic Orthologous groups (KOG) classes by differentially expressed genes in different data sets. Significantly enriched KOG classes (FDR < 0.05) with up- (red) or down-regulated (blue) genes are identified by an asterisk. For data references, see the text. Different letters after *Elysia chlorotica* indicate unstable symbiosis (S), transient symbiosis (U), and stable symbiosis (T), as defined earlier (Pelletreau et al. 2012).

*Protein import into mitochondria decreases during starvation*

Many enriched and down-regulated GO and KOG terms were assigned to energy metabolism. Thus, we subsequently analyzed the gene expression (not only of the differentially expressed genes) associated with the mitochondria in more detail. In starved specimens, independent of the light condition, the oxidative phosphorylation (OXPHOS) was predominantly down-regulated, especially genes belonging to complex I, II, and V (Online Resource 5).

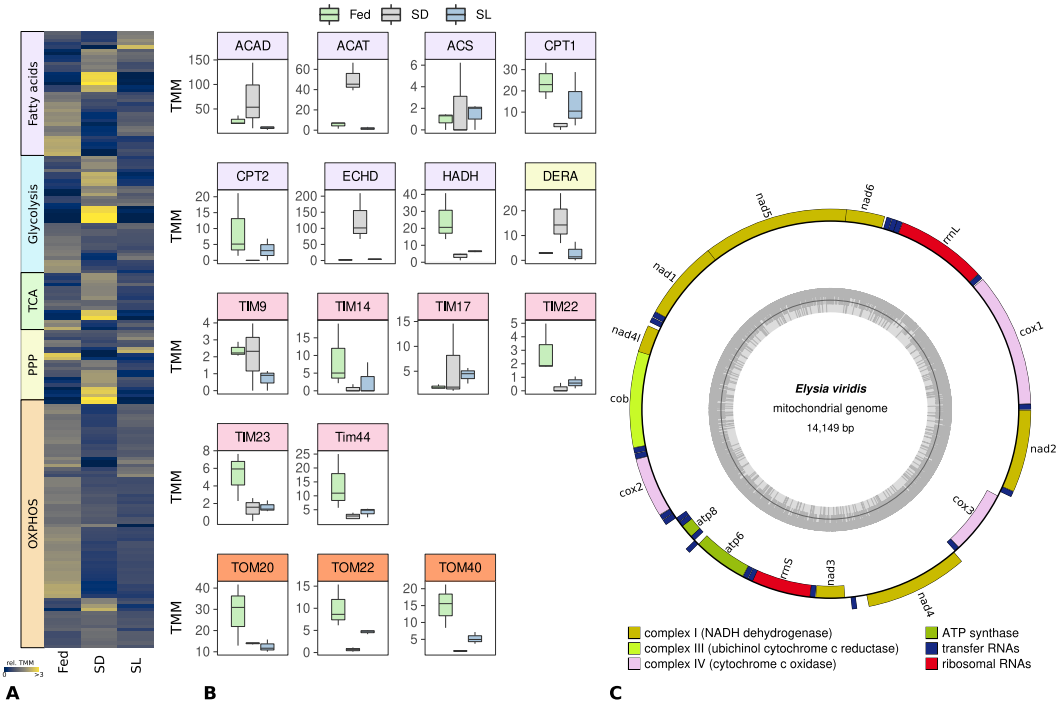


**Fig. 4. (A)** Overview of the expression of mitochondrial energy-related genes in *E. viridis* freshly fed (FED), starved in darkness (SD), and starved in light (SL). The average TMM of the replicates of each condition in relation to the average TMM of the respective gene across all samples is shown. **(B)** Specific expression profile of important genes related to fatty acid metabolism (purple), Pentose Phosphate Pathway (yellow), genes of the Translocase of the inner (TIM, pink) and outer (TOM, orange) mitochondrial membrane. Boxplot whiskers show the minimum and maximum TMM values, and the centers correspond to the medians. **(C)** Circular map of the mitochondrial genome of *Elysia viridis*. The annotations of the tRNAs were omitted for displaying reasons. ACAD: acyl-CoA dehydrogenase; ACAT: acetyl-CoA acetyltransferase; ACS: acetyl-CoA synthetase; CPT1, CPT2: Carnitine palmitoyltransferase 1, 2; ECHD: enoyl-CoA hydratase; HADH: hydroxyacyl-CoA dehydrogenase; DERA: deoxyribose-phosphate aldolase; TMM: Trimmed means of M value; TCA: tricarboxylic acid cycle; OXPHOS: oxidative phosphorylation; PPP: pentose phosphate pathway.

In SD animals, various genes of the Pentose Phosphate Pathway (e.g., deoxyribose-phosphate aldolase), the tricarboxylic acid cycle (e.g., oxogluterate dehydrogenase), and glycolysis (e.g., 6-phosphofructinase, pyruvate dehydrogenase) were up-regulated (Fig. 4 A; Online Resource 5). Most genes belonging to the fatty acid metabolism were down-regulated in starved animals (Fig. 4 A),

including carnitine O-palmitoyltransferase I and II (CPT1 and CPT2). However, several genes involved in fatty acid beta-oxidation in SD animals were up-regulated (Fig. 4 B).

The mitochondrial genome of *E. viridis* (GenBank accession no.: ON065001) has a length of 14,149 bp and consists of 13 protein-coding genes, 2 rRNAs, and 21 tRNAs, a standard set of genes among Heterobranchia, except for a missing tRNA-S2 gene. A comparison with the published mitochondrial genomes of four other *Elysia* species did not reveal sufficient sequence/structure conservation to annotate this gene. Most proteins relevant to mitochondrial metabolism are nuclear-encoded and have to be imported. Yet, core components of the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) complexes were down-regulated in starved slugs (Fig. 4 B).

*Autophagy as primary source to obtain kleptoplast nutrients*

Afterwards, we examined how starving slugs may obtain nutrients by analyzing the abundance of lysosomes and the expression of genes involved in lysosome formation and autophagy. Independent of the light condition, starvation for one week led to an increase in the abundance of lysosomes and a simultaneous decrease in the abundance of kleptoplasts in specimens of *E. viridis* (Fig. 5 A-C). SD animals had a higher lysosomal abundance (39.9 % ± 5.7 %) after one week of starvation than SL animals (17.3 % ± 1.7 %) and FED animals (11.4 % ± 2.1 %; Fig. 5 B). There was a substantial decrease in kleptoplasts abundance during starvation compared to FED animals (42.9 % ± 5.1 %; Fig. 5 B). Kleptoplast abundance in the starvation conditions was low, albeit a considerable variation in SD animals was observed (SD: 15.7 % ± 7.9 %, SL 19.7 % ± 1.7 %; Fig. 5 C). Interestingly, SL animals slightly increased in length after one week of starvation (102 % ± 4 % of the initial length), while in SD animals, the length was reduced by about 3 % (97.0 % ± 2.0 % of the initial length; Fig. 5 D). The maximum quantum yield ($F_v/F_m$) of kleptoplasts decreased similarly in SD and SL specimens (Fig. 5 E).

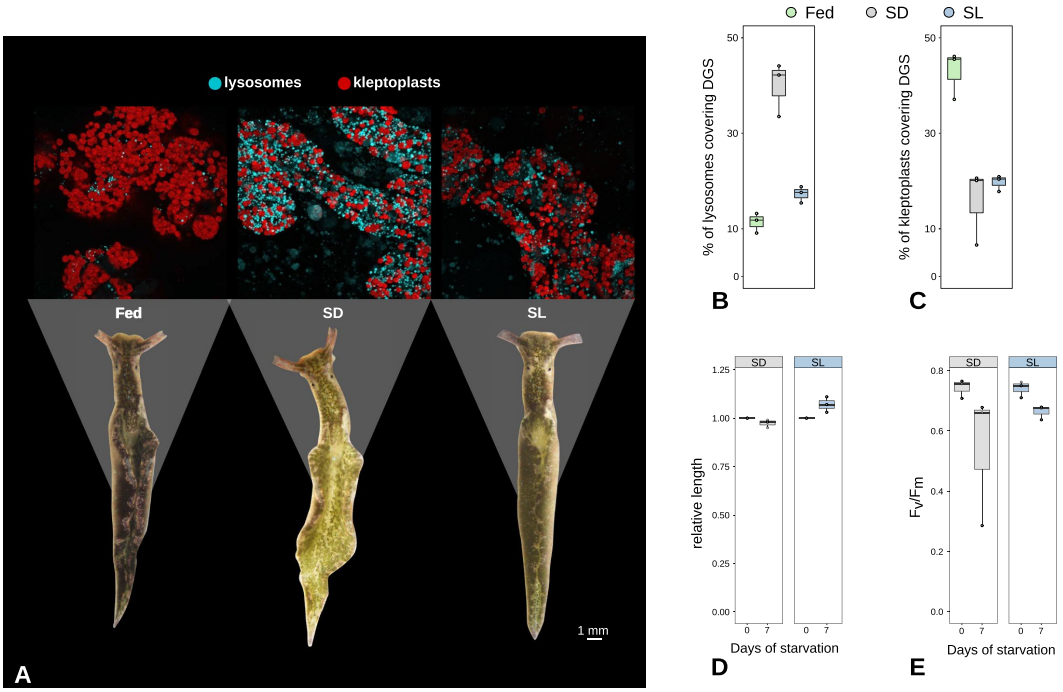


**Fig. 5. (A)** Confocal images of the digestive gland tubules of a lateral part of the parapodia and microscopic images of single individuals of *E. viridis* freshly fed (FED), starved for one week in continuous darkness (SD), and starved for one week in light (SL). The auto-fluorescence of chlorophyll *a* of kleptoplasts (red) and the fluorescence of acridine orange depicting lysosomal abundance (blue) are shown. Percentage of lysosomes **(B)** and kleptoplasts **(C)** coverage in the digestive gland tubules (DGS) of 5 different regions in the parapodia of individuals of *E. viridis* freshly fed (FED) and after one week of starvation in continuous darkness (SD) or light (SL). **(D)** Relative length of individuals of *E. viridis* during one week of starvation in continuous darkness (SD) or light (SL), and **(E)** the maximum quantum yield ($F_v/F_m$) of the kleptoplasts in the same individuals. Boxplot whiskers show the minimum and maximum values, and the center lines correspond to the medians.

Consistent with an increased lysosomal abundance, the serine/threonine-protein kinase TOR (target of rapamycin) was down-regulated in starved animals, which may initiate autophagy. Beclin-1 (BECN) was highly up-regulated in SD animals but only slightly in SL animals. Additionally, VPS34 was down-regulated in SD slugs, which can also trigger autophagy (Fig. 6 A). Several lysosomal cathepsins (CTSB, CTSD, and CTSF), serine carboxypeptidase (CPVL), and NPC intracellular cholesterol transporter 2 (NPC2) were up-regulated during starvation, independent of the light conditions (Fig. 6 A). On the contrary, lysosomal genes like alpha-glucosidase (GAA) and alpha-mannosidase (MANB) were down-regulated in starved animals (Fig. 6 A).

Glutamine is generated from ammonia by glutamine synthetase (GS), which was up-regulated in SL and SD slugs (Fig. 6 B). The glutamate synthase (GOGAT) and the catabolic glutamate dehydrogenase (GLUD2) were also up-regulated, especially in SD animals, while the anabolic glutamate dehydrogenase (GLUD1) was primarily down-regulated in SL animals (Fig. 6B).

There was no up-regulation of bicarbonate, carbonic anhydrases, or glucose transporters regardless of the light condition, but only a significant up-regulation of ammonium transporters in SD animals (Online Resource 5).

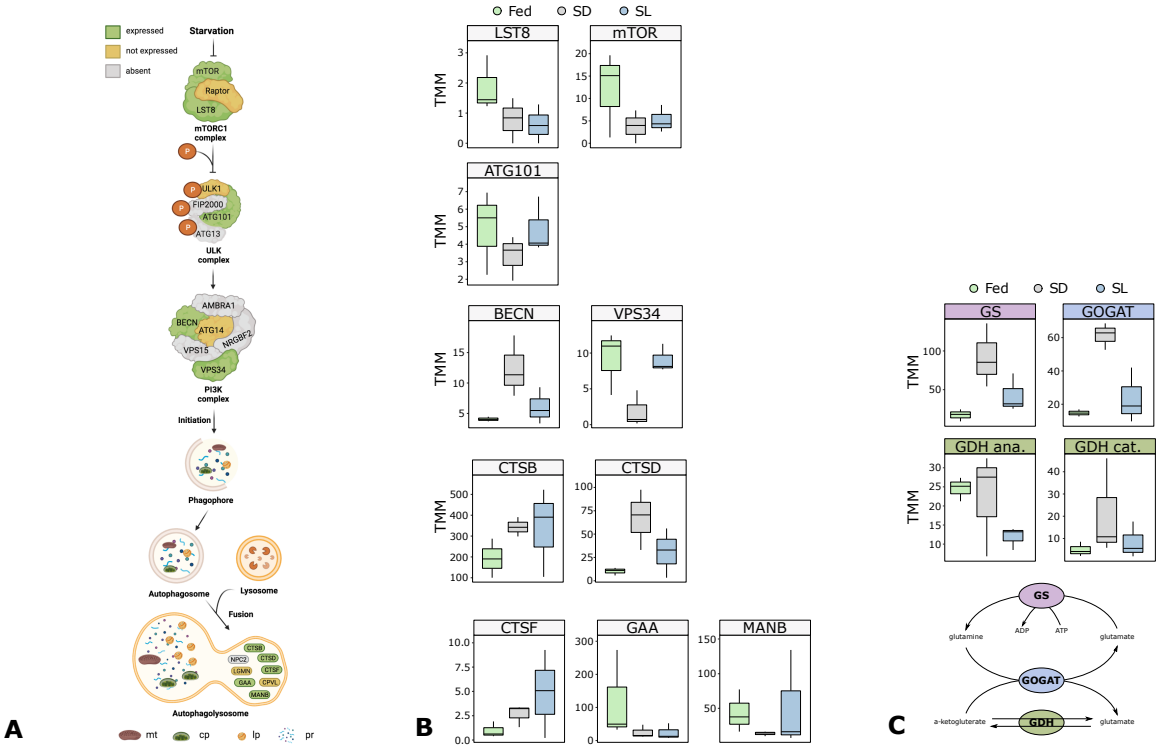


**Fig. 6.** **(A)** Simplified pathway and **(B)** expression of genes involved in autophagy-related components and downstream formation of autophagolysosomes of *E. viridis* freshly fed (FED) during one week of starvation in continuous darkness (SD) or the light (SL). The pathway was created with BioRender.com. mt: mitochodria; cp: chloroplasts; lp: lipids; pr: proteins. **(C)** Expression of genes of the GS-GOGAT cycle of *E. viridis* freshly fed (FED) during one week of starvation in continuous darkness (SD) or the light (SL). Boxplots show the expression values in TMM (Trimmed means of M values), where the whiskers are the minimum and maximum values, and the centers correspond to the medians. mTOR: mechanistic target of rapamycin kinase; ATG101: autophagy related 101; BECN: beclin; VPS34: also PIK3C3: phosphatidylinositol 3-kinase catalytic subunit type 3; CTSB, CTSD, CTSF: cathepsin B, D, F; CPVL: carboxypeptidase vitellogenic like; GAA: alpha glucosidase; MANB: phosphomannomutase; NPC2: NPC intracellular cholesterol transporter 2; GS: glutamine synthetase; GOGAT: glutamate synthase; GDH ana.: glutamate dehydrogenase anabolic; GDH cat: glutamate dehydrogenase catabolic. Pathway created with BioRender.com.

## Discussion

Even after decades of research, several aspects of functional kleptoplasty in Sacoglossa remain unclear. Of particular interest is the contribution of kleptoplasts to the metabolism of the slugs and how the slugs can access the photosynthates of the kleptoplasts. Continuous darkness is often used to assess the contribution of photosynthetically active kleptoplasts to the slug's metabolism (Casalduero and Muniain 2008; Yamamoto et al. 2013; Christa et al. 2014a; Cartaxana et al. 2017). However, it remains unexplored if and how darkness affects the metabolism of sacoglossan sea slugs. This is the first study to compare the gene expression of a plastid-bearing sea slug starved in

light and continuous darkness to shed light on the effects of starvation in darkness. We found that darkness primarily leads to glucose starvation and a general down-regulation of the metabolism. However, in conjunction with an increased autophagy rate regardless of the light condition, our results support the hypothesis that kleptoplasts function as a sort of reserve or larder in *Elysia viridis*.

Dark-starved slugs reduce their metabolic activity, cellular processes, and development. Further, starvation in darkness leads to a metabolic switch towards fatty acid beta-oxidation and deoxyribose-phosphate aldolase (DERA) expression. Interestingly, both are induced by glucose starvation in organisms (Salleron et al. 2014; Weber et al. 2020), which is observed in dark-starved specimens and could be related to a lack of glucose produced through photosynthesis. Starvation also caused the down-regulation of the translocase complexes TIM and TOM and fatty acid translocase receptors (CPTI and CPTII) of mitochondria. This down-regulation reduces the capacity to import nuclear-encoded proteins and translocate fatty acid into the mitochondria. Particularly during periods without food, CPTII deficiency prevents using certain fats for energy production (Joshi and Zierz 2020). Hence, starvation in darkness seems to induce a metabolic switch towards fatty acid degradation, but energy production through this pathway is highly reduced and independent of the light condition. Despite this, starvation in darkness might not only induce glucose starvation. Among others, constant darkness could inhibit the slug's diurnal rhythm, leading to a stress response similar to that observed in other gastropods (Shirley and Findley 1978). Darkness could also induce a resting phase, irrespective of food availability. Our KOG analyses further suggest a comparable stress response in different taxa to diverse factors like starvation, higher temperature, or darkness. Hence, at this stage, an unambiguous assignment of what triggers the reduction in the metabolic and developmental processes in dark-starved slugs is complicated. Nevertheless, the absence of photosynthates, specifically glucose, may serve as a starvation enhancer, if not an inducer. Thus, kleptoplast photosynthesis can avoid metabolic reduction by providing glucose, at least for one week of starvation in *Elysia viridis*.

It has been shown that kleptoplasts accumulate starch in some species (Evertsen and Johnsen 2009; Laetz et al. 2017b). During feeding, the kleptoplasts are exchanged (Maeda et al. 2012; Frankenbach et al. 2021), and an increased lysosomal abundance during starvation points towards digestion of the kleptoplasts (Laetz et al. 2017a). Therefore, kleptoplasts may export a few photosynthates to slugs actively or not at all. The increase in lysosomal abundance and decrease in

chloroplasts abundance that we observed here, provide further support that *E. viridis* digests its chloroplasts to acquire energy when autophagy is initiated.

Autophagy is probably the primary energy source during starvation, irrespective of starvation in light or darkness, as previously suggested (de Vries et al. 2014), but there are notable differences between starvation in light and darkness. The increased lysosomal abundance in darkness compared to light is likely based on a lower nutritional value of the kleptoplasts in non-photosynthetic conditions. Hence, more slug tissue is needed to satisfy the energetic demands. In light, kleptoplasts accumulate photosynthates (Laetz et al. 2017a) and provide sufficient amounts of glucose (or starch) to prevent physiological responses to glucose starvation and increased need for slug tissue degradation. This could also explain the slight increase in the length of the slugs in light and the decrease in the length of slugs in darkness. Similar observations were made in juveniles of *Elysia chlorotica* in which a slight increase in length was observed shortly after entering starvation, followed by a decrease in length in the longer term (Pelletreau et al. 2012). As long as the kleptoplasts are fully functional, as suggested by our Chl *a* fluorescence measurement, they can provide a benefit to the slugs, at least for a couple of days to one week, by acting as some sort of food depot.

Our results suggest that the autophagic response could be initiated via the down-regulation of VPS34 due to glucose starvation (Corona Velazquez and Jackson 2018). Since SL animals might not enter glucose starvation, a different pathway is likely involved, but the exact mechanism needs to be further examined. Moreover, even under feeding conditions, we could not find an expression of transporters relevant for nutrient exchange in other photosymbioses, including glucose, ammonium, and bicarbonate transporters (Zoccola et al. 2015; Matthews et al. 2017; Roberty et al. 2020) further supporting the lack of an active nutrient exchange.

Apart from glucose, ammonium is a crucial nutrient that connects hosts and their symbionts in photosymbioses via the GS/GOGAT cycle. It was recently suggested that functional kleptoplasts under constant feeding and light could putatively help to retrieve additional ammonium via photosynthesis in *Elysia viridis* (Cruz et al. 2020). Our results revealed an up-regulation of both GS and GOGAT during starvation in *E. viridis,* indicating that the slugs indeed receive an increased amount of ammonium. The primary source of ammonium under starvation conditions are macromolecules, such as proteins, that are degraded by autophagy (Liu et al. 2021). Hence,

starvation for one week probably does not induce a nitrogen limitation, and the kleptoplasts may serve as a nitrogen source.

Furthermore, in other photosymbioses, the translocation of bicarbonate to the symbionts is vital to ensure a high photosynthetic rate (Matthews et al. 2017; Roberty et al. 2020). In some sacoglossans, the maximum quantum yield ($F_v/F_m$) of kleptoplasts is higher compared to the $F_v/F_m$ of the chloroplasts in their natural host algae (Serôdio et al. 2014). Because we could not identify an up-regulation of relevant bicarbonate transporters, this increased $F_v/F_m$ could be based on a higher partial $CO_2$ pressure inside the animal cells that would induce a higher $CO_2$ availability for the kleptoplasts through diffusion (Serôdio et al. 2014).

**Conclusion**

Kleptoplasts in sacoglossans synthesize photosynthates when provided light and can act as a valuable source of nutrients during starvation at least for one week. Under non-photosynthetic conditions, the lack of nutritional support induces length reduction of the slug. Here we show that autophagy is probably a primary source of nutrients for the slugs during starvation in light or darkness. Starvation in darkness reduces energy-consuming processes, such as cell cycle and development, which is probably caused by glucose starvation. The photosynthetic activity of kleptoplasts may help to overcome some energetic shortcomings, but photosynthates are probably not actively exported to the slug. Kleptoplasts in *E. viridis* more likely function as larders, whose energy can be utilized following their degradation during autophagy and can support the slugs under photosynthetic conditions in the short term.

**Conflict of Interest Statement**

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

**Ethics approval**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Author Contributions**

SF, JS, and GC planned and set up the experiments. SF, JM, MB, CG, CS, LP, EL, and GC performed the experiments. GG and CL prepared the sequencing libraries and performed sequencing. SF, JM, MB, AD, CS, LP, EL, and GC analyzed the data and prepared the figures. All authors drafted and edited the manuscript.

## Data availability

All raw reads of the RNA sequencing and the DNA sequencing are available at GenBank under the Bioproject ID PRJNA810987. The RNA assembly is available as Online Resource.

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## Supplementary Material

The supplementary material of this publication is organized in Online Resources according to the style of the journal *Marine Biology* and is available online. In this section only the short tables are displayed for reading purposes.

### Online Resource 1:

**Supplementary Table 1.1.** Sequencing stats of *Elysia viridis* transcriptome.

| Sample | Treatment | Sample_ID | Raw reads | Raw data [GB] | Effective(%) | Error(%) | Q20(%) | Q30(%) | GC(%) | Biosample ID | Accession Nr. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Elvir_1 | Fed | FED01 | 67993830 | 10.2 | 97.05 | 0.03 | 97.05 | 91.9 | 35.29 | SAMN26303207 | SRR18163437 |
| Elvir_2 | Fed | FED02 | 106544212 | 16 | 97.3 | 0.03 | 97.77 | 93.75 | 37.16 | SAMN26303208 | SRR18163436 |
| Elvir_3 | Fed | FED03 | 70371612 | 10.6 | 96.34 | 0.03 | 96.27 | 90.92 | 37.46 | SAMN26303209 | SRR18163435 |
| Elvir_4 | Starved in light | SL01 | 66015288 | 9.9 | 98.94 | 0.03 | 97.28 | 92.43 | 35.13 | SAMN26303210 | SRR18163434 |
| Elvir_5 | Starved in light | SL02 | 82108314 | 12.3 | 96.19 | 0.03 | 97.73 | 93.32 | 37.45 | SAMN26303211 | SRR18163433 |
| Elvir_6 | Starved in light | SL03 | 86360294 | 13 | 95.79 | 0.03 | 97.81 | 93.89 | 38.26 | SAMN26303212 | SRR18163432 |
| Elvir_7 | Starved in darkness | SD01 | 85840148 | 12.9 | 97.55 | 0.03 | 97.52 | 92.99 | 38.44 | SAMN26303213 | SRR18163431 |
| Elvir_8 | Starved in darkness | SD02 | 71828554 | 10.8 | 97.01 | 0.03 | 96.81 | 91.7 | 36.78 | SAMN26303214 | SRR18163430 |
| Elvir_9 | Starved in darkness | SD03 | 72541412 | 10.9 | 97.53 | 0.03 | 97.93 | 93.76 | 37.28 | SAMN26303215 | SRR18163429 |

**Online Resource 6:**

**Supplementary Table 6.1.** Gene Ontology (GO) term among DEGs of SD vs FED conditions of *Elysia viridis*.

| Delta.rank | pval | Level | #seqs | GO Term | Name | p.adj | GO Category |
|---|---|---|---|---|---|---|---|
| -140 | 3.3E+06 | 1 | 72 | GO:0007049 | cell cycle | 1.6E+08 | BP |
| -120 | 2.3E+08 | 2 | 71 | GO:0009790 | embryo development | 5.7E-05 | BP |
| -119 | 1.6E-05 | 1 | 59 | GO:0007010 | cytoskeleton organization | 2.6E-04 | BP |
| 155 | 7.8E-05 | 2 | 28 | GO:0006399 | tRNA metabolic process | 7.7E-04 | BP |
| -102 | 6.4E-05 | 1 | 70 | GO:0051276 | chromosome organization | 7.7E-04 | BP |
| -119 | 1.1E-04 | 2 | 47 | GO:0000278 | mitotic cell cycle | 8.7E-04 | BP |
| -95 | 1.6E-04 | 1 | 73 | GO:0040011 | locomotion | 1.1E-03 | BP |
| -92 | 2.0E-04 | 1 | 76 | GO:0042592 | homeostatic process | 1.2E-03 | BP |
| -103 | 3.3E-04 | 2 | 54 | GO:0006397 | mRNA processing | 1.8E-03 | BP |
| -128 | 5.0E-04 | 2 | 32 | GO:0006913 | nucleocytoplasmic transport | 2.3E-03 | BP |
| -184 | 5.2E-04 | 2 | 15 | GO:0030705 | cytoskeleton-dependent intracellular transport | 2.3E-03 | BP |
| 88 | 7.7E-04 | 2 | 66 | GO:0006520 | cellular amino acid metabolic process | 3.1E-03 | BP |
| 79 | 1.1E-03 | 2 | 79 | GO:0006412 | translation | 3.9E-03 | BP |
| -111 | 1.3E-03 | 1 | 37 | GO:0007005 | mitochondrion organization | 3.9E-03 | BP |
| -95 | 1.2E-03 | 1 | 52 | GO:0048646 | anatomical structure formation involved in morphogenesis | 3.9E-03 | BP |
| -97 | 1.3E-03 | 1 | 49 | GO:0050877 | nervous system process | 3.9E-03 | BP |
| -89 | 2.3E-03 | 2 | 53 | GO:0048870 | cell motility | 6.5E-03 | BP |
| -94 | 3.3E-03 | 1 | 43 | GO:0007568 | aging | 9.0E-03 | BP |
| -99 | 4.3E-03 | 1 | 36 | GO:0061024 | membrane organization | 1.1E-02 | BP |
| -95 | 5.2E-03 | 1 | 38 | GO:0000902 | cell morphogenesis | 1.2E-02 | BP |
| -118 | 5.2E-03 | 1 | 24 | GO:0007059 | chromosome segregation | 1.2E-02 | BP |
| -77 | 1.7E-02 | 1 | 43 | GO:0008283 | cell population proliferation | 3.7E-02 | BP |
| 54 | 6.7E-02 | 1 | 52 | GO:0005975 | carbohydrate metabolic process | 1.4E-01 | BP |
| -74 | 1.0E-01 | 1 | 21 | GO:0007155 | cell adhesion | 2.0E-01 | BP |
| -52 | 1.0E-01 | 1 | 44 | GO:0008219 | cell death | 2.0E-01 | BP |
| -70 | 1.0E-01 | 1 | 23 | GO:0030198 | extracellular matrix organization | 2.0E-01 | BP |
| -76 | 1.2E-01 | 1 | 18 | GO:0021700 | developmental maturation | 2.0E-01 | BP |
| -86 | 1.2E-01 | 1 | 14 | GO:0140014 | mitotic nuclear division | 2.0E-01 | BP |
| 50 | 1.2E-01 | 2 | 42 | GO:0006351 | transcription. DNA-templated | 2.1E-01 | BP |
| -67 | 1.8E-01 | 1 | 17 | GO:0003013 | circulatory system process | 3.0E-01 | BP |
| -50 | 1.9E-01 | 1 | 29 | GO:0051301 | cell division | 3.0E-01 | BP |
| -50 | 2.1E-01 | 2 | 27 | GO:0006605 | protein targeting | 3.3E-01 | BP |
| -26 | 2.9E-01 | 2 | 77 | GO:0006259 | DNA metabolic process | 4.0E-01 | BP |
| -90 | 2.8E-01 | 1 | 6 | GO:0019748 | secondary metabolic process | 4.0E-01 | BP |
| -77 | 2.9E-01 | 1 | 8 | GO:0034330 | cell junction organization | 4.0E-01 | BP |
| -45 | 2.7E-01 | 1 | 26 | GO:0040007 | growth | 4.0E-01 | BP |
| -31 | 3.6E-01 | 1 | 39 | GO:0044403 | biological process involved in symbiotic interaction | 4.7E-01 | BP |
| -29 | 3.7E-01 | 2 | 42 | GO:0055085 | transmembrane transport | 4.8E-01 | BP |
| 49 | 4.5E-01 | 2 | 10 | GO:0007034 | vacuolar transport | 5.7E-01 | BP |
| 20 | 5.2E-01 | 1 | 46 | GO:0006457 | protein folding | 6.4E-01 | BP |
| 14 | 5.7E-01 | 1 | 76 | GO:0006629 | lipid metabolic process | 6.8E-01 | BP |
| 15 | 6.0E-01 | 1 | 53 | GO:0006091 | generation of precursor metabolites and energy | 6.8E-01 | BP |
| 22 | 6.0E-01 | 1 | 24 | GO:0051604 | protein maturation | 6.8E-01 | BP |
| -16 | 6.2E-01 | 3 | 42 | GO:0022618 | ribonucleoprotein complex assembly | 6.9E-01 | BP |
| 9 | 8.2E-01 | 1 | 29 | GO:0007267 | cell-cell signaling | 8.9E-01 | BP |
| 7 | 8.5E-01 | 1 | 37 | GO:0006790 | sulfur compound metabolic process | 9.0E-01 | BP |
| 4 | 8.8E-01 | 1 | 60 | GO:0042254 | ribosome biogenesis | 9.2E-01 | BP |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| -6 | 9.0E-01 | 2 | 21 | GO:0006914 | autophagy | 9.2E-01 | BP |
| 1 | 9.8E-01 | 2 | 47 | GO:0034655 | nucleobase-containing compound catabolic process | 9.8E-01 | BP |
| 130 | 9.7E+08 | 1 | 35 | GO:0016874 | ligase activity | 3.0E-04 | MF |
| -99 | 8.0E-04 | 1 | 35 | GO:0008092 | cytoskeletal protein binding | 1.2E-02 | MF |
| 96 | 1.5E-03 | 1 | 33 | GO:0016829 | lyase activity | 1.6E-02 | MF |
| -83 | 2.8E-03 | 1 | 40 | GO:0008134 | transcription factor binding | 2.1E-02 | MF |
| 69 | 9.5E-03 | 1 | 44 | GO:0016853 | isomerase activity | 5.9E-02 | MF |
| -54 | 4.6E-02 | 2 | 41 | GO:0003729 | mRNA binding | 1.4E-01 | MF |
| 76 | 4.2E-02 | 1 | 21 | GO:0016779 | nucleotidyltransferase activity | 1.4E-01 | MF |
| -100 | 3.4E-02 | 1 | 13 | GO:0016791 | phosphatase activity | 1.4E-01 | MF |
| -109 | 3.4E-02 | 1 | 11 | GO:0016798 | hydrolase activity. acting on glycosyl bonds | 1.4E-01 | MF |
| -62 | 4.6E-02 | 1 | 31 | GO:0030234 | enzyme regulator activity | 1.4E-01 | MF |
| 93 | 5.8E-02 | 2 | 12 | GO:0004386; GO:0140657 | helicase activity | 1.6E-01 | MF |
| -130 | 6.1E-02 | 1 | 6 | GO:0030674 | protein-macromolecule adaptor activity | 1.6E-01 | MF |
| -57 | 8.4E-02 | 1 | 28 | GO:0008289 | lipid binding | 2.0E-01 | MF |
| -124 | 1.0E-01 | 1 | 5 | GO:0042393 | histone binding | 2.3E-01 | MF |
| -37 | 1.2E-01 | 1 | 58 | GO:0005198 | structural molecule activity | 2.4E-01 | MF |
| 41 | 1.9E-01 | 2 | 31 | GO:0003735 | structural constituent of ribosome | 3.7E-01 | MF |
| 27 | 2.1E-01 | 1 | 67 | GO:0008233 | peptidase activity | 3.8E-01 | MF |
| 38 | 4.1E-01 | 2 | 14 | GO:0019843 | rRNA binding | 7.0E-01 | MF |
| -58 | 4.4E-01 | 1 | 5 | GO:0016765 | transferase activity. transferring alkyl or aryl (other than methyl) groups | 7.3E-01 | MF |
| 26 | 4.8E-01 | 1 | 22 | GO:0016301 | kinase activity | 7.4E-01 | MF |
| -21 | 5.1E-01 | 1 | 30 | GO:0016746 | acyltransferase activity | 7.6E-01 | MF |
| 19 | 6.0E-01 | 1 | 24 | GO:0003924 | GTPase activity | 7.6E-01 | MF |
| 20 | 5.7E-01 | 2 | 25 | GO:0008135 | translation factor activity. RNA binding | 7.6E-01 | MF |
| -35 | 6.2E-01 | 1 | 6 | GO:0016757 | glycosyltransferase activity | 7.6E-01 | MF |
| -16 | 5.9E-01 | 1 | 34 | GO:0022857 | transmembrane transporter activity | 7.6E-01 | MF |
| 16 | 7.0E-01 | 1 | 17 | GO:0008168 | methyltransferase activity | 8.2E-01 | MF |
| 19 | 7.1E-01 | 1 | 11 | GO:0016810 | hydrolase activity. acting on carbon-nitrogen (but not peptide) bonds | 8.2E-01 | MF |
| -5 | 8.2E-01 | 1 | 66 | GO:0003677 | DNA binding | 8.2E-01 | MF |
| 11 | 7.5E-01 | 1 | 24 | GO:0003700 | DNA-binding transcription factor activity | 8.2E-01 | MF |
| -10 | 7.7E-01 | 1 | 25 | GO:0004518 | nuclease activity | 8.2E-01 | MF |
| -8 | 8.2E-01 | 1 | 25 | GO:0051082 | unfolded protein binding | 8.2E-01 | MF |
| 64 | 8.3E-05 | 2 | 37 | GO:0005840 | ribosome | 1.3E-03 | CC |
| -59 | 1.2E-03 | 2 | 29 | GO:0005929 | cilium | 9.7E-03 | CC |
| -55 | 2.2E-03 | 1 | 30 | GO:0005815 | microtubule organizing center | 1.1E-02 | CC |
| -36 | 3.3E-03 | 2 | 75 | GO:0005856 | cytoskeleton | 1.3E-02 | CC |
| 27 | 4.7E-02 | 2 | 57 | GO:0005764; GO:0005773 | vacuole | 1.5E-01 | CC |
| 21 | 1.8E-01 | 3 | 43 | GO:0005768 | endosome | 4.1E-01 | CC |
| -35 | 1.6E-01 | 2 | 15 | GO:0005777 | peroxisome | 4.1E-01 | CC |
| 16 | 2.2E-01 | 1 | 66 | GO:0005615 | extracellular space | 4.4E-01 | CC |
| -12 | 4.7E-01 | 3 | 32 | GO:0000228 | nuclear chromosome | 7.0E-01 | CC |
| -10 | 4.6E-01 | 2 | 63 | GO:0005694 | chromosome | 7.0E-01 | CC |
| -29 | 4.9E-01 | 2 | 5 | GO:0009536 | plastid | 7.0E-01 | CC |
| 12 | 5.2E-01 | 2 | 29 | GO:0031012; GO:0030312 | external encapsulating structure | 7.0E-01 | CC |
| 17 | 6.6E-01 | 2 | 6 | GO:0005618 | cell wall | 8.1E-01 | CC |
| -2 | 9.2E-01 | 1 | 26 | GO:0005635 | nuclear envelope | 9.5E-01 | CC |
| -2 | 8.6E-01 | 2 | 57 | GO:0005794 | Golgi apparatus | 9.5E-01 | CC |
| 3 | 9.5E-01 | 2 | 6 | GO:0005811 | lipid droplet | 9.5E-01 | CC |

**Supplementary Table 6.2.** Gene Ontology (GO) term among DEGs of SD vs SL conditions of *Elysia viridis*.

| Delta.rank | pval | Level | #seqs | GO Term | Name | p.adj | GO Category |
|---|---|---|---|---|---|---|---|
| 86 | 1.5E-07 | 2 | 32 | GO:0006412 | translation | 6.2E-06 | BP |
| -76 | 8.7E-07 | 2 | 37 | GO:0009790 | embryo development | 1.7E-05 | BP |
| 99 | 2.2E-05 | 2 | 15 | GO:0006399 | tRNA metabolic process | 2.2E-04 | BP |
| -70 | 1.9E-05 | 1 | 32 | GO:0007010 | cytoskeleton organization | 2.2E-04 | BP |
| -63 | 5.2E-05 | 1 | 37 | GO:0040011 | locomotion | 4.2E-04 | BP |
| -75 | 1.9E-04 | 1 | 21 | GO:0000902 | cell morphogenesis | 1.2E-03 | BP |
| -80 | 3.0E-04 | 1 | 17 | GO:0007267 | cell-cell signaling | 1.7E-03 | BP |
| -62 | 6.5E-04 | 1 | 26 | GO:0050877 | nervous system process | 3.3E-03 | BP |
| -51 | 1.4E-03 | 2 | 35 | GO:0015031 | protein transport | 5.8E-03 | BP |
| -56 | 1.5E-03 | 2 | 28 | GO:0048870 | cell motility | 5.8E-03 | BP |
| -63 | 2.1E-03 | 1 | 20 | GO:0007049 | cell cycle | 7.5E-03 | BP |
| -46 | 3.5E-03 | 1 | 36 | GO:0042592 | homeostatic process | 1.2E-02 | BP |
| 44 | 7.9E-03 | 2 | 31 | GO:0006520 | cellular amino acid metabolic process | 2.4E-02 | BP |
| -39 | 9.3E-03 | 1 | 39 | GO:0002376 | immune system process | 2.6E-02 | BP |
| 54 | 1.1E-02 | 1 | 18 | GO:0006457 | protein folding | 3.0E-02 | BP |
| -58 | 1.6E-02 | 2 | 14 | GO:0000278 | mitotic cell cycle | 3.9E-02 | BP |
| -59 | 2.3E-02 | 1 | 12 | GO:0061024 | membrane organization | 5.4E-02 | BP |
| 41 | 2.8E-02 | 1 | 25 | GO:0006091 | generation of precursor metabolites and energy | 5.7E-02 | BP |
| -51 | 2.9E-02 | 1 | 15 | GO:0040007 | growth | 5.7E-02 | BP |
| -40 | 2.8E-02 | 1 | 26 | GO:0048646 | anatomical structure formation involved in morphogenesis | 5.7E-02 | BP |
| -51 | 3.0E-02 | 1 | 15 | GO:0007568 | aging | 5.8E-02 | BP |
| -59 | 3.8E-02 | 1 | 10 | GO:0003013 | circulatory system process | 6.9E-02 | BP |
| 60 | 4.5E-02 | 2 | 9 | GO:0022618 | ribonucleoprotein complex assembly | 7.9E-02 | BP |
| -42 | 5.6E-02 | 2 | 17 | GO:0006397 | mRNA processing | 9.3E-02 | BP |
| 41 | 6.3E-02 | 2 | 17 | GO:0006351 | transcription, DNA-templated | 1.0E-01 | BP |
| -47 | 8.4E-02 | 1 | 11 | GO:0007155 | cell adhesion | 1.3E-01 | BP |
| -51 | 9.0E-02 | 1 | 9 | GO:0021700 | developmental maturation | 1.3E-01 | BP |
| 40 | 1.1E-01 | 1 | 13 | GO:0042254 | ribosome biogenesis | 1.6E-01 | BP |
| -33 | 1.2E-01 | 1 | 19 | GO:0051276 | chromosome organization | 1.6E-01 | BP |
| -33 | 1.6E-01 | 1 | 15 | GO:0051301 | cell division | 2.1E-01 | BP |
| -31 | 2.2E-01 | 1 | 13 | GO:0008219 | cell death | 2.8E-01 | BP |
| -30 | 2.5E-01 | 1 | 12 | GO:0007005 | mitochondrion organization | 3.0E-01 | BP |
| 29 | 2.5E-01 | 1 | 13 | GO:0044403 | biological process involved in symbiotic interaction | 3.0E-01 | BP |
| 15 | 3.2E-01 | 1 | 37 | GO:0005975 | carbohydrate metabolic process | 3.8E-01 | BP |
| 24 | 3.5E-01 | 1 | 12 | GO:0051604 | protein maturation | 4.0E-01 | BP |
| 16 | 4.7E-01 | 2 | 18 | GO:0034655 | nucleobase-containing compound catabolic process | 5.2E-01 | BP |
| -17 | 4.9E-01 | 2 | 14 | GO:0055085 | transmembrane transport | 5.3E-01 | BP |
| -10 | 7.0E-01 | 1 | 11 | GO:0030198 | extracellular matrix organization | 7.4E-01 | BP |
| -8 | 7.3E-01 | 1 | 14 | GO:0006790 | sulfur compound metabolic process | 7.5E-01 | BP |
| 2 | 9.2E-01 | 2 | 24 | GO:0006259 | DNA metabolic process | 9.2E-01 | BP |
| 58 | 8.4E-04 | 1 | 19 | GO:0016874 | ligase activity | 2.4E-02 | MF |
| -50 | 4.6E-03 | 1 | 18 | GO:0008092 | cytoskeletal protein binding | 4.5E-02 | MF |
| 46 | 3.4E-03 | 1 | 24 | GO:0016853 | isomerase activity | 4.5E-02 | MF |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 50 | 1.5E-02 | 2 | 13 | GO:0003735 | structural constituent of ribosome | 6.6E-02 | MF |
| 67 | 1.6E-02 | 1 | 7 | GO:0003924 | GTPase activity | 6.6E-02 | MF |
| 73 | 1.5E-02 | 2 | 6 | GO:0019843 | rRNA binding | 6.6E-02 | MF |
| 63 | 1.5E-02 | 1 | 8 | GO:0051082 | unfolded protein binding | 6.6E-02 | MF |
| 57 | 2.1E-02 | 2 | 9 | GO:0008135 | translation factor activity, RNA binding | 7.7E-02 | MF |
| -50 | 3.3E-02 | 1 | 10 | GO:0016798 | hydrolase activity, acting on glycosyl bonds | 1.1E-01 | MF |
| -36 | 3.9E-02 | 1 | 19 | GO:0030234 | enzyme regulator activity | 1.1E-01 | MF |
| -38 | 4.7E-02 | 1 | 15 | GO:0008289 | lipid binding | 1.2E-01 | MF |
| -36 | 6.2E-02 | 2 | 15 | GO:0003729 | mRNA binding | 1.5E-01 | MF |
| 28 | 9.5E-02 | 1 | 20 | GO:0016829 | lyase activity | 2.1E-01 | MF |
| -22 | 1.3E-01 | 1 | 27 | GO:0003677 | DNA binding | 2.7E-01 | MF |
| -48 | 1.4E-01 | 1 | 5 | GO:0032182 | ubiquitin-like protein binding | 2.7E-01 | MF |
| -29 | 2.4E-01 | 1 | 9 | GO:0008134 | transcription factor binding | 4.2E-01 | MF |
| -31 | 2.3E-01 | 1 | 8 | GO:0016791 | phosphatase activity | 4.2E-01 | MF |
| 26 | 2.7E-01 | 1 | 10 | GO:0016779 | nucleotidyltransferase activity | 4.3E-01 | MF |
| -20 | 3.4E-01 | 1 | 13 | GO:0022857 | transmembrane transporter activity | 5.3E-01 | MF |
| -18 | 5.8E-01 | 1 | 5 | GO:0016757 | glycosyltransferase activity | 8.4E-01 | MF |
| 7 | 7.7E-01 | 1 | 9 | GO:0003700 | DNA-binding transcription factor activity | 9.0E-01 | MF |
| 9 | 7.4E-01 | 1 | 7 | GO:0004518 | nuclease activity | 9.0E-01 | MF |
| -15 | 6.5E-01 | 1 | 5 | GO:0008168 | methyltransferase activity | 9.0E-01 | MF |
| -5 | 7.4E-01 | 1 | 24 | GO:0008233 | peptidase activity | 9.0E-01 | MF |
| -6 | 7.5E-01 | 1 | 15 | GO:0016746 | acyltransferase activity | 9.0E-01 | MF |
| 3 | 9.3E-01 | 2 | 6 | GO:0004386; GO:0140657 | helicase activity | 9.6E-01 | MF |
| 1 | 9.2E-01 | 1 | 28 | GO:0005198 | structural molecule activity | 9.6E-01 | MF |
| -3 | 9.0E-01 | 1 | 11 | GO:0016301 | kinase activity | 9.6E-01 | MF |
| 2 | 9.6E-01 | 1 | 5 | GO:0016810 | hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds | 9.6E-01 | MF |
| 58 | 8.7E-06 | 2 | 16 | GO:0005840 | ribosome | 1.2E-04 | CC |
| -26 | 4.7E-02 | 2 | 15 | GO:0005694 | chromosome | 2.2E-01 | CC |
| -23 | 4.8E-02 | 3 | 20 | GO:0005768 | endosome | 2.2E-01 | CC |
| -21 | 6.8E-02 | 2 | 21 | GO:0005929 | cilium | 2.4E-01 | CC |
| 32 | 8.9E-02 | 1 | 7 | GO:0005635 | nuclear envelope | 2.5E-01 | CC |
| -15 | 1.2E-01 | 2 | 31 | GO:0005794 | Golgi apparatus | 2.7E-01 | CC |
| 19 | 1.4E-01 | 1 | 17 | GO:0030312 | external encapsulating structure | 2.7E-01 | CC |
| -19 | 1.8E-01 | 1 | 13 | GO:0005815 | microtubule organizing center | 3.1E-01 | CC |
| 13 | 3.8E-01 | 2 | 12 | GO:0031012 | extracellular matrix | 5.8E-01 | CC |
| 11 | 4.2E-01 | 2 | 15 | GO:0005730 | nucleolus | 5.9E-01 | CC |
| -4 | 6.6E-01 | 1 | 31 | GO:0005615 | extracellular space | 7.1E-01 | CC |
| 4 | 6.4E-01 | 2 | 33 | GO:0005739 | mitochondrion | 7.1E-01 | CC |
| 9 | 6.3E-01 | 2 | 7 | GO:0005777 | peroxisome | 7.1E-01 | CC |
| 3 | 8.0E-01 | 2 | 27 | GO:0005773; GO:0005764 | vacuole | 8.0E-01 | CC |

**Supplementary Table 6.3.** Gene Ontology (GO) term among DEGs of SL vs FED conditions of *Elysia viridis*.

| Delta.rank | pval | Level | #seqs | GO Term | Name | p.adj | GO Category |
|---|---|---|---|---|---|---|---|
| 16 | 0.03 | 1 | 8 | GO:0007010 | cytoskeleton organization | 0.66 | BP |
| -13 | 0.09 | 1 | 7 | GO:0007005 | mitochondrion organization | 0.70 | BP |
| -17 | 0.06 | 1 | 5 | GO:0030198 | extracellular matrix organization | 0.70 | BP |
| -8 | 0.26 | 2 | 9 | GO:0006259 | DNA metabolic process | 0.82 | BP |
| -6 | 0.48 | 2 | 6 | GO:0006397 | mRNA processing | 0.82 | BP |
| -8 | 0.40 | 2 | 5 | GO:0006605 | protein targeting | 0.82 | BP |
| -5 | 0.44 | 1 | 9 | GO:0006790 | sulfur compound metabolic process | 0.82 | BP |
| 10 | 0.15 | 1 | 8 | GO:0007049 | cell cycle | 0.82 | BP |
| -7 | 0.47 | 1 | 5 | GO:0007155 | cell adhesion | 0.82 | BP |
| -8 | 0.26 | 1 | 9 | GO:0008219 | cell death | 0.82 | BP |
| -8 | 0.31 | 3 | 6 | GO:0022618 | ribonucleoprotein complex assembly | 0.82 | BP |
| -9 | 0.29 | 1 | 6 | GO:0042254 | ribosome biogenesis | 0.82 | BP |
| -8 | 0.33 | 1 | 7 | GO:0048646 | anatomical structure formation involved in morphogenesis | 0.82 | BP |
| -6 | 0.50 | 1 | 6 | GO:0050877 | nervous system process | 0.82 | BP |
| 5 | 0.54 | 1 | 6 | GO:0005975 | carbohydrate metabolic process | 0.83 | BP |
| 4 | 0.62 | 1 | 6 | GO:0008283 | cell population proliferation | 0.89 | BP |
| -1 | 0.90 | 2 | 5 | GO:0006351 | transcription, DNA-templated | 0.98 | BP |
| 2 | 0.84 | 2 | 7 | GO:0006520 | cellular amino acid metabolic process | 0.98 | BP |
| -1 | 0.94 | 1 | 9 | GO:0007568 | aging | 0.98 | BP |
| 2 | 0.81 | 1 | 5 | GO:0040007 | growth | 0.98 | BP |
| -2 | 0.75 | 1 | 7 | GO:0040011; GO:0048870 | locomotion | 0.98 | BP |
| -1 | 0.92 | 1 | 6 | GO:0051276 | chromosome organization | 0.98 | BP |
| 0 | 1.00 | 2 | 6 | GO:0006913 | nucleocytoplasmic transport | 1.00 | BP |
| -4 | 0.33 | 1 | 7 | GO:0003677 | DNA binding | 0.77 | MF |
| 6 | 0.25 | 1 | 5 | GO:0008092 | cytoskeletal protein binding | 0.77 | MF |
| 7 | 0.18 | 1 | 4 | GO:0016829 | lyase activity | 0.77 | MF |
| -3 | 0.48 | 1 | 7 | GO:0005198 | structural molecule activity | 0.83 | MF |
| -2 | 0.68 | 1 | 5 | GO:0030234 | enzyme regulator activity | 0.95 | MF |
| 0 | 0.98 | 1 | 8 | GO:0008134 | transcription factor binding | 0.98 | MF |
| 1 | 0.85 | 1 | 7 | GO:0008289 | lipid binding | 0.98 | MF |
| -6 | 0.11 | 2 | 8 | GO:0031012; GO:0030312 | extracellular matrix | 0.43 | CC |
| 4 | 0.24 | 1 | 7 | GO:0005815 | microtubule organizing center | 0.48 | CC |
| 2 | 0.69 | 3 | 7 | GO:0005768 | endosome | 0.91 | CC |
| 0 | 0.93 | 2 | 6 | GO:0005694 | chromosome | 0.93 | CC |

**Online Resource 7:**

**Supplementary Table 7.1.** EuKaryotic Orthologous Groups (KOG) term among DEGs of SD vs FED conditions of *Elysia viridis*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Signal transduction mechanisms | 85 | -237 | 1.2E-08 | 2.8E-07 |
| Cell wall/membrane/envelope biogenesis | 30 | 335 | 9.7E-07 | 9.7E-06 |
| Energy production and conversion | 119 | 172 | 1.3E-06 | 9.7E-06 |
| Intracellular trafficking, secretion, and vesicular transport | 59 | -223 | 5.9E-06 | 3.4E-05 |
| Nucleotide transport and metabolism | 47 | 223 | 5.0E-05 | 2.3E-04 |
| RNA processing and modification | 64 | -177 | 1.9E-04 | 7.2E-04 |
| Cytoskeleton | 40 | -219 | 2.3E-04 | 7.7E-04 |
| Amino acid transport and metabolism | 91 | 133 | 9.5E-04 | 2.7E-03 |
| Posttranslational modification, protein turnover, chaperones | 203 | -76 | 7.4E-03 | 1.9E-02 |
| Carbohydrate transport and metabolism | 67 | 108 | 2.0E-02 | 4.5E-02 |
| Chromatin structure and dynamics | 11 | -217 | 5.3E-02 | 1.1E-01 |
| Extracellular structures | 15 | -182 | 5.8E-02 | 1.1E-01 |
| Cell motility | 2 | -468 | 7.4E-02 | 1.3E-01 |
| Coenzyme transport and metabolism | 26 | 123 | 9.2E-02 | 1.5E-01 |
| Inorganic ion transport and metabolism | 36 | 88 | 1.6E-01 | 2.5E-01 |
| Transcription | 62 | 43 | 3.7E-01 | 5.2E-01 |
| Lipid transport and metabolism | 67 | 40 | 3.9E-01 | 5.2E-01 |
| Secondary metabolites biosynthesis, transport and catabolism | 27 | 30 | 6.8E-01 | 8.7E-01 |
| Translation, ribosomal structure and biogenesis | 155 | 10 | 7.6E-01 | 8.9E-01 |
| Cell cycle control, cell division, chromosome partitioning | 13 | 28 | 7.9E-01 | 8.9E-01 |
| Replication, recombination and repair | 60 | 12 | 8.1E-01 | 8.9E-01 |
| Nuclear structure | 1 | 71 | 8.5E-01 | 8.9E-01 |
| Defense mechanisms | 9 | 12 | 9.2E-01 | 9.2E-01 |

**Supplementary Table 7.2.** EuKaryotic Orthologous Groups (KOG) term among DEGs of SD vs SL conditions of *Elysia viridis*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Signal transduction mechanisms | 53 | -125 | 0.00 | 0.00 |
| Cell wall/membrane/envelope biogenesis | 22 | 136 | 0.00 | 0.00 |
| RNA processing and modification | 22 | -129 | 0.00 | 0.00 |
| Energy production and conversion | 63 | 76 | 0.00 | 0.00 |
| Cytoskeleton | 26 | -100 | 0.00 | 0.01 |
| Intracellular trafficking, secretion, and vesicular transport | 23 | -77 | 0.03 | 0.11 |
| Amino acid transport and metabolism | 53 | 50 | 0.04 | 0.12 |
| Translation, ribosomal structure and biogenesis | 61 | 39 | 0.08 | 0.22 |
| Nucleotide transport and metabolism | 15 | 65 | 0.14 | 0.33 |
| Cell motility | 1 | -240 | 0.15 | 0.33 |
| Extracellular structures | 8 | -80 | 0.18 | 0.33 |
| Coenzyme transport and metabolism | 14 | 60 | 0.18 | 0.33 |
| Transcription | 24 | 41 | 0.23 | 0.38 |
| Replication, recombination and repair | 18 | 47 | 0.24 | 0.38 |
| Chromatin structure and dynamics | 4 | -90 | 0.28 | 0.42 |
| Lipid transport and metabolism | 33 | 28 | 0.35 | 0.48 |
| Inorganic ion transport and metabolism | 24 | -30 | 0.38 | 0.49 |
| Defense mechanisms | 2 | -65 | 0.58 | 0.71 |
| Posttranslational modification, protein turnover, chaperones | 69 | -8 | 0.70 | 0.81 |
| Carbohydrate transport and metabolism | 33 | 8 | 0.78 | 0.86 |
| Cell cycle control, cell division, chromosome partitioning | 6 | -14 | 0.84 | 0.88 |
| Secondary metabolites biosynthesis, transport and catabolism | 8 | -3 | 0.96 | 0.96 |

**Supplementary Table 7.3.** EuKaryotic Orthologous Groups (KOG) term among DEGs of SL vs FED conditions of *Elysia viridis*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Chromatin structure and dynamics | 2 | -53 | 0.04 | 0.64 |
| Secondary metabolites biosynthesis, transport and catabolism | 4 | -33 | 0.06 | 0.64 |
| Cell wall/membrane/envelope biogenesis | 3 | 32 | 0.12 | 0.64 |
| Cytoskeleton | 5 | 25 | 0.12 | 0.64 |
| Translation, ribosomal structure and biogenesis | 9 | -15 | 0.23 | 0.89 |
| Inorganic ion transport and metabolism | 3 | -19 | 0.35 | 0.89 |
| Nucleotide transport and metabolism | 2 | -21 | 0.40 | 0.89 |
| Amino acid transport and metabolism | 10 | 10 | 0.40 | 0.89 |
| Carbohydrate transport and metabolism | 10 | 9 | 0.43 | 0.89 |
| Extracellular structures | 5 | -11 | 0.49 | 0.89 |
| Defense mechanisms | 1 | -24 | 0.50 | 0.89 |
| Coenzyme transport and metabolism | 3 | 14 | 0.51 | 0.89 |
| RNA processing and modification | 6 | 8 | 0.61 | 0.90 |
| Replication, recombination and repair | 2 | 13 | 0.62 | 0.90 |
| Posttranslational modification, protein turnover, chaperones | 16 | 4 | 0.66 | 0.90 |
| Cell cycle control, cell division, chromosome partitioning | 2 | -10 | 0.71 | 0.90 |
| Transcription | 5 | 6 | 0.73 | 0.90 |
| Signal transduction mechanisms | 18 | -2 | 0.81 | 0.94 |
| Energy production and conversion | 6 | 1 | 0.95 | 1.00 |
| Intracellular trafficking, secretion, and vesicular transport | 3 | -1 | 0.97 | 1.00 |
| Lipid transport and metabolism | 6 | 0 | 1.00 | 1.00 |

**Supplementary Table 7.4.** EuKaryotic Orthologous Groups (KOG) term among DEGs of Day 5 of development and feeding of *Elysia chlorotica*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Signal transduction mechanisms | 731 | -933 | 3.9E-76 | 1.0E-74 |
| Inorganic ion transport and metabolism | 184 | -1051 | 3.8E-29 | 5.0E-28 |
| Translation, ribosomal structure and biogenesis | 237 | 875 | 7.0E-26 | 6.1E-25 |
| Replication, recombination and repair | 130 | 951 | 9.4E-18 | 6.1E-17 |
| RNA processing and modification | 200 | 627 | 3.6E-12 | 1.9E-11 |
| General function prediction only | 783 | -281 | 1.1E-08 | 4.6E-08 |
| Energy production and conversion | 135 | 499 | 4.5E-06 | 1.7E-05 |
| Extracellular structures | 90 | -595 | 7.3E-06 | 2.4E-05 |
| Function unknown | 396 | 282 | 1.7E-05 | 5.1E-05 |
| Cell cycle control, cell division, chromosome partitioning | 136 | 463 | 2.0E-05 | 5.1E-05 |
| Nuclear structure | 31 | 928 | 3.5E-05 | 8.4E-05 |
| Posttranslational modification, protein turnover, chaperones | 438 | 253 | 5.6E-05 | 1.2E-04 |
| Cell wall/membrane/envelope biogenesis | 56 | -624 | 2.0E-04 | 3.9E-04 |
| Cytoskeleton | 169 | -328 | 7.9E-04 | 1.5E-03 |
| Coenzyme transport and metabolism | 41 | 585 | 2.7E-03 | 4.7E-03 |
| Lipid transport and metabolism | 192 | 238 | 9.5E-03 | 1.5E-02 |
| Defense mechanisms | 50 | -383 | 3.0E-02 | 4.7E-02 |
| Nucleotide transport and metabolism | 76 | 276 | 5.5E-02 | 8.0E-02 |
| Cell motility | 13 | -616 | 7.5E-02 | 1.0E-01 |
| Amino acid transport and metabolism | 170 | -158 | 1.0E-01 | 1.4E-01 |
| Translation, ribosomal structure and biogenesis | 1 | 1932 | 1.2E-01 | 1.5E-01 |
| Chromatin structure and dynamics | 70 | 221 | 1.4E-01 | 1.7E-01 |
| Intracellular trafficking, secretion, and vesicular transport | 253 | 74 | 3.6E-01 | 4.0E-01 |
| Secondary metabolites biosynthesis, transport and catabolism | 101 | -42 | 7.4E-01 | 7.5E-01 |
| Transcription | 237 | 27 | 7.4E-01 | 7.5E-01 |
| Carbohydrate transport and metabolism | 155 | -32 | 7.5E-01 | 7.5E-01 |

**Supplementary Table 7.5.** EuKaryotic Orthologous Groups (KOG) term among DEGs of Day 7 of development and feeding of *Elysia chlorotica*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Signal transduction mechanisms | 1012 | -1143 | 1.4E-99 | 3.6E-98 |
| Translation, ribosomal structure and biogenesis | 218 | 1314 | 1.0E-34 | 1.3E-33 |
| Inorganic ion transport and metabolism | 220 | -1143 | 6.4E-27 | 5.4E-26 |
| Replication, recombination and repair | 165 | 1170 | 1.0E-21 | 6.4E-21 |
| RNA processing and modification | 221 | 980 | 2.7E-20 | 1.3E-19 |
| Energy production and conversion | 140 | 790 | 2.4E-09 | 1.0E-08 |
| Posttranslational modification, protein turnover, chaperones | 517 | 420 | 4.3E-09 | 1.5E-08 |
| General function prediction only | 1028 | -301 | 2.0E-08 | 6.3E-08 |
| Function unknown | 515 | 398 | 2.7E-08 | 7.6E-08 |
| Cell cycle control, cell division, chromosome partitioning | 156 | 612 | 1.1E-06 | 2.7E-06 |
| Cytoskeleton | 223 | -503 | 2.0E-06 | 4.5E-06 |
| Nuclear structure | 31 | 1311 | 2.5E-06 | 5.2E-06 |
| Extracellular structures | 106 | -657 | 1.5E-05 | 2.9E-05 |
| Coenzyme transport and metabolism | 46 | 809 | 4.1E-04 | 7.3E-04 |
| Nucleotide transport and metabolism | 85 | 544 | 1.3E-03 | 2.2E-03 |
| Cell motility | 20 | -854 | 1.4E-02 | 2.1E-02 |
| Cell wall/membrane/envelope biogenesis | 71 | -411 | 2.6E-02 | 3.8E-02 |
| Chromatin structure and dynamics | 84 | 284 | 9.5E-02 | 1.3E-01 |
| Secondary metabolites biosynthesis, transport and catabolism | 123 | -220 | 1.2E-01 | 1.6E-01 |
| Amino acid transport and metabolism | 195 | -162 | 1.5E-01 | 1.9E-01 |
| Defense mechanisms | 67 | -191 | 3.1E-01 | 3.7E-01 |
| Carbohydrate transport and metabolism | 202 | -72 | 5.2E-01 | 5.8E-01 |
| Intracellular trafficking, secretion, and vesicular transport | 314 | 56 | 5.3E-01 | 5.8E-01 |
| Transcription | 306 | 51 | 5.7E-01 | 6.0E-01 |
| Lipid transport and metabolism | 231 | 2 | 9.8E-01 | 9.8E-01 |

**Supplementary Table 7.6.** EuKaryotic Orthologous Groups (KOG) term among DEGs of Day 10 of development and feeding of *Elysia chlorotica*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Signal transduction mechanisms | 717 | -386 | 1.1E-16 | 2.8E-15 |
| Replication, recombination and repair | 121 | 809 | 7.3E-15 | 9.2E-14 |
| Inorganic ion transport and metabolism | 171 | -583 | 3.7E-11 | 3.1E-10 |
| Nuclear structure | 25 | 745 | 9.9E-04 | 6.2E-03 |
| Posttranslational modification, protein turnover, chaperones | 397 | 184 | 2.1E-03 | 8.8E-03 |
| Cell cycle control, cell division, chromosome partitioning | 115 | 328 | 2.1E-03 | 8.8E-03 |
| RNA processing and modification | 162 | 253 | 5.1E-03 | 1.8E-02 |
| Amino acid transport and metabolism | 157 | -242 | 8.3E-03 | 2.6E-02 |
| Nucleotide transport and metabolism | 70 | 307 | 2.4E-02 | 6.6E-02 |
| Translation, ribosomal structure and biogenesis | 159 | 183 | 4.5E-02 | 1.1E-01 |
| Coenzyme transport and metabolism | 34 | 341 | 7.9E-02 | 1.8E-01 |
| Energy production and conversion | 115 | 179 | 9.3E-02 | 1.9E-01 |
| Extracellular structures | 101 | 170 | 1.3E-01 | 2.6E-01 |
| Function unknown | 321 | 87 | 1.8E-01 | 3.3E-01 |
| General function prediction only | 735 | -58 | 2.1E-01 | 3.5E-01 |
| Cell motility | 18 | -314 | 2.4E-01 | 3.7E-01 |
| Carbohydrate transport and metabolism | 163 | 97 | 2.8E-01 | 4.1E-01 |
| Lipid transport and metabolism | 189 | 72 | 3.9E-01 | 5.4E-01 |
| Chromatin structure and dynamics | 58 | 77 | 6.1E-01 | 8.0E-01 |
| Transcription | 197 | -38 | 6.4E-01 | 8.0E-01 |
| Defense mechanisms | 50 | -59 | 7.1E-01 | 8.5E-01 |
| Cell wall/membrane/envelope biogenesis | 61 | -20 | 8.9E-01 | 9.7E-01 |
| Secondary metabolites biosynthesis, transport and catabolism | 117 | -13 | 9.0E-01 | 9.7E-01 |
| Cytoskeleton | 160 | -8 | 9.3E-01 | 9.7E-01 |
| Intracellular trafficking, secretion, and vesicular transport | 201 | -1 | 0.99 | 0.99 |

**Supplementary Table 7.7.** EuKaryotic Orthologous Groups (KOG) term among DEGs of aposymbiotic vs symbiotic state of *Exaiptasia diaphana*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Inorganic ion transport and metabolism | 71 | 90 | 0.01 | 0.21 |
| Signal transduction mechanisms | 225 | -53 | 0.02 | 0.21 |
| Defense mechanisms | 11 | -195 | 0.03 | 0.25 |
| Amino acid transport and metabolism | 78 | -71 | 0.04 | 0.26 |
| Translation, ribosomal structure and biogenesis | 35 | 93 | 0.07 | 0.33 |
| Extracellular structures | 28 | -97 | 0.09 | 0.33 |
| Intracellular trafficking, secretion, and vesicular transport | 46 | 72 | 0.11 | 0.33 |
| Function unknown | 60 | -64 | 0.11 | 0.33 |
| Nucleotide transport and metabolism | 7 | 167 | 0.14 | 0.38 |
| Lipid transport and metabolism | 61 | 52 | 0.18 | 0.40 |
| Replication, recombination and repair | 9 | -129 | 0.19 | 0.40 |
| Cell cycle control, cell division, chromosome partitioning | 15 | -97 | 0.21 | 0.40 |
| Transcription | 53 | -53 | 0.21 | 0.40 |
| Cell wall/membrane/envelope biogenesis | 6 | 131 | 0.28 | 0.45 |
| Posttranslational modification, protein turnover, chaperones | 113 | -32 | 0.28 | 0.45 |
| Carbohydrate transport and metabolism | 60 | 42 | 0.29 | 0.45 |
| Cell motility | 1 | -286 | 0.33 | 0.49 |
| Chromatin structure and dynamics | 8 | -91 | 0.38 | 0.53 |
| RNA processing and modification | 29 | -37 | 0.51 | 0.65 |
| Cytoskeleton | 28 | -36 | 0.52 | 0.65 |
| Energy production and conversion | 27 | 22 | 0.70 | 0.81 |
| Nuclear structure | 4 | -54 | 0.72 | 0.81 |
| Secondary metabolites biosynthesis, transport and catabolism | 26 | 16 | 0.79 | 0.86 |
| General function prediction only | 200 | 3 | 0.88 | 0.92 |
| Coenzyme transport and metabolism | 17 | 0 | 1.00 | 1.00 |

**Supplementary Table 7.8.** EuKaryotic Orthologous Groups (KOG) term among DEGs of heat stressed *Stylophora pistillata*.

| KOG-term | #seqs | Delta.rank | pval | padj |
|---|---|---|---|---|
| Carbohydrate transport and metabolism | 50 | -170 | 0.00 | 0.04 |
| Posttranslational modification, protein turnover, chaperones | 155 | 91 | 0.00 | 0.05 |
| Signal transduction mechanisms | 324 | 63 | 0.01 | 0.07 |
| Cell wall/membrane/envelope biogenesis | 13 | 237 | 0.02 | 0.13 |
| Nucleotide transport and metabolism | 18 | -196 | 0.03 | 0.13 |
| Cell motility | 4 | 387 | 0.04 | 0.15 |
| Replication, recombination and repair | 31 | -130 | 0.05 | 0.17 |
| Intracellular trafficking, secretion, and vesicular transport | 48 | 104 | 0.06 | 0.17 |
| Inorganic ion transport and metabolism | 60 | -76 | 0.12 | 0.33 |
| Secondary metabolites biosynthesis, transport and catabolism | 27 | -92 | 0.20 | 0.40 |
| General function prediction only | 246 | 32 | 0.22 | 0.40 |
| Chromatin structure and dynamics | 27 | 87 | 0.22 | 0.40 |
| Defense mechanisms | 24 | -92 | 0.23 | 0.40 |
| Function unknown | 97 | 46 | 0.24 | 0.40 |
| Amino acid transport and metabolism | 71 | -53 | 0.24 | 0.40 |
| Coenzyme transport and metabolism | 6 | -152 | 0.31 | 0.49 |
| RNA processing and modification | 38 | 59 | 0.33 | 0.49 |
| Extracellular structures | 45 | -45 | 0.42 | 0.57 |
| Energy production and conversion | 48 | -40 | 0.46 | 0.57 |
| Cell cycle control, cell division, chromosome partitioning | 48 | 39 | 0.47 | 0.57 |
| Lipid transport and metabolism | 51 | 37 | 0.48 | 0.57 |
| Nuclear structure | 5 | 109 | 0.51 | 0.58 |
| Translation, ribosomal structure and biogenesis | 16 | 42 | 0.65 | 0.68 |
| Transcription | 50 | -24 | 0.65 | 0.68 |
| Cytoskeleton | 59 | -6 | 0.91 | 0.91 |

## Chapter 2.5

# The complete mitochondrial genome of the photosymbiotic sea slug *Berghia stephanieae* (Valdés, 2005) (Gastropoda, Nudibranchia).

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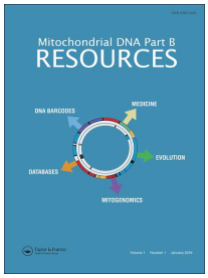


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# The complete mitochondrial genome of the photosymbiotic sea slug *Berghia stephanieae* (Valdés, 2005) (Gastropoda, Nudibranchia)

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MITOGENOME ANNOUNCEMENT



# The complete mitochondrial genome of the photosymbiotic sea slug *Berghia stephanieae* (Valdés, 2005) (Gastropoda, Nudibranchia)

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**ABSTRACT**

*Berghia stephanieae* (Nudibranchia, Cladobranchia) is a photosymbiotic sea slug that feeds exclusively on sea anemones from the genus *Exaiptasia*. It then specifically incorporates dinoflagellates belonging to the Symbiodiniaceae obtained from their prey. Here, we present the complete mitochondrial genome sequence of *B. stephanieae* combining Oxford Nanopore long read and Illumina short-read sequencing data. The mitochondrial genome has a total length of 14,786 bp, it contains the 13 protein-encoding genes, 23 tRNAs, and two rRNAs and is similar to other nudibranchs except for the presence of a duplicated *tRNA-Ser 1*.

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The aeolid nudibranch *Berghia stephanieae* (Valdés 2005) (Nudibranchia, Cladobranchia) is a stenophagous species that preferentially preys on the photosymbiotic sea anemone *Exaiptasia diaphana* (Rapp 1829). The slug digests all the anemones' tissue and incorporates the dinoflagellate symbionts Symbiodiniaceae Fensome et al. 1993, in epithelial cells of the digestive gland system (Valdés 2005; Carmona et al. 2014). Once ingested, the symbionts are retained photosynthetically active for about 10 days (Mies et al. 2017), but the slugs are even able to overcome prolonged starvation periods up to 48 days apo-symbiotically (symbiont-free) (Bleidissel 2010). Further, because apo-symbiotic adults lose their biomass in the same manner as photosymbiotic ones, the photosymbiotic relationship of *B. stephanieae* and Symbiodiniaceae is rather considered as non-mutalistic than a stable one (Mies et al. 2017; Monteiro et al. 2019). Nevertheless, *B. stephanieae* is an important species to understand the evolution of photosymbiosis in Cladobranchia, because the species seems to be in a transitional state between non-photosymbiotic and photosymbiotic. To better understand the genomic adaptations needed to evolve a stable photosymbiosis (Melo Clavijo et al. 2018), the metabolism of the mitochondrial genome can give valuable insights into a potential connectivity of the host and the symbiont (Rauch et al. 2017). As a first step toward more comprehensive studies, we sequenced the mitochondrial genome of *B. stephanieae* using a combination of Oxford Nanopore long-read and Illumina short-read sequencing.

Specimens of *B. stephanieae* were purchased from a local provider (Seepferdchen24 Meeresaquaristik GmbH, Posthausen) in February 2019 and cultivated in our lab at 25 °C, at a day/night cycle of 12 h/12h. Voucher material was preserved in 96% ethanol and stored in the Biobank at the Zoological Research Museum Alexander Koenig (Bonn, Germany, voucher no. ZFMK-TIS-53240, biobank@leibniz-zfmk.de). Seven specimens of *B. stephanieae* were frozen in liquid nitrogen and total DNA was extracted using a modified protocol based on the E.Z.N.A®Mollusc DNA Kit, Omega (Georgia, USA) and after Schalamun et al. (2019) (Supplementary material S1). The genomic library preparation was performed using the 1 D Ligation Sequencing Kit SQK-LSK109, Oxford Nanopore Technologies (Oxford, UK) for long-read sequencing on a MinION device, using a modified manufacturer's protocol (Supplementary material S1) generating about 13 GB of long reads. An additional library (insert size 100 bp, single end) was prepared using the Nextera DNA Library Prep Kit (California, USA) for Illumina sequencing on a HiSeq2500 platform resulting in approximately 5.5 GB of data. A hybrid assembly was done using SPAdes V3.14.1 (Nurk et al. 2013; Antipov et al. 2016), the assembled genome was annotated using the MITOS2 webserver (Bernt et al. 2013; Donath et al. 2019), and annotations were manually edited

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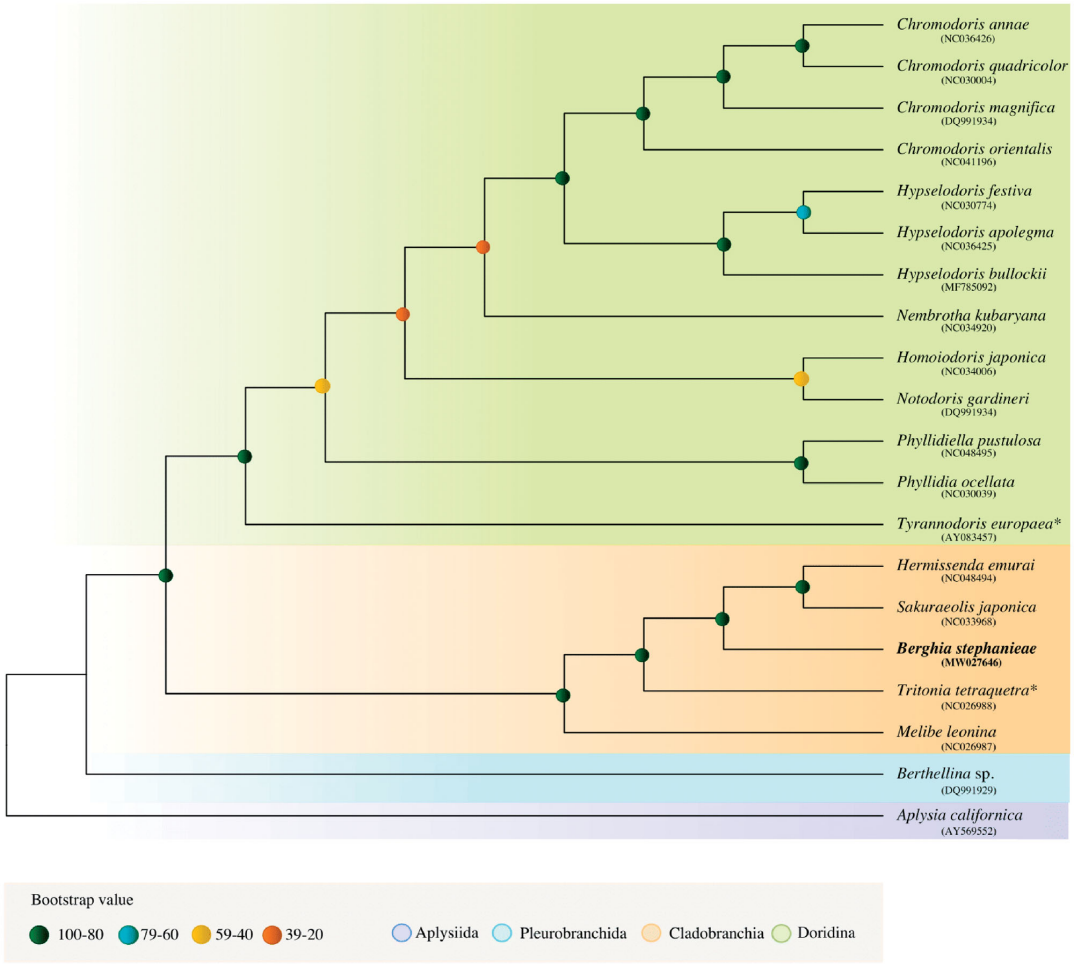


**Figure 1.** The molecular phylogeny of *Berghia stephanieae* and other nudibranchs based on the whole mitochondrial genome. The phylogenetic tree was calculated under the maximum-likelihood optimality criterion and 1,000 bootstrap replicates using *Aplysia californica* as outgroup. The accepted names (WoRMS Editorial Board, 2020) for *Tyrannodoris europaea* (synonym *Roboastra europaea*) and *Tritonia tetraquetra* (synonym *Tritonia diomedea*) were used (*).

using Geneious 9.1.5. (https://www.geneious.com). Duplicated tRNAs were further confirmed with ARWEN v.1.2 (Laslett and Canback 2008).

The mitochondrial genome of *B. stephanieae* (GenBank accession number: MW027646) has a total length of 14,786 bp and consists of 13 protein-coding genes, two ribosomal RNA (rRNA) genes, and 23 tRNA genes. The base composition of the mitogenome is 26% A, 15% C, 21% G, and 38% T. The gene order is as follows: *tRNA-Lys (aaa), cox1, tRNA-Val (gta),* the large-subunit rRNA *(rrnL), tRNA-Leu (cta) 1, tRNA-Ala (gca), tRNA-Pro (cca), nad6, nad5, nad1, tRNA-Tyr (tac), tRNA-Trp (tga), nad4L, cob, tRNA-Asp (gac), tRNA-Phe (ttc), cox2, tRNA-Gly (gga), tRNA-His (cac), tRNA-Cys (tgc), -tRNA-Gln (caa), -tRNA-Leu (tta) 2, -atp8, -tRNA-Asn (aac), -atp6, -tRNA-Arg (cga), -tRNA-Glu (gaa),* -the small-subunit rRNA *(rrnS), -tRNA-Met (atg), -nad3, -tRNA-Ser (tca) 2, tRNA-Ser (agc) 1, tRNA-Ser (aga) 1, nad4, -tRNA-Thr (aca), -cox3, tRNA-Ile (atc), nad2.* The mitogenome of *B. stephanieae* is similar in size, base composition, has the same coding regions and gene arrangement compared to all publicly available nudibranch mitochondrial genomes (Sevigny et al. 2015; Karagozlu, Sung, Lee, Kim, et al. 2016; Karagozlu, Sung, Lee, Kwak, et al. 2016; Xiang, Lin, Wang, et al. 2016; Xiang, Lin, Zhao, et al. 2016; Lin et al. 2017; Yu et al. 2018; Dinh Do, Choi, et al. 2019; Dinh Do, Kim, et al. 2019) and only differs in the presence of a duplicated *tRNA-Ser 1*.

Full-length mitochondrial genome sequences of 20 Nudipleura species were downloaded from NCBI and aligned using MAFFT (Auto mode) V7.222 (Katoh and Stanley 2013). A phylogenetic tree was built based on the maximum likelihood criterion using IQ-TREE version 2.0.5 (Minh et al. 2020) with the Model Finder Plus option (-m TEST), 1000 bootstrap replicates, and *Aplysia californica* J. G. Cooper 1863 set as outgroup. *Berghia stephanieae* clustered with the other Cladobranchia species, and forms a monophyletic clade with *Sakuraeolis japonica* (Baba 1937) and *Hermissenda emurai* (Baba 1937), that corresponds to the superfamily Aeolidioidea (Figure 1).

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## Disclosure statement

The authors declare there are no relevant financial or non-financial competing interests to report.

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## Data availability statement

The data that support the findings of this study are openly available in Figshare (www.figshare.com) at http://doi.org/10.6084/m9.figshare.12994064

## Data accessibility statement

The mitogenome sequences of *Chromodoris annae* Bergh 1877 (NC_036426) (Lin et al. 2017), *Chromodoris quadricolor* (Rüppell and Leuckart 1830) (NC_030004) (Xiang, Lin, Zhao, et al. 2016), *Chromodoris magnifica* (Quoy and Gaimard 1832) (DQ991931) (Medina et al. 2011), *Chromodoris orientalis* Rudman 1983 (NC_041196) (Yu et al. 2018), *Hypselodoris festiva* (Adams 1861) (NC_030774) (Karagozlu, Sung, Lee, Kwak, et al. 2016), *Hypselodoris apolegma* (Yonow 2001) (NC_036425) (Lin et al. 2019), *Hypselodoris bullockii* (Collingwood 1881) (MF785092) (Lin et al. 2019), *Nembrotha kubaryana* Bergh 1877 (NC_034920) (Xiang et al. 2017), *Homoiodoris japonica* Bergh 1882 (NC_034006) (Liu et al. 2016), *Notodoris gardineri* Eliot 1906 (DQ991934) (Medina et al. 2011), *Phyllidiella pustulosa* (Cuvier 1804) (NC_048495) (Dinh Do, Choi, et al. 2019), *Phyllidia ocellata* Cuvier 1804 (NC_030039) (Xiang, Lin, Wang, et al. 2016), *Tyrannodoris europaea* (García-Gómez 1985) (registered as its synonym *Roboastra europaea* García-Gómez 1985 AY083457) (Grande et al. 2002), *Hermissenda emurai* (NC_048494) (Dinh Do, Kim, et al. 2019), *Sakuraeolis japonica* (NC_033968) (Karagozlu, Sung, Lee, Kim, et al. 2016), *Tritonia tetraquetra* (Pallas 1788) (registered as its synonym *Tritonia diomedea* Bergh 1894 NC_026988) (Sevigny et al. 2015), *Melibe leonina* (Gould 1852) (NC_026987) (Sevigny et al. 2015), *Berthellina* sp. Gardiner 1936 TLT-2006 (DQ991929) (Medina et al. 2011), and *Aplysia californica* (AY569552) (Knudsen et al. 2006) are accessible via download from GenBank. Mitogenome data supporting this study are openly available in GenBank at: https://www.ncbi.nlm.nih.gov/nuccore/MW027646. Associated BioProject, SRA, and BioSample accession numbers are https://www.ncbi.nlm.nih.gov/bioproject/PRJNA684024, SRR13242883, SRR13242884, SRR13242885, and SAMN17050363, respectively.

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## Appendix 1.

## DNA extraction protocol and library preparation 1 D SQK-LSK109.

**Supplementary material**

**Appendix 1. DNA extraction protocol and library preparation 1DSQK-LSK109 for nanopore sequencing.**

Seven specimens of *Berghia stephanieae*, 17 mg each, were frozen in liquid nitrogen. The tissue was homogenized using 350 $\mu$l of the lysis buffer ML1 from the E.Z.N.A® Mollusc DNA Kit, Omega (Georgia, USA) and 25 $\mu$l of Proteinase K and heated to 60 °C for 1 h. The samples were then cleaned adding 350 $\mu$l of chloroform: isoamyl (24:1) and the phases were separate by centrifugation for 5 min at 8000 g at room temperature. The aqueous phase was collected and transferred to a new tube. One volume of buffer BL of the E.Z.N.A® Mollusc DNA Kit and 10 $\mu$l RNase A/T (Thermo Scientific, Massachusetts, USA) were added and incubated at 70 °C for 10 min. A second cleaning step was done by adding one volume of chloroform: isoamyl (24:1) to the samples and centrifuged them at 8000 g at room temperature and the aqueous phase recovered. The DNA in the aqueous phase was precipitated adding 0.1 volume 3M sodium acetate, and 1 ml 96% ethanol, and further centrifuged for 5 min at 5000 g at room temperature. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and centrifuged for 10 min at 8000 g at room temperature. After a second washing step with 1 ml 70% ethanol, the pellet was air dried and eluted overnight at room temperature in 100 $\mu$l of Elution Buffer E.Z.N.A® Mollusc DNA Kit. The concentration and purity of the isolated total DNA was measured with Nanodrop and Qubit, respectively.

Library preparation for long-read sequencing with the MinION (Oxford Nanopore, Oxford, UK) was performed using the 1D SQK-LSK109 ligation kit, following the manufacturers' recommendations. Briefly, 2 $\mu$g of pre-warmed (50 °C) isolated genomic DNA were end-repaired and prepared using the NEBNext FFPE DNA repair Mix (New England Biolabs, Massachusetts, USA) and NEBNext End repair/dA-tailing Module (New England Biolabs, Massachusetts, USA) for one hour at 20 °C followed by one hour at 65 °C. The sample was then incubated for 15 min at room temperature with AMPure XP beads (1:1, v/v) (Beckman Coulter, California, USA) previously acclimated at room temperature for 30 min. The sample was then briefly spinned down using a mini centrifuge (LMS, Tokyo, Japan), and the tube subsequently placed on a magnetic rack allowing the beads to concentrate at the side of the magnet for 5 min. Afterwards, the supernatant was removed and the beads washed by adding 500 $\mu$l of freshly prepared 70% ethanol. The washing step was repeated twice. The cleaned pellet was then eluted in 62 $\mu$l of nuclease-free water at 37 °C

for 30 min. Subsequently, the concentration and purity of the DNA was checked using a Nanodrop and only proceeded if the A260/A280 ratio was 1.8. Then, 25 $\mu$l of LNB, 10 $\mu$l of NEBNext Quick T4 DNA Ligase, and 5 $\mu$l of the Adapter Mix AMX were added to the DNA and incubated at room temperature for 1 h. Next, 40 $\mu$l of AMPure XP beads were added and mixed by inversion for 15 min at room temperature, and placed on the magnetic rack for 5 min. The supernatant was discarded and 250 $\mu$l of L Fragment Buffer LFB were added to the beads mix in order to select fragments longer than 3 kbp. This washing step was repeated once. After removing all the remaining LFB buffer from the second washing step, the pellet was resuspended in 15 $\mu$l Elution Buffer (pre-warmed at 50 °C) and incubated at 37 °C for 20 min. For sequencing, 15 $\mu$l of final DNA were mixed with 36 $\mu$l of sequencing buffer SQB, and 24 $\mu$l of loading beads LB, mixed immediately before use, having in total 75 µl DNA in solution to be loaded onto the flow cell. The flow cell was primed with a priming solution made out of 30 µl Flush Tether FLT directly mixed with 1 ml of Flush Buffer FLB. First, 800 µl of the priming solution were added into the sample port with a closed SpotON port. After 5 min the remaining 200 µl of the priming solution were added, but with an open SpotON port. Next, the prepared DNA library was loaded drop-by-drop into the open SpotON port. The sequencing run was monitored on a Ubuntu 18.04. platform using MINKNOW v.2.2. basecalling was performed using Guppy v.3.0.3.

## Chapter 2.6

## Unpublished data

# Reduction of the innate immune response might promote the establishment of functional kleptoplasty in juveniles of the slug *Elysia chlorotica* Gould, 1870

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**Abstract**

Photosymbiosis, the symbiosis of animals with unicellular phototrophic partners (photobionts), is taxonomically widespread among metazoans. The key elements to a successful interpartner maintenance are mechanisms for conflict mediation that involve the translocation of organic and inorganic compounds by the host to the photobiont. The photobiont in return provides the host, for instance, with nitrogen and energy in the form of glucose and ATP. In some marine slugs belonging to the Sacoglossa, a functionally different photosymbiotic system is found. Here, the photobionts are only chloroplasts stolen from their algal prey (kleptoplasts) and kept photosynthetically active in cells of the slug's digestive system. In Sacoglossa, little is known regarding the mechanisms of conflict mediation, photobiont maintenance, and the metabolic exchange. Yet, this could provide important insights into potential convergently evolved mechanisms in different photosymbiotic taxa, for example in comparison to cnidarians. Here, we analyzed the transcriptomic response of the sea slug *Elysia chlorotica* with regard to signatures of such mechanisms involved in maintaining the alien organelle. It appears that kleptoplast maintenance in Sacoglossa is dependent on the reduction of the innate immune response, the prevention of apoptosis, an increased ROS scavenging capacity, and an increased DNA repair, which is in parts similar to what is known in cnidarians. However, in *E. chlorotica* genes related to organic and inorganic compound translocation to the photobiont, like carbonic anhydrases and ammonium transporters, were not expressed during feeding, questioning an active involvement of the slugs to support the kleptoplasts' photosynthesis. Thus, it seems that in Sacoglossa the kleptoplast maintenance is not based on mechanisms involving an exchange of compounds.

**Keywords.** *Elysia*, functional kleptoplasty, gene expression, photosymbiosis, Sacoglossa

## Introduction

Photosymbiosis is a special form of a symbiosis between a heterotrophic host and a phototrophic symbiont (photobiont) and is taxonomically widespread in animals (Melo Clavijo et al., 2018). The host supports the photobiont with inorganic and organic nutrients, while the translocation of assimilates into the host's cytosol by the photobiont might supersede the nutritional demands of the host. This is in particular important for reef building Scleractinia cnidarians (Stanley & Lipps, 2011). Although the view on photosymbiosis as being mutualistic is challenged in some cnidarians (Peng et al., 2020), and might also be referred to as controlled parasitism by the animal host (Wooldridge, 2010), both the host and the photobiont evolved certain features that are involved in conflict mediation to maintain the symbiosis (Blackstone & Golladay, 2018). For instance, cnidarian hosts express certain carbonic anhydrases and ammonium transporters to ensure a high photosynthetic activity of the photobiont, reduce the innate immune response to tolerate the symbiont, and increase the scavenging of elevated levels of reactive oxygen species formed due to the photosynthesis of the photobiont (Lehnert et al., 2014; Mohamed et al., 2016; Matthews et al., 2017). In return, the photobiont provides the animal host with amino acids and energy, mainly in form of fixed carbon and ATP that is also needed to fuel the transporters for compound translocation to the photobiont (Roberty et al., 2020). If photosynthesis would cease, for instance due to photodamage, this exchange-cycle would eventually cease and would probably favor the expulsion of photobionts leading to coral bleaching (Blackstone & Golladay, 2018).

The mutualistic nature of photosymbiosis is not only challenged in corals, but also recently in protists (Decelle, 2013), acoel worms (Androuin et al., 2020), the yellow-spotted salamander *Ambystoma maculatum* (Shaw, 1802) (Burns et al., 2017; 2020), the *Exaiptasia*-eating nudibranch *Berghia stephanieae* (Valdés, 2005) (Monteiro et al., 2019), and in some species of marine slugs belonging to the Sacoglossa (Rauch et al., 2017). In the latter, the photobionts are only photosynthetically active chloroplasts obtained from their food algae and incorporated intracellularly in specific cells of the digestive gland system (Händeler et al., 2009). Because the interaction is not between two organisms, this photosymbiotic system is called functional kleptoplasty (Rumpho et al., 2011) and in Metazoa only found additionally in a couple of rhabdocoel worms (Van Steenkiste et al., 2019). It is still unknown whether these taxonomically

different photosymbiotic animal hosts evolved the ability to recognize and maintain the diverse photobionts convergently or if these hosts evolved different mechanisms (Melo Clavijo et al., 2018). Only recently, it has been proposed for Sacoglossa that the recognition of the chloroplasts (Melo Clavijo et al., 2020) and an increased reactive oxygen quenching ability of the slugs (de Vries et al., 2015; Chan et al., 2018) are potentially important factors to engulf and maintain the kleptoplasts. Further, the synthesis of complex polyketides (Torres et al., 2020) might additionally enhance kleptoplast longevity in the slugs. But if there are further mechanism of conflict mediation or metabolic exchange between the slugs and the chloroplasts to maintain this special symbiosis, remains unknown.

To get an insight into potential mechanisms of conflict mediation by Sacoglossa, we analyzed the transcriptomic response of *Elysia chlorotica* Gould 1870 in more detail and compared it to known mechanisms in cnidarians. In many aspects, the molecular mechanisms to maintain functional kleptoplasty are different from those known in the cnidarian-photosymbiont model. These differences are mainly based on the absence of key components of important pathways. However, similarities with regard to the decrease of the innate immune response were found. Although there is evidence that an increased amount of glucose might be transported to the host, kleptoplast digestion proceeds, while ammonium transporters are not expressed. Thus, in Sacoglossa it seems that conflict mediation is absent favoring the hypothesis that the slugs keep the kleptoplasts as some kind of larder.

## Material and Methods

*Transcriptome annotation*

The publicly available assembled transcriptomic data set of *Elysia chlorotica* was downloaded from http://cyanophora.rutgers.edu/Elysia-expression (Chan et al., 2018). The data set was generated by extracting total RNA of > 20 pooled individuals each from unfed juveniles (aposymbiotic) and from juveniles feeding for five, seven, and ten days post metamorphosis (Chan et al., 2018). The 130,413 contigs of the assembled transcriptome were translated into amino acids sequences using the LongOrf function implemented in TransDecoder v5.5.0 (Haas et al., 2015) resulting in 49,345 potential proteins. The proteins were subsequently annotated by a BLASTP (part of the BLAST+ package v2.9.0) search against the Swiss-Prot database (The UniProt Consortium, 2019) including functional annotations of gene ontology (GO) terms and Kyoto Encyclopedia of Genes and

Genomes (KEGG) identifiers. The E-value was set to $1e^{-10}$ and annotations taxonomically filtered for Metazoa, which resulted in 13,237 annotated proteins. This strategy was employed because the original annotation of the transcriptome included annotations of bacteria, fungi, and plants, which were excluded here. The annotations were then screened for core elements of the transforming growth factor ß (TGF-ß) signaling pathway, genes associated with immune suppression, apoptosis, phagosome maturation, nutrient transporters, and reactive oxygen species (ROS) quenching, which are all known to be relevant for photobiont maintenance in cnidarians (Detournay et al., 2012; Lehnert et al., 2014; Mohamed et al., 2016; Matthews et al., 2017). Additionally, KEGG pathways were reconstructed using the online KEGG mapper tool (https://www.genome.jp/kegg/tool/map_pathway.html) and pathways related to apoptosis and TGF-ß inspected in more detail.

*Analysis of the differential gene expression*

To analyze the gene expression, reads of the different developmental stages from *Elysia chlorotica* were downloaded from the NCBI short read archive (SRA accession number: SRS3101883). The short reads were mapped onto the transcriptome using Bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) and the transcript abundance of sequences was estimated using RSEM (Li & Dewey, 2011) as implemented in Trinity v2.9.0 (Grabherr et al., 2011). Differential gene expression analyses were performed using edgeR v1.26.0 (Robinson et al., 2010) and were $\log_2$ fold-change (L2FC) transformed. We compared the expression of all feeding stages to the aposymbiotic juveniles. Expression values of *E. chlorotica* were filtered for an adjusted p-value of $\leq 0.05$ and only considered as significantly differentially expressed if the L2FC was $\leq -1$ or $\geq 1$. Gene ontology enrichment tests of significantly differentially expressed genes were performed using the GOMWU package (Wright et al., 2015) and filtered for an adjusted p-value of 0.01.

## Results

*Core components of the TGF-ß sensu stricto pathway are missing*

The first step to successfully establish a photosymbiosis is the recognition of the symbiont, which is likely carried out by scavenger receptor class B members (SR-B) and thrombospondin-type-1-repeat proteins (TSRs) in sacoglossan sea slugs (Melo Clavijo et al., 2020). Particularly, in *E. chlorotica* SR-B is up-regulated at 5 days post-metamorphosis, down-regulated at 7 days, and up-regulated again at 10 days of feeding and development (Melo Clavijo et al., 2020). The TSRs-SR-B

binding might initiate a downstream cascade including the TGF-ß *sensu stricto* (ss) pathway to tolerate the alien organelle (Figure 1A). Yet, while in the transcriptome of *E. chlorotica* homologues of members belonging to the TGF-ß family (BMP ligands, Myostatin (GDF8), Nodal, and SMAD 1, 2, 4, and 6) could be identified, no TGF-ß sensu stricto (ss) ligand could be found (Supplementary Table 1). Receptors belonging to the type I and type II TGF-ß receptor class, including TGF-ß receptor type I, Activin receptor type I, BMP receptor type I, as well as Activin type II, and BMP type II receptors were present, but TGF-ß receptor type II and type III could not be identified (Supplementary Table 1). Thus, major components of the TGF-ß ss pathway are missing. However, the phosphorylation of SMAD2 might also be governed through the Activin,

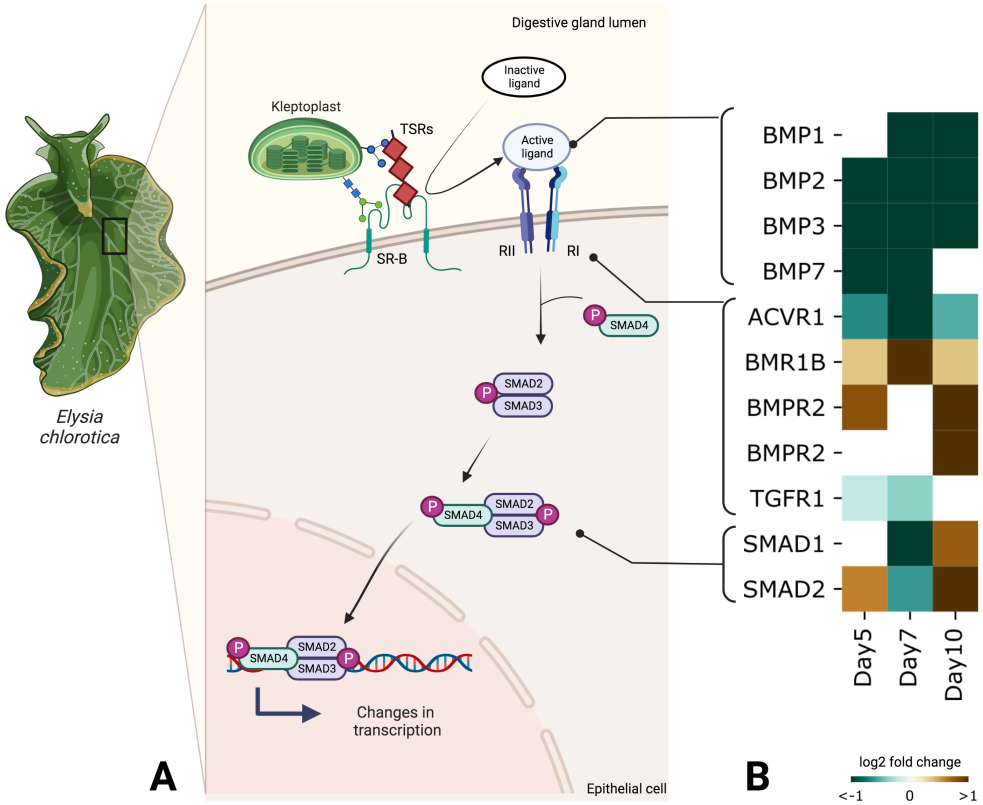


**Figure 1.** Model of the recognition and downstream cascade involving the TGF-ß family in *E. chlorotica*. **(A)** TGF-ß signaling pathway. Generally, ligands of the TGF-ß family are hypothesized to be activated through the binding of extracytosolic thrombospondin-type-1 repeat proteins (TSRs) to a symbiont (in the case of Sacoglossa to the chloroplasts) that increases the recognition (or the infection success) by scavenger receptor class B (SR-B) members. The exact mode of this activation is still unknown. The ligand is activated and binds to TGF-ß type II receptors (RII)(Li et al., 2006; Detournay et al., 2012). These receptors then form a complex with TGF-ß type I receptors (RI; TGFR1) leading to a downstream activation of SMAD2/3. SMAD2/3 forms a dimer with SMAD4 and can subsequently suppress transcription. The formation of the SMAD2/3-SMAD4 complex can be inhibited by SMAD6. The figure was redrawn from the human KEGG pathway map (hsa04350) with BioRender.com. **(B)** Expression of TGF-ß family components. Only genes that were significantly expressed (padj < 0.05) in any feeding condition are shown. BMP: bone morphogenic protein; ACVR1: activin receptor type I; BMR1B/BMPR2: bone morphogenic protein receptor type 1B, receptor type 2, respectively. Created with BioRender.com

BMP, or myostatin signaling cascade. Yet, BMP ligands were continuously down-regulated, while BMP receptors, as well as SMAD1 and SMAD2 were significantly up-regulated only after 10 days of feeding and development (Figure 1B).

*General down-regulation of the immune response*

Besides the TGF-ß pathway, further genes might be involved in immune suppression leading to a kleptoplast tolerance. For example, homologues of the mammalian pancreatic secretory granule membrane major glycoprotein GP2 (GP2) are supposed to play a major role in initiating symbiont tolerance (Mohamed et al., 2016), but in *E. chlorotica* GP2 was not expressed under any condition.

To get a further overview on the innate immune response of *E. chlorotica* during feeding, we screened the transcriptome for gene ontology (GO) annotations of the biological process "immune response" (Supplementary Table 1). Overall, 270 genes could be identified, out of which 70 were not differentially expressed in any condition. After five days of feeding and development the number of up-regulated (75 genes) and down-regulated (72 genes) innate immune system relevant genes was comparable. After seven days of feeding and developing, however, the majority of genes were down-regulated (120) and only 66 genes were up-regulated. After 10 days of feeding and development, the vast majority of genes (151 genes) was not differentially regulated, while 88 genes were down-regulated and 52 were up-regulated. Thus, after seven days of feeding the innate immune system seems to be predominantly down-regulated. Among the 10 most down-regulated genes under any condition is a homologue of the Baculoviral

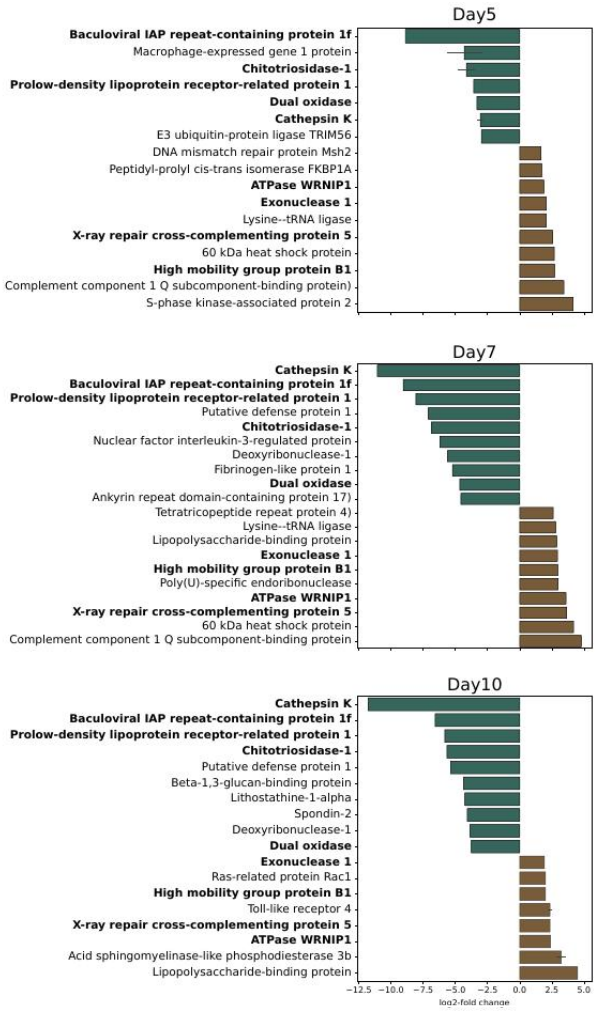


**Figure 2.** Expression of the 10 most up- and 10 most down-regulated genes that had "innate immune system" gene ontology annotations in *E. chlorotica*. Highlighted in bold are genes that are expressed in all of the three feeding periods (fed for 5, 7, and 10 days, respectively). In case of multiple copy homologs, the mean value and the standard deviation of the expression value are shown.

IAP repeat-containing protein 1f, homologues of a Chitotriosidase-1, a Prolow-density lipoprotein receptor-related protein 1 homologue, Dual oxidase homologues, and Cathepsin K homologues (Figure 2).

*Maturation and arrest of phagosomes*

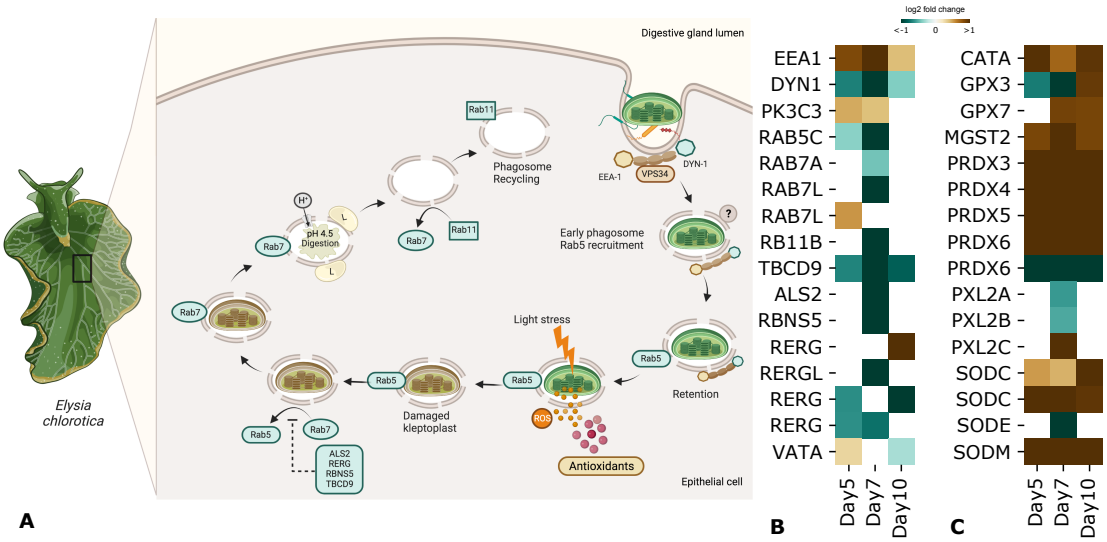


**Figure 3.** Model of phagosomal maturation and potential arrested maturation in *E. chlorotica*. **(A)** After recognition of the chloroplasts, the alien organelles are most likely enclosed by a phagosomal membrane on which Rab5 binds, guided by VPS-34, DYN-1, and EEA-1. As long as Rab5 is bound to the membrane, a replacement by Rab7 is inhibited and thus kleptoplasts could be maintained. ALS2, RBNS5, RERG, and TBCD9 are supposed to inhibit Rab7 from replacing Rab5. Prolonged presence of the kleptoplasts in the cytosol might lead to increased ROS formation in the kleptoplasts and an export into the animals' cytosol, for instance triggered through photodamage. As a result, increased ROS quenching would be necessary to cope with the elevated intracellular ROS level. Ultimately, the kleptoplasts will be damaged, which might result in a dislocation of Rab5, although it is currently unknown how this might be triggered. Once Rab5 is dislocated, Rab7 can bind to the damaged kleptoplasts and V-ATPase (VATA) pump H+ ions into the phagosomal lumen for acidification and kleptoplasts degradation. Then Rab11 binds to recycle the phagosomal membrane. The pathway is drawn after (Fransolet et al., 2012; Wright et al., 2015). **(B and C)** Gene expression of genes involved in phagosomal maturation and of ROS quenchers, respectively. Only genes that were expressed in at least one feeding period are shown. EEA1: early endosome antigen; DYN1: dynamin; PK3C3/VPS34: phosphatidylinositol 3-kinase; TBCD9: TBC1 domain family member 9; ALS2: alcin; RBNS5: Rabenosyn-5; RERG: Ras-related and estrogen-regulated growth inhibitor; L: lysosomes; GPX: Glutathione peroxidase; MGST: microsomal gluthatione S- transferase; PRDX: peroxiredoxin; PXL: peroxiredoxin-like; SODC: superoxide dismutase [Cu-Zn]; SODE: extracellular superoxide dismutase [Cu-Zn]; SODM: superoxide dismutase [Mn]. Created with BioRender.com

After the successful photobiont recognition, the kleptoplasts are likely engulfed by a phagosome membrane (Martin et al., 2013). The first step in phagosome maturation then includes the translocation of Rab5 to the phagosome, which is conducted through the Phosphatidylinositol 3-kinase (PIK3C3 or VPS-34), the early endosome antigen 1 (EEA-1), and Dynein (DYN-1) (Figure 3A) (Kinchen & Ravichandran, 2008).

Yet, only EEA-1 was significantly up-regulated after seven days of feeding and development and none of the three genes was significantly regulated under any other feeding period (Figure 3B). Further, Rab5, Rab7, and Rab11 were down-regulated only after seven days of feeding and developing. For further maturation, Rab5 needs to be removed from the phagosome, paving the way for Rab7 to bind. Rabenosyn-5 (RBNS5), Alsin (ALS2), Ras-related and estrogen-regulated growth inhibitor (RERG), and TBC1 domain family member 9 (TBCD9) are supposed to inhibit the maturation of the phagosome by blocking the translocation of Rab5 from the phagosome (Mohamed et al., 2016). Yet, almost all of these genes were either not or significantly down-regulated and only one RERG homologue is up-regulated after ten days of feeding and development (Figure 3B).

*Response to oxidative stress is elevated*

The degradation of kleptoplasts might be triggered, among others, by photodamage induced through exposure to high levels of photosynthetically active radiation. This results in the increased formation of reactive oxygen species (ROS) that need to be detoxified to prevent damage to the host cell (de Vries et al., 2015). In *E. chlorotica,* homologues of the cytosolic peroxiredoxin-4 (PRDX4, sequence ID: c127224_g1_i1_2-2380; mean L2FC: 1.71 ± 0.68), the cytosolic peroxiredoxin-6 (PRDX6, c119266_g1_i1_67-2469; mean L2FC: 2.10 ± 0.58), and the cytosolic copper, zinc superoxide dismutase (SODC, c123617_g1_i2_1-1601, mean L2FC: 1.52 ± 0.69) were significantly up-regulated. Yet, another homologue of a peroxiredoxin-6 (c183435_g1_i1_1-409; mean L2FC: -5.50 ± 0.44) was significantly down-regulated (Figure 3C). Additionally, a homologue of the mitochondrial peroxiredoxin-5 (PRDX5, c118907_g1_i1_69-2194; mean L2FC: 2.37 ± 0,58) and a homologue of the mitochondrial manganese superoxide dismutase (SODM, c116278_g1_i1_1-3283; mean L2FC: 1.42 ± 0.48) were significantly up-regulated (Figure 3C). Besides these two ROS quenchers, a homologue of a catalase (CATA, c124802_g2_i1_1-2074) was up-regulated, but not significantly (mean L2FC: 0.89 ± 0.15).

*Apoptosis is prevented*

Apoptosis might be induced either through the extrinsic receptor mediated pathway (Figure 4A) or the intrinsic mitochondria mediated pathway. Key for the extrinsic pathway are death related receptors, such as the Tumor Necrosis Factor Receptors (TNFR) that bind to Tumor Necrosis Factor Related Apoptosis Inducing Ligands (TRAIL), or the Fas ligand that binds to the FAS receptor (cluster of differentiation 95, CD95) that then initiate a downstream cascade through the adapter

protein Fas-associated death domain (FADD) (Figure 4A). However, in *E. chlorotica* none of the aforementioned genes was present in the transcriptome (Figure 4B). Furthermore, caspase 8 needed for the extrinsic pathway to be activated, was significantly down-regulated after seven days of feeding (CASP8, c113476_g1_i1_4-2367; L2FC: -2.03) while its interacting partner, caspase 10 was not expressed at all. For both, the extrinsic and intrinsic pathway, caspase 3 and caspase 7 (CASP3, CASP7) inevitable induce apoptosis. While caspase 3 was only significantly up-regulated after seven days of feeding (c122193_g2_i2_2-3789; L2FC 1.31), caspase 7 was significantly down-regulated in all conditions (c121339_g1_i1_12-1270; mean L2FC: -1.72 ± 0.26) (Figure 4B). Additionally caspase 9, needed for apoptosis execution, was not expressed in any condition. Thus, both the extrinsic and the intrinsic apoptosis pathway seem to be silenced in fed *E. chlorotica*. Furthermore, the p53 protein, the core component of the cellular tumor antigen (p53) signaling pathway and important for inducing apoptosis, was not present in the transcriptome.

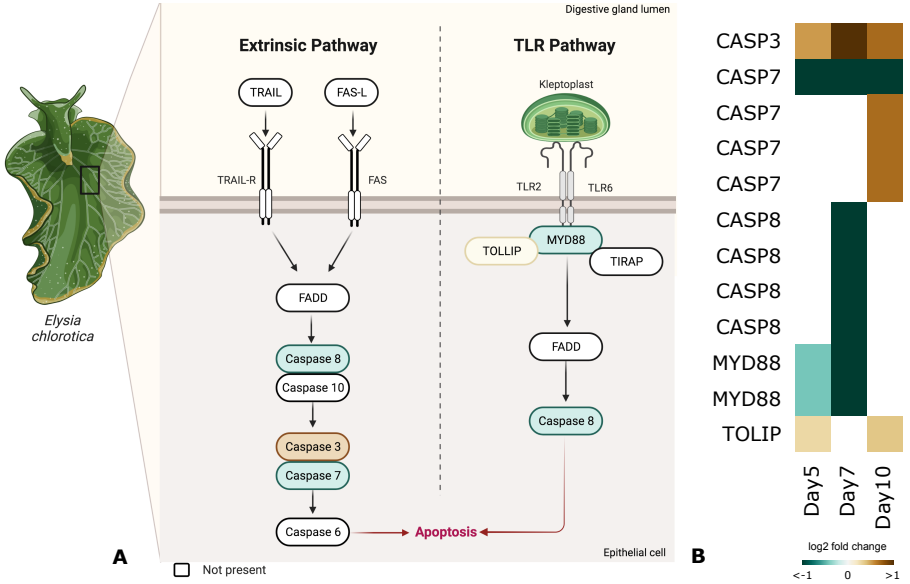


**Figure 4.** Apoptosis pathways in *E. chlorotica*. **(A)** Extrinsic apoptosis pathway and toll-like receptor (TLR) signaling pathway. The figure is based on the human KEEP pathways for apoptosis (hsa04210) and toll-like receptor signaling (hsa04620) created with BioRender.com. Genes not present in the transcriptome are colored in white, genes present in the transcriptome are colored depending on their expression. **(B)** Expression of genes involved in the extrinsic apoptosis and toll-like receptor signaling. Only genes that were expressed in at least one feeding period are shown. CASP: caspases; MYD88: Myeloid differentiation primary response 88; TOLIP: toll interacting protein. Created with BioRender.com

Apoptosis may also be triggered by the toll-like receptor signaling pathway through the signal transducer MyD88 (myeloid differentiation primary response 88) and FADD, leading to the expression of caspase 8 and the innate immune activated apoptosis (Figure 4A). Yet, none of the

needed toll-like receptors (TLR2 and TLR6), or FADD were present in the transcriptome and MyD88 was down-regulated.

*Cell cycle and DNA repair is increased*

During five to seven days of feeding, GO terms associate to cell cycle and DNA and RNA metabolism were significantly enriched ($p < 0.01$) and up-regulated (Figure 5). After ten days of feeding, especially the GO terms DNA repair, DNA recombination, and DNA metabolic processes were significantly enriched ($p < 0.01$) and up-regulated (Supplementary Table 2).

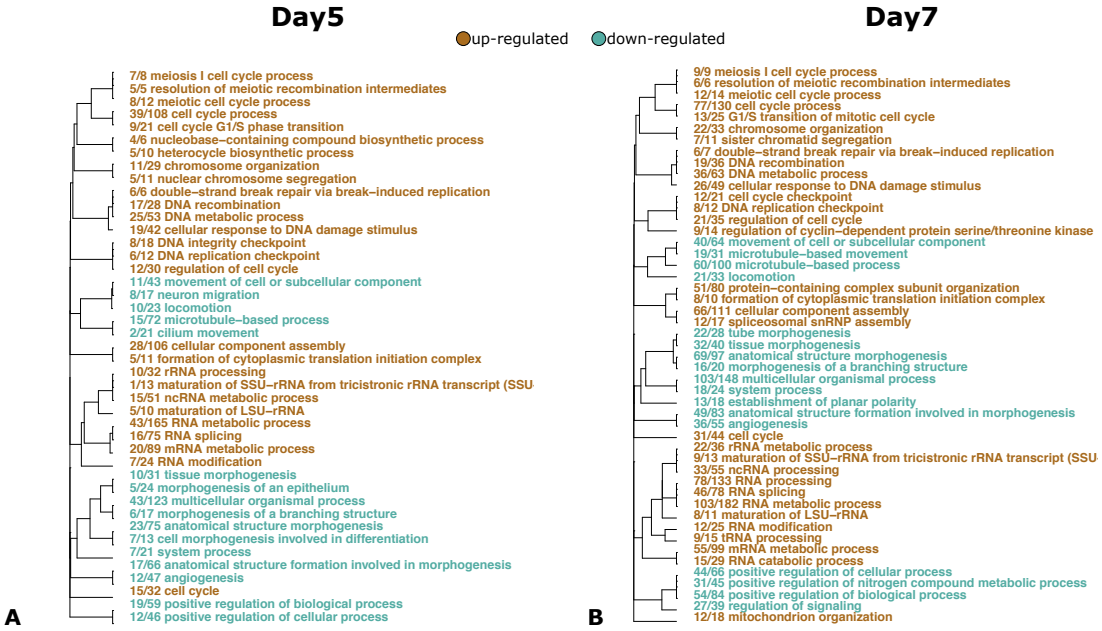


**Figure 5.** Enriched gene ontology (GO) terms after **(A)** five and **(B)** seven days of feeding of juveniles of *E. chlorotica*. Brown font displays up-regulated, green font down-regulated GO terms. The number represent the amount of statistically relevant genes included in the respective GO category and the amount of all genes in this category. Only categories with an adjusted p-value of 0.01 are displayed.

*Nutrient transporters and the Vitamin K cycle*

The kleptoplasts need to be provided with organic nutrients to guarantee a high photosynthetic capacity. Among these nutrients are $CO_2$ and ammonium. $CO_2$ might be converted to the less freely diffusion bicarbonate by carbonic anhydrases (CH) (Figure 6A). However, we could only identify one CH 2 homologue that was up-regulated during all feeding time points (c127490_g3_i1_1-2189; mean L2FC: 1.80 ± 0.85), while four CH 4 homologues (mean L2FC: -2.71 ± 1.08) and one CH 8 homologue (c119528_g1_i3_1-4769; mean L2FC: -1.38 ± 0.38) were significantly down-regulated in all conditions (Figure 6). We only considered as significantly differentially expressed if the L2FC was ≤ -1 or ≥ 1; only one AMT1 appears to be up-regulated at 10$^{th}$ day according (Figure 6B), but

the L2FC of this gene was 0.79. None of the ammonium transporters were significantly up-regulated, but one putative ammonium transporter 3 was significantly down-regulated (c95919_g1_i1_1-1720: mean L2FC: -2.92 ± 1.59) (Figure 6B). It is likely, that the slugs obtain their nutritional support from the photobiont through glucose, which needs to be exported into the potential symbiosome and then exported into the cytosol. In *E. chlorotica* one homologue of a glucose transporter 1 (c125382_g1_i4_1-2771; mean L2FC: 1.19 ± 0.18) was significantly up-regulated during the feeding period, and one (c128647_g1_i2_7-4137; L2FC: 3.52) was significantly up-regulated after 10 days of feeding (Figure 6B).

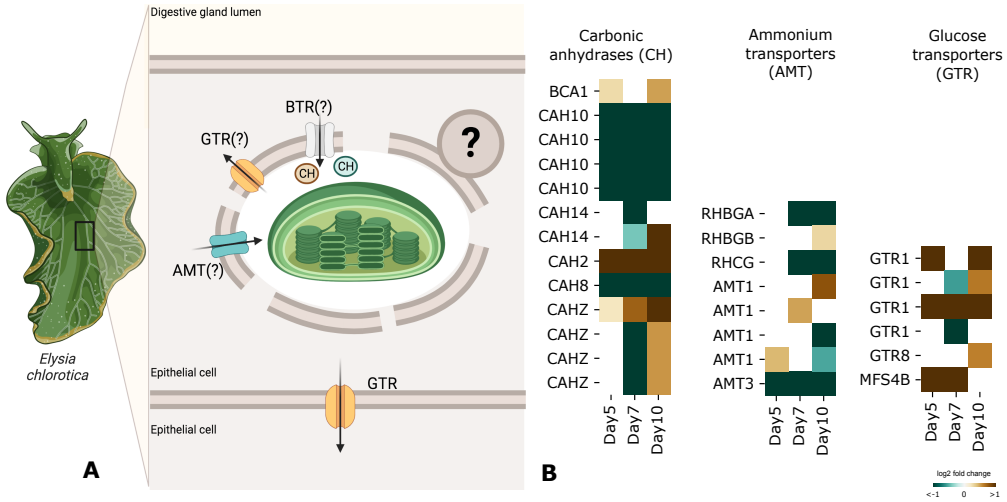


**Figure 6.** Overview of potential transporters involved in the exchange of inorganic and organic compounds between the animals' cytosol and the kleptoplasts in *E. chlorotica*. **(A)** Bicarbonate transporters (BTR) and ammonium transporters (AMT) might be located at the phagosomal membrane to support the kleptoplasts with sufficient inorganic and organic compounds relevant for photosynthesis. Carbonic anhydrases (CHs) might be present inside the phagosome to convert the transported bicarbonate ions into $CO_2$. Fixed carbon, synthesized by kleptoplast photosynthesis, might be exported through glucose transporters (GTRs) that might be located at the phagosomal membrane. Additionally, GTRs connect the epithelial cells with the sub-epithelial cell layer to distribute glucose throughout the animal. **(B)** Expression of carbonic anhydrases, ammonium transporters, and glucose transporters, respectively. Only genes that were expressed in at least one feeding period are shown. BCA: Beta carbonic anhydrase; CAH/CAHZ: carbonic anhydrase; RHBGA/B: Ammonium transporter Rh type B-A, B-B; MFS4B: sodium-dependent glucose transporter. Created with BioRender.com

A further receptor often associated with photosymbiotic cnidarians is the cell adhesion protein sym32, which contains a transmembrane domain and two fasciclin domains (Ganot et al., 2011). To function properly, sym32 has to be carboxylated, which is governed through the vitamin K cycle. For the vitamin K cycle a vitamin K epoxide reductase (VKOR) is needed, that might be in turn inhibited by calumenin. In the transcriptome of *E. chlorotica* no sym32 homologue could be

identified. Yet, we found a homologue of a transforming growth factor-beta-induced protein (TGFBI) that contained a transmembrane domain and two fasciclin domains, that was highly down-regulated during the feeding period (c103353_g1_i1_1-1283; mean L2FC: -4.25 ± 1.62). Both, VKOR (c110310_g1_i1_54-1172 and c118998_g1_i5_1-1648; mean L2FC: 2.30 ± 0.73) and calumenin (c112469_g1_i2_12-2309 and c127853_g1_i1_1-3480; mean L2FC: 1.14 ± 0.90) were up-regulated throughout the feeding period, but the Vitamin K-dependent gamma-carboxylase, needed to carboxylate a potential sym32, was significantly down-regulated (c100672_g1_i2_35-2203; mean L2FC: -1.76 ± 0.95) (Supplementary Table 1).

**Discussion**

The mechanisms for photobiont maintenance and the host-photobiont conflict mediation in functional kleptoplasty in Sacoglossa are still largely unknown. To tackle this gap of knowledge, the current study focused on the expression of potentially relevant genes and pathways of the host that might pave the way for the establishment of a successful kleptoplastic association. We found transcriptional evidence that the tolerance of kleptoplasts might depend on the ability of the host to decrease the expression of certain genes related to the innate immune system response to microbial invasion and degradation. It also should be noted that the pathways here mentioned could be regulated before or after the samples were taken. Likewise some results that differ from the original data (Chan et al., 2018) might be largely based on the methodology implemented here based on detailed pathway expression analysis and rank-based gene ontology analysis with adaptive clustering (GOMWU), instead of superclusters.

For instance, the down-regulation of dual oxidases 2, that are involved in the antimicrobial defense at the mucosa surface in humans (Sarr et al., 2018), and cathepsins, that are potentially involved in the lysosomal degradation of pathogens and induction of apoptosis (Leist & Jäättelä, 2001), might support the successful incorporation and tolerance of the kleptoplasts within the host cell. In combination with a reduced expression, or the lack of expression of apoptosis core genes, for instance caspases, cells with kleptoplasts might be prevented from undergoing apoptosis. Apoptosis is involved in removal of heterologous Symbiodiniaceae strains in some cnidarians (Dunn & Weis, 2009), while in others several genes related to apoptosis are highly expressed in the symbiotic state (Lehnert et al., 2014). Thus, the exact role of apoptosis might vary depending on the species and condition investigated. However, it is feasible that in the LtR species *Elysia chlorotica*, the

inhibition of apoptosis might be essential to maintain the kleptoplasts. Additionally, the absence of core components of the toll-like receptor signaling pathway might further be necessary for maintaining the kleptoplasts. This pathway not only induces apoptosis (Wiens et al., 2007; Salaun et al., 2007; Rauta et al., 2014), but it is also important for inflammatory response (Fitzgerald & Kagan, 2020) and the anti-microbial defense systems (Carpenter & O'Neill, 2007). In cnidarians, the toll-like signaling pathway is involved in the removal of damaged or harmful symbionts (DeSalvo et al., 2010; Wolfowicz et al., 2016; Mansfield et al., 2017). In mollusks there is an expansion of the toll-like receptor genes and their signaling components, which are relevant in the immune response against pathogens (Brennan & Gilmore, 2018). The lack of expression of core components of this pathway in *Elysia chlorotica* might thus facilitate the maintenance of the kleptoplasts by suppressing immune response and apoptosis.

Different than in cnidarians, the TGF-ß pathway and GP2 are not involved in the establishment and maintenance of functional kleptoplasty in sacoglossan sea slugs. The absence of the TGF-ß ss components in *E. chlorotica* is not surprising, because these are also not encoded in the genomes of some mollusks (Herpin, 2004). If other components of the TGF-ß pathway, like the BMP or Activin pathway, would be involved in kleptoplast tolerance, a constant significant up-regulation of their components would have been expected, yet it is rather not the case. Further, none of the other TGF-ß family pathways are known to be activated through the interaction of receptors involved in symbiont recognition, as is the case for TGF-ß ss (Li et al., 2006); Nor have they been linked to photosymbiosis (Mansfield & Gilmore, 2019). The down-regulation of GP2 seems relevant in the establishment of cnidarian photosymbiosis (Kuo et al., 2010; Mohamed et al., 2016, 2020) and might be evidence of the attenuated microbial response during the initiation of the association. This glycoprotein serves as the first line of defense against bacterial pathogens in mammalian guts (Kurashima et al., 2021), but their function in invertebrates is currently unknown. Whether GP2 is present in other sacoglossans or whether it plays a role in bacterial recognition in mollusks is unknown, but its role in the kleptoplasty in Sacoglossa may be excluded at least based on the transcriptional level.

Similar to Chan et al., (2018) we identified a suppression of the phagosome maturation but provide further details in the regulation of the genes involved in this process. The results observed here could point to the distinction of two phases in the establishment of the kleptoplasty proposed by Pelletreau et al., (2012). During the first five days of feeding and development known as "transient

kleptoplasty" there is kleptoplast incorporation but constant kleptoplast digestion, based on Rab7 expression. After seven days of feeding and developing, there is an up-regulation of the genes responsible for the phagosome initiation, but a down-regulation of Rab proteins hinting to the inhibition of the phagosome maturation. This phase is considered as the permanent kleptoplasty (Pelletreau et al., 2012). However, after 10 days there is no regulation of Rab Proteins. If the permanent kleptoplasty state continues, the expression pattern would be similar between the 7$^{th}$ day and the 10$^{th}$ day, but this is not the case. An alternative explanation according to some observations is based on the presumed absence of the phagosome membrane surrounding some kleptoplasts in histological section of *E. chlorotica* adults. Here, kleptoplasts seem to be directly in contact with the animal cytosol and are maintained for up to 8 months of starvation (Graves et al., 1979; Mujer et al., 1996; Rumpho et al., 2001). Such is also the case in *E. chlorotica* juveniles (Pelletreau et al., 2014). However, the fact that the membrane was not detected in all kleptoplasts in these studies does not necessarily imply that the membrane is not present. There is also the possibility that a cellular signal not based on Rab proteins promotes the inhibition of the phagosome maturation, but this requires further investigation.

We could provide further evidence that the successful maintenance of kleptoplasts, might depend on the ability of the animal host to efficiently quench ROS, supporting previous works on Sacoglossa (de Vries et al., 2015; Chan et al., 2018). Increased ROS formation in kleptoplasts could be triggered through photodamage (Christa et al., 2018; Cartaxana et al., 2019a) and a potential photoprotective behavior of the slugs (Jesus et al., 2010; Schmitt & Wägele, 2011), or kleptoplast photoprotection mechanisms (Cartaxana et al., 2019a; Havurinne & Tyystjärvi, 2020) might not be enough to compensate net photodamage to the kleptoplasts (Vieira et al., 2009). In order to maintain healthy cells, ROS production needs to be kept at bay to prevent an excessive accumulation, that reaches toxic levels triggering an apoptosis response (Simon et al., 2000). Thus, an efficient ROS scavenging system combined with the inhibition of apoptosis might be crucial for kleptoplast retention. But if photoprotection mechanisms of the kleptoplasts are subdued and the kleptoplasts become a source of increased ROS stress that could not be scavenged properly, as seen in species not able to maintain the kleptoplasts in the long-term (de Vries et al., 2015), apoptosis might nevertheless be triggered.

In developing juveniles genes involved in cell cycle and DNA metabolism are important to ensure growth. Following Chan et al., (2018), our analysis also showed that genes related to these

processes are enriched and highly up-regulated. Because the slugs were developing, the gene expression cannot be solely attributed to the kleptoplastic association, as noted by Chan et al., (2018).

The question whether kleptoplasts actively provide the slugs with nutrients (Yamamoto et al., 2013; Akimoto et al., 2014; Cartaxana et al., 2017; Cruz et al., 2020; Donohoo et al., 2020; Lopes et al., 2022) or if the kleptoplasts rather function as a larder (Christa et al., 2014; de Vries et al., 2015; Laetz et al., 2017) is probably the most enigmatic research topic to unravel, independent on the exact amount of nutritional support the slugs might get. For years several studies have attempted to show evidence that the kleptoplasts are vital for the slugs development, survival, and fitness (e.g. Middlebrooks et al 2012; Yamamoto et al., 2013; Akimoto et al., 2014; Cartaxana et al., 2017; Cruz et al., 2020; Donohoo et al., 2020; Lopes et al., 2022), yet the data of these studies do not show a nutrient exchange between the alien organelles and the slug confirming this hypothesis. The most important factor for growing and reproduction seems to be the constant availability of food and the light acclimation state of the algae (Baumgartner et al., 2015; Cartaxana et al., 2019b; Shiroyama et al., 2020). Often, the latter is interpreted as if the kleptoplasts would then have a higher photosynthetic capacity and thus could provide more assimilates to the host. Yet, the slugs feed primarily on the cytosol of the algae, which will be of higher nutritional value when photosynthesis is increased. Thus, the effects seen in feeding slugs might rather depend on a higher nutritional value of the algal cytosol and might be independent on the photosynthetic activity of the kleptoplasts. The present study showed that digestion and maintenance of kleptoplasts is probably simultaneously occurring when the slugs are feeding, especially in the "transient kleptoplasty" state (5 days post-metamorphosis). This is to be expected, because kleptoplasts are degrading and being digested even in species that maintain them over long periods (Evertsen & Johnsen, 2009; Maeda et al., 2012; Frankenbach et al., 2021). Thus, a discrimination between an active export of assimilates by the kleptoplasts and assimilates obtained through digestion is currently not possibly, and hence, whether the kleptoplasts are somehow involved in conflict mediation. However, the increased expression of glucose transporters in feeding slugs points towards an increased translocation of glucose. But at this point, it is not possible to determine whether these transporters are located at the phagosomal membrane surrounding the kleptoplasts to transport glucose from the kleptoplasts into the host cytosol, and/or at the basal cell membrane to distribute glucose obtained by feeding, as is also speculated for corals (Lehnert et al., 2014). Immunohistological examination of these transporters and other carbohydrate products might be able to shed light on this matter.

Ammonium and bicarbonate are the limiting nutrients to ensure a high photosynthetic rate (Roberty et al., 2020). In Sacoglossa it is often observed that the photosynthetic capacity of the kleptoplasts in freshly fed animals is higher than in the algae, potentially due to a higher partial $CO_2$ pressure in the animal's cytosol (Serôdio et al., 2014). In this line, it is interesting that at least one carbonic anhydrase (CH) was up-regulated during the feeding period. This might point towards a support of $CO_2$ to the enslaved organelle, if a bicarbonate transporter would be located on a phagosomal membrane, but the exact location of this transporter in the host cells is not yet possible to determine. Alternatively, TEM of the kleptoplast containing cells (Mondy & Pierce 2003; Pelletreau et al., 2014) shows the cells have a lot of mitochondria, often directly adjacent to the plastids. So, a diffusion of $CO_2$ could be happening given the significant production coming out of the mitochondria.

Because none of the ammonium transporters are up-regulated, it could be assumed that the animal host is probably not actively supporting the kleptoplasts with ammonium. Yet, the kleptoplasts are still photosynthetically active, and a passive translocation of ammonium or other forms of nitrogen through diffusion might support the alien organelle with the needed nutrients.

Sym32 was identified as a symbiosis-specific protein located in the symbiosome surrounding Symbiodiniaceae (Mohamed et al., 2016). It is highly expressed in symbiotic state mainly in the gastroderm of some photosymbiotic cnidarians and it might serve an interpartner signaling function. Yet, how sym32 participates in cell signaling and recognition remains unclear. The lack of sym32 in *E. chlorotica* and the vitamin K cycle suggest that this pathway might not be relevant for functional kleptoplasty, and that the interpartner communication might be based on other pathways or proteins; or there is not such interaction and the slugs rather use the kleptoplasts as a larder as suggested earlier (Christa et al., 2014; Laetz et al., 2017).

**Author contributions**

JM and GC sourced and processed the data. JM, SF, and GC performed data analyses. JM, SF, and GC edited the figures. JM, SF, JS, AP, and GC discussed and interpreted the results. JM, JS, AP, and GC wrote the manuscript and JM, SF, JS, AP, and GC revised the final version of the manuscript.

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**Conflict of Interest Statement**

The authors declare no competing interests.

**Availability of data and materials**

Raw reads are accessible via download from GenBank from the short read archive (SRS3101883). Transcriptome assembly was downloaded from http://cyanophora.rutgers.edu/Elysia-expression/.

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## Supplementary material

Supplementary material can be found at online at: 10.6084/m9.figshare.22492651

**Supplementary Table 1.** Annotation list of genes identified for each pathway analyzed in this study. (See online).

**Supplementary Table 2.** GOMWU results of the biological process categories.

Day 5

| Delta.rank | pval | Level | # seqs | GO term | GO Name | p.adj |
|---|---|---|---|---|---|---|
| 333 | 1.24E-17 | 2 | 165 | GO:0016070;GO:0006396 | RNA metabolic process | 2.51E-15 |
| -362 | 9.96E-11 | 2 | 75 | GO:0009653 | Anatomical structure morphogenesis | 1.01E-08 |
| 403 | 1.09E-09 | 2 | 53 | GO:0006259 | DNA metabolic process | 7.34E-08 |
| 394 | 5.03E-09 | 3 | 51 | GO:0034470;GO:0034660 | ncRNA metabolic process | 2.55E-07 |
| 410 | 3.07E-08 | 3 | 42 | GO:0006281;GO:0006974;GO:0033554;GO:0051716 | Cellular response to DNA damage stimulus | 1.25E-06 |
| 474 | 1.57E-07 | 2 | 28 | GO:0000724;GO:0000725;GO:0006302;GO:0006310 | DNA recombination | 4.94E-06 |
| -383 | 1.70E-07 | 2 | 43 | GO:0006928 | Movement of cell or subcellular component | 4.94E-06 |
| 439 | 5.08E-07 | 4 | 30 | GO:0051726 | Regulation of cell cycle | 1.29E-05 |
| 423 | 5.86E-07 | 3 | 32 | GO:0006364;GO:0016072 | rRNA processing | 1.32E-05 |
| 257 | 6.67E-07 | 2 | 89 | GO:0016071 | mRNA metabolic process | 1.35E-05 |
| 416 | 2.72E-06 | 5 | 29 | GO:0051276 | Chromosome organization | 5.02E-05 |
| 251 | 7.54E-06 | 2 | 75 | GO:0000398;GO:0000377;GO:0006397; GO:0000375;GO:0008380 | RNA splicing | 1.27E-04 |
| -312 | 8.84E-06 | 2 | 47 | GO:0001525 | Angiogenesis | 1.38E-04 |
| -379 | 1.04E-05 | 2 | 31 | GO:0048729 | Tissue morphogenesis | 1.51E-04 |
| -501 | 1.48E-05 | 2 | 17 | GO:0001658;GO:0048754;GO:0061138;GO:0001763 | Morphogenesis of a branching structure | 2.00E-04 |
| 200 | 2.52E-05 | 3 | 106 | GO:0022618;GO:0034622;GO:0071826; GO:0065003;GO:0043933;GO:0022607 | Cellular component assembly | 3.20E-04 |
| 554 | 5.44E-05 | 2 | 12 | GO:1903046 | Meiotic cell cycle process | 6.50E-04 |
| 673 | 6.09E-05 | 2 | 8 | GO:0061982 | Meiosis I cell cycle process | 6.87E-04 |
| -414 | 6.95E-05 | 2 | 21 | GO:0003008 | System process | 7.06E-04 |
| 448 | 6.68E-05 | 4 | 18 | GO:0031570;GO:0000075;GO:0045786 | DNA integrity checkpoint | 7.06E-04 |
| 186 | 8.41E-05 | 2 | 108 | GO:1903047;GO:0022402 | Cell cycle process | 8.13E-04 |
| -397 | 1.38E-04 | 3 | 21 | GO:0003341;GO:0007018 | Cilium movement | 1.27E-03 |
| 514 | 1.79E-04 | 7 | 12 | GO:0000076 | DNA replication checkpoint | 1.35E-03 |
| 394 | 1.56E-04 | 4 | 21 | GO:0000082;GO:0044843 | Cell cycle G1/S phase transition | 1.35E-03 |
| 318 | 1.69E-04 | 3 | 32 | GO:0000278;GO:0007049 | Cell cycle | 1.35E-03 |
| 728 | 1.73E-04 | 7 | 6 | GO:0000727 | Double-strand break repair via break-induced replication | 1.35E-03 |
| -375 | 1.67E-04 | 2 | 23 | GO:0016477;GO:0048870;GO:0040011 | Locomotion | 1.35E-03 |
| -358 | 2.40E-04 | 3 | 24 | GO:0035239;GO:0002009 | Morphogenesis of an epithelium | 1.74E-03 |
| -163 | 2.53E-04 | 1 | 123 | GO:0032501 | Multicellular organismal process | 1.77E-03 |
| 355 | 2.67E-04 | 3 | 24 | GO:0009451 | RNA modification | 1.81E-03 |
| 510 | 3.72E-04 | 4 | 11 | GO:0001732 | Formation of cytoplasmic translation initiation complex | 2.44E-03 |
| -201 | 4.24E-04 | 2 | 72 | GO:0007017 | Microtubule-based process | 2.69E-03 |
| 500 | 4.87E-04 | 3 | 11 | GO:0000070;GO:0000819;GO:0098813;GO:0007059 | Nuclear chromosome segregation | 2.99E-03 |
| 500 | 8.71E-04 | 10 | 10 | GO:0000463;GO:0000470 | Maturation of LSU-rRNA | 5.20E-03 |
| -382 | 9.47E-04 | 4 | 17 | GO:0001764 | Neuron migration | 5.38E-03 |
| -197 | 9.54E-04 | 2 | 66 | GO:0048646 | Anatomical structure formation involved in morphogenesis | 5.38E-03 |
| 633 | 1.10E-03 | 4 | 6 | GO:0034654 | Nucleobase-containing compound biosynthetic process | 6.03E-03 |
| -229 | 1.23E-03 | 2 | 46 | GO:0031325;GO:0009893;GO:0048522;GO:0010604 | Positive Regulation of cellular process | 6.58E-03 |
| -199 | 1.54E-03 | 3 | 59 | GO:0048518 | Positive Regulation of biological process | 8.00E-03 |
| 410 | 1.89E-03 | 7 | 13 | GO:0000462;GO:0030490 | Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) | 9.34E-03 |
| -411 | 1.85E-03 | 2 | 13 | GO:0000904 | Cell morphogenesis involved in differentiation | 9.34E-03 |
| 657 | 1.98E-03 | 3 | 5 | GO:0000712 | Resolution of meiotic recombination intermediates | 9.42E-03 |
| 465 | 2.00E-03 | 2 | 10 | GO:0018130;GO:0019438;GO:1901362 | Heterocycle biosynthetic process | 9.42E-03 |
| -318 | 2.26E-03 | 2 | 21 | GO:0001501;GO:0048731 | System development | 1.02E-02 |
| 353 | 2.25E-03 | 4 | 17 | GO:0007005 | Mitochondrion organization | 1.02E-02 |
| 415 | 2.50E-03 | 4 | 12 | GO:0000079;GO:1904029 | Regulation of cyclin-dependent protein kinase activity | 1.10E-02 |
| 144 | 3.30E-03 | 4 | 99 | GO:0006464;GO:0036211;GO:0043412 | Macromolecule modification | 1.43E-02 |
| -438 | 3.57E-03 | 4 | 10 | GO:0001736;GO:0007164 | Establishment of planar polarity | 1.51E-02 |
| 321 | 4.21E-03 | 4 | 18 | GO:0000387 | Spliceosomal snRNP assembly | 1.75E-02 |
| -192 | 5.11E-03 | 3 | 49 | GO:0048583 | Regulation of response to stimulus | 2.08E-02 |
| -249 | 5.77E-03 | 3 | 28 | GO:0000902 | Cell morphogenesis | 2.28E-02 |
| 245 | 5.83E-03 | 1 | 29 | GO:0022414 | Reproductive process | 2.28E-02 |
| -260 | 6.55E-03 | 5 | 25 | GO:0000187;GO:0043406;GO:0043410;GO:0071902; GO:1902533;GO:0009967;GO:0010647;GO:0023056 | Positive Regulation of signaling | 2.46E-02 |
| -220 | 6.54E-03 | 3 | 35 | GO:0043405;GO:0043408;GO:1902531;GO:0009966; GO:0010646;GO:0023051 | Regulation of signaling | 2.46E-02 |
| -407 | 6.77E-03 | 3 | 10 | GO:0009887 | Animal organ morphogenesis | 2.50E-02 |
| 331 | 7.11E-03 | 2 | 15 | GO:0008033;GO:0006399 | tRNA processing | 2.58E-02 |
| 245 | 7.64E-03 | 4 | 27 | GO:0000956;GO:0006402;GO:0006401;GO:0034655; GO:0019439;GO:0044270;GO:0046700;GO:1901361 | Cellular nitrogen compound catabolic process | 2.72E-02 |
| -555 | 8.95E-03 | 2 | 5 | GO:0003382 | Epithelial cell morphogenesis | 3.13E-02 |
| 352 | 1.04E-02 | 2 | 12 | GO:0000723;GO:0032200;GO:0060249 | Anatomical structure homeostasis | 3.57E-02 |
| 314 | 1.06E-02 | 2 | 15 | GO:0000184 | Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay | 3.60E-02 |
| 363 | 1.14E-02 | 3 | 11 | GO:0006400 | tRNA modification | 3.72E-02 |
| -208 | 1.13E-02 | 8 | 34 | GO:0032147;GO:0045860;GO:0001934;GO:0033674; GO:0031401;GO:0042327;GO:0032270;GO:0045937; GO:0010562;GO:0051247;GO:0051173;GO:0051347; GO:0043085;GO:0044093 | Positive Regulation of nitrogen compound metabolic process | 3.72E-02 |

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| -523 | 1.38E-02 | 3 | 5 | GO:0003013 | Circulatory system process | 4.44E-02 |
| -439 | 1.46E-02 | 4 | 7 | GO:0003429;GO:0003414;GO:0003422;GO:0090171; GO:0060536 | Cartilage morphogenesis | 4.55E-02 |
| -520 | 1.43E-02 | 4 | 5 | GO:0030030 | Cell projection organization | 4.55E-02 |
| 424 | 1.83E-02 | 4 | 7 | GO:0006959;GO:0001867;GO:0006956;GO:0045087; GO:0098542 | Innate immune response | 5.63E-02 |
| 381 | 2.32E-02 | 2 | 8 | GO:0000479;GO:0000478;GO:0000469;GO:0090502; GO:0090501 | Cleavage involved in rRNA processing | 7.04E-02 |
| -380 | 2.37E-02 | 3 | 8 | GO:0001504;GO:0006836;GO:0098657 | Neurotransmitter transport | 7.09E-02 |
| -436 | 2.45E-02 | 4 | 6 | GO:0001659;GO:0048871 | Multicellular organismal homeostasis | 7.11E-02 |
| -323 | 2.44E-02 | 2 | 11 | GO:0002831;GO:0031347;GO:0032101;GO:0080134 | Regulation of response to external stimulus | 7.11E-02 |
| -475 | 2.53E-02 | 3 | 5 | GO:0003127;GO:0009726;GO:0009719 | Detection of nodal flow | 7.23E-02 |
| 371 | 2.74E-02 | 7 | 8 | GO:0000413;GO:0018208;GO:0018193 | Peptidyl-amino acid modification | 7.72E-02 |
| 463 | 2.90E-02 | 4 | 5 | GO:0000491 | Small nucleolar ribonucleoprotein complex assembly | 8.03E-02 |
| -179 | 2.93E-02 | 2 | 34 | GO:0048584 | Positive Regulation of response to stimulus | 8.03E-02 |
| -149 | 3.03E-02 | 4 | 49 | GO:0000226;GO:0007010 | Microtubule cytoskeleton organization | 8.21E-02 |
| 387 | 3.11E-02 | 2 | 7 | GO:0001522 | Pseudouridine synthesis | 8.30E-02 |
| -355 | 3.43E-02 | 2 | 8 | GO:0007155;GO:0022610 | Cell adhesion | 9.05E-02 |
| 317 | 3.48E-02 | 2 | 10 | GO:0000266;GO:0048285 | Organelle fission | 9.07E-02 |
| 437 | 3.95E-02 | 2 | 5 | GO:0000154 | rRNA modification | 1.01E-01 |
| -395 | 4.16E-02 | 2 | 6 | GO:0008610;GO:0072330 | Lipid biosynthetic process | 1.06E-01 |
| 317 | 4.55E-02 | 2 | 9 | GO:0002098;GO:0002097 | tRNA wobble base modification | 1.14E-01 |
| 272 | 4.74E-02 | 6 | 12 | GO:0000054;GO:0033750;GO:0071428;GO:0071426; GO:0006405;GO:0006611;GO:0031503;GO:0050658; GO:0051168;GO:0050657;GO:0051236;GO:0015931; GO:0006913;GO:0051169 | Nucleocytoplasmic transport | 1.17E-01 |
| 280 | 5.06E-02 | 5 | 11 | GO:0090305 | Nucleic acid phosphodiester bond hydrolysis | 1.24E-01 |
| 348 | 5.27E-02 | 4 | 7 | GO:0001510;GO:0043414;GO:0032259 | Macromolecule methylation | 1.25E-01 |
| 87 | 5.49E-02 | 3 | 117 | GO:0006996 | Organelle organization | 1.25E-01 |
| 144 | 5.43E-02 | 2 | 41 | GO:0044271;GO:0043604;GO:1901566;GO:0043603 | Cellular nitrogen compound biosynthetic process | 1.25E-01 |
| 132 | 5.29E-02 | 3 | 50 | GO:0044772;GO:0044770 | Cell cycle phase transition | 1.25E-01 |
| -139 | 5.48E-02 | 2 | 44 | GO:0048869 | Cellular developmental process | 1.25E-01 |
| -324 | 5.37E-02 | 2 | 8 | GO:0051606 | Detection of stimulus | 1.25E-01 |
| 134 | 5.83E-02 | 2 | 46 | GO:0044249;GO:1901576;GO:0009058 | Organic substance biosynthetic process | 1.31E-01 |
| -88 | 6.11E-02 | 3 | 109 | GO:0006810;GO:0051234;GO:0051179 | Localization | 1.36E-01 |
| -312 | 6.29E-02 | 3 | 8 | GO:0050877 | Nervous system process | 1.37E-01 |
| -313 | 6.23E-02 | 2 | 8 | GO:0051239 | Regulation of multicellular organismal process | 1.37E-01 |
| -355 | 6.67E-02 | 4 | 6 | GO:0003333;GO:1905039;GO:0098656;GO:1903825; GO:0034220;GO:0055085 | Carboxylic acid transmembrane transport | 1.44E-01 |
| 375 | 7.77E-02 | 2 | 5 | GO:0000002 | Mitochondrial genome maintenance | 1.64E-01 |
| 374 | 7.80E-02 | 5 | 5 | GO:0006486;GO:0043413;GO:0070085 | Protein glycosylation | 1.64E-01 |
| -162 | 7.84E-02 | 3 | 27 | GO:0030154 | Cell differentiation | 1.64E-01 |
| 243 | 8.95E-02 | 2 | 11 | GO:0006952 | Defense response | 1.83E-01 |
| 76 | 8.99E-02 | 2 | 122 | GO:0044267;GO:0019538;GO:1901564 | Organonitrogen compound metabolic process | 1.83E-01 |
| 104 | 8.99E-02 | 2 | 62 | GO:1901575;GO:0009056;GO:0044248 | Catabolic process | 1.83E-01 |
| 75 | 9.17E-02 | 1 | 123 | GO:0050896;GO:0006950 | Response to stimulus | 1.84E-01 |
| -219 | 9.70E-02 | 3 | 13 | GO:0048598 | Embryonic morphogenesis | 1.93E-01 |
| -235 | 1.01E-01 | 3 | 11 | GO:0032879 | Regulation of localization | 1.99E-01 |
| 343 | 1.06E-01 | 2 | 5 | GO:0001731 | Formation of translation preinitiation complex | 2.05E-01 |
| -272 | 1.05E-01 | 5 | 8 | GO:0006865;GO:0046942;GO:0015711;GO:0015849; GO:0006820 | Organic acid transport | 2.05E-01 |
| 309 | 1.11E-01 | 4 | 6 | GO:0000077 | DNA damage checkpoint | 2.13E-01 |
| 236 | 1.17E-01 | 4 | 10 | GO:0000245 | Spliceosomal complex assembly | 2.20E-01 |
| -333 | 1.16E-01 | 2 | 5 | GO:0051050 | Positive Regulation of transport | 2.20E-01 |
| -101 | 1.19E-01 | 2 | 55 | GO:0065008 | Regulation of biological quality | 2.22E-01 |
| -197 | 1.21E-01 | 4 | 14 | GO:0051128 | Regulation of cellular component organization | 2.24E-01 |
| -209 | 1.29E-01 | 3 | 12 | GO:0000272;GO:0005976;GO:0016052;GO:0005975 | Carbohydrate metabolic process | 2.35E-01 |
| 267 | 1.37E-01 | 4 | 7 | GO:0001507;GO:0006581;GO:0008291;GO:0042135; GO:0042133;GO:1900619 | Acetylcholine metabolic process | 2.48E-01 |
| -63 | 1.40E-01 | 2 | 135 | GO:0048856 | Anatomical structure development | 2.51E-01 |
| -309 | 1.45E-01 | 2 | 5 | GO:0002697 | Regulation of immune effector process | 2.56E-01 |
| 219 | 1.46E-01 | 2 | 10 | GO:0003006 | Developmental process involved in reproduction | 2.56E-01 |
| -261 | 1.46E-01 | 1 | 7 | GO:0007610 | Behavior | 2.56E-01 |
| 108 | 1.54E-01 | 6 | 40 | GO:0001701;GO:0043009;GO:0009792;GO:0009790; GO:0007275 | Multicellular organism development | 2.66E-01 |
| -106 | 1.55E-01 | 2 | 41 | GO:0044255;GO:0006629 | Cellular Lipid metabolic process | 2.68E-01 |
| -178 | 1.61E-01 | 3 | 14 | GO:0000302;GO:0006979;GO:1901700;GO:0042221 | Response to chemical | 2.73E-01 |
| -157 | 1.61E-01 | 4 | 18 | GO:0050776;GO:0002682 | Regulation of immune system process | 2.73E-01 |
| -209 | 1.64E-01 | 2 | 10 | GO:0048585 | Negative Regulation of response to stimulus | 2.75E-01 |
| -293 | 1.68E-01 | 2 | 5 | GO:0044057 | Regulation of system process | 2.80E-01 |
| -154 | 1.70E-01 | 2 | 18 | GO:0001822 | Kidney development | 2.80E-01 |
| -163 | 1.72E-01 | 5 | 16 | GO:0001523;GO:0016101;GO:0006721;GO:0006720 | Terpenoid metabolic process | 2.81E-01 |
| 260 | 1.81E-01 | 5 | 6 | GO:0000050;GO:0019627;GO:0071941 | Urea cycle | 2.89E-01 |
| -165 | 1.79E-01 | 2 | 15 | GO:0001649 | Osteoblast differentiation | 2.89E-01 |
| 150 | 1.81E-01 | 4 | 18 | GO:0006886;GO:0046907 | Intracellular transport | 2.89E-01 |
| -277 | 1.91E-01 | 4 | 5 | GO:0001667 | Ameboidal-type cell migration | 3.02E-01 |
| -276 | 1.93E-01 | 2 | 5 | GO:0001775 | Cell activation | 3.02E-01 |

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|---|---|---|---|---|---|---|
| 102 | 1.94E-01 | 2 | 37 | GO:0044281 | Small molecule metabolic process | 3.02E-01 |
| -249 | 1.99E-01 | 2 | 6 | GO:0002833;GO:0032103 | Positive Regulation of response to biotic stimulus | 3.08E-01 |
| -104 | 2.04E-01 | 1 | 34 | GO:0002376 | Immune system process | 3.14E-01 |
| -95 | 2.11E-01 | 2 | 40 | GO:0006811 | Ion transport | 3.22E-01 |
| 264 | 2.13E-01 | 7 | 5 | GO:0000055 | Ribosomal large subunit export from nucleus | 3.23E-01 |
| 120 | 2.28E-01 | 3 | 23 | GO:0000165;GO:0023014;GO:0035556;GO:0006468; GO:0016310;GO:0006796;GO:0006793 | Phosphorus metabolic process | 3.40E-01 |
| 113 | 2.27E-01 | 4 | 26 | GO:0015031;GO:0015833;GO:0045184;GO:0042886; GO:0008104;GO:0033036 | Protein localization | 3.40E-01 |
| -255 | 2.31E-01 | 6 | 5 | GO:0002090;GO:0048259;GO:0030100;GO:0060627 | Regulation of receptor-mediated endocytosis | 3.42E-01 |
| 183 | 2.48E-01 | 3 | 9 | GO:0001889;GO:0048732 | Liver development | 3.65E-01 |
| -157 | 2.51E-01 | 2 | 12 | GO:0006814;GO:0015672 | Monovalent inorganic cation transport | 3.67E-01 |
| 176 | 2.66E-01 | 2 | 9 | GO:0000038 | Very long-chain fatty acid metabolic process | 3.82E-01 |
| -200 | 2.65E-01 | 2 | 7 | GO:0051049 | Regulation of transport | 3.82E-01 |
| 199 | 2.68E-01 | 3 | 7 | GO:0000338;GO:0070646 | Protein modification by small protein removal | 3.83E-01 |
| -209 | 2.81E-01 | 2 | 6 | GO:0001816;GO:0010467 | Cytokine production | 3.99E-01 |
| -85 | 2.90E-01 | 3 | 36 | GO:0048513 | Animal organ development | 4.09E-01 |
| 155 | 3.01E-01 | 2 | 10 | GO:0009605;GO:0051707;GO:0043207;GO:0044419; GO:0009607 | Response to external stimulus | 4.22E-01 |
| -196 | 3.12E-01 | 4 | 6 | GO:0007166 | Cell surface receptor signaling pathway | 4.30E-01 |
| 89 | 3.11E-01 | 2 | 30 | GO:0034645;GO:0009059;GO:0002181;GO:0006412; GO:0043043;GO:0006518 | Cellular macromolecule biosynthetic process | 4.30E-01 |
| -106 | 3.19E-01 | 2 | 20 | GO:0000045;GO:0070925;GO:1905037;GO:0007033 | Organelle assembly | 4.38E-01 |
| -191 | 3.25E-01 | 4 | 6 | GO:0000186 | Activation of MAPKK activity | 4.43E-01 |
| -186 | 3.38E-01 | 5 | 6 | GO:0002576;GO:0045055;GO:0006887;GO:0032940; GO:0046903;GO:0140352 | Secretion by cell | 4.55E-01 |
| 186 | 3.38E-01 | 2 | 6 | GO:0022412 | Cellular process involved in reproduction in multicellular organism | 4.55E-01 |
| -58 | 3.41E-01 | 5 | 64 | GO:0001932;GO:0031399;GO:0042325;GO:0019220; GO:0051174;GO:0032268;GO:0051246 | Regulation of phosphorus metabolic process | 4.55E-01 |
| -92 | 3.46E-01 | 4 | 24 | GO:0001666;GO:0036293;GO:0070482;GO:0009628 | Response to decreased oxygen levels | 4.59E-01 |
| -69 | 3.66E-01 | 2 | 40 | GO:0007165 | Signal transduction | 4.79E-01 |
| -162 | 3.67E-01 | 2 | 7 | GO:0016192 | Vesicle-mediated transport | 4.79E-01 |
| -104 | 3.68E-01 | 6 | 17 | GO:0030001;GO:0006812 | Metal ion transport | 4.79E-01 |
| 130 | 3.85E-01 | 5 | 10 | GO:0000244 | Spliceosomal tri-snRNP complex assembly | 4.98E-01 |
| 183 | 3.89E-01 | 2 | 5 | GO:0003002;GO:0007389 | Regionalization | 5.00E-01 |
| 81 | 3.96E-01 | 3 | 25 | GO:0042592 | Homeostatic process | 5.06E-01 |
| -59 | 4.02E-01 | 4 | 46 | GO:0045859;GO:0043549;GO:0051338;GO:0050790; GO:0065009;GO:0071900 | Regulation of transferase activity | 5.07E-01 |
| -141 | 4.01E-01 | 3 | 8 | GO:0048468;GO:0002064 | Cell development | 5.07E-01 |
| 149 | 4.06E-01 | 2 | 7 | GO:0000028 | Ribosomal small subunit assembly | 5.09E-01 |
| -98 | 4.10E-01 | 3 | 16 | GO:0001505 | Regulation of neurotransmitter levels | 5.10E-01 |
| -137 | 4.13E-01 | 3 | 8 | GO:0001654;GO:0007423 | Eye development | 5.12E-01 |
| -157 | 4.19E-01 | 4 | 6 | GO:0001558;GO:0040008 | Regulation of cell growth | 5.16E-01 |
| 89 | 4.27E-01 | 4 | 18 | GO:0000027 | Ribosomal large subunit assembly | 5.19E-01 |
| -114 | 4.25E-01 | 2 | 11 | GO:0006869 | Lipid transport | 5.19E-01 |
| -124 | 4.33E-01 | 3 | 9 | GO:0001568 | Blood vessel development | 5.23E-01 |
| -81 | 4.37E-01 | 2 | 21 | GO:0001503 | Ossification | 5.25E-01 |
| -134 | 4.54E-01 | 6 | 7 | GO:0000188;GO:0043407;GO:0043409;GO:0071901; GO:1902532;GO:0009968;GO:0010648;GO:0023057; GO:0006469;GO:0033673;GO:0051348;GO:0043086; GO:0044092 | Negative Regulation of kinase activity | 5.39E-01 |
| 73 | 4.53E-01 | 3 | 24 | GO:0051656;GO:0051640 | Organelle localization | 5.39E-01 |
| 156 | 4.63E-01 | 3 | 5 | GO:0001958;GO:0036075 | Endochondral ossification | 5.46E-01 |
| -62 | 4.84E-01 | 2 | 29 | GO:0000086;GO:0044839 | G2/M transition of mitotic cell cycle | 5.66E-01 |
| 51 | 4.86E-01 | 2 | 44 | GO:0009057;GO:0044265 | Macromolecule catabolic process | 5.66E-01 |
| 94 | 4.93E-01 | 2 | 12 | GO:0002252 | Immune effector process | 5.72E-01 |
| -54 | 5.02E-01 | 2 | 36 | GO:0000209;GO:0016567;GO:0032446 | Protein ubiquitination | 5.79E-01 |
| -112 | 5.04E-01 | 2 | 8 | GO:0044283;GO:0046394;GO:0016053 | Small molecule biosynthetic process | 5.79E-01 |
| -81 | 5.08E-01 | 5 | 15 | GO:0001933;GO:0031400;GO:0042326;GO:0032269; GO:0051248;GO:0045936;GO:0010563 | Negative Regulation of phosphorylation | 5.79E-01 |
| 75 | 5.17E-01 | 3 | 17 | GO:0006508 | Proteolysis | 5.86E-01 |
| -23 | 5.23E-01 | 5 | 198 | GO:0000122;GO:0006357;GO:0045892;GO:0006355; GO:1903506;GO:2000112;GO:2001141;GO:0010556; GO:0031326;GO:0009889;GO:0051252;GO:0019219; GO:0010629;GO:1903507;GO:2000113;GO:1902679; GO:0010558;GO:0031327;GO:0009890;GO:0031324; GO:0051253;GO:0045934;GO:0051172 | Regulation of nucleobase-containing compound metabolic process | 5.86E-01 |
| -85 | 5.22E-01 | 6 | 13 | GO:0000132;GO:0040001;GO:0051294;GO:0051293; GO:1902850;GO:0051653 | Establishment of spindle orientation | 5.86E-01 |
| -92 | 5.39E-01 | 2 | 10 | GO:0002931 | Response to ischemia | 6.01E-01 |
| 64 | 5.58E-01 | 7 | 19 | GO:0000381;GO:0048024;GO:0043484;GO:0050684; GO:1903311 | Regulation of mRNA processing | 6.20E-01 |
| 109 | 5.73E-01 | 2 | 6 | GO:0035148 | Tube formation | 6.32E-01 |
| -92 | 5.82E-01 | 2 | 8 | GO:0002250 | Adaptive immune response | 6.39E-01 |
| 110 | 6.06E-01 | 5 | 5 | GO:0000012 | Single strand break repair | 6.62E-01 |
| -80 | 6.55E-01 | 4 | 7 | GO:0003143;GO:0060562 | Epithelial tube morphogenesis | 7.11E-01 |
| -59 | 7.43E-01 | 4 | 7 | GO:0002224;GO:0002221 | Pattern recognition receptor signaling pathway | 8.00E-01 |
| -45 | 7.45E-01 | 4 | 12 | GO:0002253;GO:0050778;GO:0002684 | Positive Regulation of immune system process | 8.00E-01 |
| -66 | 7.55E-01 | 2 | 5 | GO:0000380 | Alternative mRNA splicing, via spliceosome | 8.06E-01 |
| -44 | 8.22E-01 | 2 | 6 | GO:0001936;GO:0050678;GO:0042127 | Regulation of cell population proliferation | 8.69E-01 |
| -41 | 8.19E-01 | 3 | 7 | GO:0046395;GO:0016054;GO:0044282;GO:0001561;GO:0 009062;GO:0019395; GO:0044242;GO:0072329;GO:0034440;GO:0016042;GO:0 030258;GO:0055114 | Carboxylic acid catabolic process | 8.69E-01 |
| -16 | 8.32E-01 | 4 | 42 | GO:0071702 | Organic substance transport | 8.76E-01 |
| -41 | 8.47E-01 | 2 | 5 | GO:0009791 | Post-embryonic development | 8.87E-01 |
| -12 | 8.70E-01 | 6 | 43 | GO:0070647 | Protein modification by small protein conjugation or removal | 9.06E-01 |
| 11 | 8.98E-01 | 4 | 30 | GO:0071705 | Nitrogen compound transport | 9.30E-01 |
| 8 | 9.46E-01 | 2 | 16 | GO:0006955 | Immune response | 9.70E-01 |
| 5 | 9.46E-01 | 2 | 38 | GO:0051641;GO:0051649 | Establishment of localization in cell | 9.70E-01 |
| -5 | 9.66E-01 | 4 | 19 | GO:0000281;GO:0061640;GO:0000910 | Mitotic cytokinesis | 9.86E-01 |
| 4 | 9.84E-01 | 2 | 5 | GO:0001947 | Heart looping | 9.89E-01 |
| 5 | 9.79E-01 | 4 | 7 | GO:0006511;GO:0019941;GO:0043632;GO:0051603 | Modification-dependent protein catabolic process | 9.89E-01 |
| -2 | 9.80E-01 | 5 | 27 | GO:0006631;GO:0032787;GO:0019752;GO:0043436; GO:0006082 | Carboxylic acid metabolic process | 9.89E-01 |
| -1 | 9.95E-01 | 5 | 7 | GO:0001678;GO:0042593;GO:0055082;GO:0033500; GO:0048878;GO:0019725 | Chemical homeostasis | 9.95E-01 |

Day 7

| Delta.rank | pval | Level | # seqs | GO term | GO Name | p.adj |
|---|---|---|---|---|---|---|
| 490 | 8.79E-27 | 4 | 182 | GO:0016070 | RNA metabolic process | 1.9E-24 |
| 509 | 5.80E-22 | 5 | 133 | GO:0006396 | RNA processing | 6.4E-20 |
| 563 | 2.72E-12 | 2 | 55 | GO:0034470;GO:0034660 | ncRNA processing | 2.0E-10 |
| 421 | 3.87E-12 | 2 | 99 | GO:0016071 | mRNA metabolic process | 2.1E-10 |
| 448 | 4.37E-11 | 3 | 78 | GO:0000398;GO:0000377;GO:0006397;GO:0000375; GO:0008380 | RNA splicing | 1.9E-09 |
| 486 | 1.11E-10 | 2 | 63 | GO:0006259 | DNA metabolic process | 4.1E-09 |
| -377 | 7.39E-10 | 2 | 97 | GO:0009653 | anatomical structure morphogenesis | 2.3E-08 |
| 410 | 1.03E-09 | 3 | 80 | GO:0022618;GO:0034622;GO:0071826;GO:0065003; GO:0043933 | protein-containing complex subunit organization | 2.9E-08 |
| 493 | 7.24E-09 | 5 | 49 | GO:0006281;GO:0006974;GO:0033554;GO:0051716 | cellular response to DNA damage stimulus | 1.8E-07 |
| 562 | 1.40E-08 | 3 | 36 | GO:0006364;GO:0016072 | rRNA metabolic process | 3.1E-07 |
| 524 | 1.18E-07 | 2 | 36 | GO:0000724;GO:0000725;GO:0006302;GO:0006310 | DNA recombination | 2.4E-06 |
| 525 | 3.82E-07 | 2 | 33 | GO:0051276 | chromosome organization | 7.0E-06 |
| -359 | 1.57E-06 | 2 | 64 | GO:0006928 | movement of cell or subcellular component | 2.3E-05 |
| 276 | 1.57E-06 | 2 | 111 | GO:0022607 | cellular component assembly | 2.3E-05 |
| 484 | 1.43E-06 | 4 | 35 | GO:0051726 | regulation of cell cycle | 2.3E-05 |
| -232 | 3.90E-06 | 1 | 148 | GO:0032501 | multicellular organismal process | 5.4E-05 |
| -604 | 5.08E-06 | 4 | 20 | GO:0001658;GO:0048754;GO:0061138;GO:0001763 | morphogenesis of a branching structure | 6.6E-05 |
| -425 | 6.13E-06 | 2 | 40 | GO:0002009;GO:0048729 | tissue morphogenesis | 7.5E-05 |
| 518 | 1.22E-05 | 2 | 25 | GO:0009451 | RNA modification | 1.4E-04 |
| 832 | 2.36E-05 | 2 | 9 | GO:0061982 | meiosis I cell cycle process | 2.6E-04 |
| 654 | 3.45E-05 | 2 | 14 | GO:1903046 | meiotic cell cycle process | 3.6E-04 |
| 213 | 6.69E-05 | 2 | 130 | GO:1903047;GO:0022402 | cell cycle process | 6.7E-04 |
| -479 | 7.38E-05 | 2 | 24 | GO:0003008 | system process | 7.1E-04 |
| -291 | 7.69E-05 | 2 | 66 | GO:0031325;GO:0009893;GO:0048522;GO:0010604 | positive regulation of cellular process | 7.1E-04 |
| 540 | 1.09E-04 | 2 | 18 | GO:0007005 | mitochondrion organization | 9.6E-04 |
| 712 | 1.35E-04 | 6 | 10 | GO:0001732 | formation of cytoplasmic translation initiation complex | 1.1E-03 |
| 673 | 1.57E-04 | 3 | 11 | GO:0000070;GO:0000819;GO:0098813;GO:0007059 | sister chromatid segregation | 1.3E-03 |
| 338 | 1.67E-04 | 2 | 44 | GO:0000278;GO:0007049 | cell cycle | 1.3E-03 |
| -304 | 1.61E-04 | 2 | 55 | GO:0001525 | angiogenesis | 1.3E-03 |
| -420 | 1.78E-04 | 2 | 28 | GO:0035239 | tube morphogenesis | 1.3E-03 |
| -246 | 1.75E-04 | 3 | 84 | GO:0048518 | positive regulation of biological process | 1.3E-03 |
| 634 | 2.00E-04 | 2 | 12 | GO:0000076 | DNA replication checkpoint | 1.3E-03 |
| -397 | 1.98E-04 | 3 | 31 | GO:0003341;GO:0007018 | microtubule-based movement | 1.3E-03 |
| 595 | 2.81E-04 | 2 | 13 | GO:0000462;GO:0030490 | maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) | 1.8E-03 |
| 637 | 3.48E-04 | 4 | 11 | GO:0000463;GO:0000470 | maturation of LSU-rRNA | 2.2E-03 |
| 456 | 4.20E-04 | 3 | 21 | GO:0031570;GO:0000075;GO:0045786 | cell cycle checkpoint | 2.6E-03 |
| 828 | 5.79E-04 | 2 | 6 | GO:0000712 | resolution of meiotic recombination intermediates | 3.3E-03 |
| 765 | 6.01E-04 | 8 | 7 | GO:0000727 | double-strand break repair via break-induced replication | 3.3E-03 |
| 525 | 5.77E-04 | 2 | 15 | GO:0008033;GO:0006399 | tRNA processing | 3.3E-03 |
| -327 | 5.89E-04 | 4 | 39 | GO:0043405;GO:0043408;GO:1902531;GO:0009966 ;GO:0010646;GO:0023051 | regulation of signaling | 3.3E-03 |
| 403 | 6.80E-04 | 2 | 25 | GO:0000082;GO:0044843 | G1/S transition of mitotic cell cycle | 3.7E-03 |
| -301 | 7.08E-04 | 4 | 45 | GO:0001934;GO:0031401;GO:0042327;GO:0032270; GO:0045937;GO:0010562;GO:0051247;GO:0051173 | positive regulation of nitrogen compound metabolic process | 3.7E-03 |
| 472 | 9.88E-04 | 5 | 17 | GO:0000387 | spliceosomal snRNP assembly | 5.1E-03 |
| 516 | 1.08E-03 | 3 | 14 | GO:0000079;GO:1904029 | regulation of cyclin-dependent protein serine/threonine kinase activity | 5.4E-03 |
| 354 | 1.30E-03 | 3 | 29 | GO:0000956;GO:0006402;GO:0006401 | RNA catabolic process | 6.4E-03 |
| -443 | 1.48E-03 | 3 | 18 | GO:0001736;GO:0007164 | establishment of planar polarity | 7.1E-03 |
| -321 | 1.87E-03 | 2 | 33 | GO:0016477;GO:0048870;GO:0040011;GO:0001764 | locomotion | 8.8E-03 |
| -186 | 2.05E-03 | 2 | 100 | GO:0007017 | microtubule-based process | 9.4E-03 |
| -203 | 2.10E-03 | 2 | 83 | GO:0048646 | anatomical structure formation involved in morphogenesis | 9.5E-03 |
| -347 | 2.34E-03 | 5 | 27 | GO:0000187;GO:0043406;GO:0043410;GO:0071902; GO:1902533;GO:0009967;GO:0010647;GO:0023056 | positive regulation of signaling | 1.0E-02 |
| -787 | 2.85E-03 | 3 | 5 | GO:0003382 | epithelial cell morphogenesis | 1.2E-02 |
| -712 | 3.10E-03 | 4 | 6 | GO:0001659;GO:0048871 | multicellular organismal homeostasis | 1.3E-02 |
| 520 | 3.47E-03 | 2 | 11 | GO:0006400 | tRNA modification | 1.4E-02 |
| -311 | 3.52E-03 | 5 | 31 | GO:0032147;GO:0045860;GO:0033674;GO:0051347; GO:0043085;GO:0044093 | positive regulation of protein kinase activity | 1.4E-02 |
| -458 | 5.14E-03 | 2 | 13 | GO:0000904 | cell morphogenesis involved in differentiation | 2.1E-02 |
| -476 | 5.25E-03 | 2 | 12 | GO:0009887 | animal organ morphogenesis | 2.1E-02 |
| -118 | 5.69E-03 | 3 | 211 | GO:0000122;GO:0006357;GO:0045892;GO:0006355; GO:1903506;GO:2000112;GO:2001141;GO:0010556; GO:0031326;GO:0009889;GO:1903507;GO:2000113; GO:1902679;GO:0010558;GO:0031327;GO:0051253; GO:0009890;GO:0045934;GO:0051172 | negative regulation of nitrogen compound metabolic process | 2.2E-02 |
| -334 | 5.71E-03 | 2 | 24 | GO:0001501;GO:0048731 | system development | 2.2E-02 |

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| -216 | 5.90E-03 | 2 | 58 | GO:0048583 | regulation of response to stimulus | 2.2E-02 |
| 443 | 6.88E-03 | 2 | 13 | GO:0018130;GO:0019438;GO:1901362 | heterocycle biosynthetic process | 2.5E-02 |
| -480 | 7.06E-03 | 1 | 11 | GO:0007610 | behavior | 2.6E-02 |
| 644 | 7.48E-03 | 2 | 6 | GO:0001731 | formation of translation preinitiation complex | 2.7E-02 |
| 382 | 7.67E-03 | 5 | 17 | GO:0000723;GO:0032200;GO:0060249 | anatomical structure homeostasis | 2.7E-02 |
| 510 | 9.57E-03 | 2 | 9 | GO:0001510;GO:0043414;GO:0032259 | macromolecule methylation | 3.3E-02 |
| 510 | 9.52E-03 | 3 | 9 | GO:0034654 | nucleobase-containing compound biosynthetic process | 3.3E-02 |
| 449 | 1.17E-02 | 6 | 11 | GO:0000054;GO:0033750;GO:0071428;GO:0071426;GO:0006405;GO:0006611;GO:0031503;GO:0050658;GO:0051168;GO:0050657;GO:0051236;GO:0006913;GO:0015931;GO:0051169 | nucleocytoplasmic transport | 3.9E-02 |
| 446 | 1.22E-02 | 3 | 11 | GO:0018193;GO:0000413;GO:0018208 | peptidyl-amino acid modification | 4.0E-02 |
| -368 | 1.27E-02 | 3 | 16 | GO:0000272;GO:0005976;GO:0016052;GO:0005975 | carbohydrate metabolic process | 4.1E-02 |
| 126 | 1.26E-02 | 2 | 145 | GO:0006996 | organelle organization | 4.1E-02 |
| 651 | 1.36E-02 | 2 | 5 | GO:0000491 | small nucleolar ribonucleoprotein complex assembly | 4.3E-02 |
| 513 | 1.39E-02 | 4 | 8 | GO:0002098;GO:0002097 | tRNA wobble base modification | 4.3E-02 |
| -548 | 1.39E-02 | 2 | 7 | GO:0003013 | circulatory system process | 4.3E-02 |
| 316 | 1.43E-02 | 8 | 21 | GO:0000184 | nuclear-transcribed mRNA catabolic process nonsense-mediated decay | 4.3E-02 |
| 588 | 1.46E-02 | 3 | 6 | GO:0001522 | pseudouridine synthesis | 4.3E-02 |
| 230 | 1.45E-02 | 2 | 40 | GO:0034655;GO:0044265;GO:0019439;GO:0044270;GO:0046700;GO:1901361 | cellular macromolecule catabolic process | 4.3E-02 |
| 123 | 1.57E-02 | 2 | 143 | GO:0006464;GO:0036211;GO:0043412 | macromolecule modification | 4.6E-02 |
| 434 | 2.00E-02 | 3 | 10 | GO:0000245 | spliceosomal complex assembly | 5.5E-02 |
| 487 | 1.96E-02 | 2 | 8 | GO:0000479;GO:0000478;GO:0000469;GO:0090502;GO:0090501 | cleavage involved in rRNA processing | 5.5E-02 |
| -214 | 1.95E-02 | 2 | 42 | GO:0048584 | positive regulation of response to stimulus | 5.5E-02 |
| -184 | 2.00E-02 | 2 | 57 | GO:0048869 | cellular developmental process | 5.5E-02 |
| -197 | 2.05E-02 | 1 | 49 | GO:0002376 | immune system process | 5.6E-02 |
| 515 | 2.08E-02 | 3 | 7 | GO:0000154 | rRNA modification | 5.6E-02 |
| 539 | 2.51E-02 | 3 | 6 | GO:0000002 | mitochondrial genome maintenance | 6.7E-02 |
| -144 | 2.80E-02 | 4 | 84 | GO:0001932;GO:0031399;GO:0042325;GO:0032268;GO:0019220;GO:0051246;GO:0051174 | regulation of phosphorylation | 7.4E-02 |
| 403 | 3.08E-02 | 3 | 10 | GO:0000266;GO:0048285 | organelle fission | 7.9E-02 |
| 208 | 3.07E-02 | 1 | 38 | GO:0022414 | reproductive process | 7.9E-02 |

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| -113 | 3.16E-02 | 2 | 135 | GO:0051234;GO:0051179;GO:0006810 | localization | 8.0E-02 |
| 170 | 3.44E-02 | 2 | 55 | GO:0044249;GO:1901576;GO:0009058 | biosynthetic process | 8.6E-02 |
| 193 | 3.56E-02 | 3 | 42 | GO:0034645;GO:0009059;GO:0044271 | cellular nitrogen compound biosynthetic process | 8.8E-02 |
| -195 | 4.36E-02 | 2 | 38 | GO:0030154 | cell differentiation | 1.1E-01 |
| -465 | 5.33E-02 | 3 | 6 | GO:0030030 | cell projection organization | 1.3E-01 |
| -456 | 5.85E-02 | 5 | 6 | GO:0003429;GO:0003414;GO:0003422;GO:0090171;GO:0060536 | growth plate cartilage chondrocyte morphogenesis | 1.4E-01 |
| 238 | 5.95E-02 | 5 | 22 | GO:0000381;GO:0048024;GO:0043484;GO:0050684;GO:1903311 | regulation of mRNA processing | 1.4E-01 |
| -496 | 6.02E-02 | 3 | 5 | GO:0001919 | regulation of receptor recycling | 1.4E-01 |
| 350 | 6.08E-02 | 3 | 10 | GO:0006959;GO:0001867;GO:0006956;GO:0045087;GO:0098542 | defense response to other organism | 1.4E-01 |
| -387 | 6.39E-02 | 2 | 8 | GO:0007155;GO:0022610 | cell adhesion | 1.5E-01 |
| 408 | 6.71E-02 | 2 | 7 | GO:0000338;GO:0070646 | protein modification by small protein removal | 1.5E-01 |
| 178 | 6.88E-02 | 2 | 37 | GO:0044281 | small molecule metabolic process | 1.6E-01 |
| -260 | 6.96E-02 | 3 | 17 | GO:0001649 | osteoblast differentiation | 1.6E-01 |
| -421 | 8.01E-02 | 3 | 6 | GO:0002092;GO:0048260;GO:0045807;GO:0051130 | positive regulation of cellular component organization | 1.8E-01 |
| -210 | 8.18E-02 | 2 | 24 | GO:0001503 | ossification | 1.8E-01 |
| -82 | 8.34E-02 | 2 | 167 | GO:0048856 | anatomical structure development | 1.8E-01 |
| -387 | 8.28E-02 | 2 | 7 | GO:0050877 | nervous system process | 1.8E-01 |
| 291 | 8.79E-02 | 2 | 12 | GO:0090305 | nucleic acid phosphodiester bond hydrolysis | 1.9E-01 |
| 379 | 8.93E-02 | 5 | 7 | GO:0000050;GO:0019627;GO:0071941 | urea cycle | 1.9E-01 |
| -408 | 9.03E-02 | 2 | 6 | GO:0006633;GO:0008610;GO:0072330 | lipid biosynthetic process | 1.9E-01 |
| -347 | 9.58E-02 | 5 | 8 | GO:0003333;GO:0006865;GO:1905039;GO:0046942;GO:0098656;GO:1903825;GO:0015711;GO:0015849;GO:0006820;GO:0034220;GO:0055085 | organic acid transport | 2.0E-01 |
| -144 | 9.65E-02 | 4 | 47 | GO:0006811 | ion transport | 2.0E-01 |
| 118 | 9.84E-02 | 2 | 71 | GO:1901575;GO:0009056;GO:0044248 | catabolic process | 2.0E-01 |
| -134 | 1.04E-01 | 2 | 52 | GO:0007165 | signal transduction | 2.1E-01 |
| -255 | 1.06E-01 | 4 | 14 | GO:0002831;GO:0031347;GO:0032101;GO:0080134 | regulation of response to external stimulus | 2.1E-01 |
| 154 | 1.09E-01 | 4 | 38 | GO:0001701;GO:0043009;GO:0009792;GO:0009790 | embryo development ending in birth or egg hatching | 2.2E-01 |
| -214 | 1.25E-01 | 2 | 18 | GO:0048598 | embryonic morphogenesis | 2.5E-01 |
| -111 | 1.28E-01 | 2 | 67 | GO:0000226;GO:0007010 | microtubule cytoskeleton organization | 2.5E-01 |
| 184 | 1.27E-01 | 2 | 24 | GO:0051656;GO:0051640 | organelle localization | 2.5E-01 |

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| -204 | 1.33E-01 | 7 | 19 | GO:0001933;GO:0031400;GO:0042326;GO:0032269; GO:0051248;GO:0045936;GO:0010563 | negative regulation of phosphate metabolic process | 2.5E-01 |
| -247 | 1.32E-01 | 2 | 13 | GO:0002250 | adaptive immune response | 2.5E-01 |
| 110 | 1.30E-01 | 4 | 68 | GO:0044772;GO:0044770 | mitotic cell cycle phase transition | 2.5E-01 |
| 76 | 1.33E-01 | 1 | 144 | GO:0050896;GO:0006950 | response to stimulus | 2.5E-01 |
| 389 | 1.40E-01 | 2 | 5 | GO:0006520 | cellular amino acid metabolic process | 2.6E-01 |
| -263 | 1.39E-01 | 2 | 11 | GO:0048585 | negative regulation of response to stimulus | 2.6E-01 |
| -354 | 1.42E-01 | 4 | 6 | GO:0003351;GO:0006858;GO:0099111 | extracellular transport | 2.6E-01 |
| -164 | 1.51E-01 | 2 | 27 | GO:0000902 | cell morphogenesis | 2.7E-01 |
| -280 | 1.55E-01 | 4 | 9 | GO:0002576;GO:0045055;GO:0006887 | exocytosis | 2.8E-01 |
| 370 | 1.60E-01 | 2 | 5 | GO:0048609 | multicellular organismal reproductive process | 2.8E-01 |
| -287 | 1.69E-01 | 3 | 8 | GO:0001504;GO:0006836;GO:0098657 | neurotransmitter transport | 3.0E-01 |
| 361 | 1.71E-01 | 2 | 5 | GO:0000012 | single strand break repair | 3.0E-01 |
| -248 | 1.84E-01 | 2 | 10 | GO:0001568 | blood vessel development | 3.1E-01 |
| -350 | 1.84E-01 | 2 | 5 | GO:0001667 | ameboidal-type cell migration | 3.1E-01 |
| -320 | 1.84E-01 | 2 | 6 | GO:0001775;GO:0002263 | cell activation | 3.1E-01 |
| -275 | 1.88E-01 | 5 | 8 | GO:0002833;GO:0032103 | positive regulation of response to external stimulus | 3.2E-01 |
| -292 | 1.90E-01 | 2 | 7 | GO:0051050 | positive regulation of transport | 3.2E-01 |
| -157 | 1.95E-01 | 2 | 24 | GO:0000045;GO:0070925;GO:1905037;GO:0007033 | organelle assembly | 3.2E-01 |
| -152 | 2.01E-01 | 4 | 25 | GO:0051128 | regulation of cellular component organization | 3.3E-01 |
| -248 | 2.07E-01 | 4 | 9 | GO:0000188;GO:0043407;GO:0043409;GO:0071901; GO:1902532;GO:0006469;GO:0033673;GO:0051348; GO:0043086;GO:0044092;GO:0009968;GO:0010648; GO:0023057 | negative regulation of signaling | 3.4E-01 |
| 60 | 2.07E-01 | 4 | 169 | GO:0044267;GO:0019538;GO:1901564 | organonitrogen compound metabolic process | 3.4E-01 |
| 300 | 2.13E-01 | 3 | 6 | GO:0046395;GO:0016054;GO:0044282 | organic acid catabolic process | 3.4E-01 |
| -93 | 2.23E-01 | 2 | 61 | GO:0065008 | regulation of biological quality | 3.6E-01 |
| 269 | 2.28E-01 | 6 | 7 | GO:0000028 | ribosomal small subunit assembly | 3.6E-01 |
| -143 | 2.28E-01 | 4 | 25 | GO:0001822 | kidney development | 3.6E-01 |
| 113 | 2.34E-01 | 2 | 39 | GO:0043604;GO:1901566;GO:0043603 | organonitrogen compound biosynthetic process | 3.7E-01 |
| -129 | 2.40E-01 | 3 | 29 | GO:0001666;GO:0036293;GO:0070482;GO:0009628 | response to hypoxia | 3.7E-01 |
| -154 | 2.43E-01 | 2 | 20 | GO:0006814;GO:0015672 | monovalent inorganic cation transport | 3.7E-01 |
| 97 | 2.43E-01 | 2 | 52 | GO:0007275 | multicellular organism development | 3.7E-01 |
| -220 | 2.38E-01 | 2 | 10 | GO:0048468 | cell development | 3.7E-01 |
| -260 | 2.44E-01 | 2 | 7 | GO:0051606 | detection of stimulus | 3.7E-01 |
| -274 | 2.55E-01 | 2 | 6 | GO:0002064 | epithelial cell development | 3.8E-01 |

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|---|---|---|---|---|---|---|
| 221 | 2.62E-01 | 4 | 9 | GO:0001889;GO:0048732 | liver development | 3.9E-01 |
| -231 | 2.69E-01 | 7 | 8 | GO:0000186 | activation of MAPKK activity | 4.0E-01 |
| 217 | 2.70E-01 | 2 | 9 | GO:0000244 | spliceosomal tri-snRNP complex assembly | 4.0E-01 |
| 212 | 2.80E-01 | 2 | 9 | GO:0000077 | DNA damage checkpoint | 4.0E-01 |
| 241 | 2.81E-01 | 4 | 7 | GO:0001507;GO:0006581;GO:0008291;GO:0042135; GO:0042133;GO:1900619 | acetylcholine metabolic process | 4.0E-01 |
| -101 | 2.79E-01 | 2 | 41 | GO:0015031;GO:0015833;GO:0045184;GO:0042886; GO:0071705;GO:0008104;GO:0033036 | nitrogen compound transport | 4.0E-01 |
| -88 | 2.77E-01 | 4 | 54 | GO:0071702 | organic substance transport | 4.0E-01 |
| -278 | 2.92E-01 | 2 | 5 | GO:0001541;GO:0048608 | ovarian follicle development | 4.1E-01 |
| -197 | 2.91E-01 | 2 | 10 | GO:0001558;GO:0040008 | regulation of cell growth | 4.1E-01 |
| 140 | 2.90E-01 | 3 | 20 | GO:0006886;GO:0046907 | intracellular transport | 4.1E-01 |
| -152 | 2.91E-01 | 3 | 17 | GO:0032879 | regulation of localization | 4.1E-01 |
| -97 | 2.99E-01 | 2 | 41 | GO:0044255;GO:0006629 | cellular lipid metabolic process | 4.1E-01 |
| 270 | 3.06E-01 | 3 | 5 | GO:1901565 | organonitrogen compound catabolic process | 4.2E-01 |
| -265 | 3.16E-01 | 2 | 5 | GO:0003014 | renal system process | 4.3E-01 |
| 241 | 3.18E-01 | 2 | 6 | GO:0000038 | very long-chain fatty acid metabolic process | 4.3E-01 |
| 222 | 3.20E-01 | 2 | 7 | GO:0003002;GO:0007389 | pattern specification process | 4.3E-01 |
| -220 | 3.24E-01 | 4 | 7 | GO:0001816;GO:0010467 | gene expression | 4.3E-01 |
| -81 | 3.24E-01 | 6 | 53 | GO:0045859;GO:0043549;GO:0051338;GO:0050790; GO:0065009;GO:0071900 | regulation of kinase activity | 4.3E-01 |
| -170 | 3.38E-01 | 2 | 11 | GO:0051239 | regulation of multicellular organismal process | 4.5E-01 |
| -250 | 3.42E-01 | 5 | 5 | GO:0031349 | positive regulation of defense response | 4.5E-01 |
| -135 | 3.46E-01 | 3 | 17 | GO:0001523;GO:0016101;GO:0006721;GO:0006720 | terpenoid metabolic process | 4.6E-01 |
| 125 | 3.82E-01 | 2 | 17 | GO:0006508 | proteolysis | 5.0E-01 |
| -97 | 3.87E-01 | 2 | 28 | GO:0002682 | regulation of immune system process | 5.0E-01 |
| -99 | 4.11E-01 | 2 | 24 | GO:0006955 | immune response | 5.3E-01 |
| -181 | 4.18E-01 | 2 | 7 | GO:0008016;GO:1903522;GO:0044057 | regulation of blood circulation | 5.4E-01 |
| -208 | 4.31E-01 | 6 | 5 | GO:0001676 | long-chain fatty acid metabolic process | 5.5E-01 |
| 145 | 4.39E-01 | 4 | 10 | GO:0001843;GO:0060606;GO:0035148 | tube formation | 5.6E-01 |
| 83 | 4.44E-01 | 3 | 30 | GO:0042592 | homeostatic process | 5.6E-01 |
| -132 | 4.58E-01 | 3 | 11 | GO:0003143;GO:0060562 | embryonic heart tube morphogenesis | 5.8E-01 |
| -96 | 4.69E-01 | 2 | 20 | GO:0002252 | immune effector process | 5.9E-01 |
| 95 | 4.72E-01 | 6 | 20 | GO:0000027 | ribosomal large subunit assembly | 5.9E-01 |
| -64 | 4.77E-01 | 2 | 43 | GO:0000086;GO:0044839 | G2/M transition of mitotic cell cycle | 5.9E-01 |
| -171 | 4.77E-01 | 5 | 6 | GO:0002224;GO:0002221 | toll-like receptor signaling pathway | 5.9E-01 |
| -110 | 4.87E-01 | 2 | 14 | GO:0000302;GO:0006979;GO:1901700;GO:0042221 | response to chemical | 6.0E-01 |

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|---|---|---|---|---|---|---|
| -82 | 4.99E-01 | 2 | 24 | GO:0002684;GO:0050776;GO:0050778 | regulation of immune response | 6.0E-01 |
| -115 | 4.99E-01 | 4 | 12 | GO:0002697 | regulation of immune effector process | 6.0E-01 |
| 121 | 4.97E-01 | 3 | 11 | GO:0006486;GO:0043413;GO:0070085 | protein glycosylation | 6.0E-01 |
| 109 | 5.04E-01 | 2 | 13 | GO:0009605;GO:0051707;GO:0043207;GO:0044419; GO:0009607 | response to external stimulus | 6.0E-01 |
| -148 | 5.08E-01 | 4 | 7 | GO:0002443;GO:0002449;GO:0002460 | adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains | 6.0E-01 |
| 166 | 5.30E-01 | 6 | 5 | GO:0000722;GO:0006312 | telomere maintenance via recombination | 6.2E-01 |
| 167 | 5.28E-01 | 3 | 5 | GO:0002091;GO:0048261;GO:0045806;GO:0051051; GO:0051129 | negative regulation of receptor internalization | 6.2E-01 |
| -101 | 5.53E-01 | 3 | 12 | GO:0002090;GO:0048259;GO:0030100;GO:0060627; GO:0051049 | regulation of transport | 6.4E-01 |
| -145 | 5.48E-01 | 2 | 6 | GO:0007166 | cell surface receptor signaling pathway | 6.4E-01 |
| -68 | 5.53E-01 | 6 | 27 | GO:0030001;GO:0006812 | metal ion transport | 6.4E-01 |
| -95 | 5.95E-01 | 2 | 11 | GO:0002931 | response to ischemia | 6.8E-01 |
| 42 | 6.10E-01 | 4 | 53 | GO:0009057 | macromolecule catabolic process | 6.9E-01 |
| -46 | 6.09E-01 | 2 | 43 | GO:0048513 | animal organ development | 6.9E-01 |
| -81 | 6.23E-01 | 3 | 13 | GO:0016192;GO:0032940;GO:0046903;GO:0140352 | vesicle-mediated transport | 7.1E-01 |
| -103 | 6.45E-01 | 2 | 7 | GO:0051603;GO:0006511;GO:0019941;GO:0043632 | proteolysis involved in cellular protein catabolic process | 7.3E-01 |
| -33 | 6.56E-01 | 6 | 66 | GO:0000209;GO:0016567;GO:0032446;GO:0070647 | protein modification by small protein conjugation or removal | 7.3E-01 |
| -98 | 6.60E-01 | 3 | 7 | GO:0002164;GO:0009791 | post-embryonic development | 7.3E-01 |
| -117 | 6.57E-01 | 2 | 5 | GO:0008283 | cell population proliferation | 7.3E-01 |
| 55 | 7.17E-01 | 2 | 15 | GO:0006952 | defense response | 7.9E-01 |
| -51 | 7.39E-01 | 3 | 15 | GO:0001505 | regulation of neurotransmitter levels | 8.1E-01 |
| 70 | 7.39E-01 | 4 | 8 | GO:0001654;GO:0007423 | eye development | 8.1E-01 |
| -56 | 7.33E-01 | 5 | 13 | GO:0006869 | lipid transport | 8.1E-01 |
| 71 | 0.7504828 | 2 | 7 | GO:0001947 | heart looping | 8.1E-01 |
| 36 | 7.64E-01 | 5 | 25 | GO:0006631;GO:0032787;GO:0019752;GO:0043436; GO:0006082 | carboxylic acid metabolic process | 8.2E-01 |
| -58 | 7.68E-01 | 2 | 9 | GO:0044283;GO:0046394;GO:0016053 | small molecule biosynthetic process | 8.2E-01 |
| 47 | 8.00E-01 | 5 | 10 | GO:0001936;GO:0050678;GO:0042127 | regulation of cell population proliferation | 8.5E-01 |
| 37 | 8.19E-01 | 6 | 13 | GO:0000132;GO:0040001;GO:0051294;GO:0051293; GO:1902850;GO:0051653 | establishment of mitotic spindle localization | 8.7E-01 |
| 41 | 8.35E-01 | 3 | 9 | GO:0002244 | hematopoietic progenitor cell differentiation | 8.8E-01 |
| 41 | 8.44E-01 | 2 | 8 | GO:0022412 | cellular process involved in reproduction in multicellular organism | 8.9E-01 |
| 38 | 8.73E-01 | 2 | 6 | GO:0001825 | blastocyst formation | 9.1E-01 |
| 24 | 8.70E-01 | 2 | 16 | GO:0003006 | developmental process involved in reproduction | 9.1E-01 |
| -17 | 8.82E-01 | 5 | 27 | GO:0002181;GO:0006412;GO:0043043;GO:0006518 | peptide metabolic process | 9.1E-01 |
| -34 | 8.87E-01 | 2 | 6 | GO:0002699 | positive regulation of immune effector process | 9.2E-01 |
| 32 | 9.05E-01 | 3 | 5 | GO:0034613;GO:0070727 | cellular macromolecule localization | 9.3E-01 |
| 7 | 9.37E-01 | 3 | 44 | GO:0051649;GO:0051641 | establishment of localization in cell | 9.6E-01 |
| -9 | 9.47E-01 | 4 | 21 | GO:0000281;GO:0061640;GO:0000910 | cytoskeleton-dependent cytokinesis | 9.6E-01 |
| -15 | 9.55E-01 | 2 | 5 | GO:0002700 | regulation of production of molecular mediator of immune response | 9.7E-01 |
| 3 | 9.78E-01 | 6 | 34 | GO:0000165;GO:0023014;GO:0035556;GO:0006468; GO:0016310;GO:0006796;GO:0006793 | phosphorus metabolic process | 9.9E-01 |
| 6 | 9.81E-01 | 2 | 5 | GO:0030258 | lipid modification | 9.9E-01 |
| 0 | 1.00E+00 | 3 | 13 | GO:0002253 | activation of immune response | 1.0E+00 |

## Day 10

| Delta.rank | pval | Level | # seqs | GO Term | GO Name | p.adj |
|---|---|---|---|---|---|---|
| 441 | 9.8E-06 | 2 | 20 | GO:0000724;GO:0000725;GO:0006302;GO:0006310 | DNA recombination | 0.0 |
| 315 | 9.4E-06 | 2 | 40 | GO:0006259 | DNA metabolic process | 0.0 |
| 518 | 5.5E-05 | 2 | 12 | GO:1903046 | meiotic cell cycle process | 0.0 |
| 354 | 2.0E-04 | 5 | 22 | GO:0006281 | DNA repair | 0.0 |
| 630 | 1.8E-04 | 2 | 7 | GO:0061982 | meiosis I cell cycle process | 0.0 |
| 291 | 3.7E-04 | 4 | 30 | GO:0006974;GO:0033554;GO:0051716 | cellular response to DNA damage stimulus | 0.0 |
| -322 | 5.6E-04 | 2 | 23 | GO:0003008 | system process | 0.0 |
| 157 | 5.6E-04 | 3 | 102 | GO:1903047;GO:0022402 | cell cycle process | 0.0 |
| 325 | 6.5E-04 | 5 | 22 | GO:0051276 | chromosome organization | 0.0 |
| 431 | 7.9E-04 | 2 | 12 | GO:0002224;GO:0002221 | pattern recognition receptor signaling pathway | 0.0 |
| 277 | 1.0E-03 | 2 | 28 | GO:0051726 | regulation of cell cycle | 0.0 |
| 237 | 1.5E-03 | 2 | 36 | GO:0000278;GO:0007049 | cell cycle | 0.0 |
| -337 | 3.4E-03 | 2 | 15 | GO:0001763 | morphogenesis of a branching structure | 0.1 |
| 268 | 4.0E-03 | 2 | 23 | GO:0000082;GO:0044843 | G1/S transition of mitotic cell cycle | 0.1 |
| 393 | 5.2E-03 | 7 | 10 | GO:0000076 | DNA replication checkpoint | 0.1 |
| -434 | 5.8E-03 | 4 | 8 | GO:0003333;GO:1905039;GO:0098656;<br>GO:1903825;GO:0034220;GO:0055085 | organic acid transmembrane transport | 0.1 |
| -286 | 6.5E-03 | 3 | 18 | GO:0048731 | system development | 0.1 |
| 453 | 7.0E-03 | 2 | 7 | GO:0000727 | double-strand break repair via break-induced replication | 0.1 |
| -344 | 7.4E-03 | 3 | 12 | GO:0001658;GO:0048754;GO:0061138 | branching morphogenesis of an epithelial tube | 0.1 |
| -296 | 7.9E-03 | 3 | 16 | GO:0000272;GO:0005976;GO:0016052;GO:0005975 | carbohydrate metabolic process | 0.1 |
| 220 | 9.5E-03 | 1 | 28 | GO:0022414 | reproductive process | 0.1 |
| -111 | 9.7E-03 | 1 | 116 | GO:0032501 | multicellular organismal process | 0.1 |
| 518 | 9.2E-03 | 3 | 5 | GO:0034654 | nucleobase-containing compound biosynthetic process | 0.1 |
| -465 | 1.0E-02 | 2 | 6 | GO:0030030 | cell projection organization | 0.1 |
| -313 | 1.1E-02 | 2 | 13 | GO:0001501 | skeletal system development | 0.1 |
| -418 | 1.3E-02 | 3 | 7 | GO:0003013 | circulatory system process | 0.1 |
| -156 | 1.8E-02 | 2 | 47 | GO:0006928 | movement of cell or subcellular component | 0.1 |
| 296 | 1.7E-02 | 2 | 13 | GO:0006952 | defense response | 0.1 |
| 97 | 1.8E-02 | 2 | 129 | GO:0016070 | RNA metabolic process | 0.1 |
| 252 | 1.7E-02 | 3 | 18 | GO:0031570;GO:0000075;GO:0045786 | negative regulation of cell cycle | 0.1 |
| -207 | 2.1E-02 | 3 | 25 | GO:0016477;GO:0048870;GO:0040011 | locomotion | 0.1 |
| 317 | 2.4E-02 | 4 | 10 | GO:0000079;GO:1904029 | regulation of cyclin-dependent protein<br>serine/threonine kinase activity | 0.2 |
| -246 | 2.7E-02 | 2 | 16 | GO:0000027 | ribosomal large subunit assembly | 0.2 |
| 329 | 2.6E-02 | 2 | 9 | GO:0000070;GO:0000819;GO:0098813;GO:0007059 | mitotic sister chromatid segregation | 0.2 |
| 293 | 2.9E-02 | 7 | 11 | GO:0001732 | formation of cytoplasmic translation initiation complex | 0.2 |
| 291 | 3.0E-02 | 4 | 11 | GO:0006959;GO:0001867;GO:0006956;GO:0045087;<br>GO:0002253;GO:0098542 | activation of immune response | 0.2 |
| -221 | 3.6E-02 | 2 | 18 | GO:0001764 | neuron migration | 0.2 |
| 293 | 3.7E-02 | 2 | 10 | GO:0060249;GO:0000723;GO:0032200 | anatomical structure homeostasis | 0.2 |
| 189 | 3.8E-02 | 3 | 24 | GO:0000956;GO:0006402;GO:0006401 | RNA catabolic process | 0.2 |
| -110 | 4.1E-02 | 2 | 72 | GO:0009653 | anatomical structure morphogenesis | 0.2 |
| -282 | 4.5E-02 | 5 | 10 | GO:0006865;GO:0046942;GO:0015711;GO:0015849;<br>GO:0006820 | organic acid transport | 0.2 |
| -196 | 6.2E-02 | 3 | 18 | GO:0000045;GO:0070925;GO:1905037;GO:0007033 | organelle assembly | 0.3 |
| 131 | 5.7E-02 | 3 | 43 | GO:0001701;GO:0043009;GO:0009792;GO:0009790;<br>GO:0007275 | multicellular organism development | 0.3 |
| 102 | 6.0E-02 | 2 | 70 | GO:0016071 | mRNA metabolic process | 0.3 |
| -295 | 6.0E-02 | 4 | 8 | GO:0048585 | negative regulation of response to stimulus | 0.3 |
| -156 | 6.1E-02 | 2 | 29 | GO:0048729 | tissue morphogenesis | 0.3 |
| -369 | 6.3E-02 | 3 | 5 | GO:0048871 | multicellular organismal homeostasis | 0.3 |
| -177 | 7.7E-02 | 3 | 20 | GO:0002009;GO:0035239 | morphogenesis of an epithelium | 0.3 |
| -161 | 7.7E-02 | 6 | 24 | GO:0043405;GO:0043408;GO:1902531;GO:0009966;<br>GO:0010646;GO:0023051 | regulation of signaling | 0.3 |
| -293 | 8.1E-02 | 2 | 7 | GO:0050877 | nervous system process | 0.3 |
| 243 | 8.4E-02 | 2 | 10 | GO:0051707;GO:0043207;GO:0044419;GO:0009605;<br>GO:0009607 | response to external stimulus | 0.3 |
| -223 | 9.7E-02 | 4 | 11 | GO:0001736;GO:0007164 | establishment of planar polarity | 0.4 |
| 75 | 9.8E-02 | 2 | 102 | GO:0006996 | organelle organization | 0.4 |
| 174 | 1.2E-01 | 7 | 16 | GO:0000184 | nuclear-transcribed mRNA catabolic process<br>nonsense-mediated decay | 0.4 |
| -167 | 1.2E-01 | 2 | 17 | GO:0001503 | ossification | 0.4 |
| 146 | 1.3E-01 | 2 | 22 | GO:0002682 | regulation of immune system process | 0.4 |
| -148 | 1.2E-01 | 3 | 22 | GO:0003341;GO:0007018 | cilium movement | 0.4 |
| 194 | 1.2E-01 | 3 | 13 | GO:0007005 | mitochondrion organization | 0.4 |
| -118 | 1.2E-01 | 2 | 35 | GO:0044255;GO:0006629 | cellular lipid metabolic process | 0.4 |
| -143 | 1.2E-01 | 4 | 23 | GO:0071705 | nitrogen compound transport | 0.4 |
| -170 | 1.3E-01 | 3 | 16 | GO:0000187;GO:0043406;GO:0043410;GO:0071902;<br>GO:1902533;GO:0009967;GO:0010647;GO:0023056 | positive regulation of signaling | 0.4 |
| 223 | 1.3E-01 | 2 | 9 | GO:0018130;GO:0019438;GO:1901362 | heterocycle biosynthetic process | 0.4 |
| -171 | 1.5E-01 | 2 | 14 | GO:0001649 | osteoblast differentiation | 0.5 |
| 174 | 1.6E-01 | 7 | 13 | GO:0000387 | spliceosomal snRNP assembly | 0.5 |

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|---|---|---|---|---|---|---|
| 141 | 2.9E-01 | 2 | 11 | GO:0003006 | developmental process involved in reproduction | 0.7 |
| -202 | 3.1E-01 | 3 | 5 | GO:0007166 | cell surface receptor signaling pathway | 0.7 |
| -68 | 3.2E-01 | 2 | 44 | GO:0048869 | cellular developmental process | 0.7 |
| 143 | 3.3E-01 | 8 | 9 | GO:0000462;GO:0030490 | maturation of SSU-rRNA | 0.7 |
| -81 | 3.4E-01 | 2 | 28 | GO:0030154 | cell differentiation | 0.7 |
| -83 | 3.6E-01 | 2 | 25 | GO:0000086;GO:0044839 | G2/M transition of mitotic cell cycle | 0.7 |
| 131 | 3.5E-01 | 5 | 10 | GO:0000245 | spliceosomal complex assembly | 0.7 |
| 187 | 3.5E-01 | 3 | 5 | GO:0001731 | formation of translation preinitiation complex | 0.7 |
| 183 | 3.6E-01 | 5 | 5 | GO:0002699 | positive regulation of immune effector process | 0.7 |
| 176 | 3.8E-01 | 5 | 5 | GO:0002027 | regulation of heart rate | 0.7 |
| 43 | 3.7E-01 | 2 | 90 | GO:0006464;GO:0036211;GO:0043412 | macromolecule modification | 0.7 |
| -150 | 3.7E-01 | 1 | 7 | GO:0007610 | behavior | 0.7 |
| -93 | 4.1E-01 | 3 | 16 | GO:0000904 | cell morphogenesis involved in differentiation | 0.8 |
| 143 | 3.9E-01 | 2 | 7 | GO:0001510;GO:0043414;GO:0032259 | macromolecule methylation | 0.8 |
| 165 | 4.1E-01 | 3 | 5 | GO:0001654;GO:0007423 | eye development | 0.8 |
| -56 | 4.0E-01 | 4 | 47 | GO:0006811 | ion transport | 0.8 |
| -28 | 4.1E-01 | 6 | 199 | GO:0010629;GO:0000122;GO:0060337;GO:0043892; GO:0006355;GO:1903506;GO:2000112;GO:2001141; GO:0010556;GO:0031326;GO:0051252;GO:0009889; GO:0019219;GO:1903507;GO:2000113;GO:1902679; GO:0010558;GO:0031327;GO:0051253;GO:0009890; GO:0031324;GO:0045934;GO:0051172 | regulation of nucleobase-containing compound metabolic process | 0.8 |
| 33 | 4.1E-01 | 2 | 132 | GO:0048856 | anatomical structure development | 0.8 |
| -163 | 4.1E-01 | 2 | 5 | GO:0051050 | positive regulation of transport | 0.8 |
| 36 | 4.2E-01 | 2 | 106 | GO:0006950;GO:0050896 | response to stimulus | 0.8 |
| -106 | 4.3E-01 | 2 | 11 | GO:0051239 | regulation of multicellular organismal process | 0.8 |
| -86 | 4.5E-01 | 3 | 15 | GO:0001523;GO:0016101;GO:0006721;GO:0006720 | diterpenoid metabolic process | 0.8 |
| 109 | 4.6E-01 | 2 | 9 | GO:0001889;GO:0048732 | liver development | 0.8 |
| -132 | 4.7E-01 | 4 | 6 | GO:0001936;GO:0050678;GO:0042127 | regulation of cell population proliferation | 0.8 |
| -61 | 4.4E-01 | 1 | 32 | GO:0002376 | immune system process | 0.8 |
| 64 | 4.6E-01 | 3 | 27 | GO:0006364;GO:0016072 | rRNA processing | 0.8 |
| -94 | 4.6E-01 | 2 | 12 | GO:0032879 | regulation of localization | 0.8 |
| -84 | 4.6E-01 | 2 | 15 | GO:0042221;GO:0000302;GO:0006979;GO:1901700 | response to chemical | 0.8 |
| -52 | 4.7E-01 | 2 | 39 | GO:0044271;GO:1901566;GO:0043604;GO:0043603 | cellular nitrogen compound biosynthetic process | 0.8 |
| 56 | 4.7E-01 | 2 | 34 | GO:0044281 | small molecule metabolic process | 0.8 |
| -45 | 4.7E-01 | 2 | 53 | GO:0065008 | regulation of biological quality | 0.8 |
| 120 | 4.8E-01 | 3 | 7 | GO:0046395;GO:0016054;GO:0044282 | carboxylic acid catabolic process | 0.8 |

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|---|---|---|---|---|---|---|
| -103 | 1.6E-01 | 4 | 37 | GO:0071702 | organic substance transport | 0.5 |
| -117 | 1.7E-01 | 4 | 28 | GO:0002181;GO:0006412;GO:0034645;GO:0043043; GO:0009059;GO:0006518 | macromolecule biosynthetic process | 0.5 |
| 228 | 1.8E-01 | 6 | 7 | GO:0000054;GO:0033750;GO:0071428;GO:0071426; GO:0006405;GO:0006611;GO:0031503;GO:0050658; GO:0051168;GO:0050657;GO:0051236;GO:0006913; GO:0015931;GO:0051169 | nucleocytoplasmic transport | 0.5 |
| 214 | 1.7E-01 | 5 | 8 | GO:0000413;GO:0018208;GO:0018193 | peptidyl-amino acid modification | 0.5 |
| -245 | 1.8E-01 | 2 | 6 | GO:0001816;GO:0010467 | cytokine production | 0.5 |
| 162 | 1.7E-01 | 5 | 14 | GO:0009451 | RNA modification | 0.5 |
| 87 | 1.8E-01 | 3 | 48 | GO:0044772;GO:0044770 | cell cycle phase transition | 0.5 |
| -139 | 1.9E-01 | 6 | 18 | GO:0032147;GO:0045860;GO:0033674;GO:0051347; GO:0043085;GO:0044093 | activation of protein kinase activity | 0.5 |
| -136 | 2.0E-01 | 4 | 18 | GO:0001666;GO:0036293;GO:0070482;GO:0009628 | response to decreased oxygen levels | 0.5 |
| -98 | 2.1E-01 | 8 | 33 | GO:0001934;GO:0031401;GO:0042327;GO:0032270; GO:0045937;GO:0031325;GO:0051247;GO:0010562; GO:0009893;GO:0010604;GO:0051173 | positive regulation of metabolic process | 0.6 |
| 61 | 2.1E-01 | 2 | 89 | GO:0006396 | RNA processing | 0.6 |
| 208 | 2.2E-01 | 4 | 7 | GO:0001507;GO:0006581;GO:0008291;GO:0042135; GO:0042133;GO:1900619 | acetylcholine metabolic process | 0.6 |
| -167 | 2.1E-01 | 3 | 11 | GO:0002250 | adaptive immune response | 0.6 |
| -244 | 2.2E-01 | 2 | 5 | GO:0007155;GO:0022610 | cell adhesion | 0.6 |
| 126 | 2.3E-01 | 4 | 18 | GO:0002684;GO:0050776;GO:0050778 | regulation of immune response | 0.6 |
| -54 | 2.3E-01 | 3 | 104 | GO:0006810;GO:0051234;GO:0051179 | localization | 0.6 |
| -229 | 2.5E-01 | 3 | 5 | GO:0006308 | DNA catabolic process | 0.6 |
| 83 | 2.5E-01 | 2 | 39 | GO:0007165 | signal transduction | 0.6 |
| 82 | 2.7E-01 | 5 | 37 | GO:0034470;GO:0034660 | ncRNA metabolic process | 0.6 |
| 198 | 2.8E-01 | 4 | 6 | GO:0000050;GO:0019627;GO:0071941 | urea cycle | 0.6 |
| -212 | 2.9E-01 | 6 | 5 | GO:0000188;GO:0043407;GO:0043409;GO:0071901; GO:1902532;GO:0009968;GO:0010648;GO:0023057; GO:0006469;GO:0033673;GO:0051348;GO:0043086; GO:0044092 | negative regulation of intracellular signal transduction | 0.6 |
| 159 | 2.8E-01 | 3 | 9 | GO:0001568 | blood vessel development | 0.6 |
| 124 | 2.8E-01 | 2 | 15 | GO:0002252 | immune effector process | 0.6 |
| -69 | 2.8E-01 | 2 | 50 | GO:0009057 | macromolecule catabolic process | 0.6 |
| 81 | 2.7E-01 | 2 | 37 | GO:0034655;GO:0044265;GO:0019439;GO:0044270; GO:0046700;GO:1901361 | cellular macromolecule catabolic process | 0.6 |

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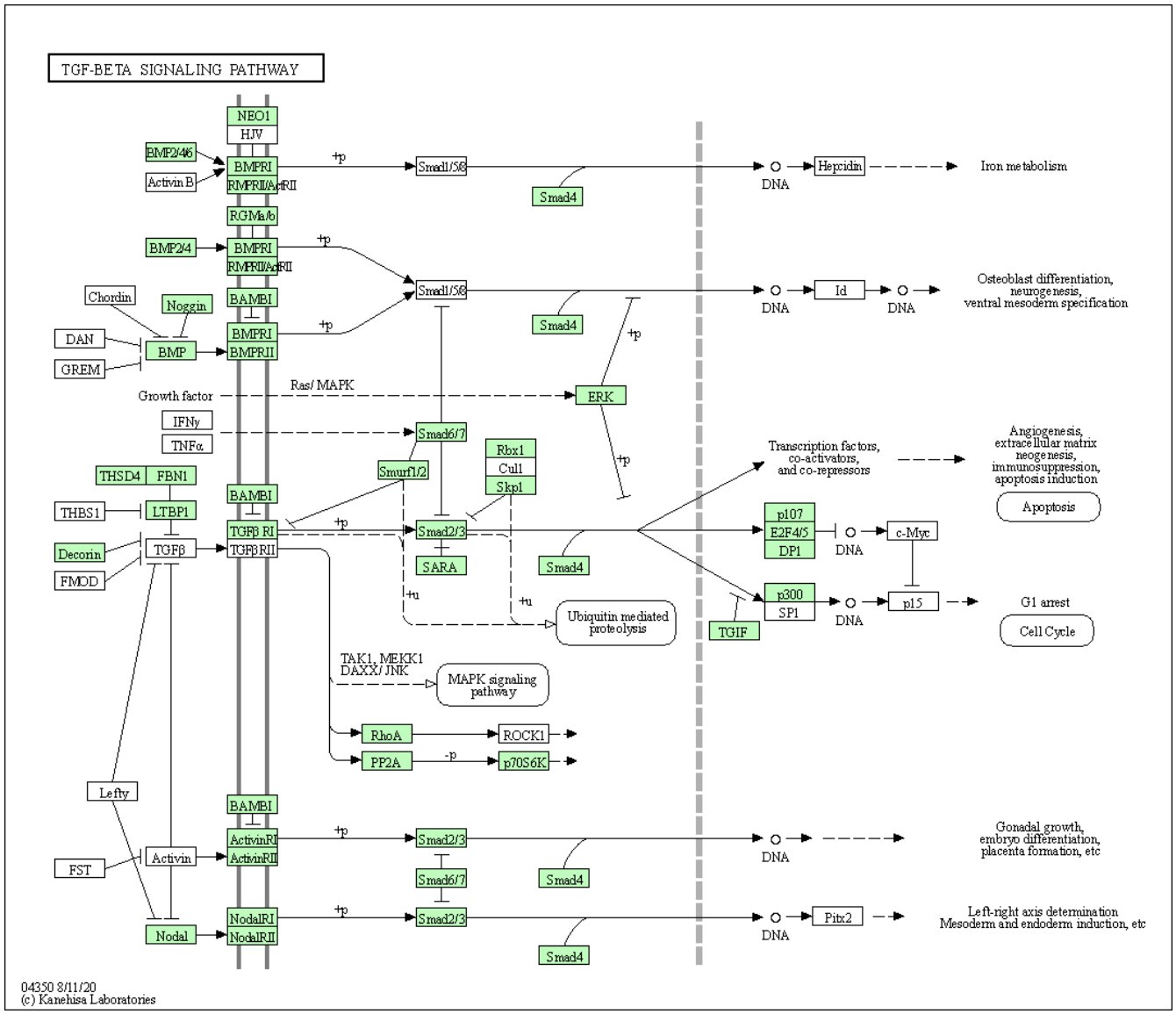
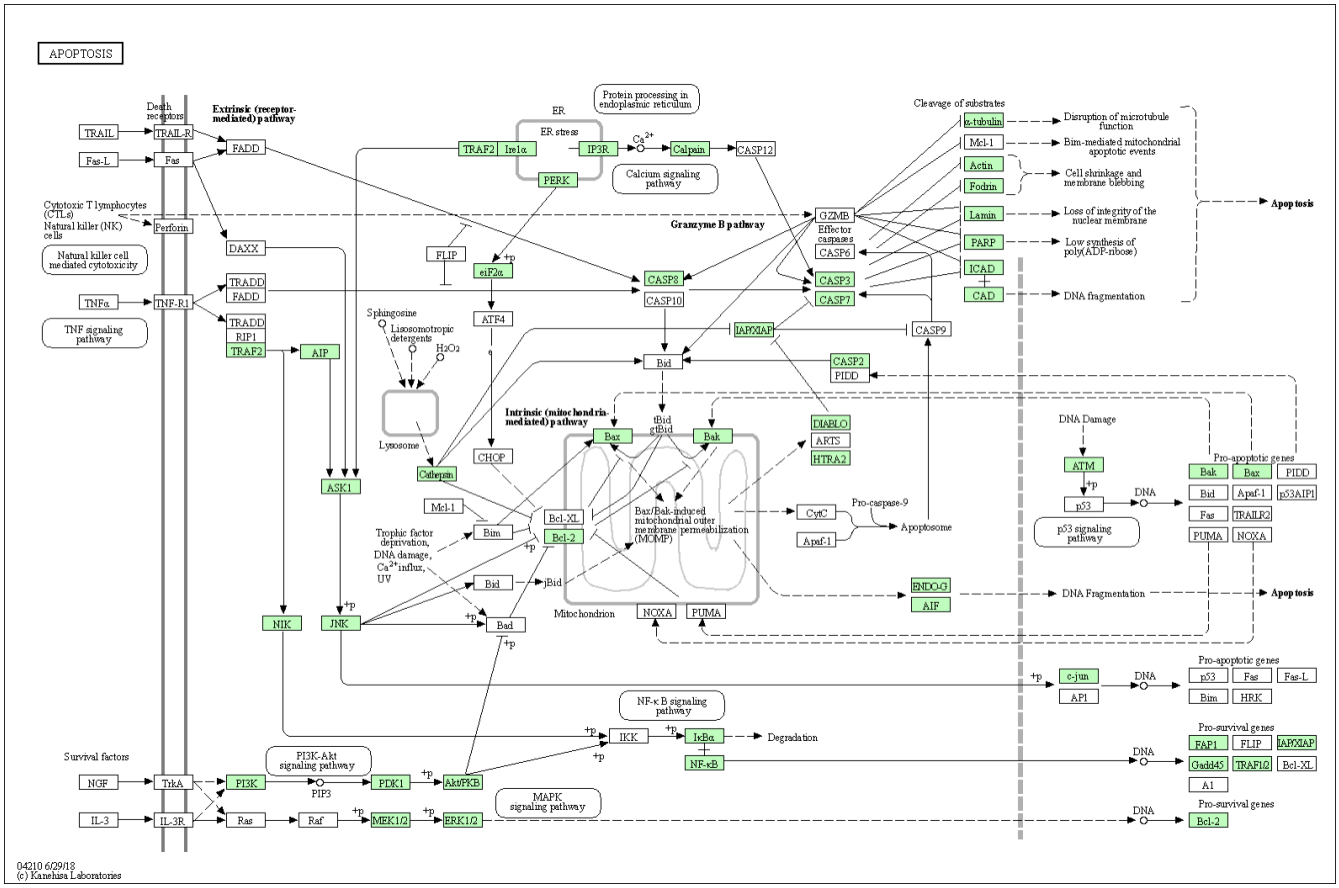
|---|---|---|---|---|---|---|
| -117 | 4.9E-01 | 3 | 7 | GO:0044283;GO:0046394;GO:0016053 | small molecule biosynthetic process | 0.8 |
| -114 | 5.0E-01 | 2 | 7 | GO:0055082;GO:0048878;GO:0019725 | chemical homeostasis | 0.8 |
| -122 | 5.0E-01 | 2 | 6 | GO:0002449;GO:0002460;GO:0002443 | adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains | 0.8 |
| 85 | 5.1E-01 | 4 | 12 | GO:0002697;GO:0002831 | regulation of response to biotic stimulus | 0.8 |
| 40 | 5.2E-01 | 3 | 53 | GO:0000398;GO:0000377;GO:0006397;GO:0000375;GO:0008380 | RNA splicing | 0.8 |
| -101 | 5.2E-01 | 2 | 8 | GO:0008016;GO:1903522;GO:0044057 | regulation of blood circulation | 0.8 |
| -63 | 5.2E-01 | 5 | 21 | GO:0030001;GO:0006812 | cation transport | 0.8 |
| -97 | 5.4E-01 | 3 | 8 | GO:0001504;GO:0006836;GO:0098657 | neurotransmitter transport | 0.8 |
| 88 | 5.3E-01 | 3 | 10 | GO:0048468 | cell development | 0.8 |
| 70 | 5.3E-01 | 3 | 16 | GO:0051656;GO:0051640 | establishment of organelle localization | 0.8 |
| 96 | 5.7E-01 | 2 | 7 | GO:0000244 | spliceosomal tri-snRNP complex assembly | 0.8 |
| 88 | 5.8E-01 | 6 | 8 | GO:0000266;GO:0048285 | organelle fission | 0.8 |
| -112 | 5.7E-01 | 2 | 5 | GO:0001678;GO:0042593;GO:0033500 | cellular glucose homeostasis | 0.8 |
| 107 | 5.6E-01 | 3 | 6 | GO:0003143;GO:0060562 | embryonic heart tube morphogenesis | 0.8 |
| -99 | 5.6E-01 | 5 | 7 | GO:0006511;GO:0019941;GO:0043632;GO:0051603 | modification-dependent protein catabolic process | 0.8 |
| 111 | 5.8E-01 | 2 | 5 | GO:0006520 | cellular amino acid metabolic process | 0.8 |
| 96 | 5.7E-01 | 2 | 7 | GO:0022412 | cellular process involved in reproduction in multicellular organism | 0.8 |
| -39 | 5.7E-01 | 2 | 43 | GO:0044249;GO:1901576;GO:0009058 | organic substance biosynthetic process | 0.8 |
| 51 | 5.4E-01 | 3 | 29 | GO:0048513 | animal organ development | 0.8 |
| 48 | 5.6E-01 | 2 | 30 | GO:0048584 | positive regulation of response to stimulus | 0.8 |
| 87 | 6.1E-01 | 3 | 7 | GO:0000077 | DNA damage checkpoint | 0.8 |
| -103 | 6.1E-01 | 3 | 5 | GO:0001825 | blastocyst formation | 0.8 |
| 76 | 6.1E-01 | 3 | 9 | GO:0002244 | hematopoietic progenitor cell differentiation | 0.8 |
| 83 | 6.0E-01 | 3 | 8 | GO:0008033;GO:0006399 | tRNA processing | 0.8 |
| -100 | 6.1E-01 | 3 | 5 | GO:0001945 | lymph vessel development | 0.8 |
| -79 | 6.4E-01 | 2 | 7 | GO:0002931 | response to ischemia | 0.9 |
| -64 | 6.3E-01 | 4 | 11 | GO:0003429;GO:0003414;GO:0003422;GO:0090171;GO:0060536;GO:0009887 | animal organ morphogenesis | 0.9 |
| -53 | 6.4E-01 | 3 | 16 | GO:0006814;GO:0015672 | monovalent inorganic cation transport | 0.9 |
| 45 | 6.6E-01 | 2 | 19 | GO:0000281;GO:0061640;GO:0000910 | mitotic cytokinesis | 0.9 |
| 66 | 7.2E-01 | 3 | 6 | GO:0000338;GO:0070646 | protein modification by small protein removal | 0.9 |
| -38 | 7.2E-01 | 6 | 18 | GO:0000381;GO:0048024;GO:0043484;GO:0050684;GO:1903311 | regulation of alternative mRNA splicing, via spliceosome | 0.9 |
| 77 | 7.0E-01 | 4 | 5 | GO:0000447 | endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) | 0.9 |
| 57 | 7.2E-01 | 2 | 8 | GO:0000479;GO:0000478;GO:0000469;GO:0090502;GO:0090501 | cleavage involved in rRNA processing | 0.9 |
| 42 | 7.2E-01 | 3 | 15 | GO:0001505 | regulation of neurotransmitter levels | 0.9 |
| 25 | 7.0E-01 | 2 | 50 | GO:0001525 | angiogenesis | 0.9 |
| 24 | 6.9E-01 | 3 | 55 | GO:0001932;GO:0031399;GO:0042325;GO:0032268;GO:0019220;GO:0051246;GO:0051174 | regulation of phosphorylation | 0.9 |
| 68 | 6.9E-01 | 3 | 7 | GO:0006486;GO:0043413;GO:0070085 | protein glycosylation | 0.9 |
| -36 | 7.0E-01 | 4 | 24 | GO:0006631;GO:0032787;GO:0019752;GO:0043436;GO:0006082 | oxoacid metabolic process | 0.9 |
| -80 | 6.9E-01 | 2 | 5 | GO:0009791 | post-embryonic development | 0.9 |
| 60 | 6.7E-01 | 2 | 10 | GO:0031347;GO:0032101;GO:0080134 | regulation of response to external stimulus | 0.9 |
| -16 | 7.1E-01 | 4 | 123 | GO:0044267;GO:0019538;GO:1901564 | organonitrogen compound metabolic process | 0.9 |
| 59 | 6.9E-01 | 2 | 9 | GO:0048598 | embryonic morphogenesis | 0.9 |
| -46 | 7.0E-01 | 2 | 14 | GO:0051128 | regulation of cellular component organization | 0.9 |
| -53 | 6.7E-01 | 4 | 13 | GO:0090305 | nucleic acid phosphodiester bond hydrolysis | 0.9 |
| 23 | 7.3E-01 | 4 | 48 | GO:0000226;GO:0007010 | microtubule cytoskeleton organization | 0.9 |
| 19 | 7.2E-01 | 2 | 71 | GO:0048646 | anatomical structure formation involved in morphogenesis | 0.9 |
| 33 | 7.3E-01 | 2 | 21 | GO:0006955 | immune response | 0.9 |
| -60 | 7.4E-01 | 5 | 6 | GO:0001561;GO:0009062;GO:0019395;GO:0044242;GO:0072329;GO:0034440;GO:0016042;GO:0030258;GO:0055114 | lipid modification | 0.9 |
| 33 | 7.4E-01 | 3 | 21 | GO:0042592 | homeostatic process | 0.9 |
| 64 | 7.5E-01 | 2 | 5 | GO:0006400 | tRNA modification | 0.9 |
| -53 | 7.7E-01 | 5 | 6 | GO:0000028 | ribosomal small subunit assembly | 0.9 |
| -51 | 7.8E-01 | 2 | 6 | GO:0000038 | very long-chain fatty acid metabolic process | 0.9 |
| -24 | 7.7E-01 | 2 | 29 | GO:0000902 | cell morphogenesis | 0.9 |
| -56 | 7.8E-01 | 4 | 5 | GO:0001667 | ameboidal-type cell migration | 0.9 |
| -34 | 7.8E-01 | 8 | 14 | GO:0001933;GO:0031400;GO:0042326;GO:0032269;GO:0045936;GO:0051248;GO:0010563 | negative regulation of protein phosphorylation | 0.9 |
| 59 | 7.7E-01 | 2 | 5 | GO:0002833 | positive regulation of response to biotic stimulus | 0.9 |
| -15 | 7.8E-01 | 2 | 72 | GO:0007017 | microtubule-based process | 0.9 |
| 29 | 7.9E-01 | 5 | 17 | GO:0015833;GO:0042886;GO:0008104;GO:0033036 | protein localization | 0.9 |
| -53 | 7.9E-01 | 3 | 5 | GO:0016192 | vesicle-mediated transport | 0.9 |
| 16 | 7.9E-01 | 2 | 55 | GO:0048522;GO:0048518 | positive regulation of biological process | 0.9 |
| 26 | 8.0E-01 | 5 | 20 | GO:0000165;GO:0023014;GO:0035556;GO:0006468;GO:0016310;GO:0006796;GO:0006793 | phosphorus metabolic process | 0.9 |
| -19 | 8.1E-01 | 2 | 32 | GO:0000209 | protein polyubiquitination | 0.9 |
| -32 | 8.1E-01 | 5 | 12 | GO:0006869 | lipid transport | 0.9 |
| 27 | 8.2E-01 | 2 | 14 | GO:0001822 | kidney development | 0.9 |
| 16 | 8.5E-01 | 2 | 27 | GO:0051641;GO:0051649 | establishment of localization in cell | 0.9 |
| 8 | 8.6E-01 | 3 | 88 | GO:0022618;GO:0034622;GO:0071826;GO:0065003;GO:0022607;GO:0043933 | cellular component assembly | 0.9 |
| 18 | 8.7E-01 | 2 | 15 | GO:0006508 | proteolysis | 0.9 |
| -12 | 8.8E-01 | 6 | 33 | GO:0045859;GO:0043549;GO:0051338;GO:0050790;GO:0065009;GO:0071900 | regulation of kinase activity | 0.9 |
| -20 | 8.9E-01 | 5 | 10 | GO:0000132;GO:0040001;GO:0051294;GO:0051293;GO:1902850;GO:0051653 | establishment of mitotic spindle orientation | 0.9 |
| -19 | 8.9E-01 | 2 | 10 | GO:0000463;GO:0000470 | maturation of LSU-rRNA | 0.9 |
| -23 | 9.1E-01 | 3 | 5 | GO:0003360 | brainstem development | 0.9 |
| -15 | 9.2E-01 | 2 | 8 | GO:0051606 | detection of stimulus | 1.0 |
| -11 | 9.3E-01 | 4 | 12 | GO:0006886;GO:0015031;GO:0046907;GO:0045184 | protein transport | 1.0 |
| 3 | 9.5E-01 | 2 | 69 | GO:0044248;GO:0009056;GO:1901575 | catabolic process | 1.0 |
| -5 | 9.8E-01 | 6 | 9 | GO:0002090;GO:0048259;GO:0030100;GO:0060627;GO:0051049 | regulation of transport | 1.0 |
| -2 | 9.7E-01 | 3 | 44 | GO:0016567;GO:0032446;GO:0070647 | protein modification by small protein conjugation or removal | 1.0 |
| 2 | 9.7E-01 | 3 | 44 | GO:0048583 | regulation of response to stimulus | 1.0 |
| 2 | 9.9E-01 | 2 | 6 | GO:0002064 | epithelial cell development | 1.0 |
| 1 | 1.0E+00 | 2 | 6 | GO:0035148;GO:0001843;GO:0060606 | tube formation | 1.0 |




**Supplementary Figure 1.** KEGG map of the TGF-beta signaling pathway. Genes highlighted in green were present in the analyzed transcriptome.




**Supplementary Figure 2.** KEGG map of apoptosis. Genes highlighted in green were present in the analyzed transcriptome.

## Unpublished data

# Starvation coupled with excessive light or darkness causes oxidative stress in *Berghia stephanieae* (Valdés, 2005).

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### Introduction

Reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{*}OH$), and hydrogen peroxide ($H_2O_2$) are naturally produced as byproducts of biochemical reactions, for instance in the mitochondria, chloroplasts, and the endoplasmic reticulum (Halliwell & Gutteridge, 2015). The imbalance between the production of ROS and the antioxidant defense activity is considered as the cause for oxidative stress in the cells (Richier et al., 2005). Starvation is thought to be responsible for increased ROS production leading to harmful cellular effects like lipid peroxidation, protein oxidation, and DNA degradation (Domenicali et al., 2001; Pascual et al., 2003; Lesser, 2006). Like starvation, abiotic factors such as light intensity can also induce oxidative stress, especially in photosymbiotic animals (Roth, 2014). Prolonged exposure to high light intensity can cause photodamage in the photosynthetic apparatus of the photobiont. Due to the prolonged high absorption of excitation energy, the reduction in photosynthetic electron transport, and the ongoing creation of ROS, photoinhibition may also result from light stress (Lesser, 2006). Likewise, darkness can induce photoinhibition, hypoxia, and endoplasmic reticulum stress (DeSalvo et al., 2012), and long-term darkness might even lead to telomere length shortening (Rouan et al., 2021). Dark stress yields an accumulation of ROS, dark-induced bleaching, cell death, and the breakdown of the photosymbiosis.

An accumulation of ROS induces the host enzymatic defense response to act and prevent the damaging effects of ROS via quenching (Richier et al., 2005; Lesser, 2006). Enzymes like superoxide dismutase (SOD), peroxidases, glutathione reductase, glutathione S-transferase, and catalase (CAT), and non-enzymatic compound like reduced glutathione (GSH) participate in the antioxidant defense. SODs are the first defense against oxidative damage converting toxic $O_2^{\bullet-}$

radicals into $H_2O_2$ (Fridovich, 1995; Chung, 2017). Catalases are responsible for the reduction of $H_2O_2$ into water (García-Caparrós, 2021). Glutathione reductase is a flavo-protein oxidoreductase that reduces glutathione disulfide (GSSH) to glutathione (GSH), and is mainly generated in mitochondria, chloroplasts, and cytosol (Schulz et al., 1978). GSH is a critical molecule composed of thiol tripeptide (γ-glutamylcysteinyl-glycine) that acts as a reducing agent, scavenges ROS via the ascorbate-glutathione cycle (Hasanuzzaman et al., 2017), glutathiolation, or by reduction resulting in GSSH (García-Caparros, 2021).

Oxidative stress in the context of photosymbiosis has been studied through the effects of light intensity and the symbiotic state in corals (Lesser, 2006; DeSalvo et al., 2012; Roth, 2014; Rouan et al., 2021). Marine slugs have been the subjects of studies addressing the effects of starvation and oxidative stress, especially in the frame of functional kleptoplasty (e.g. Christa et al., 2014; de Vries et al., 2015; Rey et al., 2020). In these studies, starvation under darkness is commonly used to determine the kleptoplast contribution to the animal host. Likewise, in photosymbiotic cladobranchs the symbiont retention and the nutritional support of the dinoflagellate (Symbiodiniaceae) to its host have been evaluated during starvation in the presence and absence of light (Kempf, 1984; Burghardt & Wägele, 2004, 2006, 2014; Burghardt et al., 2005, 2008a,b; Wägele & Johnsen, 2001). Yet, the effect of different light intensities, continuous darkness, and starvation on the slugs in terms of oxidative stress and antioxidant defense remains unknown.

The aim of this study was to evaluate the effect of starvation and light intensity in the nudibranch *Berghia stephanieae* (Valdés, 2005). For this, we analyzed the antioxidant defense of the slug under different light conditions and starvation, focusing in three major players in ROS scavenging: GSH, SOD, and CAT.

## Materials and methods

### Exaiptasia diaphana *culture*

The culture of the animals in this study was done as described in Melo Clavijo et al., (2022). Individuals of *Exaiptasia diaphana* (Rapp, 1829) were kept in 55 l tanks (60 cm x 30 cm x 30 cm) filled with circulating artificial seawater (ASW) (ABReef Salt, Aqua Medic, Germany) with a salinity of 1.02 to 1.03 s.g., temperature of 25 °C, pH 8, and light intensity of 30 mol photons m-2 s-1 provided by RGB top light for reef tanks (Daylight Sunrise 520). Every two weeks 2 BactoBalls (Fauna Marin GmbH, Germany) were added and replaced to ensure a stable microbiome. Brine

shrimp *Artemia* Leach, 1819 were grown using the Artemio Set and 16 g of Artemio Mix Eggs+Salt (JBL, Germany). Two to three times per week, the anemones were fed freshly hatched brine shrimp nauplii *Artemia*.

Berghia stephanieae *culture*

The animals in this study were cultured in the way described by Melo Clavijo et al., (2022). In short, individuals of *B. stephanieae* cultivated in our lab at 23°C, at a day/night cycle of 12 h/12 h were kept in a 50 ml plastic container with lid (75mm, FAUST, Germany) with ASW. Freshly prepared ASW with salinity between 1.02 and 1.03 s.g., pH 8.0, and at 23 °C was used for water changes (50%) three times per week. Three times per week, the slugs were fed small *E. diaphana* anemones (7 mm foot and 4 mm oral disk or 4 mm foot and 3 mm oral disk).

*Experimental design and sample collection*

Forty-five specimens of *B. stephanieae* were randomly selected from the culture and each individual was placed in a 25 ml container with ASW. The animals were separated in three main groups: feeding (Experiment 1), starvation after light acclimation (Experiment 2), and direct starvation (Experiment 3). All the slugs were fed with anemones acclimated at low light. In the first group the slugs were feeding under high light, low light, and darkness. In the second group the slugs were feeding during an acclimation week under high light, low light and darkness, and starved for another week under the respective light intensity. Slugs in the third group were feeding under low light and starved directly for seven days under different light conditions. In each group, three subgroups were created according to the light condition: high light (90 µmol quanta $m^{-2}$ $s^{-1}$), low light (25 µmol quanta $m^{-2}$ $s^{-1}$), and constant darkness. Except for darkness, the slugs under high light and low light were kept at a day/night cycle of 12 h/12 h. For each light condition five slugs were used (Figure 1).

During the starvation period for both starvation groups, the chlorophyll *a* fluorescence of the dinoflagellates in the slugs was measured using a Diving Pulse Amplitude Modulated (PAM) fluorometer (Walz, Effeltrich Germany). After the slugs were dark acclimated for 5 min, the maximum quantum yield ($F_v/F_m$; $F_v = F_m - F_o$; where $F_o$ and $F_m$ are the minimum and maximum fluorescence emitted by dark adapted samples, respectively) was determined by applying a saturation pulse (pulse duration 0.8 ms, white light, >5,000 $\mu$mol photons $m^{-2}$ $s^{-1}$). For length measurements, five images of each slug were taken with a phone camera during movement to

obtain the point of maximum elongation, and the average length of the individual images was calculated using ImageJ v2.3.0. Both measurements (length and maximum quantum yield) were taken at the beginning of the experiment (Day 0), at Day 2, Day 5, and after one week of starvation (Day 7). At Day 7 of starvation, the animals were snap frozen and stored at -80 °C until processing.

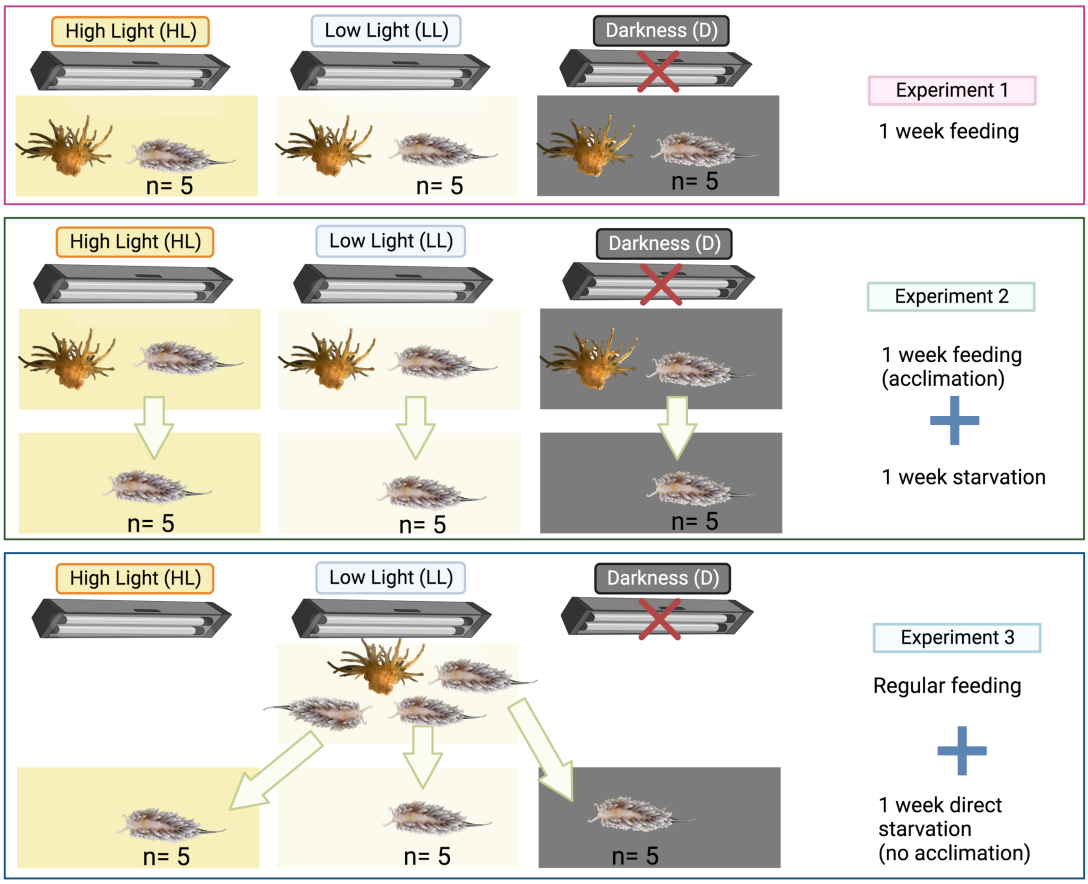


**Figure 1.** Experimental design. The animals were divided in three major groups according to the experiment. Experiment 1: the slugs regularly fed with symbiotic anemones under high light, low light (control), and darkness. Experiment 2: the slugs fed during one week under high light, low light, and darkness and starved for one week. Experiment 3: the slugs fed under low light and starved under high light, low light, and darkness for one week. Each experiment was done with 15 slugs, 5 per light intensity. Created with BioRender.com

*Glutathione quantification*

The quantification of total glutathione was done following Bornhorst protocol. First the KPE Buffer ($KH_2PO_4$ 15.9 mM, $K_2HPO_4$ 81.9 mM, EDTA 8.78 mM), the glutathione in reduced form (GSH) and oxidized glutathione (GSSG) stock solutions (1mg/ml in KPE buffer), and extraction buffer (20 µl Triton X-100, 120 mg sulfosalicylic acid, 20 ml KPE buffer) were prepared and stored at 4 °C

until use. The GSH and GSSG standards were prepared by serial dilutions from 8 µM to 30 nM in extraction buffer, starting from a stock solution of 1 mg/ml in extraction buffer.
The samples were homogenized in 300 µl ice-cold extraction buffer using a homogenizer (Janke & Kunkel Ika-Werk Ultra Turrax, Heinrich Faust GmbH, Germany), and then centrifuged at 3000 g for 4 min at 4 °C. The pellets were discarded and 120 µl supernatant were subsequently used. Solutions of DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) (2 mg DTNB in 3 ml KPE buffer), NADPH (2 mg NADPH in 3 ml KPE buffer), and glutathione reductase (GR) (40 µl of GR in 3 ml KPE buffer) were freshly prepared and stored on ice until use. A Corning™ Costar™ 96-well plate (Thermo Fischer, USA) was used for the quantification of total GSH and GSSG. For the plate set up 20 µl of KPE buffer for blank, 20 µl of standards, or 20 µl of the samples' supernatant were added into each well. Equal volumes of DTNB and GR solutions were mixed together at the same time, and 120 µl of the mixed DTNB/GR was added to each well. Exactly 30 s after, 60 µl of NADPH solution was added to each well. The absorbance was quantified at 412 nm using an Infinite® 200 microplate reader (Tecan, Switzerland) and the software iControl 2.0.10.

The rate of 2-nitro-5-thiobenzoic acid formation for samples and standards was calculated by the change in absorbance per min. Parallel, an 1:1 ethanol/KPE solution was prepared and kept on ice until use. Then 5 µl 2-vinylpyridine were added to the EtOH/KPE mix. For the GSSG quantification, 2 µl of the 2-vinylpyridine/EtOH/KPE mix were added to 100 µl of the samples' supernatant and the GSSG standards, and were kept under a fume hood for 1 hour. The plate set up and the GSSG measurement was done exactly like the GSH as previously described. The samples' supernatant was subsequently used for the quantification of total protein concentration following the Lowry (1951) protein assay. Briefly, the Lowry reagent mix solution was prepared using 100 ml of Lowry A (4 g/l NaOH, 20 g/l $Na_2CO_3$), 1 ml of Lowry B (1% $CuSO_4$ x 5 $H_2O$), and 1 ml of Lowry C (2% NaK-Tartrate). Then, 10 µl of each sample were mixed with 90 µl $ddH_2O$ and 2000 µl of Lowry reagent mix and incubated for 10 min at room temperature in darkness.
After, 100 µl of Folin & Ciocalteu′s phenol reagent (1:2 diluted in $ddH_2O$) were added to the mix and incubated for 30 min at room temperature in darkness. The absorbance was quantified at 578 nm with a Genesys 10UV spectrophotometer (ThermoFisher, Germany). Bovine albumin serum (BSA, Sigma-Aldrich, Germany) dilutions (0–1000 µg/ml) were used as standards, starting from a stock solution of 1 mg/ml (Supplementary Figure 1, Supplementary Table 1).

Lowry results were then used to normalize GSH and GSSG results to total protein content in nmol/mg protein. The concentration of GSH and GSSG in the samples was calculated using the linear regression for the standard curve. However, based on prior tests on other slug samples that yielded a negative or undetectable GSSG value, the quantification of GSSG was not performed for the samples of these experiments. So the subsequent analysis was only done using GSH values. Yet, the procedure is described in detail for replication purposes in further studies.

*Superoxide dismutase enzymatic activity*

The enzymatic activity of the superoxide dismutase was measured using the stored frozen samples following Sun et al. (1988) protocol and adapted to smaller volumes. In summary, SOD activity involves nitroblue tetrazolium (NBT) reduction inhibition, with xanthine-xanthine oxidase acting as a superoxide producer (Sun et al., 1988). First, a reactive solution was prepared using xanthin 0.3 mM as substrate, EDTA 0.6 mM, NBT 150 µg/L, $Na_2CO_3$ 400 nM, and BSA 1g/L. The stock solution of the enzyme xanthin oxidase (XOD) was prepared with a concentration of 0.167 U/ml and was stored at 4 °C. To prepare the XOD solution, 9 µl (0.167 U/ml) of the XOD stock solution were mixed with 1.5 ml of 2M $(NH_4)_2SO_4$ and kept in ice until use. For the blank measure 178 µl of the reactive solution and 3 µl of the XOD solution were mixed. For the target measure 178 µl of the reactive solution, 3 µl of the XOD solution, and 6.25 µl of the sample were mixed. Blank and targets were mix by vortex and incubated for 20 min at 25 °C. Subsequently, 6.25 µl of 0.8 mM $CuCl_2.2H_2O$ to the blank and the targets to stop the reaction. Then, 3 µl of sample were added to the blank. Blank and targets were mix by vortex and placed in separate wells in a Corning™ Costar™ 96-well plate (Thermo Fischer, USA). The absorbance was measured at 560 nm against distilled water using a Infinite® 200 microplate reader (Tecan, Switzerland) and the software iControl 2.0.10. The enzymatic activity (U/mg Protein) was calculated as follows, considering the total protein content of the supernatant and negative values as no (0) enyzmatic activity:

$$Enzymatic\ activity = \frac{Blank\ Absorption - Target\ Absorption}{Blank\ Absorption} X\ \frac{20\ U/ml\ SOD}{mg\ Protein\ (sample)} X\ dilution\ factor$$

*Catalase enzymatic activity*

The enzymatic activity of catalases was measured using the stored frozen samples following Lartillot et al., (1988) protocol and adapted to smaller volumes. The protocol is based on the determination of the residual $H_2O_2$ by spectrophotometry. First, the phosphate buffer solution (50

mM $H_3PO_4$ 85%, pH 6.8) and the substrate solution (10 mM $H_2O_2$ in 50 mM $H_3PO_4$, pH 6.8) were freshly prepared. Then, 625 µl of the substrate buffer were mixed with 5 µl of the sample by vortex and incubated exactly for 2 min at 37 °C. To stop the reaction, 125 µl of 1N HCl were added to the mix. The absorbance (Ar) was then quantified at 240 nm with a Genesys 10UV spectrophotometer (ThermoFisher, Germany). For the control, 625 µl of the phosphate buffer mixed with 125 µl of 1N HCl were used. The absorbance was quantified at time zero (As) on a solution of 625 µl of the substrate buffer with 125 µl of 1N HCl. To eliminate the absorbance of the protein sample, the absorbance of a solution containing 625 µl of the phosphate buffer, 5 µl of the sample, and 125 µl of 1N HCl (At) was measured. The absorbance variation due to enzymatic activity was calculated as: A= (As+At)-Ar. The enzymatic activity (U/ml) was calculated according to Lartillot et al., (1988) with the following equation:

$$Enzymatic\ activity = \frac{A * Vt}{\epsilon * t * Vs}$$

Where A is the absorbance variation due to enzymatic activity, Vt is the total reaction volume, ϵ is the specific absorbance coefficient of $H_2O_2$ (0.0396 $cm^2$/µmol), t is the time of the reaction (2 min), and Vs is the sample volume (5 µl). Specific enzymatic activity was then calculated considering the total protein content of the supernatant (U/mg protein). Negative values were considered as no (0) enyzmatic activity.

*Statistical analyses*

Kruskal-Wallis tests were done to determine if there were significant differences between the conditions for each assay. Wilcoxon tests were used for pairwise comparisons using the Benjamini & Hochberg (1995) (BH) correction for adjusting the p value. All statistical analyses were performed with R (R Core Team, 2022). Results were considered statistically significant at a significance level of 0.05.

## Results

*Direct starvation induces higher concentration of glutathione*

The glutathione concentration was quantified in slugs feeding under different light conditions (Experiment 1), in slugs feeding and acclimated to each light condition for a week and starved for seven days under the same light condition (Experiment 2), and in slugs that were feeding under low

light and starved directly for seven days with no acclimation period under different light conditions (Experiment 3) (Supplementary Table 2).

The slugs that fed constantly under high light, low light, and darkness showed a lower concentration of GSH than the slugs acclimated and starved, and the slugs directly starved (Figure 2). During feeding, the GSH concentration was significantly higher under darkness ($3.06x10^6$ nM GSH $nmol^{-1}$ $mg^{-1}$ Protein ± $1x10^6$) compared to high light ($1.39x10^6$ nM GSH $nmol^{-1}$ $mg^{-1}$ Protein ± $6x10^5$, p = 0.048), and compared to low light ($1.12x10^6$ nM GSH $nmol^{-1}$ $mg^{-1}$ Protein ± $3x10^5$, p = 0.024) (Figure 2). There was no significant difference between the GSH concentration in slugs feeding under low light and high light (p=0.69). In general, the slugs increased in length regardless of the light condition they were exposed to (high light: 109 % ± 2 % of the initial length; low light: 108 % ± 1%; darkness: 116% ± 3%) (Figure 3).

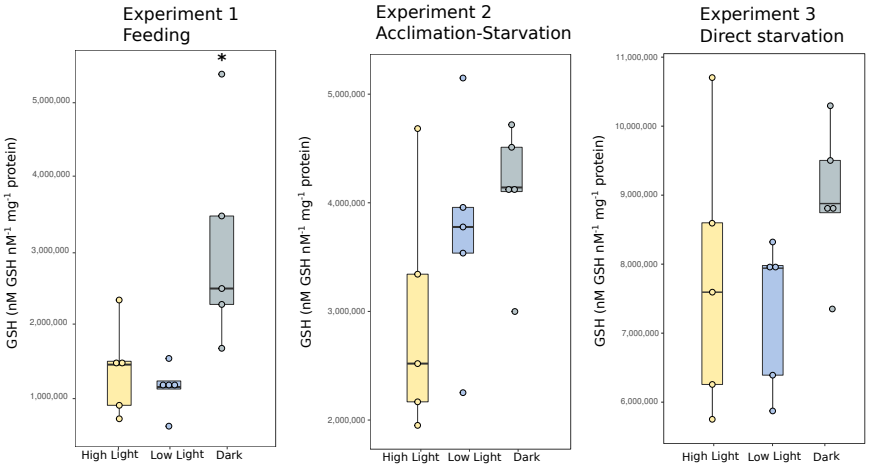


**Figure 2.** Glutathione concentration in *B. stephanieae*. Boxplots show the concentration values of GSH, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. The asterisk (*) represents significant values.

The GSH concentration in slugs that were feeding and acclimated to each light condition for a

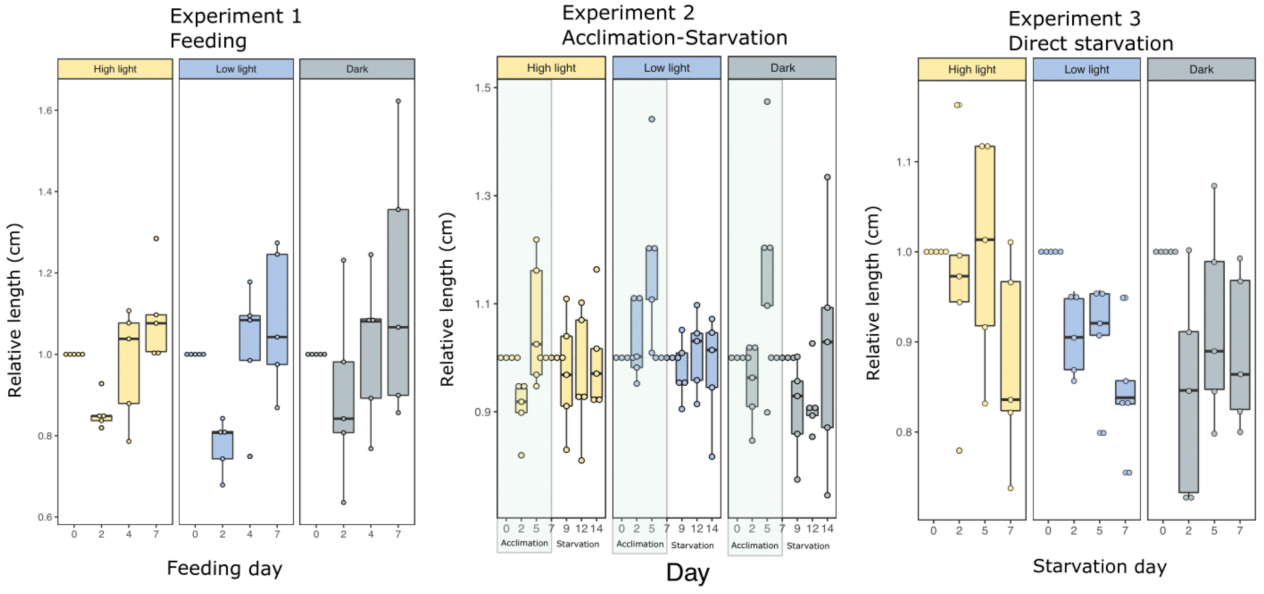


**Figure 3.** Relative length of *B. stephanieae*. Boxplots show the relative length (cm) of the animals in each experiment under high light, low light, and darkness, where the whiskers are the minimum and maximum values, and the centers correspond to the medians.

week, and then starved under the same light conditions for seven days was similar in all conditions (Experiment 2; High light: 2.9 x $10^6$ nM GSH $nmol^{-1}$ $mg^{-1}$ Protein ± 1 x $10^6$; Low light: 3.8 x $10^6$ ± 1 x $10^6$; Darkness: 4.2 x $10^6$ ± 7 x $10^6$) (Figure 2). No significant differences were found between the GSH concentrations under different light conditions (Kruskal-Wallis p=0.18). The length of starved animals under high light (99.9 % ± 9.9 % of the initial length) and darkness (101 % ± 22.45 %) seemed to stay constant, while in low light it decreased 2 % (98 % ± 10.2 %)(Figure 3). The maximum quantum yield (Fv/Fm) of Symbiodiniaceae in these slugs dropped notably after the fifth day of starvation (Figure 4).

Likewise, in slugs that starved directly without an acclimation period, there were no significant differences in the GSH concentration under different light conditions (Experiment 3; High light: 7.8 x $10^6$ nM GSH $nmol^{-1}$ $mg^{-1}$ Protein ± 2 x $10^6$; Low light: 7.3 x $10^6$ ± 1 x $10^6$; Darkness: 8.9 x $10^6$ ± 1 x $10^6$; Wilcoxon p=0.46 D-HL and D-LL, p=0.17 HL-LL) (Figure 2). The length of the animals decreased around 12 % in all conditions (High light: 88 % ± 11.2 %; Low light: 85% ± 6.9 %; Darkness: 89% ± 8.6%) (Figure 3). The maximum quantum yield (Fv/Fm) of Symbiodiniaceae in these slugs declined to 0 after the fifth day of starvation (Figure 4).

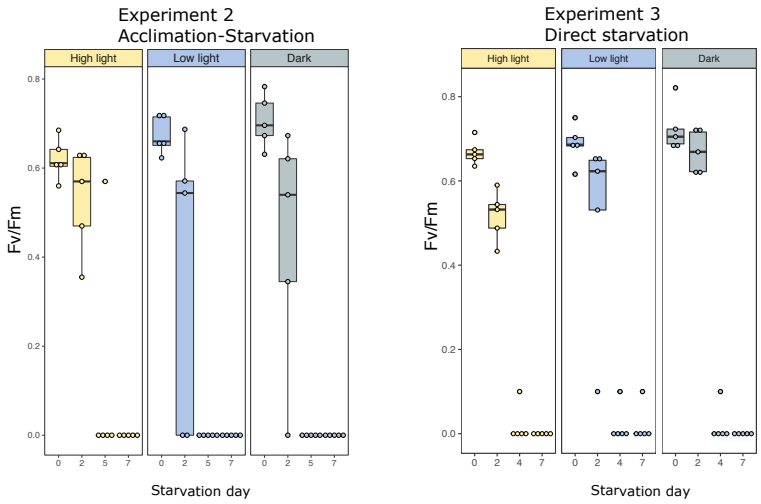


**Figure 4.** Maximum quantum yield of Symbiodiniaceae in *B. stephanieae*. Boxplots show the Fv/Fm of the photobionts within the animal host in experiments 2 and 3 under high light, low light, and darkness, where the whiskers are the minimum and maximum values, and the centers correspond to the medians.

GSH concentrations of slugs under high light, low light, and darkness were also compared between acclimation-starvation (Experiment 2) and direct starvation treatments (Experiment 3). Overall, the GSH concentration in each light intensity condition almost duplicated in slugs directly starved compared to acclimated and starved slugs (Figure 2). There were significant differences between the GSH concentrations of acclimation-starvation and direct starvation treatments in all light conditions (Wilcoxon p=0.024 HL Exp2-Exp3, p=0.017 LL Exp2-Exp3, p=0.017 D Exp2-Exp3).

*Direct starvation might induce higher SOD enzymatic activity under high light*

The enzymatic activity of SOD and catalase were only measured in starved slugs of experiments 2 and 3. In general SOD enzymatic activity ranged between 0.06 and 7.8 IU/mg protein in starved slugs. For the experiment 2, the levels of SOD activity in light-acclimated-starved slugs where almost undetectable in all light conditions and no significant differences were found between treatments (High light: 0.16 IU/ mg Protein ± 0.28; Low light: 0.31 ± 0.70; Darkness: 0.38 ± 0.58; Wilcoxon p=0.8 D-HL, p=0.8 D-LL and HL-LL).

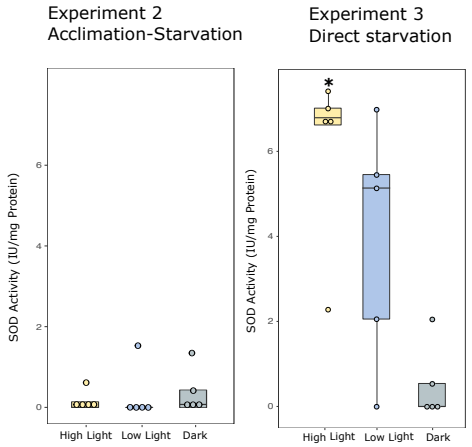


**Figure 5.** SOD activity in *B. stephanieae*. Boxplots show the SOD activity (IU/mg protein) in the slug in experiments 2 and 3 under high light, low light, and darkness, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. Asterisks (*) show significant values. Negative values were taken as 0 activity for plotting.

Starved slugs of the experiment 3 with no light acclimation showed significantly higher levels of SOD activity, especially under high light (High light: 6.33 IU/ mg Protein ± 2.24; Low light: 4.11± 2.98; Darkness: 0.54 ± 0.93; Wilcoxon p=0.033 D-HL, p=0.1 D-LL, p=0.151 HL-LL) (Figure 5). Overall, the SOD activity was higher in directly starved slugs under high light and low light compared to light-acclimated-starved slugs (Figure 5). Yet, these SOD activity values were significant only in high light (Kruskal-Wallis p=0.004, Wilcoxon p=0.045 HL Exp2-Exp3, p=0.135 LL Exp2-Exp3, p=1 D Exp2-Exp3) (Supplementary Table 3).

*Starvation induces similar CAT enzymatic activity regardless of the light intensity or light acclimation*

The levels of catalase activity in starved slugs ranged from 0.5 to 36.9 IU/mg protein. Acclimated starved slugs (experiment 2) showed almost undetectable levels of catalase activity in all light conditions and no significant differences were found between treatments (High light: 2.63 IU/ mg Protein ± 2.39; Low light: 0.11 ± 0.24; Darkness: 4.74 ± 8.95; Wilcoxon p=0.59 D-HL and D-LL, p=0.13 HL-LL). Slugs starved directly (experiment 3)

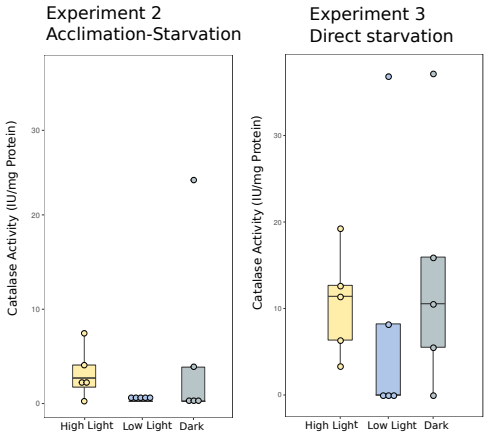


**Figure 6.** Catalase activity in *B. stephanieae*. Boxplots show the CAT activity (IU/mg protein) in the slug in experiments 2 and 3 under high light, low light, and darkness, where the whiskers are the minimum and maximum values, and the centers correspond to the medians. Negative values were taken as 0 activity for plotting.

showed higher levels of catalase activity under high light and undetectable levels in low light, but no significant differences were found (High light: 11.1 IU/ mg Protein ± 6.43; Low light: 9.45 ± 18.78; Darkness: 14.56 ± 15.08; Wilcoxon p=1 D-HL, p=0.5 D-LL and HL-LL) (Figure 6). Further, the CAT activity was higher in slugs directly starved under high light and darkness compared to acclimated and starved slugs (Figure 6). However, no significant differences were found between the CAT activity values of acclimation-starvation and direct starvation treatments in all light conditions (Kruskal-Wallis p=0.06; Wilcoxon p=0.12 HL Exp2-Exp3, p=0.60 LL Exp2-Exp3, p=0.51 D Exp2-Exp3) (Supplementary Table 4).

**Discussion**

In the present study we provide some insight on the effect of starvation and light intensity on photosymbiotic cladobranch slugs. Our results show that with a steady food supply, darkness acts as more damaging stressor than higher light intensities. Starvation induces symptoms of oxidative stress as indicated by the increased levels of antioxidant activity. Food depletion combined with light stress and no light acclimation might trigger a higher antioxidant defense in *Berghia stephanieae* especially under high light intensity. Thus, starvation alone is a pro-oxidant condition that together with external abiotic factors like light enhances the stress response in the slugs.

The effect of starvation and light stress has been previously evaluated in photosymbiotic scleractinian coral species like *Stylophora pistillata* (Esper, 1792). Bleached corals resisted light stress (500 μmoles photons $m^{-2}s^{-1}$) better when fed; they exhibit less oxidative damage and protein degradation than starved individuals (Levy et al., 2016). Heterotrophy protects organisms from oxidative damage and aids in protein and DNA repair. Such is also the case of *B. stephanieae,* the slugs tolerated high light intensity and darkness better during feeding than under starvation (Figure 2). Here, the levels of GSH incremented almost twice during starvation compared with fed slugs.

Starvation also contributes to the bleaching response (the symbiont expulsion) in photosymbiotic animals. High light intensity induces the loss of *ca* 80% of the symbionts in starved corals, which is reduced to 15% loss in well-fed corals (Levy et al., 2016). The photosynthetic activity of Symbiodiniaceae in *B. stephanieae* dropped to 0 after 2 days of starvation regardless of the light condition or the light acclimation period (Figure 4). The symbionts in the slug are being digested and/or expelled in the feces during starvation, which also happens when the food is not limited

under regular light conditions. Previous studies have shown that this slug can retain the symbionts for up to 10 days during starvation under regular light intensity (80 µmol photons $m^{-2} s^{-1}$), losing 90% of symbiont cells by day 5 of starvation (Monteiro et al., 2019). However no maximum quantum yield values of the symbionts have been recorded in these reports, so the slug might looked pigmented and some symbiont cells might be visible, but the dinoflagellates might no longer be photosynthetically active even before the 5 day threshold.

Our results show that the combination of two stressors, starvation with no light acclimation is more damaging to the slugs. Slugs directly starved showed almost six times more GSH than the fed slugs, and twice or three times more GSH than light-acclimated-starved slugs. We found differences in GSH levels between light-acclimation and direct starvation, but not within the treatments. Thus, the GSH levels seen in light-acclimated slugs are more likely linked to starvation stress rather than light stress. Hence, the GSH levels detected in directly starved slugs are the result of the synergistic effects of starvation and light stress, similar to the combined effect seen in light-stressed starved corals (Levy et al., 2016). The light-acclimated starved slugs under low light (Experiment 2) should have had similar GSH concentration than the slugs directly starved under low light (Experiment 3). Yet, the GSH levels significantly doubled, and SOD and CAT activity increased but not significantly in the slugs of experiment 3 compared to the ones in experiment 2. A possible explanation could be based on the individual fitness, since their length decrease more than the slugs in experiment 2.

High light intensity combined with starvation seems to induce symptoms of oxidative stress, as indicated by the enhanced SOD activity in the slugs. This might be explained by the fact that SOD is the first line of antioxidant defense (Lesser, 2006; Richier, 2003, 2005), and could suggest that toxic $O_2^{\bullet-}$ levels increase faster than other types of ROS under high light intensities. Similarly, in light-stressed corals SOD genes were also highly up-regulated as an oxidative stress symptom (Levy et al., 2016). However, in the slugs SOD activity levels were much lower than the ones detected on other photosymbiotic mollusks (unstressed 11-105 U/mg protein depending on the tissue: Schick & Dykens, 1985; 200 U/mg protein in heat-stressed *Tridacna crocea* Lamarck, 1819 after 12 h, Zhou et al., 2018). The difference could be based on the different methods used or could also indicate an effect of the sample storage on the activity measurement. Here, the quantification was carried out on frozen samples after sample preparation, and not immediately after protein

extraction. To confirm the effect of high light on SOD activity in the slugs, a replication of these methods would be required.

Catalase activity was similar in all light and starvation treatments in the slugs. Contrary to SOD, the CAT activity values obtained here were comparable to the ones detected in heat-stressed mollusks (approx. 10 U/mg protein; Zhou et al., 2018) and other photosymbiotic animals (20-40 U/mg protein; Dykens & Shick, 1984; Shick & Dykens, 1985; Merle et al., 2007). Starvation induces an increase in SOD and CAT activity (Morales et al., 2004; Suda et al., 2015). SOD activity can lead to an enhanced $H_2O_2$ generation, which can be normally catabolized by peroxidases. When the $H_2O_2$ dose is higher and potentially harmful, catalases are more efficient $H_2O_2$ quenchers (Halliwell & Gutteridge, 2015). For instance, in marine mollusks like the Antarctic limpet *Nacella concinna* (Strebel, 1908), CAT activity increased in animals starved for a week compared to fed ones. In prolonged starvation, SOD activity increased first, followed by an increment in the CAT activity in these limpets (Suda et al., 2015). In the slugs, starvation induces a higher CAT gene expression (Melo Clavijo et al., 2022) and CAT enzymatic activity regardless of the light condition, but further comparisons against fed slugs are needed to have a more complete picture.

Darkness seems to have an additional stress effect during feeding, indicated by the significantly higher GSH concentration (Figure 2: Experiment 1). In corals, prolonged darkness treatment causes hypoxia and likely photodamage in the symbiont photosynthetic machinery (DeSalvo et al., 2012). The anemones given to the slugs were acclimated to low light, so the symptoms of oxidative stress seen in slugs feeding under darkness might no be a result of prior stress of the anemone's symbiont. However, the change from low light to continuous darkness could have induced a stress response in the symbiont once in the slug, becoming a stressor for its host. Other cladobranch species fed in darkness show a decrease on the number of photosynthetically active symbiont cells, which could be due to symbiont digestion and a switch in the symbiont to heterotrophic state (Burghardt et al., 2005). Harboring symbionts in darkness seems to be less beneficial for other photosymbiotic hosts. Such is the case of *Paramecium bursaria* (Ehrenberg) Focker, 1836 and its symbiont *Chlorella* Beijerinck, 1890; the host can grow faster symbiont-free than in symbiotic state under darkness (Lowe et al., 2016; Sørensen et al., 2019). Darkness also affects circadian rhythm of the animal host (Shirley & Findley, 1978), so the higher GSH concentration detected in darkness during feeding might be a result of stress induced by the symbiont combined with alterations in the circadian rhythm in the slugs.

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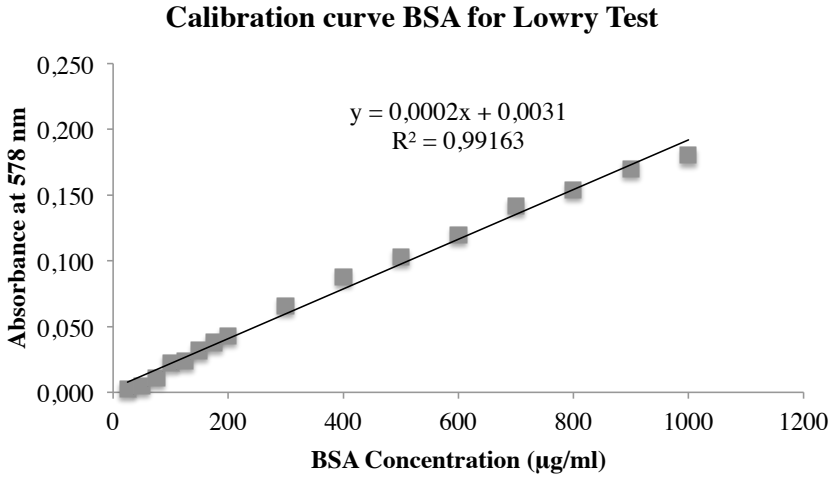
## Supplementary Material




**Supplementary Figure 1.** Calibration curve BSA for Lowry protein assay. BSA was used as a reference protein.

**Supplementary Table 1.** Calibration values of BSA for Lowry protein assay. R1, R2, and R3: replicates 1, 2, and 3.

| | Absorbance (578 nm) | | | | |
|---|---|---|---|---|---|
| **BSA Concentration (µg/ml)** | **R1** | **R2** | **R3** | **Average** | **Standard deviation** |
| 25 | 0.001 | 0.003 | 0.003 | 0.002 | 0.001 |
| 50 | 0.004 | 0.005 | 0.006 | 0.005 | 0.001 |
| 75 | 0.015 | 0.01 | 0.009 | 0.011 | 0.003 |
| 100 | 0.024 | 0.022 | 0.022 | 0.023 | 0.001 |
| 125 | 0.025 | 0.026 | 0.022 | 0.024 | 0.002 |
| 150 | 0.031 | 0.033 | 0.032 | 0.032 | 0.001 |
| 175 | 0.039 | 0.038 | 0.037 | 0.038 | 0.001 |
| 200 | 0.045 | 0.041 | 0.043 | 0.043 | 0.002 |
| 300 | 0.063 | 0.065 | 0.070 | 0.066 | 0.004 |
| 400 | 0.085 | 0.085 | 0.093 | 0.088 | 0.005 |
| 500 | 0.093 | 0.108 | 0.108 | 0.103 | 0.009 |
| 600 | 0.117 | 0.121 | 0.121 | 0.120 | 0.002 |
| 700 | 0.141 | 0.138 | 0.146 | 0.142 | 0.004 |
| 800 | 0.157 | 0.153 | 0.153 | 0.154 | 0.002 |
| 900 | 0.174 | 0.168 | 0.168 | 0.170 | 0.003 |
| 1000 | 0.180 | 0.186 | 0.175 | 0.180 | 0.006 |

**Supplementary Table 2.** GSH concentration values. SD: Standard deviation.

| | Light condition | Samples | Concentration GSH [g/ml] | Concentration Average | Concentration Total Protein (g/ml) | Concentration GSH (mM) | Concentration GSH (nM) | nM GSH/Total Protein | Average nM GSH/ nmol/mg Protein | SD nM GSH/ nmol/mg Protein |
|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 1 Feeding | Low Light | B1 | 2.35E-06 | 1.85E-06 | 0.006 | 0.008 | 7657.048 | 1197349.232 | 1121251.091 | 318918.331 |
| | | B2 | 1.83E-06 | | 0.005 | 0.006 | 5958.363 | 1094281.625 | | |
| | | B3 | 8.23E-07 | | 0.004 | 0.003 | 2676.853 | 651038.317 | | |
| | | B4 | 2.19E-06 | | 0.005 | 0.007 | 7123.114 | 1544585.722 | | |
| | | B5 | 2.07E-06 | | 0.006 | 0.007 | 6745.708 | 1119000.560 | | |
| | High Light | B6 | 2.39E-06 | 2.42E-06 | 0.005 | 0.008 | 7762.079 | 1503793.178 | 1390314.776 | 619250.316 |
| | | B7 | 1.34E-06 | | 0.005 | 0.004 | 4371.546 | 924542.715 | | |
| | | B8 | 1.87E-06 | | 0.008 | 0.006 | 6093.649 | 740569.075 | | |
| | | B9 | 3.68E-06 | | 0.005 | 0.012 | 11973.132 | 2327139.424 | | |
| | | B10 | 2.80E-06 | | 0.006 | 0.009 | 9114.040 | 1455529.486 | | |
| | Dark | B11 | 2.60E-06 | 2.66E-06 | 0.005 | 0.008 | 8456.480 | 1676210.177 | 3061384.314 | 1450084.540 |
| | | B12 | 2.16E-06 | | 0.003 | 0.007 | 7023.919 | 2281728.028 | | |
| | | B13 | 2.42E-06 | | 0.003 | 0.008 | 7879.621 | 2492236.380 | | |
| | | B14 | 3.55E-06 | | 0.002 | 0.012 | 11546.837 | 5383140.790 | | |
| | | B15 | 2.56E-06 | | 0.002 | 0.008 | 8319.287 | 3473606.196 | | |
| Experiment 2 Acclimation-Starvation | Low Light | B1 | 6.42E-06 | 5.27E-06 | 0.006 | 0.021 | 20890.312 | 3594547.586 | 3816116.980 | 1061194.195 |
| | | B2 | 4.32E-06 | | 0.004 | 0.014 | 14063.887 | 3876128.764 | | |
| | | B3 | 6.29E-06 | | 0.004 | 0.020 | 20456.742 | 5274621.900 | | |
| | | B4 | 2.80E-06 | | 0.004 | 0.009 | 9098.650 | 2306375.277 | | |
| | | B5 | 6.51E-06 | | 0.005 | 0.021 | 21198.789 | 4028911.373 | | |
| | High Light | B6 | 5.00E-06 | 4.36E-06 | 0.006 | 0.016 | 16269.007 | 2557349.743 | 2993157.540 | 1141030.800 |
| | | B7 | 4.08E-06 | | 0.004 | 0.013 | 13281.594 | 3424562.376 | | |
| | | B8 | 3.05E-06 | | 0.005 | 0.010 | 9930.672 | 1988122.456 | | |
| | | B9 | 6.44E-06 | | 0.004 | 0.021 | 20949.317 | 4784769.812 | | |
| | | B10 | 3.24E-06 | | 0.005 | 0.011 | 10527.966 | 2210983.312 | | |
| | Dark | B11 | 5.43E-06 | 5.38E-06 | 0.006 | 0.018 | 17665.600 | 3048421.091 | 4189720.307 | 691257.078 |
| | | B12 | 5.37E-06 | | 0.004 | 0.017 | 17489.147 | 4236369.777 | | |
| | | B13 | 5.91E-06 | | 0.004 | 0.019 | 19220.162 | 4831209.449 | | |
| | | B14 | 5.07E-06 | | 0.004 | 0.016 | 16495.024 | 4631265.410 | | |
| | | B15 | 5.14E-06 | | 0.004 | 0.017 | 16714.314 | 4201335.809 | | |
| Experiment 3 Direct Starvation | Low Light | B1 | 6.04E-06 | 4.92E-06 | 0.002 | 0.020 | 19638.591 | 7977762.195 | 7302506.866 | 1091638.519 |
| | | B2 | 5.93E-06 | | 0.002 | 0.019 | 19285.233 | 7941756.970 | | |
| | | B3 | 5.74E-06 | | 0.002 | 0.019 | 18685.223 | 8323039.269 | | |
| | | B4 | 3.72E-06 | | 0.002 | 0.012 | 12117.044 | 6394218.474 | | |
| | | B5 | 3.18E-06 | | 0.002 | 0.010 | 10351.126 | 5875757.421 | | |
| | High Light | B6 | 3.90E-06 | 4.76E-06 | 0.002 | 0.013 | 12700.872 | 5742669.915 | 7780267.425 | 1983649.148 |
| | | B7 | 3.00E-06 | | 0.002 | 0.010 | 9775.170 | 6259447.477 | | |
| | | B8 | 5.36E-06 | | 0.002 | 0.017 | 17428.789 | 7594243.363 | | |
| | | B9 | 4.70E-06 | | 0.002 | 0.015 | 15287.649 | 8596615.860 | | |
| | | B10 | 6.84E-06 | | 0.002 | 0.022 | 22255.543 | 10708360.511 | | |
| | Dark | B11 | 3.38E-06 | 5.01E-06 | 0.001 | 0.011 | 10989.184 | 7350624.596 | 8954433.647 | 1085423.374 |
| | | B12 | 3.21E-06 | | 0.001 | 0.010 | 10461.333 | 8878075.736 | | |
| | | B13 | 5.86E-06 | | 0.002 | 0.019 | 19058.261 | 8749010.615 | | |
| | | B14 | 5.87E-06 | | 0.002 | 0.019 | 19112.054 | 9500606.547 | | |
| | | B15 | 6.73E-06 | | 0.002 | 0.022 | 21908.746 | 10293850.739 | | |

**Supplementary Table 3.** SOD concentration values. SD: Standard deviation.

| | Light Condition | Sample | Absorbance Blank (560 nm) | Absorbance Target (560 nm) | Abs Blank-Target/Blank | Protein concentration (g/ml) | Protein concentration (mg/ml) | Activity (IU/mg Protein) | Activity (IU/mg) Protein positive values) | Activity (IU/mg) Average | Activity (IU/mg) SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 2 Acclimation-Starvation | Low Light | B1 | 0.092 | 0.098 | -0.060 | 0.006 | 5.812 | -0.205 | 0.000 | | |
| | | B2 | 0.132 | 0.094 | 0.285 | 0.004 | 3.628 | 1.568 | 1.568 | | |
| | | B3 | 0.094 | 0.150 | -0.596 | 0.004 | 3.878 | -3.073 | 0.000 | 0.314 | 0.701 |
| | | B4 | 0.104 | 0.146 | -0.414 | 0.004 | 3.945 | -2.096 | 0.000 | | |
| | | B5 | 0.097 | 0.159 | -0.646 | 0.005 | 5.262 | -2.454 | 0.000 | | |
| | High Light | B6 | 0.093 | 0.095 | -0.023 | 0.006 | 6.362 | -0.071 | 0.000 | | |
| | | B7 | 0.097 | 0.095 | 0.027 | 0.004 | 3.878 | 0.138 | 0.138 | | |
| | | B8 | 0.104 | 0.112 | -0.083 | 0.005 | 4.995 | -0.331 | 0.000 | 0.159 | 0.284 |
| | | B9 | 0.112 | 0.113 | -0.010 | 0.004 | 4.378 | -0.045 | 0.000 | | |
| | | B10 | 0.136 | 0.115 | 0.156 | 0.005 | 4.762 | 0.656 | 0.656 | | |
| | Dark | B11 | 0.094 | 0.092 | 0.021 | 0.006 | 5.795 | 0.074 | 0.074 | | |
| | | B12 | 0.090 | 0.094 | -0.042 | 0.004 | 4.128 | -0.203 | 0.000 | | |
| | | B13 | 0.095 | 0.100 | -0.052 | 0.004 | 3.978 | -0.260 | 0.000 | 0.378 | 0.588 |
| | | B14 | 0.100 | 0.092 | 0.078 | 0.004 | 3.562 | 0.438 | 0.438 | | |
| | | B15 | 0.131 | 0.095 | 0.274 | 0.004 | 3.978 | 1.378 | 1.378 | | |
| Experiment 3 Direct Starvation | Low Light | B1 | 0.101 | 0.113 | -0.122 | 0.003 | 2.462 | -0.993 | 0.000 | | |
| | | B2 | 0.169 | 0.125 | 0.260 | 0.003 | 2.428 | 2.139 | 2.139 | | |
| | | B3 | 0.258 | 0.094 | 0.638 | 0.002 | 2.245 | 5.682 | 5.682 | 4.116 | 2.978 |
| | | B4 | 0.320 | 0.156 | 0.511 | 0.002 | 1.895 | 5.394 | 5.394 | | |
| | | B5 | 0.269 | 0.094 | 0.649 | 0.002 | 1.762 | 7.363 | 7.363 | | |
| | High Light | B6 | 0.130 | 0.096 | 0.263 | 0.002 | 2.212 | 2.381 | 2.381 | | |
| | | B7 | 0.259 | 0.100 | 0.615 | 0.002 | 1.562 | 7.871 | 7.871 | | |
| | | B8 | 0.498 | 0.094 | 0.811 | 0.002 | 2.295 | 7.072 | 7.072 | 6.328 | 2.236 |
| | | B9 | 0.536 | 0.184 | 0.657 | 0.002 | 1.778 | 7.388 | 7.388 | | |
| | | B10 | 0.551 | 0.154 | 0.720 | 0.002 | 2.078 | 6.927 | 6.927 | | |
| | Dark | B11 | 0.192 | 0.207 | -0.081 | 0.002 | 1.495 | -1.080 | 0.000 | | |
| | | B12 | 0.099 | 0.096 | 0.034 | 0.001 | 1.178 | 0.581 | 0.581 | | |
| | | B13 | 0.116 | 0.150 | -0.286 | 0.002 | 2.178 | -2.629 | 0.000 | 0.545 | 0.929 |
| | | B14 | 0.179 | 0.209 | -0.165 | 0.002 | 2.012 | -1.643 | 0.000 | | |
| | | B15 | 0.169 | 0.130 | 0.228 | 0.002 | 2.128 | 2.145 | 2.145 | | |

**Supplementary Table 4.** CAT concentration values. SD: Standard deviation.

**Absorbance time zero (As)= 0.003**

| | Light Condition | Sample | Absorbance solution (At) | Absorbance Protein sample (Ar) | Absorbance variation (A) | Activity (IU/ml) | Total protein concentration (mg/ml) | Activity (IU/mg Protein) | Activity (IU/mg Protein) positive values | Activity (IU/mg Protein) Average | Activity (IU/mg Protein) SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 2 Acclimation-Starvation | Low Light | B1 | 0.056 | 0.066 | -0.007 | -14.681 | 5.812 | -2.526 | 0.000 | | |
| | | B2 | 0.046 | 0.051 | -0.002 | -4.194 | 3.628 | -1.156 | 0.000 | | |
| | | B3 | 0.063 | 0.065 | 0.001 | 2.097 | 3.878 | 0.541 | 0.541 | 0.108 | 0.242 |
| | | B4 | 0.045 | 0.051 | -0.003 | -6.292 | 3.945 | -1.595 | 0.000 | | |
| | | B5 | 0.060 | 0.073 | -0.010 | -20.972 | 5.262 | -3.986 | 0.000 | | |
| | High Light | B6 | 0.046 | 0.096 | -0.047 | -98.569 | 6.362 | -15.494 | 0.000 | | |
| | | B7 | 0.048 | 0.047 | 0.004 | 8.389 | 3.878 | 2.163 | 2.163 | | |
| | | B8 | 0.061 | 0.049 | 0.015 | 31.458 | 4.995 | 6.298 | 6.298 | 2.627 | 2.389 |
| | | B9 | 0.053 | 0.049 | 0.007 | 14.681 | 4.378 | 3.353 | 3.353 | | |
| | | B10 | 0.055 | 0.055 | 0.003 | 6.292 | 4.762 | 1.321 | 1.321 | | |
| | Dark | B11 | 0.066 | 0.080 | -0.011 | -23.069 | 5.795 | -3.981 | 0.000 | | |
| | | B12 | 0.047 | 0.078 | -0.028 | -58.722 | 4.128 | -14.224 | 0.000 | | |
| | | B13 | 0.120 | 0.084 | 0.039 | 81.792 | 3.978 | 20.559 | 20.559 | 4.744 | 8.946 |
| | | B14 | 0.052 | 0.092 | -0.037 | -77.597 | 3.562 | -21.787 | 0.000 | | |
| | | B15 | 0.060 | 0.057 | 0.006 | 12.583 | 3.978 | 3.163 | 3.163 | | |
| Experiment 3 Direct Starvation | Low Light | B1 | 0.056 | 0.049 | 0.010 | 20.972 | 2.462 | 8.520 | 8.520 | | |
| | | B2 | 0.062 | 0.089 | -0.024 | -50.333 | 2.428 | -20.728 | 0.000 | | |
| | | B3 | 0.065 | 0.099 | -0.031 | -65.014 | 2.245 | -28.959 | 0.000 | 9.451 | 16.781 |
| | | B4 | 0.086 | 0.054 | 0.035 | 73.403 | 1.895 | 38.735 | 38.735 | | |
| | | B5 | 0.062 | 0.073 | -0.008 | -16.778 | 1.762 | -9.524 | 0.000 | | |
| | High Light | B6 | 0.072 | 0.068 | 0.007 | 14.681 | 2.212 | 6.638 | 6.638 | | |
| | | B7 | 0.054 | 0.047 | 0.010 | 20.972 | 1.562 | 13.429 | 13.429 | | |
| | | B8 | 0.060 | 0.050 | 0.013 | 27.264 | 2.295 | 11.880 | 11.880 | 11.133 | 6.434 |
| | | B9 | 0.063 | 0.063 | 0.003 | 6.292 | 1.778 | 3.538 | 3.538 | | |
| | | B10 | 0.083 | 0.066 | 0.020 | 41.944 | 2.078 | 20.182 | 20.182 | | |
| | Dark | B11 | 0.056 | 0.051 | 0.008 | 16.778 | 1.495 | 11.223 | 11.223 | | |
| | | B12 | 0.072 | 0.053 | 0.022 | 46.139 | 1.178 | 39.156 | 39.156 | | |
| | | B13 | 0.073 | 0.070 | 0.006 | 12.583 | 2.178 | 5.777 | 5.777 | 14.567 | 15.081 |
| | | B14 | 0.063 | 0.050 | 0.016 | 33.556 | 2.012 | 16.680 | 16.680 | | |
| | | B15 | 0.059 | 0.138 | -0.076 | -159.389 | 2.128 | -74.889 | 0.000 | | |

## General Discussion

The present dissertation aimed to understand the molecular mechanisms behind the onset and the maintenance of the photosymbiosis in heterobranch sea slugs. A special focus was given to the role of the host's innate immune system in these associations. Moreover, this work also explored the stress response induced by starvation and abiotic factors such as light intensity and continuous darkness in sea slugs, and the implications on the photosymbiotic association. Lastly, it provides new genomic resources that serve as useful molecular tools to understand these unique and fascinating photosynthetic symbioses.

*Initiation of photosymbiosis: photobiont recognition*

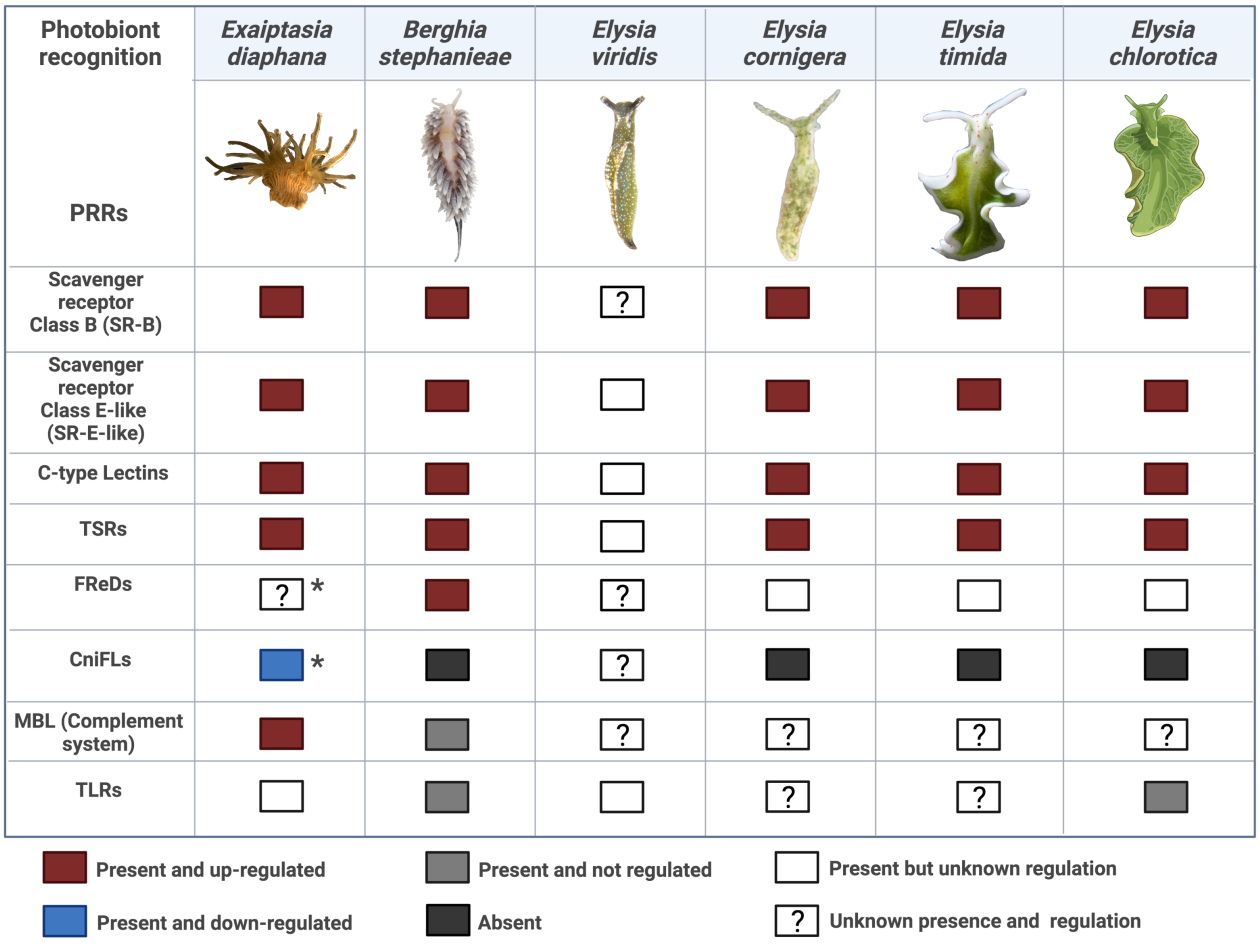



**Figure 1.** Summary of PRRs potentially involved in photobiont recognition in marine slugs. *E. diapahana* data serves as a reference (reviewed in Mansfield & Gilmore, 2019). Based on Chan et al. (2018); Melo Clavijo (2018); Melo Clavijo et al. (2020, 2022); Mendoza et al. (2023). The color code of the rectangles is based on the presence of the PRRs in transcriptomes or genomes (when available) and their regulation. Cnidarian ficolin-like receptors (CniFLs) also contain fibrinogen domain in combination with collagen and immunoglobulin domains, they were not classified as FReDs, but as an entire different group (*) (Baumgarten et al., 2015). Photos: *E. diaphana, B. stephanieae, E. viridis*: Jenny Melo; *E. timida, E. cornigera*: Heike Wägele. Created with BioRender.com.

A central finding of this study is that distantly related animals seem to recognize different symbionts using conserved mechanisms of the innate immune system. Whether the animal host engulfs an organelle or an entire organism, similar receptors are present and are highly expressed during the initial contact between the animal host cell and the potential symbiont (Melo Clavijo et al., 2020, 2022), at least at the transcriptional level. Scavenger receptors from the class B, class E-like, C-type lectins, and TSRs are candidates for the symbiont recognition in sacoglossans (Melo Clavijo et al., 2020), in cladobranchs (Melo Clavijo et al., 2022), and in photosymbiotic cnidarians (reviewed in Mansfield & Gilmore, 2019) (Figure 1). So far, no studies have examined the receptors potentially involved in the symbiont recognition in photosymbiotic bivalves or other dinoflagellate-bearing animals. Nevertheless, based on the hypothesis that innate elements are conserved across distantly divergent taxa, it would be expected that homologue receptors could be involved in the dinoflagellate recognition in bivalves and other animals hosting Symbiodiniaceae.

The present study is the first to highlight that fibrinogen-related domain-containing proteins (FReDs) seem to be relevant for the selective uptake of dinoflagellates, particularly for cladobranchs (Melo Clavijo et al., 2022). The FReDs are involved in non-self recognition, especially relevant for the pathogen recognition and defense against them (Hanington & Zhang, 2011; Adema, 2015; Gordy et al., 2015). This group of proteins is characterized by the fibrinogen domain at the C-terminal, followed by different domains at the N-terminal (Romero et al., 2011). Cnidarians have similar domain architectures, but they are classified as a separate group of proteins called cnidarian ficolin-like proteins (CniFLs) (Baumgarten et al., 2015). These CniFLs contain fibrinogen domain, immunoglobulin, collagen at the N-terminal, transmembrane regions, and in some cases whey acidic protein domain (WAP) (Baumgarten et al., 2015; van der Burg et al., 2016). However, more evidence is needed to confirm their involvement in the symbiosis onset. In gastropods, fibrinogen-containing proteins have the C-terminal fibrinogen domain followed by one or two immunoglobulin domains at the N-terminal and are classified as FREPs or IgSF-FREPs (Adema et al., 1997; Gorbushin et al., 2010; Adema, 2015). They have been identified and characterized only in Heterobranchia in the freshwater snail *Biomphalaria glabrata* (Say, 1818) (Adema et al., 1997), the sea hare *Aplysia californica* Cooper, 1863 (Gorbushin et al., 2010), and the marine gastropod *Littorina littorea* (Linnaeus, 1758) (Gorbushin & Borisova, 2015). Within heterobranchia, FReDs have been identified in the sacoglossan transcriptomes of the non-retention species *Placida dendritica* (Alder & Hancock, 1843), the StR species *Elysia cornigera*, and the LtR species *E. timida* and *E. chlorotica* (Melo Clavijo, 2018). However, expression analyses of these

PRRs need to be conducted to understand if they are involved in the onset of functional kleptoplasty. The fibrinogen-like proteins identified in *B. stephanieae* only had one fibrinogen domain and were annotated as fibroleukin or fibrinogen-like protein 2 (Melo Clavijo et al., 2022). These could be protein sequences that only contain this domain as in other mollusks, and thus would be assigned as FReDs (Adema, 2015). Alternatively, they could be incomplete sequences lacking the IgSF domain and would still belong to the IgSF-FREPs, similar to the FREPs 1, 5, 6, 8, 9, 10, and 11 identified in *Biomphalaria glabrata* (Hanington & Zhang, 2011). Due to their function as PRRs and their expression pattern, they are possible candidates in the initiation of the photosymbiosis and their involvement warrants further evidence. Additional studies targeting these proteins would be needed to clarify their evolution within Heterobranchia, and if they are present as IgSF-FREPs or only as FReDs in other cladobranchs. In the context of photosymbiosis, more research into the role of these PRRs in recognizing Symbiodiniaceae in species with stable symbiosis is essential. This also applies to the other PRRs aforementioned. Immunohistology assays and in situ hybridization (ISH) could clarify the location of these receptors within the animal cell. Combined with the gene expression at a specific time, this approach could reveal valuable information on the mechanisms of symbiont recognition.

In the symbiont recognition process, not only the host's PRRs are relevant, but also the symbiont's MAMPs. When the symbiont is an entire organism like Symbiodiniaceae, there is more evidence regarding the type of MAMPs that it produces, specifically the glycome. This information is crucial for their potential role in the host colonization. For instance, N-glycans seem to influence the speed and success of the host colonization by Symbiodiniaceae (Tivey et al., 2020). Further, the abundance of N-glycans present in Symbiodiniaceae cell surface has a direct effect on the uptake by the host (Tivey et al., 2020). For instance, D-galactose residues are more abundant in the cell surface of compatible symbionts like *Breviolum minutum* and *Cladocopium goreaui* than in incompatible and free-living species like *Fugacium kawagutii* (Tortorelli et al., 2022). Not only N-glycans abundance is relevant for the onset of symbiosis, but also the proportion of these glycans within a specific range. For example, low and high abundance of high-mannose glycans reduces colonization success (Lin et al., 2000; Wood-Charlson, et al., 2006; Tivey et al., 2020). Several studies support the interaction of Symbiodiniaceae glycans with the animal host lectins (reviewed in Davy et al., 2012). For instance, D-galactose residues interact with cnidarian host lectins like the SLL2 (Jimbo et al., 2000, 2005, 2013; Koike et al., 2004) and the CeCL (Jimbo et al., 2010). In particular, β-D-galactose residues are crucial for the recognition of suitable symbionts, and other

sugar residues like L-fucose, D-xylose, and D-galacturonic acid are likely involved in the photosymbiosis onset in cnidarians (Tortorelli et al., 2022). Similar glycan-lectins interactions may be also present between Symbiodiniaceae and cladobranchs.

In the case of functional kleptoplasty, where the symbiont is a stolen organelle, the MAMPs have been only hypothesized based on the chloroplast's cyanobacterial past. It is unclear if MAMPs are present in the chloroplasts, or if they interact with the sacoglossan host lectins (Melo Clavijo et al., 2020). To date, no study has examined the chloroplast glycome of any of the algae sacoglossan slugs feed on. Nonetheless, there is a potential lectin-glycan interaction based on the abundance and presence of galactolipids in the outer membranes of primary and secondary chloroplasts and the higher expression of lectins during the initial contact between the sacoglossan slug and the algae food source. Therefore, functional studies must be carried out from the perspective of both the animal host PRRs and the kleptoplasts.

*Photobiont tolerance and maintenance*

Identifying the receptors potentially involved in the symbiont recognition is the first step to understanding photosymbiosis in sea slugs. This study shed first light on potential mechanisms of photobiont recognition in sea slugs, but the subsequent steps leading to symbiont maintenance are still unclear. The first assumption is that recognition triggers phagocytosis of the potential symbiont. A phagosome would then engulf the symbiont. In intracellular symbioses, the phagosome serves as the membrane that connects the host and its symbiont, also known as symbiosome (Neckelmann & Muscatine, 1983; Hinde & Trautman, 2001; Kazandjian et al., 2008). However, the mechanisms behind the symbiosome formation are still poorly understood. In cladobranchs, for instance in *B. stephanieae,* a membrane surrounds Symbiodiniaceae (Figure 2D in Rola et al., 2022). As reported in other cladobranchs such as *Baeolidia moebii* Bergh, 1888 (formerly known as *Berghia major)*, species of *Melibe*, and *Pteraeolidia ianthina,* Symbiodiniaceae reside in vacuoles within the "carrier" cells of the digestive gland as the result of phagocytosis (Kempf, 1984). In some cladobranch species like *P. ianthina* and *M. engeli,* Symbiodiniaceae are located not only within the digestive glandular epithelium, but also in tubules and cisternae originated from the digestive gland (Rudman, 1981a; Wägele & Johnsen, 2001; Burghardt & Wägele, 2014). Carrier cells arrange closely together forming these fine tubules in *P. ianthina*. The membrane of these carrier cells surrounds three to five Symbiodiniaceae cells rather than a single cell (Wägele & Johnsen, 2001). This particular case shares structural similarities with the zooxanthellal tubular

system in photosymbiotic clams, where epithelial siphonal mantel cells line and form the tubules housing large numbers of Symbiodiniaceae cells extracellularly (Norton et al., 1992).
In Sacoglossa, the membrane matter is still debated. Several authors have reported the presence of the host phagosome membrane surrounding some kleptoplasts but not all, in *Elysia viridis*, *Costasiella ocellifera, E. timida* juveniles, and *Placida dendritica*; While other studies found that a phagosome membrane is absent and the kleptoplast is in direct contact with the animal cytosol, for instance in *E. timida* adults and *E. chlorotica* (reviewed in Wägele & Martin, 2014). These observations led to the hypothesis that the presence of a phagosome membrane is correlated with the kleptoplast digestion, where kleptoplasts within a phagosome membrane are quickly digested and kleptoplasts without this membrane are retained longer (Marín & Ros, 1993; Evertsen & Johnsen, 2009; Wägele & Martin, 2014; Martin et al., 2015; Schmitt, 2020). However, the apparent absence of the membrane in some kleptoplasts does not necessarily imply that it does not exist. During fixation and tissue processing for electron microscopy, the membrane can rupture. Other studies have confirmed the presence of such membrane tightly surrounding the kleptoplasts (e.g. Mondy & Pierce, 2003; Curtis et al., 2006). Even more enigmatic is what happens to the kleptoplasts in some slug species that consume *Vaucheria litorea*, like *E. chlorotica*. The chloroplasts of this xanthophyte alga are surrounded by four membranes, indicating a secondary endosymbiosis event. When they are phagocytized, these kleptoplasts lose two of their four membranes (Rumpho et al., 2001). It is still unknown why this occurs and whether it also the case in other sacoglossan species that also feed on *Vaucheria* and incorporate their chloroplasts, such as *Alderia modesta* (Lóven, 1844).

In cnidarians, once the symbiont is engulfed, several cellular mechanisms promote the maintenance of the symbiont within the host cell, establishing a stable symbiosis. Usually, early phagosomes mature into functional ones by fusing with lysosomes. Yet, cnidarian phagosomes containing healthy symbionts do not fuse with lysosomes, inhibiting maturation and leading to the symbiosis maintenance (e.g. Fitt & Trench, 1983; Fransolet et al., 2012). Thus, a crucial step in symbiont maintenance is to stop the phagosome maturation and prevent symbiont digestion (symbiophagy). Rab proteins mediate this process. In photosymbiotic cnidarians like *E. diaphana,* Rab5 marks phagosomes with healthy symbionts (Chen et al., 2004), while Rab7 labels phagosomes with damaged ones. The damage can be caused by heat or photosynthesis blockers like DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Chen et al., 2003). Similar to Rab7, Rab11 is only present in

phagosomes containing damaged symbionts and acts during the endosome recycling (Zerial & McBride, 2001; Chen et al., 2005) (Figure 2).

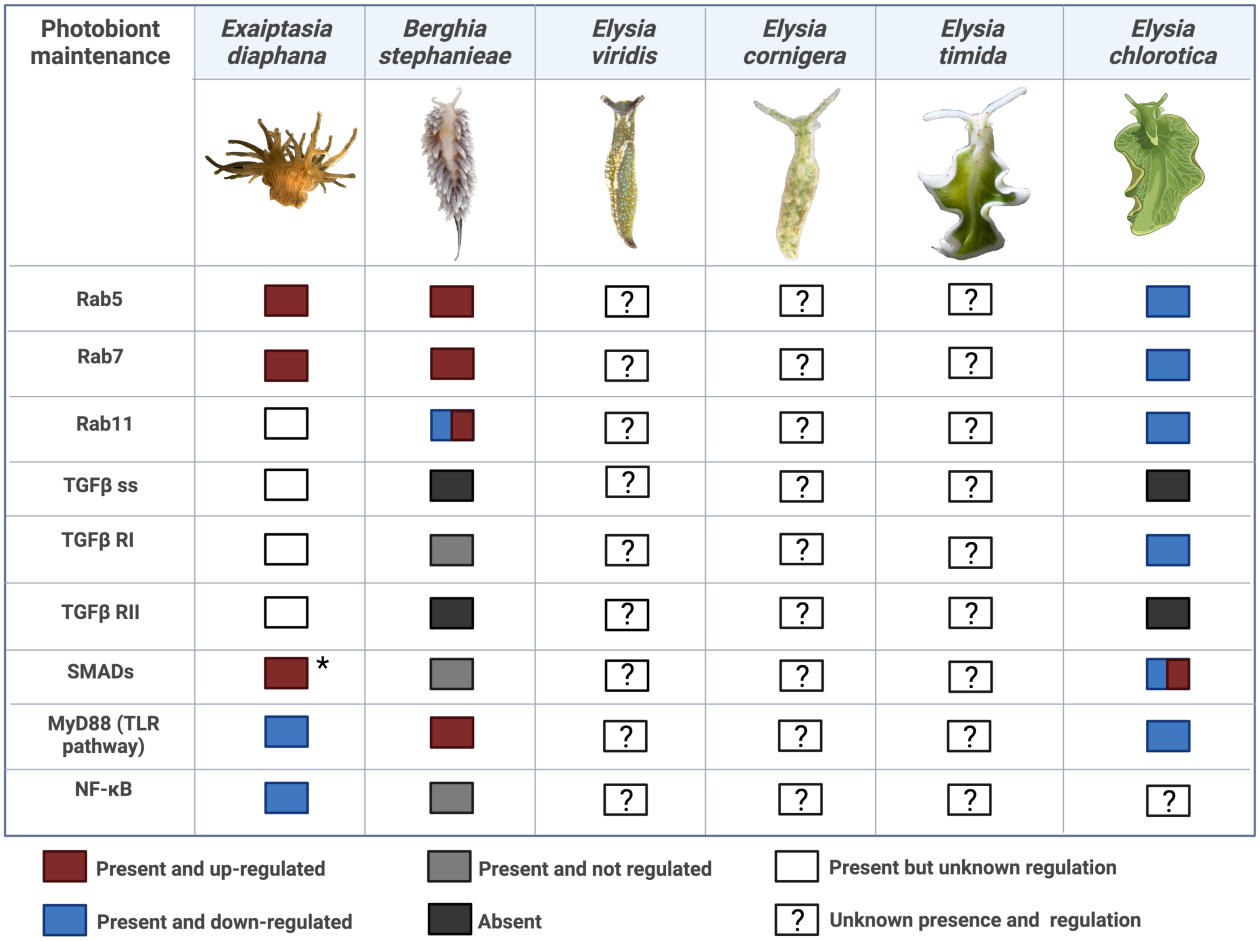



**Figure 2.** Summary of some of the players in photobiont maintenance in marine slugs. *E. diapahana* data serves as a reference (reviewed in Mansfield & Gilmore, 2019). Based on Chan et al., (2018); Melo Clavijo et al., (2022). The color code of the rectangles is based on the presence of the elements in transcriptomes or genomes (when available) and their regulation. Phosphorylation evidence (*) (Detournay et al., 2012). Photos: *E. diaphana, B. stephanieae, E. viridis*: Jenny Melo; *E. timida, E. cornigera*: Heike Wägele. Created with BioRender.com.

In sea slugs, the mechanisms related to the phagosome maturation are only beginning to be explored (Figure 2). In the unstable symbiosis between *B. stephanieae* and the dinoflagellate *Breviolum minutum,* Rab5, Rab7, and Rab11 are up-regulated together with lysosome proteins LAMP1 and 2 in the presence of the symbiont. This expression pattern indicates an ongoing symbiophagy, rather than an inhibition of the phagosome maturation (Melo Clavijo et al., 2022). Several possibilities can explain this expression pattern. For instance, the slugs cannot distinguish phagosome content in the long term and digest symbionts even if they are healthy. Moreover, *B. minutum* might be unable to change the chemical properties of the phagosome membrane, to interfere with the endosome

recycling, or to prevent the phagosome maturation and its own digestion. Many other intracellular microbial pathogens like *Leishmania donovani* (Laveran & Mesnil, 1903) Ross, 1903, *Tripanosoma cruzi* Chagas, 1909, *Mycobacterium* Lehmann & Neumann, 1896, and *Salmonella* Lignières, 1900 can selectively retain Rab5 and prevent Rab7 and Rab11 from marking their phagosomes inhibiting the fusion with lysosomes (Scianimanico et al., 1999; Duclos et al., 2000; Hashim et al., 2000; Machado et al., 2000). Similarly, apicomplexan parasites, the sister taxon of dinoflagellates (Janouškovec et al., 2010), are able to manipulate host's Rab functions and expression levels (Coppens & Romano, 2020). To date, it remains unknown how Symbiodiniaceae adapts to the highly acidic phagosome environment, and how it survives or evades the phagosome maturation. Likewise, there is no information about the inhibition of the phagosome maturation in cladobranch species with stable symbiosis.

In Sacoglossa, the expression of these Rab proteins has only been addressed in *E. chlorotica* juveniles, where there were not regulated in day 5 and 10 of development, but only down-regulated after seven days of feeding and development (Chapter 2.6 unpublished data, Chan et al., 2018). This could be based on the adaptions of symbionts like Symbiodiniaceae to persist within their host (e.g. chemical signals and modulation of the host phagosome machinery), which might be lacking in sequestered kleptoplasts. Similar to cladobranchs, the mechanisms to prevent kleptoplast digestion in Sacoglossa are unknown.

Another crucial step to ensure photobiont maintenance is the suppression of the host immune response against the photobiont (Mansfield & Gilmore, 2019; Jacobovitz et al., 2021) (Figure 5). In cladobranchs, this process has only been explored in *B. stephanieae* (Melo Clavijo et al., 2022). Contrary to stable symbioses in photosymbiotic cnidarians, *B. stephanieae* cannot suppress the immune attack against its symbiont *B. minutum*. Innate immune pathways involved in the symbiont tolerance in stable symbioses like TGF-β, TLR, NF-κB are either incomplete or not regulated in *B. stephanieae* (Melo Clavijo et al., 2022). In cladobranchs with stable symbioses, one would expect the opposite: an active immunosuppression response in the presence of Symbiodiniaceae to maintain the symbiotic relationship. However, expression studies are necessary to verify this hypothesis. In the sacoglossan LtR species *E. chlorotica,* the gene expression analysis of juveniles suggests this may be the case (Chan et al., 2018; Chapter 2.6 unpublished data). Here, genes related to the immune response were predominantly down-regulated in slugs that were feeding and developing. Also, the number of down-regulated genes was higher after seven days of feeding and developing than after only five days. This follows the idea of two stages of early development in *E.*

*chlorotica* proposed by Pelletreau et al., (2012), where the first five days are considered transient kleptoplasty, and the later phase (from the 7th day of development) is known as permanent kleptoplasty. Similar to unstable symbiosis in cladobranchs, the TGF-β pathway was not involved in kleptoplast immunotolerance; it was rather incomplete and not regulated (Chapter 2.6 unpublished data). However, contrary to unstable symbiosis, the signal transducer MyD88 from the TLR pathway was down-regulated preventing apoptosis and expulsion of kleptoplasts via vomocytosis (Jacobovitz et al., 2021) (Figure 5). Further innate immune pathways from the complement system or NF-κB activation have not been yet examined in any sacoglossan slugs.

Further, apoptosis can act as a post-phagocytic sorting process in cnidarians even at the early stages of development, where incompatible Symbiodiniaceae strains can be recognized by host PRRs, but are later removed by the host apoptotic response (Davy et al., 2012). Here, the gastrodermis exhibits high caspase activity, similar to the activity in some mollusks' digestive gland in response to parasite damage or environmental toxins (Sokolova, 2009; Kiss, 2010; Romero et al., 2015). In unstable cladobranchs, only one of the apoptosis activators, the adaptor protein Fas-associated death domain (FADD), was highly up-regulated in the presence of the symbiont (Melo Clavijo et al., 2022). But none of the downstream elements involved in apoptosis were regulated, for example the initiator caspases-8 and -9, the executioner caspases-3, -6, and -7 (McIlwain et al., 2013), or the transcription factor NF-κB. This lack of regulation suggests that the elimination of the symbiont is not done via apoptosis, at least at the transcriptional level (Melo Clavijo et al., 2022).
In sacoglossans, it seems that the apoptotic sorting process following phagocytosis is not carried out when the animals are feeding, which contributes to the retention of kleptoplasts (Chapter 2.6 unpublished data). Additionally, no evidence of apoptotic activity has been found in the digestive tissue of healthy adults (Pierce et al., 1999; Mondy & Pierce, 2003). Kleptoplasts are digested in animals feeding constantly after a food switch (Frankenbach et al., 2021). However, it is currently not possible to determine whether apoptosis or digestion is responsible for kleptoplast turnover. Only after prolonged starvation and at the end of the animal's life cycle, the apoptotic activity increases with the concurrent expression of an endogenous retrovirus (Pierce et al., 1999; Mondy & Pierce, 2003).

Moreover, the host's ability to cope with increased levels of ROS has been identified as an essential factor in maintaining the symbiont and hence the stability of the symbiosis (Lesser, 2006; Weis, 2008; Ishikawa et al., 2016; Rosset et al., 2021). ROS can be generated as a byproduct of the

symbiont's photosynthesis *in hospite* (Dykens & Shick, 1982; Niyogi, 1999; Richier et al., 2003; Lesser, 2006; Roth, 2014; Parrin et al., 2017). Comparative studies between symbiotic and non-symbiotic cnidarians show that the response to oxidative stress varies greatly. Symbiotic animals have a more diverse repertoire of antioxidant enzymatic isoforms than non- symbiotic ones (Richier et al., 2003; Furla et al., 2005). The expression and activity of these enzymes is increased compared to the aposymbiotic state (Dykens & Shick, 1982; Richier et al., 2005, 2006). Conversely, some anemones show decreased antioxidant activity in the symbiotic state compared to the aposymbiotic state (Rodriguez-Lanetty et al., 2006; Ganot et al., 2011; Lehnert et al., 2014; Oakley et al., 2016). Particularly for superoxide dismutases (SODs), this contradiction may be explained by the expression, activity, location, and specific function of different isoforms in the cnidarian host (Richier et al., 2003; Furla et al., 2005; Rodriguez-Lanetty et al., 2006). For instance, in some anemones, MnSODs and FeSODs are found in the mitochondrial of the symbiont and host endodermal cells (Furla et al., 2005), and their activity increase only in the symbiotic state (Richier et al., 2005; Rodriguez-Lanetty et al., 2006). Whereas other SODs like CuZnSOD are thought to be specific to the animal host (Furla et al., 2005), are located in both endoderm and ectoderm, and their activity decreases in symbiotic state (Richier et al., 2005; Rodriguez-Lanetty et al., 2006). It has been suggested that the symbiont may be producing these enzymes as an additional antioxidant defense for the holobiont, explaining why SOD activity in the host decreased in the symbiotic state in some cnidarians (Rodriguez-Lanetty et al., 2006; Oakley et al., 2016). This hypothesis hasn't been fully verified yet, not for SODs or for the other antioxidants that exhibit this expression in cnidarians.

The expression of ROS scavengers radically changes in the early stages of the symbiosis breakdown, where the animal host increases its antioxidant defense (Richier et al., 2006; Weis, 2008; Louis et al., 2017). There are also differences in ROS scavenging mechanisms in unstable and stable symbioses. Species with a stable symbiosis, like *Hydra viridissima* Pallas, 1766, scavenge ROS more effectively than unstable symbiotic species, such as *Hydra vulgaris* Pallas, 1766 (Ishikawa et al., 2016). Similarly, LtR sacoglossan species seem to cope with elevated ROS in their cells more efficiently than StR species (de Vries et al., 2015). This activation of antioxidant defense has been evidenced since early stages of the development in the presence of newly acquired kleptoplasts in *E. chlorotica* (Chan et al., 2018; Chapter 2.6 unpublished data). Unstable photosymbiotic cladobranchs show an elevated antioxidant activity in the presence of the symbiont during feeding (Melo Clavijo et al., 2022), which follows the hypothesis that the host first

establishes the endosymbiosis and the ROS tolerance emerges later (Kawano et al., 2004). In this line, maintaining a symbiosis can become costly because the symbiont might be a burden, as seen also in *H. vulgaris* (Ishikawa et al., 2016). To date, no comparison of the efficiency of the antioxidant defense has been made between unstable and stable photosymbiotic cladobranch species. Whether species with stable symbioses are more resistant to oxidative stress and have a higher tolerance to ROS remains to be seen.

Finally and yet equally important for the maintenance and stability of the symbiosis, is the role of other host-associated microorganisms (microbiome), aside the photobionts (reviewed in Bourne et al., 2016; Garrido et al., 2021). Especially the host-associated bacterial component known as bacteriome has been the focus of recent research. In these tripartite relationships (photobiont-host-bacteriome) each partner influences the ability of the others to function and survive (Bernasconi et al., 2019). In cnidarians, the bacteriome diversity may be affected by abiotic factors (temperature, pH, salinity, and organic compounds) (Ahmed et al., 2019) and by the presence of photobionts (Bourne et al., 2013, 2016; Röthig et al., 2016; Bathia et al., 2022; Xiang et al., 2022). A change in the symbiotic state significantly varies the bacteriome composition and its interaction with the photobiont. For example, dimethylsulfoniopropionate (DMSP) is a sulfur compound that mediates the interactions between host, photobiont, and microbiome (Garrido et al., 2021). The photobiont, the coral-host, and even some bacterial symbionts (members of the Alphaproteobacteria) can generate DMSP, a crucial substrate for bacterial sulfur cycle (Raina et al., 2013; Kuek et al., 2022). But in some cases, the absence of the photobiont, as well as thermal stress, may cause a shift in the DMSP-degrading bacterial communities related to the available DMSP concentration in the host (Van Alstyne et al., 2009; Frade et al., 2015; Röthig et al., 2016). Opportunistic pathogen populations like *Vibrio coralliilyticus* Ben-Haim et al., 2003 may increase, while beneficial symbionts such as *Endozoicomonas* Kurahashi & Yokota, 2007 decrease (Maher et al., 2022).

Even though there has been an increase in microbiome studies in sea slugs (e.g. Doepke et al., 2012; Abdelrahman et al., 2021; Zhukova et al., 2022; Rosani, 2022), it is still only the beginning in the context of photosymbiosis. For instance, there is evidence that nudibranch predators and their cnidarian prey share similarities in the bacterial composition present in the cerata tips and tentacles, respectively (Doepke et al., 2012). This interrelationship can be further explored in species that engage in a photosymbiosis and have restricted diet preferences. To date, only one study has addressed the bacteriome composition in photosymbiotic cladobranchs (Sickinger et al., 2022).

In Sacoglossa, only a few studies have shown the bacterial diversity present in these slugs (Devine et al., 2012; Davis et al., 2013; Mahadevan & Middlebrooks, 2020). So far, the role of the microbiome in sea slug photosymbiosis and kleptoplasty is not yet fully understood.

*Symbiotic vs aposymbiotic: how to generate symbiont-free hosts*

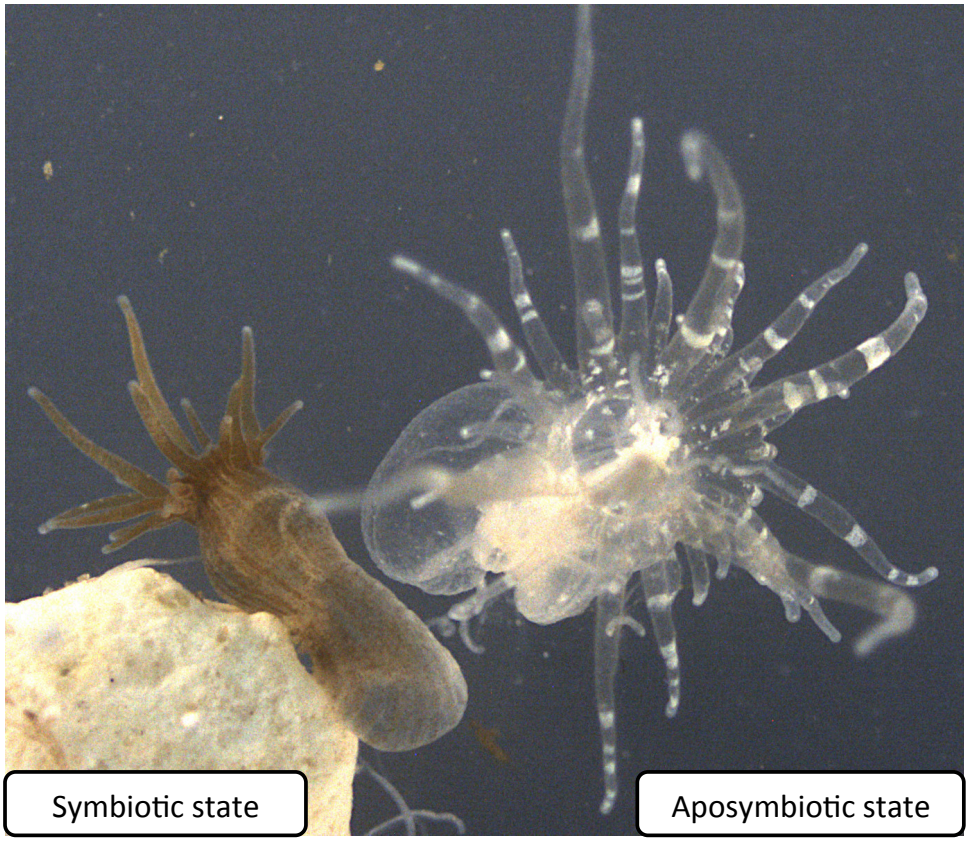



**Figure 3.** Comparison between symbiotic anemones and aposymbiotic ones. Symbiont-free anemones were obtained following Matthews et al., (2016) menthol treatment protocol.

The different aspects of the photosymbiosis are usually studied comparing symbiotic individuals to aposymbiotic ones. Thermal treatments like heat-shock or cold-shock, menthol induced bleaching, continuous darkness, and/or chemical inhibition of photosynthesis with DCMU are methods often used to generate symbiont-free individuals (Wang et al., 2012; Dani et al., 2016; Matthews et al., 2016; Schmidt et al., 2022). Menthol treatment has proven to be the fastest and most effective in terms of less mortality and better host fitness in cnidarians (Matthews et al., 2016) (Figure 3).

Because most sea slugs acquire their photobionts via food ingestion, the easiest way to generate aposymbiotic individuals is to starve them. Alternatively, they can be fed with aposymbiotic prey,

but this requires producing and maintaining symbiont-free prey. This approach can be applied to photosymbiotic cladobranchs, and it has been successfully used in *Berghia stephanieae* adults (Leal et al., 2012; Monteiro et al., 2019; Melo Clavijo et al., 2022), and cultured juveniles that reached adulthood and produced offspring (García Galera, 2021) (Figure 4). But, feeding on aposymbiotic prey may also induce a shift in the composition of fatty acids in the slug and its offspring. For example, embryos from slugs fed with aposymbiotic prey had lower content of docosahexaenoic acid (DHA) and higher levels of alpha-linolenic acid (ALA) compared to embryos of symbiotic-fed parents (Leal et al., 2012). This effect might be only relevant depending on the question one seeks to answer, but it is worth to take it into consideration. For instance, questions regarding the nutritional impact of fatty acids on the development, fitness, and foraging behavior of the offspring from parents fed on bleached anemones (Silva et al., 2023). This matter may be extended to trophic ecological questions about the impact of prolonged consumption of bleached prey on the predator population growth (e.g. Poor quality diet with low DHA content may slow or even stop population growth in crustaceans like *Daphnia* (Martin-Creuzburg & von Elert, 2009)).

In comparison, obtaining aposymbiotic individuals of sacoglossan slugs can be more challenging. One way is to use juveniles post-metamorphosis as aposymbiotic state, but they have to feed constantly to develop and survive. Thus, any differences seen between symbiotic (kleptoplastic) and symbiont-free state can be attributed to the development and the constant feeding and not solely to the photobiont presence (Chan et al., 2018; Chapter 2.6 unpublished data). In this context, the polyphagous *Elysia viridis* becomes a very useful model, since it can be classified as non-incorporation (aposymbiotic), StR, or LtR, depending on what it feeds on (Rauch et al., 2018). The common alternative methods have been the photosynthesis inhibition of the kleptoplasts with DCMU or monolinuron, starvation, and continuous darkness, but these come with additional effects that are just being explored.

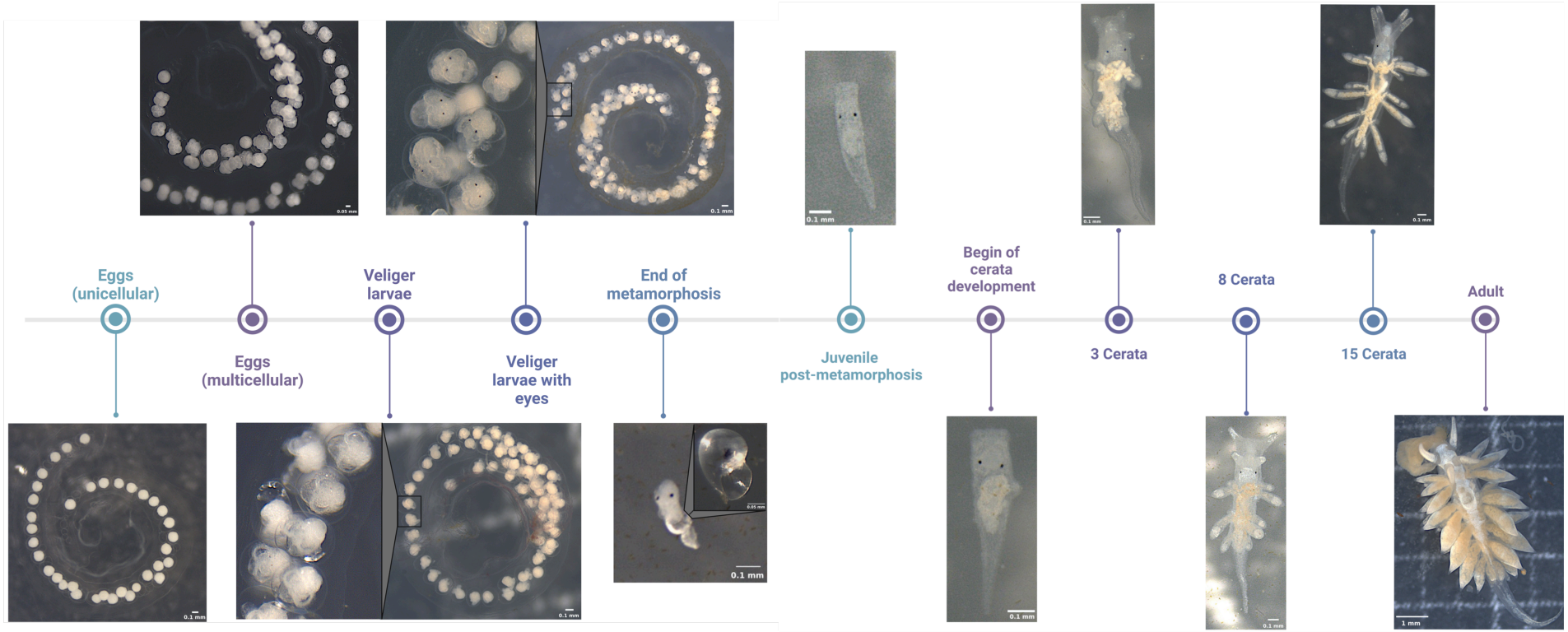



**Figure 4.** Development of aposymbiotic *Berghia stephanieae*. Symbiont-free slug were obtained by feeding individuals with aposymbiotic anemones. The anemones were obtained following the menthol treatment protocol proposed by Matthews et al. (2016). These aposymbiotic slugs reached maturity and were able to reproduced. The eggs obtained were further cultivated. The eggs masses are formed by two mucouid layers: one capsule that surrounds each embryo and another gelatinous layer that protects all the capsules. The time line shows the development of the slug (Kristof & Klussmann-Kolb et al., 2010) and it starts with the egg mass containing unicellular embryos. Within hours the embryos start to divide asynchronously. This can be observed in the second point of the time line, where some embryos are still in one-cell stage, while others already are in 4-cell division stage (See Appendix for a time-lapse). The third point in the time-line corresponds to the veliger larvae stage, detectable by the presence of the larval shell and the velum. This is followed by the eye and foot development. Afterwards, the larvae hatch and are ready to leave the shell, marking the end of the metamorphosis stage. In this point the early juveniles start the benthic life style (juveniles post-metamorphosis). At this point the juveniles are provided with pieces of aposymbiotic anemones. After a few days the cerata start to develop. The ramification of the digestive system can be observed. The adult specimen in the figure is feeding on pieces of aposymbiotic anemone.

*What happens to the animal host during starvation?*

In photosymbiotic cnidarians, starvation induces symbiophagy and autophagy to compensate for nutritional deficiencies (Chera et al., 2009; Dani et al., 2016; Klionsky et al., 2021). These processes are considered common mechanisms to control the photobiont populations, but are enhanced during starvation and can be stopped after food reintroduction (Davy et al., 2012; Dani et al., 2016). The main survival strategy in food depletion conditions seems to be autophagy, which occurs independently of the photobiont presence (Chera et al., 2009; Klionsky et al., 2021).

When studying photosymbiotic sea slugs, starvation alone, or combined with darkness, are commonly used to gain insight into the nutritional support that the photobiont may provide to its host. Yet, little is known about what happens in the slug during starvation, and even less when they starve under darkness. The response to food depletion also changes depending on the stability of the symbiosis. For instance, cladobranch species that form a stable symbiosis are able to survive, maintain their biomass and photosynthetically active and dividing Symbiodiniaceae cells. The slugs still lay eggs during starvation periods, most likely due to the photobiont contribution. Although, it is still unclear how nutrient exchange would occur (Burghardt et al., 2005; Burghardt & Wägele, 2014). Whether these eggs can further developed and reach reproductive maturity has not yet been explored. In contrast, unstable symbiotic species like *B. stephanieae* lose biomass and their symbionts in short time during starvation periods. Even though they lay eggs, the majority of these are not fertile. Such is also the case of sacoglossan sea slugs that lose biomass or length during starvation, regardless on the retention form (StR or LtR) (Hinde & Smith, 1975; Green et al., 2005; Casalduero & Mundain, 2008; Yamamoto et al., 2013; Christa et al., 2014; Cartaxana et al., 2017; Donohoo et al., 2020; Frankenbach et al., 2023), which is a sign that the contribution of the kleptoplast to the host is simply not enough to maintain nor gain biomass (Rauch et al., 2017a).

In general, starvation can have pro-oxidant effects and may cause major oxidative damage (e.g. Robinson et al., 1997; Domenicali et al., 2001; Morales et al., 2004; Chen et al., 2023). In particular, the response at the transcriptional level of starved animals like cnidarians and slugs, is very similar to the stress response induced by abiotic factors like light, acidification, or temperature (Levy et al., 2016; Melo Clavijo et al., 2022). This stress response doesn't change if the slug feeds on symbiotic or aposymbiotic prey before the starvation period (Melo Clavijo et al., 2022). So far, the effect of starvation at the molecular level has not been studied in stable symbiotic cladobranch species, nor the combined stress of starvation and abiotic factors like light intensity, temperature, or continuous darkness.

Darkness affects the survival of stable symbiotic cladobranch species despite being fed (Burghardt & Wägele, 2014), and may cause symptoms of oxidative stress in unstable symbiotic species even if they are feeding (Chapter 2.6 unpublished data). A shift to heterotrophy of the dinoflagellate symbiont might be the cause of these effects in cladobranchs hosts (Burghardt et al., 2005). To date, the effect of darkness during feeding in sacoglossan slugs has not yet been investigated.

Starvation and darkness have synergistic effects in the slug host, meaning the stress response heightens. Here, the photobiont is not only affected by photosynthesis inhibition, but also by the host stress response of symbiophagy and autophagy induced by starvation. The photosynthetic activity of the photobiont (Symbiodiniaceae and kleptoplasts) and the abundance in the slug host decreases drastically in dark starved individuals (Burghardt et al., 2005; Frankenbach et al., 2023). Cladobranch slugs starved in darkness shrink much faster, become paler, develop local swellings, lose more cerata, and are less active than the ones starved in light. These effects are much stronger in juveniles than in adult individuals (Burghardt et al., 2005). Similar effects are seen in sacoglossan sea slugs, where starvation in darkness induces an energy metabolic change, a decrease in protein import into mitochondria, and an increase of lysosomal abundance (Frankenbach et al., 2023). As a response to starvation, authophagy in the digestive gland is triggered in sacoglossan slugs (Laetz et al., 2017b), as seen in other mollusks that can withstand fasting for months (Klionsky et al., 2021), but it is more dramatic under darkness (Frankenbach et al., 2023).

*New genomic resources available of photosymbiotic slugs*

Genomic resources such as genomes and transcriptomes are vital tools to study many aspects of the photosymbiosis. Up to 2018, only 33 of these resources were publicly available of different photosymbiotic animals; one of them belongs to photosymbiotic cladobranchs and 5 to sacoglossan slugs (Melo Clavijo et al., 2018). To date, many more have been generated including the ones in this study (Maeda et al., 2021; Melo Clavijo et al., 2021; Frankenbach et al., 2023; Melo Clavijo et al., 2022; Mendoza et al., 2023). Transcriptomes can provide insight of the genes actively expressed in a certain tissue of an organism occurring at a specific time. Thus, if a certain gene is not present in the transcriptome does not necessarily mean the gene is not encoded in the genome of the organism. This doubt can be resolved if the genome of the certain species is available, but as mentioned before this is not the case in many sea slugs involved in photosymbiosis or kleptoplasty. Also, conclusions can be drawn only at the transcriptional level, which means the regulation might be happening in other stages pre- transcription, post-transcriptional, posttranslational

(phosphorylation, ubiquitination, acetylation), epigenetically for instance by methylation (Feder & Walser, 2005).

To date 8 mitochondrial genomes belonging to Cladobranchia are publicly available (Sevigny et al., 2015; Karagozlu et al., 2016a; Dinh et al., 2019, 2020; Melo Clavijo et al., 2021; Galià-Camps et al., 2023; Mizobata et al., 2023), but only two of photosymbiotic species. Whereas 9 mitogenomes of Sacoglossa have been published (Grande et al., 2008; Rumpho et al., 2008; Medina et al., 2011; Fan et al., 2013; Karagozlu et al., 2016b; Greve et al., 2017; Rauch et al., 2017b; Frankenbach et al., 2023). The mitochondrial genomes of Cladobranchia and Sacoglossa are very similar in size (roughly 14Kb) and in gene arrangements like other heterobranch mitogenomes (Wägele et al., 2008). Mitogenomes are useful tools not only to infer phylogenetic relationships, but also to study further effects of starvation stress in symbiosis, and the relationship between photobionts and host mitochondria (Rauch et al. 2017b).

**Outlook**

Research on photosymbioses in heterobranch slugs, specifically regarding the molecular events that take place to establish and maintain this association is in its infancy. This study provides a reference framework about how the initial contact between host and symbiont might occur, specifically which candidate genes might be involved in this process. Further, which immune pathways seem to be relevant in the establishment and the maintenance of the photosymbiosis. This work offers molecular tools like transcriptomes, mitogenomes, and a sorted list of candidate genes as reference for targeted functional studies at the cellular and molecular level.

This work is only the beginning, as many questions remain unexplored and many others emerge from this starting point. Future research could focus on pivotal questions regarding the metabolic exchange between the slug and the photobiont, particularly for the cladobranch system: How much photosynthetically fixed carbon is translocated to the animal host? In what forms? How is it translocated (control mechanisms), and what is the contribution in the nutrition, survival, and fitness of the slug? Further topics need attention, such as the role of the microbiome, the part mitochondria play in the photosymbiosis, the autophagy process during starvation in plastid/symbiont bearing slugs, and the effect of heterologous Symbiodiniaceae genotypes, as well

as abiotic stressors like temperature and acidification in the photosymbiotic and kleptoplastic association.

In this work the focus was more towards the animal host, but in the photosymbiosis two other players are vital for the stability of the association: the photosynthetic symbiont and the rest of the microbiome (protists, endolitic algae, eubacteria, archaea, and viruses). Further studies could focus on the symbiont perspective like its contribution and benefit in the association, and also the role of the microbiome in the initiation and maintenance, and the effect of its alteration in the photosymbiotic association.

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# Appendix

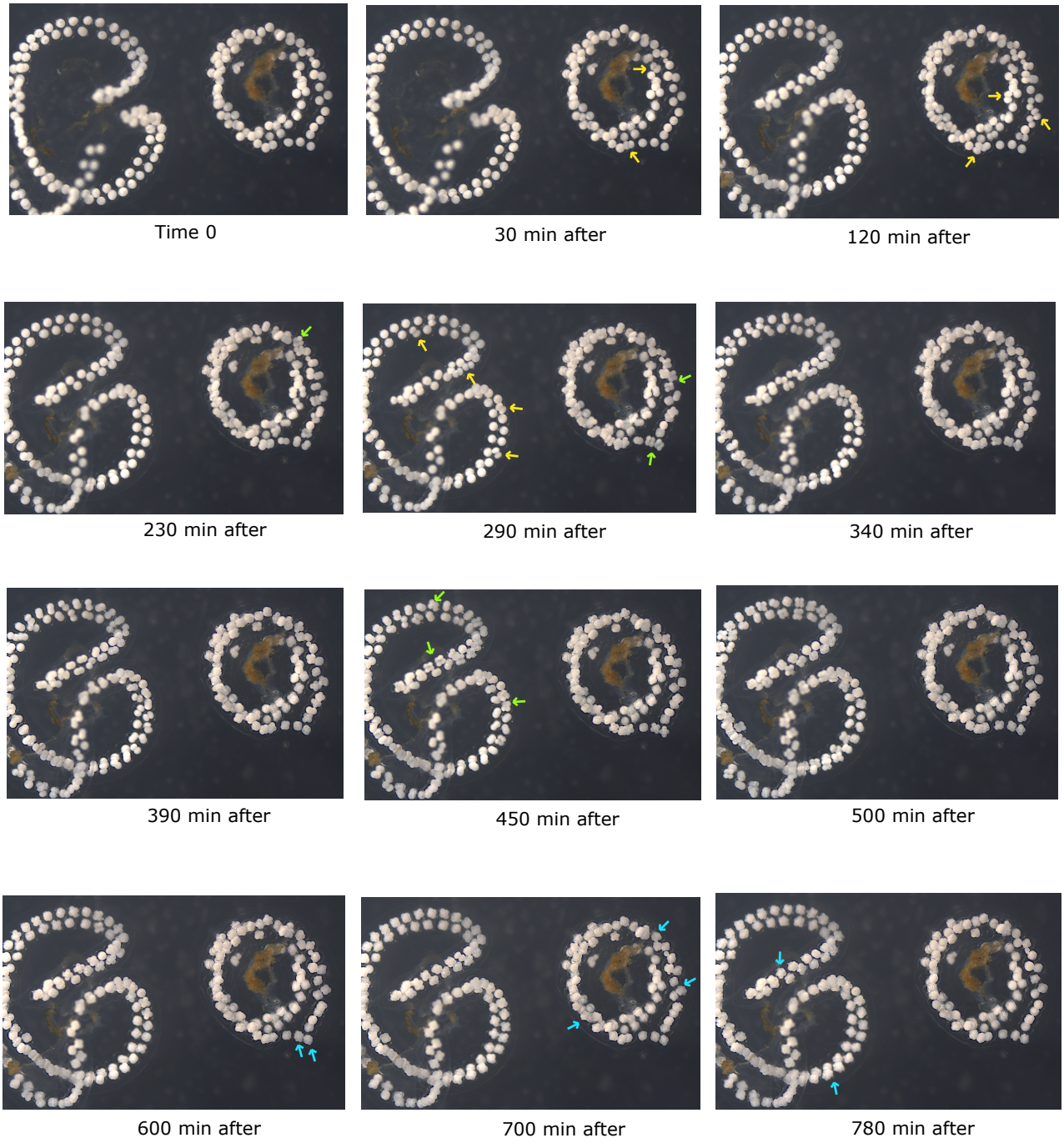


**Figure 1.** Asynchronous embryonic development of *Berghia stephanieae*. The time-lapse shows two egg masses with embryos dividing at different time. Yellow arrows point to 2-cell division stage. Green arrows show 4-cell division stage. Blue arrows indicate multicellular stage, where the divisions are difficult to count.

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