



Unraveling the *Phidiana* paradox: *Phidiana lynceus* can retain algal symbionts but its nocturnal tendencies prevent benefits from photosynthesis

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Abstract

Numerous marine invertebrates form symbiotic relationships with single-celled algae, termed “photosymbioses”, and the diversity of these interactions is likely underestimated. We examined *Phidiana lynceus*, a cladobranch sea slug that feeds on photosymbiotic hydrozoans. We assessed its ability to acquire/retain algal symbionts by examining specimens in starvation, finding that *P. lynceus* is able to incorporate and retain symbionts for up to 20 days. Examining body size during starvation revealed that *P. lynceus* does not receive enough energy from hosting symbionts to maintain its body mass let alone grow. Intact symbionts were still present in deceased specimens, indicating that *P. lynceus* does not digest all of its symbionts, even when starving to death. We also examined slug behavior in the field and lab to determine if it seeks light to facilitate photosynthesis, which could provide energetic and oxygenic benefits. In the field, slugs were always observed hiding under stones during the day and they displayed light avoidance in the lab, suggesting this species actively prevents photosynthesis and the benefits it could receive. Lastly, we measured their metabolic rates during the day and night and when treated with and without a photosynthetic inhibitor. Higher metabolic rates at night indicate that this species displays nocturnal tendencies, expending more energy when it emerges at night to forage. Paradoxically, *P. lynceus* has evolved all of the requisite adaptations to profit from photosymbiosis but it chooses to live in the dark instead, calling into question the nature of this symbiosis and what each partner might receive from their interaction.

Keywords Photosymbiosis · Cladobranchia · Nudibranchia · Unstable symbiosis · Metabolic rate · Phototaxis

1 Introduction

Since the discovery of animal-algae symbioses (termed photosymbiosis), researchers have sought to understand why these partnerships evolved (Stanley Jr 2006; Rumpho et al. 2011; Melo Clavijo et al. 2018). Stony corals are probably the most famous group known to form photosymbioses and their relationship is generally considered to be mutualistic in that the coral host offers a protective and suitable environment for single-celled algae from the family Symbiodiniaceae (called zooxanthellae when *in hospite*), while

the zooxanthellae produce metabolites and oxygen for the animal host via photosynthesis (Davy et al. 2012). In return, the algal cells receive nitrogenous waste products from the corals and carbon dioxide (Trench 1993; Yellowlees et al. 2008). Recent findings, however, suggest that some corals feed upon their sequestered zooxanthellae, calling into question the mutualistic nature of this relationship (Wiedenmann et al. 2023). Other non-coral photosymbioses (e.g. some species in Porifera, Mollusca, etc.) have not been investigated in as much depth and it remains unclear whether these relationships are mutualistic, or commensal, or parasitic/predator-prey.

Numerous molluscan species also form symbiotic relationships with zooxanthellae. Increasingly, researchers are focusing on sea slugs (Nudibranchia, Gastropoda) due to the diverse adaptations they exhibit that allow some species to incorporate zooxanthellae into their own digestive tissues and remain there for varying lengths of time (Rola

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et al. 2022). The amount of time symbionts can be retained inside a slug's digestive tissues and whether or not each member benefits from the symbiosis depends on the slug species and potentially the zooxanthellae species. Kempf (1991) characterized nudibranch/zooxanthellae symbioses in six types ranging from non-symbiotic (the cnidarian and algae are digested as prey) to mutualistic (a long-term interaction between zooxanthellae and slugs that facilitates nutrient exchange between the two partners and where the slug has "specialized cells or organs for symbiont maintenance". These mutualistic symbioses are often referred to as "stable" symbioses and found in nudibranch species such as *Melibe engeli* and *Phyllodesmium briareum* (Burghardt and Wägele 2014). Both species are able to grow and maintain their reproductive capacity when deprived of cnidarian prey, indicating they receive enough nutrition from these symbionts to continue metabolic function. Unstable photosymbioses comprise relationships where a slug can maintain zooxanthellae for a few days to a few weeks before being expelled or digested by the slug and nutrient exchange is not always clear (Types 3 to 5 in Kempf 1991). The energetic benefits these slugs could receive from this interaction are debated (reviewed in Rola et al. 2022). Cladobranch species that directly digest or expel zooxanthellae are known as non-retaining, and are thus not considered photosymbiotic.

Answering evolutionary questions about the potential gain and loss of photosymbiotic abilities in cladobranch slugs is currently impossible due to the limited number of species that have been examined. For example, the family Facelinidae Bergh 1889 contains both photosymbiotic (*Pteraeolidia semperi*) and non-photosymbiotic (*Cratena peregrina*) (Rola et al. 2022), yet these two species are the only two facelinid species in which photosymbiotic

capacity has been examined. This constitutes < 1% of facelinid listed in the World Register of Marine Species (WoRMS 2023). Since many facelinid species feed on photosymbiotic cnidarians which provides access to symbiotic algae (Goodheart et al. 2016), and have brown coloration in their digestive gland tissues which could be due to incorporated symbionts containing photosynthetic pigments (Rudman 1981; Carroll and Kempf 1990), the number of photosymbiotic facelinids might be vastly underestimated.

To further understand photosymbiosis in cladobranch species, we examined *Phidiana lynceus* Bergh 1867, a whitish and transparent slug that contains brown coloration throughout its digestive tissues, which could indicate that it forms a photosymbiotic relationship with zooxanthellae. To date, nothing has been published about its specific diet or photosymbiotic capacity. *Phidiana lynceus* has, however, been filmed feeding on the hydrozoan *Myrionema amboinense* (Coral Morphologic 2010), which is photosymbiotic (Fitt 2000), indicating that the slug has access to symbionts via at least one species of cnidarian prey (Fig. 1). We first determined if the dark coloration in the cerata (dorsal appendages containing digestive gland tissue) of *P. lynceus* are zooxanthellae by looking for chlorophyll fluorescence. We then examined their ability to retain the symbionts they acquire by placing specimens in starvation and measuring (a) the amount of time they retain symbionts, (b) if they gain or lose biomass over that time since growth in starvation would indicate their energetic needs are met by the symbionts, as is observed in nudibranchs capable of stable photosymbioses (Burghardt and Wägele 2014), and (c) the duration they can survive without access to cnidarian prey. We then examined specimens throughout the starvation period by imaging chlorophyll fluorescence to determine if

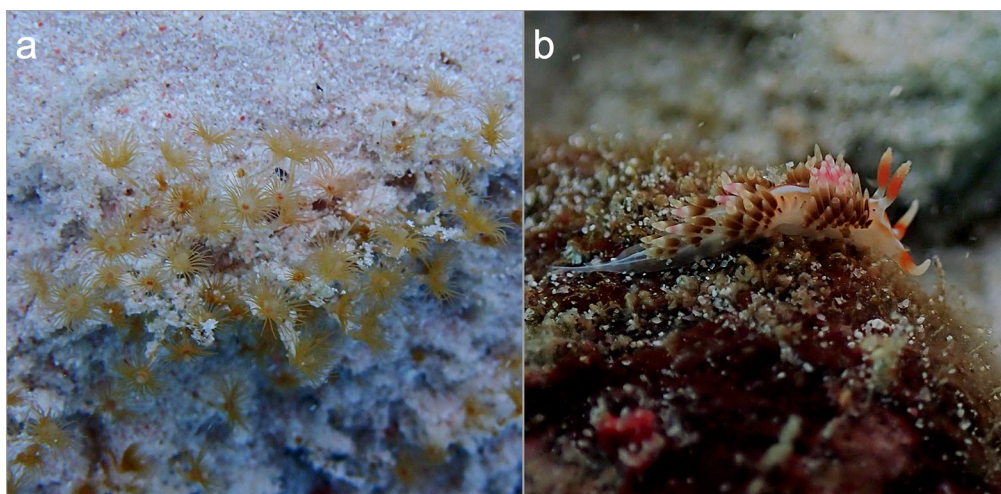


Fig. 1 *Myrionema amboinense* and *Phidiana lynceus* in the field. **a** *Myrionema amboinense* was only observed inhabiting the upper facing side of the stones in Piscadera Bay. Individuals were < 1 cm tall from the base of the stolon to the tips of the outstretched tentacles,

and its polyps were ~2–4 mm in diameter, when the tentacles were outstretched. **b** *Phidiana lynceus* was found on the underside of the rocks covered in *M. amboinense*, and ranged in size from ~0.3–1.5 cm in length

they show signs of symbiont digestion (i.e. degraded symbionts and/or decreasing symbiont density). We also examined their behavior in the field and in the lab, to determine if they display positive phototaxis which could indicate they seek out light, allowing their symbionts to photosynthesize, or if they display negative phototaxis, limiting the light available for photosynthesis. In the lab, phototaxis was tested using both living rocks (the same rocks on which they were observed inhabiting in the field) and sun-bleached rocks to remove the possibility that any movements toward the rock were actually foraging behavior driven by the smell of prey. Lastly, we examined their metabolic rates (oxygen consumption) when exposed to light and to darkness, when treated and not treated with a photosynthetic blocker, to determine if they are more active during the day or night and if this activity could be correlated to the ability to photosynthesize. Together, these investigations demonstrate a comprehensive look at photosymbiotic capacity and behavior in a previously uninvestigated facelinid sea slug.

2 Materials and methods

2.1 Specimen collection and husbandry

Thirty-five specimens were collected in Piscadera Bay, Curaçao (12°07'18.8"N 68°58'10.8"W) in November and December of 2022 by flipping over rocks on a sandy flat filled with coral rubble and stones at 1 - 3 m depth. All specimens were transported to the wet lab at Caribbean Research and Management of Biodiversity Institute (CARMABI) where they were housed separately to avoid cannibalism and other aggressive behaviors (as observed in Sales et al. 2019). Partial water changes were conducted every other day with new seawater from Piscadera Bay. Slugs were provided with indirect, full-spectrum lighting at an intensity of $700 \mu\text{mol m}^{-2}\text{s}^{-1}$, which was measured with a SQ-500-SS Full-spectrum Quantum sensor attached to a 30 cm cable and microCache Bluetooth micro-logger (Apogee Instruments Inc., USA). This light intensity was chosen based on measurements taken in the field where *P. lynceus* were naturally found, using the same light meter enclosed in a water-tight camera flash housing (Olympus PFL-E01 underwater housing for FL36 flash) as detailed in Burgués Palau et al. (2024). Each tank was provisioned with a small piece of sun-bleached coral rubble (~2 cm diameter) from the beach to provide shelter yet prevent feeding, so the amount of time they are able to withstand starvation and the duration they are able to retain symbionts could be assessed.

2.2 Symbiont retention

At the end of each of the starvation time points (0, 4, 8, 12, 16, 20 days), 1 to 2 specimens were photographed using an Olympus Tough TG-6 camera (Olympus Worldwide) before fixation in 4% formaldehyde and transported to the University of Groningen for imaging. During the fixation process, every specimen autotomized cerata, which were then collected and mounted on slides for imaging using a Nikon Eclipse E800 epi-fluorescent microscope with a custom filter cube that had a peak excitation at 400–440 nm, a dichroic at 515 nm, and a long-pass emission at 610 nm (Chroma, USA). Image settings were standardized for each magnification in order to accurately compare any potential loss in symbiont abundance over time. Violin plots using the *ggplot2* package (Wickham 2016) in RStudio (v. 2023.03.0+386, Posit Team 2023) were made to demonstrate the distribution in the longevity of all the specimens. The mean age reached and standard deviations were also calculated in RStudio.

2.3 Phototaxis in field and lab

To examine positive or negative phototaxis, we placed individual specimens ($n=6$) in a rectangular tray (40 cm x 60 cm) and provided a gradient of Photosynthetically Active Radiation (PAR) ranging from 0 to $1,396 \mu\text{mol m}^{-2}\text{s}^{-1}$ using 30 W full spectrum LED lamps, (OSRAM GmbH, Germany), according to the protocol described in Burgués Palau et al. (2024). This range matches the range of light intensities recorded at the collection site, on around and below the coral rubble, as measured by Burgués Palau et al. (2024). All of the data for this experiment was collected in one afternoon on the day after specimens were collected to allow for 24 h of acclimation to laboratory conditions. The trays were divided into cells measuring 2 cm x 2 cm. A light intensity value was assigned to each cell using the 500-SS Full-spectrum Quantum meter. Every tray was supplied with two air hoses, on opposite sides of the tray, constantly oxygenating and mixing the seawater at room temperature (26 °C). Each tank was provided with a freshly collected piece of coral rubble that was covered with algae and hydrozoans from the collection site (hereafter termed “living” stones). These stones were placed on the darker side of the tray to create a shelter under which specimens would be exposed to the dark (no light penetrated through the stone). In order to avoid measuring the effects of relocation stress, each slug was placed in the center of the tray with the lights off for five minutes. After this acclimation time, the lights were turned on and the initial position of the slug was recorded (position at time zero) together with its corresponding light intensity in that position. The position

was recorded every five minutes for a period of 30 min, according to the protocol established by Burgués Palau et al. (2024), which demonstrated that similarly sized sea slugs are capable of crossing the tray in < 10 min when trying to limit their light exposure. After 30 min, the light was turned off and placed on the opposite side of the tray to control for any effect of the lamp's position in the tray. The experiment was repeated following the same aforementioned procedure. Each tray was thoroughly scrubbed in between each new individual and test. The final position after every 30-minute trial was considered to be their preferred light intensity.

Trials were also conducted with similarly-sized pieces of sun-bleached coral rubble (hereafter called “dead” stones) instead of living stones, to ensure that slugs choosing to crawl toward the shelter were exhibiting light avoidance behavior rather than foraging behavior. Dead stones were thoroughly rinsed in fresh water, dried and then placed in the same location as was used for the living stones. Trials with dead stones were conducted as described above for living stones. Each specimen was tested once with a living stone and once with a dead stone and the order in which they were tested was randomized to avoid confounding effects of repeated measures. The light position was also changed after 30 min as described above.

To examine changes in preferred light intensity throughout the phototaxis experiment, we built a model using “light_position”, “dead_living_stone”, and “time” as fixed effects, “id” as a random effect to account for repeat measures, and “light_intensity” as the response variable. Models were compared using Akaike Information Criteria (AIC) and the model with the lowest AIC was selected as the best fit model. This model contained interactions between all of the fixed effects, and explained 39.76% of the variation (conditional $r^2=0.3976$). A simplified model was tested by removing the predictor “dead_living_stone” since it did not significantly explain variation, but the resulting AIC was higher, so this predictor was left in the final model. Post hoc testing was performed by calculating Estimated Marginal Means (EMMs) via the *emmeans* package (Lenth 2022). Means and standard deviations were then calculated by pooling data from living and dead stones (since this predictor did not have explanatory power) for the initial (time=0) and final positions (time=30). The trajectory of each slug in the tray throughout the experiment, their position over time and the change in light intensity between initial and final positions were visualized using plots generated in the *ggplot2* package (Wickham 2016).

2.4 Comparing metabolic rate during day and night

Metabolic rates were calculated by monitoring oxygen saturation in the seawater surrounding a specimen that was

enclosed in a sealed respiratory chamber. Most animals take up oxygen meaning the saturation decreases over time, however some photosymbiotic animals oxygenate the water around them when the oxygen produced during photosynthesis exceeds the amount required by the animal to sustain aerobic respiration and the excess diffuses into the seawater (e.g. Al-Horani et al. 2003). This means that their metabolic rates appear to be negative when they are simply obscured by O₂ production, and expectedly, this only occurs when specimens are exposed to light and photosynthesis can function. To account for the masking effects of oxygen production due to photosynthesis and determine the “true” metabolic rate of *P. lynceus* (i.e. amount of oxygen it consumes from the surrounding seawater to sustain aerobic respiration), we measured specimens exposed to 700 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light and treated with a 10 μM solution of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea ($n=6$) (DCMU, Thermo Fisher Scientific, Worldwide) diluted in seawater, following the protocol established by Havurinne and Tyystjärvi (2020). DCMU is a highly selective photosynthetic inhibitor that prevents plastoquinone binding and thus electron flow in photosystem II (Jones 2004). We also measured six specimens that were not exposed to DCMU to measure the amount of oxygen that is produced due to photosynthesis. Lastly, we re-measured all of these specimens in the dark to determine if they are more metabolically active during the day (indicating they show diurnal patterns of activity) or at night (indicating they are more active nocturnally). Both DCMU and untreated specimens were measured at 10:00 and 22:00 to ensure they were measured during and after the photoperiod. The order in which specimens were measured (i.e. day then night or night then day) was randomized for both untreated and DCMU-treated specimens.

To measure their metabolic rates (oxygen consumption), individual slugs were enclosed in respirometry chambers (glass jars with volume of 2 ml) filled with aerated seawater ($n=6$) or aerated seawater+DCMU ($n=6$). Each chamber contained an oxygen microsensor (sensor type PSt3, PreSens, GmBH, Germany). All chambers were placed in a water bath to maintain temperature stability at 26 °C, matching the water temperature at which they were collected. A Compact Oxygen Transmitter OXY-1 ST (PreSens, GmBH, Germany) was used to measure the temperature, pressure and the oxygen saturation within the chamber. The sensor was calibrated using nitrogen gas (0% oxygen saturation) and aerated seawater (100% oxygen saturation). Specimens measured during the day were exposed to full-spectrum lighting at an intensity of $\sim 700 \mu\text{mol m}^{-2}\text{s}^{-1}$ (to minimize stress due to high light intensities, but ensure that photosynthesis could still function) and were measured in the dark at night. Measurements were taken every five minutes for

a total of 30 min. The difference in initial and final oxygen saturation measurements for DCMU-treated and therefore photosynthetically inhibited (non-oxygen-producing) specimens reflects the actual metabolic rate for each individual, while the difference in initial and final oxygen saturation measurements for untreated specimens indicates the metabolic rate minus any oxygen that was produced and used by the slug. The initial and final oxygen concentrations, along with temperature, salinity and pressure, were taken into account when calculating oxygen consumption per hour. To account for possible oxygen offset caused by microbial communities in the sea water, one chamber was measured with seawater but no slug for each treatment group. The oxygen consumption per hour calculated from the blank data was subtracted from the individual organism's consumption, obtaining a final metabolic rate value.

All statistical analysis was carried out in R Studio using the *tidyverse* package (Wickham et al. 2019) for data handling. First, the raw metabolic rate (in $\text{mgO}_2\text{g}^{-1}\text{h}^{-1}$) was \log_{10} transformed to account for the non-linear relationship between mass and rates of oxygen consumption (allometric scaling). Two linear mixed effects models were generated to analyze the metabolic rate data using the *lme4* package (Bates et al. 2015). The first contained additive effects and the second allowed an interaction between the fixed effects, "night_day" (whether a sample was measured during the night or during the day) and "DCMU" (treated or untreated) ($n=6$). Since each slug was measured twice (10:00 and 22:00), "Specimen" identification was included as a random effect. Both models had a conditional r^2 of 0.93, however the additive model generated a lower AIC of -37.23. This model was simplified using the "step" function of the *stats* package (R Core Team 2023), which removed "DCMU" and resulted in an improved AIC of -45.27 and a conditional $r^2 = 0.94$, explaining 94% of the variation. Post-hoc testing

was performed by calculating EMMs, as described above. Data was plotted using the *ggplot2* package (Wickham 2016).

3 Results

3.1 Symbiont retention

Phidiana lynceus specimens decreased in length during the starvation period (Fig. 2). The mean longevity of the 15 total individuals was 16.73 ± 2.46 days, ranging from 13 to 21 days (Fig. 3). For all of the starvation points except the last (20 days), we did not observe a decrease in symbiont density, despite a decrease in overall body size, and in the number and size of the cerata (Fig. 4). After 20 days in starvation, chlorophyll was still present in dense aggregations in the cerata.

3.2 Phototaxis in the field and lab

Phidiana lynceus specimens were consistently found underneath rocks and pieces of coral rubble that were resting in the sand during the day. Individuals were occasionally observed on top of these rocks at night. These rocks were often covered with *Myrionema amboinense*, the photosymbiotic hydrozoan on which *P. lynceus* feeds (Fig. 1a) (Coral Morphologic 2010). These hydrozoans were not observed on the underside of the rocks and when we overturned rocks looking for *P. lynceus*, individuals immediately started crawling to the other side of the rock (Fig. 1b). In the laboratory experiments to assess phototaxis, all but one specimen in one trial moved toward parts of the tray with lower light intensities. Since no significant differences were found between rocks containing hydrozoans (living)



Fig. 2 *Phidiana lynceus* individuals at the different starvation time points (in days). Specimen length at 0 days = approx. 7 mm and at 20 days = < 1 mm

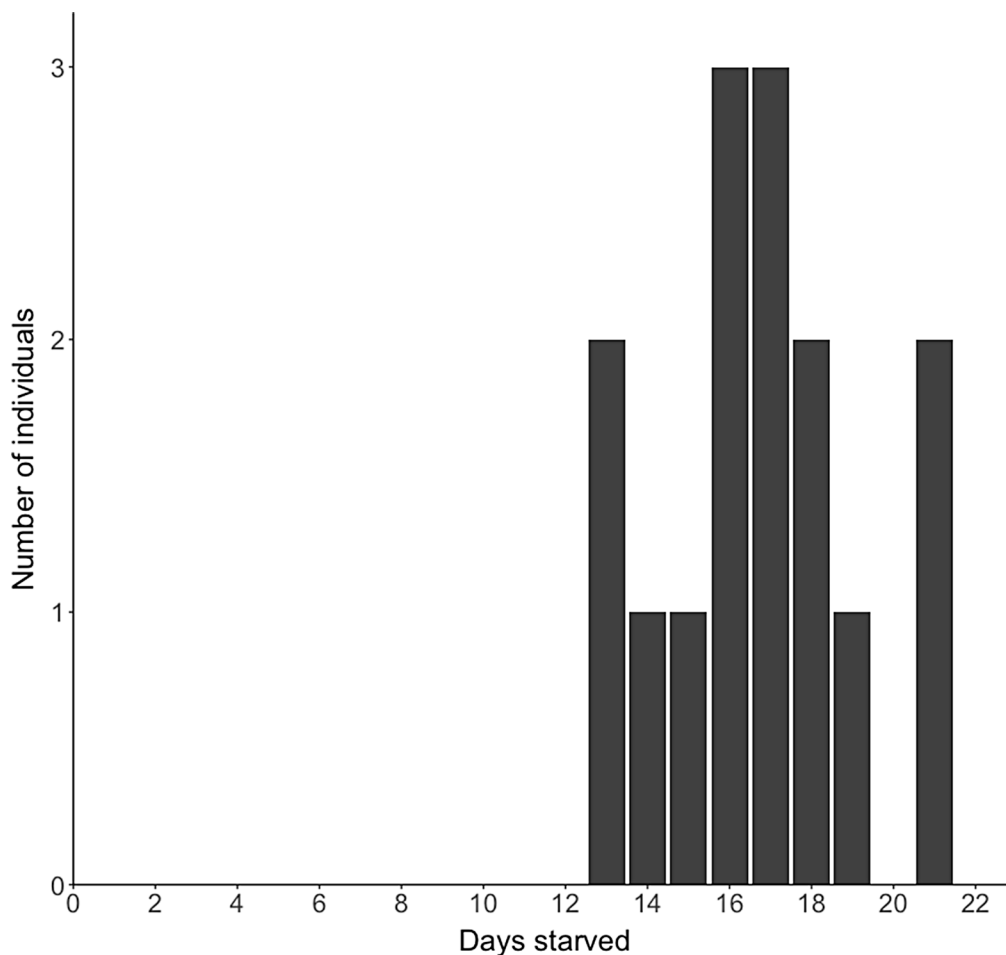


Fig. 3 Frequency plot showing the number of days *P. lynceus* individuals ($n=15$) were able to survive in starvation (their longevity), which averaged 16.73 ± 2.46 days (mean \pm standard deviation)

and sun-bleached rocks (dead) ($p > 0.05$), we pooled the two types of stone and presented the pooled values here ($n=12$). The position of the light (whether it was at the front of the tray or back) affected the speed at which specimens moved toward the less illuminated side of the experimental chamber but it did not influence their overall trajectory, i.e. they still moved toward the darker side of the chamber. When the light was in the front of the tray, specimens were exposed to an average of $254.67 \pm 250.65 \mu\text{mol m}^{-2}\text{s}^{-1}$ light at the beginning of the experiment, and $10.67 \pm 19.72 \mu\text{mol m}^{-2}\text{s}^{-1}$ at the end of the experiment. When the light was in the back of the tray, specimens were exposed to $508.83 \pm 266.17 \mu\text{mol m}^{-2}\text{s}^{-1}$ light at the beginning of the experiment, and $8.42 \pm 15.23 \mu\text{mol m}^{-2}\text{s}^{-1}$ at the end of the experiment. Regardless of the light position over the tray, 75% of specimens crawled beneath the living or dead piece of coral rubble within 20 min and the remaining 25% of

specimens moved to areas containing light intensities less than $52 \mu\text{mol m}^{-2}\text{s}^{-1}$ after the full 30 min (Fig. 5b & e, Supplementary Fig. 1). The light intensity to which specimens were exposed at the beginning of the trial (its initial location at time=0) and at the end (final location at time=30) differed significantly for both light positions (EMMs, $t_{130}=4.022$, $p=0.0018$ and EMMs, $t_{130}=8.249$, $p < 0.0001$ for the light positions “front” and “back” respectively). A full statistical summary can be viewed in Supplementary Tables 1 and the EMM output of the best-fit model can be viewed in Supplementary Table 2.

3.3 Comparing metabolic rate during day and night

All *P. lynceus* specimens consumed oxygen from the surrounding seawater (the net rate of uptake was always positive), regardless of the treatment condition (i.e.

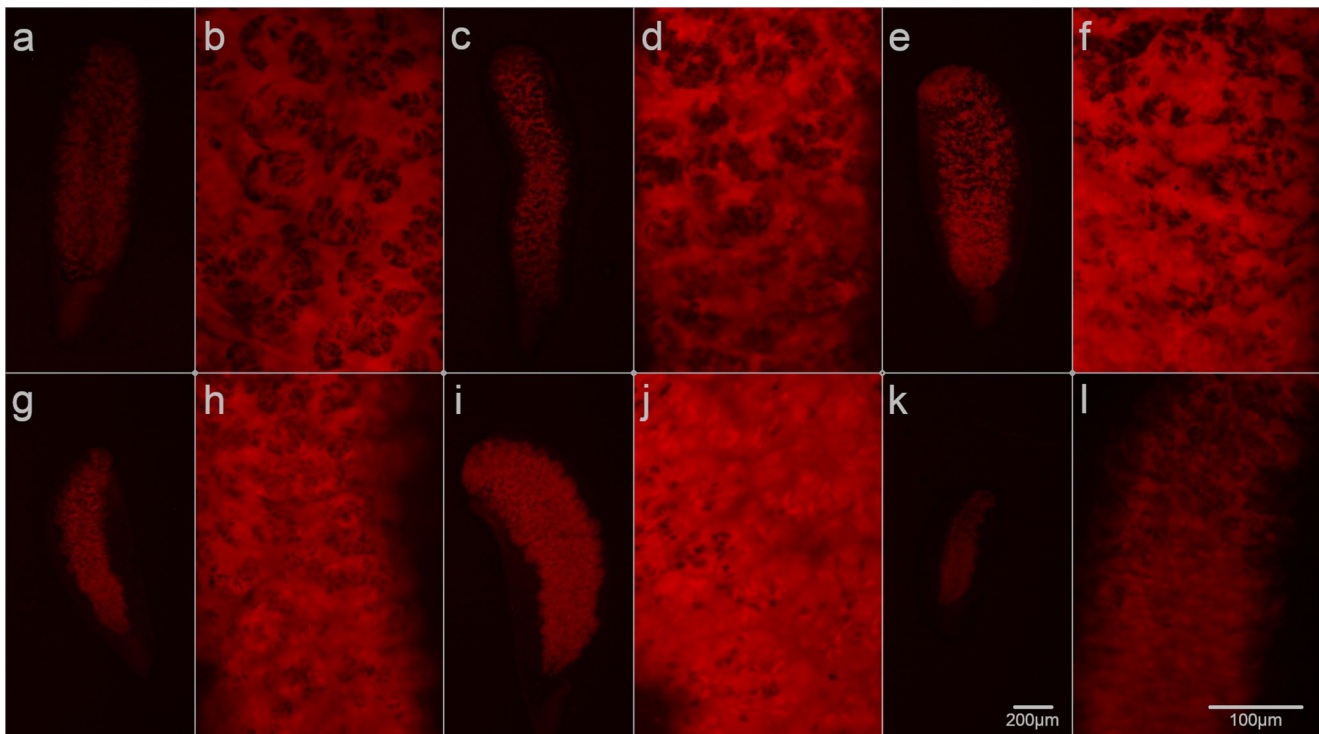


Fig. 4 Chlorophyll fluorescence in *P. lynceus* cerata (singular cerata) taken after each starvation time point. **a–b** A single cerata before starvation, **c–d** after 4 days in starvation (ds), **e–f** after 8 days ds, **g–h** after

12 ds, **i–j** after 16 ds, **k–l** after 20 ds. The 200 μm scale bar applies to panels **a, c, e, g, i** and **k**, while the 100 μm scale bar applies to panels **b, d, f, h, j** and **l**

DCMU-treatment and time (day or night)). The metabolic rates of *P. lynceus* individuals differed between day and night (ANOVA, $t_{17}=2.128$, $p=0.048$), and 75% of individuals ($n=12$) showed an increase in oxygen consumption at night, but exposure to DCMU did not change their metabolic rates (Fig. 6a, b). A statistical summary of the final best-fit model can be viewed in Supplementary Table 3.

4 Discussion

4.1 Symbiont retention

During the 20 days *P. lynceus* was able to survive starvation, all specimens lost considerable biomass yet retaining a high density of symbionts in the remaining digestive tissue (Figs. 2 and 4), indicating that *P. lynceus* cannot form a stable symbiosis with zooxanthellae. It is therefore best classified as a Type 5 unstable photosymbiosis according to Kempf (1991). The ability to retain symbionts until its eventual death by starvation distinguishes *P. lynceus* from other cladobranchs that form unstable Type 5 symbioses but excrete or digest their symbiotic algae, appearing bleached (symbiont-free) when deprived of food for a few days and dying shortly thereafter (e.g. *Berghia stephanieae*) (Kempf 1991; McFarland and Muller-Parker 1993; Monteiro et al.

2019; Silva et al. 2021). However, other aspects of the *P. lynceus*/algae photosymbiosis align with observations of other cladobranch species. Like *B. stephanieae* and *Pteraeolidia ianthina* (Nudibranchia: Facelinidae), for example, (Kempf 1984, 1991), *Phidiana lynceus* is unable to maintain its body mass when starved, indicating that any nutritive benefits that its symbiotic algae could provide are not enough to maintain a starving slug. This includes both energy that could be translocated from the symbionts to the slug (e.g. carbohydrates produced via photosynthesis) and energy that could be attained by digesting the symbionts themselves.

4.2 Phototaxis in the field and lab

In the field, individuals of this *P. lynceus* population were consistently found underneath rocks and coral rubble that were embedded in the sand during the day, although a few individuals were observed on top of these rocks at night. When we flipped rocks and exposed them, *P. lynceus* demonstrated a strong interest in escaping this exposed position by rapidly crawling to the new underside of the rock (Fig. 1b). Additionally, the absence of hydrozoan prey on the underside of the rocks suggests that *P. lynceus* likely forages nocturnally and then returns to the underside of the rocks for shelter during the day. Despite the clear light avoidant behaviors observed in this study, *P. lynceus* has

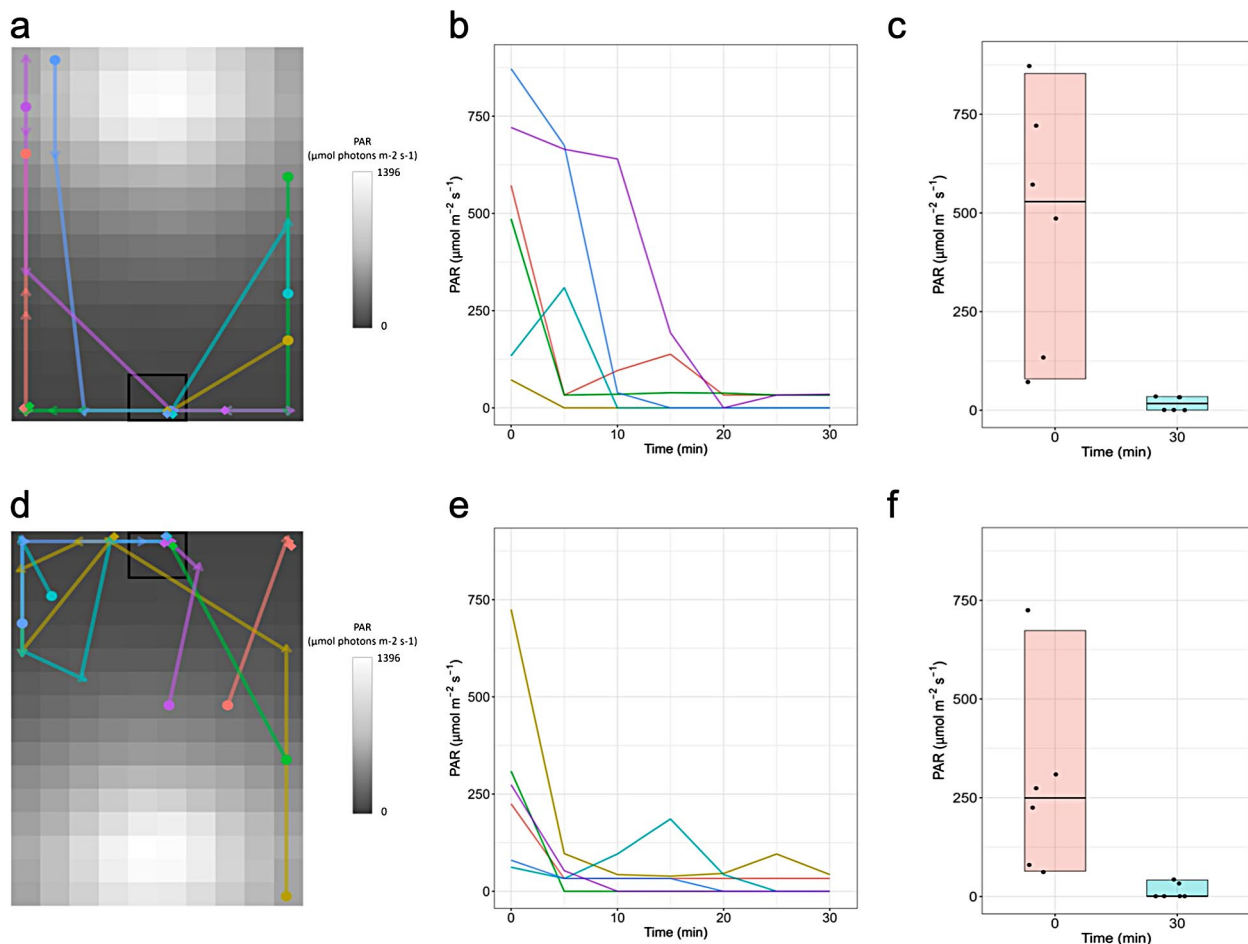


Fig. 5 Phototactic behavior of *P. lynceus* individuals during the trial using “dead” rocks. **a** Trajectory plot showing a bird’s eye view of each slug’s movements at each time when the lamp is positioned at the back and the **d** front of the tank. The starting point is indicated with the colored circle and the end point with the associated colored diamond, and arrows indicate the direction they traveled. The black square represents the dead rock under which there is no light ($0 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)

been photographed by SCUBA divers exposed to light and feeding during the day (e.g. Ianniello 2009), but this was not observed in the population we examined during the 40+ hours in which we surveyed this habitat.

The phototaxis experiments in this study support the assertion that *P. lynceus* avoids light, since every individual reduced the light intensity to which it was exposed by moving toward the darker side of the tray and almost all specimens sought shelter under their stones, regardless of if the stone was “living” or “dead”. None of the slugs we examined crawled on top of the stone which we would expect if they were intent on foraging. Instead, most crawled directly underneath the stones and the rest chose positions with low light intensity levels near the stones within the given 30 min (Fig. 5b & e). This suggests their attraction to the stone is not due to chemical cues from prey or a desire to forage from the stone. We therefore concluded that *P. lynceus* was

$\text{m}^{-2}\text{s}^{-1}$). **b** The light intensity to which each individual (depicted with different colors) was exposed during the experiment when the light was positioned at the back of the tray and **e** the front of the tray. **c** Box plot displaying the light intensity to which each specimen was exposed at the start of the experiment and end when the light is positioned over the back of the tray, and **f** over the front of the tray

using these stones for shelter, as has been observed in other phototaxis experiments on light-avoidant slugs (Burgués Palau et al. 2024).

4.3 Comparing metabolic rate during day and night

The net rate of oxygen uptake was never negative, indicating that photosynthetic activity did not generate more oxygen than the slug needed to maintain its aerobic scope, which would allow oxygen to diffuse into the surrounding seawater. This means *P. lynceus* still took up oxygen from the seawater, unlike some other photosymbiotic species (e.g. Schutter et al. 2010). *Phidiana lynceus* consumed more oxygen from its surrounding seawater at night than during the day, indicating that they are more metabolically active at night (Fig. 6a, b), however the overlapping ranges between oxygen consumption rates during the day and night

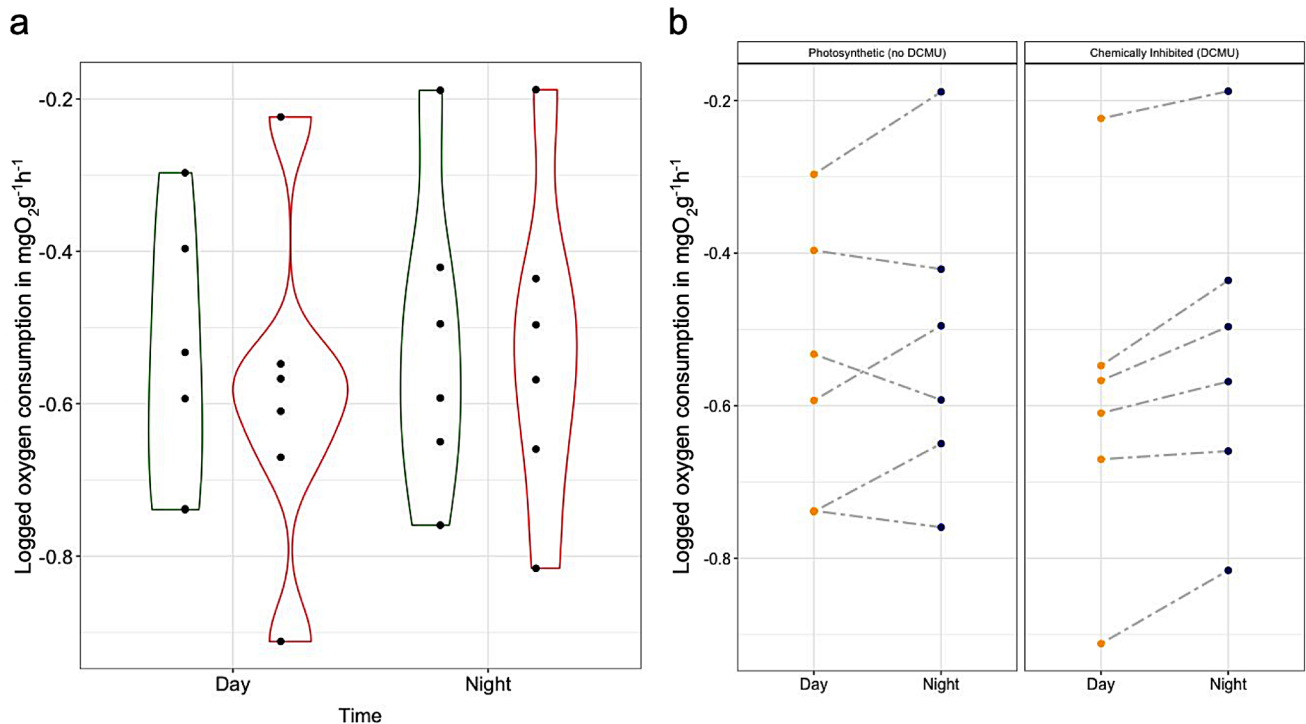


Fig. 6 **a** Logged rates of *P. lynceus* oxygen consumption (in $\text{mgO}_2\text{g}^{-1}\text{h}^{-1}$) during the day and night. Rates measured in DCMU-treated slugs are depicted in red and rates in untreated (photosynthetic) slugs are colored dark green. Individual data points represented by dots. **b** Paired scatter plot comparing the rates of oxygen consumption

from day to night for individuals allowed to photosynthesize (no DCMU exposure) and those whose photosynthetic capabilities were chemically inhibited (exposed to DCMU). For clarity, day is always depicted first (on the left) even though the order in which day and night were assessed was randomized

suggests that *P. lynceus* does not display major swings in metabolic activity between day and night, as is observed in other species (i.e. humans, many reef fish, etc.). This suggests that energy allocation during the night and day likely differ in this species. *Phidiana lynceus* are mobile predators that need to actively forage for their prey. Since we did not observe them hunting during the day in the field, we conclude that they hunt only at night, a behavior associated with nocturnal organisms. Many nudibranch species exhibit nocturnal feeding activity, so this finding aligns with previous reports (Gochfeld and Aeby 1997; Slattery et al. 1998; Vermeij 2010; Kirouac et al. 2012). While metabolic rates were lower during the day, they were not drastically different than at night, suggesting that *P. lynceus* is still metabolically active during the day, but likely expends energy on metabolic processes that are not related to foraging and locomotion, such as growth, mating, or digestion.

Contrary to our expectations, DCMU-treated individuals did not take up or consume more or less oxygen from the seawater to make up for the lack of photosynthetically produced oxygen, which could be due to numerous factors such as symbiont density between DCMU-treated and non-treated slugs, an ineffective DCMU concentration, or the actual amount of oxygen that was produced via photosynthesis in non-DCMU-treated individuals. Visual inspections

of symbiont density and chlorophyll concentration did not reveal a noticeable difference between the cerata of specimens used in the DCMU and non-DCMU treatments, so this is an unlikely explanation of why no difference in oxygen uptake was observed. An ineffective DCMU concentration is also unlikely since this concentration has been used effectively in slugs before (Havurinne and Tyystjärvi 2020) and various other organisms (cnidarians, slugs) in our laboratory (EMJL unpublished results). Therefore, we interpret the similarity in oxygen uptake by DCMU-treated and untreated specimens as most likely due to the low amount of oxygen that is produced via photosynthesis, i.e. very little oxygen is produced in untreated specimens, so very little oxygen production is inhibited when they are exposed to DCMU. Confirming this interpretation, however, will require further examination, but should this interpretation prove accurate, our results indicate that *P. lynceus* is not receiving measurable oxygenic benefits from photosynthesis performed by its incorporated zooxanthellae.

4.4 The *Phidiana* paradox

Contrary to our expectations, *P. lynceus* does not seem to profit energetically or oxygenically from forming an unstable photosymbiosis with zooxanthellae, despite having the

adaptations needed to acquire and retain these symbionts. This is best demonstrated by the lack of symbiont digestion and loss of biomass that we observed in specimens that were prevented from feeding. The strong desire to hide that we observed in the field and lab during the day effectively prevents photosynthesis and any energetic or oxygenic benefits it would confer. The increase in metabolic activity at night further indicates that this species demonstrates nocturnal tendencies. These observations make *P. lynceus* a paradox: it evolved the requisite adaptations needed to retain algal symbionts and reap the energetic and/or oxygenic benefits of photosynthesis, yet chooses not to receive these benefits from this symbiosis, and numerous evolutionary explanations could exist. For example, photosymbiosis could be an vestigial trait in *P. lynceus*, that formerly granted a benefit, but is no longer advantageous due to selective pressures from predation or other factors. Alternatively, symbiont uptake and retention in a nocturnal species could be incidental and the lack of strong positive or negative selective forces could explain symbiont retention. It is also possible that retaining algal symbionts confers benefits that extend beyond the nutritive and oxygenic options explored in this study.

Our observations of *P. lynceus* demonstrate that unstable photosymbioses have multiple facets that need to be examined in order to understand the benefits and evolution of photosymbiosis in cladobranch slugs. These include, the amount of time symbionts can be retained in a slug, what eventually happens to those symbionts (digestion or excretion), and whether or not a slug displays behaviors (like positive phototaxis) that facilitate photosynthesis and the benefits it could provide. Only then can the evolution and radiation of photosymbiosis within the Cladobranchia be evaluated. For now, the relationship between *P. lynceus* and its zooxanthellae should be considered a neutralism, where neither species benefits nor is harmed by their interaction, at least until the *Phidiana* paradox is resolved.

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Author contributions EMJL conceptualized and designed this study. EMJL, LBP and BAP collected the specimens. NMB conducted and analyzed the microscopy data, LBP conducted and analyzed the phototaxis experiments, EMJL collected the metabolic rate data and BAP, NMB and EMJL analyzed this data. NMB and EMJL wrote the first

draft and all authors were involved in editing and revisions. EMJL acquired funding and managed this project. All authors read and approved the final manuscript.

Data availability All data will be made available upon acceptance of this manuscript via DataverseNL. During review, the following (temporary) link can be accessed: <https://dataverse.nl/privateurl.xhtml?token=8159a563-b1d7-4d7e-9932-bb00e3cc0d73>.

Declarations

Ethical approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with unregulated invertebrate species.

Consent to participate Not applicable.

Consent for publication All authors approved the submitted draft of this manuscript and agree to be responsible for their contributions to this publication.

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