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CORRECTION

Correction: Critical thermal maxima and oxygen uptake in *Elysia viridis*, a sea slug that steals chloroplasts to photosynthesize

Elise M. J. Laetz, Can Kahyaoglu, Natascha M. Borgstein, Michiel Merkx, Sancia E. T. van der Meij and Wilco C. E. P. Verberk

There was an error in *J. Exp. Biol.* (2024) **227**, jeb246331 (doi:10.1242/jeb.246331).

In Fig. 3, the numbers on the *y*-axis were incorrect. The corrected and original versions of the figure are shown below. In Fig. 7, the units for net oxygen consumption (mg O_2 $g^{-0.792}$ h^{-1}) were missing from the figure legend.

Both the online full text and PDF versions of the paper have been corrected. We apologise to the authors and readers for this error and any inconvenience it may have caused.

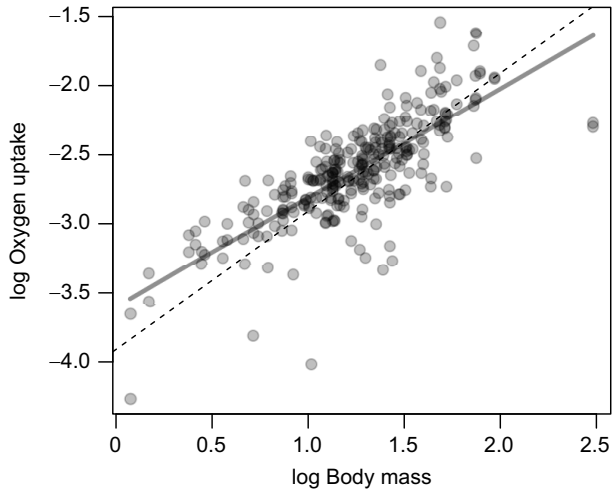

Fig. 3 (corrected). The allometric scaling relationship (log_{10}–log_{10}) of body mass (mg) and oxygen uptake (mg O_2 h^{-1}). The solid line indicates the empirically derived scaling exponent of 0.792+0.05 (mean+s.d.) and the dotted line indicates isometry, i.e. a scaling exponent of 1. This empirical scaling exponent was used to calculate the mass-specific rates of oxygen uptake rates.

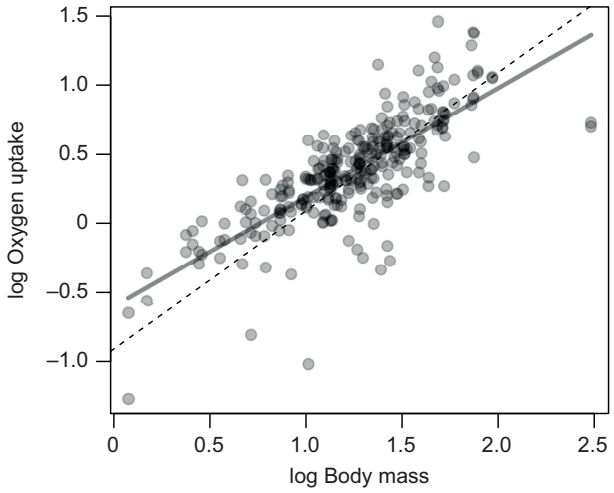

Fig. 3 (original). The allometric scaling relationship (log_{10}–log_{10}) of body mass (mg) and oxygen uptake (mg O_2 h^{-1}). The solid line indicates the empirically derived scaling exponent of 0.792+0.05 (mean+s.d.) and the dotted line indicates isometry, i.e. a scaling exponent of 1. This empirical scaling exponent was used to calculate the mass-specific rates of oxygen uptake rates.

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RESEARCH ARTICLE

Critical thermal maxima and oxygen uptake in *Elysia viridis*, a sea slug that steals chloroplasts to photosynthesize

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ABSTRACT

Photosynthetic animals produce oxygen, providing an ideal lens for studying how oxygen dynamics influence thermal sensitivity. The algivorous sea slug *Elysia viridis* can steal and retain chloroplasts from the marine alga *Bryopsis* sp. for months when starved, but chloroplast retention is mere weeks when they are fed another green alga, *Chaetomorpha* sp. To examine plasticity in thermal tolerance and changes in net oxygen exchange when fed and starving, slugs fed each alga were acclimated to 17°C (the current maximum temperature to which they are exposed in nature) and 22°C (the increase predicted for 2100) and measured at different points during starvation. We also examined increased illumination to evaluate a potential tradeoff between increased oxygen production but faster chloroplast degradation. Following acclimation, we subjected slugs to acute thermal stress to determine their thermal tolerance. We also measured net oxygen exchange before and after acute thermal stress. Thermal tolerance improved in slugs acclimated to 22°C, indicating they can acclimate to temperatures higher than they naturally experience. All slugs exhibited net oxygen uptake, and rates were highest in recently fed slugs before exposure to acute thermal stress. Oxygen uptake was suppressed following acute thermal stress. Under brighter light, slugs exhibited improved thermal tolerance, possibly because photosynthetic oxygen production alleviated oxygen limitation. Accordingly, this advantage disappeared later in starvation when photosynthesis ceased. Thus, *E. viridis* can cope with heatwaves by suppressing metabolism and plastically adjusting heat tolerance; however, starvation influences a slug's thermal tolerance and oxygen uptake such that continuous access to algal food for its potential nutritive and oxygenic benefits is critical when facing thermal stress.

KEY WORDS: CT_{max}, Kleptoplasty, Metabolism, Sacoglossa, Starvation

INTRODUCTION

Temperature and oxygen availability are perhaps the most critical abiotic factors for multicellular life and both have pervasive effects across all levels of biological organization. Oxygen availability is limited in aquatic habitats because oxygen diffuses ∼300,000 times slower in water than in air (Dejours, 1981). Temperature modulates the availability of oxygen by changing the solubility and diffusivity of oxygen in water and it also changes the thickness of boundary layers via the effects on the viscosity of water (Graham, 1990; Verberk and Atkinson, 2013; Verberk et al., 2011). Rising global temperatures due to climate change can deoxygenate aquatic habitats because oxygen solubility in water decreases with elevated temperatures, while metabolic rates increase, which in turn increases the demand for oxygen to maintain aerobic respiration. Hypoxia is widely acknowledged to amplify the detrimental effects of warming (Woods et al., 2022) and reductions in heat tolerance under hypoxia have been reported for a range of aquatic invertebrates, including crustaceans, insects and mollusks (Davenport and Davenport, 2007; Ern et al., 2016; Koopman et al., 2016; Verberk et al., 2016; 2018). However, species differ in their sensitivity to the combined stressors of heat and hypoxia, with some species showing strong reductions in heat tolerance under hypoxia whereas others do not (e.g. Verberk and Bilton, 2013).

Exposure to heat varies across aquatic and terrestrial realms (Pinsky et al., 2019), and ectotherms inhabiting the intertidal and uppermost subtidal zones are particularly exposed to heat stress (Cereja, 2020; Marshall et al., 2011; Pörtner, 2001). The maximum temperatures present in intertidal habitats are strongly associated with the upper thermal limits observed in their ectotherm inhabitants (Stillman, 2003; Stillman and Somero, 2000), although these limits and the effects of thermal stress vary by taxa and latitude (Sunday et al., 2019). In other words, many intertidal ectotherms already experience conditions that approach the tolerance limits that define their thermal windows (Cereja, 2020; Vinagre et al., 2019).

Critical thermal maxima are often investigated by monitoring various biological changes (such as running performance or metabolic rate/oxygen uptake) in individuals exposed to increasingly warmer temperatures (Clusella-Trullas et al., 2011; Vasseur et al., 2014). An animal's upper thermal limit or critical thermal maximum, is usually defined as the temperature at which it loses voluntary muscle control and is thus no longer capable of trying to escape from conditions that will result in death (Cowles and Bogert, 1944) or the temperature at which it enters a heat-induced coma (Armstrong et al., 2019; Lutterschmidt and Hutchison, 1997). Although critical thermal maxima are often portrayed as being fixed properties of a species, they can be modulated via acclimation and via heat hardening. In the case of thermal acclimation, prolonged exposure to somewhat elevated temperatures results in physiological changes that allow an individual to better tolerate heat (e.g. Gunderson and Stillman, 2015). In the case of heat hardening, a short exposure to intense heat

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triggers a heat-shock response (Feder and Hofmann, 1999), which can further improve survival of individuals subjected to subsequent acute heat stress (Verberk and Calosi, 2012), but can have long-term fitness costs (Krebs and Loeschcke, 1995).

Since climate change is implicated in both rising average temperatures and the increase in extreme weather events such as heatwaves, the effects of both chronic warming and acute heatwaves are relevant when trying to predict whether species can cope with climate change. Thermal effects on both oxygen availability and demand have been used to argue that insufficient oxygen may be a defining factor in an ectotherm's thermal window (Pörtner and Farrell, 2008). However, the generality and testability of this oxygen limitation hypothesis are debated (summarized in Jutfelt et al., 2018; Pörtner et al., 2018; Verberk et al., 2016). Surveying a wide range of species from different clades and studying model organisms with unique physiological attributes can help to disentangle specific aspects of the relationships between temperature, oxygen demand and availability.

Animals that share a symbiotic relationship with photosynthetic organisms could provide a lens with which to study the interactions between temperature, oxygen demand and oxygen availability because the photosynthesis occurring in the symbionts produces oxygen that can potentially be used to sustain the animal's aerobic respiration and oxygenate the environment. Some species of sea slugs (Gastropoda: Plakobranchoidea) stand out as ideal study subjects for such experiments because they can steal and retain functional chloroplasts from their algal food sources in a process called functional kleptoplasty (Rumpho et al., 2010). These kleptoplasts are stored intracellularly in the digestive gland, a highly branched organ that radiates throughout the animal's body, and replaced periodically by fresh chloroplasts as long as the animal continues to have access to algal food (Frankenbach et al., 2021). In many species, chloroplast densities can be so high that they give the slug a solid green appearance, which accordingly disappears if the animal is then prevented from feeding (Laetz and Wägele, 2018).

In some species of slugs, incorporated kleptoplasts can continue to function, sometimes for months, producing both oxygen and photosynthates such as carbohydrates (Hinde and Smith, 1975; Laetz and Wägele, 2018; Laetz et al., 2017; Trench et al., 1973). This ability gives them the moniker 'solar-powered sea slugs'. The photosynthates they receive can provide nutrition to starving slugs during periods of food unavailability (due to the pelagic stages of some algal life cycles) or food inaccessibility (due to calcification of the algal thallus that prevents slugs from feeding) (Marín and Ros, 1992). How much nutrition is provided by photosynthesis is debated (Cartaxana et al., 2017; Laetz and Wägele, 2018; Rauch et al., 2017) and highly dependent on the algal and slug species/populations involved (Laetz et al., 2017; Wägele and Martin, 2014). However, kleptoplasty does not produce enough photosynthates to fully support a starving slug, i.e. the slug is not photoautotrophic (Cartaxana et al., 2017; Hinde and Smith, 1975; Rauch et al., 2018).

The emerald sea slug *Elysia viridis* (Montagu 1804) has been a model species for understanding functional kleptoplasty for over 50 years (Taylor, 1968). It inhabits the uppermost subtidal zone from the Mediterranean Sea to the British Isles and the northeastern Atlantic coast of Scandinavia (Baumgartner et al., 2015; Laetz et al., 2016). *Elysia viridis* is known to feed on a multiple, chlorophyte genera including *Codium*, *Bryopsis*, *Chaetomorpha*, *Cladophora* and the rhodophyte genus *Griffithsia* (Baumgartner et al., 2015; Händeler and Wägele, 2007; Rauch et al., 2018). The amount of time *E. viridis* can retain functional chloroplasts ranges from 1–2 months when fed *Codium* spp. and *Bryopsis* spp., down to a few weeks when fed *Chaetomorpha* spp. (Rauch et al., 2018; Trowbridge et al., 2008). Chloroplast retention has not been observed in *E. viridis* feeding on *Cladophora* spp. or *Griffithsia* spp. Since oxygen is produced as a byproduct of photosynthesis within the slug's body, it is available for aerobic respiration. Internal oxygen production could be especially important when demand is high, such as in warmer waters, but this remains uninvestigated.

In this study, we investigated the critical thermal maxima for *E. viridis* specimens under varying biotic and abiotic conditions to examine if and how environmental stress affects slug heat tolerance. We also measured their net oxygen exchange before and after assessing their critical thermal maxima to determine if and how their energy metabolism changed after exposure to acute heat stress. Since photosynthetic activity decreases during starvation as a result of chloroplast digestion (Frankenbach et al., 2021; Laetz et al., 2016), and periods of food unavailability have been observed for this species (Baumgartner and Toth, 2014), we measured heat tolerance and net oxygen exchange at different points during a starvation period. We identified and present here, environmental factors that underpin a solar-powered slug's response to acute and prolonged exposure to hyperthermal stress and our predictions for its ability to handle the warmer, deoxygenated oceans of the future.

MATERIALS AND METHODS

Specimen acquisition

Elysia viridis specimens were collected from a shallow reef at Bommenede in Zeeland, The Netherlands (51°43′47.5″N 3°58′35.3″E) at a depth of 20–50 cm in July 2020 (for preliminary experimentation) and in October 2020 (for the following experiments) (Fig. 1A). Rocks covered in *Bryopsis plumosa* were also collected (Fig. 1B). *Chaetomorpha* cf. *linum* was donated by Frits Kuiper Groningen, a local aquarium store. Above the thermocline, the temperature reached 23°C in June and August, the warmest months at this collection site in 2020 (Rijkswaterstaat Water Info; https://waterinfo.rws.nl/#!/details/publiek/watert). During these months, *E. viridis* migrated below the thermocline to ∼1.5 m depth where they were exposed to a maximum of 17°C, so 17°C was considered the maximum temperature under present conditions (without the +5°C predicted increase due to global warming). All specimens were transported to the laboratory at the University of Groningen for experimentation since cultivation in the laboratory under controlled conditions is not possible for this species due to their complex pelagic larval stages. All experiments were conducted in the four months following collection.

Ethical care

All specimens were treated according to best practices, which aim to minimize animal suffering whenever possible. Following each trial, specimens were allowed to recover. When necessary, animals were killed via flash freezing to minimize suffering.

Initial acclimation to lab conditions

All slugs were allowed to adjust to lab conditions for a minimum of 2 weeks before experimentation began to avoid measuring stress from relocation. During this time, slugs were housed in multiple glass tanks with 10 individuals per tank and provided with a continuous supply of *B. plumosa*, on which they were observed feeding. Throughout the entire acclimation and experimental periods, partial water changes (∼50%) were performed weekly, providing specimens with fresh, artificial sea water (Instant Ocean, Spectrum Brands, Inc). Each tank was scrubbed every month and given a complete water change to prevent biofilm and/or feces

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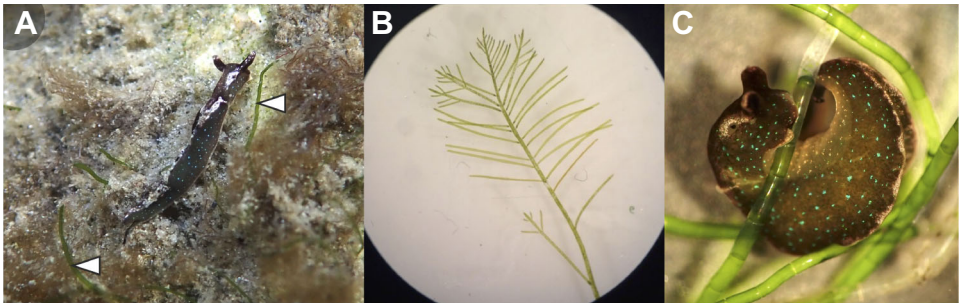

Fig. 1. *Elysia viridis* and its food algae. (A) *E. viridis* and small *Bryopsis plumosa* thalli (indicated by white arrowheads) under natural conditions at the collection site. *E. viridis* specimen length ∼7 mm. (B) *Bryopsis plumosa* thallus growth after grazers, primarily *E. viridis*, were removed. Each algal thallus grew to ∼7 cm in length and the specimen pictured is ∼5 cm in length. (C) An *E. viridis* specimen feeding on *Chaetomorpha* cf. *linum*. Specimen length ∼15 mm.

accumulation. Tanks were also outfitted with aerators to ensure the water was mixed and that O_2 was consistently at full saturation. Each aquarium was provided with 100 µmol m^{-2} s^{-1} full spectrum light (Superfish SLIM LED 45) and the temperature of the climate chamber (Clima Temperatur Systeme T600) where they were housed was maintained at 17°C to match the maximum temperature to which they were exposed in 2020. An overview of the acclimation phase, designs for experiments 1 and 2 and the treatments used in each experiment can be found in Fig. 2.

Experiment 1

Acclimation period: varying temperature and algal food sources

To examine the effects of prolonged exposure to increased temperatures, a change in algal food source, and starvation on oxygen uptake and critical thermal maxima (experiment 1), approximately 100 slugs were randomly selected and moved to another climate chamber (Clima Temperatur Systeme T600). In this new climate chamber, the temperature was gradually increased to 22°C, which reflects the 5°C temperature increase predicted for the North Sea by 2100 due to global warming (Klein Tank and Lenderink, 2009). Temperature was increased slowly at a rate of 1°C per day to prevent rapid temperature changes causing stress. Slugs were then given 2 weeks to acclimate and feed on *B. plumosa* upon reaching 22°C. After the change in temperature, half of the specimens maintained under each temperature regime (17°C and 22°C) were switched to a diet of *C.* cf. *linum*. The diet switch was done 3 weeks prior to the start of the experiment to ensure they no longer contained *B. plumosa* chloroplasts, but only *C.* cf. *linum* chloroplasts (Fig. 1C), as recommended by Frankenbach et al. (2021).

Elysia viridis can retain chloroplasts from other algal species within each of these genera, but these specific algal species have not been assessed before. When fed *Bryopsis hypnoides*, stolen chloroplasts can remain functional for up to 21 days (Rauch et al., 2018). Slugs have been observed feeding on *Chaetomorpha melagonium* (Baumgartner and Toth, 2014) but the duration chloroplasts remain active has not been assessed in this species and some authors doubt whether chloroplasts from *Chaetomorpha* sp. can be retained at all (Clark et al., 1990; Trowbridge and Todd, 2001). By examining slugs fed these two algal species, we were able to assess how differences in chloroplast retention time and nutritive quality affected thermal tolerance and net oxygen exchange. Following these periods where slugs were acclimated to each temperature and diet, each specimen was placed in a clean tank without algae and it entered the experimental phase (described below).

Measuring critical thermal maxima (CT_{first} and CT_{last})

The protocol for measuring critical thermal maxima was adapted from Armstrong et al. (2019) and applied to every slug in every treatment. Each slug (n=8 intended per treatment) was placed in an individual bottle containing aerated seawater. These bottles were placed in a circulating water bath to control temperature. The same light intensity to which they had been acclimated during the acclimation phase (100 µmol m^{-2} s^{-1}) was also provided. The temperature was increased by 1°C every 15 min (i.e. 0.0667°C min^{-1}) and slugs were observed continuously during this thermal ramping.

Preliminary trials were conducted to define two stages of heat stress in sea slugs, since definitions of the critical thermal maximum differ in the literature. In the first stage, starting from the initial exposure temperature (17°C or 22°C), the slugs were observed as they tried to escape by climbing up the walls of the bottle as the temperature increased. The end of the first stage was defined as the temperature at which a slug could no longer attempt to flee by climbing the walls of the test chamber, indicating it had lost voluntary muscle control. The temperature at this point is defined here as CT_{first}. We also recorded a second stage of heat stress. This point was most reliably determined by gently prodding each slug's rhinophores with a probe every few minutes. The temperature at which they could no longer contract a rhinophore when stimulated, i.e. they lost involuntary muscle control and nervous system function, was termed CT_{last}. This point most closely corresponds to a heat-induced coma described by Lutterschmidt and Hutchison (1997).

Immediately upon reaching CT_{last}, slugs were removed from the water bath and allowed to cool down by 1°C every 10 min to a temperature that facilitated recovery. Slugs that were initially acclimated to 17°C during the acclimatory period were cooled down to 27°C for recovery and slugs initially acclimated to 22°C during the acclimatory period were cooled down to 29°C for recovery. These temperatures were based on the average temperature at which all of the specimens recovered and none died of hyperthermia during a set of preliminary investigations. Heat tolerance plasticity was calculated using acclimation response ratios (ARRs), which express how much heat tolerance is gained per degree of warm acclimation. ARRs were calculated as the increase in CT for every degree of warm acclimation, using the formula: $ARR=(CT_{22°C}–CT_{17°C})/5$, as described by Gunderson and Stillman (2015).

Analyzing CT_{first} and CT_{last}

All analyses were conducted in Rstudio based on R version 3.6.1 (https://www.r-project.org/) using the packages matrixstats (https://cran.r-project.org/package=matrixStats), lme4 (https://cran.r-project.org/package=lme4; Bates et al., 2015), MuMin (https://CRAN.R-project.org/package=MuMIn), lmerTest (https://CRAN.R-project.org/package=lmerTest; Kuznetsova et al., 2017), ggplot2 (https://ggplot2.tidyverse.org/), dplyr (https://dplyr.tidyverse.org/) and visreg (https://CRAN.R-project.org/package=visreg; Breheny and Burchett, 2017).

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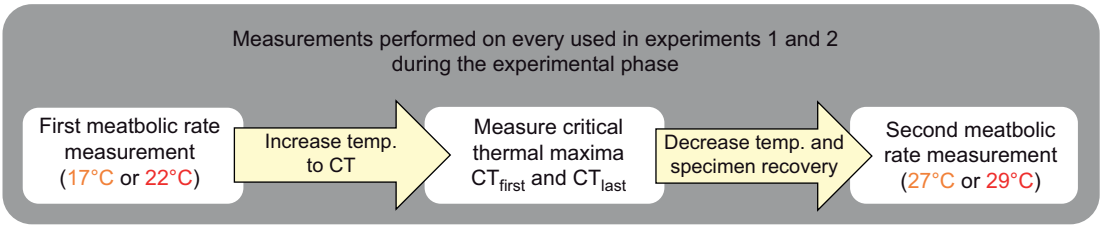
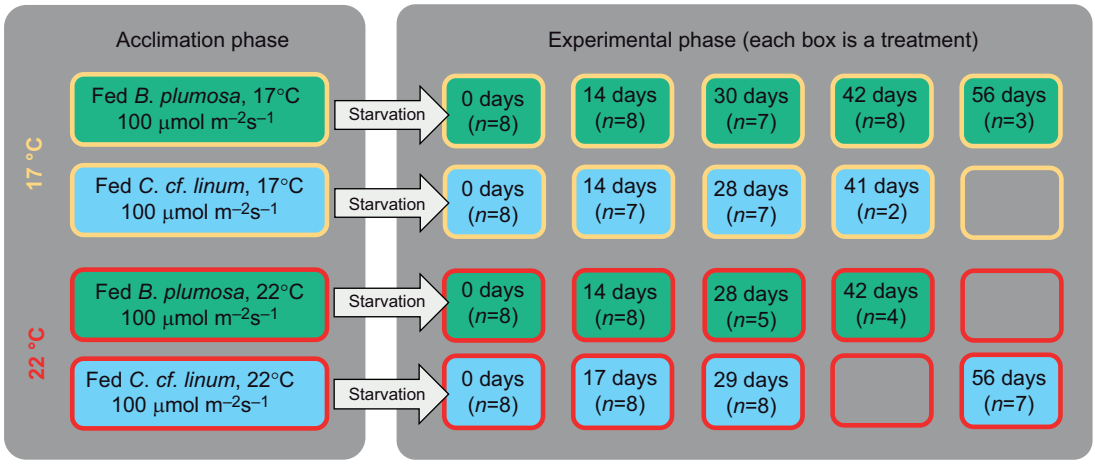


Experiment 1: higher temperature, starvation and algal food source



Experiment 2: higher light levels and starvation

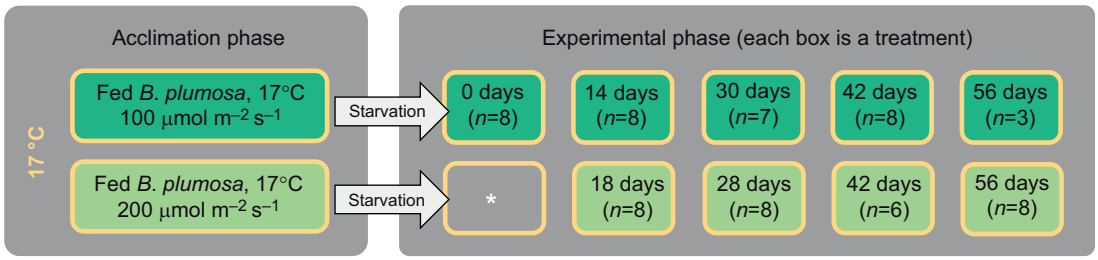


Fig. 2. Overview of the design and sampling points for experiments 1 and 2. Every slug in every treatment was measured according to the same procedure. First, its net oxygen exchange was measured. Then its critical thermal maxima (CT_{first} and CT_{last}) were measured. It was then allowed to cool down by 1°C every 10 min until it reached 27°C (for specimens that had been acclimated to 17°C) or 29°C (for specimens that were acclimated to 22°C), before oxygen exchange was measured again (top panel). Specimens were fed and acclimated to laboratory conditions during the 'acclimation phase', depicted in the panels on the left. The temperature and light intensity were maintained when specimens were removed from the algae and placed in clean tanks for starvation and experimentation during the 'experimental phase' (right panels). Treatments that are labeled '0 days starved' include specimens that were measured right after the acclimation phase and had therefore fed *ad libitum* until a few hours before experimentation. The number of individuals (*n*) tested at each sampling point is shown below the corresponding sampling point and all replicates are biological replicates. Empty boxes indicate sampling points we were unable to take due to specimen mortality or logistical reasons (*).

The R-script we generated and all datasheets are available in DataverseNL (https://doi.org/10.34894/AUFQE7). We used general linear models to investigate how our treatments affected each slug's critical thermal maxima. The two stages of heat stress (CT_{first} and CT_{last}) were positively correlated (B=0.221; $t_{1,111}$=4.66; P<0.001, where B is the slope; Fig. S1A). Preliminary analyses revealed that effects of mass, temperature and starvation differed between our two critical thermal maxima metrics, so, for the sake of clarity, we analyzed variation in CT_{first} and CT_{last} in separate models.

We analyzed CT_{first} by building a model that included the temperature at which slugs were tested, algal food source and starvation period as fixed effects. To account for non-linear effects, we also included a quadratic term for starvation duration. We also included the body mass of each individual slug, as previous studies have demonstrated that heat tolerance may vary with body size in aquatic ectotherms (Leiva et al., 2019). In addition to the main effects, we considered interactions between experimental conditions, notably starvation duration and algal source to test whether slugs fed with different algae responded

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differently to starvation. To prevent overfitting, we considered only 2-way interactions and simplified the model by excluding non-significant interactions and interactions that contributed little to the explained variation. The best fit model was chosen by comparing Akaike Information Criteria (AIC) values and by examining the variation that was explained. This resulted in a final model that explained 43.2% of the variation. The same process was repeated for CT_{last} and its best fit model explained 41.3% of the variation. These models are listed in Table 1 and the statistical summaries resulting from these models are displayed in Tables S1 and S2.

Measuring the rate of net oxygen exchange

The net oxygen exchange for each specimen was determined before and after the critical thermal maxima trials described in the previous sections. Rates were measured via closed-system respirometry with a Fibox 3 LCD Trace oxygen meter and PSt3 optical sensors that were glued into glass vials (Presens GmBH, Germany). Prior to the measurements, we calibrated the O_2 sensor spot using a 2-point calibration (100% O_2 saturated seawater and 0% oxygen:nitrogen gas). The values we report represent net rates of oxygen exchange and are an underestimation of the total oxygen needed to support aerobic respiration. This is because each specimen also produced oxygen via photosynthesis, although despite such oxygen production all slugs exhibit net uptake of oxygen (and we therefore refer to net oxygen exchange as oxygen uptake hereafter). We did not quantify the relative contribution of oxygen produced via photosynthesis and total oxygen consumed by each specimen. This would require measuring a specimen's oxygen uptake before and after applying a photosynthetic inhibitor, but this was incompatible with our experimental design as the photosynthetic inhibition cannot be undone and we wanted to measure oxygen uptake rates twice, once before and once after the critical thermal maxima assessment. Note also that measuring specimens in the dark is not a viable way to measure a lack of photosynthetically produced oxygen because these slugs have a circadian rhythm like other gastropods (Sandison, 1966; Sokolova and Pörtner, 2001) and exposure to darkness induces differences in their metabolic activity (Frankenbach et al., 2023; Shirley and Findley, 1978). All oxygen exchange measurements were conducted at the same time of day (11:00 h–12:00 h) to facilitate comparison.

To measure oxygen uptake, each specimen was first placed in a respiratory chamber filled with 100% O_2 saturated seawater at the same temperature to which it was acclimated during the acclimation phase (detailed above). One chamber was filled with aerated seawater only to control for background microbial O_2 consumption. The chambers were closed to prevent water exchange and placed in a water bath maintained at one of the four experimental temperatures (17°C or 22°C for measurements taken before the critical thermal limits were assessed or 27°C and 29°C for measurements taken afterward). Different temperatures were used for the oxygen uptake measurements before and after the critical thermal maxima were assessed because we wanted to measure the oxygen uptake directly following the critical thermal maxima trials. However, maintaining slugs at their critical thermal maximum temperatures was lethal in preliminary trials, so lowering the temperatures and allowing slugs to recover was crucial. Decreasing the temperature down to their initial exposure temperatures (17°C and 22°C) would have taken multiple hours and we were concerned this delay would result in a measurement that missed the window when the animals were incurring the greatest metabolic costs of acute thermal stress. Therefore, they were allowed to cool down to the highest temperature at which 100% of specimens showed signs of recovery from heat shock in the preliminary trials. Specimens acclimated to 17°C recovered at 27°C, while those acclimated to 22°C recovered at 29°C.

Each respiratory chamber was placed under the same light intensity to which the specimens were initially exposed to maintain the photoacclimation status of that specimen's chloroplasts (100 µmol m^{-2} s^{-1}) and ensure that the slugs did not experience light stress during the experiment. Because we measured rates of oxygen uptake when exposed to light, each measurement comprises the net demand for oxygen (i.e. total oxygen demand minus photosynthetically-produced oxygen). The decrease in O_2 saturation, i.e. the amount of O_2 that was extracted from the water by the slug, was measured every 10 min for 1 h, or until the oxygen saturation dipped below 70%, to prevent inadvertently measuring oxygen uptake under hypoxic conditions. These parameters were

Table 1. The final models used in each analysis

Response variable	Fixed effects in best model	Random effect
Experiment 1		
CT_{first}	acclim_temp.+log_body_mass+algae+ poly(starvation_days,2)+acclim_temp: log_body_mass+ acclim_temp:poly(starvation_days,2)+ algae: poly(starvation_days,2)	None, since each individual had a single measurement of CT_{first} or CT_{last}
CT_{last}	acclim_temp+log_body_mass+algae+ poly(starvation_days,2)+acclim_temp: log_body_mass+ acclim_temp:poly(starvation_days,2)+ algae: poly(starvation_days,2)	Not needed
$\dot{M}_{O_2}$	acclim_temp+test_temp+heat_shock* poly(starvation_days,2)+algae+ poly(starvation_days,2)*algae-heat_shock:algae	specimen_ID (to account for multiple measurements for each individual)
Experiment 2		
CT_{first}	log_body_mass+irradiance+poly(starvation_days,2)+ log_body_mass: poly(starvation_days,2)	Not needed
CT_{last}	log_body_mass+irradiance+poly(starvation_days,2)+ irradiance: poly(starvation_days,2)	Not needed
Temperature-corrected $\dot{M}_{O_2}$	irradiance+heat_shock*starvation_days	In the first models, specimen_ID was used but removed in the best model since it did not contribute explanatory power

Note that heat_shock is a dummy variable created to distinguish if a metabolic rate was measured before or after heat shock. The other fixed effects are all predictor variables used in the experimental design.

In experiment 2, slugs were not acclimated to two acclimation temperatures so the $\dot{M}_{O_2}$ before heat shock (17°C) and after (27°C) could be compared with temperature-corrected $\dot{M}_{O_2}$ values to account for differences due to thermodynamics

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based on preliminary experiments that indicated that 1 h was a sufficient time span to see oxygen uptake by the slug and ensure the control vials which did not contain slugs displayed stable oxygen saturations (Fig. S2). Prior to each measurement, the light was briefly turned off to avoid any interference with the optical sensor. Directly before each measurement, each vial was gently inverted to mix the water and prevent any oxygen gradients from developing. Each respiratory chamber was sterilized with ethanol and thoroughly rinsed before and after each use. At the end of the experiment, the wet mass of each slug was measured by placing it on a sheet of aluminium foil. Excess water was removed with a tissue before each slug was transferred into a pre-massed Petri dish filled with seawater on a scale.

Analyzing the rate of net oxygen exchange

To calculate the amount of seawater (and therefore oxygen) in each respiratory chamber, the volume of each slug was computed and then subtracted from the total volume of each chamber. Respiratory chambers of different sizes were used (2 ml or 22 ml), depending on the size of the slug. Since directly and accurately measuring the volume of each *E. viridis* specimen via displacement was impossible due to their small size, we used their wet mass to estimate their volume. Rather than assuming that their density equaled 1 g ml^{-1}, we measured the volume and wet mass of the larger congeneric species (*Elysia crispata*, n=10) to calculate its average density (1.25 g ml^{-1}), which we used to convert wet mass into volume for *E. viridis* and then subtracted from the total volume of the respiratory chamber. Then, the initial and final oxygen saturation measurements (%) were converted to oxygen concentrations in mg O_2 l^{-1} seawater for each experimental temperature using an online calculator (https://water.usgs.gov/cgi-bin/dotables). These values were then used to calculate the rates of oxygen uptake before and after the critical thermal maxima trials in mg O_2 taken up by each individual per hour.

We then corrected these values for differences in individual body mass, which varied across two orders of magnitude. Observed variation in body mass arose from initial differences in size associated with acclimation temperature, and from decreases in body mass during starvation. Since rates of oxygen uptake scale allometrically with body mass (Verberk et al., 2020; White et al., 2007), we could not express rates of oxygen uptake on a per gram basis. Instead, we corrected rates of oxygen uptake using an empirically derived scaling exponent. Because body mass was statistically related to acclimation temperature and starvation duration, we needed to ensure that our empirically derived scaling exponent did not reflect variation in body mass across these treatment conditions. Therefore, we calculated the exponent by fitting a random intercept for each combination of acclimation temperature and starvation period (i.e.=2 temperatures×5 starvation intervals=10 levels), thus focusing on the effect of within-treatment variation in body mass. This yielded a scaling exponent of 0.792+0.05 s.d. (Fig. 3), which agrees well with known values for ectotherms in the literature (Verberk et al., 2020; White et al., 2007). Effects of body mass explained ∼55% of the variation in absolute oxygen uptake rates.

Next, we analyzed these mass-corrected rates of oxygen uptake (mg O_2 $mg^{-0.792}$ h^{-1}), using linear mixed effects models. For each individual slug we measured oxygen uptake rates twice, before and after exposure to acute thermal stress where critical thermal maxima were assessed (Fig. 2). Both measurements were included in a single model, adding 'HS' (heat shock) as a binary variable to distinguish between oxygen uptakes measurements made before and after the critical thermal maxima trials. This approach allowed us to better disentangle the effects of the initial temperature to which they were acclimated and the temperature at which they were tested because different test temperatures were employed before (17°C and 22°C) and after (27°C and 29°C) their critical thermal maxima were measured.

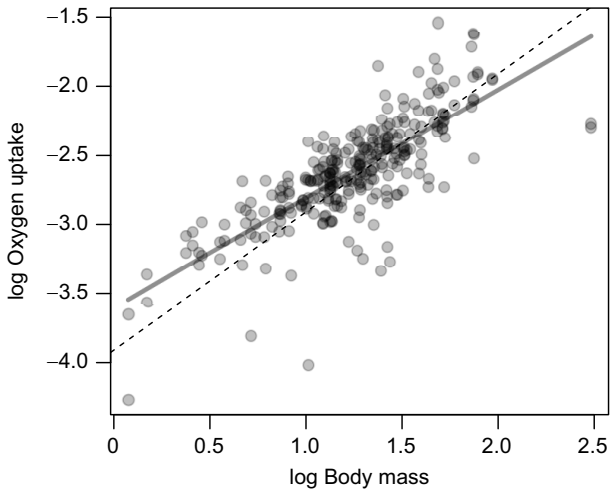

Fig. 3. The allometric scaling relationship (log_{10}–log_{10}) of body mass (mg) and oxygen uptake (mg O_2 h^{-1}). The solid line indicates the empirically derived scaling exponent of 0.792+0.05 (mean+s.d.) and the dotted line indicates isometry, i.e. a scaling exponent of 1. This empirical scaling exponent was used to calculate the mass-specific rates of oxygen uptake rates.

In experiment 1, we started with a simple additive model that included the fixed effects: acclimation temperature (17°C or 22°C), the test temperature (17°C, 22°C, 27°C or 29°C), a quadratic term for starvation duration to account for non-linear effects, the algal food source and our heat shock binary variable indicating if the measurement for oxygen uptake was taken before or after critical thermal maxima were measured. We also included slug identity as a random factor because each individual was measured twice (before and after their critical thermal maxima were measured). This additive model explained 27% of the variation (conditional R^2=0.27). Next, we also considered interactions between starvation period and algal source to test whether slugs fed with different algae responded differently to starvation, which we predicted due to the different amounts of time *E. viridis* can retain chloroplasts from these algal genera. We also tested whether responses to algal source and starvation differed for oxygen uptake rates measured before or after the thermal tolerance assay (i.e. pre- and post-heat-shock). To do this, we compared a model that included interactions between heat-shock and both starvation and algal source against a model that did not include these interactions. The best model explained 49% of the variation (conditional R^2=0.49), is displayed in Table 1 and reported in our results.

Experiment 2

Acclimation period: increasing light intensity

In a second experiment, we examined the effect of light intensity on a subset of our specimens maintained at 17°C and fed *B. plumosa* (Fig. 2). Other than light intensity, all other conditions matched those described above for experiment 1. The specimens used in experiment 2 were provided with either 100 µmol m^{-2} s^{-1} or 200 µmol m^{-2} s^{-1} of full spectrum light (Superfish SLIM LED 45) for 12 h each day. These light intensities were chosen because

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natural light intensity varies considerably and is influenced by a multitude of environmental factors (solar exposure, time of year, shade by other objects in the habitat, etc.). Algae can adjust their photoacclimation status to modulate light harvesting under these naturally fluctuating conditions, but this requires nuclear transcription and regulation. *E. viridis* lacks algal nuclei, meaning it lacks the ability to photoacclimate and repair/replace many photosystem components when they get damaged by excessive light (Rauch et al., 2015). This means that *E. viridis* hosts kleptoplasts whose photoacclimation status was determined by the light intensities to which the algae they consumed were exposed and themselves photoacclimated (Vieira et al., 2009), which occurred during the acclimation phase (i.e. 100 or 200 µmol m^{-2} s^{-1}) in our study. The higher light level is expected to increase photosynthetic capacity and oxygen production, at least in the short-term, but could, in the long-term, also lead to an increased breakdown of incorporated chloroplasts due to eventual photodamage and the inability of a slug to repair its chloroplasts (Christa et al., 2018; de Vries et al., 2013;

Vieira et al. (2009) demonstrated that exposure to 140 µmol m^{-2} s^{-1} markedly decreased photosystem II activity in *E. viridis*, shortening the time kleptoplasts remained functional when compared with specimens acclimated to 30 µmol m^{-2} s^{-1}, although photosynthetic efficiency decreased in both light treatments. We chose 100 µmol m^{-2} s^{-1} as our 'normal' light condition because 30 µmol m^{-2} s^{-1} is far lower than these species would regularly experience in the field, which often exceeds 1000 µmol m^{-2} s^{-1} at >1 m depth, (E.M.J.L., unpublished results from the same collections site in July 2021; Burgués Palau et al., 2024, in the tropics). We chose 200 µmol m^{-2} s^{-1} as our high light treatment because it exceeds the amount of light previously reported to markedly decrease photosystem II activity (Vieira et al., 2009). Light intensity was measured with an SQ-500 full-spectrum quantum sensor (Apogee Instruments, Inc., USA) using corrections for underwater measurements as instructed by the manufacturer. The quantum sensor was placed underwater in each seawater-filled aquarium and the water bath where each experiment was conducted (described below) and the light intensity measurements in µmol m^{-2} s^{-1} were recorded. After this acclimation phase in which the slugs were acclimated to either 100 µmol m^{-2} s^{-1} or 200 µmol m^{-2} s^{-1} for a minimum of 2 weeks, the slugs were transitioned to the experimental phase (detailed below).

Measuring critical thermal maxima

Critical thermal limits were measured following the protocol outlined above for experiment 1. The only difference is that the slugs were exposed to either 100 µmol m^{-2} s^{-1} or 200 µmol m^{-2} s^{-1} during the critical thermal maxima trials, to match the light intensity to which they had been exposed during the acclimation phase. To analyze CT_{first} and CT_{last} for slugs in experiment 2, we used the same method that is described above for experiment 1. To determine the best fit model, we included starvation period, body mass and light intensity as fixed effects when evaluating CT_{first} and CT_{last} (since the other predictors were not used in this experiment). The best fit models were selected based on AIC values and explained variation. As in experiment 1, both CT_{first} and CT_{last} were best explained with models containing interactions, explaining 45.9% and 37.3% of the variation for CT_{first} and CT_{last} respectively. Both models are described in Table 1.

The two stages of heat stress (CT_{first} and CT_{last}) were also positively correlated in experiment 2 (B=0.234; $t_{1,61}$=3.468; P<0.001, Fig. S1B).

Measuring the rate of net oxygen exchange

Each respiratory chamber was placed under the same light intensity to which the specimens were initially exposed to maintain the photoacclimation status of that specimen's chloroplasts (100 µmol m^{-2} s^{-1} or 200 µmol m^{-2} s^{-1}) and ensure that the slugs did not experience light stress during the experiment. The rest of the protocol was conducted as described for experiment 1.

To analyze net oxygen exchange in experiment 2, we built a model with the following fixed effects: light intensity, starvation days (again as a 2nd order polynomial), and the dummy variable (HS) to distinguish between measurements of oxygen uptake before and after critical thermal maxima were measured. We also included slug identity as a random factor since each slug was measured twice (before and after its critical thermal maxima were reached). Since the slugs in this experiment were only fed *B. plumosa* and acclimated to 17°C, algal food source and acclimation temperature were not included in this analysis. In addition, rather than including test temperature (17°C or 27°C), rates of oxygen uptake were standardized to an average temperature of 22°C, using the thermal sensitivity coefficient derived from experiment 1. This solved the problem that the effect of test temperature and heat shock were collinear in experiment 2 (slugs were measured at 17°C before the heat-shock and at 27°C after the heat shock). This initial model explained 64% of the variation (adjusted R^2) and had an AIC value of −29.40. We then simplified this model by removing non-significant interactions, producing a simpler model that also explained 64% of the variation and had a lower AIC value (–32.55). This simpler model is reported in our results and contains light intensity, HS and starvation days as fixed effects with an interaction between HS and starvation days (Table 1).

Measuring color and photosynthetic efficiency

The color of each specimen was recorded before its oxygen uptake and thermal maxima were recorded. *Elysia viridis* get their dark green coloration from their kleptoplasts and they fade to a terracotta color as kleptoplasts are digested during starvation (Rauch et al., 2018). Four distinct color stages were observed (dark green, pale green, pale brown, terracotta). Five specimens of each color were dark-acclimated for 15 min before being measured with a pulse amplitude modulated (PAM) fluorometer (Mini-PAM, Walz GmBH, Germany), to determine the average photosynthetic efficiency (F_v/F_m) at each color stage and to assess if specimens had ingested chloroplasts that could photosynthesize and the photosynthetic efficiency of these chloroplasts, as has been observed in previous studies (see e.g. Laetz and Wägele, 2018; Serôdio et al., 2014; Vieira et al., 2009).

RESULTS

Specimen mass, color and photosynthetic activity during starvation

All slugs had a dark green color due to the chloroplasts they had previously incorporated when they entered the lab and began the acclimation phase. They remained dark green throughout the acclimation phase during which they were allowed to feed *ad libitum*. Once they entered the starvation period, color changes were observed. After an extended period of starvation, many of the slugs had terracotta coloration, indicating they had lost most of their chloroplasts (Fig. 4A). During starvation, the chloroplasts within all slugs became less efficient, as indicated by decreasing F_v/F_m (PAM fluorometry) measurements (Fig. 4B). Slugs also exhibited weight loss and shrinkage during the starvation period (Fig. 4B). In Experiment 1, slugs fed *C.* cf. *linum* were larger than slugs fed only

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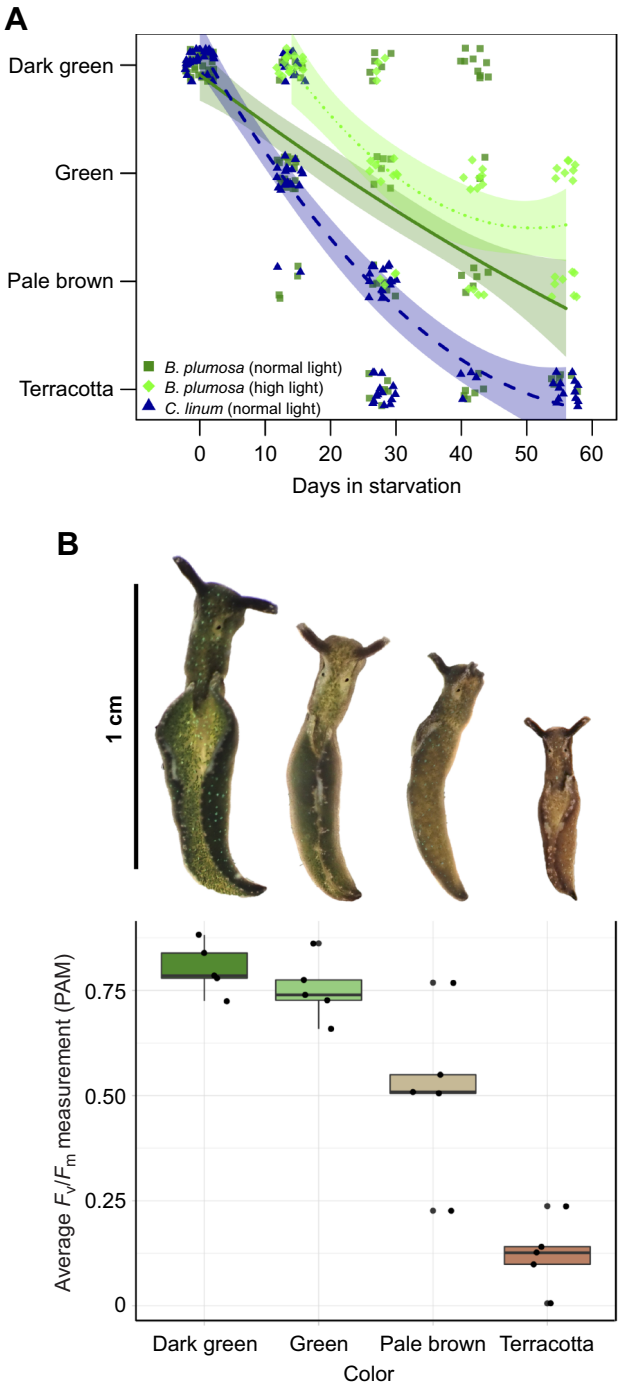

Fig. 4. Change in color during starvation and photosynthetic efficiency (F_v/F_m) at each color stage for specimens acclimated to 17°C. (A) Loss of chloroplasts during starvation causes a color change from dark green to green to pale brown and finally terracotta. The rate of bleaching and hence loss of chloroplasts differed between the two algal food sources at 17°C. Specimens fed *B. plumosa* under normal light levels (100 µmol m^{-2} s^{-1}) are depicted in dark green squares, specimens fed *B. plumosa* under high light levels (200 µmol m^{-2} s^{-1}) are shown in light green diamonds and specimens fed *C.* cf. *linum* are illustrated in dark blue triangles. Data points are jittered on both axes to increase visibility. Model predictions are from a model with starvation period as a quadratic function. The lines are regression lines and the shaded bands are 95% confidence intervals. (B) An example of a slug depicting each color and the average F_v/F_m value measured by pulse amplitude modulated (PAM) fluorometry for specimens assigned to each color category (n=5). The horizontal lines indicate the median, each box depicts the interquartile range and the dots indicate the measured values (jittered on the x-axis only for increased visibility). Each box represents the PAM values measured for slugs in each of the four color categories.

B. plumosa after the acclimation phase, making them larger when they commenced the starvation period (Fig. S3). However these slugs also lost mass faster than slugs fed *B. plumosa* during the starvation period. For example, at 28 days in starvation, slugs fed *C.* cf. *linum* averaged 18.91±8.08 mg (mean±s.d.) while those fed *B. plumosa* averaged 27.29±14.56 mg (mean±s.d.) (at 17°C and 100 µmol m^{-2} s^{-1}).

Heat tolerance in experiment 1

Experiment 1 examined how thermal acclimation, starvation and algal food source impact a slug's critical thermal maxima. Variation in both measures of critical thermal maxima (CT_{first} and CT_{last}) could be related to effects of acclimation temperature (Fig. 5), starvation, body mass and algae (Fig. 6, Tables S1 and S2). Slugs acclimated to 22°C (the higher temperature) generally showed improved heat tolerance compared with slugs acclimated to 17°C, but the exact magnitude differed between CT_{first} and CT_{last} (Fig. 5; Tables S1,S2).

The acclimation response ratios (ARRs, which expresses how much heat tolerance in °C is gained for every degree of warm acclimation) was on average higher for CT_{first} (ARR=0.218), than for CT_{last} (ARR=0.068). Differences in heat tolerance with acclimation temperature were more pronounced in larger slugs. Larger-bodied slugs exhibited reduced heat tolerance, a pattern that was most evident for CT_{last} and especially pronounced in specimens kept at the lower acclimation temperature (Fig. 6A,B). The amount of time spent in starvation also affected the thermal tolerance of the slugs. Halfway through starvation we found the largest difference between acclimation temperatures, with slugs acclimated to colder temperatures having lower heat tolerance, and this was most evident for CT_{first} (Fig. 6C,D). In contrast, at the start (0 days starved) and at the end (60 days starved) of the starvation period, heat tolerance was

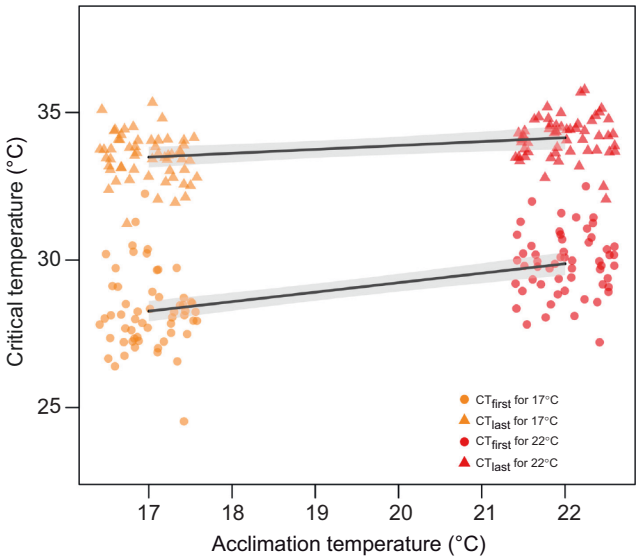

Fig. 5. Improvements in critical thermal maxima in *E. viridis* with acclimation temperature. Values for CT_{first} and CT_{last} are shown separately with circles and triangles, respectively, whereas colors indicate different acclimation temperatures (17°C in orange, 22°C in red).

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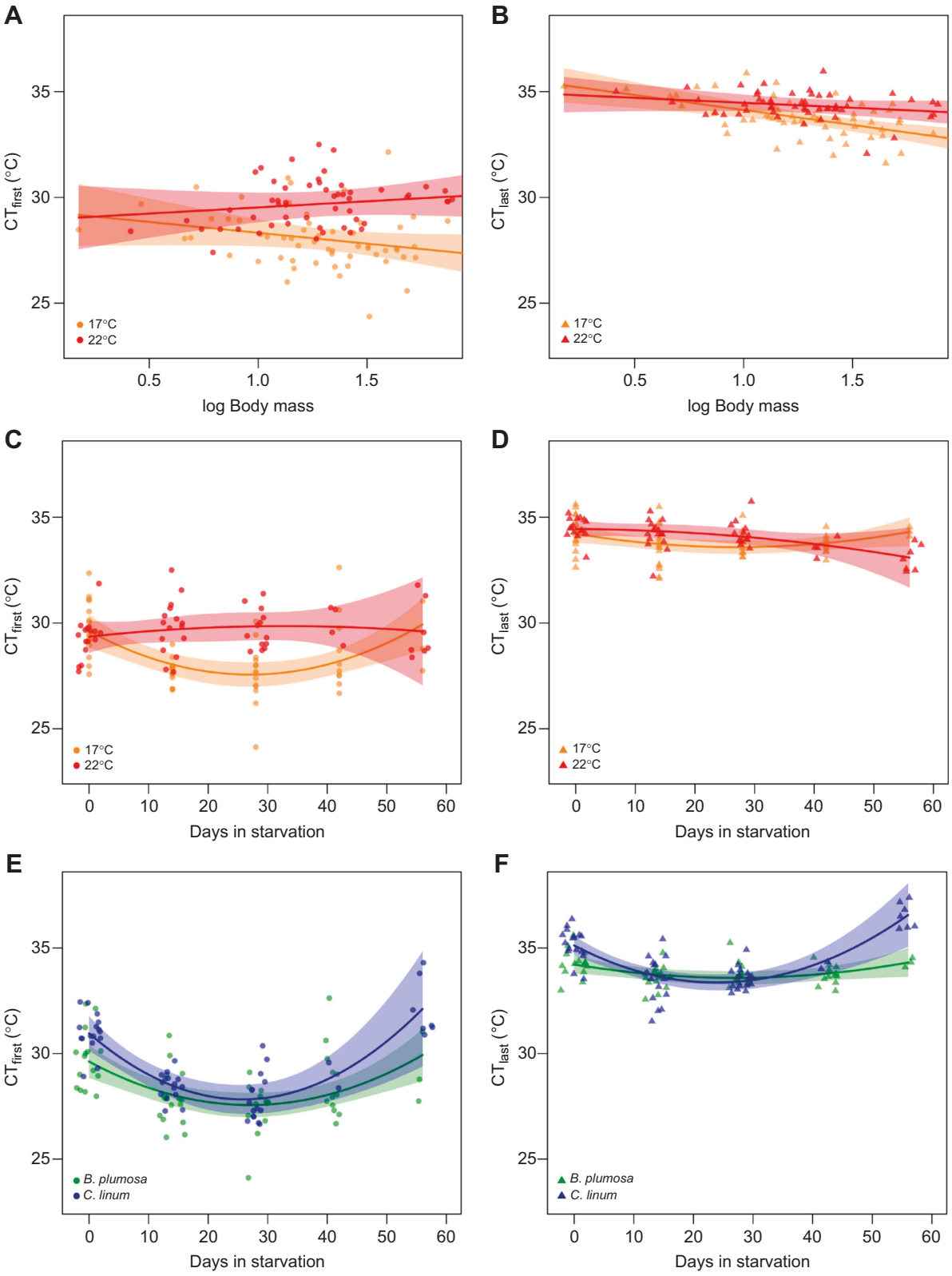

Fig. 6. Variation in critical thermal maxima in *E. viridis*. CT_{first} (A,C,E) and CT_{last} (B,D,F). Improvements in heat tolerance with log_{10}-transformed body mass (A,B) and with acclimation temperature varied with starvation duration (C,D). Thermal tolerance also varied owing to interactive effects of starvation duration and the algal food source that was provided (E,F). Colors for acclimation temperature and symbols for CT type are the same as in Fig. 5. Slugs fed with *B. plumosa* are shown in dark green, and those fed with *C.* cf. *linum* are represented in dark blue in E and F (all under normal light). Note that all panels with starvation duration on the *x*-axis (C–F) contain data points that are slightly jittered along the *x*-axis. All panels represent partial residual plots, which illustrate the relationship between the response variable and a given independent variable while accounting for the effects of other independent variables in the model (Tables S1, S2). The lines are regression lines and the shaded bands are 95% confidence intervals.

generally greater (Fig. 6C). This pattern of greater heat tolerance at the start and end of the starvation period was further accentuated by the algal food source they had ingested prior to experimentation: slugs feeding on *C.* cf. *linum* displayed higher thermal tolerances than those fed on *B. plumosa* (Fig. 6E,F; Table S1).

Net oxygen exchange in experiment 1

The oxygen concentration decreased in every respirometry chamber throughout the experimental period, indicating that oxygen consumption via aerobic respiration outpaces oxygen production via photosynthetic activity, resulting in a net oxygen uptake across all animals and treatments. A strong allometric scaling relationship was evident between rates of oxygen uptake and body mass (Fig. 3), so our subsequent analyses focused on mass-corrected rates of oxygen uptake (mg O2 g-0.792 h-1; see Materials and Methods). Rates of oxygen uptake increased with measurement temperature (Fig. 7A; t1,109=3.476; P<0.001), and, for a given measurement temperature, slugs acclimated to a warmer temperature exhibited reduced rates of oxygen uptake (Fig. 7B, t1,109=−3.309; P=0.001). Oxygen uptake rates were depressed after the critical thermal maxima trials, i.e. the slugs took up less oxygen after exposure to an acute heat shock (Fig. 7C, t1,109=−4.036; P<0.001). Slugs fed with *C.* cf. *linum* and measured at the start of the starvation period exhibited higher rates of oxygen uptake than those fed with *B. plumosa*. However, following starvation, differences in rates of oxygen uptake due to algal food source disappeared (Fig. 7D,E; Table S3).

Heat tolerance in experiment 2

Experiment 2 examined the effect of light intensity on thermal tolerance in slugs acclimated to 17°C and fed *B. plumosa* (the species whose chloroplasts can be retained for a longer time span). Slugs exposed to 200 µmol m^{-2} s^{-1} (the higher light intensity) displayed higher critical thermal maxima (∼2°C) than those exposed to 100 µmol m^{-2} s^{-1}. This increase in thermal tolerance was observed throughout most of the starvation period for CT_{first} (Fig. 8A), but for CT_{last} it was most evident during the middle of the starvation period (Fig. 8B). For both CT_{first} and CT_{last} the effect of light on critical thermal maxima disappeared at the end of the starvation period when all slugs had lost most of their chloroplasts.

Net oxygen exchange in experiment 2

As in experiment 1, the oxygen concentration decreased in every respirometry chamber throughout the experimental period, despite ongoing photosynthetic activity at the beginning of the starvation period. This resulted in a net oxygen uptake across all animals and treatments. Differences in light intensity (experiment 2) did not affect oxygen uptake rates (P=0.194).

DISCUSSION

Heat tolerance and oxygen

Elysia viridis heat tolerance improved following exposure to 22°C, when compared with slugs acclimated to 17°C. This acclimatory ability to improve heat tolerance is widely investigated in ectotherms (Gunderson and Stillman, 2015), including sea slugs (Armstrong et al., 2019). The ARR values that we found (0.218 and 0.068 for CT_{first} and CT_{last}) fall within the range of values previously reported for sea slugs (Armstrong et al., 2019). Perfect acclimation (i.e. an ARR value of 1) would mean that the animal can keep increasing its heat tolerance in tandem with warming of its habitat. Since acclimation was not perfect (ARR<1), there appears to be a limit in how plasticity in heat tolerance can help buffer *E. viridis* against the highly variable temperature fluctuations that they experience in their uppermost subtidal habitats. Note also that improvements in heat tolerance following thermal acclimation are likely magnified on longer timescales (Verberk et al., 2023). Our findings of interactions between acclimation temperature and body size also align with the findings by Rohr et al. (2018) who reported greater acclimation capacity in larger animals, especially when animals were given sufficient time to acclimate, as was the case in this study. Acclimation temperature had a stronger effect on CT_{first} (an endpoint associated with lower temperatures that reflects loss of voluntary muscle control) whereas CT_{last} (an endpoint associated with higher temperatures that reflects neuronal dysfunction) was less responsive to acclimation temperature (Fig. 5A). One way to explain this is that with increasing intensity of heat stress, more and more physiological mechanisms (e.g. oxygen provisioning, protein structure, membrane function) will become prone to failure and physiological adjustment of all these mechanisms will become increasingly difficult. Thus, earlier critical points, which are recorded at lower temperatures, may involve fewer mechanisms, which makes them more malleable in terms of physiological acclimation; however, other explanations remain possible as well.

Several observations are consistent with the hypothesis that oxygen becomes limiting when animals reach their thermal maxima, necessitating a switch to anaerobic metabolism. Oxygen availability in water has a demonstrable effect on heat tolerance in gastropods (Davenport and Davenport, 2007; Hoefnagel and Verberk, 2017; Koopman et al., 2016). While most marine gastropods use both gills and their epidermis for gas exchange, most sacoglossans including *E. viridis* take up oxygen exclusively through their epidermis (Graham, 1990; Neusser et al., 2019; Weaver and Clark, 1981), a mode of respiration that is especially prone to suffer from oxygen limitation at thermal extremes (Verberk and Bilton, 2013). Moreover, smaller individuals have higher surface area to volume ratios, which provides more area for O_2 diffusion, and we observed that in slugs acclimated to 17°C, smaller individuals were more heat tolerant than larger individuals, at least for CT_{last} (Fig. 5B). A previous meta-analysis also showed that larger aquatic ectotherms exhibited lower heat tolerance, especially in longer ramping trials where the efficacy of anaerobic metabolism is reduced (Leiva et al., 2019). In this study, we increased the temperature by 1°C every 15 min so the ramping trials took multiple hours. We also observed an increase in heat tolerance for individuals exposed to high light intensity (∼2°C higher than slugs in low light conditions), which was most pronounced after 30 days of starvation and disappeared towards the end. Internal oxygen production from photosynthesis throughout the animal in the highly branched digestive gland could alleviate oxygen limitation. Sugars produced via photosynthesis could also boost thermal tolerance and future experiments to examine this will be required.

As starvation progressed, the slugs modulated their heat tolerance. The algal food source slugs consumed had larger effects at the start of the starvation period, whereas acclimation temperature had stronger effects in the middle of the starvation period. While we have no definitive explanation for these patterns, it does suggest that different mechanisms may be governing thermal tolerance each operating at specific temporal windows. For example, recently fed slugs could have larger pools of sugar and a subsequent larger capacity to recruit anaerobic metabolism, erasing any difference due to acclimation temperature, whereas starved slugs may rely more on aerobic metabolism, depressing oxygen demand (see below), possibly contingent upon acclimation temperature, rendering them more responsive to acclimation temperature.

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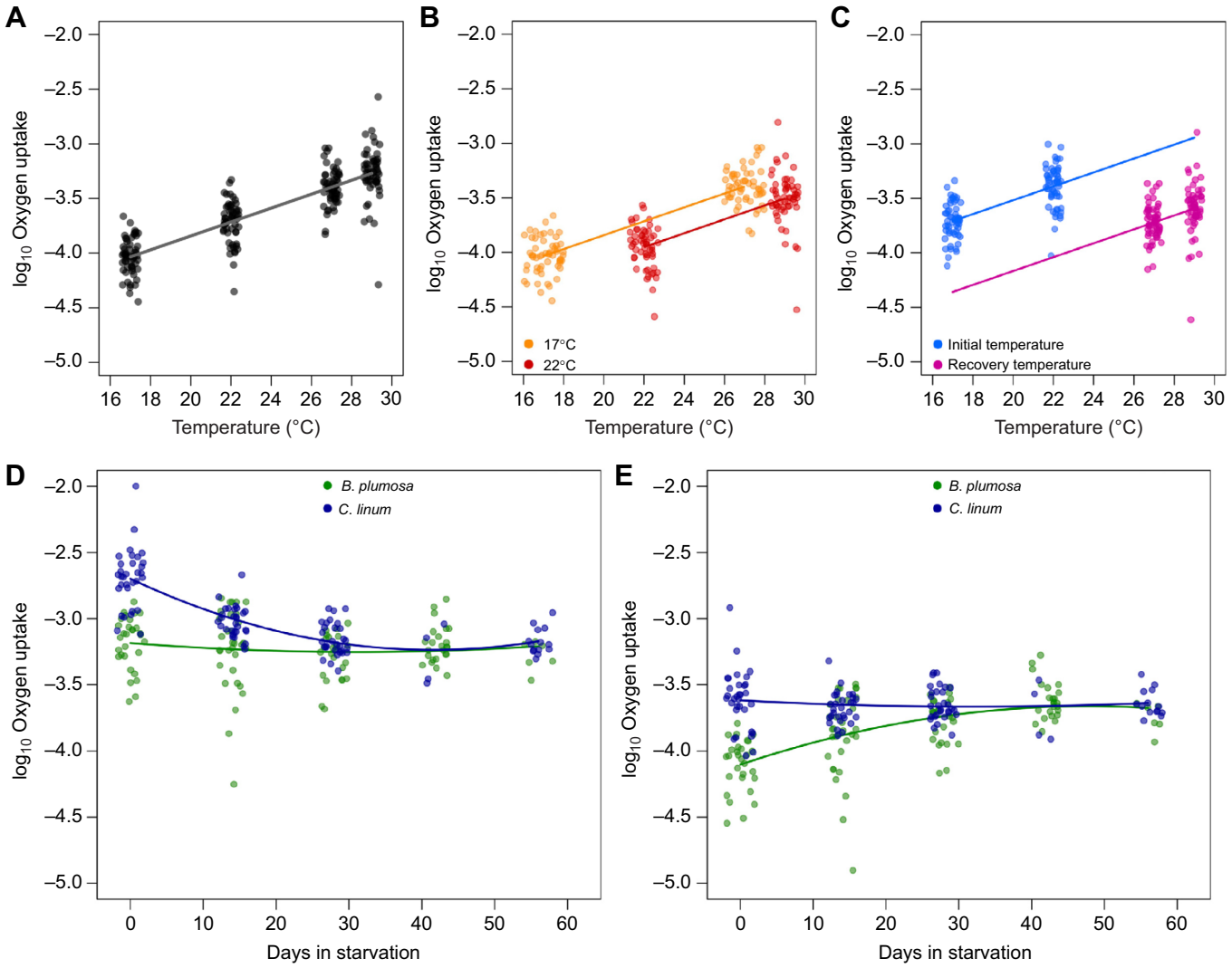

Fig. 7. Net oxygen exchange between *E. viridis* and the seawater in the respirometry chamber. Note that all individuals exhibited net oxygen consumption (measured in mg O_2 $g^{-0.792}$ h^{-1}) and that negative values are below 1 (which then become negative after $\log_{10}$ transformation). (A) The rate of oxygen consumption by specimens at each of the temperatures at which they were tested (i.e. the 17°C and 22°C acclimation temperatures and the 27°C and 29°C recovery temperatures after the CT trials). (B) Rates of oxygen exchange for each of the acclimation temperatures (17°C in orange, 22°C in red) and (C) for each temperature before the CT trials (blue) and after the CT trials (magenta). Rate of oxygen exchange was also affected by the algal food source with which slugs were fed and this differed during starvation both (D) before the CT trials and (E) afterward. Slugs fed *B. plumosa* are depicted in dark green, and specimens fed *C.* cf. *linum* are depicted in dark blue. Note that individual data points are slightly jittered on the *x*-axis and the lines/curves are regression lines/curves. These partial residual plots illustrate the relationship between the response variable and a given independent variable while accounting for the effects of other independent variables in the model.

Food and light intensity

In their natural habitats, *E. viridis* can be found in great abundance near or on their food algae (Hinde and Smith, 1975; Trowbridge et al., 2008). This study demonstrates that the Dutch population of *E. viridis* from Bommenede naturally feeds on *B. plumosa* and that it can retain chloroplasts from *B. plumosa* for more than a month, which expands the list of algal species whose chloroplasts can be retained by these kleptoplastic slugs. The abundance and availability of algal species on which these slugs feed can vary greatly throughout the year in the North Sea (Baumgartner and Toth, 2014; E.M.J.L., unpublished observation). Data on the year-round abundance of *B. plumosa* in Zeeland is however lacking, and algal species can be periodically unavailable if conditions for germination are unfavorable (Rietema, 1969; 1970). Food availability may therefore be a limiting factor for temperate, stenophagous species such as *E. viridis* and this could have selected for the evolution of kleptoplasty or the ability to feed on multiple algal food sources (Wägele and Martin, 2014).

Almost every specimen acclimated to 22°C had a yellowish appearance due to chlorophyll breakdown by 28 days in starvation, whereas many specimens acclimated to 17°C retained bright green coloration from chlorophyll for more than 42 days. Since kleptoplasts provide some energy to starving *E. viridis* via photosynthesis and this energy may help them withstand periods of food unavailability (Cartaxana et al., 2019), the premature breakdown of chlorophyll earlier in the starvation period at 22°C could have serious energetic consequences for starving slugs during times of food unavailability in a warmer world.

Our results demonstrate that *E. viridis* fed exclusively *C.* cf. *linum* could retain functional chloroplasts for more than 17 days in starvation, contrasting with reports hypothesizing that *E. viridis* could not retain *C.* cf. *linum* chloroplasts (Clark et al., 1990;

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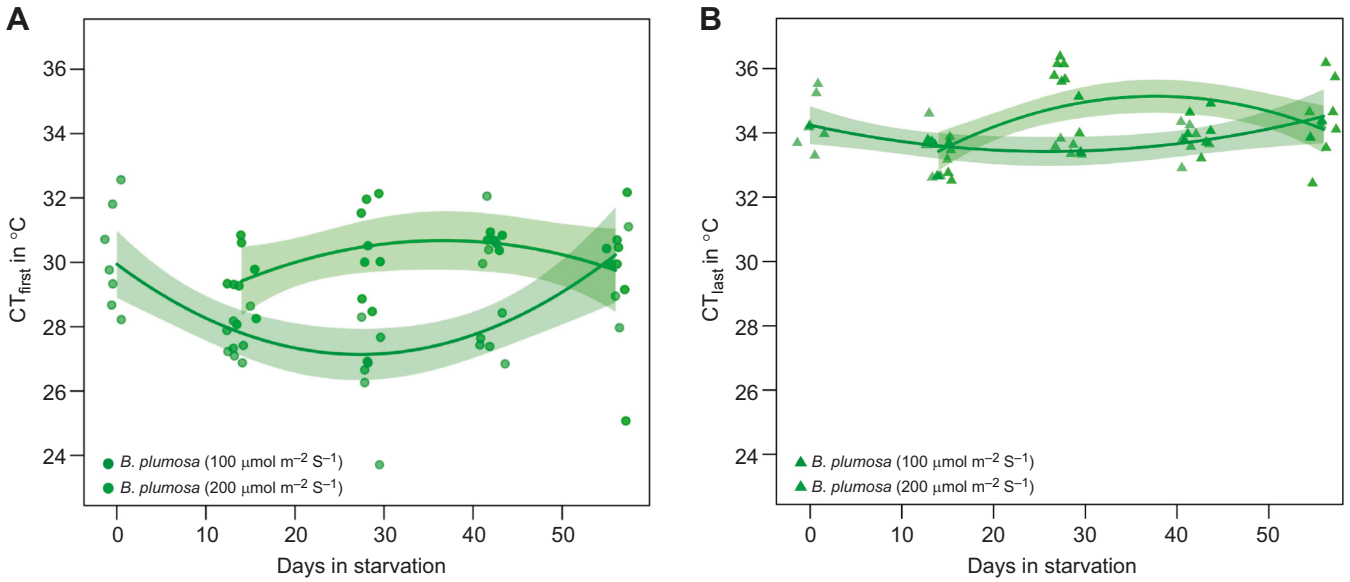

Fig. 8. Critical thermal limits under different light intensities for *E. viridis* kept at 17°C and fed *B. plumosa*. (A) For CT_{first}, slugs exposed to 200 µmol m^{-2} s^{-1} had a higher thermal tolerance than slugs exposed to 100 µmol m^{-2} s^{-1} throughout the starvation period. (B) Slugs exposed to high light levels of 200 µmol m^{-2} s^{-1} initially exhibited the same heat tolerance for CT_{last}, which increased in the middle of the starvation period before returning to the same levels as observed in specimens exposed to normal light levels of 100 µmol m^{-2} s^{-1}. Note that individual data points are slightly jittered along the *x*-axis. This partial residual plot illustrates the relationship between the response variable and a given independent variable while accounting for the effects of other independent variables in the model. The lines are regression lines/curves and the shaded bands are 95% confidence intervals.

Trowbridge and Todd, 2001). Moreover, slugs fed exclusively on *C.* cf. *linum* grew almost twice as long and they were ∼3 times heavier than members of the same population that were maintained on *B. plumosa*. Baumgartner and Toth (2014) found that *E. viridis* gained more mass when fed *C. melagonium*, aligning with our observations and indicating that *Chaetomorpha* spp. are particularly nutritious for *E. viridis*, even if certain populations rarely consume this alga in nature (Trowbridge et al., 2008). Other sacoglossan species have also been reported to thrive on food algae on which they are seldom observed eating in nature (Barber et al., 2021; Clark, 1975). Before their critical thermal maxima were measured, slugs fed with *C.* cf. *linum* exhibited higher rates of (mass-corrected) oxygen uptake than those fed with *B. plumosa*, possibly due to the increased growth and locomotion we observed in these *C.* cf. *linum*-fed specimens. These differences disappeared with longer starvation duration, probably due to the faster rate of chloroplast breakdown in specimens fed *C.* cf. *linum*. Despite their large initial size, *E. viridis* specimens fed *C.* cf. *linum* lost both mass and photosynthetic capacity faster than specimens fed *B. plumosa*, suggesting that kleptoplasts from *C.* cf. *linum* are less advantageous in terms of nutrition and oxygen production during extended starvation. In contrast, specimens fed *B. plumosa* and exposed to high light levels retained functional chloroplasts the longest and exhibited the lowest weight loss during starvation.

Oxygen uptake

Slug metabolism was strongly related to body mass according to a power law with a mass scaling exponent of 0.79, which agrees with empirical estimates for ectotherms (Verberk et al., 2020; White et al., 2007). We adjusted oxygen uptake rates using this empirical mass scaling exponent to enable a comparison across large and small slugs. Note that simply using mass specific oxygen uptake (i.e. dividing respiration rates by mass) would have caused us to underestimate metabolism in larger individuals. Several factors in our experiment (e.g. food, starvation, temperature) affected body mass and by using the empirical mass correction, we accounted for indirect effects of these factors on metabolic rate via changes in body size and instead isolated any direct effects. In our model, test temperature and acclimation temperature affected slug metabolism in opposite ways, confirming the known thermodynamic effect of increasing respiration at acutely higher test temperatures and the subsequent response of downregulated oxygen uptake rates following acclimation to warmer conditions (22°C) (Fig. 6B) to compensate for this thermodynamic effect (Seebacher et al., 2015). In every trial, we measured net oxygen uptake, indicating that photosynthesis in *E. viridis* did not produce enough oxygen to fully support aerobic respiration. It was not possible to determine how much oxygen was produced via photosynthesis in each individual, since examining each animal in the dark or subjecting to photochemical inhibition would have required repeat measures or doubling the sample size, both of which were not feasible during this study. This means that the ‘true’ amount of oxygen needed to support aerobic respiration will require further study. Previous work by Dionísio et al. (2018) on *E. viridis* showed net oxygen production via photosynthesis which contrasts our results. However, it is not clear on which algal species this population of *E. viridis* specimens was feeding since they were provided both *B. plumosa* and *Codium tomentosum*. Thus, the discrepancy between our study and theirs could be due to the incorporation of chloroplasts from *C. tomentosum*.

During starvation, *E. viridis* digest their incorporated chloroplasts (Laetz et al., 2016; Rauch et al., 2018) so the sampling points that occurred late in the starvation period (42 and 56 days) surveyed animals that contained fewer chloroplasts than they had at the onset of starvation, as recorded by the change in coloration we observed. The functionality of the remaining chloroplasts is uncertain; however, the extremely low or complete lack of photosystem II activity we measured with PAM fluorometry suggests that the remaining chloroplasts display a low photosynthetic efficiency. This indicates that oxygen production due to photosynthetic activity has

likely decreased late in starvation. We expected to observe increased oxygen uptake rates as starvation progressed as slugs take up more oxygen from the seawater to compensate for the declining oxygen availability from photosynthesis; however, oxygen uptake rates decreased during starvation for individuals fed *C.* cf. *linum* and stayed constant for individuals fed *B. plumosa* in non-heat shocked individuals. When facing starvation, animals may conserve ATP by metabolic suppression (de Vries et al., 2015; Semsar-Kazerouni et al., 2020), which likely explains the abrupt decline in oxygen uptake we observed as starvation began (Fig. 3C). Metabolic suppression is a survival strategy in which an organism suppresses non-critical cellular processes to reduce its oxygen uptake and conserve energy in an attempt to ensure long-term survival during acute environmental stress (Sokolova and Pörtner, 2001). Locomotion, growth and reproduction may all be curtailed to save energy. Metabolic suppression likely also explains the decrease in oxygen uptake observed when comparing oxygen uptake rates measurements before and after the heat tolerance assay (compare Fig. 6D,E). This strategy is often observed in intertidal gastropods that have shells in which to retreat during periods of thermal stress (Marshall et al., 2011; Sokolova and Pörtner, 2001, 2003), but examinations of slugs are limited.

Conclusions

The data presented here indicate that *E. viridis* will be able to cope with gradual and acute warming of their habitat. To deal with temperature increases, they can downregulate their metabolism and increase their heat tolerance via plasticity. All but three of the *E. viridis* specimens that experienced acute heat stress were able to recover from the loss of voluntary muscle control and neuronal dysfunction associated with heat shock, indicating that exposure to their thermal maxima (CT_{last}) is rarely lethal. This species lives in shallow coastal waters where exposure to heat and high light intensity will be positively correlated. Internal oxygen production via photosynthesis may help alleviate some degree of oxygen limitation, even if it does not provide all of the oxygen needed to fuel aerobic respiration. Provided their algal food species remain plentiful and do not experience dramatic seasonality shifts due to warmer conditions so the slugs are not faced with excessive starvation periods, protracted warm periods should not be a problem for *E. viridis*. Finally, the surface temperature at our collection site varied seasonally between ∼5 and 23°C (Rijkswaterstaat Water Info; https://waterinfo.rws.nl/#!/details/publiek/watert). *Elysia viridis* live above the thermocline for most of the year, but migrated below it in July when the surface temperature exceeded 17°C. This avoidance behavior and the physiological adjustments in oxygen uptake and heat tolerance suggest that *E. viridis* with access to their food algae are capable of withstanding the warmer waters of the future.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.M.J.L.; Methodology: E.M.J.L., C.K., W.C.E.P.V.; Software: E.M.J.L.; Validation: E.M.J.L., W.C.E.P.V.; Formal analysis: E.M.J.L., W.C.E.P.V.; Investigation: E.M.J.L., C.K., N.M.B., M.M.; Resources: E.M.J.L., S.E.T.v.d.M., W.C.E.P.V.; Data curation: E.M.J.L., C.K., N.M.B., M.M., W.C.E.P.V.; Writing - original draft: E.M.J.L., C.K., N.M.B., M.M., W.C.E.P.V.; Writing - review & editing: E.M.J.L., N.M.B., M.M., S.E.T.v.d.M., W.C.E.P.V.; Visualization: E.M.J.L., W.C.E.P.V.; Supervision: E.M.J.L., S.E.T.v.d.M., W.C.E.P.V.; Project administration: E.M.J.L., S.E.T.v.d.M.; Funding acquisition: E.M.J.L., W.C.E.P.V.

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Data availability

All of our data and R-scripts are publicly available in the online data repository, DataverseNL, under a CC-BY license: https://doi.org/10.34894/AUFQE7.

ECR Spotlight

This article has an associated ECR Spotlight interview with Elise Laetz.

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