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One year of warming leads to the total loss of productivity in a widespread photosymbiosis

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ABSTRACT: Photosymbioses, in which photosynthetic microorganisms reside within heterotrophic hosts, are important components of aquatic ecosystems and are under threat from environmental warming. The immediate ecological consequences of acute warming for archetypal photosymbioses, such as those between corals and zooxanthellae, are well documented. In contrast, understanding of the evolutionary responses of photosymbioses to persistent warming remains limited and direct observations of evolution in response to warming are scarce, as many associations are slow-evolving and do not enable observations on a tractable timescale. To address this knowledge gap, we exposed the widespread microbial *Paramecium bursaria*–*Chlorella* spp. photosymbiosis to 295 d of continuous growth under +5°C of persistent warming. We subsequently quantified the thermal responses of traits associated with symbiosis persistence and ecological function (growth rate, symbiont density [the number of symbionts within hosts], and metabolic rates) compared with cultures maintained at ambient temperature and cultures exposed to −5°C of cooling for the same time period. Strikingly, while growth rate thermal optimum increased with warming, net photosynthesis and carbon-use efficiency (the proportion of photosynthetic carbon available for growth) both strongly declined to zero. These data suggest a significant change in ecological function with persistent warming. We also detected larger autonomous symbiont populations following 295 d of warming, and symbionts from the warm-adapted symbiosis demonstrated a 'switch' from exclusive growth on organic to inorganic nitrogen, suggesting that symbionts could have evolved increased autonomy from hosts. Thus, warming could erode the ecological function and promote symbiont autonomy in photosymbiosis over evolutionary timescales.

KEY WORDS: Evolution · Warming · Mutualism · Photosymbiosis · Photosynthesis

1. INTRODUCTION

Symbiotic mutualisms are critical ecological interactions that have long been studied in the context of conservation (Bronstein 2009). Some well-known mutualisms are exemplified by plant–pollinator relationships upon which most flowering plants depend (Devaux et al. 2014), human gut microbiota that are critical for host survival (Marchesi et al. 2016), and the associations between chemosynthetic bacteria and marine annelids that enable non-photosynthetic primary production in deep-sea ecosystems (Smith preprint https://doi.org/10.5194/bgd-9-17037-2012). However, in the present era of climate change — within the warmest multi-century period in more than 100 000 yr — warming-induced degradation has been observed across many important mutualisms, risking the critical ecosystem services and biodiversity that they support (Kiers et al. 2010, Hom & Penn 2021, IPCC 2023, Toullec et al. 2024). This trend is set to increase in the future as global mean temperatures could rise by an increment of up to ~5°C within the century (IPCC 2023). Accordingly, understanding the possible future impacts of temperature-

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driven changes on mutualisms and their wider ecosystems remains a major research challenge for ecologists today.

An important class of symbiotic mutualism known to be under threat from warming is photosymbiosis (Weis 2010, Lesser 2011, Davy et al. 2012, Bailly et al. 2014). These keystone interactions are responsible for ~50% of all marine photosynthesis and thus a significant portion of the total global carbon budget, representing significant biodiversity and ecologically important ecosystem engineers such as corals and giant clams (Decelle 2013, Bailly et al. 2014, Decelle et al. 2015, Schmidt et al. 2016). Although acute thermal stress has clear impacts on photosymbiosis in the short term, particularly well characterised in corals, it is also likely that persistent warming will impact photosymbioses over evolutionary timescales (Moran & Wernegreen 2000, Sachs & Simms 2006, Sachs et al. 2011, Frederickson 2017, Baker et al. 2018, Werner et al. 2018). The evolutionary trajectories along which these associations may travel are diverse (Sachs & Simms 2006); for example, evolutionary responses may sustain symbiosis if selection drives thermal niche adaptation, but other outcomes are possible, such as extinction of one or both partners, reversion of partners to autonomy, and shifts from mutualism to parasitism (Sachs & Simms 2006, Sachs et al. 2011, Werner et al. 2018).

Empirical measurements are required to support the theoretical responses (Baker et al. 2008, Kiers et al. 2010, Chakravarti & van Oppen 2018). While the thermal responses of autonomous symbionts of corals have been cultivated and studied over multiple generations — research that has revealed their capacity for rapid evolution (Chakravarti & van Oppen 2018), paralleling evolutionary findings in free-living phytoplankton (Padfield et al. 2016, Schaum et al. 2017) — studies involving the experimental evolution of hosts together with their resident symbionts appear to be scarce (Salsbery & DeLong 2021). This is likely because evolutionary studies are complicated by slow-growing and difficult-to-cultivate photosymbiotic hosts (Chakravarti & van Oppen 2018). As a result, evolutionary responses remain challenging to observe directly in these associations, with predictions derived from theory (Sachs & Simms 2006) or induced from fossil records (Davis et al. 2022).

To help assess the thermal responses of photosymbiosis following a relatively long time period in the laboratory (295 d), we selected a tractable photosymbiosis that is capable of rapid growth in the laboratory, as has been used in a recent evolutionary study (Salsbery & DeLong 2021). *Paramecium bursaria* is a widespread, globally present and abundant freshwater ciliate that harbours symbiotic algae such as *Chlorella* spp. (Minter et al. 2018, Greczek-Stachura et al. 2021, Spanner et al. 2022). While a useful model of 'classical' photosymbiosis that has been studied for at least 50 yr (Karakashian 1975, Sørensen et al. 2019), it is also representative of ecologically relevant microbial photo-mixotrophs, now recognised to be highly abundant and diverse in aquatic ecosystems (Sanders 1991, 2011, Summerer et al. 2008, Sonntag et al. 2011, Decelle et al. 2015, Greczek-Stachura et al. 2021, Spanner et al. 2022). Furthermore, the freshwater environment is known to be particularly susceptible to climate warming for a number of reasons, including the limited capacity for organisms to disperse, tight coupling between temperature and environment, and compounding contemporary stressors such as microplastic contamination (Woodward et al. 2010, Makin 2023), with aquatic protists being ubiquitous constituents of these ecosystems (Weisse et al. 2016). This means that this microbial association is both a tractable model organism and is ecologically relevant in its own right.

In this study, we exposed the *P. bursaria–Chlorella* spp. photosymbiosis to persistent +5°C warming, approximating the upper predicted temperature increase increment within the century by the IPCC (IPCC 2023) while maintaining an absolute temperature that is tolerable and realistic for this association (Możdżeń et al. 2017). We maintained cultures under warming for 295 d and assessed the responses of traits associated with symbiosis maintenance and ecological function (growth rate, symbiont density, photosynthesis, and carbon-use efficiency [CUE]) and compared our findings with those from control cultures (in which ambient temperature was maintained) and cultures subjected to −5°C of cooling. Specifically, our aim was to examine (1) the possible evolutionary trajectories for photosymbioses under warming, (2) the capacity of the association to rapidly adapt to warming, and (3) any changes in the ecological function of the photosymbiosis (with particular focus on net primary productivity, given the clear importance of photosymbiotic primary production; Bailly et al. 2014) and thus the wider ecological consequences of warming with respect to photosymbiosis. We suspected that whether the photosymbiosis is able to increase its thermal performance, maintain its productivity, and ultimately persist in the face of warming will depend on whether it is able to compensate for temperature-induced impacts on metabolism (Padfield et al. 2016, Barton et al. 2020) and therefore the metabolite trade central to the symbiosis (Johnson 2011b).

2. MATERIALS AND METHODS

Fig. 1 provides a general overview for the experimental design. Notably, many of the specific methodological procedures (Makin 2023, 2024) and statistical methods (Padfield et al. 2016) have been previously published.

2.1. Cultures and temperature treatments

The *Paramecium bursaria* strain (HA1g, National BioResource Project) used in this study was originally isolated in Hirosaki, Japan, in 2010 and was maintained in our laboratory at 25°C prior to the experiments for ~1 yr, as is typical of *P. bursaria* laboratory strains (Sørensen et al. 2021). *P. bursaria* cells (i.e. symbiotic hosts with their intracellular resident symbionts living inside them) were originally established in 6 replicate 200 ml stock cultures at each of 3 temperatures at approximately 100 cells per ml: 20°C (cooled), 25°C (ambient), and 30°C (warmed). Each culture was subcultured into fresh growth medium every 2 wk to allow continuous growth, at which point a cell count was performed and a volume was transferred into fresh medium such that the starting density following each transfer was approximately 100 cells per ml in a final culture volume of 200 ml. The growth medium into which cells were transferred comprised protozoan pellet/Volvic medium inoculated with a clonal strain of *Serratia marcescens* for 48 h prior to use (grown at 25°C to standardise growth conditions) to provide an abundance of bacterial prey. Cultures were kept under a 12 light:12 h dark cycle (~75 µmol photosynthetically active radiation [PAR] m^{-2} s^{-1}) and were gently shaken to maintain uniform mixing (~60 rotations min^{-1}). Cultures were transferred and maintained in this manner for 295 d, after which measurements were taken for each experimental replicate (see below).

The assays at 295 d as described below were conducted to enable comparison of the thermal performance curves (TPCs) at each of the temperatures (20, 25, and 30°C; hereafter referred to as the 'long-term' temperature to refer to the 295 d pre-assay temperature). The temperature of 25°C, as the ambient temperature at which the study culture had been maintained prior to the experiment, was treated as the control against which we could compare the responses to warming. This means that any potential impacts of adaptation to the specific controlled experimental conditions beyond the studied temperature change during the 295 d temperature treatment period were controlled for, enabling assessment of the potential evolutionary responses that could underly any differences between the TPCs. As documented below, we also used acclimation periods over several days at each assay temperature prior to measurement, such that differences between the TPCs are likely to have been driven by the 295 d of warming and not any short-term acclimation responses.

2.2. Thermal responses: metabolism

To characterise the metabolic thermal responses of cultures following long-term temperature treatments, we measured net photosynthesis (NP) via oxygen evolution at different light intensities, and we measured respiration (R) (via oxygen evolution in the dark) in 1 ml aliquots of each experimental replicate (n = 6) following previously published methodology (Makin 2023, 2024). Prior to measurements, *P. bursaria* cells were washed on 10 µm filters using mineral water (Volvic alone) to remove bacteria and free-living algae and then acclimatised to the assay temperature (20, 25, and 30°C) for 30 min in the dark. Briefly, we used a Clark-type oxygen electrode (Hansatech; Chlorolab2) to measure R as the uptake of oxygen by respiring organisms in the dark. NP was measured over ~10 min at increasing light intensities following the previously published photosynthetic photon flux density [PFD] range (Makin 2024); this range deliberately increases beyond likely natural values to control for any interaction between temperature and light intensity on NP; i.e. it enables optimal PFD for all measurements (see below). Together, these data deliver a photosynthesis—irradiance curve at each assay temperature; the curves were then fitted to a photoinhibition model using the statistical methods published in Padfield et al. (2016). The maximum oxygen evolution in the light (i.e. at the optimum light intensity) was taken as the maximum NP (P_{max}). P_{max} is used to control for any potential interactions between light intensity and temperature in measuring the thermal response of NP. Gross photosynthesis (GP) was then estimated as $GP = P_{max} + R$ (absolute), and CUE was estimated as $CUE = 1 - R / GP$.

CUE and NP were each fit to linear mixed models (LMEs) using the 'lme' function in the 'lme4' package (Bates et al. 2015) in R statistical software (v.3.2.0) (R Core Team 2015), where each was the dependent variable in their respective model. In both models, assay temperature and long-term treatment were the explanatory variables. Random effects were determined at the level of replicates nested within long-term

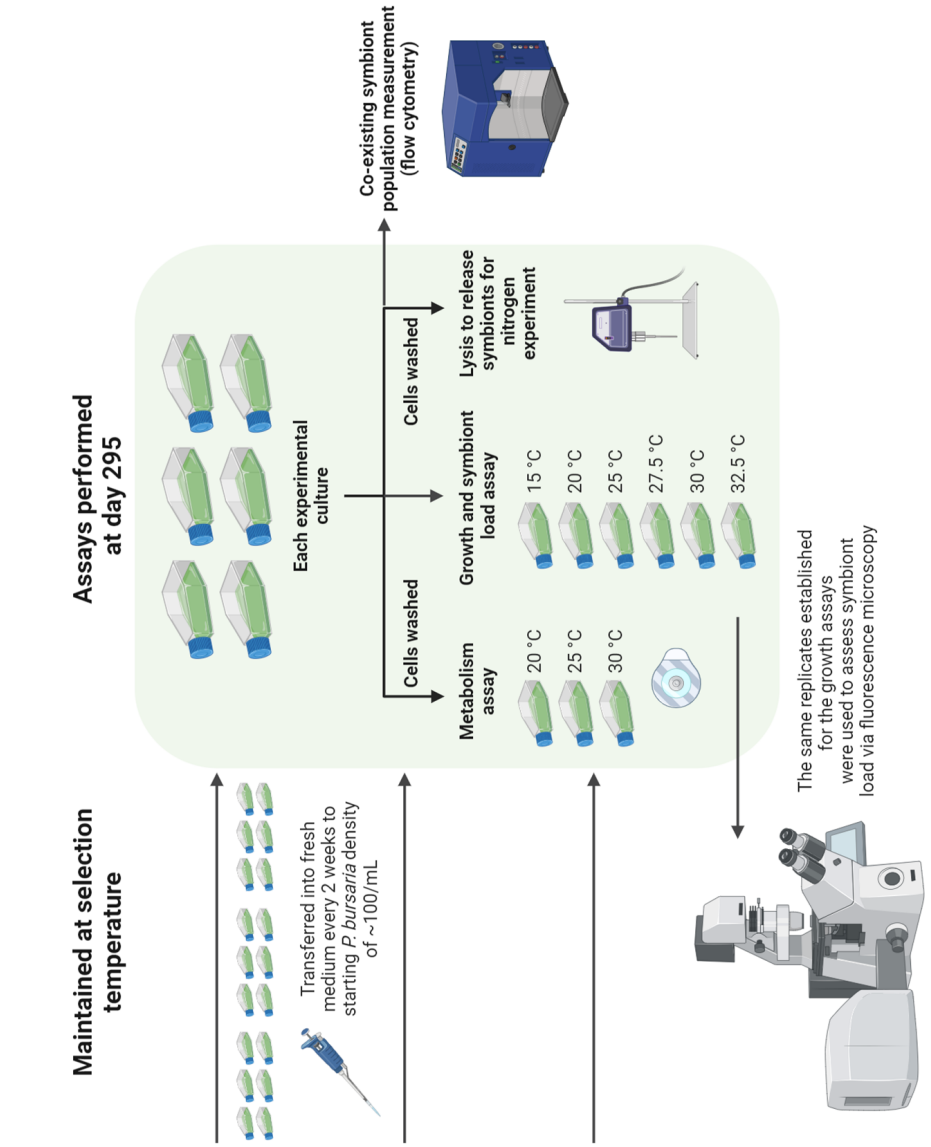

Fig. 1. Study schematic. A single 'ancestral' culture of HA1g was used, maintained before the experiment for at least 1 yr in our laboratory. At study initiation, we allocated 6 replicate cultures from this single ancestral culture to each of 3 temperatures that we refer to as 'long-term' temperature, to refer to the fact that they were maintained at this temperature for 295 d. Maintenance involved regular transferral into fresh medium to achieve a constant approximate cell density, enabling continual growth at the different temperature treatments. After 295 d, assays were performed to measure the various traits of interest (see Section 2.1). Notably, acclimation periods over several days were used for growth experiments (see Section 2.3) such that the resultant thermal performance curves (TPCs) could be compared. The temperature at which the ancestral culture was kept prior to the experiment (25°C) was used as the control temperature and was used to compare the long-term temperature treatments, to control for any potential adaptation to the specific experimental design and conditions

treatment. We also fit CUE and NP at long-term growth temperature to general linear models, where the respective trait was the dependent variable and long-term treatment the explanatory variable.

2.3. Thermal responses: growth

Each replicate was established separately at each of 6 assay temperatures (15, 20, 25, 27.5, 30, and 32.5°C) under a 12 h light:12 h dark cycle (~100 µmol PAR m^{-2} s^{-1}) in 40 ml cultures using the same media reported in Section 2.1. Cultures were first incubated under the above conditions for 4 d (to allow for sufficient cell densities and to avoid abrupt temperature change affecting growth or causing population crashes, as well as to enable any short-term acclimation responses to occur). Cultures were then diluted with bacterised medium as per Section 2.1 until the starting cell concentration was ~100 cells ml^{-1} and then incubated for a further 4 d at the assay temperature; samples were fixed in 3% glutaraldehyde and 0.3% formaldehyde at the start and end of this 4 d incubation period, enumerated via manual imageJ analysis (i.e. manual counts conducted on each image) of fluorescence microscopy images at 10× magnification (Leica TCS SP8; Leica Microsystems), and the growth rate was calculated using the decadic logarithm of cell counts (assuming exponential growth, a biologically plausible assumption; Smith 1999) using the 'lmList' function in the 'lme4' package in R. Cultures were also photographed and cell counts performed after 6 d in the same manner as described above.

Following previous work, growth rates were then fitted to a modified Sharpe-Schoolfield equation for high-temperature inactivation, as this model has effectively been used to capture the TPCs of a range of aquatic photosynthetic organisms (Schoolfield et al. 1981, Padfield et al. 2016, Schaum et al. 2017, Barton et al. 2020), using non-linear least squares regression; fits were determined using the 'nls_multstart' function in the 'nls.multstart' package in R. This package compares Akaike information criterion (AIC) values to identify the parameter set, drawn from a uniform distribution, which best characterises the data. The goodness-of-fit values of the selected models were examined graphically and via assessment of pseudo-R^2 values. These parameter sets were used to calculate an optimum temperature value for each experimental replicate (i.e. the temperatures where growth rate is highest), which could be compared between long-term temperature treatments. Optimum temperature was calculated using the following formula:

$$\text{Optimum temperature (in K)} = (\text{Eh} \times \text{Th}) / \left(\text{Eh} + \{8.62\text{e}{-}05 \times \text{Th} \times \log[(\text{Eh} / \text{Ea}) - 1]\}\right) \quad (1)$$

where Eh is the value of the deactivation energy (in eV), Th is the temperature at which the substrate is half high-temperature suppressed (in K), and Ea is the activation energy (in eV). Optimum temperature values and maximum fitted growth rate estimates from these fitted Sharpe-Schoolfield curves were compared between long-term temperature treatments using general linear models.

2.4. Thermal responses: symbiont load (number of symbionts within hosts)

The same replicates established for the growth measurements were used to assess symbiont load. Samples at the 6 d time point were photographed at 10× using fluorescence microscopy; these images were analysed for mean fluorescence intensity using the 'mean gray value' parameter obtained with the 'analyze particles' base function in ImageJ. The returned intensity values were then converted into symbiont load values (i.e. symbiont density within hosts, expressed as symbionts μm^{-1}) using the following empirically derived formula: $L = (m \times I) + c$, where L is the symbiont load (μm^{-1}), $m = 1.3 \times 10^{-5}$, $c = -5.4 \times 10^{-5}$, and I is intensity (mean gray value). The constants m and c were empirically derived by lysing reference populations of *P. bursaria* of known intensities, directly enumerating symbionts within each host via flow cytometry (see below), and constructing a linear model within which fluorescence intensity was the explanatory variable and symbiont load was the dependent variable.

Direct symbiont density counts within hosts were achieved by sonicating 3 *P. bursaria* stocks kept at each of a selection of assay temperatures (15, 20, 25, 30, and 35°C) during mid-log growth phase (using 3 pulses of 10 s at 90% amplitude over ice; each culture was 10 ml) in order to rupture host cell membranes; we enumerated the resultant released symbiotic algal cells using flow cytometry (BD Accuri C6). Flow cytometry involved simply passing these lysed cultures through a flow cytometer in which the number of algae detected in a known volume (10 µl) of the sample was based on chlorophyll fluorescence while the culture was penetrated by a laser. The system was first calibrated as recommended and detailed by the manufacturer, and negative controls (i.e. where growth medium alone was run through the flow cyto-

meter) were first compared with algal cultures in trial runs to confirm that isolated algae in our experiments could be successfully detected and counted. Direct symbiont density counts were normalised by the density of *P. bursaria* hosts by dividing the number of counted algae by the number of hosts (enumerated via ImageJ analysis using the 'blur' followed by 'find maxima' base functions; the number of hosts lysed per replicate was typically ~1000) using fluorescence microscopy images at 10× magnification (Leica TCS SP8; Leica Microsystems). Symbiont population sizes per host were also divided by mean cell volume estimates in order to control for changes in host cell volume; measurements of *P. bursaria* cell volume (μm^3) were performed manually on bright field microscopy images using the provided manufacturer software (LAS X; Leica Microsystems). Length and width measurements were taken and volume was calculated assuming that cells were prolate spheroids (volume = $4/3\pi a^2 c$; where a and c are the polar radii; the number of hosts measured per replicate was 25).

To allow for a non-linear response, we fitted the resultant symbiont load estimates (converted from cell fluorescence intensities as detailed above) to a generalised additive mixed model (GAM) using the 'gam' function in the 'mgcv' package in R, in which assay temperature and long-term treatment were explanatory variables and symbiont load was the dependent variable. Random effects on the intercept were determined at the level of replicates nested within long-term treatment. The most suitable GAM model was selected using AIC and corrected AIC (AIC_C). We also fit symbiont load at long-term growth temperature to a general linear model, in which the respective trait was the dependent variable and long-term treatment the explanatory variable.

2.5. Autonomous symbiont growth and co-existing symbiont population size

A 10 ml sample from each long-term temperature treatment replicate was first washed following the same procedure outlined above and then mechanically lysed via sonication to release symbiotic algae from hosts. These algae were then established separately in 2 different growth media: one containing inorganic nitrogen (i.e. nitrate) as the nitrogen source and the other containing organic nitrogen (i.e. bacto-peptone) established at equimolar concentrations for nitrogen. Both media also contained modified Bold's basal medium (BBM) from which the nitrogen source had been omitted. Algal counts at 0 and 4 d after incubation at the respective long-term temperature were conducted via flow cytometry (as previously described) and the growth rate was calculated using the decadic logarithm of cell counts (assuming exponential growth). Algal growth on inorganic and organic nitrogen was fit to linear models where growth on the appropriate source was the dependent variable and the long-term temperature treatment was the explanatory variable.

A 1 ml sample from each long-term temperature treatment was also used to estimate the co-existing symbiont population size via flow cytometry (as outlined above).

2.6. Assessing the number of generations passed during the experiment

The number of generations was calculated following Padfield et al. (2016) using the following formula:

$$g = \frac{\Delta T}{\ln(2)/\mu} \quad (2)$$

where ΔT is the time interval of the transfer (days), $\ln(2)/\mu$ is the doubling time (days) and μ is the growth rate (d^{-1}).

2.7. Additional statistical procedures

Each measured trait was analysed using the model type as described above in R statistical software. Model assumptions were tested as per Makin (2023) using the 'plot' function in R. As per this past work, we assessed the assumptions based on the quantile–quantile plot alongside plots of residuals versus fitted data, scale-location, and residuals versus leverage data. No data transformations were used following these diagnostic plot tests. Where *F*-statistics (for GLMs) or *L*-ratios (for LMEs) are reported (see Section 3.4), the model was compared with a null model using ANOVA testing in R to uncover whether long-term temperature significantly explained differences in the dependent variables (the assessed traits). A significance level of 0.05 (5%) was used.

3. RESULTS

3.1. Cultures and selection temperatures

For thermal selection experiments, replicate cultures (n = 6) of *Paramecium bursaria–Chlorella* spp. (strain HA1g; National Bio-Resource Project) were established at 3 temperatures corresponding to am-

bient (25°C), cooled (20°C), and warmed (30°C) conditions for growth and maintained for 295 d. *P. bursaria* populations completed, on average (±SE), 55.5 ± 1.07 (20°C), 57.7 ± 0.98 (25°C), and 21.1 ± 0.22 (30°C) generations. Two of the 6 replicates in the 30°C treatment died and so were not included in the assays.

3.2. Growth responses

Following the long-term temperature experiment, the responses of host growth and symbiont density within hosts were assayed for each temperature treatment (Fig. 2A) across a thermal gradient of 15–35°C. Growth thermal responses were assumed to be unimodal and were analysed by fitting Sharpe-Schoolfield thermal response curves to the data and comparing model parameters as described above.

Long-term warming resulted in an increase in the thermal optimum for growth (i.e. the growth thermal optima for cells selected at 30°C was ~3°C higher than for those grown at 20 and 25°C; $F_{2,11} = 5.002$, p = 0.035; Fig. 2B), but there were no differences between long-term temperature treatments in growth rates at the thermal optima ($F_{1,15} = 0.8766$, p = 0.365; Fig. 2C). Similarly, there was no evidence for an increase in the growth rate of cultures from the long-term warming treatment assayed at 30°C ($F_{2,14} = 0.668$, p = 0.531; Fig. 2D).

3.3. Symbiont density responses

Symbiont density responses were analysed by using a GAM framework and assessing shape and intercept parameters. The response of symbiont density to assay temperature varied as a result of temperature selection (Fig. 3A). Overall, symbiont densities were lower and the response of symbiont density to assay temperature showed reduced variation in the cultures exposed to warming. Notably, decreases in symbiont density were most apparent at lower, not higher, assay temperatures in the long-term warming cultures and were statistically indistinguishable between the long-term temperature treatments assayed at 30°C ($F_{2,15} = 1.176$, p = 0.339; Fig. 3B).

3.4. Metabolic responses

To examine the impact of long-term warming on photosymbiosis metabolism, we assayed cultures from the long-term temperature treatments for rates of R and NP at assay temperatures of 20, 25, and 30°C. Analyses using LMEs revealed that the thermal responses were significantly different between the long-term temperature treatments ($L.Ratio_{4,11} = 33.267$, p < 0.0001 and $L.Ratio_{4,11} = 21.707$, p < 0.001 for R and photosynthesis, respectively) with a notable apparent increase in R at 30°C in the long-term warming cultures (Fig. 4A,B). Consequently, for long-term warming cultures assayed at their long-term temperature (30°C), *t*-tests to compare means with zero revealed that NP (i.e. GP − R) and CUE (1 − R / GP, the relative proportion of carbon fixed by photosynthesis available for growth) were not significantly different from zero ($T_3 = 0.480$, p = 0.664 and $T_3 = 0.180$, p = 0.869 for NP and CUE, respectively; Fig. 4C,D).

3.5. Co-existing symbiont populations and autonomous symbiont growth

Co-existing symbiont (i.e. symbionts co-existing in the growth medium) populations increased in size with long-term temperature ($F_{2,15} = 5.139$, p = 0.023; Fig. 5A).

Autonomous symbiont (i.e. symbionts extracted from hosts) growth rate was significantly affected by the interaction between nitrogen source and long-term temperature treatment ($F_2 = 56.868$, p < 0.0001; Fig. 5B). Growth on organic nitrogen was higher compared to inorganic nitrogen in the long-term ambient culture and the opposite pattern was observed in the long-term warming culture, such that growth rate was significantly higher on the organic nitrogen source.

4. DISCUSSION

Current understanding of the long-term evolutionary responses of photosymbioses to persistent warming remains limited and empirical measurements of evolution are required to support theory (Sachs & Simms 2006, Kiers et al. 2010, Salsbery & DeLong 2021). To address this need, we exposed the widespread *Paramecium bursaria–Chlorella* spp. photosymbiosis to ~1 yr (295 d) of +5°C of persistent warming and quantified the subsequent thermal responses of traits associated with symbiosis persistence and ecological function (growth rate, symbiont density [the number of symbionts within hosts], and metabolic rates) in comparison with control (i.e. ambient) cultures maintained at the pre-experimental temperature of 25°C as well as cultured subjected to −5°C of cooling.

In agreement with past work (Salsbery & DeLong 2021), the association appeared to 'broaden' its ther-

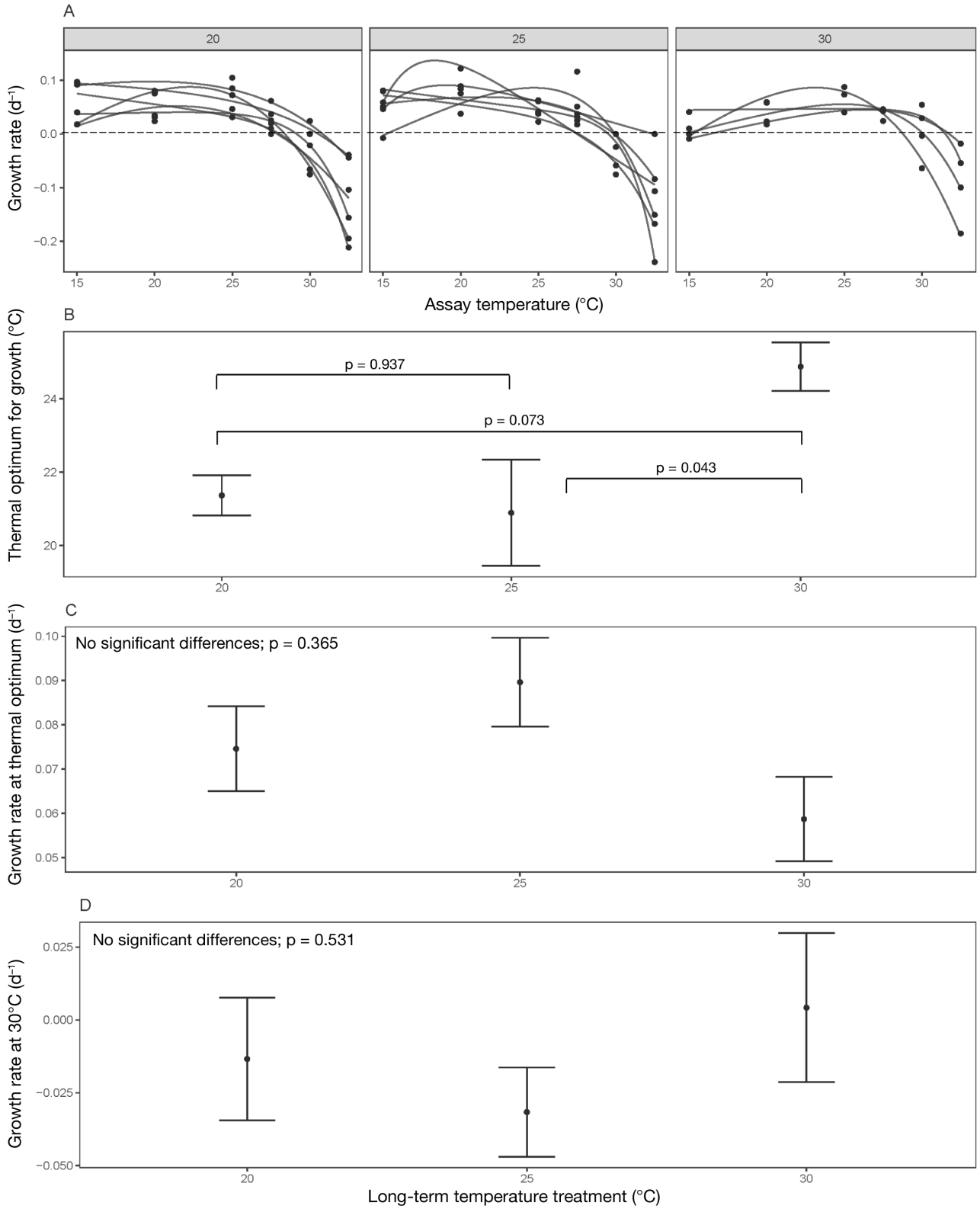

Fig. 2. (A) *Paramecium bursaria* growth rate with fitted Sharpe-Schoolfield curves (see Section 2.3) for each long-term replicate in response to assay temperature. Temperature label in grey block indicates the long-term treatment temperature. (B) Thermal optima for growth for each long-term treatment. These parameters were derived from the fitted Sharpe-Schoolfield equations in (A) (see Section 2.3). (C) Growth rate measured at optimum temperature (i.e. maximum growth rate) for each long-term selection temperature. (D) Growth rate measured at 30°C for each long-term selection temperature. Number of replicates = 6 (n = 4 in the long-term warming treatment due to death of 2 replicates). Error bars: ±SE; p-values shown are from Tukey's post hoc testing within the long-term temperature treatment factor (in B) or represent the significance of the long-term temperature treatment factor itself where significance was not achieved, calculated using ANOVA comparison of generalised linear models compared with their null models (in C and D); see Section 2.7

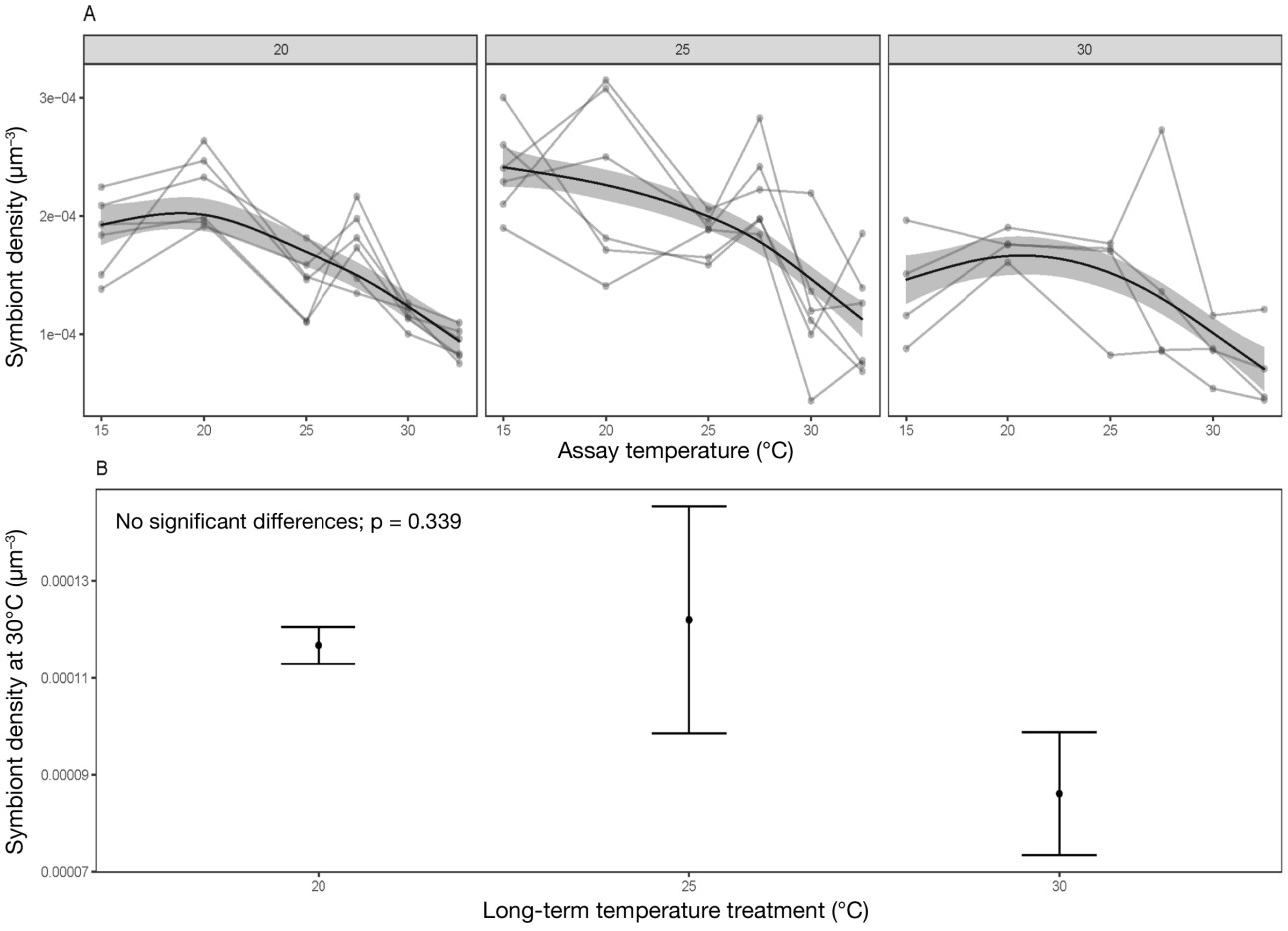

Fig. 3. (A) *Paramecium bursaria* symbiont load in response to assay temperature. Temperature label in grey block indicates the long-term treatment temperature. Black lines indicate fitted generalised additive model; grey shaded area represents predicted error margins. (B) Symbiont load measured at 30°C for each long-term temperature treatment. Number of replicates = 6 (n = 4 in the long-term warming treatment due to death of 2 replicates). Error bars: ±SE; p-value shown represents the significance of the long-term temperature treatment factor, calculated using ANOVA comparison of generalised linear models compared with their null models; see Section 2.7

mal response curve in response to warming, albeit with an increase in thermal optimum and a trend towards increased growth rate at the +5°C temperature. Strikingly, this could be the result of modest adaptation that occurred following only 1 yr (and ~20 generations) of warming, broadly comparable with the absolute time periods assessed previously in an evolutionary experiment (Salsbery & DeLong 2021). The rapid growth responses are also supported by the wider literature. Growth rate in free-living *Chlorella* at initially stressful temperatures has been shown to increase over ~100 generations of warming under similarly controlled conditions (Padfield et al. 2016), and studies of free-living *Symbiodinium* symbionts of coral photosymbioses have also demonstrated comparable results (Chakravarti & van Oppen 2018). Together, these findings support the notion that photosymbiosis can rapidly evolve thermal tolerance. It is notable that the evolutionary processes occurred using a clonal stock of known *P. bursaria* strain, which presumably limited the pre-existing pool of variation upon which selection could draw and suggests that this thermal adaptation can rapidly emerge de novo in photosymbiosis. However, we also observed that 2 of the 6 replicates established at long-term warming died before the end of the experiment. This suggests that while rapid adaptation is possible, extinction of the symbiosis can also occur.

While rapid thermal evolution would be encouraging for the long-term persistence of photosymbiosis, there are clearly numerous other ways in which the association could be impacted by long-term warming, arguably the most impactful of which lies in its ecological outcome (Kiers et al. 2010, Bailly et al. 2014, Baker et al. 2018). In particular, symbioses have long been known to be strongly context-dependent; that

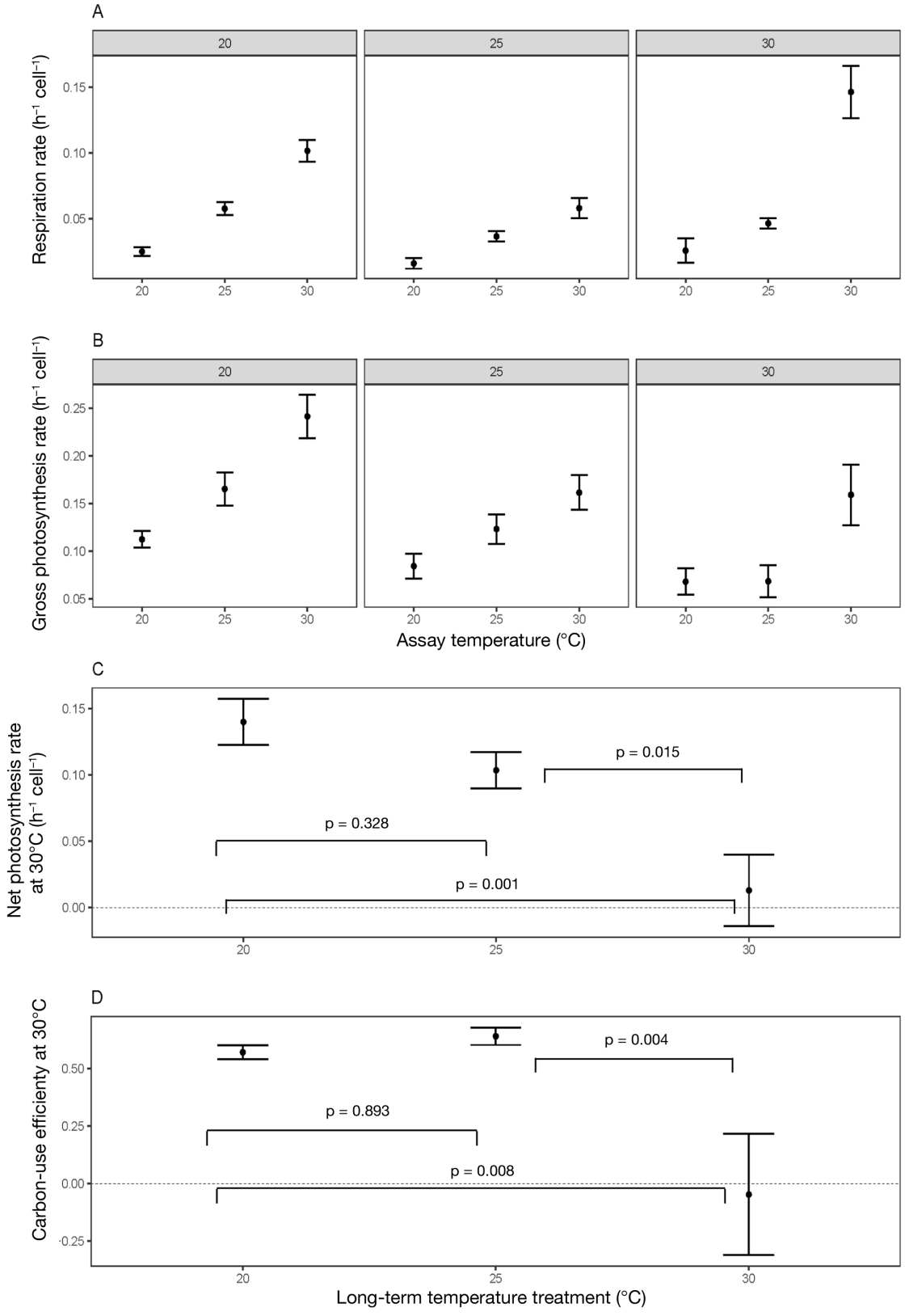

Fig. 4. (A) Respiration and (B) gross photosynthesis measured at each assay temperature for each long-term temperature treatment in *Paramecium bursaria*. (C) Net photosynthesis and (D) carbon-use efficiency measured at 30°C for each long-term temperature treatment. Number of replicates = 6 (n = 4 in the long-term warming treatment due to death of 2 replicates). Error bars: ±SE; p-values shown in (C) and (D) are from Tukey's post hoc testing within the long-term temperature treatment factor for the trait

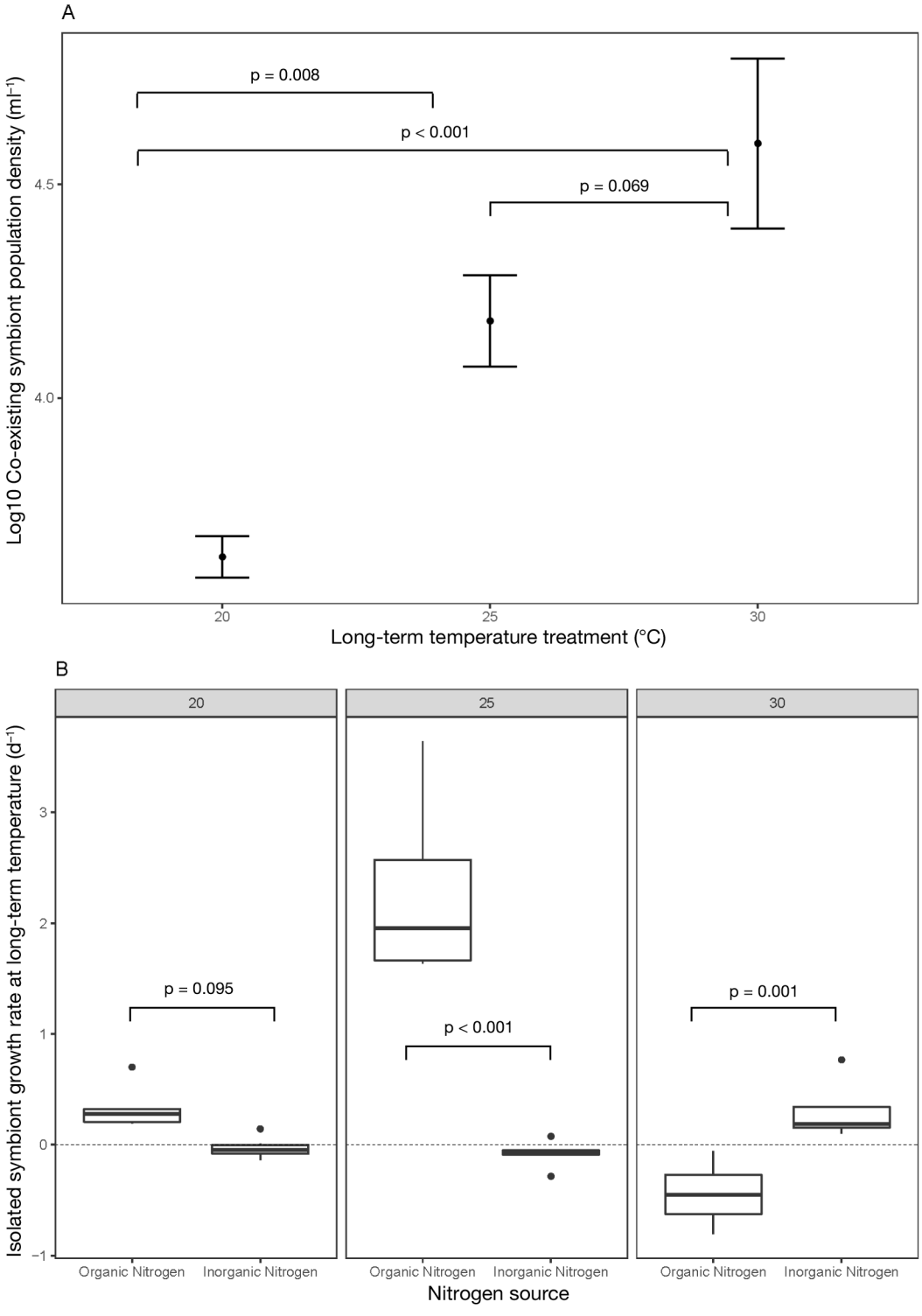

Fig. 5. Autonomous symbiont population size and growth on organic versus inorganic nitrogen sources. (A) Symbionts co-existing autonomously (i.e. outside of hosts) within long-term temperature treatments measured at the end of the experimental period Error bars: ±SE. (B) Boxplots (median, first and third quartile, and 95% confidence interval of median) for symbionts isolated from each long-term temperature treatment growth rate on organic and inorganic nitrogen. Number of replicates = 6 (n = 4 in the long-term warming treatment due to death of 2 replicates); p-values shown are from Tukey's post hoc testing within the long-term temperature treatment factor for the trait (in A) or within the nitrogen source-long-term temperature interaction term in the generalised linear model (in B)

is, mutualisms may become parasitic and vice versa, with their dynamic outcome existing on a 'slider' between parasitism and mutualism (Bronstein 1994). Indeed, while established in theory, studies have also directly observed the transitioning of mutualistic towards parasitic interactions—including in photosymbiosis (Moran & Wernegreen 2000, Kiers et al. 2010, Salsbery & DeLong 2018, Baker et al. 2018, Drew et al. 2021). Most relevant is recent work showing that *Chlorella* symbionts become costly to their *P. bursaria* hosts with warming and are maintained in symbiosis despite a fitness cost to hosts (Salsbery & DeLong 2018, 2021), a paradoxical finding given the ability of hosts to adaptively regulate their symbiont loads observed in other work (Lowe et al. 2016). In support of the idea that warming may favour symbiont maintenance regardless of the cost–benefit balance for hosts, we observed no significant decline in symbiont density with long-term warming, with symbiont density being statistically indistinguishable between the long-term temperature treatments assayed at 30°C. It is also notable that symbiont load appeared to be variable, with a relatively wide skew in values, especially in the long-term warming cultures (see Fig. 3). While some of these values could be explained by outliers and/or random noise, it suggests that there may be competing dynamics and/or loose control by hosts (Lowe et al. 2016) in this system.

Unique to this study, we also assessed metabolic responses to shed light on photosymbiosis dynamics and provide greater context to the cost–benefit balance experienced by hosts. Photosymbioses are shaped by metabolic trade between host and symbionts (Karakashian 1963, Johnson 2011b); thus, adaptation in key metabolic processes such as respiration and photosynthesis may be key drivers of long-term responses, as established in free-living algae (Padfield et al. 2016). The greater thermal sensitivity of respiration over photosynthesis observed in photosynthetic organisms (Padfield et al. 2016, Schaum et al. 2017, Barton et al. 2020) is likely to reduce NP in symbionts and increase the respiratory demand of hosts in the short term, eroding the metabolite trade central to photosymbiosis. Whether the photosymbiosis can rapidly evolve metabolic traits to compensate for such degradation, as in autonomous phytoplankton (Padfield et al. 2016, Schaum et al. 2017, Barton et al. 2020), is therefore critical. However, we recorded a notable increase in R at 30°C in the long-term warming cultures, and both NP and CUE (the proportion of photosynthate available for growth processes; Padfield et al. 2016) were not significantly different from zero, suggesting that the photosymbiosis failed to compensate for the warming-induced metabolic impacts. Therefore, taken together with past work (Salsbery & DeLong 2018, 2021), this total loss in NP and CUE strongly supports the emerging view that long-term warming drives the maintenance of costly symbionts and the degradation of photosymbiosis dynamics.

It is challenging to reconcile these findings with other work that has shown that hosts can regulate their symbionts to their own benefit, suggestive of a relationship skewed towards host exploitation of symbionts (Kiers & West 2016, Lowe et al. 2016, Salsbery & DeLong 2021). What could be the reason for this apparent discrepancy? One explanation could lie in changes to the capacity for regulation of symbionts with long-term warming. Organic nitrogen provision is thought to be a key way in which hosts regulate the abundance of symbionts; i.e. they restrict nitrogen supply to control symbiont load (Lowe et al. 2016, He et al. 2019). Since CUE and NP are zero in the long-term warming treatment, the host might be expected to impose tight sanctions in the form of nitrogen control on their symbionts as a result of the strongly eroded photosynthetic benefit and photosynthate provision (Foster et al. 2017); theoretically, this could place a strong selection pressure on symbionts to 'escape' from host control via the evolution of an alternative growth strategy independent of organic nitrogen. Notably, such a mechanism could realistically occur, given that symbionts can be released into the environment (e.g. following host cell lysis) and they can be taken up from the environment by *P. bursaria* (Kodama & Fujishima 2009) — theoretically enabling exposure of the symbionts to both organic and inorganic nitrogen. To determine whether symbionts changed their nitrogen use strategy in response to long-term warming in symbiosis, we isolated symbionts from washed *P. bursaria* cells from long-term temperature cultures and assayed their growth autonomously (i.e. outside hosts) on organic and inorganic nitrogen sources. At ambient temperature, we found that growth was higher on organic nitrogen compared to inorganic nitrogen, where growth on inorganic nitrogen was ~zero. This suggests that, in the absence of long-term warming, symbionts are dependent on organic nitrogen provided by hosts for growth. Strikingly, for the warmed treatment, the pattern was reversed, such that the growth rate was significantly higher on the inorganic nitrogen source. These data provide intriguing initial evidence that warm-adapted symbionts might evolve independence from host organic nitrogen provision, leading to their maintenance in symbiosis with warming despite lack of clear benefit for hosts.

Furthermore, growth on nitrate is a strong indicator of free-living capacity (Minter et al. 2018) and is therefore likely to be indicative of the ability for symbionts to 'abandon' symbiosis over evolutionary timescales (Werner et al. 2018). In support of this idea, we also found that co-existing symbiont populations (i.e. symbionts living outside hosts in the *P. bursaria* cultures) increased in size with long-term temperature (although we note that our data do not reveal whether this was an evolved response). Taken together, the data suggest an increased capacity for symbiont autonomy mediated by resource acquisition strategy, which is poised to be a key enabler for mutualism breakdown over evolutionary timescales (Werner et al. 2018). Such abandonment of the symbiosis is plausible: the host is known to retain the ability to live symbiont-free (Tonooka & Watanabe 2002) and/or acquire new symbionts from their surroundings via uptake into vacuoles (Kodama & Fujishima 2009), while some symbiont strains can also retain the ability to grow autonomously (Minter et al. 2018). Indeed, phylogenetic evidence suggests a turbulent history of symbiosis maintenance and abandonment: the *Chlorella* group shows signs of repeated loss and gain of symbiosis, and *P. bursaria* phylogeny is characterised by loss, re-acquisition, and switching of symbiont strains (Hoshina & Imamura 2008, 2009).

In addition, the total loss of NP following the potential thermal adaptation clearly represents a substantial change in the ecological function of photosymbiosis, which plays a major role in global productivity (Bailly et al. 2014). This finding raises concern over the ecological function of keystone photosymbioses as the world becomes warmer and supports an evolutionary trajectory towards ecosystem service decline in the future in addition to the known acute effects in these associations (Baker et al. 2008). While general trends are reported, including the reduction of oceanic NP with rising temperatures, how ecosystem-level NP rates will respond to warming remains a complex picture of biodiversity, individual species responses, and the capacity for thermal evolution, among other factors (Yvon-Durocher et al. 2012, Tait & Schiel 2013, Schaum et al. 2017, Bestion et al. 2020). Therefore, understanding the responses of key players in these rates, such as photosymbioses, will be necessary. For example, our findings suggest that in contrast with findings in phytoplankton showing rapid evolution to compensate for warming-induced effects (Padfield et al. 2016), the same level of adaptability may not exist when partners are involved in photosymbiosis.

There are several key limitations of this study. Chiefly, while the *P. bursaria* strain used in this experiment is well characterised, is reported to contain *Chlorella* spp., and is clonal, there exists significant variation between strains of hosts and symbionts (Minter et al. 2018). Phylogenetically, *P. bursaria* differentiated early from the 5 *Paramecium* species (He et al. 2019) and followed a complex co-evolutionary history with its symbionts (Hoshina & Imamura 2008, 2009). Although not carried out in the current study, future evolutionary studies would ideally carefully define the populations to place the findings into the context of this complex variation. For instance, while it is generally assumed that the HA1g strain contains *Chlorella* spp., we did not specifically identify the symbionts in our study — and the results should therefore be interpreted with caution. Furthermore, the laboratory environment was clearly a simplified simulation of the real world, and numerous other factors will play a role in defining the real-world evolutionary outcomes. Notably, real-world symbionts receive protection from viral threats (Kodama & Fujishima 2010); in native freshwater, the titre of the *Chlorella* virus PBCV-1, which infects free-living *Chlorella*, can reach 100 000 plaque-forming units per ml and would therefore be expected to strongly influence symbiosis dynamics (Grimsley et al. 2012, Kiers & West 2016). There are also likely to be other benefits of symbionts to hosts and vice versa that complicate the simplified view of symbiotic mutualism; for example, symbionts may provide UV protection to their hosts (Summerer et al. 2009). While we focused on key traits and metabolic processes in this study, various other possible benefits of the association could be explored in further work. Finally, it is possible that the number of generations assessed in our study was underestimated, for example, because of high mortality; indeed, we observed that 2 of the 6 replicates established at the long-term warming condition died before the end of the experiment.

Regardless, our study demonstrates a modest capacity for photosymbiosis to rapidly adapt to environmental warming on timescales similar to autonomous phototrophs, including coral–zooxanthellae symbionts and a wide range of freshwater and marine phytoplankton (Padfield et al. 2016, Schaum et al. 2017, Chakravarti & van Oppen 2018, Barton et al. 2020), despite concerns raised by past work on the persistence of photosymbiosis in a warmer world (Baker et al. 2008, 2018) and more broadly on the persistence of symbiotic mutualisms in the face of climate change (Kiers et al. 2010, Werner et al. 2018). In general, this finding is in agreement with another recent evo-

lutionary study in the *P. bursaria* photosymbiosis (Salsbery & DeLong 2021). However, unique to our study was the finding that long-term exposure to warming led to total loss of NP, likely disrupting the metabolite trade that underpins the association (Karakashian 1963, Johnson 2011a,b). This finding supports an emerging view that symbionts become costly and shift the mutualism towards parasitism with long-term warming (Salsbery & DeLong 2021) and also raises the significant contributions of photosymbioses in general to global primary production (Bailly et al. 2014) as an issue of serious concern. Furthermore, warming apparently selected for symbionts with an increased capacity for autonomy in terms of nitrogen use and increased existence outside of hosts, further suggestive of changed symbiosis dynamics and providing a foundation for an evolutionary trajectory towards breakdown over longer timescales (Werner et al. 2018). Lastly, the fact that 2 of 6 populations subject to long-term warming died is evidence that responses are uncertain and multiple outcomes are possible — including extinction (Sachs & Simms 2006). Consequently, even if photosymbioses persist in a warmer world, exposure to chronic warming may substantially change their ecological function, degrade the mutualistic interaction, and leave them susceptible to evolutionary breakdown. These findings raise concern about photosymbiosis persistence and function in response to global warming over evolutionary timescales.

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