FEATURE ARTICLE

Physiological response to temperature, light, and nitrates in the giant kelp *Macrocystis pyrifera* from Tasmania, Australia

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ABSTRACT: Climate change is characterised by multiple abiotic forcings acting simultaneously on biotic systems. In marine systems, temperature appears to drive much of the observed change in biotic communities subject to climate change, but this may reflect the focus of most studies only on temperature without consideration of other environmental variables affected by climate change. The giant kelp *Macrocystis pyrifera* was once abundant in eastern Tasmania, forming extensive habitats of ecological and economic importance, but recent extensive population decline has occurred. Southerly incursion of warm oligotrophic East Australian Current (EAC) water has increased in frequency and intensity into this region, which has warmed ~4 times the global average, and the warming trend is predicted to continue. This study investigated the single and combined effects of temperature, light, and nitrate availability on the physiology of juvenile *M. pyrifera* sporophytes in a laboratory experiment. Determination of relative growth rate, photosystem II characteristics, pigments, elemental chemistry, and nucleic acid characteristics over 28 d showed that all experimental factors affected sporeling physiology. Temperature and light drove much of the observed variation related to performance characteristics, and rapid deterioration of kelp tissue was a consequence of temperature stress (high temperature), photoinhibition (high light), and low light, accompanied by impaired photosynthetic efficiency and increased RNA concentration, presumably associated with production of photoprotective proteins. Surprisingly, higher relative growth rates were observed in low-nitrate treatments. These findings suggest that negative effects of temperature on *M. pyrifera* populations will be mediated by local variation in light and nutrient conditions.

Vanishing underwater forest: One of the last remaining patches of giant kelp on the east coast of Tasmania.

Photo: Cayne Layton

KEY WORDS: Kelp · Climate change · *Macrocystis pyrifera* · Relative growth · Photosynthesis · Physiology · Morphology

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1. INTRODUCTION

Climate change is transforming marine ecosystems, causing shifts in species ranges, declines in biodiversity, and changes to ecosystem structure and functioning (Poloczanska et al. 2007). Negative impacts of climate change on marine foundation species or ‘ecosystem engineers’ (see Jones et al. 1994) that support extensive economic and social wellbeing, such as corals, seagrasses, mangroves, and seaweeds, are likely to be particularly important as these species form the basis of hierarchically organised, species-rich communities (Crain & Bertness 2006, Schiel & Foster 2015). Although temperature appears to be a major factor impacting the performance and physiology of marine organisms, climate change encompasses shifts in other environmental factors (i.e. light and nutrient supply) that are important for biological functioning (e.g. biosynthesis and metabolism) and which may act on their own or in combination with temperature (Poloczanska et al. 2007). Multiple forcing factors complicate the ability to predict, test, and interpret the impacts of climate change, creating uncertainty for adaptive management strategies.

Habitat-forming seaweeds are a dominant feature of temperate marine ecosystems and form the basis of productive and diverse communities (Steneck et al. 2002). The south-east region of Australia is an ocean-warming ‘hotspot’ (Hobday & Pecl 2014) and encompasses a substantial portion of the Great Southern Reef, one of the most productive temperate reef zones in the world (Bennett et al. 2016). Historically, this region has been warming rapidly since 1950 (Hobday & Pecl 2014) and is predicted to continue to warm at almost 4 times the global average (Ridgway 2007) due to shifting wind patterns and ocean currents which cause more frequent and intense southerly incursions of the East Australian Current (EAC) (Cai et al. 2005, Oliver et al. 2014). The warm waters of the EAC are oligotrophic, with nitrate (NO₃⁻) levels typically <0.5 μm and often undetectable (Harris et al. 1987). As seaweeds rely on seasonal nutrient loading for growth and other metabolic processes (Chapman & Craigie, 1977, Gerard 1982, Wheeler & Srivastava 1984), increased exposure to EAC conditions are likely to exert both temperature and nutrient stress on canopy-forming kelps.

*Macrocystis pyrifera* (C. Agardh) is the world’s largest and fastest-growing seaweed. It is found from the low intertidal zone to ~30 m depth in all continents in the southern hemisphere (except Antarctica) and the west coast of Northern America (Graham et al. 2007), where it forms giant kelp ‘forests’ in cool-temperate waters. Its large-scale distribution is determined by temperatures between 4 and 20°C and nitrate concentrations >1 μM NO₃⁻ (Schiel & Foster 2015), while local-scale processes such as grazing, storms, and upwelling events play major roles in driving kelp forest dynamics (Dayton et al. 1984, 1999, Ebeling et al. 1985, Steneck et al. 2002). In Australia, *M. pyrifera* is confined to the south-eastern part of the continent with the largest population occurring in Tasmania, where it was once a prominent habitat type in eastern Tasmania at depths of 8 to 22 m. However, forests forming dense surface canopies have declined by up to 95% in the past 60 yr (Johnson et al. 2011), associated with the increased influence of warm oligotrophic EAC waters in the region. In response to the decline, *M. pyrifera* was listed as an endangered marine habitat type under the federal Environmental Protection and Biodiversity Conservation Act 1999 (EPBC Act 1999). The need for better predictions of the future condition of giant kelp forests under climate change is important for management and/or adaptation by humans, and resolving impacts of climate change on the physiological functioning of *M. pyrifera* in eastern Tasmania is one element of this goal.

In general, increasing temperatures lead to reduced fitness in temperate seaweeds (Hatcher et al. 1987, Andersen et al. 2013), and further temperature-driven declines of *M. pyrifera* populations are predicted in south-east Australia (Johnson et al. 2011). However, as light and nutrients also play key roles in seaweed physiology, these factors must be addressed alongside temperature and the potential interactions among them considered. Multifactor climate impact studies with seaweeds reveal both synergistic or antagonistic effects between factors (e.g. among temperature, salinity, UV intensity, desiccation, wave action, and light) on the growth and photosynthesis in *Fucus* spp. (see review by Wahl et al. 2011) but also show additive effects in some cases (e.g. temperature and UV inhibits *E. radiata* growth and photosynthesis rates; Xiao et al. 2015). Increased influence of EAC waters in Tasmania will likely lead to kelp canopy destabilisation and thinning and thus increased light levels (Wernberg et al. 2010). Adult *M. pyrifera* engineer the understory light environment, and thus for juvenile sporophytes, light can be either limiting under a thick canopy (Dean & Jacobsen 1984, Kinlan et al. 2003) or high and photo-inhibitory under a low density or absent canopy (Graham 1996), and therefore, resolving how temperature and light are likely to interact is important. Understanding how these fac-
tors impact the juvenile sporophyte stage is important as its early survival and growth will ultimately determine productivity and biomass (Graham et al. 2007). Additionally, little is known of *Macrocystis pyrifera* nutrient utilisation dynamics in Tasmania although in California, this species exhibits growth response proportional to nutrient availability (Deyscher & Dean 1986, Dayton et al. 1999). The growth rate hypothesis (Elser et al. 2003) would posit that seaweeds at higher latitudes will be more susceptible to nutrient limitation because selection favours higher instantaneous growth rates in shorter growing seasons, requiring higher P-rich RNA concentrations and a greater reliance on N for stoichiometry balance to maintain photosynthesis machinery. Assuming DNA tissue concentration is constant, organisms with higher growth potential should have higher RNA:DNA ratios and higher N-limitation thresholds (Dortch et al. 1983, Elser et al. 2003). Thus, it is useful to assess how nutrients and light interact with temperature to affect *Macrocystis pyrifera* physiology under climate change scenarios.

This study examines the interaction of temperature, light, and nitrate on the physiology of juvenile *M. pyrifera* sporophytes. The aims were to test (1) whether the effects of increased temperature on *M. pyrifera* physiology will be particularly severe in south-east Australia due to synergistic effects of reduced nitrates and increased light due to a decline in canopy cover and (2) whether RNA concentration, and thus RNA:DNA ratios, will correlate with growth rates due to increased protein synthesis.

2. MATERIALS AND METHODS

2.1. Collection and in situ measurements

Juvenile *Macrocystis pyrifera* sporophytes (50 to 150 mm in length) were collected from Fortescue Bay, Tasmania (43.1230° S, 147.9764° E) in September 2012 at a depth of ~10 m. At the time of collection, in situ water temperature was ~13°C, and photosynthetically active radiation (PAR) under the canopy was ~30 μmol photons m⁻² s⁻¹ (Odyssey PAR logger, Dataflow Systems). The juvenile sporophytes were transported in seawater-filled coolers to the IMAS laboratory facility in Hobart and held at 13°C for 24 h under low light (12 h light:12 h dark at 10 μmol photons m⁻² s⁻¹) to slow respiration and photosynthesis rates and reduce the risk of temperature, oxygen, and nutrient stress (Peckol 1983). During collections, in situ baseline physiology was measured from 3 haphazardly selected juvenile sporophytes using a PAM fluorometer, and an additional 4 juvenile sporophytes were collected for baseline measurements of pigments, chemistry, and nucleic acids back in the laboratory (see Section 2.3).

2.2. Experimental design and growth conditions

The response of juvenile *M. pyrifera* sporophytes to temperature, light, and nitrate levels were determined using a 3-way factorial design, with main effects of temperature (12, 17, and 22°C), irradiance (6, 30, and 80 μmol photons m⁻² s⁻¹), and nitrate concentration (0.5 and 3.0 μM NO₃⁻). This range of temperatures was approximated to reflect those currently experienced by *M. pyrifera* in Tasmania (~12°C winter and ~18°C summer) and projected summer maxima (22°C) under the highest possible Representative Concentration of Pathways of greenhouse gas emissions (RCP8.5) scenarios at current CO₂ loadings (CSIRO and Bureau of Meteorology 2015). The incident irradiance simulated light levels in the field under dense, partial, and absent kelp canopy (Tatsumi & Wright 2016, Dayton et al. 1999, E. Flukes et al. unpubl. data), while nitrate levels were chosen based on normal (high) and EAC-influenced (low) ranges observed at Maria Island, Tasmania (Rochford 1984).

The juvenile sporophytes were grown for up to 28 d in the experimental treatments in the laboratory, which is sufficient time to see changes in seaweed growth and elemental stoichiometry (Flukes et al. 2015), with 3 independent replicates of each temperature–irradiance–nitrate combination.

Upon arrival at the laboratory, juvenile *M. pyrifera* sporophytes were divided between 3 holding tanks containing 0.2 μm filtered seawater, aerated, and shaded with flyscreen mesh, and assigned 1 of 3 temperature-controlled rooms. Incremental changes to temperature (~2.0°C d⁻¹) were made until experimental target conditions were reached. An earlier pilot trial indicated acute physiological stress in juvenile sporophytes under sudden increases in temperature and light. Consequently, incremental changes to temperature (2°C d⁻¹) were made until the 3 experimental target temperatures were reached. After 3 d acclimation, thalli were haphazardly selected from the tubs, and 2 individuals were placed into each glass beaker (2000 ml) containing growth media and subject to a particular treatment. Thalli were free-floating, as pilot trials showed no difference in physiological performance between vertically oriented
and free-floating thalli. The media was aerated to ensure sufficient mixing and disturbance of the diffusion boundary layer around thalli. Beakers were shaded by multiple layers of flyscreen to achieve irradiance of 10 μmol photons m$^{-2}$ s$^{-1}$. Irradiance was increased for high-light treatments by removal of layers of flyscreen over an additional 2 d by 10 μmol photons m$^{-2}$ s$^{-1}$ was reached, and similarly more flyscreen was added to achieve the low light treatment of 6.0 μmol photons m$^{-2}$ s$^{-1}$. Media was replenished every 2 to 3 d for the duration of the experiment to ensure nutrient levels were maintained.

Growth media (Wright’s Chu #10 [WC]) comprised autoclaved, nutrient-depleted, 0.2 μm filtered seawater with added base stocks of vitamins, trace metals, potassium hydrogen sulphate (K$_2$HPO$_4$), and sodium nitrate (NaNO$_3$) (Guillard & Lorenzen 1972). Manipulation of nitrate was achieved by modifying the volume of NaNO$_3$ added to WC media to achieve target concentrations, whilst keeping the ratio of phosphorus to nitrate stoichiometrically balanced at 20:1 by also manipulating the addition of K$_2$HPO$_4$ (Guillard & Lorenzen 1972), to avoid limitation of macronutrients. Aeration of the media in each beaker was used to promote circulation of media around the thalli, with air passing through a 0.2 μm syringe filter to minimise media contamination risk. Temperature-controlled rooms were set to 12, 17, and 22°C, while light was provided by cool white fluorescent tubes (Sylvania 36W/w41) on a 12 h light:12 h dark (L:D) cycle.

### 2.3. Physiological measurements

Physiological responses requiring tissue sacrifice were measured from one of the pair of thalli in each beaker at $T = 0$ d ($T_0$: post-acclimation) and at the end of experiment ($T_{end}$: up to 28 d after $T_0$). On both occasions, PAM fluorometry measurements were taken, and then tissue was sacrificed for chlorophyll, tissue chemistry, and nucleic acid analysis. At $T_0$, the second thallus was weighed to enable determination of relative growth when the same individual was weighed at the end of the experiment. Once PAM fluorometry was conducted, thalli were cut into sections of 10 to 20 mm$^2$ for nucleic acid analysis and stoichiometric analysis, while larger sections (20 to 40 mm$^2$) were required for pigment analysis (see Section 2.5). All sections were handled with gloves, tweezers, and a scalpel after being washed in dH$_2$O, then patted dry and prepared for storage before processing. Tissue chemistry sections were immediately placed into a −20°C freezer for further analysis, and pigment sections were immediately frozen in darkness at −20°C. Nucleic acid sections were placed in RNA later (Ambion), a non-toxic, rapid stabilisation buffer that preserves total tissue RNA and DNA, then frozen at −20°C.

#### 2.4. PAM fluorometry

Relative photosynthetic performance was estimated from rapid light curves (RLCs) obtained by measuring variable chlorophyll a (chl a) fluorescence in photosystem II (PSII) as a function of PAR using a blue-light diving PAM fluorometer (Walz). A ‘leaf clip’ with closable window was attached to thalli just above the meristematic region to maintain uniform spacing between the fibre optic light source and thallus tissue and to eliminate ambient light interference to ensure consistency of fluorescence measurements. RLCs were generated by an internal PAM software routine where actinic light intensity increased in 8 steps of 10 s each, measuring effective quantum yield of PSII ($\Phi_{PSII}$) as a function of PAR at each step. Relative electron transport rate (rETR) was determined by multiplying $\Phi_{PSII}$ by the respective PAR, which estimates the rate of electrons pumped through the photosynthetic chain (Beer et al. 2001). Estimates of electron transport dynamics were determined by plotting rETR against the respective PAR and fitted to a double exponential decay model (Eq. 1).

RLCs were determined twice on single thalli under pre-treatments of ambient light and dark-acclimation (~15 min or more). Dark-acclimation duration was determined by pilot studies to be ~15 min, after which more time under dark conditions showed no further increases in maximum fluorescence ($F_m$) or maximum quantum yield ($F_v/F_m$), indicating re-oxidation of the electron transport chain and relaxation of the photoprotective mechanisms. Ambient light RLCs reflect the immediate light history and can be affected by ambient irradiance conditions (i.e. cloud cover, canopy shading, water turbidity, etc.), whereas dark-acclimated RLCs indicate the inherent state of the photosystem (Ralph & Gademann 2005). When conducting PAM fluorometry with a leaf clip, local areas of tissue are subject to saturating pulses of actinic light, so it was ensured that the 2 PAM measurements (i.e. light-acclimated and dark-acclimated) came from different tissue, by moving the PAM leaf clip approximately 1 to 2 cm from the first measurement position between readings.
RLCs can be described and compared by characterising the photosynthetic response (raw rETR data) as a function of light, determined by the initial linear response and the region of photoinhibition (Ralph & Gademann 2005). RLC parameters were derived by fitting the raw data to the Platt et al. (1980) double exponential decay function to calculate maximum electron transport rate (rETR\text{max}) and saturating light intensity (E\text{k}) using the following equation:

\[
P = P_0 \left[ 1 - \exp\left( -\frac{\alpha E_d}{P_0} \right) \right] \times \exp\left( -\beta E_d \right)
\]  

(1)

where \(P\) is the photosynthetic rate (rETR), \(\alpha\) is the initial slope before the onset of saturation, \(E_d\) is the incident downwelling irradiance of the PAM internal halogen light, \(\beta\) characterises the slope region where PSII declines after photoinhibition (Henley 1993), and \(P_0\) is a scaling factor defined as the maximum potential rETR. The parameters rETR\text{max} and E\text{k} were estimated as per the Platt et al. (1980) equation using a nonlinear least-squares function in the ‘R’ software environment (v 3.0.0) to fit the models. To ensure convergence, the regression model settings were as follows: iterations = 100; step size = 1/1024; tolerance = 0.00001; initial seed value for \(P_0\) = rETR\text{max} derived from raw data, \(\alpha\) = slope of linear regression fitted to the first 3 points of raw data (typically in the range 0.7 to 1.0).

\(F_r/F_m\) was determined from dark-acclimated measurements where minimum fluorescence (\(F_0\)) and \(F_m\) were used to calculate variable fluorescence (\(F_v\)), then used to determine the intrinsic potential quantum efficiency of PSII.

2.5. Pigments

Frozen samples were thawed and patted dry, and 100 to 150 mg of tissue was weighed (to the nearest 0.01 mg) and placed into a 15 ml vial containing 5 ml of N,N-Dimethylformamide (DMF, Sigma Aldrich) to facilitate pigment extraction. Extraction vials were pre-wrapped in aluminium foil, and samples were processed rapidly in a fume hood under low light to avoid pigment damage from ambient light then placed in the freezer under total darkness at −20°C for 96 h for pigment extraction to occur.

2.6. Chl \(a\) and \(c\)

A 3 ml aliquot of pigment extract was pipetted into a centrifuge tube and centrifuged for 8 min at 8000 rpm. Chl \(a\) and \(c\) content of the supernatant was determined spectrophotometrically (wavelengths: 664.5, 631, and 582 nm) using a DynaMax HALO RB-10 Spectrophotometer and processed using UV Detector software (v.1.1). Blanks of 100% DMF were read every 10 readings. In accordance with manufacturer’s instructions, where absorbance read above 1000, supernatant was diluted with DMF until absorbance dropped below 1000. Dilution factor was recorded and factored into the final calculations for pigment concentration. Chl \(a\) and \(c\) concentrations were calculated using the recommended absorption coefficients following Inskeep & Bloom (1985) and Seely et al. (1972).

2.7. Fucoxanthin

Fucoxanthin content was determined from the remaining ~2 ml extractant. Aliquots of 2 μl of extract were injected using Waters Acquity H-series Ultra High Performance Liquid Chromotography (UPLC) coupled to a Waters Acquity Photodiode Array detector with a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) C18 column (2.1 mm × 100 mm × 1.7 μm particles). Mobile phases comprised a gradient mixture of 3 solvents prepared by Merck Chemicals, viz. acetic acid (1%), acetonitrile, and 80:20 methanol:hexane (Merck). Initial conditions were held for 3.5 min in a 20% acetic acid solvent and 80% acetonitrile solvent, followed immediately by 80% acetonitrile and 20% methanol:hexane solvent which was then held for a further 5.5 min, followed by 3 min re-equilibration to original conditions. The column was held at 35°C, and the flow rate was 0.35 ml min\(^{-1}\). The photodiode array detector was monitored continuously over the range 230 to 500 nm. Under these conditions, fucoxanthin eluted at 2.3 min. Initial calibration of the visible ultra-violet response at 440 nm for fucoxanthin was carried out on a freshly prepared standard solution (Sigma Aldrich) made up at 1.26 μg ml\(^{-1}\) in methanol. Chromatograms were extracted at 440 nm from the raw data using Waters TargetLynx v.4.1 software, and the area of the fucoxanthin peak was recorded and converted to mg ml\(^{-1}\) fucoxanthin before conversion to mg g\(^{-1}\) wet weight.

2.8. Elemental stoichiometry (N, C)

Frozen samples were thawed, patted dry, and then freeze-dried, with weighing every 12 to 24 h (to 0.01 mg) until deemed anhydrous when no further weight loss was detected. Samples were ground and homogenised in a mortar and pestle, and ~5 mg of
powder was placed into tin cups, which were folded gently prior to analysis. Carbon, nitrogen, and isotope signatures (δ13C and δ15N) were measured using a Thermo gas chromatograph coupled to a Finnigan Mat Delta S isotope ratio mass spectrometer in continuous flow mode at CSIRO, Hobart. Results were calculated as follows and are presented in standard sigma notation:

\[ \delta^{13}C \text{ or } \delta^{15}N (\%) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \]  

(2)

where \( R = \frac{^{13}C}{^{12}C} \text{ or } \frac{^{15}N}{^{14}N} \). Standards were replaced and run every 12 cycles with Pee Dee Belemnite (PDB) used as a standard for carbon and air as a standard for nitrogen.

2.9. Nucleic acids

Absolute RNA and DNA levels were determined to obtain RNA:DNA ratios as a proxy for ‘growth potential’ by extraction of total nucleic acids and RNA/DNA components from a single tissue sample (Flukes et al. 2015). A piece of algal tissue preserved in RNAlater weighing between 1 and 5 mg (w/w) was patted dry, weighed and homogenised in a round-bottom 2 μl centrifuge tube (Eppendorf Safe-lock microcentrifuge tube) using a drill pestle in an extraction buffer comprising 500 μl urea (4 M), 1% sodium dodecyl sulphate (SDS), trisodium citrate (1 mM), sodium chloride (0.2 M), and 5 μl of proteinase K (Urea/SDS buffer). To ensure stabilisation of nucleic acid, digestion of RNAses by proteinase K and complete cell lysis, the homogenised solution was held at 37°C for 10 min then placed immediately onto ice. Impurities (e.g. chlorophyll, phenolic compounds, salts, and detergents in urea/SDS) were removed by vortexing the solution with 750 μl of ammonium acetate, followed by centrifugation for 5 min at 14 100 × g. The resulting supernatant was decanted into a 1.5 ml tube to which 700 μl isopropanol was added, and the tube was gently inverted 40 times to aid complete mixing of total nucleic acids (tNA) and isopropanol. The total tNA was pelleted by centrifuge (10 min at 14 100 × g), and the pellet was washed twice in 75% ethanol (EtOH) solution, then resuspended in 200 μl molecular grade H2O at 55°C for 10 min and separated into two 100 μl aliquots.

To isolate RNA, a solution of 80 μl molecular grade water, 5 μl DNase (DNase I – Biolabs M0303L) with 20 μl buffer (New England Biolabs B0303S) was added to 1 aliquot for total DNA digestion, whilst 100 μl water and 5 μl RNAse (Sigma Aldrich R6148-25ML) was added to the second aliquot to digest RNA for total DNA isolation. To facilitate digestion, aliquots were incubated at 37°C for 20 min then stabilised on ice. Isolated nucleic acids were stabilised and extracted by vortexing (10 s) with 400 μl of urea/SDS buffer, vortexing (15 s) and centrifuging (10 min at 14 000 × g) with 200 μl ammonium acetate (7.5 M), decanting supernatant into 1.5 ml tubes, and binding and pelleting with isopropanol as described in the previous step. RNA and DNA pellets were washed twice in 75% EtOH and resuspended into 100 μl of molecular grade water (RNA) and EB buffer (DNA). RNA and DNA concentrations were measured by fluorescence assays using a Quibit assay probe and fluorometer and expressed as total RNA and total DNA (μg g−1 wet weight tissue). These values were used to calculate the RNA:DNA ratios.

2.10. Growth

Absolute growth and relative growth rates were determined from wet weight measures after first drying the specimen on absorbent paper towel before placing on the scale. Daily relative growth rate \( R \) was calculated as

\[ R = \frac{\log_{e} W_{T} - \log_{e} W_{T_{0}}}{T - T_{0}} \]  

(3)

where \( W \) is weight in grams, and \( T \) is time in days.

2.11. Statistical analyses

Tissue necrosis during the experiment led to poor tissue condition in some juvenile sporophytes, which resulted in unrealistic physiological measurements (PSII, chemistry, and nucleic acid), and a number of individuals, particularly from high-temperature and high-light treatments, were not able to be measured for certain metrics. Therefore, the planned fully factorial univariate analyses testing for temperature × light × nitrate effects after 28 d of experimental treatments were not possible for some metrics. Data were partitioned into 3 different analyses:

(1) at low temperature (12°C), 2-way ANOVAs determined the effects of light (fixed: 6, 30, and 80 μmol photons m−2 s−1) and nitrate (fixed: 0.5 and 3.0 μM NO3−) at \( T_{0} \) and \( T_{\text{end}} \) on PSII, pigments, elemental chemistry, nucleic acids, and relative growth rates;

(2) at \( T_{0} \) 3-way ANOVAs tested the effects of temperature (fixed: 12, 17, and 22°C), light (fixed: 6 and
30 μmol photons m\(^{-2}\) s\(^{-1}\), i.e. 80 μmol photons m\(^{-2}\) s\(^{-1}\) was not included), and nitrate (0.5 and 3.0 \(μM\) NO\(_3\)\(−\)). The same analyses were done at \(T_{\text{end}}\) except for treatments subject to high water temperature (22°C), which was dropped for all response variables except for pigments for which sufficient material was available;

(3) at \(T_{\text{end}}\), moderate light (30 μmol photons m\(^{-2}\) s\(^{-1}\)) occurred across all levels of temperature and nitrate, and thus a 2-way ANOVA was conducted across temperature and nitrate for all metrics except pigments, which were incorporated into the second set of analyses.

Assumptions of ANOVA were checked and transformations determined using the Box-Cox method. Tukey’s HSD post-hoc tests were conducted where there were significant overall results to determine the source of differences between treatment groups.

The multivariate physiological response to treatments was analysed using permutational multivariate ANOVA (PERMANOVA) at both \(T_0\) and \(T_{\text{end}}\) to determine overall treatment effects on joint distributions of response variables. In 5 instances, determination of \(r\text{ETR}_{\text{max}}\) and \(E_k\) was not possible due to degraded tissue, and so these variables were not included in the multivariate analyses. The design was unbalanced since 1 replicate could not be included because of missing nucleic acid data. PERMANOVA was conducted on Gower similarity matrices (Gower 1971) generated from raw data, with 9999 permutations to calculate pseudo \(F\)-statistics. Terms with negative estimates of components of variation were pooled (Anderson et al. 2008).

3. RESULTS

3.1. Short-term response to acclimation: low-temperature treatment (12°C)

Following short-term acclimation (i.e. at \(T_0\)) at 12°C, 2-way ANOVA revealed no effects of nitrate or light separately, nor any light × nitrate interaction, for \(F_v/F_m\), pigments, elemental chemistry, nucleic acids and relative growth at \(T_0\) (Table 1A, Figs. 1–4). \(r\text{ETR}_{\text{max}}\) and \(E_k\) were significantly higher at high light compared to low light (80 > 6 μmol photons m\(^{-2}\) s\(^{-1}\); Fig. 1). Juvenile sporophytes in moderate-light (30 μmol photons m\(^{-2}\) s\(^{-1}\)) treatments had significantly higher carbon concentration (compared to other light
treatments) and C:N ratio (compared to high light only; Table 1A, Fig. 3). In low-light treatments, stable isotope ratios of sporeling tissue were less negative for $\delta^{13}C$ (compared to moderate light) and $\delta^{15}N$ (compared to high light; Table 1A, Fig. 3).

### 3.2. Short-term response to acclimation: multiple temperature treatments

Three-way ANOVAs (excluding the high-light treatment) showed no treatment effects at $T_0$ for chl a, fucoxanthin, and $\delta^{15}N$ (Table 2A, Figs. 2 & 3). $\text{rETR}_{\text{max}}$ and $E_k$ were significantly higher at moderate light (vs. low) and 17°C (vs. 12°C) (Table 2A, Fig. 1). At 22°C, there were no further increases in PSII traits but significantly lower $F_v/F_m$ under low light (Table 2A, Fig. 1), indicating a reduction in PSII integrity at high temperature and low light (temperature × light interaction). Under low light, chl c was significantly higher (Table 2A, Fig. 2) while carbon concentration and C:N ratios were lower, associated with a less negative carbon isotope signature (Table 2A, Fig. 3). At high temperature (22°C), juvenile sporophytes subject to low-light treatments had higher nitrogen concentrations (Table 2A, Fig. 3) but were lower in RNA content (Table 2A, Fig. 4) than when grown under moderate light. RNA concentrations were higher at 12°C (vs. 22°C, but only at low light; temperature × light interaction) and 0.5 μM...
NO$_3^-$ (vs. 3.0 μM, but only in moderate light; nitrate × light interaction; Table 2A, Fig. 4). There were significant main effects affecting absolute DNA concentration (light: 30 > 6 μmol photons m$^{-2}$ s$^{-1}$) and RNA:DNA ratios (light and temperature: 6 > 30 μmol photons m$^{-2}$ s$^{-1}$ and 22 > 17°C; Table 2A, Fig. 4).

### 3.3. Longer-term responses: low temperature treatment (12°C)

#### 3.3.1. PSII and pigments

At $T_{end}$, juvenile sporophytes grown at low temperature (12°C) revealed no treatment effects for $F_v/F_m$ or fucoxanthin (Table 1B, Figs. 1 & 2). $E_k$ was significantly higher when juvenile sporophytes were grown under high light than at moderate light levels (Table 1B, Fig. 1). Pigment concentrations were higher under low-nitrate treatments (chl a only) and low-light treatments compared to moderate light (chl c only; Table 1B, Fig. 2).

#### 3.3.2. Growth, nucleic acids, and tissue chemistry

At $T_{end}$ and 12°C, no treatment effects were found for $\delta^{15}$C, RNA concentration, or RNA:DNA ratios (Table 1B, Figs. 3 & 4). Across all light treatments, high light yielded more negative $\delta^{15}$N signatures and higher concentrations of DNA (Table 1B, Figs. 3 & 4) compared to other light treatments. Surprisingly, relative growth was significantly higher under the lowest nitrate treatment (Table 1B, Fig. 4).

Fig. 2. As in Fig. 1 but for pigment content. Plots show tissue concentrations of (A,D) chl a; (B,E) chl c; (C,F) fucoxanthin. Bars indicate mean values ± SE (n = 3)
3.4. Longer-term responses: multiple temperature treatments

3.4.1. PSII and pigments

At the end of the experiment, 3-way ANOVA showed no treatment effects for \(F_v/F_m\) (Table 2B, Fig. 1). Temperature effects on \(rETR_{\text{max}}\) (17 > 12°C) were restricted to moderate light levels only (temperature \(\times\) light interaction), whilst the effect of temperature on \(E_k\) was not dependent on the light level (Table 2B, Fig. 1). Low light yielded significantly higher chl \(a\) and \(c\) concentrations, but there was no such change in fucoxanthin levels (Table 2B, Fig. 2). Higher concentrations of chl \(a\) and fucoxanthin at 22°C (vs. 12°C) only occurred at high nitrate concentrations (temperature \(\times\) nitrate interaction). Chl \(c\) concentration was higher at 12°C (vs. 22°C) when nitrate levels were low and was higher at 0.5 \(\mu\text{M}\) NO\(_3\) treatments (vs. 3.0 \(\mu\text{M}\)) but only at 22°C (temperature \(\times\) nitrate interaction, Table 2B, Fig. 2).
3.4.2. Growth, nucleic acids, and tissue chemistry

Three-way ANOVA revealed no treatment effects on nucleic acid levels (Table 2B, Fig. 4). Carbon concentration was significantly higher at 30 μmol photons m⁻² s⁻¹ light treatments (vs. 6 μmol photons m⁻² s⁻¹) but only at low nitrate availability (Table 2B, Fig. 3). Nitrate treatments significantly influenced nitrogen concentration (3.0 > 0.5 μM NO₃⁻) and C:N ratio (0.5 > 3.0 μM NO₃⁻) in low-light treatments only (light × nitrate interaction). Additionally, the C:N ratio was significantly higher at 30 μmol photons m⁻² s⁻¹ (vs. 6 μmol photons m⁻² s⁻¹) but only at high nitrate levels (light × nitrate interaction; Table 2B, Fig. 3). Significant temperature and nitrate effects were additive for relative growth rates which were higher at 12°C and at low nitrate levels (Table 2B, Fig. 4).

3.5. Longer-term responses: moderate light treatment

At Tend, under moderate light levels (30 μmol photons m⁻² s⁻¹), 2-way ANOVA showed no treatment effects on carbon or nucleic acid concentrations (Table 3, Figs. 3 & 4). rETRmax and Eₖ increased with temperature from 12 to 17°C but not to 22°C and were associated with significantly lower Fv/Fm at 22°C, suggesting down-regulation and compromised integrity.
Table 2. F-test statistics for 3-way factorial ANOVA testing for effects of temperature (3 levels: 12, 17, and 22°C), except at $T_{end}$ for nucleic acids, elemental chemistry, and relative growth rate, where there were 2 levels (12 and 17°C), light (2 levels: 6 and 30 μmol photons m$^{-2}$ s$^{-1}$), and nitrate (2 levels: 0.5 and 3.0 μM l$^{-1}$) on Tasmanian-sourced juvenile *Macrocystis pyrifera* PSII, pigments, elemental chemistry, and nucleic acids at (A) $T_{0}$ and (B) $T_{end}$. Results of Tukey’s HSD tests for significant results are given below the table. Only significant differences are shown (i.e. levels not included in table are not significantly different to all other levels). See Table 1 for abbreviations; df shown for treatment effects (vertical) and denominator (horizontal); df = 2 (#: Table 2B only). ***p < 0.001; **p < 0.01; *p < 0.05

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of photosystem II (Table 3, Fig. 1). In low-nitrate treatments, there were temperature effects on nitrogen concentration (12°C = 17°C > 22°C; significant temperature × nitrate interaction), while at 22°C, nitrate levels affected the C:N ratio (0.5 > 3.0 μM NO$_3^-$). δ$^{15}$N values were less negative under 22°C (vs. at 12 and 17°C) and 0.5 μM NO$_3^-$ (vs. 3.0: Table 3, Fig. 3). Relative growth was significantly lower at 22°C (vs. 12 and 17°C; Table 3, Fig. 4).

3.6. Multivariate phenotype response

Three-way PERMANOVA revealed significant variation in the multivariate physiological response of juvenile *M. pyrifera* sporophytes at both $T_0$ and $T_{	ext{end}}$ due to interaction effects of various combinations, although there was no evidence of a 3-way interaction (Table 4). Short-term ($T_0$) multivariate response differentiated 12 and 17°C from 22°C under low light only (a temperature × light interaction: Pseudo-$F_{1,21} = 2.29$, $p = 0.036$). The first 2 eigenvalue correlations of the canonical analysis of principle co-ordinates (CAP) were 76% and 29% and suggested that the variables contributing most to the variation under different treatments were $F_v/F_m$, DNA, and accessory pigments, which increased in the direction of benign environments, whereas RNA and elemental chemistry increased in the direction of stressful environments (Fig. 5). By the end of the experiment ($T_{	ext{end}}$), the multivariate response indicated significant 2-way interactions involving all 3 factors (temperature × nitrate: Pseudo-$F_{2,21} = 2.89$, $p = 0.004$; temperature × light: Pseudo-$F_{1,21} = 3.14$, $p = 0.005$; and nitrate × light: Pseudo-$F_{2,21} = 2.36$, $p = 0.007$) (Fig. 6). The first 4 eigenvalue correlations of the CAP were 96, 89, 80, and 77% and suggested that the variables contributing most to the variation under different treatments were relative growth rate and $F_v/F_m$ and carbon concentration, which increased in favourable environments, whilst RNA, pigments, and DNA increased under stressful environments.

4. DISCUSSION

This study found that differential temperature, light, and nitrate levels lead to significant responses in a range of variables describing the physiology of juvenile *Macrocystis pyrifera* sporophytes. Warming seawater negatively affected multiple traits in juvenile sporophytes, consistent with previous studies in which elevated temperatures reduce growth and im-

| Table 3. F-test statistics for 2-way factorial ANOVA testing for effects of temperature (3 levels: 12, 17 and 22°C) and nitrate (2 levels: 0.5 and 3.0 μM l$^{-1}$) at 30 μmol photons m$^{-2}$ s$^{-1}$ on Tasmanian-sourced juvenile *Macrocystis pyrifera* photosynthesis, pigments, elemental chemistry, nucleic acids and relative growth at $T_{	ext{end}}$. Results of Tukey’s HSD tests for significant results are given below table. Only significant differences are shown (i.e. levels not included in table are not significantly different to all other levels). See Table 1 for abbreviations; df shown for treatment effects (vertical) and denominator (horizontal). ***p < 0.001; **p < 0.01; *p < 0.05.
pair biological function in most temperate seaweeds (Harley et al. 2012). Additionally, light and nitrates provided further impacts either independently, additively, or synergistically. These results illustrate the impacts on physiological processes that will likely shape the performance and distribution of *M. pyrifera* under future climate change in south-eastern Australia whilst showing that globally distributed species exhibit locally evolved nutrient utilisation dynamics.

4.1. Additive effects of temperature and light on *M. pyrifera* condition

Different temperature and light treatments caused significant variation in multiple physiological variables. Chronic macroscopic tissue deterioration and sporeling mortality in high temperature (22°C) and high light (80 μmol photons m−2 s−1) treatments were observed. Relationships between temperature and light and PSII are well documented for photoautotrophs, as PSII-associated enzyme-catalysed reactions (RuBisCO and Calvin cycle activity) are thermally labile (Davison & Davison 1987) and electron transfer rates are light-dependent (Ramus 1981). This kind of change in enzymatic activity might explain the general pattern of increases in rETR\textsubscript{max} and *E*\textsubscript{k} with increasing temperature (12 to 17°C) and increasing light. Conversely, high temperature stress impairs biochemical pathways (Davison & Pearson 1996) and can cause denaturation of proteins and degradation of thylakoid membrane properties (Ma-

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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Residual</td>
<td>33</td>
<td>248.75</td>
<td>21</td>
<td>161.43</td>
</tr>
</tbody>
</table>

Table 4. PERMANOVA results comparing the multivariate phenotype of *Macrocystis pyrifera* between orthogonal treatments of temperature (12, 17 and 22°C), light (10 and 30 μmol photons m\textsuperscript{2} s\textsuperscript{-1}), and nitrate (0.5 and 3.0 μM NO\textsubscript{3}–) at *T*\textsubscript{0} and *T*\textsubscript{end}. Interaction terms with negative coefficients of variation were dropped from the analysis. Significant *p* values given in **bold**.

Fig. 5. Canonical analysis of principal coordinates (CAP) ordination of physiological response metrics of *Macrocystis pyrifera* to combined effects of temperature and light (based on a Gower similarity matrix of raw data for 12 traits) post-acclimation (*T*\textsubscript{0}). The CAP analysis was constrained to differentiate among treatments of temperature and light levels and shows clustering of light treatments and distinct separation of temperature effects. The vector overlay represents Pearson correlations between the ordination axes and direction and magnitude of trait influence. Symbols: temperature (12, 17, and 22°C) and light (low: LLT, medium: MLT, high: HLT).
Inhibiting key photoprotective processes such as the production of chaperones (heat-shock proteins; Wahl et al. 2011). Moreover, nutrient uptake and gas exchange capabilities are impacted by high temperature, causing downregulation of photosynthetic activity (i.e. a drop in $r_{\text{ETR}}$) and impaired PSII ($F_v/F_m$) (Andersen et al. 2013), and ultimately tissue degradation and mortality as was observed at 22°C. Light energy absorption is independent of temperature and this energy is diverted into production of proteins, enzymes, and photoprotective mechanisms (Franklin et al. 2003). Light absorbed in excess of photosynthetic demand generates reactive oxygen species that alter the permeability of chloroplast membranes and loss of electron transport capacity (Kyle et al. 1984), disrupting carbon fixation and protein synthesis (Murata et al. 2007), leading to chronic photoinhibition and photodamage where photoprotection fails to mitigate photoinactivation (Franklin et al. 2003).

In the high-light treatments, the increased energetic requirements of photosynthetic activity at temperatures above 12°C may have undermined photoprotective capabilities, leading to rapid mortality in these treatment combinations. This follows temperate algal assemblages that exhibit increased compensating irradiance and reduced primary productivity and respiration under elevated temperature and irradiance (Tait & Schiel 2013). These findings highlight the vulnerability of *M. pyrifera* at temperatures above 12°C under a reduced canopy where irradiance can fall within the range of 30 to 280 μmol photons m$^{-2}$ s$^{-1}$ in Tasmanian kelp forests (Tatsumi & Wright 2016) and depends on depth, season, and degree of canopy loss.

Conversely, in low-light conditions, investment in pigment synthesis can lead to an ‘energy crisis’, where energy is diverted from carbohydrate and lipid production to producing light-harvesting pigments (Falkowski & LaRoche 1991). In this context, the results suggest a synergistic interaction between temperature and light in that short-term exposure (at $T_0$) to low light and high temperature at the same time quickly led to impaired PSII function (evidenced by low $F_v/F_m$) and significantly greater pigment content (the only determinable metric) in tissue subject to low light compared to moderate light. This suggests an increased vulnerability to ocean warming for *M. pyrifera* recruits in low-light environments such as dense canopy understory, at depth, or locations subject to high sediment loads.

Elevated RNA:DNA ratios under high temperature and low light were also associated with impaired PSII (low $F_v/F_m$) after short-term exposure ($T_0$) and reflected both an overall increase in RNA concentration and a decrease in DNA levels in treatments with low light. This association between PSII and nucleic acid levels may indicate elevated RNA synthesis of protective proteins in response to temperature-induced photosynthetic stress (as found in corals: Hauri et al. 2010) and/or low-light stress where downregulation of RuBisCO gene expression, transcription, and protein production is associated with lower cellular DNA concentrations, as has been identified in cucumber leaves (Sun et al. 2014). The nature of association between RNA:DNA and $F_v/F_m$ found in *M. pyrifera* was contrary to that found in juvenile *Phyllospora comosa* (Flukes et al. 2015), where a precondition for elevated RNA:DNA ratios was a healthy, functioning PSII (high $F_v/F_m$). These differences are possibly attributable to the extremely different light...
environments in which these species proliferate, with *P. comosa* occurring in much shallower waters than *M. pyrifera*.

In the context of the growth rate hypothesis, there was no evidence to support any relationship between RNA:DNA ratio and lineal growth relationship. This reflects the finding that ratios could change with changes in either the absolute concentration of DNA as found in *Phyllospora* (Flukes et al. 2015), RNA, or both. Variable DNA concentration is known in plants and seaweeds and can be influenced by cell packing density (Dortch et al. 1983), increasing cell size or cell wall thickness (Kraemer & Chapman 1991), and size of chloroplasts (Rauwolf & Chapman 2010), characteristics that were undetectable by the measurements. Consequently, these results support that RNA:DNA ratio may be a more useful indicator of recent stress as RNA synthesises for stress-related protein complexes. Identification and quantification of particular stress proteins (i.e. heat-shock proteins) using qPCR methods could be used to indicate the type and severity of stress within populations.

### 4.2. Additive effects of temperature and nitrates on *M. pyrifera* growth

Sporeling growth rates were highest at low temperature and low nitrate levels. Lower growth rates at temperatures above 12°C suggest a temperature ‘growth boundary’ (van den Hoek 1982) between 12 and 17°C for *M. pyrifera* in Fortescue Bay. Hence, it is probable that sustained positive temperature anomalies such as the recent 130 d heatwave event in south-east Australia (Hobday et al. 2016, E. Oliver pers. comm.) restrict opportunity for sporeling growth and development.

Annual variation of ocean nitrate levels in south-eastern Australia generally follow a seasonal trend, ranging from ~0.5 μM NO\(_3^-\) and sometimes undetectable levels during the mid-late summer growth season to up to ~3.0 μM NO\(_3^-\) in the winter (Harris et al. 1987). *M. pyrifera* and other kelps can actively regulate nitrogen uptake, assimilation, storage, and use and display locally adapted nitrogen-utilisation strategies (Gagné et al. 1982, Stephens & Hepburn 2016), although relative to other Laminarian species, *M. pyrifera* has poor capacity for nitrogen storage (Gerard 1982, 1997). Interestingly, juvenile *M. pyrifera* sporophytes in low-nitrate treatments had higher relative growth rates, whilst those in high-nitrate treatments displayed preference for uptake and storage of N (higher N concentrations and more negative \(^{15}\)N signatures) rather than growth *per se*. Juvenile sporophytes with higher growth rates had lower nitrogen tissue concentrations, indicating that nitrogen use outstripped replenishment rates, which is typical of Laminarians in low-nitrate conditions (Gerard 1982). However, this does not explain the observed lower growth rates in high-nitrate treatments. Plastic response to stressors via the coupling of physiology and morphology is well documented in kelps (Druehl & Kemp 1982, Fowler-Walker et al. 2006). Resource acquisition influences morphology in macroalgae, and nutrient stress (low ambient nutrients) may trigger a shift in resource allocation to thallus growth, increasing total surface area to volume ratio and improving nutrient uptake kinetics (Hein et al. 1995). Plants subjected to nutrient starvation can shift growth allocation from leaves to roots (Hirose & Kitajima 1986), demonstrating resource-driven changes to morphology. An inverse relationship between nutrient availability and growth has also been described in unicellular marine algae where nitrogen deprivation leads to increased competition between carbon fixation and N assimilation (Hinck et al. 1983). This observation is contrary to results for Californian *M. pyrifera*, where growth was limited at yearly minima (2 μM NO\(_3^-\)) but not limited at yearly maxima (8 μM NO\(_3^-\)) in experimental conditions (Deysher & Dean 1986, Dayton et al. 1999). This distinction between different populations of *M. pyrifera* provides some evidence that nutrient utilisation dynamics in kelp can be locally adapted and that sporophyte development may be in part triggered by seasonal environmental cues, the timing of which has implications for sporophyte fitness (Kinlan et al. 2003).

### 4.3. The future: efficacy of a multifactor approach

This study emphasises the importance of multifactor approaches to determining species and ecosystem response to climate change. Marine climate change studies up until recently have been predominantly temperature-focused (Harley et al. 2006) and have shown that temperature is clearly important for *M. pyrifera* and other marine species. However, this study demonstrates that effects of ocean warming may be substantially altered when there is simultaneous change in other environmental factors, demonstrating the importance of multifactor approaches in climate-change studies. Further to this, multivariate approaches to assessing physiology are crucial for holistic organismal-level insights into perform-
ance effects. Based on the lack of support for the growth rate hypothesis and the unexpected inverse relationship between relative growth and nitrogen treatment levels, it can be argued that predictions derived from simplified and generalised approaches, such as RNA:DNA ratios and relative growth rates, may come with high levels of uncertainty (Schiel & Foster 2015).

Yet a further layer of complexity is associated with ‘ecosystem engineers’ such as kelp whereby biotic structures modify the abiotic environment, altering conditions for recruitment, which in turn may influence patch dynamics and stability, which may come with high levels of uncertainty (Schiel & Foster 2002). It is likely that the treatment effects observed here will differ for adult and microscopic stages, as is the case in other species of brown algae (Wahl et al. 2011). Hence, more research into the impacts of climate change on other stages is required to determine if and how populations may adapt. Future research must also consider genetic and non-genetic drivers that determine adaptability (Schiel & Foster 2015), such as genetic diversity and phenotypic plasticity (Reusch 2014). Nevertheless, juvenile sporophytes are an important link in sustaining *Macrocystis pyrifera* populations, and as demonstrated here, they exhibit negative responses and susceptibility across a range of physiological parameters. At the very least, temperature, canopy destabilisation (increased light), and disruption of nutrient regimes are certain to provide further distributional declines in *M. pyrifera*. Examining multiple physiological traits allows for a more comprehensive interpretation of the effects of climate change on overall physiology compared to predictions based on one or a few types of metrics.

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