



Not only warming: The consequences of thermal variability in the growth of *Fucus serratus*

Ángela Fernández García

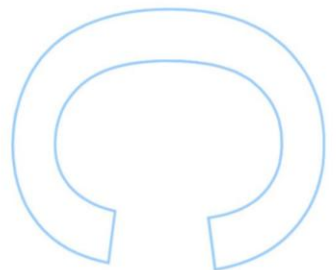
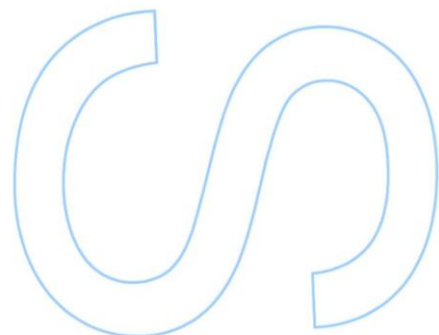
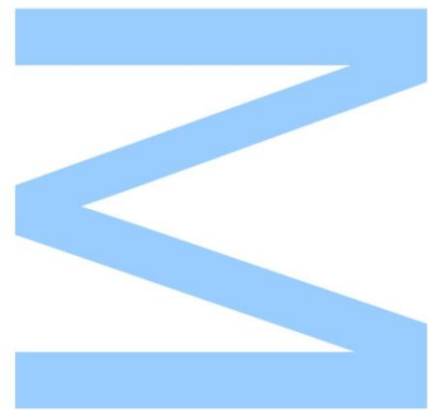
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Orientador

Francisco Arenas Parra, Investigador auxiliar, CIIMAR

Co-orientador

Brezo Martinez Díaz-Caneja, Professora, URJC



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ABSTRACT

Despite the current and future climate change scenarios forecast an increase in the intensity and variability in thermal and temporal stress events, most of the studies have focused on the impact of increases or decreases in average stress intensity. For this reason and in order to know the *Fucus serratus* resilience to temperature variability, we performed two manipulative experiments to evaluate the potential changes in the physiological and growth responses of this specie to extreme events of temperature variability. The first experiment analyzed variation in mean temperature seawater (optimal: 18°C; sublethal: 22°C and lethal: 26°C) at three levels of thermal variability (control, low and high; $\delta = 0, 2$ or 4 °C respectively). In the second experiment, we examined simultaneously thermal variability (two levels, low and high; $\delta = 2$ or 4 °C) and temporal variance (two levels: low and high, i.e. stress events distribution homogeneously or not, respectively) in a sublethal temperature. Results showed that thermal variability of seawater have significant effects in addition to changes in mean temperature, suggesting that high thermal variability buffers negative effects of high temperature in the growth and some photosynthetic parameters. These results suggest that the mechanisms that govern this interaction could be involved in the current distributional patterns of *F. serratus* in the Iberian Peninsula. Results also suggest that it is important to consider the capacity to survive at short and repeated periods of extreme conditions. This study has significant implications for understanding macroalgae responses to climate change, but more studies may be done under climate change variability scenarios.

Key words: Climate change, *Fucus serratus*, thermal variability, temporal variance, stress.

RESUMO

Apesar do que os cenários atuais e futuros de alterações climáticas prevêm um aumento na intensidade e variabilidade nos eventos de estresse térmico e temporal, a maioria dos estudos têm-se centrado no impacto dos aumentos ou diminuições da intensidade média deste stress. Por esta razão, e a fim de conhecer a resiliência de *Fucus serratus* a variabilidade da temperatura, foram realizadas duas experiências para avaliar as possíveis variações nas respostas fisiológicas e de crescimento desta espécie aos eventos extremos de variação térmica. A primeira experiência analisou a variação da temperatura média em água do mar (ótima: 18 °C; subletal: 22 °C e letal: 26 °C) com três níveis de variabilidade térmica (controlo, baixa e alta; $\delta = 0, 2$ ou 4 °C, respectivamente). Na segunda experiência, foram examinados simultaneamente a variabilidade térmica (dois níveis, baixo e alto; $\delta = 2$ ou 4 °C) e variação temporal (dois níveis: baixa e alta, ou seja, se a distribuição de eventos de estresse era de forma homogênea ou não, respectivamente) em uma temperatura subletal. Os resultados mostraram que a variabilidade térmica da água do mar tem efeitos significativos, além das mudanças na temperatura média, sugerindo que altos níveis de variabilidade térmica amortiça os efeitos negativos da alta temperatura no crescimento e alguns parâmetros fotossintéticos. Estes resultados sugerem que os mecanismos que regem essa interação poderia ser envolvido nos atuais padrões de distribuição de *F. serratus* na Península Ibérica. Os resultados também sugerem que é importante ter em conta a capacidade de sobreviver em períodos curtos e repetidos de condições de stress. Este estudo tem implicações significativas para a compreensão das respostas das macroalgas nas alterações climáticas, mas mais estudos devem ser feitos em cenários variáveis de alterações climáticas.

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1.3. Abbreviations

°C: centigrade's degrees

δ: thermal variability

N: north

W: west

μM: micromole

N: nitrogen

P: phosphorus

NaNO₃: sodium nitrate

NaH₃PO₄: sodium phosphate

FW: fresh water

SD: standard deviation

g: grams

h: hour

l: liter

var: variance / variability

seq: sequence

W: weight

t: time

Fv/Fm: maximal quantum yield of photosynthesis

RLC: rapid light curves

ETR: electron transport rate

P-I curves: photosynthesis- irradiance curves

m: meter

s: second

F_{II} : fraction of chlorophyll associated to PSII

E: incident actinic irradiance

A: absorptance

E_0 : incident irradiance

E_t : transmitted irradiance

ETR_m: maximum electron transport rate

E_k : saturation irradiance

α : maximal light utilization efficiency

β : photoinhibition rate

AIC: Akaike information criterion

HSD: Honestly Significant Difference

SNK: Student Newman Keuls

df: degrees of freedom

MS: Mean Square

SS: Sum of Square

temp: temperature

2. INTRODUCTION

The relationship between latitudinal species' distribution and climate is a central concept in biogeography (Hutchins 1947, Araújo & Luoto 2007) and a timeless research topic. Mounting and unambiguous evidence indicates that climate change is altering the distributional patterns of many species worldwide (Beardall et al. 1998, Parmesan & Yohe 2003). In the last decade several studies found a relationship between global warming and shifts in species' distribution in marine systems (eg. Lima et al. 2007, Wernberg et al. 2011, Harley et al. 2012). However, our understanding of the processes underpinning these reported species distribution shifts are still limited, particularly on the role of extreme events, hindering our ability to predict future geographic range shifts. Actually, changes in mean intensity, and temporal and thermal variability of climatic extreme events have been described all over the globe (Eastirling et al. 2000; Lima & Wethey 2012). However, when exploring causality behind species distribution or abundance, ecologists have usually focused on studying the variation of species response to changes on the mean intensity of predictor variables, e.g. environmental or climatic factors (Kappelle et al. 1999, Hughes 2000, Walther et al. 2002), while little attention has been paid to the variability of predictor variables as causal explanation for the species response (Jentsch et al. 2007, Bozinovic et al. 2011). Environmental time series are complex (Helmuth et al. 2006) and temporal and spatial variability of environmental factors is a wide recognized trait of natural systems (Horne & Schneider 1995). Thus different aspects of an environmental signal, including extremes, range, and patterns of variability, will have different biological consequences (Parmesan et al. 2000).

When predicting future ecological patterns – and when designing experiments to validate those predictions – it is tempting to treat environmental change as a steady shift in mean conditions. However designing experiments including variability in the predictor variables may in fact lead to more realistic results rather than those experiments using mean constant values of the predictor variables (Benedetti-Cecchi 2003, Bertocci et al. 2005, 2007, Jentsch et al. 2007, Bozinovic et al. 2011). These approaches may provide better mechanistic knowledge needed to reduce the uncertainty of predicted contractions and expansions of distributional ranges under climate change (eg. Buckley et al. 2010).

In the last centuries coastal areas have been increasingly threatened by anthropogenic activities (Barbier et al. 2011), but now they are facing the additional consequences of climate change, especially an increase in the intensity and frequency of extreme weather events (Cardoso et al. 2008; Lima & Wetthey 2012). In particular, intertidal areas are highly variable environments with tides causing accentuate daily physical gradients (Bertness 1999, Harley & Helmuth 2003). Intertidal organisms living in the interplay between land and sea experience consecutive periods of emersion and immersion that may take them close to their physiological tolerance thresholds (Helmuth et al. 2002, Tomanek & Helmuth 2002). Thus, they are suitable model organisms for detecting the early warning signals of climate change-related impacts (Barry et al. 1995, Southward et al. 1995, Thompson et al. 2002, Hawkins et al. 2003, Harley et al. 2006, Helmuth et al. 2006).

Seaweeds are key structuring organisms, providing food, habitat and harboring highly diverse communities at intertidal and subtidal zones (Graham 2004, Christie et al. 2009). Particularly large canopy species are primary habitat for many marine animals including commercial fisheries (Jones et al. 1997, Viejo et al. 2011). Many large seaweeds are harvested for a wide range of uses such as food additives, pharmaceutical and cosmetic applications, biofuels and human consumption. In our study we used *Fucus serratus* Linnaeus as model intertidal species. *F. serratus* is a cold-temperature furoid with a fragmented distribution along the Brittany coast and north of Iberian Peninsula, probably resulting from the upwelling events of cold water present in Galicia and north of Portugal and the high sea surface temperatures in the Bay of Biscay, particularly in summer (Gómez-Gesteira et al. 2008, Michel et al. 2009). In the Cantabrian Sea, ocean warming has been associated with the recent decline of this species (Viejo et al. 2011, Duarte et al. 2013). However, this has not occurred at its southern limit of distribution, in north of Portugal. These two limits, Asturias and Viana do Castelo (Alcock 2003, Arrontes 1996), cannot be explained by the traditional models that associate these southern limits with the August oceanic isotherm (reviewed in Lüning 1990) as Martínez et al. 2012 observed in their study. We used *Fucus serratus* to test for physiological effects of stressors variability and with the appreciation that it would serve as a model species for other cold-temperature macroalgae.

With heat waves and other extreme events increasing on intensity and frequency (Easterling et al. 2000, Beniston et al. 2007, Jentsch et al. 2007), investigating the ability to acclimate of these structuring intertidal species is indispensable in order to

better understand the potential impacts of climate change (Chown et al. 2010). For that reason, and because data on intertidal rockpool temperature variations suggest that intertidal seaweeds have to deal with large daily temperature differences (Metaxas & Scheibling 1993, Araujo et al. 2006), our objective was to examine potential changes in the physiological and growth responses of *Fucus serratus* to extreme events of thermal and temporal variability at the same time, since according to climate change predictions. So we aimed to test the general hypothesis that the temperature variability may affect species resilience to seawater warming by simultaneously examining temporal (two levels: low and high, i.e. stress events distribution homogeneously or not, respectively) and intensity variance on temperature variability (three levels: control, low and high; $\delta = 0, 2$ or 4 °C respectively) at three different mean temperature scenarios (optimal: 18°C; sublethal: 22°C and lethal: 26°C).

3. MATERIALS AND METHODS

3.1. Algae collection and acclimation

For experiments of thermal and temporal variability, 260 vegetative fronds of *F. serratus* (Fig 1a) were collected during low tide on the 20th of November 2013 at Las Margaritas beach in Oleiros, A Coruña (43°21'43" N, 8°20'49"W) (Fig 1b). After collection, fronds were immediately transported to the laboratory at CIIMAR (Centro de Investigação Marinha e Ambiental, Porto) in a cool box in darkness. Then were kept in an outdoor shaded tank of 360 L at 16 °C during 2 days to allow for the acclimation of the algae. Seawater was enriched to avoid nutrient limitation by adding inorganic N (NaNO₃) and P (NaH₃PO₄) to a final concentration of over 50 µM N and 5 µM P, respectively every two days. The initial weights of all fronds were similar in both experiments (1.3 ± 0.08 g FW, mean \pm SD, n = 260). Each replicate was individually labelled and hold in the culturing chambers using plastic pegs marked in numbers from lines attached to plastic frames (Fig 1c).



Figure 1. a) *Fucus serratus* frond; b) View of Las Margaritas beach, A Coruña; c) Replicates individually labelled and hold from plastic frames.

3.2. Experimental design

Ambient variability and selection of stress

Regarding the selection of temperatures for stress levels, we decided to choose the intensity of our stress treatments based on the results obtained in a previous experiment that was performed in an outdoor area during May and June of 2012. Individual fronds of *Fucus serratus* were maintained at 12 different temperatures in an interval between 8 and 30°C during 6 weeks in order to determinate the lethal, sub-lethal and optimal water temperature of *Fucus serratus* performance, through the increase or decrease of species growth rates (Figure 2) (Trilla 2013).

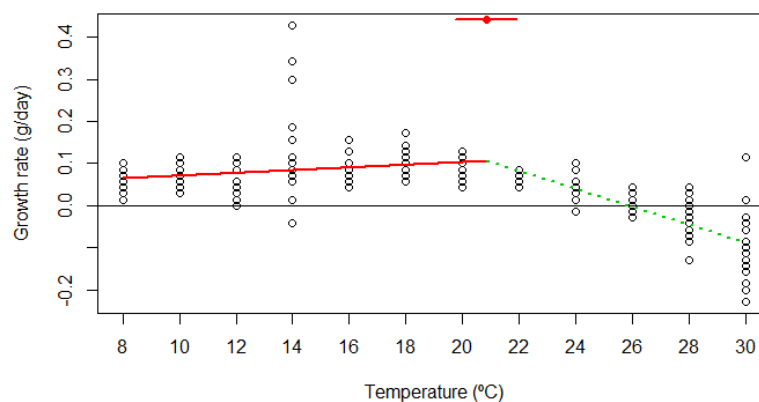


Figure 2. Piece-wise regression (segmented package for R from V. Muggeo, 2012) of the growth response of *Fucus serratus* after 14 days of laboratory culture at different temperatures, from 8 to 30°C. Red line represents the positive slope of the curve and the green dotted line represents the negative slope. The red point is the breakpoint with SD.

Based on these results we chose 18°C as a low intensity level, temperature at which algae grow; 22°C as medium intensity level, where algae starts to decrease, i.e. sub-lethal temperature and which coincides in turn with the maximum summer SST in the mid-Cantabrian sea, where *Fucus serratus* seems to be retracting (Viejo et al. 2011) and 26°C as the high intensity level that matches the lethal temperature of *Fucus serratus*.

Similarly variability levels were selected taking into account daily seawater variability found in rockpools in the North Portugal. Data collected in Viana do Castelo showed that daily temperatures may change up to 8 degrees in one single day (Figure 3). We decided than maximum variability of 8, i.e. +4 and -4 °C average temperature.

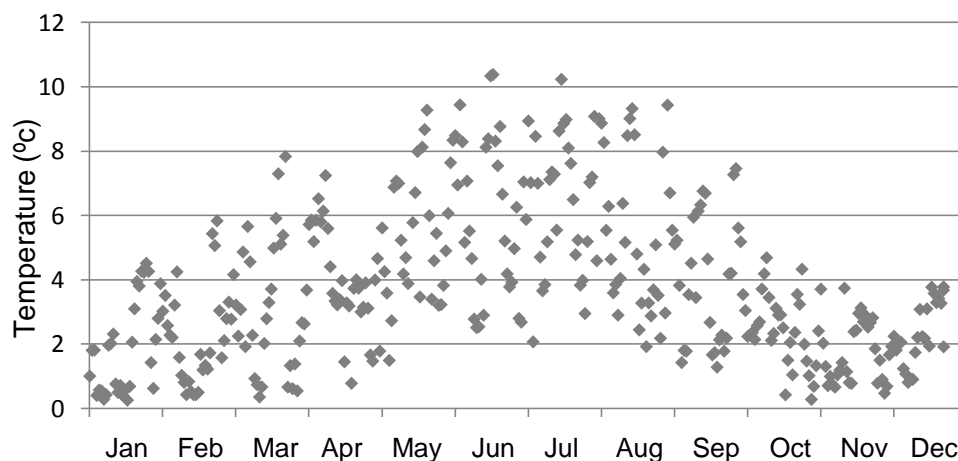


Figure 3. Daily temperature variation recorded on an intertidal rockpool at Praia Norte, Viana do Castelo during 2011.

Thermal variability experiment

The aim of the experiment was to assess the effects of mean intensity and thermal variability of water temperature extreme events on *F. serratus* physiological and growth responses. Experimental design consisted of a set of 3 units each simulated one of the three mean water temperatures (18, 22 and 26°C). In turn, each unit consisted of 6 white plastic chambers inside in which simulated individually one of three variability levels ($\delta = 0, 2$ or 4 °C) changing over a 4 days period (Fig 4). All treatments suffered 5 stress events. We used two replicated chambers for each combination of temperature treatments, i.e. 18 chambers (Fig 5). In each chamber ten fronds from *F. serratus* were placed during the experiment set on the terrace at CIIMAR and lasted 20 days.

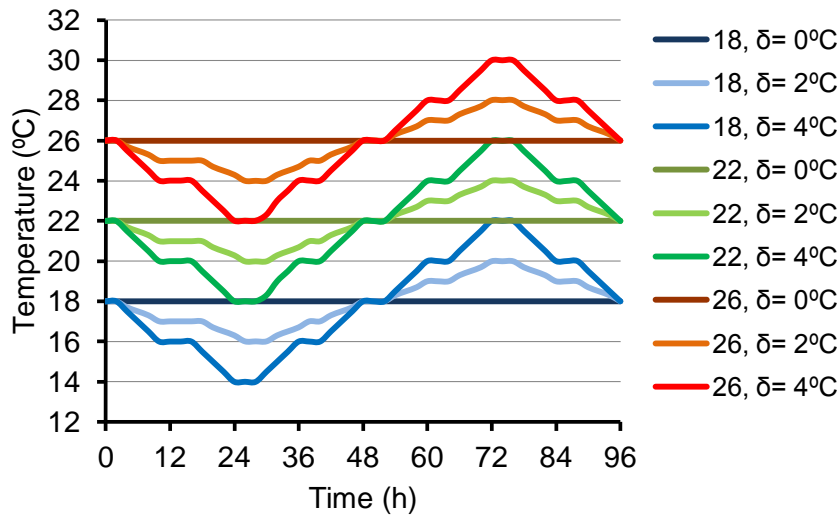


Figure 4. Temperature treatments diagram for a stress event (4 days).

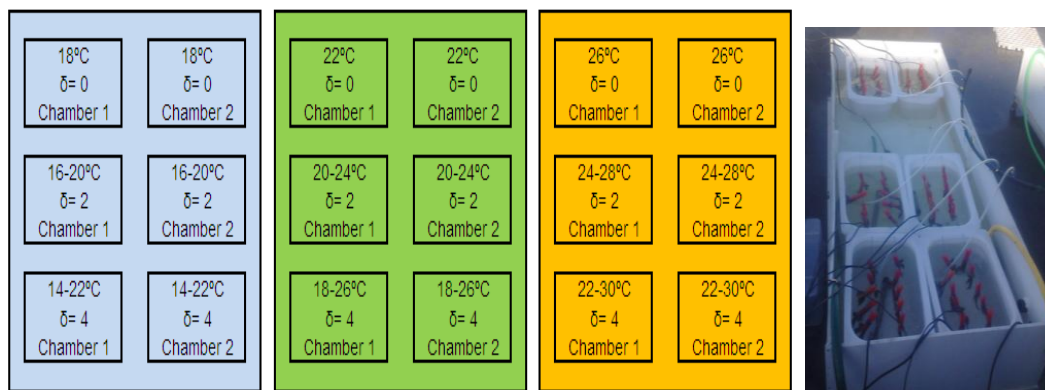


Figure 5. Representative units diagram with different mean temperature (18°C-blue, 22°C-green and 26°C-orange). Squares represent the 20 l chambers where fronds were submerged during the experiment. Temperature range reach and variability levels are shown (left). Detailed view of experimental unit with chambers submerged in a water bath system (right).

Seawater temperature was programmed and controlled using titanium heaters regulated by digital controllers and individual temperature probes (Aqua Medic® AT Control System controllers, GmbH, Bissendorf, Germany). This system allowed a continuous control and record of seawater temperature with a programmed error of 0.1°C. In order to ameliorate the effects caused by changes in air temperature and help heaters, chambers were submerged in a water bath system, set at the minimum temperature reached in each unit. Salinity was regularly monitored and chambers were refilled with freshwater to compensate for water evaporation every two days, mainly the

unit with higher temperature. Chambers were fitted within a constant aeration system to prevent stagnation and homogenize the experimental conditions.

Temporal variance experiment

The aim of the second experiment was to assess the effects of temporal variance of water temperature extreme events on *F. serratus* physiological and growth responses. Experimental design consisted of a set of 2 units at 22°C. Each simulated conditions of low (unit described above for the previous experiment) or high temporal variance (Fig. 6). High temporal variance unit consisted of 8 white plastic chambers where two different sequences were simulated, each with two different levels of variability ($\delta = 2$ or 4 °C) changing over a 2, 4 or 6 days periods (Table 1). Treatments suffered always 10 days of high stress conditions. We used two replicated chambers for each combination of temperature treatments, i.e. 12 chambers (Fig 6). In each chamber ten fronds from *F. serratus* were placed during the experiment set on the CIIMAR terrace that lasted 20 days.

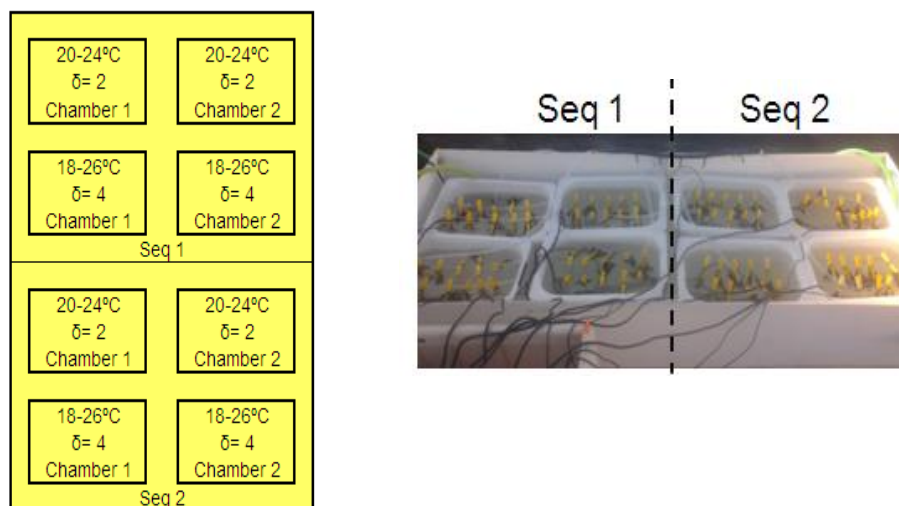


Figure 6. Representative unit diagram of high temporal variance at 22°C of temperature. Squares represent the 20 l chambers where fronds were submerged during the experiment. Temperature range reach and variability levels are shown for both sequences (left). Detailed view of experimental unit with chambers submerged in a water bath system (right).

Table 1. Distribution scheme of different stress events to low and high temporal variance treatments.

			Days																			
Temp. var.	Thermal var.	Seq	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Low	±2	1	20	20	24	24	20	20	24	24	20	20	24	24	20	20	24	24	20	20	24	24
Low	±4	1	18	18	26	26	18	18	26	26	18	18	26	26	18	18	26	26	18	18	26	26
High	±2	1	20	24	20	20	20	24	24	24	20	20	24	24	20	24	20	20	24	24	20	24
High	±4	1	18	26	18	18	18	26	26	26	18	18	26	26	18	26	18	18	26	26	18	26
High	±2	2	20	20	20	24	24	24	20	20	24	24	20	24	20	24	20	24	20	20	24	24
High	±4	2	18	18	18	26	26	26	18	18	26	26	18	26	18	26	18	26	18	18	26	26

3.3. Functional responses

Growth responses

Fronds were weighed at the beginning, in the middle (after 3 stress events, day 12 of experiment) and at the end (after the 10 days of stress events, day 20) of experiments. Fronds were weighed fully hydrated after dry it with absorbent paper to avoid possible errors associate with water excess, thus expressing growth as increase in fresh weight (FW) and determining the final size of the fronds (g). Growth was calculated as relative growth rate (Hoffmann & Pooter 2002):

$$RGR = \frac{\ln(W_2) - \ln(W_1)}{t_2 - t_1}$$

where W_2 is the fresh weight at time 2 (t_2) and W_1 is the fresh weight at time 1 (t_1)

Maximal quantum yield of photosynthesis (Fv/Fm)

To characterize the physiological status of the algae in response to the different treatments, we measured maximum quantum yield of photosynthesis (Fv/Fm) in darkness using a MiniPAM (Heinz Walz GmbH, Effeltrich, Germany) as an indicator of physiological stress following Maxwell & Johnson (2000).

To know the starting fronds conditions, an initial Fv/Fm measurement previous experiments (*predawn*) in darkness was performed. We repeated this process on days 5 and 12 of experiments to ensure that algae were alive. At the end of experiment, after 20 days, we perform rapid light curves (RLC) to determine the electron transport rate (ETR). RLCs are plots of ETRs versus actinic irradiances (red light), making P-I curves (Hill 1996, Figueroa et al. 2003). Actinic light was increased every 15 seconds by exposing the sample to 9 increasing irradiances from 2 to 186 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Withe & Critchley, 1999). ETR was calculated relating the effective quantum yield (Y II), which corresponds to the fluorescence of a frond not adapted to the darkness, and the irradiation intensity PAR according to the following modified formula from Schreiber et al. (1994):

$$ETR = Y(II) \cdot E \cdot A \cdot F_{II}$$

where, E is the incident actinic irradiance, A the absorptance and F_{II} is the fraction of chlorophyll associated to PSII being in brown algae 0.8 according to Grzymiski et al. (1997). Absorptance (A), is the fraction of light that is actually retained by a sample. It was calculated using the following equation (Beer et al. 2000):

$$A = 1 - \left(\frac{E_t}{E_0} \right)$$

where E_0 is the incident irradiance of PAR and E_t is the transmitted irradiance with the algae being located on the light sensor. We use as absorptance value of 0.956 ± 0.051 (mean \pm SE, n=90).

There are several models that relate the light intensity and the rate of photosynthesis, but not all describe the degree of photoinhibition. For this reason light curves were fitted according to the model Platt & Gallegos (1980) to obtain efficiency values (ETR, equation 1), maximum ETR (ETR max, equation 2) and saturation irradiance (E_k , equation 3).

$$ETR = P_s (1 - e^{(-\alpha E / P_s)}) \cdot e^{(\beta E / P_s)} \quad (\text{eq 1})$$

$$ETR \text{ max} = P_s (\alpha / (\alpha + \beta)) \cdot (\beta / (\beta + \alpha))^{\beta / \alpha} \quad (\text{eq 2})$$

$$E_k = ETR \text{ max} / \alpha \quad (\text{eq 3})$$

being α the maximal light utilization efficiency which coincides with the initial P-I curve slope and β the photoinhibition slope.

3.4. Statistical analysis

Growth response of fronds to the treatments was highly heterogeneous, namely in those with higher stress, resulting in large variance heterogeneity among treatments. Data transformation was unable to lower heterocedasticity preventing the use of traditional ANOVA approach. Thus to examine growth effect we used weighted mixed effects models with *mean temperature* and *thermal variability* as fixed predictors and *chamber* as a random predictor. To account for larger residual spread in highly stress treatments we include a variance covariate term in the mixed effect model (Zuur et al. 2009). The selection of the most appropriate term was done comparing the different available structures in R and comparing the resulting models with AIC (Zuur et al. 2009). To examine *a posteriori* differences among treatments we used Tukey HSD poshoc test. All analyses using mixed effects models were done with R (R Development Core Team 2013) using lme and lsmeans packages.

The effects of treatment on Fv/Fm (after 15 min and curve lights) were analysed using ANOVAs (Underwood 1997). The term *mean temperature* (optimum, 18°C; sublethal, 22°C and lethal, 26°C) was fixed factor; *thermal variability* (control, $\delta=0$; low, $\delta=2$ and high, $\delta=4$) was fixed and crossed with mean temperature and *chamber* (1 or 2) was included as a random factor nested in the interaction of both for first experiment. ANOVA were carried out with Statistica 10 (StatSoft Inc., Tulsa, OK, USA).

In return, second experiment was an asymmetric design, where *temporal variance* (low or high) was fixed factor; *sequence* (1 or 2) was random factor and repeated only with the highest level of temporal variance; *thermal variability* (low, $\delta=2$ and high, $\delta=4$) was fixed and crossed with sequence and *chamber* (1 or 2) was included as a random factor nested within the interaction of the rest. To analyse the data from this study, we used a method described in Underwood (1993) that consists of combining the sum of squares values from separate analyses of variance. We made two different ANOVAs. The first did not to distinguish between the temporal variability factor, i.e. it considered the design like symmetrical and with three different sequences (two of high temporal variability and one of low temporal variability). With this analysis we obtain the SS interaction “sequence x thermal variability” will be split in several components because

this term is always additive. The second ANOVA compared just the two high temporal variance sequences, obtaining the SS interaction "sequence x thermal variability" within sequences of high variance. Using these last analyses, we completed the first ANOVA and split the interaction "sequence x thermal variability" in two different sources of variation additive (sequence x thermal variability within high variance + sequence x thermal variability high vs. low temporal variance) (for more detail, see Glasby 1997). Analyses were carried out with GMAV (1997) statistical package (University of Sidney, Australia).

Homogeneity of variances for both experiments was tested using the Cochran's test (Underwood 1997). We considered $p < 0.05$ as threshold value when testing the significant differences in the analysis of variance, i.e. the null hypothesis was rejected at the 95% confidence level.

4. RESULTS

4.1. Thermal variability experiment

Growth

Thermal variability experiment showed that frond growth was clearly affected by the interaction of main seawater temperature and thermal variability ($F_{1,4} = 6.0421$; $p = 0.0001$ and $F_{1,4} = 7.5774$; $p < 0.0001$; growth at 12 and 20 days respectively) (Table 2). Fronds growth followed the same pattern throughout the experiment, that is, data obtained after 12 and 20 days of experiment were similar. As expected, fronds of treatments at 18 °C grow slightly more than those found at 22 °C reaching values of $0.030 \pm 0.001\text{g}$ and $0.024 \pm 0.001\text{g}$ (mean \pm SE, $n = 20$) respectively at the end of the experiment. However, fronds in treatments at 26 °C (lethal temperature) showed a decrease, $-0.019 \pm 0.007\text{g}$ (mean \pm SE, $n = 20$), i.e. lost tissue, and were dead (approx. 50% survival rate, compared with 100% in other temperature treatments). These results are in agreement with those obtained in a previous experiment, where growth fronds were tested at different seawater temperatures recorded lower growth at 18 and 22 °C ($0.022 \pm 0.001\text{g}$ and $0.021 \pm 0.001\text{g}$, mean \pm SE, $n = 20$, respectively) and higher at 26 °C ($0.0002 \pm 0.001\text{g}$) of temperature than in our experiment (data not shown). It was observed that increased thermal variability made that the effects

caused by a higher or lethal temperature being ameliorated (Fig. 7). Chamber was consistently significant, suggesting some environmental heterogeneity of the experimental prototypes.

Table 2. Growth model summary for the significance of predictors of weighted mixed effect models for the growth at 12 days and 20 days in *Fucus serratus*. Predictors included mean temperature and thermal variability.

Variables	df	Growth			
		12 days		20 days	
		F	p	F	P
Intercept	1	1508.63	<0.0001	1795.81	<0.0001
Mean Temp -T	2	247.1389	<0.0001	215.3919	<0.0001
Thermal Var. - Th v	2	1.6194	0.3334	1.0775	0.4439
Chamber - C	1	1508.631	<0.0001	1785.813	<0.0001
T x Th v	4	6.0421	0.0001	7.5774	<0.0001
Residual	168				

Significant differences at $\alpha < 0.05$ are shown in bold.

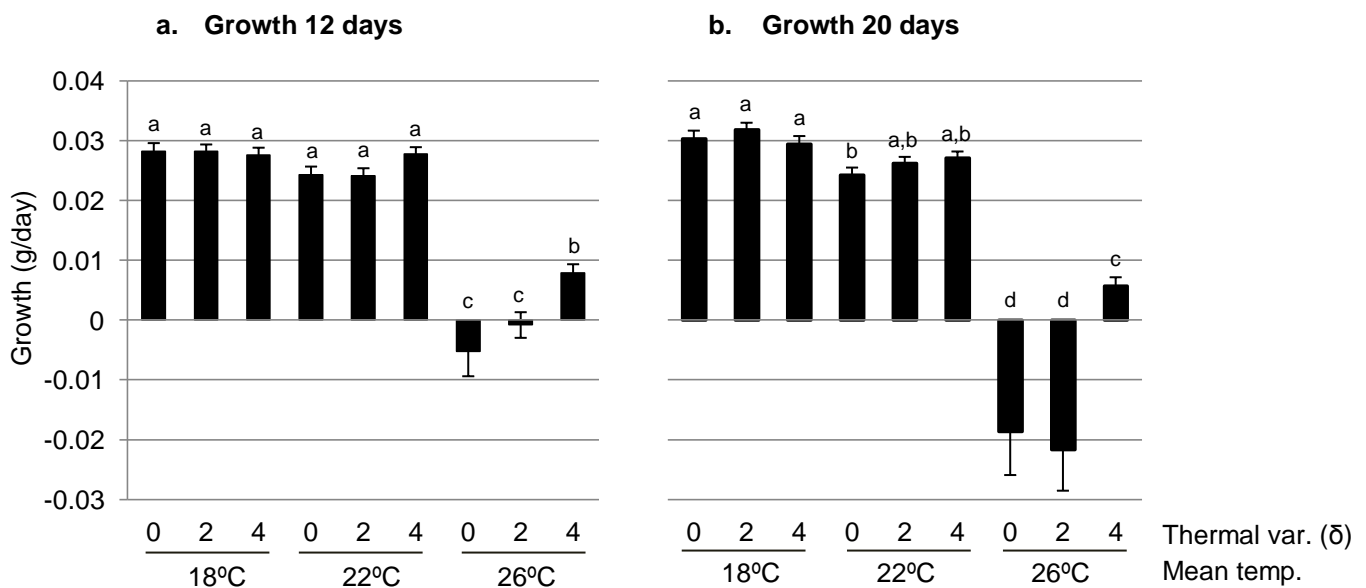


Figure 7. Averaged *growth* (mean \pm SE, n = 20) in thermal variability experiment. a) Represent values to 12 days growth and b) values to 20 days growth. Means with a common letter do not differ significantly based on Tukey HSD tests at p = 0.05 level.

Fv/Fm and RLC.

Values of F_v/F_m at *predawn* (before the experiment) and after 5 days of experiment were high, about 0.777 ± 0.004 , (mean \pm SE, $n=64$) and 0.713 ± 0.009 (mean \pm SE, $n=72$), respectively, suggesting optimal photosynthetic performance before start and at the beginning of the experiment and without trends noticeable. F_v / F_m values after 12 days were also high in general, around 0.716 ± 0.009 (mean \pm SE, $n=72$), however appeared statistically significance differences between high temperature treatment (0.635 ± 0.027 , mean \pm SE, $n=24$) and low and medium temperature treatment (0.759 ± 0.005 and 0.755 ± 0.003 , mean \pm SE, $n=24$, respectively).

The effects of mean temperature and thermal variability in terms of the photosynthesis electron transport were determined using the curves ETR vs. Irradiance (PI) as we discussed previously. Figure 8 shows a detailed summary of the photosynthetic parameters obtained from these curves. Visually nor treatment effects or trends were found in these physiological parameters. Also, no statistically significant differences were found in the analysis of variance (Table 3) for photosynthetic parameters except for photoinhibition rate (β) which showed a significant mean temperature \times thermal variability interaction ($F_{4, 54} = 8.465$, $p = 0.004$). This interaction shows that in the control treatment ($\delta=0$) photoinhibition rate shows a slight increase as we increase the mean temperature of the treatments. However, treatments with low thermal variability ($\delta=2$) shows that the rate of photoinhibition decreases drastically as we increase the mean temperature, reaching the maximum values at 18 °C and the minimum at 26 °C. Finally, the high thermal variability treatments ($\delta=4$) show very similar values of photoinhibition rate at 18 °C and 26 °C, significantly lower than values obtained for 22°C. Noteworthy that there are practically no differences in values obtained at 22 °C for all thermal variability (Figure 8.d). No significant differences in means based on SNK test at $p = 0.05$ level were observed for this interaction.

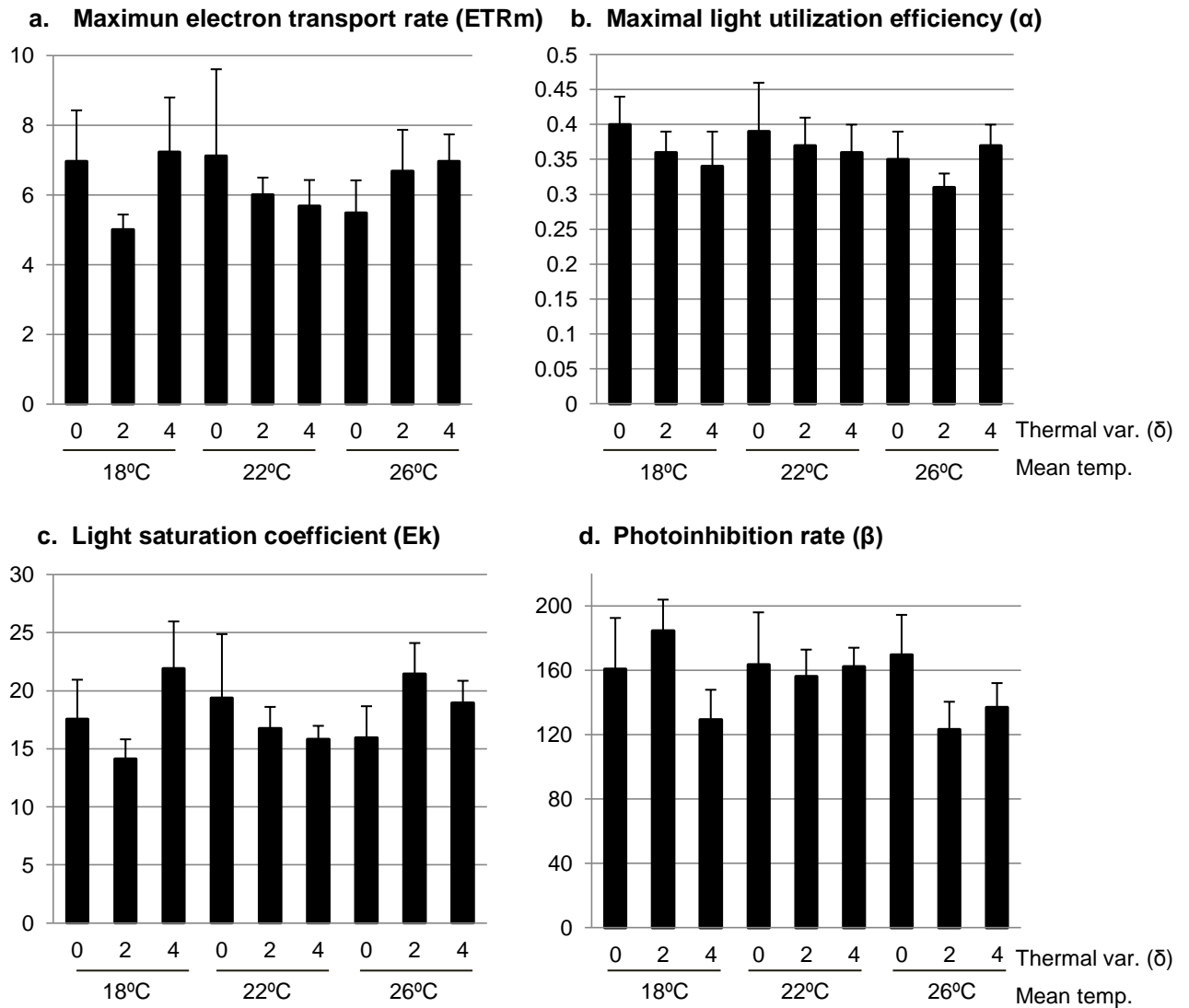


Figure 8. Photosynthetic parameters determined from ETR vs. Irradiance (PI), namely a) ETR_m (μmol e⁻ m⁻² s⁻¹), b) α [(μmol e⁻ m⁻² s⁻¹) (μmol m⁻² s⁻¹)⁻¹], c) E_k (μmol m⁻² s⁻¹) and d) β [(μmol e⁻ m⁻² s⁻¹) (μmol m⁻² s⁻¹)⁻¹] for the different treatments in thermal variability experiment. Mean ± SE (n= 20).

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Table 3. ANOVA of photosynthetic parameters determined from ETR vs. Irradiance (PI), namely ETRm, α , Ek and β for the different treatments in thermal variability experiment.

Variables	df	Fv/Fm											
		ETRm			α			Ek			β		
		MS	F	p	MS	F	p	MS	F	p	MS	F	p
Mean Temp -T	2	0,119	0,016	0,984	0,006	0,197	0,825	13,526	0,487	0,630	2130,149	4,525	0,044
Thermal Var. - Th v	2	3,692	0,508	0,618	0,008	0,263	0,774	15,091	0,543	0,599	2820,243	5,990	0,022
Chamber - C	9	7,263	1,087	0,388	0,031	3,478	0,002	27,770	0,568	0,817	470,795	0,219	0,990
T x Th v	4	8,749	1,205	0,373	0,005	0,165	0,951	96,937	3,491	0,055	3985,500	8,465	0,004
Residual	54												

Significant differences at $\alpha < 0.05$ are shown in bold.

4.2. Temporal variance experiment

Growth

In our second experience, i.e. the temporal variance experiment, we expected that *F. serratus* growth will show increased values at high thermal variability following the pattern of previous experiment at 22°C. The ANOVA performed with growth data showed a significant sequence x thermal variability within both treatment sequences with high temporal variance interaction ($F_{2, 108} = 13.791$, $p = 0.010$; growth at 12 days) and most important, the same interaction, sequence x thermal variability of low temporal variance versus high temporal variance interaction ($F_{1, 108} = 12.706$, $p = 0.012$; growth at 12 days) (Table 4, Fig 9). These significant differences between sequences and thermal variability were mitigated at the end of the experiment, no significant differences were found at 20 days (Table 4). Although it is noted that algae from sequence 1 in high temporal variance treatment grew better than those of sequence 2, which could be due to the distribution of different stress events and their duration because may not have the same effects stress events lasting six days in which the temperature change due to the thermal variability becomes more progressive than in stress events lasting two days in which temperature changes are more drastic and pronounced. Chamber was significant too in both periods, suggesting some environmental heterogeneity of the experimental prototypes.

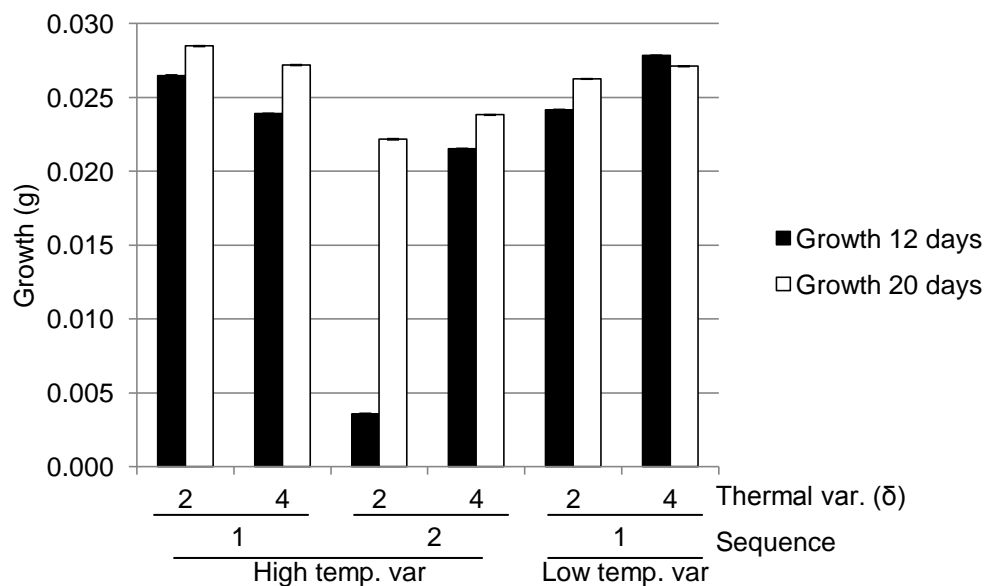


Figure 9. Averaged *growth* (mean \pm SE, $n = 20$) in temporal variance experiment. *Open bars* represent values to 12 days growth and *filled bars* correspond to 20 days growth.

Table 4. ANOVAs of effect of thermal and temporal variance with different sequences on growth at 12 days and 20 days in *Fucus serratus*.

Variables	df	Growth							
		12 days				20 days			
		SS	MS	F	p	SS	MS	F	p
Sequence - S	2	0.455	0.227	21.050	0.002	0.051	0.026	1.660	0.267
Thermal Var. -									
Th v	1	0.121	0.121	1.090	0.406	0.001	0.001	0.220	0.687
Chamber - C	6	0.065	0.011	5.460	<0.001	0.092	0.015	10.160	<0.001
S x Th v	2	0.222	0.111	10.270	0.012	0.005	0.002	0.150	0.864
S x Th v within									
high temporal variance	1	0.211	0.211	13.791	0.010	0.004	0.004	0.200	0.670
S x Th v low vs. high temporal variance	1	0.011	0.011	12.706	0.012	<0.001	<0.001	0.188	0.680
Residual	108*	0.214	0.002			0.214	0.002		

Significant differences at $\alpha < 0.05$ are shown in bold. * To achieve homogeneity of variances, 2 outliers were replaced by the mean of the group in growth at 12 and at 20 days ($df = 104$).

Fv/Fm and RLC.

Values of *predawn* and *Fv/Fm* after 5 days of experiment were high, about 0.777 ± 0.004 , (mean \pm SE, $n = 64$) and 0.732 ± 0.037 (mean \pm SE, $n = 48$), respectively, suggesting optimal photosynthetic performance before start and at the beginning of the experiment. Statistically significant differences were observed in the thermal variability x sequence interaction at 5 days, where *Fv/Fm* values of sequence 2 from the high temporal variance treatment were much lower than the rest (data not shown). Values of *Fv/Fm* at 12 days were a bit higher, around 0.748 ± 0.003 (mean \pm SE, $n = 48$). Even some smaller values emerged, no trends were noticeable for this time.

The best and worst fronds light adapted state according to the values obtained from RLC photosynthetic parameters were those from sequence 2 of high temporal variance and high ($\delta = 4$) and low ($\delta = 2$) thermal variability treatment, respectively. That is, high thermal variability treatment recorded the highest values of *ETR_m* (9.965 ± 1.173), α

(0.439 ± 0.047) and E_k (24.630 ± 2.274 , mean \pm SE, $n = 20$) parameters and the minimum of β parameter (116.010 ± 25.052 , mean \pm SE, $n = 20$). In contrast, low thermal variability treatment recorded the minimum values of these parameters, ETRm (4.000 ± 0.545), α (0.265 ± 0.021) and E_k (14.925 ± 1.325 , mean \pm SE, $n = 20$) and the second maximum value of β parameter (174.601 ± 15.712 , mean \pm SE, $n = 20$) (Figure 10). These results were partially confirmed with the ANOVA performed on photosynthetic parameters. These analyses showed a significant sequence \times thermal variability within both treatment sequences with high temporal variance interaction for ETR m ($F_{1, 36} = 6.405$, $p = 0.045$) and α ($F_{1, 36} = 26.231$, $p = 0.002$). A priori test confirmed that differences in sequences in the high variability treatment were responsible for these interactions. Chamber was significant too in some analysis, suggesting some environmental heterogeneity of the experimental prototypes (Table 5).

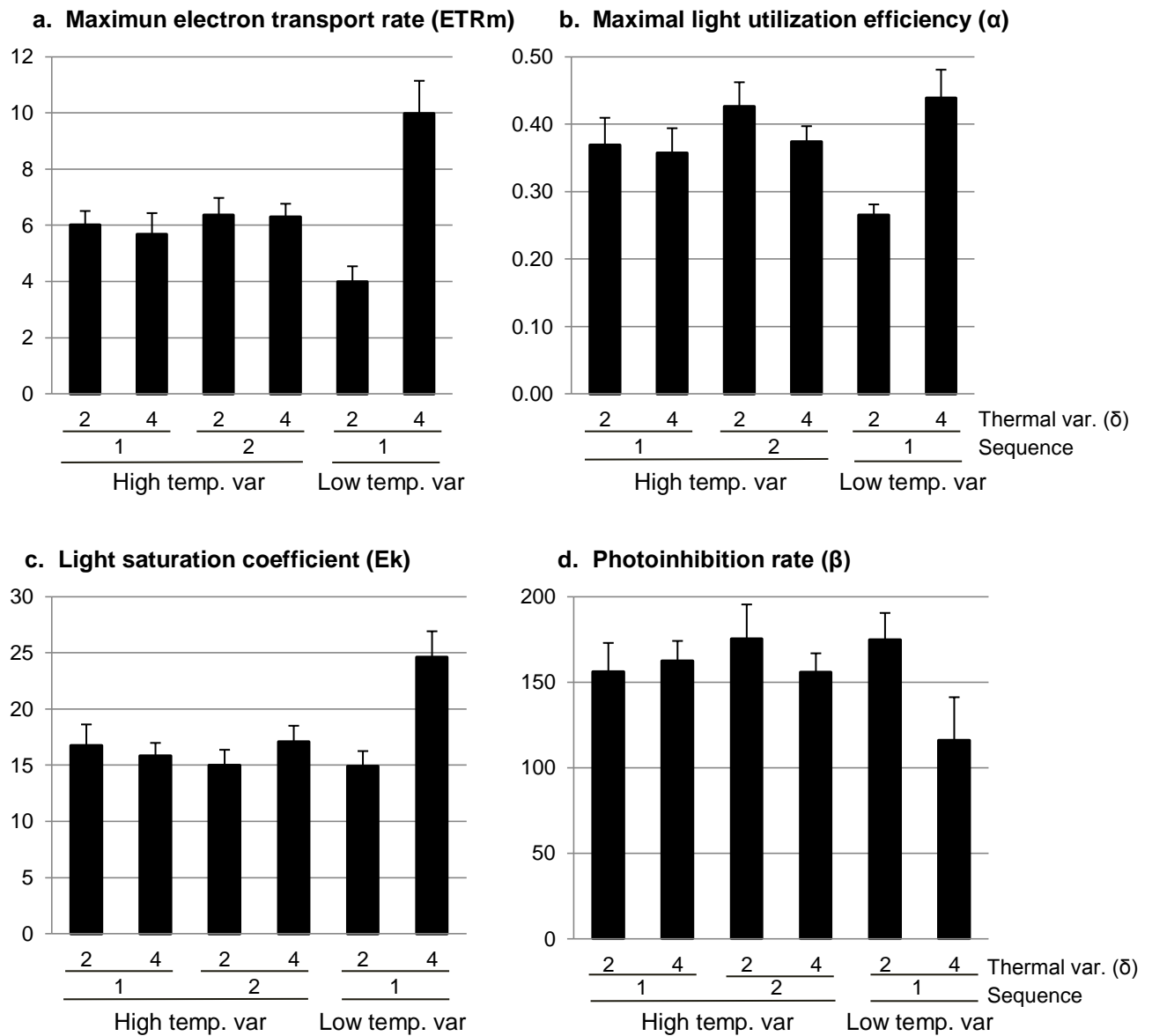


Figure 10. Photosynthetic parameters determined from ETR vs. Irradiance (PI), namely a) ETR_m (μmol e- m-2 s-1), b) α [(μmol e- m-2 s-1) (μmol m-2 s-1)-1], c) E_k (μmol m-2 s-1) and d) β [(μmol e- m-2 s-1) (μmol m-2 s-1)-1] for the different treatments in temporal variance experiment. Mean ± SE (n= 20).

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Table 5. ANOVA of photosynthetic parameters determined from ETR vs. Irradiance (PI), namely ETRm, α , Ek and β for the different treatments in temporal variance experiment.

Variables	df	Fv/Fm															
		ETRm				α				Ek				β			
		SS	MS	F	p	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Sequence - S	2	10.380	5.190	0.630	0.563	0.020	0.010	3.250	0.111	139.816	69.908	1.190	0.368	3442.615	1721.308	0.79	0.495
Thermal Var. - Th v	1	41.296	41.296	0.810	0.462	0.017	0.017	0.290	0.646	157.351	157.351	1.310	0.371	6881.811	6881.811	1.62	0.331
Chamber - C	6	49.221	8.204	3.750	0.005	0.019	0.003	0.650	0.688	353.579	58.930	4.930	0.001	13013.183	2168.863	1.69	0.151
S x Th v	2	101.576	50.788	6.190	0.035	0.116	0.058	18.860	0.003	240.286	120.143	2.040	0.211	8513.759	4256.880	1.69	0.221
S x Th v within high temporal variance	1	72.838	72.838	6.405	0.045	0.102	0.102	26.231	0.002	116.140	116.140	1.377	0.285	3074.970	3074.970	1.00	0.356
S x Th v low vs. high temporal variance	1	28.738	28.738	0.032	0.863	0.014	0.014	0.048	0.834	124.146	124.146	0.033	0.862	5438.789	5438.789	0.032	0.863
Residual	36																

Significant differences at $\alpha < 0.05$ are shown in bold.

5. DISCUSSION

Our experiment aimed to examine how thermal variability, both intensity variability and temporal variability of seawater temperature affects the growth and photosynthetic performance of the brown seaweed *Fucus serratus* from populations close to its southernmost European limit.

The most remarkable result of the first experiment on thermal variability was the big fronds' resilience to changes when the variability was high, since the algae at 26 °C with high variability treatment responded better to the stress than those with low variability, despite reaching values as high as 30 °C, that was never recorded in the field. It is also very important the existence of an interaction between of mean water temperature and thermal variability in the growth and β . This interaction result in a positive effect on growth of thermal variability under the harshest temperature conditions, suggesting that high thermal variability buffers negative effects of high temperature mainly for the growth rate and for β . None of the other photosynthetic parameters measured (ETR_m, alpha and E_k) exhibited differences were able to discriminate any of our treatments (with the exception of chamber which was a significant factor for alpha). Probably intertidal seaweed like *Fucus serratus* which suffer daily emersion-immersion periods with large changes on environmental factors like temperature, irradiance, wind, etc have their photosynthetic apparatus highly adapted to cope environmental stress and thus differences did not emerged during our experiment. Only photo-inhibition rates were clearly lower at the higher temperatures and high thermal variability.

Unlike thermal variability, in the experiment where temporal variance of thermal stress events were modified results were ambiguous and not consistent thought time, maybe because treatments were not enough variables to detect their possible effects due to the great resilience of the specie to changes. Higher temporal variability did not have positive or negative effects on fronds growth or photosynthetic capacities, but rather responses depended on the stress events distribution, i.e. sequence. Distribution of stress events seems to be very relevant because several consecutive events stress of short duration can slow the growth and resilience of species, as it causes more sudden and drastic changes in temperature that if the same change is performed in events stress of longer duration, i.e. cannot have the same effect a thermal variation of $\delta = 2$ in an event stress of 2, 4 or 6 days. The capacity to survive short and repeated periods

of extreme conditions is another aspect to consider (Bertocci et al. 2005, Overgaard et al. 2014). Our results suggest that a high temporal variability reduces the growth rate and fitness and therefore its ability to recovery or acclimation after several consecutive short-term stress events, as in sequence 2.

It is also interesting to highlight that growth rates in both experiments were similar to those suggested from the functional response to temperature in previous experiment that we use to define our treatment temperatures. That is, if we consider the average temperatures of our treatments (18, 22 or 26 ° C) and compare these growth rates with those obtained at the same temperatures in the previous experiment (Arrontes 1993, Chapman 1995, 2002, Martínez et al. 2012), we observed that the values are similar, although in small increments or decreases due to different thermal or temporal variability treatments. This suggested some predictability of species from the average temperature conditions in variables environmental systems.

Despite the growing amount of research exploring the consequences of global warming on marine ecological systems, few studies have focused on stressors variability or extreme effects. In fact, most of the work have examined the impact of increases or decreases in average intensity of stressors. This is the case of seaweeds, where most of ecophysiological studies focused on responses to changes in the average intensity of stressors (Pearson et al 2009) and their potential interactions (e.g., Martinez et al. 2012, Ferreira et al. 2014). In fact, the only research focused on the role of variability of stressors on macroalgal assemblages aimed to understand the impact of temporal variance of physical disturbance at community level responses like changes in assemblages structure or stability (Bertocci et al. 2005, 2007, Benedetti-Cechi et al. 2006, Vaselli et al. 2008). Only a recent study tried to examine the interaction between temporal variability of stressors and its intensity (Trilla 2013).

To our knowledge, no manipulative experiments have been carried out concerning a thermal variability on macroalgae, despite being a very important and novel subject in other research fields, like on insect's research (Lalouette et al. 2007, Engelbrecht et al. 2010, Folgueira et al. 2011, Williams et al. 2012, Bozinovic et al. 2011). In these studies with insects, it was found that an increase in environmental variability may have both, positive or negative effects on fitness and growth species populations. Recent models in insects indicate that thermal variance could have as much (or more) of an impact on fitness as does the mean temperature, since an increase in thermal variability when mean temperature is close to the optimal, impair performance while if

temperature is far from the optimal can improve growth and fitness population (Bozinovic et al. 2011). In particular, Terblanche et al. (2010), using a similar approach to our experiment, and Bozinovic et al. (2011) found that a large variability could limit thermal plasticity responses, thus reducing the fitness of flies, their target specie. They proposed that at low mean temperatures, the critical thermal minimum of the species, i.e. the minimum sublethal temperature, was slightly higher with a greater thermal variability. Similarly, at high mean temperatures, greater thermal variability increased the critical thermal maximum. Those results suggest that at low mean temperatures increased thermal variability had a negative effect, whereas at higher temperatures mean increasing the variability had a positive effect on the fitness species. They found that for the low thermal variability scenarios responses of acclimatization for most of their parameters measured were typical, however for high variability scenarios poorer resistance to climatic stress in some but not all parameters, was detected. Authors suggest that this response may be related to heat shock protein production (Kalosaka et al. 2009), enhanced by the heat shock response found in all living organisms, which offers an effective defense against exposure to adverse environments (Lindquist 1986). These results have some similarities to our thermal variability results. We found the expected effects of mean temperature and more interestingly under the most extreme experimental temperatures, greater thermal variability had a positive effect on growth, i.e. *Fucus serratus* grew better with high thermal variability ($\delta = 4$) than with low ($\delta = 2$) or no thermal variability ($\delta = 0$) at high temperatures. All this could be related to increase thermal variability in high temperatures treatments might cause the increase of critical thermal maximum, slowing down its decrease or tissue lost as occurred in 26 °C treatments.

The mechanisms behind this paradoxical results required more research and could be involved in the current distributional patterns of *Fucus serratus* in the Iberian Peninsula. Higher temperature variability under extreme conditions (i.e 26 °C on average) meant that fronds endured temporary the highest temperature events (i.e 30 °C) but also the lowest temperature conditions (i.e. 22 °C) of the whole set of treatments with mean temperature of 26 °C. The current area of distribution of *Fucus serratus* in Portugal (around Viana do Castelo) has in terms of mean seawater temperatures values similar to other areas in the Cantabrian Sea where there are no longer *F. serratus* populations. However coastal summer seawater temperatures are among the lowest of the whole Atlantic Iberian shores due to the persistent upwelling events from March to November

(Cacabelos 2013). These cooling events may well enable seaweeds to recover from very extreme emersion stress and reduce the impacts of increasing air temperatures.

The current and future climate change scenarios forecast an increase in the intensity and variability in thermal and temporal stress events (Easterling et al. 2000, McGregor et al. 2005, Meehl et al. 2007). In particular, up to 4 °C increase in water temperature at the end of the 21st century (Müller et al. 2009) and a higher frequency, between 5 and 10 times more of heat waves over the next 40 years (Schär et al. 2004, Barriopedro et al. 2011) is anticipated along North-Atlantic shores. Species respond to these changes with phenological changes and in their distributional ranges harboring local extinctions such as *Fucus serratus* in the north of the peninsula (Viejo et al. 2011). In order to better understand the possible species responses to this variability impact and their demographic consequences it is important to investigate and understand their phenotypic plasticity and adaptive evolvability, that in *Fucus serratus* is quite small (Bijlsma & Loeschcke 2012) because their flow and genetic diversity is low (Coyer et al. 2003, Hampe & Petit 2005, Pearson et al. 2009), since it is a perirical and isolated population after suffering the last glaciation (Hoarau et al 2007).

In conclusion, our results revealed interactive effects of mean intensity and both thermal and temporal variability of seawater stress events on *Fucus serratus* growth and physiological response. This interaction suggests that high thermal variability buffers negative effects of high temperature mainly for the growth rate and for β , although mechanism driving these responses remains still unknown. So, to predict responses to climate change, future work may take into account the patterns of thermal variation and the mechanism by which seaweed cope with this variation, i.e. their species' plasticity and acclimation.

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