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Temperature Tolerance Test Exposition with Temperate Sea Anemone *Actinia equina*, a Climatic and Environmental Changes Simulation

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Abstract

Atlantic and Mediterranean warming-related diseases outbreaks and species shifts recently have been documented. Evaluated tools of short-term effects on the health or organisms resistance are necessary to assess and understand mechanisms affecting marine biodiversity. Until now, climate warming has been studied at the population or community level. Here we offer a better understanding of such phenomena at the individual organism level, using anatomic-morphological approaches to interpret effects of natural physical stressors, according to behavioral patterns. The goal of this work was to evaluate the sea anemones behavior with temperature variance. This study takes a method of behavioral observations (morphological and anatomic parameters, with physiological implications) to identify changes in behavior, after exposure to the physical stressors temperature (10˚C, 15˚C, 20˚C, 25˚C and 30˚C) on temperate sea anemone *Actinia equina* over 96 h of exposure. Other endpoints as condition index and reproduction also assessed. Behavioral patterns analysis placed the differentially ecological functions in a wide range of categories including tentacle flexion, tentacle retraction, column cavitation, peristome depression and oral disc flexion. These parameters suggest that the "early stress response" (before result on individual death) to elevated temperature involves essentially all aspects of same chemical reactions. In this case we observed receptors functioning and the frequency of open-close oral sea anemones, tentacles and columns anatomic alterations to detect earlier the ef-

fects of physical stress induction. The superiority of results tested was that the key species reacted to different temperature ranges in order to demonstrate that species from different climatic zones could have the same behavioral pattern but have intrinsic adaptations on each climatic zone. Also some collections of parameters such as: 1) water nutrients availability, 2) reproductions rate (number of polyps), 3) survival (condition index) and 4) temperature variations were significant on behavioral answers.

Subject Areas

Animal Behavior, Marine Biology

Keywords

Temperature Tolerance, Behavior, Early Warning, Climatic Changes

1. Introduction

The coral reefs community is very rich on biodiversity. Many studies reveal that organism inhabits the reefs that are very sensitive to environmental alterations. It has been over 10 years since the phenomenon of extensive coral bleaching was first described. In most cases, bleaching has been attributed to elevated temperature, but other instances involving high solar irradiance, and sometimes disease, have also been documented.

Massive bleaching events are becoming an increasingly important cause of mortality and reef degradation on a global scale, linked by many to global climate changes. Previous studies assessing the effects of bleaching on natural populations and laboratorial exposures have investigated the effect of environmental and physical stressors, such as temperature and UV (ultraviolet) radiation, on symbiotic algae (zooxanthallae) [1].

The present study focused only on responses of sea anemones to the environmental stress (temperature variance). The increase on temperatures affects photosynthetic performance, resulting in a zooxanthellae release, as well the bleaching in sea anemones caused by oxidative stress [1] [2] [3] [4].

In addition, Programmed Cell Death (PCD) and necrosis found to occur simultaneously in both host tissues and zooxanthellae [5]. Moreover, these effects depend on the temperature and duration [5].

Low temperatures can also have severe effects on anemones which depend on the temperature and duration [6]. Decreased temperatures suggested to cause exocytosis of zooxanthellae [6] [7].

However, the behavior responses of anemones to temperature-induced bleaching remain largely unknown. In this study, the behavior responses of a temperate sea anemone were assessed using criteria that had previously been validated for sea anemones as indicators of environmental and climatic changes [8].

Anatomic and morphological parameters of physiological alterations were assessed, namely tentacles flexion, retraction, oral disc flexion, column cavitation

and peristome depression. Researchers studied behavioral reactions of the sea anemone *Anthopleura xanthogrammica* to ultraviolet and visible radiations, for the first time [9]. Each behavioral endpoint has a physiological implication and ecological interpretation. It is important to study the response of a wide variety of anemones to temperature stress, because anemones can have different responses to temperature, even if they are genetically similar, as shown by [10]. These authors reported that anemones from southern and northern California (USA), despite being genetically similar (electrophoretic method), had different oxygen consumption patterns in response to acclimation and acute changes in temperature. The two populations also differed in the extent of metabolic compensation to temperature following several weeks of acclimation [10]. In this study, we used the temperate sea anemone *Actinia equina*, collected in the northwest of Portugal, near the border with Spain. This sea anemone is a cosmopolitan species, very common on the Iberian coast and possesses physiological adaptations to body water balance [11]. Portugal is the southern geographical limit for many boreal species and the northern or western limit of subtropical and Mediterranean species [12].

On contrary, a few works that were developed were related to environmental stressors affecting sea anemones directly. In fact, temperature variations consist in an important key to understand marine and environmental coastal alterations. Sea anemones and another cnidarians groups (such as corals), are a good vehicle to study and understand the natural processes and environmental alterations (global temperature increase). A recent and innovative goal was to try anticipating these alterations and to create a database to prevent and predict the environmental and climatic changes before the damage being irreversible.

Recently the development and application of BEWS (Biological Early Warning Systems) have been reviewed using various groups of organisms (such as bacteria, algae, cladocerans, bivalve and fish) and computational methods to process the behavioral monitoring data [13]. Thus, in this work we assessed the temperature tolerance of *Actinia equina* to assess whether it is or not a suitable species for BEWS. The hypothesis tested was that key species react to different temperature ranges in order to demonstrate that species from different climatic zones could have the same behavioral pattern but have intrinsic adaptations on each climatic zone. Based on these affirmations, the sea anemones could use as an early environmental signalizing to climatic changes. The present study intends to add a new species to environmental bioindicators, using a benthonic and cosmopolitan key-species: *Actinia equina*.

The goal of this work was to investigate behavioral responses of sea anemones to the occurrence range of temperature of these species, showing a temperature/time dependence. The following behavioral parameters assessed: tentacle flexion, tentacle retraction, oral disc flexion, column cavitation, and peristome depression, have ecological interpretations and physiological implications. We simulated an induced temperature stress using sea anemones, considering the concern worldwide with coastal areas alterations and the effects of environmental

changes. We will study physical, toxicological and ecological factors involved on organism damages, to understand the regulating mechanisms of low richness species caused by environmental alterations.

2. Material and Methods

2.1. Sampling Site

Based on sea anemones distributions, the sampling site were chosen along the Portuguese coast. The site is located in the NW Portuguese coast, specifically in Vila Praia de Âncora (41˚49'13.54"N and 8˚52'26.05"W). This site is situated near small fishery villages and far from big population aggregates and potential sources of contamination (agricultural, urban and harbor). Several studies performed in the Portuguese coast indicate this site as relatively undisturbed by anthropogenic pressures and it been used as a reference site in previous studies [14].

2.2. Animal Sampling

The Taxonomical identification of anemones performed using the Handbook of the Marine Fauna of the North-West Europe [15]. It was collected *Actinia equina* specimens by hand and with the aid of mussel shells [16] during low tide, avoiding collect individuals with similarly coloration in the vicinity, since these could be clones [17]. The organisms collected had 1 - 3 cm in length. One hundred specimens were collected and transported for the lab facilities in buckets with oxygenated seawater.

The following abiotic factors were measured "*in situ*", in the rainy season in the year of 2012: salinity ($g \cdot L^{-1}$) and conductivity ($mS \cdot cm^{-1}$) (Wissenschaftlich Technische Werkstätten, LF 330 meter, Brüssel, Belgium), dissolved oxygen (percentage) saturation (Wissenschaftlich Technische Werkstätten Cell Ox 325) and pH (Wissenschaftlich Technische Werkstätten 537 meter). The abiotic factors measured in order to reproduce the same conditions during the experiments.

2.3. Acclimation in a Flow-Through System and Experimental Design

After arrival to the lab, anemones were transferred to aquaria of 2.5 L with artificial seawater in a flow-through system for depuration. The following conditions were kept: temperature (20˚C ± 1.8˚C), salinity (32 ± 0.3 $g \cdot L^{-1}$), pH (7.74 ± 0.16), dissolved oxygen (84.14% ± 6.33%) and photoperiod 16h light: 8h dark. Artificial seawater was prepared with MilliQ® complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) and pH adjusted. The sea anemones were kept at 20˚C since it is the recommended temperature for temperate scenarios [18]. They were fed every three days with "diet fish feed", a standard diet with a low lipid and protein concentration, to avoid the excessive Ammonia accumulation. The organisms were kept under these conditions during two weeks, for acclimatization [19].

During this period, water parameters, polyps survival and reproduction rate were checked regularly, only four individuals were kept in each aquarium. For these species, a specific procedure was followed in two steps: first an acclimatization and second, an acclimation, that were separated on 2 weeks and then more 4 weeks, respectively, under lab conditions [20] [21]. The second step called: "debugging" that consisted in a depuration time with a biological filter. The biological filter consisted in a mixture of natural seawater, including microorganisms, which introduced in the flow-through system, that improve the water quality. Figure 1 illustrates the flow-through system. After the depuration time, the organisms were ready to the exposure procedures (temperature tolerance). The sea anemone viability, growth, behavior and the mortality were analyzed to answer the temperature variations. Table 1 illustrates all controlled conditions during the acclimation.

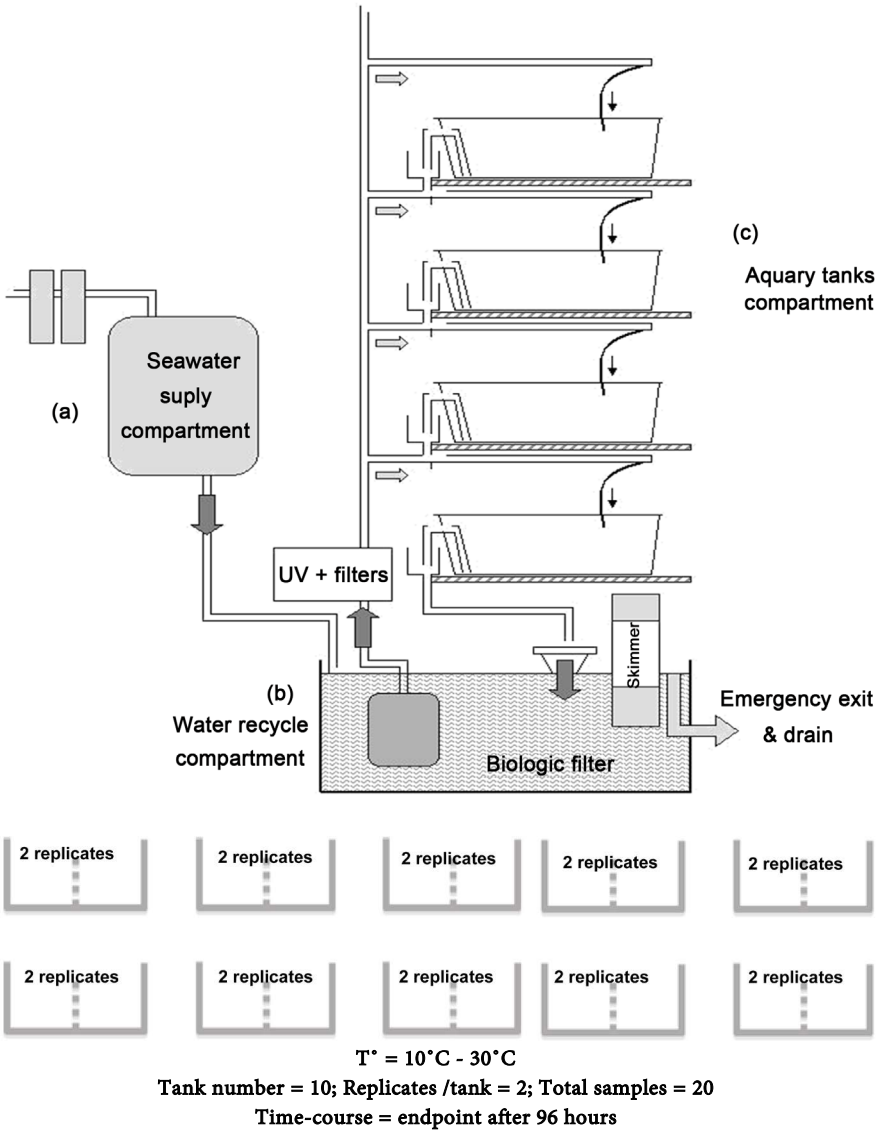


Figure 1. The flow-through system used to acclimatizing the sea anemones Actinia equine and experimental design (Abreu, S.N).

Table 1. Acclimation conditions previous the physical stressors exposition tests.

Parameters	Conditions
System	Continuously
Salinity	32 ± 0.29
Temperature	20 ± 1.75
pH	7.74 ± 0.16
Dissolved Oxygen (%)	84.14 ± 6.33
Photoperiod	16:8 (Artificial light)
Type of Aquarian	Glass, transparent, with cover
Type of Water	Artificial seawater (MiliQ® with marine salt)
Water Volume	2.5 L (each Aquarian)
Water change	Continuously
Aeration	Continuously
N° of *Actinia equina (adult)*	100
N° of *Actinia equina*	100
Number of Replicates (each Aquarian)	*2*
Diet	Each 3 days*
Monitoring	Daily (each 6 hours)
***Feed diet fish**	

*Artificial Food composition: Protein = 55.00%, Oil = 14%, Ash = 12%, Fibre = 1%, Vit A = 30,000 i.u/Kg, Vit D3 = 2500 I.U/Kg, Vit E = 700 mg/kg, Vit C = 2000 mg/kg, W3 HUFA = 30 mg/g dwt.

2.4. Experimental Design

The experimental procedures were developed following the maintenance guidelines [18] with minor adjustments for ecotoxicological test using sea anemones [20] [22] (see **Table 1**). Four specimens were incubated in aquarium with 2.5 L containing artificial seawater, under continuous aeration and the above-described conditions and exposed to the temperature ranges: 10˚C, 15˚C, 20˚C, 25˚C or 30˚C for 96 h. Tests were carried out in duplicate samples per aquaria, with 10 Aquaria (n = 20 for each treatment). It was kept constant all other factors (e.g. salinity 32 ± 0.29 ppm, pH 7.74 ± 0.16, and dissolved oxygen 84.14% ± 6.33). During the test period, some endpoints were observed such as survival, reproduction and behaviour of the anemones. The behaviour endpoint was analysed on the second and last day. Reproduction was assessed based on detachment of juvenile polyp's (asexual reproduction), which were counted in each aquarium. The anemones behaviour was assessed based on tentacle flexion, tentacle retraction, oral disc flexion, column cavitation and peristome depression. For each parameter, a scale value was registered: flected, semi-flected and extended.

Moreover, the condition index was determined at the end of the experiment. The condition index was determined based on the fresh weight of the organisms, using the following equation:

$$\mathrm{CI} = \frac{W_f}{W_i}$$

Other researchers also present an empirical and theoretical comparison of the scaled mass index and OLS residuals as C is [23]. They argue that the scaled mass index is a useful new tool for ecologists.

Where CI is the condition index; W_f is the final weight and W_i is the initial weight (at the beginning of the experiment).The weight of anemones was determined to the nearest 0.01 mg.

2.5. Chemical Analysis

The water physicochemical parameters (temperature, pH, salinity, conductivity and dissolved oxygen), chlorophylls and nutrients were analyzed on the first and last day for each treatment. Water samples for the nutrients (nitrites, nitrates and phosphates) were previously filtered with glass fiber filters (Whatman, GFC, 1.2 μm) and then analyzed using the Diazotization Method, Cadmium Reduction and Ascorbic Acid Method, and USEPA method using powder pillows, respectively [24]. Water samples for chlorophylls (a, b and c) were filtered with glass fiber filters (Whatman GF/C, 1.2 μm) and analyzed following the Jeffrey & Humphrey's tri-chromatic Equations [25].

2.6. Statistical Analysis

Statistical analysis was performed using SPSS v. 20 [26]. Data was tested for normality (Kolmogrov-Smirnov test) and homogeneity of variances (Barlett test), following [27]. For data with normally distribution, a repeated-measures ANOVA was used to assess fluctuations of condition index, physical-chemical parameters, chlorophylls (a, b and c) and nutrients in each aquarium along time. Multiple comparisons relative to the control (20˚C) were performed using the Tukey and Dunnet to the parametric data and Kruskalwallis, Dunn's test and Student Newman Keuls for the non-parametric data.

The Behaviour data were constituted non-parametric data, for this were performed statistical analysis with the Kruskall-Wallis test followed by the multiple comparisons in relation to temperature vs time. The treatment 20˚C was considered as a control in a multiple comparisons test. The Spearman's correlations were employed to identify the possible correlations between behaviour parameters and temperature/time variation. All statistical analysis based on a 0.05 significance level.

3. Results

1) Physico-chemicals parameters

The water physicochemical parameters in each treatment are depicted in **Figure 2**. There were significant differences among temperatures, but they are not ecologically relevant. The coefficient of variation calculates in order to show the variation level of each parameters, represented in **Table 2**.

Table 2. Coefficient of variation values for each physico-chemical parameters variation with temperature.

Temperature (˚C)	Parameters	**CV	
		Firstday	Lastday
10˚C	Salinity	0.099	0.104
	Condutivity	0.022	0.029
	Dissolvedoxygen	0.015	0.030
	pH	0.003	0.004
15˚C	Salinity	0.072	0.064
	Condutivity	0.072	0.063
	Dissolvedoxygen	0.024	0.030
	pH	0.003	0.004
20˚C	Salinity	0.122	0.041
	Condutivity	0.021	0.022
	Dissolvedoxygen	0.028	0.032
	pH	0.004	0.003
25˚C	Salinity	0.079	0.067
	Condutivity	0.074	0.075
	Dissolvedoxygen	0.023	0.035
	pH	0.006	0.007
30˚C	Salinity	0.056	0.064
	Condutivity	0.005	0.039
	Dissolvedoxygen	0.031	0.064
	pH	0.005	0.008

**Coefficientofvariation: 0.1 - 0.15.

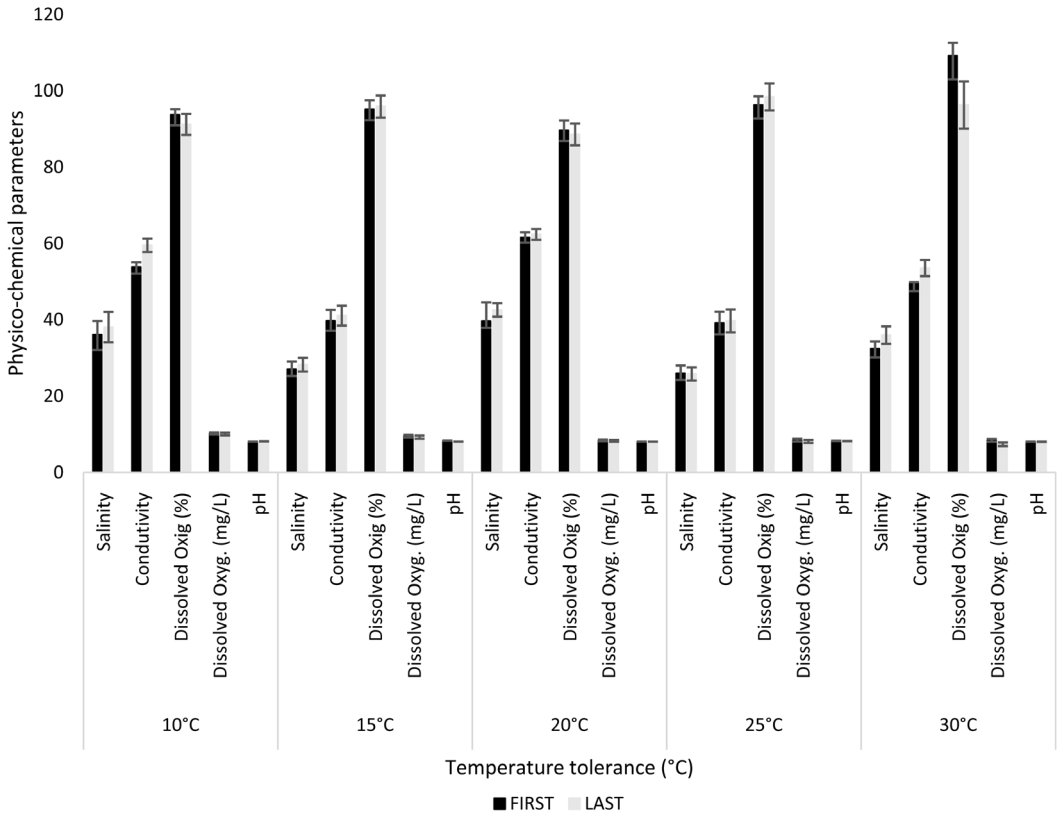


Figure 2. Physico-chemical parameters on each temperature tolerance test.

Dunnett analysis for Physic chemical parameters variation vs Temperature is significantly for all parameters within and between, $p = 0$ (see Table 3). Dunnet analysis for PFQs vs. times showed that dissolved oxygen and pH parameters varied along the 96h tests ($p = 0.02$ and 0.009, respectively).

The only parameters that not verified variation it was pH in 20˚C and 30˚C ($p = 0.99$). Tukey analysis for Physic chemical parameters variation vs. Temperature is significantly different in almost possible combinations, see Table 2. Tukey analysis for physic chemical parameters vs. time only showed significantly variation between dissolved oxygen and pH ($p = 0.02$ and 0.009) (Table 4).

The amount of chlorophylls *a*, *b* and *c* in the water was not significantly affected by temperature (Tukey and Dunnet, df = 4, $\alpha = 0.05$).

Table 3. Multiple comparison between physic-chemical parameters and temperature tolerance tests.

Multiple Comparisons							
Dunnett t (2-sided)							
Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Salinity	10˚C	30˚C	2.85000*	0.94072	**0.011**	0.5137	5.1863
	15˚C	30˚C	−6.56500*	0.94072	**0.000**	−8.9013	−4.2287
	20˚C	30˚C	6.90000*	0.94072	**0.000**	4.5637	9.2363
	25˚C	30˚C	−8.33500*	0.94072	**0.000**	−10.6713	−5.9987
Conductivity	10˚C	30˚C	5.08500*	0.83560	**0.000**	3.0098	7.1602
	15˚C	30˚C	−11.18000*	0.83560	**0.000**	−13.2552	−9.1048
	20˚C	30˚C	10.42000*	0.83560	**0.000**	8.3448	12.4952
	25˚C	30˚C	−12.15500*	0.83560	**0.000**	−14.2302	−10.0798
Dissolved Oxygen %	10˚C	30˚C	−10.28500*	1.39207	**0.000**	−13.7422	−6.8278
	15˚C	30˚C	−7.20000*	1.39207	**0.000**	−10.6572	−3.7428
	20˚C	30˚C	−13.60000*	1.39207	**0.000**	−17.0572	−10.1428
	25˚C	30˚C	−5.39000*	1.39207	**0.001**	−8.8472	−1.9328
Dissolved Oxygen mg/L	10˚C	30˚C	2.22600*	0.13723	**0.000**	1.8852	2.5668
	15˚C	30˚C	1.49000*	0.13723	**0.000**	1.1492	1.8308
	20˚C	30˚C	0.39500*	0.13723	**0.018**	0.0542	0.7358
	25˚C	30˚C	0.35600*	0.13723	**0.038**	0.0152	0.6968
pH	10˚C	30˚C	0.04050	0.02294	**0.237**	−0.0165	0.0975
	15˚C	30˚C	0.15250*	0.02294	**0.000**	0.0955	0.2095
	20˚C	30˚C	0.00700	0.02294	0.994	−0.0500	0.0640
	25˚C	30˚C	0.17700*	0.02294	**0.000**	0.1200	0.2340

*The mean difference is significant at the 0.05 level.

Table 4. Tukey analysis results to comparison physic-chemical parameters vs. temperature variation.

Tukey HSD			
Dependent Variable	(I) Temperature	(J) Temperature	Sig.
Salinity (psu)	10˚C	15˚C	0
		20˚C	0
		25˚C	0
		30˚C	0.026
	15˚C	10˚C	0
		20˚C	0
		30˚C	0
		10˚C	0
	20˚C	15˚C	0
		25˚C	0
		30˚C	0
		10˚C	0
	25˚C	20˚C	0
		30˚C	0
		10˚C	0.026
		15˚C	0
	30˚C	20˚C	0
		25˚C	0
		15˚C	0
		20˚C	0
Condutivity (μs/cm)	10˚C	25˚C	0
		30˚C	0
		10˚C	0
		20˚C	0
	15˚C	30˚C	0
		10˚C	0
		15˚C	0
		25˚C	0
	20˚C	30˚C	0
		10˚C	0
		20˚C	0
		30˚C	0
	25˚C	10˚C	0
		15˚C	0
		20˚C	0
		25˚C	0

Continued

	30˚C	25˚C	0.006
		30˚C	0
		20˚C	0
		15˚C	0
		25˚C	0
Dissolved Oxygen %	10˚C	30˚C	0
		10˚C	0.006
		20˚C	0
	15˚C	30˚C	0.002
		10˚C	0
		15˚C	0
		20˚C	0
	20˚C	25˚C	0.002
		15˚C	0
		20˚C	0
	25˚C	25˚C	0
		30˚C	0
		10˚C	0
		20˚C	0
	30˚C	25˚C	0
		30˚C	0
		10˚C	0
		15˚C	0
Dissolved Oxygen mg/L	10˚C	30˚C	0.039
		10˚C	0
		15˚C	0
		10˚C	0
	15˚C	15˚C	0
		20˚C	0.039
		15˚C	0
		25˚C	0
	20˚C	10˚C	0
		20˚C	0
		30˚C	0
		15˚C	0
	25˚C	25˚C	0
		10˚C	0
		20˚C	0
		30˚C	0

Continued

	30˚C	15˚C	0
		25˚C	0
pH	10˚C	15˚C	0
		25˚C	0
		10˚C	0
	15˚C	20˚C	0
		30˚C	0
	20˚C	15˚C	0
		25˚C	0
	25˚C	10˚C	0
		20˚C	0
		30˚C	0
	30˚C	15˚C	0
		25˚C	0

*The mean difference is significant at the 0.05 level.

The variation of nutrients (nitrites, nitrates and phosphates) among test temperatures is shown in Figure 3. Significant differences relative to the control were found for nitrites, nitrates and phosphates (test Tukey, df = 4, $p = 0.004$), nitrates/phosphates and phosphates/ nitrates (test Tukey, df = 2, $p = 0$) (Table 5).

The Tukey test showed the significant nutrients varied when compared 10˚C with 20˚C and 20˚C with 25˚C and 30˚C ($p = 0.01$, 0.02 and 0.05, respectively) (Table 5).

2) Physiological endpoints

Survival was 100% throughout the test period, except for anemones exposed to 30˚C. At this temperature, survival reduced to 55% on day 4.

Reproduction was null after 48 h of exposure, for all temperatures. After 96 h of exposure, it was higher at 10˚C and lower at 25˚C (Table 6). The reproduction, measured as the number of polyps released during 4 days, was significantly affected by temperature (H = 15.59, df = 4, $p = 0.004$) (Figure 4). Significant differences in reproduction were found between anemones at 10˚C and 25˚C (Figure 4). It was improved a comparative analysis for data and observed two distinct groups: one similar group between 20˚C and 25˚C and other group between 10˚C, 15˚C and 30˚C, revealing an interesting variation between "extremes" temperatures (10˚C and 30˚C) in relation to control temperatures (20˚C) on number of polyps. For the condition index, the comparative analysis showed two groups: one similar with 25˚C and 15˚C and other similar with 30˚C, 20˚C and 10˚C (Figure 5).

Figure 5 shows the Comparative analysis results for number of polyps and condition index values on temperature test tolerance during 96 h. It was verified significantly differences between control and multiple comparisons. It was

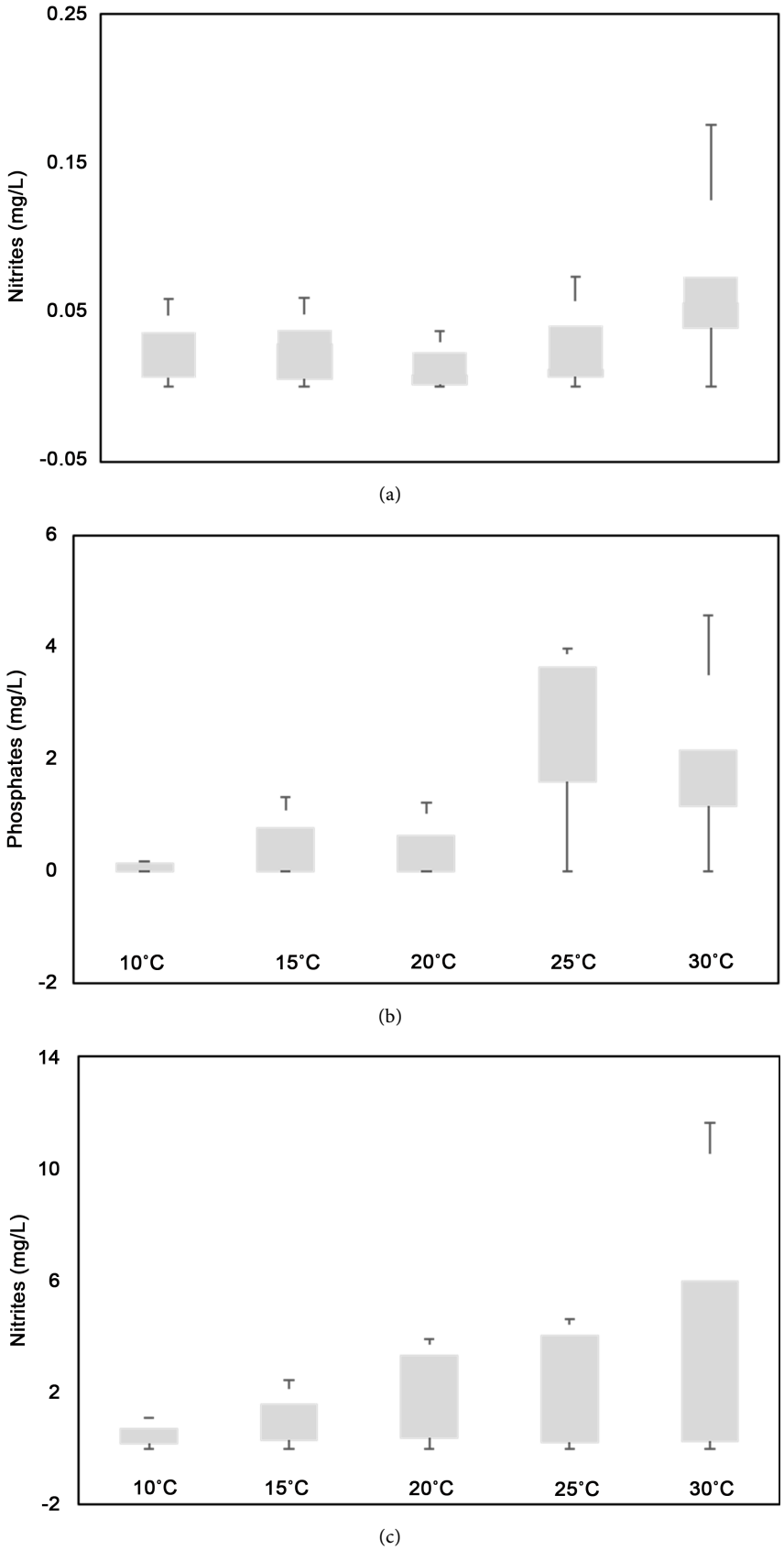


Figure 3. Nutrients on each temperature tolerance test. (a) nitrites; (b) phosphates and (c) nitrates.

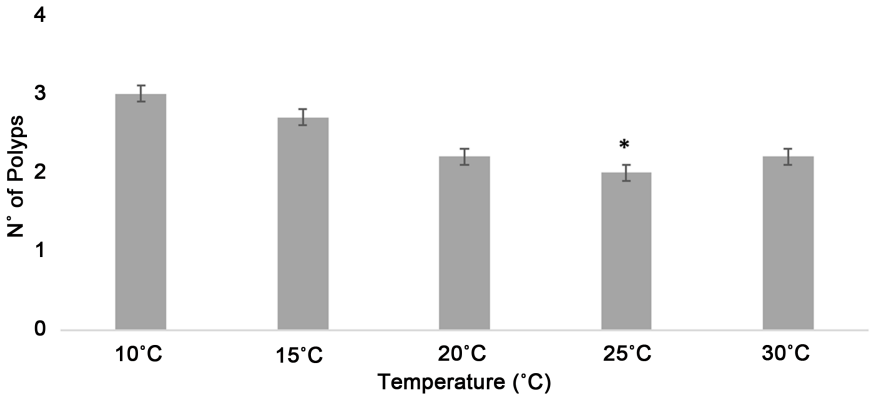
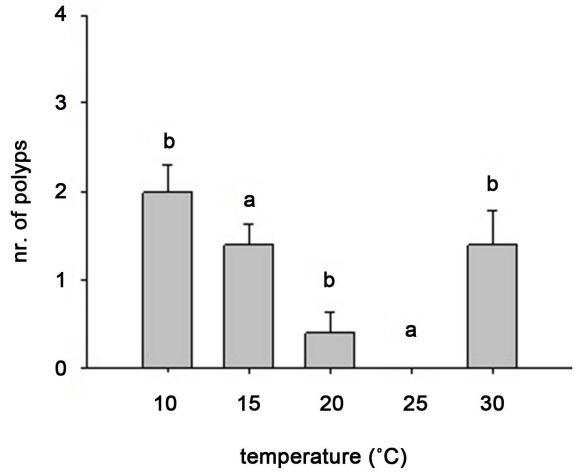
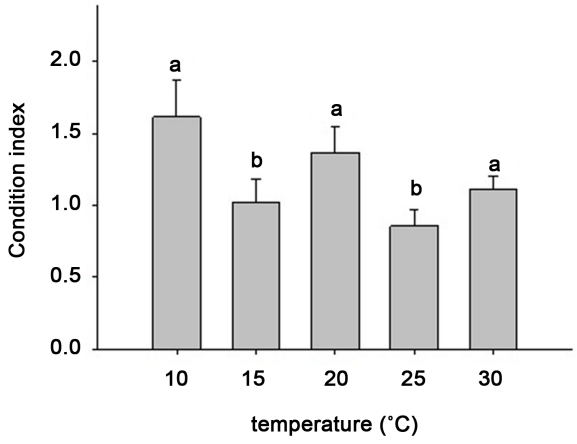


Figure 4. Number of polyps average per groups of 2 anemones (each replicate in 10 aquaria, N° = 20) exposed to 10˚C, 15˚C, 20˚C, 25˚C and 30˚C during 96 h. Error bars represent standard error. For these parameters, the data was considered the appearance of juvenile polyps since the second day of experiment until the end. (*For the treatment 25˚C, none juvenile polyps were liberated after the second day, only 2 polyps were liberated on the first day, so this data was not considered).



(a)



(b)

Figure 5. Comparative analysis results of (a). Number of juvenile polyps liberated after the 96 hours of exposure test, and (b). Condition index of sea anemones of tolerance test on each temperature. * W_f and W_i means final weight and initial weight respectively.

Table 5. The Tukey HSD results to nutrients variations between each other.

Multiple Comparisons						
Dependent Variable Nutrient value Tukey HSD						
(I) Nutrient type		Mean Difference (I–J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nitrites	PH**	−0.69778*	0.25887	0.021	−1.3107	−0.0848
	NA**	−1.63898*	0.25887	0.000	−2.2519	−1.0260
Phosphates	NI**	0.69778*	0.25887	0.021	0.0848	1.3107
	NA	−0.94120*	0.25887	0.001	−1.5541	−0.3283
Nitrates	NI	1.63898*	0.25887	0.000	1.0260	2.2519
	PH	0.94120*	0.25887	0.001	0.3283	1.5541

*The mean difference is significant at the 0.05 level. **PH, NA and NI-Phosphates, Nitrates and Nitrites, respectively.

Table 6. Survival and Reproduction of adult polyps on each temperature test tolerance, on first and last observation, along 96 hours.

	Temperature									
	10˚C		15˚C		20˚C		25˚C		30˚C	
Time	Second day	Last day	Second day	Last day	Second day	Last day	Second day	Last day	Second day	Last day
Survival	20	20	20	20	20	20	20	20	20	11
Reproduction	0	10	0	7	0	2	0	0	0	7

improved Student Newman Keuls test that was verified significantly differences for number of polyps on treatment 10˚C vs. 25˚C; 15˚C vs. 25˚C; 15˚C vs. 20˚C;30˚C vs. 25˚C; 30˚C vs. 20˚C (H = 15.59, df = 4, p = 0.004). For Condition index, the Student Newman-Keuls observed 10˚C vs. 25˚C; 10˚C vs. 15˚C; 20˚C vs. 25˚C; 20˚C vs. 15˚C; 30˚C vs. 25˚C and 30˚C vs. 15˚C (H = 10.89, df = 4, $p = 0.028$).

3) Behavioral parameters

The variation of the behavioral parameters tentacle flexion, tentacle retraction, oral disc flexion, column cavitation and peristome depression during the test period for each temperature is depicted in **Figure 6**.

a) Tentacle Flexion

Tentacle flexion (**Figure 6(a)**) was significantly affected by temperature (p = 0.012) and time (p = 0.011). Spearman's correlation showed a significant correlation between tentacle flexion and temperature/time (p = 0.05). After 24 h of exposure the highest percentage of flected tentacles found at 15˚C (55%); the highest percentage of semi-flected tentacles found at 10˚C (60%) and the highest percentage of extended tentacles found at 25˚C (45%). After 96 h of exposure to the test temperature, the results were similar, except that the highest percentage of flected tentacles found at 20˚ (80%).

b) Tentacle Retraction

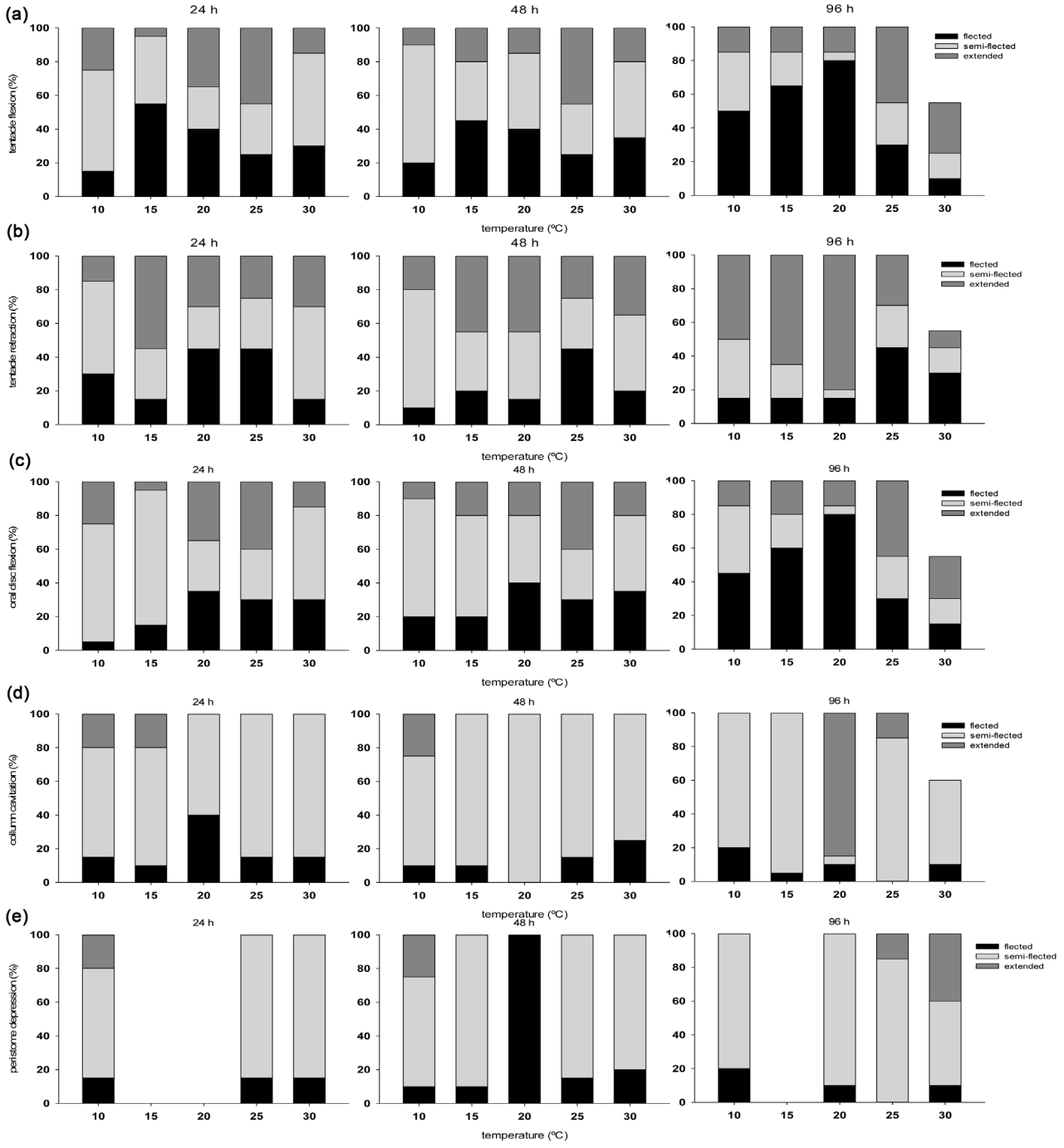


Figure 6. Behavior levels percentage variation with temperature. In a scale of flected, semi-flected and extended open/close of each behavioral parameter. (a)-Tentacle Flexion; (b)-Tentacle Retraction; (c)-Oral Disc Flexion; (d)-Collumn Cavitation and (e)-Peristome Depression.

Tentacle retraction (**Figure 6(b)**) was significantly affected by temperature (p = 0.033). The higher percentages of tentacle retraction flected was found on 20˚C and 25˚C, with the same values (45%); semi-flected on 10˚C and 30˚C, with the same values (60%); extended on 15˚C (55%). On the last day, the higher percentages of tentacle retraction flected was on 25˚C (45%), semi-flected on 10˚C (35%) and extended on 20˚C (80%).

c) Oral disc flexion

Kruskal-wallis results it was observed that rejected the nulli hypothesis, between temperature and time variations (p = 0.008 and 0, respectively). It was done Spearman's correlation and observed that exist correlation between oral disc flexion and only time (δp = 0.05). On the first day, it was higher percentages of oral disc flexion flected on 20˚C (35%), semi-flected on 15˚C (80%) and extended 25˚C (40%) (**Figure 6(c)**). On the last day, it was higher percentages of oral disc flexion flected on 20˚C (80%), with a 45% of increment in comparison to the first day. Semi-flected higher percentages on 10˚C (40%) and extended on 25˚C (45%), with only 5% of increment in relation to the first day (**Figure 6(c)**).

d) Column cavitation

Kruskal-wallis results it was a significant difference between temperature variations (p = 0.052). It was done Spearman's correlation and observed that exist correlation between tentacle flexion and only temperature (δ = 1). Post-hoc test observed significant differences on column cavitation openness/close on 10˚C and 30˚C data (p = 0.02) (**Figure 6(d)**). On the first day, it was higher percentages of column cavitation flected on 20˚C (40%), semi-flected higher percentages on 25˚C and 30˚C, with the same values (85%). The extended on 10˚C and 15˚C, with the same values (20%). On the last day, the column cavitation flected with higher percentages was on 10˚C (20%), with a decrease of 20˚C of 30%. Semi-flected higher values 15˚C (95%), almost the totally of individuals, with a decrease of 30˚C (35%). The column cavitation extended higher percentages was on 20˚C (85%), with a decrease of 10˚C and 15˚C to nuli.

e) Peristome depression

Kruskal-wallis results it was observed that rejected the nulli hypothesis, between temperature and time variations (p = 0.025 and 0.018, respectively). It was done Spearman's correlation and observed that exist correlation between oral disc flexion and only time (p = 0.008) (**Figure 6(e)**). On the first day, the higher percentages of peristome depression flected was found on 10˚C, 25˚C and 30˚C (15%), semi-flected on 25ºC and 30˚C (85%) and extended on 10˚C (20%). On the last day, the higher percentages of peristome depression flected was on 10˚C (20%), with a 5% of increment in comparision to the first day. The semi-flected on 20˚C (90%), almost the totally of individuals, with a decrease of 30˚C of 35%. The extended on 30˚C (40%), with a decrease of 10˚C to nuli.

4. Discussion

The main objective of this study was to assess the temperature tolerance of *Actiniaequina* to temperatures between 10˚C and 30˚C, and assess their responses (condition index, reproduction and behavior) over time (96 h). A wide range of temperature was test in order to establish a "worst case scenario".

The results showed that their anemones responses were significantly affect by temperature and varied during the exposure period. The results of this investigation show that transient low and high temperature stress in 96 hours causes a variation on behavior parameters in the populations of sea anemones *Actinia*

equina and a series of chemical alterations on water. Maybe results of sea anemones metabolism alteration are due to temperature variation along time.

Before the test, anemones were maintaining in a flow-through system under controlled conditions, receiving artificial fish diet feed, containing a low lipid level, but constituted of nutrients such as proteins, vitamins, fiber, ash and oil. During stress periods, sea anemones have ability to retain large quantities of food and water into the gastro vascular cavity. The uptake or release of food and other natural physiological products could explain the variations of nutrients and chlorophylls during the test [19] [28].

In relation to survival, it was only reducing at 30˚C test (almost 50% of the total number of organisms). This could be explaining by the fact that this species is natural from temperate ecosystems, thus more tolerant to decreasing temperature than increasing temperature [29].

Besides survival, also the reproduction of anemones was affect by temperature. In all temperature tolerance, tests it was observing increase on number of polyps in relation to the first day, with juvenile polyp's liberation, with a significant difference between 10˚C and 25˚C. The increased reproduction at extreme temperatures is probably due to the stress caused by temperature, leading to a reproduction investment under stress conditions, aiming to save the population. However, there could be a "temperature/time limit" to these stimuli.

The condition index was also affect by temperature, showed higher decrease at 10˚C and 30˚C. This could be explaining by the fact that ranges near the ideal temperature are not enough to implement the loss weight. At 15˚C and 25˚C it was detected an increase on condition index. The present work agrees with a previous work studying with the species tolerance temperature, in the rocky intertidal zone of the Mediterranean coast of Israel, they examined variation in polyp growth at several temperatures within the local range [30]. Under laboratory conditions, the authors observed that only polyps at low temperatures (15˚C and 20˚C) grew, whereas those at higher temperatures (25˚C and 30˚C) lost body mass [30]. Another important conclusion it was that at summer seawater temperatures along the coast of Israel (28.7˚C - 29.5˚C), polyps of *A. equina* are unable to balance their metabolic requirements with energy input, resulting in a seasonal reduction in biomass. Polyps appear to be able to acclimate to high temperatures, but not sufficiently to avoid shrinkage of tissues [30].

The physical-chemical parameters were maintained constant along to 96 hours, although they showed statistically significantly differences among temperatures, but these variations are not ecologically relevant according to [18].

The chlorophylls value not showed significantly differences between temperatures ranges. Temperature, leads to increased chlorophylls "a" and "c" levels. This research was in agree with [31], who compared the chlorophylls a and c values in relation to temperature stress on water over time, not finding any significant differences. They showed that heat stress could initiate cell death pathways that differ in rate and magnitude. The rapid response contrasts with the effect of elevated sea-surface temperatures for reef ecosystems, which is currently

determined in terms of degree heating weeks. In bleaching events in the field, relatively small diurnal fluctuations in temperature may be far more important than previously recognized and may selectively promote the rapid Programmed Cell Death response over slower necrotic cell death. The timing of the peak of apoptosis-like cell death, at around 3 h exposure, was similar in ectoderm and endoderm cells and at all temperatures, but was more pronounced (higher frequency) at higher temperatures.

Nutrients composition showed differences with temperatures variations over time. When compared to control (20˚C, supposed ideal temperature) the nutrients concentrations decreased with increasing temperature. It was maybe due to sea anemones metabolism increase/decrease. Higher consumption of oxygen and hypoxia condition on higher temperatures (30˚C), elimination of nitrogen compounds on water, increase the pH and consequently increase the nutrients reactions and consumption. Many species of marine invertebrates, such as corals, sea anemones and giant clams, harbor endosymbiotic dinoflagellates (zooxanthellae) in their tissues. Zooxanthellae release short-term photosynthetic products to their hosts and so are an important source of organic carbon for host metabolism, growth and reproduction. Zooxanthellae are also important in the recycling and conservation of essential nutrients, such as nitrogen, and enhance calcification rates in corals [6].

The thermal stress in laboratorial climate change simulation could to induce other effects on these species of sea anemones. A recently study, to conclude that the high levels of genetic polymorphism and sexual reproduction (even though rarely) documented in Israeli populations of *A. equina* result from environmental stress experienced by members of this species at their southernmost limit of distribution in the Mediterranean region [30]. Environmental stress is known to increase genetic polymorphism, through high rates of mutation and recombination. This pattern extends to populations in Croatia, where applied the same AFLP methodology to *A. equina* living in much cooler water than in Israel, and revealed locus polymorphism only about half (36.8% - 47.2%) as high as that in Israel (56.1% - 73.5%). They conclude that the exclusively sexual reproduction, high genetic diversity, and low abundance of Mediterranean populations of *A. equina* all may be cause in part by the stressful environmental conditions experienced by members of this species in this region. Under the less stressful environmental conditions in northern Europe, asexually produced offspring may form large aggregations that perpetuate parental genotypes, which are well adapted to the locally benign conditions of low temperature for this species. Sexual reproduction is thought to enhance the long-term success of organisms, since it generates a wide range of genotype escapable of "tracking" environmental changes [28] [29] [30] [32].

Researchers suggested that responses to environmental changes could be divided in four categories: passive—no response, when the stimulus is not sense or occurs too rapidly thus leading to a decrease in performance capacities or even death [33]. Behavioral reactions—when subjected to certain chemicals, animals

usually react in seconds or minutes, avoiding stress and trying to obtain a favorable position relative to the level of stimulus. Physiological responses—organisms suffer internal changes in various physiological processes, including adjustments in physiological rate functions and tolerance acclimation enhancement, which may occur within hours to weeks. Finally, biochemical responses—synthesis of new molecules like-stress/proteins in response to environmental changes, in order to restore homeostasis within genetic constrains, which may take from days to weeks. Therefore, adding behavior as an endpoint can help to formulate a quantitative minute-to-minute or hour-to-hour assessment of how tested species are. Re-acting towards the toxicant concentration, bearing in mind that behavior can be classified as the cumulative interaction of a variety of biotic and abiotic factors that represents the animal's response to internal (physiological) and external (environmental, social) factors and that relates one organism to another [34]. Behavior provides an insight into various levels of biological organization, being a result and determinant of molecular, physiological, and ecological aspects of toxicology [35]. Therefore, behavioral responses may reflect biochemical changes in the individual organism and subsequently promote alterations in communities, which can be translated into ecological consequences [36].

Behavioral parameters were significantly affect by temperature and varied over time. The ecological interpretation of each behavioral parameter could be translated as physiological functions. Tentacle flexion is a response to specific photoreceptors in that the maximum efficiency for stimulation is in the same spectral regions as for many forms with discrete photoreceptors. The analogous behavior parameters to this was tentacle retraction that is considered to be a response to absorption of energy by proteins and nucleic acids, as evidenced by it is maximum efficiency peak at 280 nm. The results showed that tentacle flexion/retraction was also affect by temperature and time. A correlation between temperatures and time variation found, meaning that the tentacle expends a large time to absorptions energy in proteins and nucleic acids forms. The oral disc flexion, column cavitation and peristome depression responses involve regional muscle action, probably by deep photoreceptors rather than by nonspecific effects on cell proteins. This answers could be translated by timing expended to producing energy to accumulate, and how much temperature elevate how energy was expended and involving on cellular reactions. However, oral disc flexion and peristome depression only presented correlations in relation to time variation. In another hand, column cavitation only showed correlation in relation to temperature variation (extreme 10˚C and 30˚C). The column contraction could activate by seawater contact and the temperature could be the first stimuli to this act. Therefore, the species *Actinia equina*, spend a more time with retractions tentacles, because they did not have symbiotic algae to help in an increase against temperatures expositions protections. They could be involved a deep photoreceptors nonspecific to develop the cell protein production as a secondary function [8].

The most of bibliography focused on symbiotic relations and they anatomic and physiological thermal stress effects. So the present work, try to find a way to explanation these environmental and global changes effects on solely organisms, to generalize to all populations [2] [3] [7] [31] [37] [38] [39].

The present research could be considering a first step to find baseline behavioral answers to environmental and climatic changes as early warning systems, using sea anemones as sentinel species. Because was tested such of behavioral (traduced as chemical effects) on thermal controlled induced effects along the short-time exposure. Behavior endpoints could be the first signals to physiological alterations and give us time to try to return or revert the first alterations and comprehend how we can proceed to explain the environmental and global alterations, focused on climatic changes.

Elevated temperatures and solar ultraviolet (UV) radiation been implicated as recent causes for the loss of symbiotic algae (*i.e.*, bleaching) in corals and other invertebrates with photoautotrophic symbionts. Until, aposymbiotic organisms could reveal water physical alterations, in relation to increase of chlorophylls levels.

The first study with behavioral reactions of the sea anemone, *Anthopleuraxanthogrammica*, to ultraviolet and visible radiations was published by [9]. They test for first time, the behavioral parameters to use on the present study and they concluded ecological interpretation for each endpoint, such as tentacle flexion appears to be a response to specific photoreceptors in that the maximum efficiency for stimulation is in the same spectral region as for many forms with discrete photoreceptors [9]. Tentacle retraction considered a response to absorption of energy by proteins and nucleic acids, as evidenced by its maximum efficiency peak at 280 nm. Oral disc, column cavitation, and peristome responses involve regional muscle action, probably induced by deep photoreceptors rather than by nonspecific effects on cell proteins [9]. The early stress response to elevated temperature involves essentially all aspects of same chemical reactions; in this case, we observed a receptors functioning and the frequency of open-close oral sea anemones, tentacles and columns anatomic alterations to detect earlier the effects of physical stress induction.

The results of these study suggest that the behavior alterations reveal that short-term effects of temperatures variations could not evoke substantial consequences, but on natural environment, the organism were expose to multiple stressors. Intertidal organisms are subject to a variety of stresses such as desiccation, water temperature, acidification, increase salinity, nutrient limitation, space competition and predation.

5. Conclusion

The present work showed that sea anemones are sensitive to environmental and global changes. The physical stressor (temperature) tested in the scope of a climatic and environmental change simulation, showing that differences on sea anemones behavioral answers, demonstrated that these organisms have potential to use as early warning systems to climatic changes.

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