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The Aquaculture of Corals

The effects of temperature and modifications in photoperiod in performance and growth of *Stylophora pistillata*.

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EN: Summary: Nowadays, corals have a large economic potential and the increasing demand places an enormous pressure on wild reefs. This issue brings new challenges for coral production in terms of increasing production and efficiency. Besides this, climate change and warmer oceans are threatening the future of corals, with several bleaching events occurring worldwide. Many factors that influence the growth and health of the corals have already been extensively studied, however, some factors, such as low temperatures and photoperiod require further research. Temperature and light also play a critical role in the phenomena of coral bleaching, which means that our knowledge about the interaction of these two factors is essential. The main objectives of this research were to find more information to improve the production protocols and better understand the physiology of stony corals under abnormal light and thermal conditions. In this study different combinations of temperatures (20º, 23º, 26º and 29ºC) and photoperiods (8L16D, 12L12D, 16L8D) were tested for a period of one month. Growth and metabolism measurements, zooxanthellae counts and pigments' analysis were conducted to evaluate the condition, calcification and photosynthetic activity of *Stylophora pistillata*. No increase in growth was achieved with the extension of the photoperiod, however, a shorter photoperiod revealed to be detrimental to growth after a significant reduction of 25% compared to control treatment. Colonies maintained at 20º and 29ºC suffered reductions on their growth rates independently of the photoperiod regime. Photosynthetic efficiency and concentration of pigments suffered a decrease under the 16h light regime while corals maintained at 8h regime kept their photosynthetic efficiency and increased their pigmentation. Zooxanthellar populations were strongly reduced by low temperatures. The interaction between photoperiod and temperature was observed in photosynthetic efficiency and pigments concentration. These results lead to conclude that the effects of photoperiod are similar to those of light intensity, cold stress presents analogous effects to heat stress, as, the combined effects of photoperiod and temperature are similar to light intensity and temperature.

PT: Resumo: Na actualidade, os corais têm um potencial económico muito elevado e o aumento da sua procura coloca uma grande pressão sobre os recifes de coral. Este problema trás novos desafios para a produção de corais em termos de aumento da produção e da sua eficiência. Para além disto as alterações climáticas e oceanos mais quentes são uma ameaça ao futuro dos corais, com vários eventos de branqueamento a ocorrer em todo o mundo. Muitos dos factores que influenciam o crescimento e a saúde dos corais foram já extensamente estudados, no entanto, alguns factores como a temperatura ou o fotoperíodo requerem mais investigação. Boas performances a temperaturas de produção mais baixas ou em fotoperíodos mais curtos podem representar uma redução no consumo energético, aumentando a viabilidade económica da produção. Os principais objectivos deste estudo serão encontrar pistas para melhorar os protocolos de produção e melhorar o nosso entendimento sobre a fisiologia dos corais sob condições anormais de temperatura e luz. O propósito do procedimento experimental é testar diferentes combinações de temperaturas (20º, 23º, 26º e 29ºC) e fotoperíodos (8L16D, 12L12D, 16L8D) com a espécie *Stylophora pistillata*. Medições de taxas de crescimento, de consumo e produção de oxigénio, contagem de zooxantelas e análises de pigmentos fotossintéticos e carotenoides serão levados a cabo para avaliar a condição, calcificação e actividade fotossintética de desta espécie de coral duro. A experiencia durou 1 mês e meio e foi interrompida devido ao surto de uma doença infeciosa e contagiosa que levou à morte dos corais. Os resultados não demonstraram incremento das taxas de crescimento em conjugação com a extensão do fotoperíodo. No entanto, uma redução do fotoperíodo demonstrou ser negativa para o crescimento com uma redução de 25% comparado com o grupo de controlo. Os corais mantidos a 20º e 29º sofreram uma redução na sua taxa de crescimento, independentemente do fotoperíodo a que estiveram expostos. A eficiência fotossintética e a concentração de pigmentos dos corais sofreram uma redução quando expostas a 16 horas de luz enquanto que corais expostos a 8 horas de luz mantiveram a sua eficiência fotossintética e aumentaram a sua pigmentação. A densidade de zooxantelas foi intensamente reduzida pelas temperaturas mais baixas. Ocorreu interação entre o fotoperíodo e as temperaturas na eficiência fotossintética e na concentração de pigmentos. Estas observação levam a concluir que os efeitos do fotoperíodo são similares aos da intensidade luminosa, que baixas temperaturas causam efeitos análogos aos das temperaturas elevadas, bem como os efeitos combinado do fotoperíodo e da temperatura são semelhantes ao da intensidade luminosa e da temperatura.

1 - State of the Art

1.1 - Introduction

Coral reefs are mainly found in tropical regions around the globe (Wilkinson, 2008). Corals typically live in large colonies of several identical polyps and many species live in symbiosis with photosynthetic unicellular dinoflagellates of the genus *Symbiodinium,* commonly known as zooxanthellae. These unicellular algae live within coral's tissue sharing a large proportion of organic carbon resultant from its photosynthetic activity with the host. In return, the coral provides shelter and nutrients (Osinga *et al*., 2011). The coral and the algae form an holobiont - assemblage of different species that form ecological units or acting like a unique organism.

Taxonomically, corals belong to the Phylum Cnidaria, class Anthozoa which is divided into three subclasses, Hexacorallia, Octocorallia, and Ceriantharia. These groups include all stony and soft corals, sea anemones, sea pens and gorgonians. A particular group, the order Scleractinia (subclass Hexacorallia), also known as stony corals, hard corals or even scleractinian corals, has an important role in coastal marine ecosystems since they build themselves a hard-calcified skeleton, and thus, they are responsible for the construction of corals reefs. Is this incredible ability, together with the symbiotic relationship with the zooxanthellae, involving recycling of nutrients and a close interaction between trophic levels that generates one of the richest, and most ecologically successful ecosystems on Earth (Smith *et al*., 2005).

Beyond their importance for the biodiversity, corals provide several benefits to humans, many of them with huge economic and social value (Wilkinson, 2008; Birkland, 2015). Coral reefs work as natural barriers that protect the shore from violent wave action and its biodiversity attracts tourism and promotes primary economic activities like fishing. These services guarantee a large support for many local populations. Also, our passion for the ocean together with its beautiful colours and shapes made corals a requested object for home aquariums and ornamental organism industry all around the globe (Delbeek, 2001). Furthermore, in the last decades an increased demand for natural products, for pharmaceutical or biotechnological purposes, also did mankind to look at marine organisms in search for new compounds and substances. Corals are one of the largest sources of these new products, giving them an increasing value for the future of our society (Leal *et al.*, 2013). All these services and economic activities around the corals and the reefs created a large pressure and negative impact in natural populations of these organisms and in the ecosystem created by them, mainly due to, the direct collection and harvesting (Brukner 2001; Wilkinson, 2008; Birkland, 2015).

1.2 – Coral Aquaculture

Aquaculture of corals or coral farming seems to be a sustainable solution to avoid harvesting of wild populations and even to help in the process of reef restoration. However, the rearing of these organisms brings several challenges in provision and control of the components required for coral's growth. In the 80s the popularity of corals in home aquaria increased. Initially, this demand was supplied by a direct collection of coral fragments from nature causing a large pressure over the wild reefs due to unsustainable practices and large harvested volumes (Brukner 2001; Dee *et al*, 2014). The first cultivations through asexual propagation were made by some public aquaria in the early 80s and rapidly by hobbyists and retailers in their own aquaria and trading among themselves. However, this early production was not enough to supply the demand and new corals were still imported from nature. Nowadays, a large amount of corals is cultivated in *in-situ*, in Asia-Pacific, and *ex-situ*, in Europe and North America and they are one of the most lucrative organisms in the ornamental trade (US$7,000 per tonne (Wabnitz *et al*, 2003)). In the recent years, new purposes motivated the production of corals, such as conservation efforts or to the production of new products for the pharmaceutical and biotechnology industries. Nevertheless, coral aquaculture still requires the development of new techniques and protocols to optimize production.

Coral aquaculture can be divided in in-situ and *ex-situ* production; *in-situ* (production in the ocean; mariculture) requires low daily work and low maintenance costs, since all the conditions are provided by the natural environment. However, the unpredictability of local conditions, the production system exposure to predation, pollutants, sedimentation, diseases, natural disasters and fluctuations in food availability, along with the geographically limited areas to operate (tropical regions) are major issues that can limit production. *Ex-situ* production (land-based facilities) is considered an alternative to overcome these constraints, but many times, especially in northern latitudes, the high energy costs of lighting and heating of indoor systems, can compromise its profitability (Delbeek, 2001; Leal *et al.*, 2013; Osinga *et al.*, 2011; Olivotto *et al*., 2011). Like any other aquaculture production, coral's aquaculture carries the risk of contracting diseases that can result in the lost of the entire production. The high density of individuals is one the main reasons for easy transmissibility of pathogens. Prevention or mitigation of potential pathologies plays a fundamental role in the survival of the production system (Sheridan *et al*. 2013).

1.3 - Coral Symbiosis: the marriage between an animal and a plant.

Scleractinian corals are colonial animals, and their basic unit is the polyp. It has a simple shape of a cilindrum with an oral disk in the upper part and a basal plate in the opposite disk. In the oral disk, there is a mouth, in the middle, surrounded by tentacles used to capture preys. The mouth opens to the gastrovascular cavity, also known as coelenteron, divided by mesenteries forming compartments. In few words, the polyp resembles the sea anemones. Like all other Cnidaria, corals are diploblastic having the basic two layers' organization; the endoderm and the ectoderm. In these organisms, the endoderm origins the gastrodermis and the ectoderm origins the epidermis. Usually, these layers are only one cell thick separated by a layer of connective tissue called mesoglea, consisting of collagen, mucus and "wandering cells". The polyps are connected by horizontal sheets of tissue known as coenosarc extending over the superficial surface of the skeleton and completely covering it. These sheets are continuous with the body wall of the polyps and include extensions of the gastrovascular cavity of each polyp forming a common gastrovascular cavity that interconnects all the colony and it might work to transfer food, zooxanthellae, and waste compounds among polyps. In addition, the upper layer of the ectoderm (epidermis) is in contact with the sea water while the lower layer (aboral) is in contact with the skeleton, being these cells the responsible to secret the materials to build up the skeleton (Muller-Parker *et al*., 2015; Titlyanov and Titlyanova, 2002; Galloway *et al*., 2007) (Figure 1).

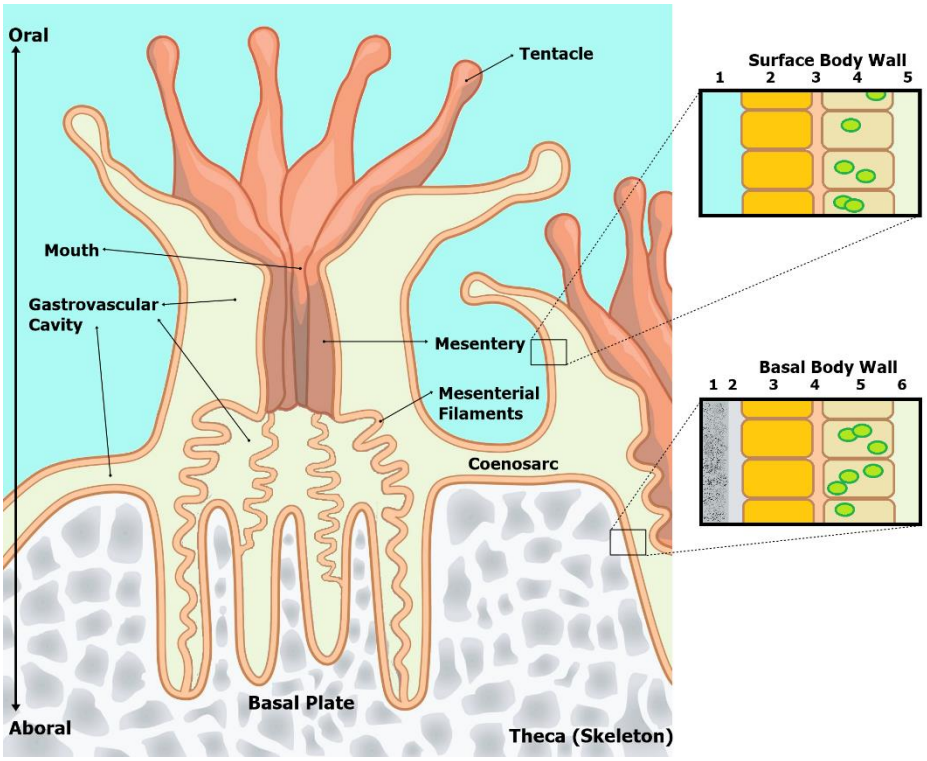


Figure 1 – Coral polyp anatomy.
Surface Body Wall: 1 – seawater; **2** – epidermis; **3** – mesoglea; **4** – gastrodermis with zooxanthellae; **5** – gastrovascular cavity.
Basal Body Wall: **1** – Corallum (skeleton); **2** – calcifying medium; **3** - epidermis; **4** – mesoglea; **5** – gastrodermis with zooxanthellae; **6** – gastrovascular cavity
Source: Adapted from http://www.dkfindout.com/uk/animals-and-nature/jellyfish-corals-and-anemones/inside-coral-polyp/

Although the name does not have taxonomic value, "zooxanthellae" is primarily used to describe the dinoflagellates that live-in symbiosis with the corals (Genus Symbiodinium), but a general use of the term is applied for every symbiont algae that live-in animals (Muller-Parker *et al*., 2015). Zooxanthellae inhabit in the endoderm cells of the coral's tissue at heterogeneous densities of 0.5 to 5 million/ cm2 of coral (Smith *et al*., 2005). They are between 8-12 µm in diameter and have all the structural elements of a typical dinoflagellate. Their chloroplast contains all the characteristic photosynthetic pigments of a dinoflagellate: *a* and *c2* chlorophylls, peridinin, diadinoxanthin, dinoxanthin, and *β*-caroten. Inside the host, the algae stay in a coccoid form, immobile, with or without a perisymbiotic membrane and reproducing by mitotic division. However, they can live freely outside of the host's body acquiring two flagella and all the structural elements of a free-living dinoflagellate (Muller-Parker *et al*., 2015, Titlyanov and Titlyanova, 2002). Initially it was thought that the zooxanthellae that inhabit the corals belonged to a single species, *Symbiodinium microadriaticum*, however, in the last years, with the advance of the new genetic analysis, it was suggested that several zooxanthellae clades exist and it was proven that they have a large genetic variety with several taxa or genotypes living in the same host species or even in the same host organism (Muller-Parker *et al*., 2015; Little *et al*., 2004).

As previously said, when their metabolic needs are satisfied, microalgae zooxanthellae translocate the photosynthetic products to the host, thus the coral can use this resource for their own growth, both for soft tissues and for the calcified skeleton. Therefore, it can be said that the zooxanthellae take a large role in the coral's growth (Shutter *et al*., 2011). In contrast, the host provides shelter and important nutrients to the microalgae's metabolism, such as nitrogenous compounds, phosphates, and CO2; that the coral itself acquires by its heterotrophic feeding (Smith et al., 2005). Furthermore, due to its optimal light reflection properties, the calcium carbonate skeleton also improves the light capture by the symbionts (Osinga *et al*., 2011a). About 90% of the food requirements of the coral can be derived from zooxanthellae photoassimilates – fatty acids, sugars, amino acids and even some vitamins (Titlyanov and Titlyanova, 2002). Corals also have heterotrophic feeding that comes from the predation of zooplankton, filtration of particles, absorption of dissolved organic substances (DOS) and the digestion of old or dead zooxanthellae (Titlyanov and Titlyanova, 2002). All these heterotrophic feeding strategies are the main source of organic compounds of nitrogen and phosphorus, nutrients that take a role in the metabolism of the coral, and of the zooxanthellae too. Additionally, it was found that nitrogen-fixing bacteria also inhabits corals and contributes as a source of nitrogen (Lesser *et al*., 2004). It was demonstrated that the coral cannot survive only by heterotrophic feeding since this source of food does not provide the amount of energy and certain substances that zooxanthellae can provide (Titlyanov and Titlyanova, 2002). Both

organisms obtain from seawater the inorganic compounds needed for their metabolism, such as CO_2, and bicarbonate for the plant symbiont's photosynthesis and ions of calcium and carbonate for the coral's skeleton. The CO_2 is acquired not only from seawater but also from the respiratory metabolism of the host. In brief, it was this close relationship, with large interchange and recycling of resources and energy that allowed corals and algae to successfully evolve and habit in an oligotrophic environment like tropical and subtropical water, and beyond that, they create a whole complex structure and ecosystem that is a true hotspot of biodiversity (Muscatine and Porter, 1977).

1.4 – Coral Bleaching - When the marriage evolves into divorce.

Under stress conditions, the coral-zooxanthellae symbiosis can be strongly affected resulting in the expulsion of the zooxanthellae and/or a loss of photosynthetic pigments, and subsequent reduction of pigmentation in corals, a phenomenon called coral bleaching. This bleaching is a reaction to abnormal environmental conditions and bleaching events have been highly correlated with the rising of seawater temperature. An increase of less than 2ºC above the average summer maxima is enough to cause coral bleaching (Jones *et al*.,1998; Goulet, 2006; Smith *et al.*, 2005). The coral relies entirely on its heterotrophic feeding being able to survive for some weeks or even months. But, if the abnormal conditions persist, it leads to the coral's death since the host is highly dependent on the food provided by the algae. Bleaching has been the biggest concern of the scientific community since global warming has caused changes in ocean's temperatures. However, it has been hypothesized, that bleaching has an adaptive function that enables the coral to switch to a more suitable and adapted population of zooxanthellae, either by colonization of new genotypes or by rebalancing the dominant genotype in their tissue's native populations (Goulet, 2006).

As referred before, bleaching is, in *sensu lato*, the interruption of the symbiotic relationship between the coral and the zooxanthellae, in the presence of stress. However, it is a complex mechanism that is triggered by a set of several factors at the metabolic, cellular and biochemical level. Zooxanthellae have the capacity to acclimatize to rapid and accentuated fluctuations on light regimes, however, when the rate of excitation (absorption of light) exceeds the photosynthetic rate the mechanisms to dissipate the excess of energy are activated to avoid damage of the photosynthetic apparatus. These mechanisms are energetically expensive, and so, the photosynthetic efficiency or yield is reduced, occurring what is called photoinhibition. But, since this energy dissipation ability is limited and other factors, such as temperature, UV radiation, pathogens, and others, can additionally increase the susceptibility to photoinhibition, then, over-excitation can lead to a total disruption of the photosynthetic system. Either when the Calvin cycle is interrupted or saturated, or the photosystem II (PSII) is damaged, or even when the fold/unfold mechanism of certain proteins of the

photosynthetic apparatus became slower, the whole system is no longer capable of dissipating or using all the energy/photons and these disruptions lead to the formation of reactive oxygen species (ROS) that are extremely harmful to the alga and the host. Both organisms are capable of reacting and neutralizing the damage, producing anti-oxidants or repairing proteins, but at a certain level, especially if there is a long-lasting stress, the host notices that is no longer advantageous to have this ROS producing symbiont and expels it. The release of zooxanthellae is also triggered if the photosynthesis is interrupted or reduced and the amount of photoassimilates shared with the host is lower than usual. This especially happens during extended darkness conditions, where none of the structures in the organisms are damaged but the photosynthesis stops (Douglas, 2003; Jones *et al*., 1998; Bhagooli and Hidaka, 2004; Smith *et al*., 2005; Baird *et al.*, 2009; Obura, 2009; Roth, 2014).

1.5 – Coral's Growth

One important physiological process of scleractinian corals is the ability to build up a hard skeleton of calcium carbonite (aragonite), a process named calcification. This skeleton provides structural support for the whole colony, and each polyp lives in a small depression in the skeleton, the corallites, that allows to shelter against predators when polyps retract into them. It is the aboral ectoderm cells, also known as calicoblastic cells, that produce the corallum, through the secretion of calcium ions. (Figure 2) This ions secretion is made by means of a Ca^{2+}/H^{+} ATP-ases from the cytoplasm of the cell to a fluid layer between the cell and the corallum, known as the calcifying medium. The ATP-ases work in an antiport system extracting H^{+} from the calcifying medium in exchange for CA^{2+}, and using ATP as energy to pump ions against the concentration gradient. The source of this ATP is the respiration of the compounds derived from the zooxanthellae's photosynthesis or heterotrophic feeding. Consequently, the increasing concentration of calcium ions and high pH in the calcifying medium results in a supersaturation of calcium carbonate. Thus, the calcium carbonate precipitates and creates aragonite crystals producing the skeleton. Otherwise, the carbonate concentration mechanism in the calcifying medium is unknown, but, by removing protons and increasing the pH in the medium the equilibrium favors the CO_3^{2-} instead of HCO_3^{-}. (Wijgerde, 2013; Osinga *et al*, 2011). Zooxanthellae also take an indirect role in growth, beyond the energy provision to its host, as they increase the internal pH, due to their regular photosynthetic activity,

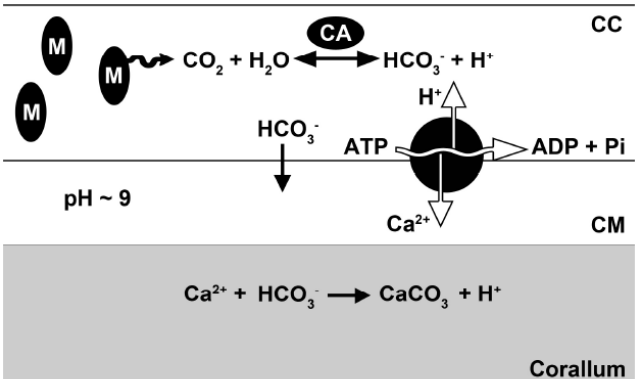


Figure 2 - Schematic overview of the calcification process in scleractinian corals. Carbon dioxide produced by calicoblastic cells' (CC) mitochondria (M) is converted to bicarbonate by the enzyme carbonic anhydrase (CA). Bicarbonate diffuses or is transported to the calcifying medium (CM). Source: Wijgerde, 2013

facilitating the formation of aragonite crystals, in what is called light-enhanced calcification (Osinga *et al*, 2011; Pearse and Muscatine, 1971).

1.6 – Factors that influence the Growth

Four major factors are recognized to be fundamental for coral growth: Light, waterflow, aragonite saturation state and nutrients. There are other factors that can negative or positively influence this process, including, pH, temperature, competition or predation, trace elements, sedimentation, oil, pollutants, sunscreens, UV radiation, dissolved oxygen, genotype, etc. (Osinga *et al,* 2011). It is important to note that each coral species has a different response to different levels of each factor and that all these factors can interact among themselves. Following, some of this factors will be briefly described.

Light

Light is a key factor on coral's growth since its symbiont is photosynthetic and that is the reason why in nature it is not usual to find photosynthetic scleractinian corals below 60m depth (Lesser *et al.*, 2010). Higher photon flux densities are positively correlated with faster skeleton growth. Up to a certain limit, an increase in light quantity will enhance photosynthetic rate that leads to more energy translocated to the host. This effect is commonly referred to as light-enhanced calcification. Usually, calcification in light is found to be 3 to 4 times higher than in darkness (Shutter *el al*., 2011). Light also affects coral quality-related aspects such as physiological condition, shape, colour and metabolite content (Leal *et al.*, 2014).

Light manipulation in *ex-situ* facilities comprises quantitative (irradiance), qualitative (light spectrum) and technological aspects (types of light sources). Changes in light regimes have demonstrated modifications in the population of zooxanthellae, the efficiency of photopigments, and all these affect the physiology and survival of the host (Rocha *et al.*, 2013; Osinga *et al.*, 2011). These photoacclimation mechanisms include modifications on zooxanthellae density, pigment concentration, pigment composition, production of photoprotectants etc (Osinga *et al.*, 2011; Leal *et al.*, 2014). Furthermore, a host growing under low light regims will try to expose more horizontal surface to the incoming light and will thus develop a more flattened shape than a specimen of the same species growing under high irradiance (Leal *et al.*, 2014). Light variations also influence the colour. Corals under low light regimes may have an increase of pigments in their symbionts resulting in brighter and more intense colours. However, in moderate or high light regimes the same can happen, since occurs the production of photoprotective molecules, such as fluorescent proteins (Leal *et al.*, 2014).

Although photoperiod is an integral part of light conditions, it will be addressed in detail in the section 1.8.

Water flow

Scleractinian corals cannot generate actively water movements; thus, they are closely dependent on ambient water flow to facilitate their metabolism. All the exchanges from the external environment are reliant on the flow, that enhances the exchange of gasses, dissolved compounds, and food. Without a proper flow, the depletion of resources and the accumulation of toxic waste products compromise the survival of the coral. In addition, flow inhibits the settlement of sessile organisms on the coral's body surface and removes any sediments and particles. Otherwise, high flows can have negative effects on corals, namely the deformation of the polyp's shape reducing its predation efficiency (Osinga *et al*, 2011; Leal *et al*, 2014).

The Aragonite Saturation State

The Aragonite Saturation State is the product of the concentration of the dissolved calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions divided by the temperature dependent solubility of the aragonite, which is represented by **Ω**. Both these crucial components to calcification are actively concentrated into the calcifying fluid as it was explained above. **Ω** and pH are, in the calcifying medium, well above the sea water levels. Thus, it is stated that the concentration of this ions is positively correlated with calcification. Although in nature Ca^{2+} concentration is stable, in an aquarium, the amount of calcium is rapidly absorbed by the corals growing. In the case of carbonate, it is absorbed even faster and contrarily to calcium, the availability of carbonate in the seawater varies depending on biological and chemical processes that occur in the environment, particularly processes that change the pH. The pH strongly determines the concentration of carbonate, and that is why the monitoring of the pH is very important in an aquarium. For this reason, ocean acidification is threatening corals in nature because lower pH decreases CO_3^{2-} availability in seawater (Osinga *et al.*, 2011; Comeau *et al.*, 2013; Ohde and Hossain, 2004; Marubini *et al.*, 2001).

Inorganic Nutrients

Organic and inorganic nutrients are very important for the metabolism and for the growth for the partners of the holobiont. The inorganic nutrients such as nitrogen and phosphorus are used as blocks for the synthesis of proteins and other organic components. Although the algae can obtain these nutrients directly from the sea water, the coral obtains them mainly by ingestion of food and by translocated compounds from zooxanthellae. But if the dissolved inorganic nitrogen (DIN) is limited the translocated food from the algae to the coral is poor in nitrogen and only provides metabolic energy to the host, missing the necessary building blocks for the biosynthesis. Many authors have

stated that the addition of DIN improves the overall performance of the holobiont by augmenting the zooxanthellae's growth and increasing the concentration of pigments. However, if the concentration of DIN increases above the natural ambient concentrations it can have a negative effect on corals skeleton growth. The dissolved inorganic phosphorus (DIP) is important to be in balance with the DIN, otherwise, if added without a corresponding increase of DIN, it leads to the formation of polyphosphate crystals that have a negative impact on the coral's growth. Beyond DIN and DIP, iron and zinc also influence the growth of the holobiont. Iron and zinc also benefit the coral's growth when their amounts increase, but like DIN, when they reach concentrations above natural environmental levels they are harmful. Both play a role as components of many enzymes. Zinc, for example, makes part of carbonic anhydrase. This enzyme, in particular; that is used to capture dissolved inorganic carbon, is important both for photosynthesis and calcification. Nevertheless, high amounts of zinc can lead to adverse effects on growth due to the formation of toxic free radicals, that are harmful to zooxanthellae (Osinga *et al.,* 2011).

Organic Nutrients

Heterotrophic feeding of scleractinian corals, as was previously summarised, takes the role of supplementing the holobiont with organic nitrogenous and phosphate compounds. Contrarily to the addition of DIN, that can inhibit the growth, feeding can stimulate positively both members of the holobiont, because organic food provides the nitrogen, carbon, and phosphorus in the right ratio and does not interfere with the nutritional balance (Osinga *et al.*, 2011; Leal *et al.*, 2014; Wijgerde, 2013). Several species were recorded to have improvements in their growth rates when fed, besides improving its resilience to stress and its tissue-skeleton ratio that is critical for drug production (Leal *et al.,* 2014; Wijgerde, 2013).

Other factors

Others factors like competition, predation, pollutants, UV radiation etc, are especially important in nature or in *in-situ* production, and so, they will not be addressed in the present document. Furthermore, genetic factors also influence coral growth rate. In aquaculture facilities, corals are reproduced asexually by propagation, producing several identical clones from the original colony. All the clones, from the same original colony, are genetically identical, having the same set of genes – it means that all them belongs to the same genotype. Each genotype grants different responses to the environmental conditions, besides having different strategies, with some genotypes investing more in growth while others invest in disease and stress resistance. In production facilities it is common to use the same genotype to produce several clonal colonies what brings advantages for replicability purposes. In addition, the genetic variability of zooxanthellae also increases the variety

of responses of the holobiont to the environment (Osinga *et al.*, 2011. Leal *et al.*, 2014; Muller-Parker *et al.*, 2015). Dissolved oxygen (DO) is a large lacuna in coral's growth knowledge mainly, due to the complexity of working with low DO levels (Osinga *et al.*, 2011). The other factors that can influence the coral's growth, such as temperature and photoperiod, that are the main topic of this project are addressed in the following sections.

1.7 – Temperature

Comparatively to other factors, the effect of temperature on coral growth and metabolism was scarcely studied as most of the studies focused on natural environment events and bleaching of reef communities (Gates *et al*., 1992; Jones *et al.*, 1998; Coles and Fadlallah, 1991; Hoegh-Guldberg and Fine, 2004; Smith *et al.*, 2005). In general, all these studies refer to a typical bleaching event associated with thermal shocks and in some severe situations, mortality of the corals. Furthermore, most of this studies are focused on the impact of high-temperature stress, with low-temperature stress mostly ignored (Kemp *et al.*, 2011). In production systems, lower temperatures can prevent possible disease outbreaks since pathogens increase their growth and virulence at higher temperatures (Sheridan et al., 2013). As stated previously (see section 1.4), the temperature shocks lead to zooxanthellae's photoinhibition and photodamage and induce its release by the host, and this seems undoubtedly to be the major consequence of thermal stress (Hoegh-Guldberg and Smith, 1989, Jones *et al.*, 1998). The same pattern was observed in other zooxanthellae symbiotic cnidarians (Steen and Muscatine, 1987; Muscatine *et al*., 1991; Hoegh-Guldberg *et al.*, 2005) High temperatures reduce the tolerance to photoinhibition (Bhagooli and Hidaka, 2004) and the resistance to heat stress depends on zooxanthellae genotype and on the capability of the host to react to that stress and its associated damages, that varies from species to species (Fitt *et al.*, 2009; Flores-Ramírez and Liñán-Cabello, 2007). The symbiotic corals are geographically limited to regions where the water temperature does not drop below 18ºC (Saxby *et al.*, 2003). Most of the conducted studies with low-temperature stress reported similar responses as for high-temperature stress (Jokiel and Coles, 1977; Saxby *et al*., 2003; Hoegh-Guldberg *et al*., 2005; Roth *et al*., 2012). Published research on the effects of low temperatures in individual colonies under controlled conditions is to our knowledge scarce (Jokiel and Coles, 1977; Coles and Jokiel, 1977; Reynaud *et al.*, 2004; Saxby *et al*., 2003; Al-horani, 2005; Roth *et al*., 2012) , however, the results are consistent with the field observations of cold condition events: bleaching effect (Hoegh-Guldberg and Fine, 2004; Coles and Fadlallah, 1991).

Overall, temperature-dependent growth in corals follows a bell-shaped curve (Figure 3) with a maximum that corresponds to the optimum and with the extreme temperatures having detrimental effects on growth. Jokiel and Coles (1977) observed lower growth rates at 21-22º C and rapid mortality at 18ºC in several Hawaiian coral species. In addition, the same authors observed strong regressions between temperature with photosynthesis and respiration (Coles and Jokiel, 1977). Saxby *et al*. (2003) observed that cold temperatures have a negative effect on *Montipora digitata*, decreasing its photosynthetic efficiency, loss of zooxanthellae and pigments, and even bleaching and death. Nonetheless, moderate cold stress resulted in acclimatory responses of the holobiont. They also observed different responses according to the light regime, being the corals exposed to low light regime less affected by thermal stress (Saxby *et al.*, 2003). Roth et al. (2012) observed similar patterns, with both, higher and lower temperatures (± 5ºC), resulting in slower growth, loss of zooxanthellae. However, they suggest that long-term high temperatures exposure have stronger negative effects on *Acropora yongei* than long-term cold temperatures; the corals at higher temperatures stopped growing and bleached while the lower temperature ones kept a good number of zooxanthellae and grew. They also observed some level of acclimation by the coral at lower temperatures (Roth *et al*., 2012). In contrast, Jokiel and Coles (1977) had opposite results when studying Hawaiian corals and observed stronger harmful effects to colder water stress than to higher temperatures (± 4º C) (Jokiel and Coles, 1977). Marshall and Clode (2004), compared the calcification rate of a zooxanthellae coral and an azooxanthellae coral along a temperature range (18-29ºC), observed a similar temperature dependence in both corals (Figure 3) and that the temperature influences calcification independently from light exposure. They suggest that the Calcium-ATPase has a temperature dependent activity and it shows consistency with the consensual thermic optimum to tropical coral's growth (25-27ºC) (Marshall and Clode, 2004 and their references). Reynaud *et al*. (2004) observed higher growth rates and Strontium/Calcium incorporation with higher temperatures (20-29ºC) in *Acropora verweyi* (Reynaud *et al*., 2004). Many of these works also indicate that the time of exposure of the organism to the stress, the magnitude of the stress factor, and the thermal history of the specimens have and important role in the response of the organism to the thermic changes, with usually the sudden and long-term changes that cause

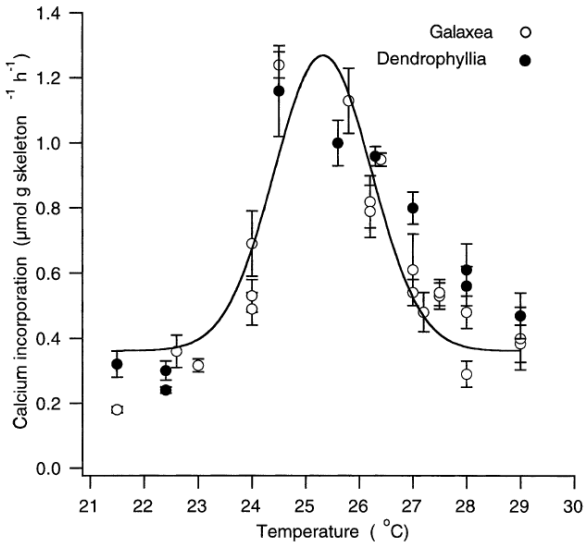


Figure 3 - Calcification rate (measured as calcium incorporation per unit mass of skeleton) along a temperature range in a zooxanthellae coral (*Galaxea fascicularis*) and an azooxanthellae coral (*Dendrophyllia* sp.). Source: Marshall and Clode, 2004

serious effects (Jokiel and Coles, 1977; Coles and Jokiel, 1977; Coles and Fadlallah, 1991; Clausen and Roth, 1975; Saxby *et al*., 2003; Edmunds, 2009; Howe and Marshall, 2002; Roth *et al*., 2012).

1.8 - Photoperiod

The positive correlation between light and growth is already well studied: larger quantity of light results in better growth, until the photoinhibition trigger point. Higher photon flux densities are positively correlated with faster skeleton growth. Up to a certain limit, an increase in light quantity will enhance photosynthetic rate that leads to more energy translocated to the host.

To provide more light to a rearing system, usually, that can be achieved by increasing the light irradiance, however, the quantity of light available for zooxanthellae is not only established by the intensity of the source but also by the length of the stimulus – the photoperiod. At the moment, almost no research has been conducted on the effects of photoperiod on coral growth and physiology and there is no information about the optimal number of hours of light per day that corals need. Schutter *et al.* (2011) did the first attempt to reveal such effects. The authors theorized that increasing the length of the photoperiod corals would increase their daily growth rate. They tried different combinations of light irradiance and photoperiods, but, their results were not conclusive, since the growth was not significantly different between *Galaxea fascicularis* grown at 8 and 16 hours light. Nonetheless, they note that the corals had the ability to adapt to extended photoperiods (Schutter *et al.*, 2011).

2 – Objectives

As was described previously, light and temperature play a strong role in the physiology of corals. According to the presented state of art, it can be observed that temperature and light regimes are closely related and interact in the coral's physiology. Understanding the interaction between these factors is very important to improve the *ex-situ* production of these organisms. The aim of this thesis project was to assess the effects of different temperatures and modifications in the photoperiod, on the growth and physiology of corals. In addition, the interaction between these two factors were also analysed.

Saxby *et al.* (2003) observed that corals exposed to cold temperatures and low light regimes had less detrimental effects than their warmer temperatures or high light counterparties. Thus, is it possible to hypothesize that low light regimes increase the tolerance of corals to thermal stress and can shorter photoperiods be an approach to achieve this tolerance? The present work will address these questions and the obtained answers could help to provide better production protocols and increase our knowledge in corals physiology and capacity of acclimation. Several approaches, beyond the growth rate measures, will be taken into account to achieve these objectives. They will be, the

zooxanthellae's density, to obtain the condition of the symbiotic relationship; chlorophyll fluorescence measurements, to assess the damage and efficiency of the photosynthetic apparatus, and pigments absorbance's will be measured to determine the amount of different pigments: chlorophylls, carotenes, and xanthophylls. Many of these pigments not only take a role in photosynthesis but also work as photo protectors and antioxidants, which allows the evaluation of the response and aclimation to light and thermal stress.

The main questions of this project are:

- **How do corals respond to different photoperiods?** – This is largely unknown in contrast to variations in light intensity.
- **How do corals respond to cold water induced stress?** – Will they respond similarly to heat stress?
- **Do photoperiods and temperatures have any interaction on coral's physiology?** – Are modified photoperiods able to neutralize the effects of thermal stress? Corals at higher latitudes are susceptible to suffer changes in these abiotic factors both in summer and in winter.
- **Can different combinations of photoperiod and temperatures optimize coral aquaculture? –** Improvement of growth rates and reduction of production costs.
-
-

3 - Materials and Methods

3.1 – The Corals

One species of stony coral was chosen to be part of this experiment - *Stylophora pistillata* (Esper, 1797)) - an Indo-Pacific species. This *S. pistillata* clone was originally from Eilat Gulf (Israel) and the colonies were at coral's lab of Wageningen University. They all belong to the same genotype. These coral colonies one year before, when nubbins (10 polyps clones), were fixed with aquarium epoxy to square shaped 5×5cm PVC plates and put in a 360L culture tank prior to start of the experiment. Light in this tank ranged from 100 to 200 μmol quanta m^{-2} $s^{-1.}$ with a 12L:12D photoperiod (12 hours light:12 hours dark). Tanks were maintained at a constant temperature of 25-26ºC and a salinity of 34.0-35.5 ppt (artificial seawater - Tropic Marin: type = Zoomix). For the present experiment, 96 colonies were used and randomly distributed into the experimental tanks. Colonies are identified by a number written in their PVC plates.

<u>Ethical Note:</u> None of the specimens used in this experiment was collected from nature. All of them were captive breed in ex-situ facilities for research and/or educational purposes.

3.2 – Experimental design

The experiment was conducted in the aquatic animal's facilities at Wageningen University. In the present work 3 different photoperiod regimes and 4 different temperatures, were combined and tested resulting in 12 different treatments. Each treatment had two replicates, resulting into 24 tanks for the whole experiment. The photoperiod regimes were 8L:16D; 12L:12D and 16L:8D and the temperatures were 20º, 23º, 26º, and 29ºC (Table 1). The treatment 26ºC 12L:12D was set as control treatment since these are the same conditions kept in the nursery tanks. All experimental tanks were part of a single recirculation system. The recirculation system consisted of 24 experimental tanks (20L), a water storage tank (300L) and a bypass for chilling water (≈20ºC). Water was pumped from the water storage tank to a height above the experimental tanks where it is distributed along the tanks. The inflow in the tanks was set to accomplish a turnover rate of the tank's volume per day (approximately 65 mL/min). The outflow was discharged by hoses to the storage tank by gravity. Water of the storage tank was maintained at ≈23ºC resorting to chilling machines. The tanks were placed in shelf and separated per photoperiod regime. Each tank was conditioned to the respective temperature. The tanks at 23ºC were at the same temperature of the storage tank water; tanks at 26º and 29ºC were heated using aquarium heaters (50W) while the 20ºC treatments were chilled by a chilling machine that makes part of a bypass in the system, specifically to chill the water for this treatment (Figure 4). To attain temperature stability, each tank was placed into a Styrofoam box, itself filled with water forming a bath (Figure 5). All the temperatures were monitored at every 2 days. The water storage tank had a foam fractionator (skimmer) and a UV filter. An irradiance level of 140-160 μmol quanta m^{-2} s^{-1} was provided by 187W Philips CoralCare LED lights and the photoperiod settled using the software from the Philips' lights. The water flow inside experimental tanks was created by an aquarium pump, compact 1000 (Eheim®, Germany). The corals were fed 3 times a week with 2 ml of *Artemia nauplii* hatched from cysts (Great Salt Lake *Artemia* cysts, *Artemia* International LLC, Fairview, USA; at a salinity of 25 gL^{-1} and a temperature of 28◦C and used immediately after hatching).

Table 1 – Experimental treatments

	Photoperiod Regime			
Temperatures		8D:16L	12L:12D (Control)	16L:8D
	20ºC	8D:16L – 20ºC	12L:12D – 20ºC	16L:8D – 20ºC
	23ºC	8D:16L – 23ºC	12L:12D – 23ºC	16L:8D – 23ºC
	26ºC (Control)	8D:16L – 26ºC	12L:12D – 26ºC (Control)	16L:8D – 26ºC
	29ºC	8D:16L – 29 ºC	12L:12D – 29ºC	16L:8D – 29ºC

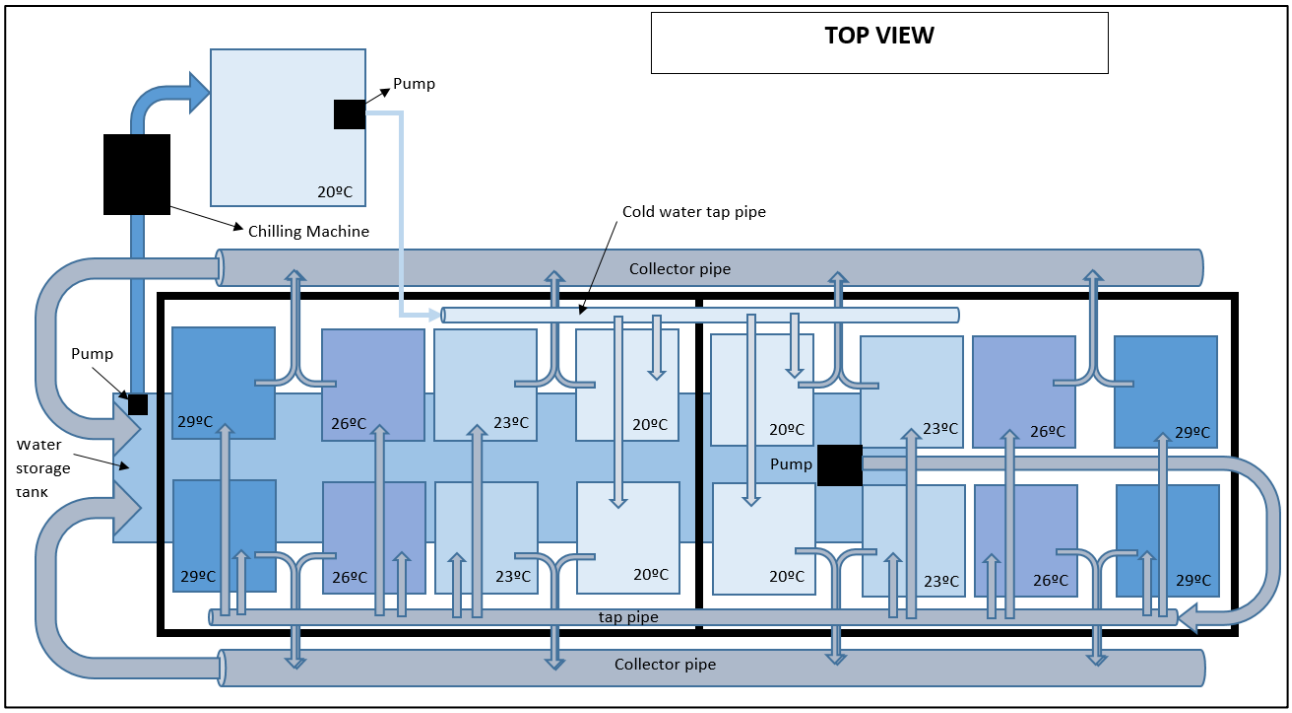


Figure 4 – Top view of the experimental setup. This scheme represents only 16 of the 24 experimental tanks. The shelf where the experiment was conducted has 2 levels. Each set of 8 tanks had a different photoperiod.

3.3 – Sampling: Parameters of coral's growth and condition

Data collection was taken at the beginning of the experiment (time 0) and according to the analysis in intervals of 2 or 4 weeks. The measurements took a whole week since it was not possible to do all the measurements for all colonies in a single day. The experiment started after a 3-week period: first, one week of acclimation of the corals to the new system without treatment conditions. One week later, was used for the initial data collection (time 0) without treatment conditions, lastly, in the final week to slow transition to each experimental treatment. The experiment was planned to last for a minimum of 3 months (or 84 days – 7 days x 4 weeks x 3 months).

Growth Rate

Buoyant weight (BW) is a practice to weight hanging the live sample by a threat (usually hooked to the PCV plate) connected to a balance. The coral is submerged in a water and the weight is recorded, avoiding stress due to air exposure. BW is good estimate of skeletal weight since coral tissue has a density which is close to that of seawater and does not contribute significantly to the buoyancy. Tissue represents only 1% of the total buoyant weight (Schutter *et al.*, 2008). To measure coral skeletal growth, the increase in buoyant mass was used. In this procedure, according to Schutter *et al.* (2008), coral colonies are taken from their experimental tank and suspended on a nylon thread with a hook, attached to an analytical balance in a predefined volume of seawater (35ppt) at a constant depth. The temperature of this seawater was at 25ºC to standardize the water density for all measurements. Averages of at least 3 measurements were used to create an estimate of buoyant mass. Prior to the attachment of coral nubbins to their PVC plate, the buoyant mass was measured and compared to the buoyant mass of coral nubbins attached to their PVC plate. This was done, in order to extract the mass of the PVC plate and glue from later measurements. Previous data of PVC plates was already available for the colonies of *S. pistillata*, to be used in this experiment. Buoyant mass was used to calculate the specific growth rate (μ) using the following formula;

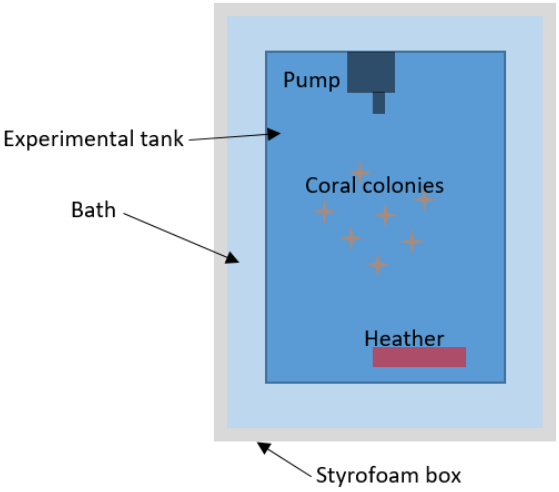


Figure 5 – Experimental tank setup.

$$\text{Specific Growth Rate } (\mu) = (\ln BM_t - \ln BM_{t0})/\Delta t$$

where the specific growth rate is expressed in BM day^{-1}. BM_t is the buoyant mass at the end of a growth interval, BM_{t0} is the buoyant mass at the start of a growth interval and Δt is the time of the growth interval in days.

Metabolic Rates

Net photosynthesis and dark respiration were measured by means of intermittent flow respirometry in a respirometric flow cell according to Schutter *et al.* (2008). Three colonies of each tank, were randomly chosen and used for the measurements. Each colony was placed in the respirometric flow cell for 60 minutes. The water in the flow cell is originated from the experimental recirculatory system and it was kept at a temperature identical to each treatment and renewed after each measurement. A magnetic stirrer was used to ensure adequate mixing and to simulate the

situation in the tank environment. Oxygen levels within the enclosure were recorded, by means of a luminescent oxygen probe (Hach). Oxygen consumption was measured in the dark and oxygen production was measured under similar light conditions to the experimental tanks (140-160 μmol quanta m^{-2} s^{-1}), during 30 minutes each (total of 60 min of incubation). Oxygen consumption/production was then be calculated using the following formula:

$$R_d/P_{net} = ((V_{cell} - V_{coral}) \text{ x slope})/ \text{ BM (mg } O_2\text{/gBW/min)}$$

Where R_d/P_{net} is the rate of dark respiration or the net photosynthesis (μmol O_2/min/gBW); V_{cell} is the volume of respirometric flowcell (l); V_{coral} is the volume of coral; the slope is the regression coefficient of dissolved oxygen against time (μmol O_2/min), and BM is the buoyance weight of coral (g). Surface-area was not used, as determination of the surface-area is believed to be very hard to measure for the branching *S. pistillata*. Later the daily photosynthesis/respiration ratio was calculated:

$$\text{Daily}\frac{\text{P}}{\text{R}}\text{ratio} = \frac{\text{Pnet x hours of ligh/day} - \text{Rd x hours on dark/day}}{\text{Rd x hours on dark/day}} \text{ (Dimensionless)}$$

The measurements were performed in the week before the beginning of the treatment conditions.

Chl a fluorescence measurements

Chl *a* fluorescence was measured using a pulse amplitude modulated (PAM) fluorometer according to Saxby *et al.* (2003). The fluorimeter was used to measure the minimal (F_0) and maximal (F_m) fluorescence yields. The measurements were done during the morning period, approximatey one hour after the start of the photoperiod. Fluorimeter optical head was placed perpendicularly adjacent to the surface of the coral and fluorescence measured. 9 measurements were randomly obtained in the colonies of each tank. The measurements were performed at the start of the experiment and then, biweekly. The fluorimeter gives the quantum yield (F_v/F_m), the ration between variable fluorescence (F_v) and maximum fluorescence (F_m). This value provides a good approximation of the maximum photochemical efficiency of Photosystem II (PSII).

Zooxanthellae density

At the end of the experiment, 3 random branches of coral from each tank were cut and used as samples. Then the tissue from each branch was removed using high pressured air. The resulting slurry from each sample was homogenized by shaking it in 10ml of seawater. Samples were then centrifuged for 10 min at 3000 rpm and the supernatant was rejected after and the pellet was re-suspended in 2ml of seawater. Zooxanthellae were counted (3 replicate counts) using a counting chamber assembled in an inverted microscope and using an image software Fiji ImageJ.

Zooxanthellae density was expressed as the number of zooxanthellae per cm^2 surface area. Surface area was determined by, covering the branches with a single layer of aluminium foil. The foil was removed and subsequently weighted. The area was then calculated comparing with the weight of a $1cm^2$ foil.

Pigment content analysis

Using the same homogenate obtained from zooxanthellae isolation, a 0.5ml aliquot homogenate was taken to determine the content of chlorophylls *a*, *b* and *c*, carotenes and xanthophylls. To obtain it, 4.5ml of acetone was addes to this aliquot and then was placed in a freezer (–20°C) for 24 h. The solution absorbance's were determined at 664, 630 and 750 nm on a spectrophotometer to determine chl-*a*, chl-*c*, and turbidity, respectively. The same method was applied to measure peridinin, diadinoxanthin, dinoxanthin, and *β*-carotene concentrations with respective wavelengths; 442, 447, 466, and 454 nm (Jeffrey and Haxo, 1968). The concentrations of chlorophyll-*a* and chlorophyll-c were calculated according to the equations given by Jeffrey and Humphrey (1975) for dinoflagellates.

3.4 – Statistical Analysis

A 3-way repeated measures mixed ANOVA was conducted in SPSS software (IBM Corp., 2016) to determine differences between treatments for P/R ratio and measurements of photosynthetic efficiency. The three factors used in this test were photoperiod, temperature and, time. A 2-way ANOVA was used to test the significance of zooxanthellae density and pigments content, as well as to growth rate and hourly production/consumption of oxygen (one test for T_0 and another for T_{30}). Before, a F-test and, subsequently, a T-test were performed to access the homogeneity of variances and differences in means, respectively, between replicates in order to evaluate the presence of tank effects. The 2-way ANOVAs used the tanks as experimental unit. Only zooxanthellae density data used each fragment sample as experimental unit, instead of the tanks, which due to, no significant tank effects were found. When significant differences were found, a Tukey's post hoc test was used to attribute differences between specific treatments. A confidence level of 95% was used in all analyses.

4 - Results

Disease outbreak

On the 8th week of the experiment, the rapid tissue necrosis (RTN) was detected in some colonies. This unexpected event led to the abortion of the experiment, and to a fast collection of samples of remaining

healthy tissue. It also led to small changes in the zooxanthellae extraction protocol, since the samples were kept frozen, after collected, at -20ºC for later extraction and analysis. It is relevant to report that the disease was firstly detected in the tanks at 29ºC. In the following days, it was detected in the tanks at 26ºC. This disease is characterized by fast tissue degradation (peeling) and death of the colony. It is suggested to be caused by *Vibrio harveyi*, a bacteria that lives in the coral tissue (Luna *et al.*, 2007).

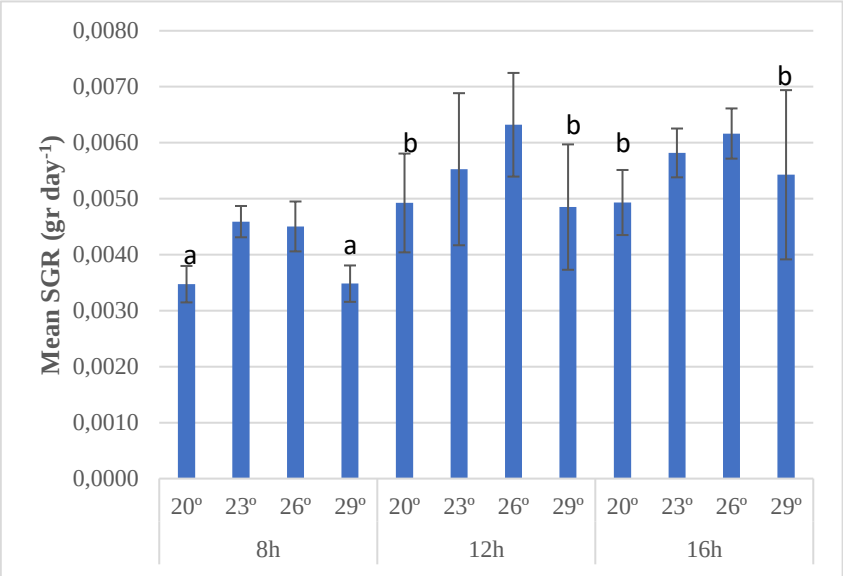


Figure 6 – Specific growth rates (SGR) for each experimental treatment after 37 days of experiment. n = 3.

Specific Growth rates

The overall mean specific growth rate (SGR) of the experiment was 0.005±0.00088 grams day^{-1} after 37 days. No later record of the BW was made because of the unexpected disease outbreak. The different experimental groups grew at different rates (Figure 6). A significant main effect of photoperiod was observed (Table 2): The 8 hours photoperiod treatments had a significant lower SGR compared with the other photoperiods; SGR at 8 hours light was 25,7% lower than at 12 hours light and 28,2% lower than at 16 hours light (p-value = 0.000 < 0.05 in Tukey's test). *S. pistillata* grew similarly (p-value = 0.804 > 0.05) at the 12 and 16h light hour regimes, showing similar SGR, respectively.

The ANOVA test indicates a significant temperature effect (Table 2). Treatments at 20º and 29º were not significantly different as p-value = 0.957 > 0.05. These temperatures induced a lower SGR in all photoperiods. Statistically the for all photoperiods. SGR at 23º and 26º were not significantly different as p-value = 0.671 > 0.05 between them and both were different from 20ºC (p-value = 0.039 < 0.05 and p-value = 0.001 < 0.05). Additionally, 26º and 29º were also significantly different: p-value = 0.007 < 0.05). The highest mean of SGR was observed in the control treatment – 26ºC 12h – however, statically it did not differ from the other 23ºC or 16h treatments (Table 2). No interaction was found between photoperiods and temperatures (p-value = 0.891 > 0.05)

Metabolic rates

In the beginning of the experiment, photoperiods were significantly different for production and O_2 consumption (Table 3). The 8h photoperiod was significantly different from 12h in terms of oxygen production (p-value = 0.039 < 0.05), while 12h and 16h were different regarding consumption (p-value = 0.013 < 0.05) (Figure 7).

After 30 days, the longer photoperiod treatments (12 and 16h light) showed a great negative effect on metabolic rates. Hourly net photosynthetic rates in both cases were strongly reduced while respiratory rates, though they also decreased, were less strongly affected. (Figure 7 and Table 4). According to Tukey's tests (Table 3) all comparisons between photoperiods were significant for O_2 production. No significant differences were detected between photoperiods regarding respiration rates. Initially, temperatures were significant for consumption and not significant for production (Table 3). Between 20º and 23ºC was detected significant differences (p-value = 0.035 < 0.05). 30 days later, temperatures were not significant for consumption and significant for production. Photosynthetic rates for treatments at 20ºC were different from 26ºC (p-value = 0.001 < 0.05)

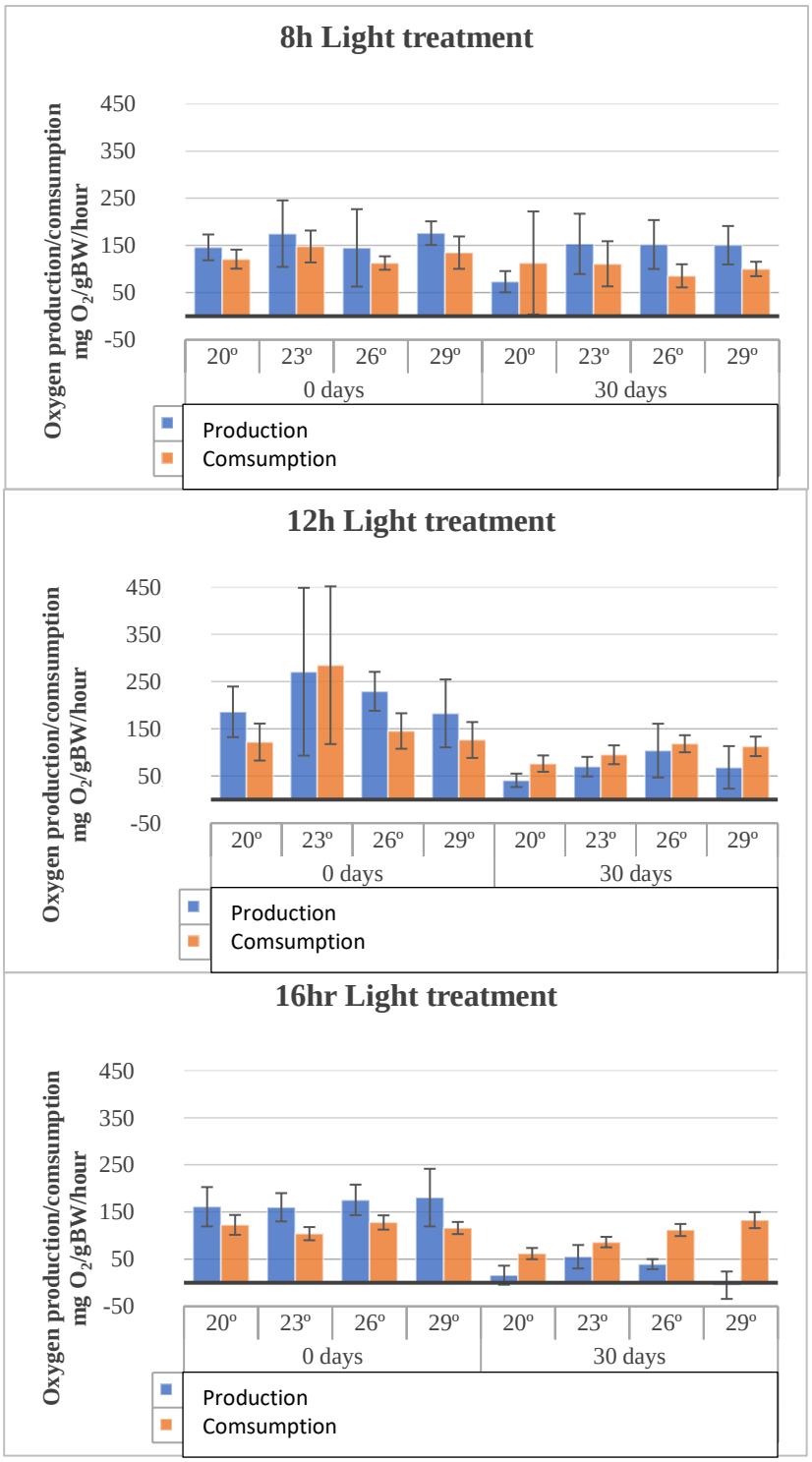


Figure 7 – Hourly net photosynthesis for every treatment at T_0 and after 30 days of experiment. In this analysis, the future treatment for each tank was predefined and they were analysed as separated treatments, though at T_0 no treatment conditions were running. *Blue = production; Orange = consumption;* n = 3.

The treatment 29ºC/16h was the only one with a negative photosynthetic rate after 30 days (Figure 7). It is also noteworthy that in the 12h photoperiod at the start of the experiment the several treatments showed strongly variable rates, and higher rates than the other photoperiods treatments. Moreover no treatments were, in fact, running at this point. Additionally, the control treatment (26ºC 12h light) was also affected, showing a

strong decrease after 30 days in both photosynthetic and respiratory rates. In the shorter photoperiod treatments(8h) the metabolic rates seem to be almost no affected. The only exception was the 20ºC treatments that had a relevant reduction in their photosynthetic rates.

Concerning the P/R ratio no significant effects both for temperature and photoperiod were observed, but there was a significant effect over time: p-value = 0.000 < 0.05. The test shows significant interaction between temperature and photoperiod with p-value = 0.012 < 0.05 (Table 3). Both values, in the beginning, and one month later, were quite variable (Figure 8). In the beginning, with no treatments running, one group displayed a negative ratio of -0.088 (23ºC 12L), and the remaining groups show a large range of values. The highest ratio was observed in the treatment 23ºC 16h with a value of 0.87. The overall mean of the ratio between net photosynthesis and dark respiration was 0.406±0.251 After 30 days the major tendency observed is that almost all the treatments got a negative ratio. Only the treatments 23ºC 16h and 26ºC 8h kept a mean positive ratio. The treatment 29ºC 16h had the most negative value. The overall mean was -0.344±0.0344.

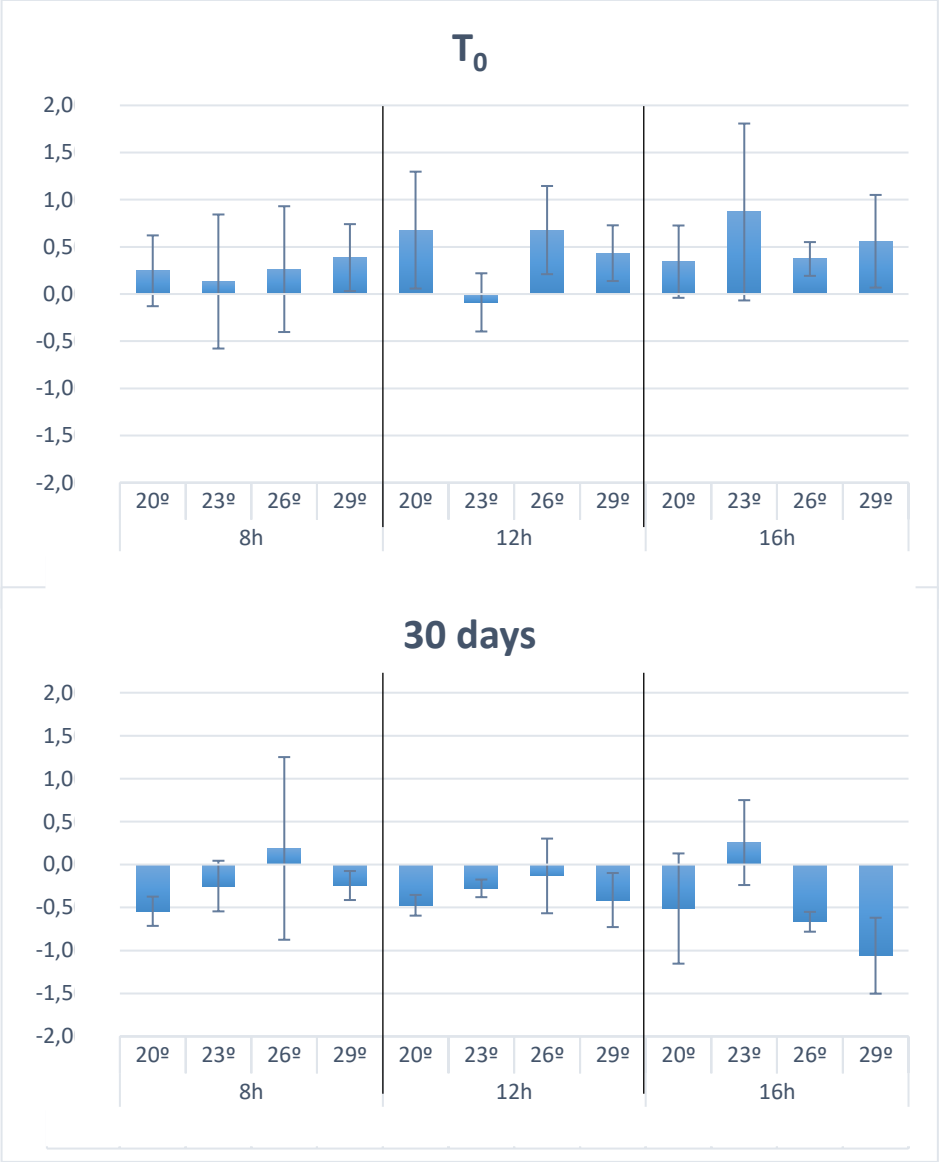


Figure 8 – Mean P/R ratio at T_0 and after 30 days of experiment for each treatment. In this analysis, the future treatment for each tank was predefined and they were analysed as separated treatments, though at T_0 no treatment conditions were running. n = 3.

Photochemical efficiency of PSII.

The effects of both temperature and photoperiod were significant and a strong interaction occurred between both: p-value = 0.000 < 0.05 and significant differences between all the photoperiods and between the temperature 20ºC with the 23º and 26ºC were observed (Table 2).

The average photochemical efficiency (PE) of the PSII obtained in the beginning of the experiment was 0.64 ±0.03. Along the experiment was observed a great decay of the PE in the 16 hours treatments (Figure 9). At 30days it reached 0.55±0.098. This decrease was already observable after 15days of the experiment. Inside the 16hours photoperiod regime, the corals that seemed to be more affected were the ones exposed to 20º and 29ºC. The remaining photoperiods kept a high PE except for the treatment 20ºC 12h, that had a great decrease, similar to the 20ºC 16h treatment.

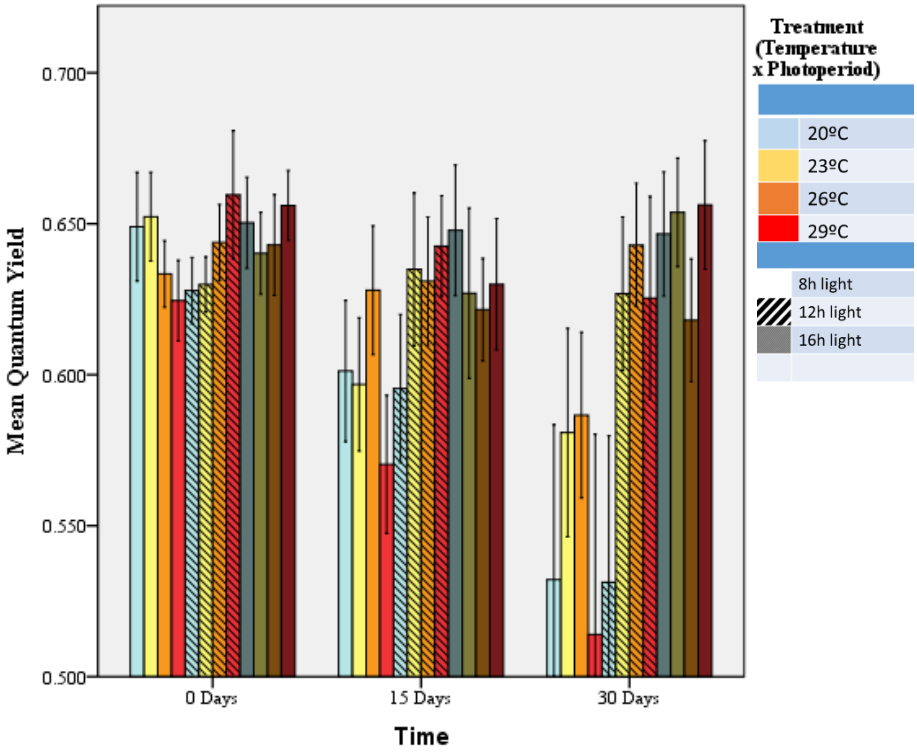


Figure 9 – Quantum yield efficiency of each treatment at T_0. 15 and 30 days of experiment. n = 9

Zooxanthellae Density.

Strong effects of both temperature and photoperiod were found p-value = 0.000 < 0.05 (Table 5). The interaction between photoperiod and temperatures was significant (p-value = 0.036 < 0.05) The shorter photoperiod was different from 12 and 16h (p-value = 0.031 and 0.000 < 0.05). Treatments at 20ºC have differed from the other temperatures (p-value = 0.000 < 0.05) as well 23ºC was different from 26º and 29ºC, and the zooxanthellae density varied significantly between treatments (Figure 10). The 8h photoperiod was the one with higher mean density – 1003939.1 cells per cm^2(±205493.81). The other photoperiods had temperature groups that suffered a large reduction in the number of cells, which lead to lower mean values; 869962,9(±306666.74) and 751184.3(±307412.62) for the 12h and 16h respectively. It represents a reduction of 13.35% and 25.18% in zooxanthellae density respectively. Regarding the temperatures, is

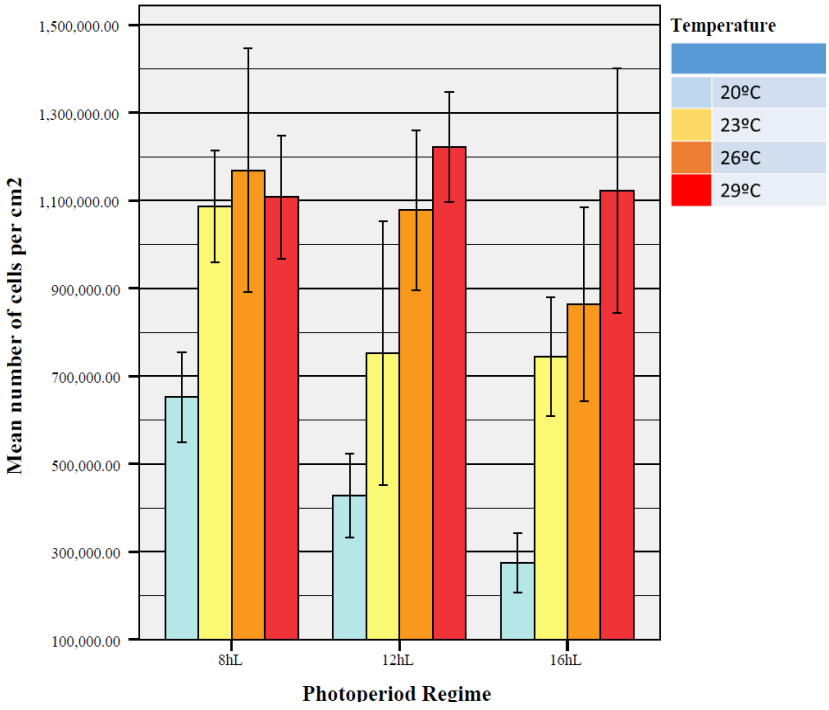


Figure 10 – Zooxanthellae density for each treatment after 30 days of experiment. n = 3

notorious that the lower temperature (20ºC) had the strongest reduction in the number of cells independently of the photoperiod. In addition, concerning this temperature it is visible the same effect of the photoperiods; the longer the photoperiod, the lower the zooxanthellae density. In the longer photoperiods (12 and 16h) a strong effect of the temperatures was observed, with a significant increase in the number of zooxanthellae following the increase in temperature.

Table 2 – Statistical results of Growth rate and Photochemical Efficiency PSII

	Growth rate	***Photochemical Efficiency PSII***
Temperature	.000**	.006*
Photoperiod	.000**	.000**
Time		.000**
Temperature x Photoperiod	.891	.000**
Tukey's test		
8h – 12h	.000**	.004*
8h – 16h	.000**	.000**
12 – 16h	.804	.000**
20º - 23º	.039*	.014*
20º - 26º	.001*	.010*
20º - 29º	.957	.258
23º - 26º	.671	1
23º - 29º	.124	.620
26º - 29º	.007*	.551

Table 3 – Statistical results of O_2 Production/Consumption and Photosynthesis/Respiration ratio. In this analysis, the future treatment for each tank was predefined and they were analysed as separated treatments, though at T_0 no treatment conditions were running

	O_2 Production hr^{-1}		***O_2 Consumption hr^{-1}***		***P/R ratio***
	T_0	**30 days**	**T_0**	**30 days**	
Temperature	.557	.001*	.022*	.146	.157
Photoperiod	.032*	.000**	.012*	.939	.966
Time					.000**
Temperature x Photoperiod	.656	.102	.007*	.155	.012*
Tukey's test					
8h – 12h	.039*	.000**	.065	.988	.991
8h – 16h	.919	.000**	.796	.933	.962
12 – 16h	.095	.001*	.013*	.976	.990
20º - 23º	.481	.003*	.035*	.752	.530
20º - 26º	.886	.001*	.988	.408	.299
20º - 29º	.936	.187	.998	.113	1
23º - 26º	.892	.976	.077	.942	.977
23º - 29º	.830	.399	.055	.575	.467
26º - 29º	.999	.203	.999	.888	.251

Table 4 - Overview of respirometric parameters of corals maintained at the different photoperiods. Presented for 26ºC

	8h				12h				16h			
	26º		All		26º		All		26º		All	
	Mean	StD	Mean	StD	Mean	StD	Mean	StD	Mean	StD	Mean	StD
T_0												
Hourly Net Photosynthesis	144,9	82,07	160.49	59,15	229,64	41.3	217,41	108,02	175,72	32,37	169,37	43.98
Dark Respiration	112,77	14,30	129,09	30.23	145,38	37,65	169,69	112,26	127,81	15,1	117,67	26,11
Daily net Photosynthesis	385,55	921.14	376,75	75,33	1011.09	775,04	572,64	443.28	574,97	284	620.44	115,62
Daily Respiration	1353.22	171.63	1549,12	362,76	3259,44	2132,64	2067,66	1428,24	1533.71	181.15	1411.99	313.36
P/R ratio	0.26	0.67	0.26	0.09	0.68	0.47	0.43	0.31	0.37	0.18	0.54	0.21
30 days												
Hourly Net Photosynthesis	151.98	51.9	132,26	58,34	103.90	57,02	70.74	44,49	39,48	10.65	26,35	31.97
Dark Respiration	85,58	24,57	102,41	62,33	118,41	18,11	100.66	25,23	111.81	12,86	98,16	29,85
Daily net Photosynthesis	-153.42	739,23	-580.5	395,34	-174,07	634,99	-359,02	134,79	-262,73	91.7	-363.62	486,61
Daily Respiration	1369,25	393.08	1638,59	997,32	1230.95	344,95	1182,08	308,34	894,47	102,88	785,29	238,83
P/R ratio	0.19	1.06	-0.21	0.26	-0.13	0.43	-0.32	0.13	-0.67	0.12	-0.49	0.48

Table 5 – Statistical results of zooxanthellae density and pigments.

	Zooxanthellae Density	***Chlorophyll-a***		***Chlorophyll-c***		***Diadinoxanthin***		***Dinoxanthin***		***Peridinin***		***β-Carotene***	
		1000 cells	cm^2	1000 cells	cm^2	1000 cells	cm^2	1000 cells	cm^2	1000 cells	cm^2	1000 cells	cm^2
Temperature	.000**	.009*	.000**	.000**	.000**	.005*	.000**	.008*	.000**	.011*	.000**	.006*	.000**
Photoperiod	.000**	.000 **	.000**	.000**	.000**	.010*	.000**	.013*	.000**	.006*	.000**	.011*	.000**
Temperature x Photoperiod	.036*	.000 **	.000**	.065	.000**	.000**	.000**	.001*	.000**	.000**	.000**	.000**	.000**
Tukey's test													
8h – 12h	.031*	.078	.000**	.020*	.000**	.042*	.000**	.059	.000**	.045*	.000**	.045*	.000**
8h – 16h	.000**	.000 **	.000**	.000**	.000**	.896	.000**	.845	.000**	.897	.000**	.884	.000**
12 – 16h	.063	.015*	.002*	.001*	.001*	.013*	.001*	.015*	.001*	.015*	.001**	.014*	.001*
20º - 23º	.000**	.032*	.000**	.003*	.000**	1	.000**	1	.000**	1	.000**	1	.000**
20º - 26º	.000**	.010*	.000**	.000**	.000**	1	.000**	1	.000**	1	.000**	1	.000**
20º - 29º	.000**	.088	.000**	.000**	.000**	.015*	.000**	.024*	.000**	.017*	.000**	.017*	.000**
23º - 26º	.023*	.975	.085	.245	.004*	1	.046*	1	.149	1	.046*	1	.063
23º - 29º	.000**	.976	.526	.478	.005	.017*	.090	.022*	.198	.019*	.094	.018*	.111
26º - 29º	.232	.838	.721	.971	1	.019*	.992	.023*	.999	.022	.990	.020*	.994

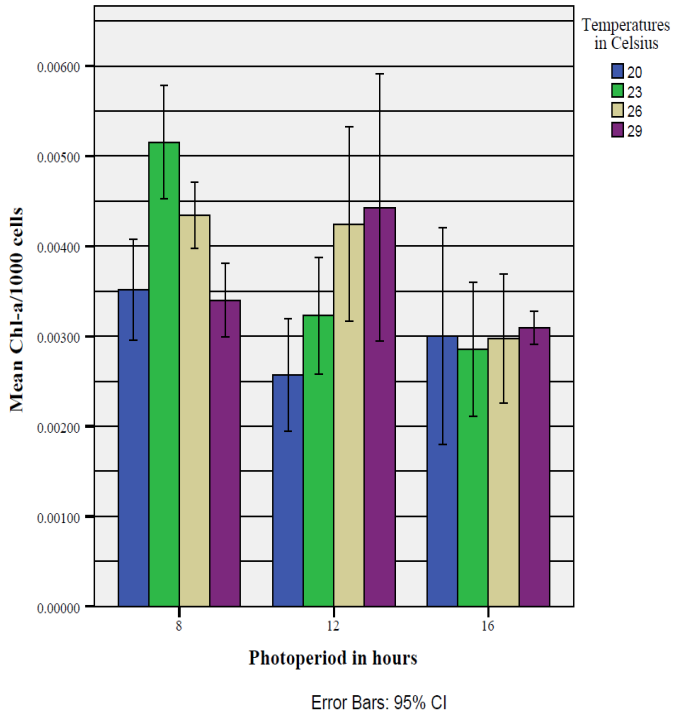
Pigments Analysis.

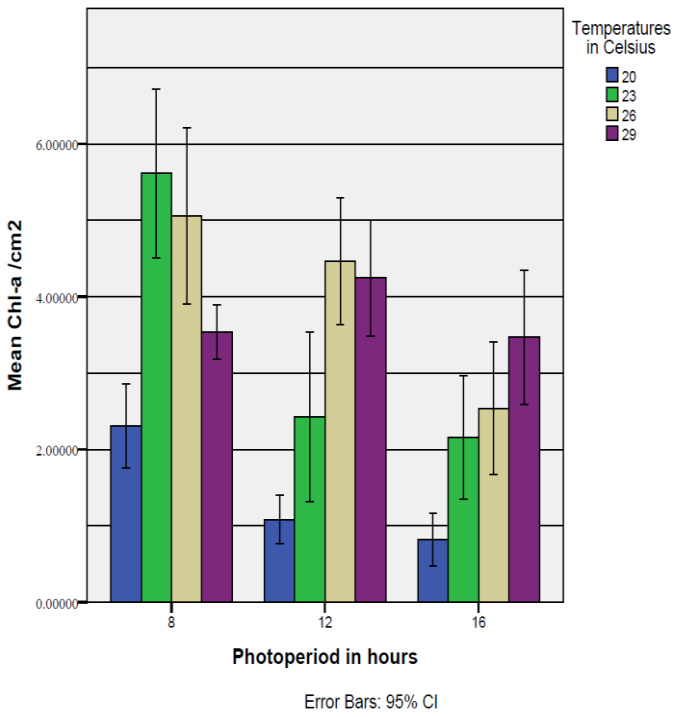
The values of chlorophyll-*a* concentration (cm^2) indicate strong effects of both temperature and photoperiod as well as a significant interaction between these two factors (table 5): All comparisons between photoperiods were significant (p-value < 0.05). The 8h photoperiod had the highest mean value of Chl-*a* (0.00410 μg/cm^2 ±0.00083). In all the photoperiods, the 20ºC had the lowest mean values which implied that this temperature was significantly different from the others (p-value = 0.000 < 0.05), while all the other comparisons were not significant. The concentration of Chl-*a* in zooxanthellae had equally significant effects for the two factors. The 16h photoperiod was different from 8h and 12h (p-value = 0.000 and 0.015 < 0.05, respectively). Regarding temperatures, 20ºC was different from 23 and 26º but not significantly different from 29ºC (p-value = 0.088 > 0.05).

The Chl-*a*/1000 zooxanthella has a photoperiod effect (Figure 11). The 16h groups had have the lowest mean value (0.00298 μg/cm2 ±0.0007), and no relevant differences between temperatures within this photoperiod were observed. The 12h photoperiod had a mean value of 0.00362 μg/cm^2 (±0.00117) and in the treatment 20ºC 12h, it presents the lowest value of the experiment (0.00269 ±0.00054) and the largegest variance between temperatures. On the other hand, in terms of μg of Chl-*a*/cm2, the temperatures seemed to be the factor that had the strongest effect, though the interaction between the two factors be notorious: 16h photoperiod is statistically different (p-value = 0.002 < 0.05). Average chlorophyll-*a* within all analysed coral samples was 3.1442 μg/cm^2 (±1.6120). Chlorophyll-*c*, compared with Chl-*a* showed much lower values both in concentrations by number of cells and by surface area. The average amount of Chl-*c* per cm^2 was 1.2225 μg (±0.6824). Again, the 20ºC temperature was the one that caused significant differences between treatments (p-value = 0.000 < 0.05). All the accessory pigments showed similar patterns of absorbances with particular focus in the lower amount of these pigments in 20ºC treatments and slightly higher amounts in the 8h photoperiod treatment. Dinoxanthin had, overall, lower values than all the other pigments.

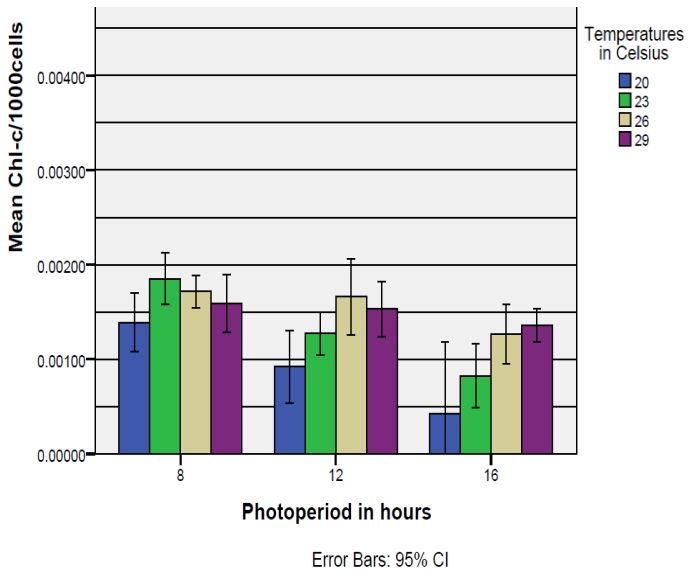
At visual observation (Figure 12) the corals evidenced different colorations after 2 months of the experiment. Corals under 16h of light were clearly paler than the ones at 12h and the colonies under 8h light were clearly darker. These differences were even visible after 30days of the experiment. Concerning temperatures, within the same photoperiod, visual observation did not show severe differences. It was notorious that the corals at 20ºC 12h light were slightly paler than the other 12h treatments.

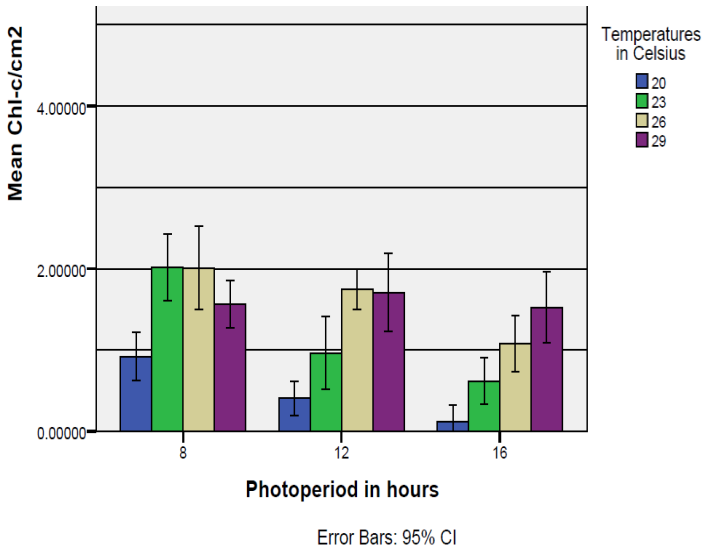
Chlorophyll-*a* (µg)





Chlorophyll-*c* (µg)





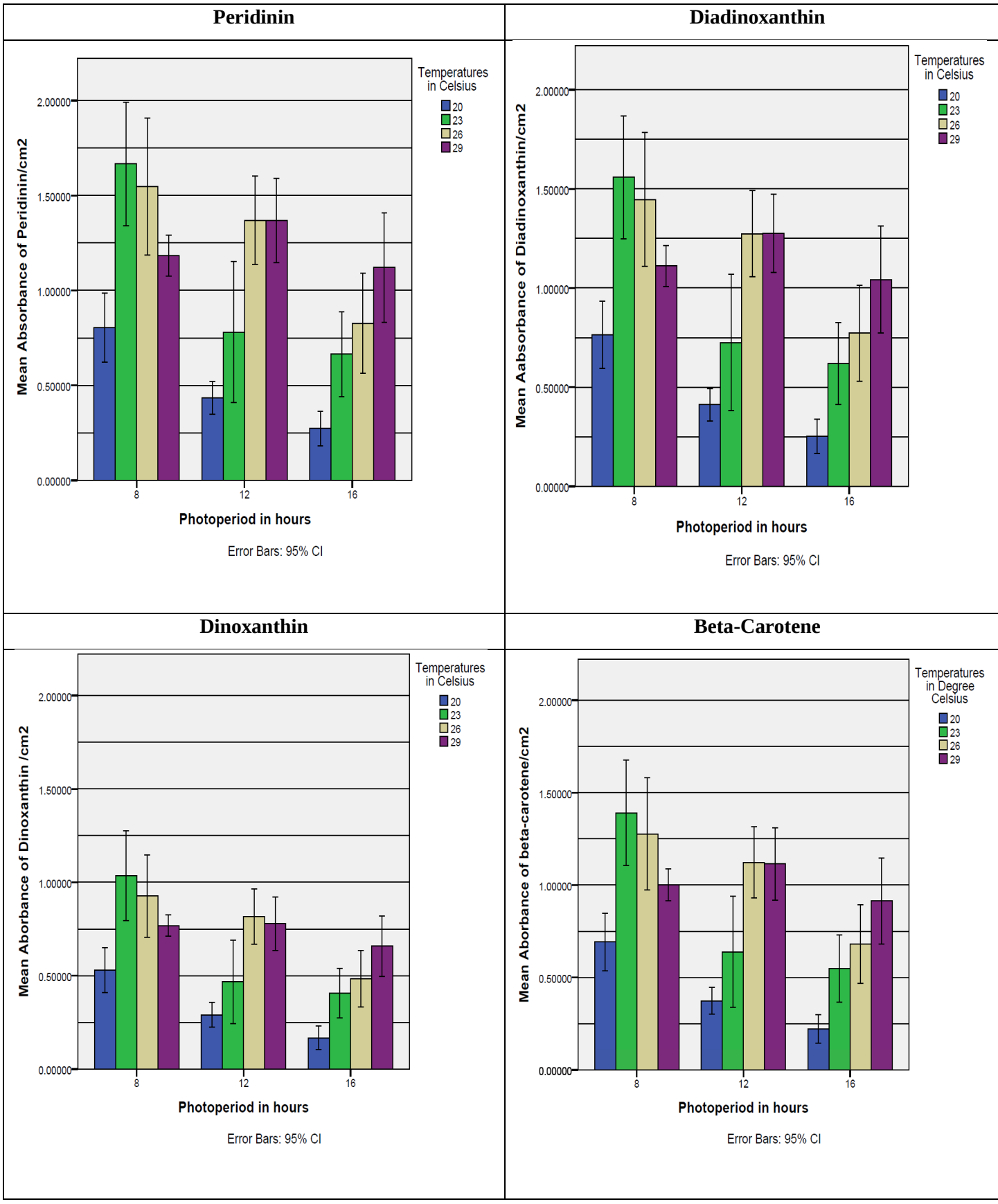
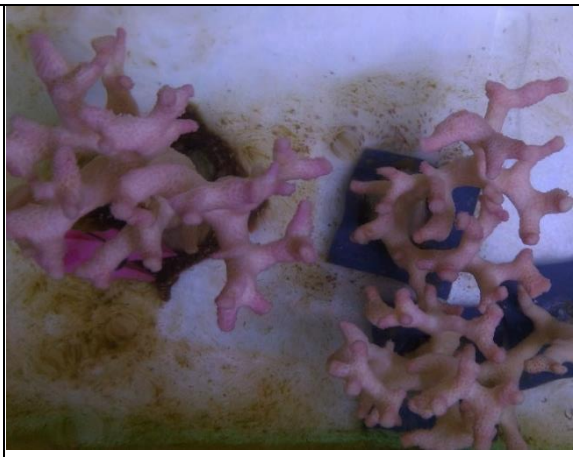


Figure 11 – Graphical representation of the concentration of Chl-*a* and -*c* and carotenoids.

23ºC 16h	23ºC 8h
	
20ºC 12h	23ºC 12h
	
26ºC 16h	20ºC 16h

	
26ºC 12h	23ºC 12h
	
23ºC 8h	26ºC 8h

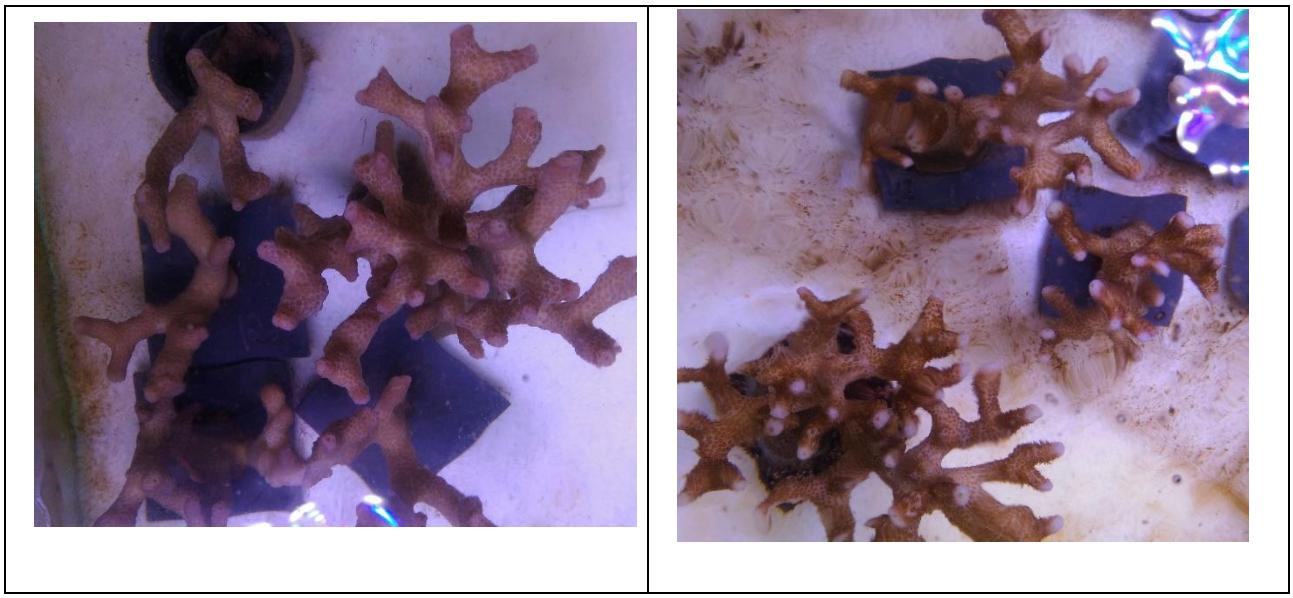


Figure 12 – Pictures of several colonies under different treatments. The pictures were taken after the detection of the rapid tissue necrosis disease.

5 - Discussion

Light is a vital factor for coral biology and for the maintenance of the symbiosis with zooxanthellae. The interaction light and temperature represents a strong modulatory influence able to change, limit or even disrupt the physiology of the holobiont - This study attempted to investigate the interaction between light and temperature, with particular emphasis on two neglected aspects of these two factors, the effects of variations in day length and lower temperatures. Saxby *et al.* (2003) stated that cold temperatures conjugated with extreme light conditions have similar effects to those caused by elevated temperatures. The knowledge of corals adaptability to temperature is of extreme importance due to the threats that they face in nature. As coral distributions tend to become more skewed towards higher latitudes under scenarios of global warming, the risk of coral populations becoming exposed to warm and/or cold shocks under either longer or shortened day lengths will increase.

Since the quantity of light determinates the photosynthetic production of zooxanthellae, a lower availability of light results in lower energy supply to the coral to build its skeleton. This fits with the enhancement of calcification mediated by photosynthesis, but this process still is relatively arguable and unknown (Osinga *et al*., 2011; Moya *et al.*, 2006; Allemand *et al*., 2004; Schutter *et al*., 2011; Marubini *et al*., 2001). In the present work, light availability was tested as temporal availability and it was hypothesized that a shorter or longer photoperiod regime would supply the holobiont with less or more energy. The expectations were to see significantly different growth rates. As the results show, the specific growth rates (SGR) of the shorter photoperiod (8h) are lower than the longer counterparts (12h and 16h). This reduced SGR under fewer hours of light can have two explanations: the reduced time offered to produce energy through photosynthesis or a reduction in their metabolic efficiency, which means, a lower production and/or consumption of energy

per hour. According to the metabolic rates' results, corals in the 8h treatments did not change their metabolic efficiency. They kept the same rates of production/consumption per hour as in the beginning of the experiment. Hence, the slower growth rate at 8h light is the consequence of less time available to photosynthesize. This response is analogous to corals acclimation to low light environments where corals reduce tissue biomass or growth rates (Roth, 2014). Increasing the photoperiod to 16 hours did not stimulate growth in comparison to a 12 hours photoperiod. This corresponds to the observed metabolic rates in these two treatments: compared to corals under 12 hours of light, corals in the 16h photoperiod treatments had their hourly net photosynthesis reduced after 30 days of the experiment while their respiration remained similar. This reduction, in photosynthetic activity, while keeping the same growth rate, can be translated as a relaxation of the photosynthesis. Having more hours of available light these corals can produce daily the same amount of metabolic energy even though they produce less on an hourly basis. But this relaxation idea can be refuted by the results of the daily net photosynthesis/dark respiration ratio (Figure 8 and Table 3). These patterns are however of difficult interpretation since almost all the treatments got a negative daily net photosynthesis after 30 days of the experiment and both, photoperiod and temperature, were not significant. This means that all these treatments did not have an energetic surplus to spend in growth. However, the growth observed is likely result of the initial weeks of the experiment when the energetic budget was still positive and corals were able to build up the skeleton. In the final weeks, besides the negative balance, the growth stagnated.

Based on results of Schutter *et. al* (2011) for *Galaxea sp.* some similarities can be observed. Their corals grown at 16h light did not differ significantly from their counterparts at 12h, and the hourly photosynthesis of these corals was also reduced under a longer photoperiod. Conversely to the present study, their experimental group of corals grown at the 8h light had the same growth rate as the other photoperiods. Possibly, *Stylophora* may respond negatively to shorter photoperiods compared to *Galaxea sp*.. However, Moya *et al*. (2006) did not find any significant difference in calcification rates between *Stylophora* exposed to longer or shorter photoperiods, thought they lack any reference to the duration of exposure of their corals to these conditions, which makes it difficult to make comparisons.

The corals of the experiment demonstrated higher SGRs compared with ones presented in Rocha *et al*., (2013). Their *Stylophora* colonies cultivated under LED at 250 μmol quanta^{-2} s^{-1} (26ºC 12L12D) had a SGR ranging between 0.002 and 0.0025 coral weight day^{-1} while in this experiment under similar abiotic conditions it was 0.0063 (±0.0009). This difference can rely on that no exogenous food was supplied during Rocha *et al*. experiment, while in this study tanks were fed with *Artemia* each two days. Hylkema (2012) obtained twice of our growth rate in his control treatments, which was 0.012 mg gr BW^{-1}. His corals were maintained at 250 μmol quanta^{-1} s^{-1} and a 12:12 h light. These differences can also be explained by genetic differences since different genotypes can have different growth rates (Osinga *et al.*, 2012).

Regarding the temperature, the results showed that the most extreme temperatures, susceptible of causing thermal stress had a negative effect on the growth of the corals. This result agrees with the observations of Marshall and Clode (2004) on calcification rates along a thermic range (Figure 3). Besides that, the lack of

significant interaction between temperature and photoperiod presented in our results maybe is in concordance with their conclusion of independence of light for calcification. Thus, regarding growth, photoperiod cannot compensate for the negative effects of thermal stress.

The excess of light has, as previously described a potential to cause damage in the photosynthetic apparatus and consequently induce a stress response in the holobiont. The resistance to this stress is strongly influenced by temperature (Smith *et al.*, 2004, Roth, 2014). In this study, the quantum yield of PSII demonstrates strong differences between both photoperiod and temperatures regimes. The treatments under longer photoperiods had their quantum yield of PSII significantly reduced after 30days. Similar observations for corals under high light conditions were reported in other studies (Bhagooli and Hidaka 2004; Jones and Hoegh-Guldberg, 2001). In addition, the decrease in the quantum yield was even larger under extreme temperatures (20ºC and 29ºC). Jones *et al*. (1998) recorded a quantum yield of their 28 °C-treated control corals ranging from 0.56 to 0.64; values that are in line with the current study. They also observed a loss of 40% in these values after exposing their corals to 34ºC. Saxby *et al*. (2003) demonstrated that water temperatures of 12ºC for 12 h or more led to the complete loss of photosynthetic efficiency by PSII and death of exposed coral. Corals under 14ºC revealed a light-dependent response, in which thermal stress increases under high light exposure. Consequently, they established that cold stress creates similar physiological symptoms in corals to those seen when they are under heat stress. These observations are in agreement with the results obtained in the present study for PSII's efficiency and growth rates. Additionally, Jones *et al*. (2000) reported in field measurements that bleached corals had a much lower F_v/F_m than healthy colonies. This is in accordance with our corals since the paler corals also had a lower photosynthetic efficiency (Figure 12).

The loss of the photosynthetic function of the symbionts is one of the first responses to thermal stress, due to the decrease of the threshold for photoinhibition (Roth, 2014; Fitt *et al*., 2009). The reduction of the PSII efficiency, which results in a smaller electron transport to the production of energy has, as consequence, lower photosynthetic rates - photoinhibition (Roth, 2014; Brown *et al*., 1999) which to some extent may be the explain for the reduction in the photosynthetic rates observed in the oxygen incubations for the corals at 16h light regime (Figure 7). The photoinhibition presented in these corals can be the result of energy dispersion mechanisms, PSII damage or both (Roth, 2014; Jones and Hoegh-Guldberg, 2001). As symbiont photosynthetic ability decrease, the level of nutrients passed from zooxanthellae to the host will also decrease (Jones et al, 1998), but probably the extended exposure to light that translates to more time available to photosynthesis, may have compensated for corals under 16h of light.

After almost 2 months of the experiment (i.e. after the disease outbreak) it was possible to observe differences in coloration between treatments of colonies that were still unaffected by the disease (Figure 12). Corals under 16h of light were clearly paler than the ones at 12h and the colonies under 8h light were clearly darker. This observation suggests that increasing the photoperiod can induce bleaching. These differences were already visible after 30days of the experiment. Concerning temperatures, within the same photoperiod, naked eye observation did not show any marked differences. It was notorious that the corals at 20ºC 12h light were

slightly paler than the other 12h treatments. These differences in coloration are the result of the loss of the symbionts and/or their associated pigments (Venn *et al.*, 2006)

Zooxanthellae density was strongly influenced by temperatures. Corals at 20ºC were the most affected in all photoperiods what indicates that low temperatures affect the survival of the microalgae or the establishment of the symbiosis. Several authors (e.g. Warner *et al.*, 1999, Baker *et al.*, 2004, Baker 2003. 2004, and LaJeunesse *et al*., 2004) have found that many types of zooxanthellae display optimum growth rates at 26ºC, a constrain that can explain such strong effect of lower temperatures. A longer exposure to light seemed to have increased this trend since the corals at the 20ºC 16h light had the lowest cell density. This suggests that the holobiont could not cope with both sources of stress together. This contrast however with the work of Schutter *et al.* (2011), who did not find significant differences in zooxanthellae density between 8h and 16h photoperiods for *Galaxea fascicularis*. In the current study the 29ºC of temperature do not had any significant effect, and it is important to refer that in many studies, the loss of zooxanthellae is only observed with temperatures above 29ºC (Bhagooli and Hidaka 2004, Fitt *et al*., 2009, Hoegh-Guldberg and Smith, 1989b, Marshall and Baird, 2000. Strychar *et al.*, 2004, Jones *et al*., 1998). One of the earlier studies about bleaching, Hoegh-Guldberg and Smith (1989) observed a reduction of zooxanthellae density in corals at 30ºC compared with corals exposed to 27ºC. Although *Stylophora* is considered a highly sensitive species to bleaching (Baird *et al*., 2008), the 29ºC used in this experiment is not expressively different from the 30ºC used by Hoegh-Guldberg and Smith (1989), which implies that the difference observed between the two studies is probably due to differences in the symbiont's resistance than host's one. Their corals grown at 32ºC had severe bleaching or death. It may suggest that 29ºC in our experiment was not a severe warm temperature. Both readings are in line with the results of other studies (Fitt et al., 2009; Jones et al., 1998). Fitt *et al.* (2009) recorded zooxanthellae density of 1.0 (±0.2) $\times 10^6$ cm^{-2} and Jones et al. (1998) recorded a zooxanthellae density ranging from 1.1-1.5 $\times 10^6$ cm^{-2} for *S. pistillata* in their control treatments, both at 28ºC. These values are similar to the ones found in present study.

The main photoactive pigments of *Symbidium* sp. can be divided into photosynthetic pigments and carotenoids. Photosynthetic pigments are Chlorophyll-*a* and -*c*. They have as main role absorbing and transferring light to the reaction centres of PSI and PSII. Carotenes as β-carotene and xanthophylls like peridinin, diadinoxanthin, and diatoxanthin are part of the carotenoids group and occasionally also called accessory pigments. They have a wide variety of roles including auxiliary absorption of light, antioxidants or sinks of an excess of energy (Roth, 2014). Therefore, all of them have a crucial function when talking about photoinhibition, stress resistance, and photosynthetic efficiency. The pigment analyses performed in this study relied on this statement. However, the host can also produce pigments, including fluorescent chromoproteins, that modulates the stress response of the all holobiont (Strychar and Sammarco, 2012), but such pigments were not analysed in this work.

A chlorophyll-*a* concentration between 3.5-5.0 μg/cm^2 was found in our control treatment (26ºC 12h). Such values are similar to the ones recorded in non-bleached *S. pistillata*; 3.5-4.0 μg/cm^2 (Nakamura, *et al*.,

2003). The concentration of Chl-*a* per cm^2 was significantly lower at 20ºC for all photoperiod regimes. This may suggest that at 20ºC this pigment becomes dysfunctional or is degraded, a similar response to when exposed to high temperatures (>30ºC) (Strychar and Samarco, 2012). Under the 12h photoperiod, the treatments 26º and 29ºC had more Chl-*a* than the colder counterparts. A similar pattern can also be seen in the 16h photoperiod. This suggests that higher temperatures favor the increase in Chl-*a* something that was also observed by Flores-Ramírez and Liñán-Cabello (2007) for Arabian Gulf's corals. Strychar and Sammarco, (2012) observed that their Great Barrier Reef's corals had a negative response to higher temperatures in their pigment concentrations. Other studies also indicate that phytopigment concentrations decrease as temperatures increase (Jokiel and Coles, 1974; Kleppel *et al*., 1989; Jones *et al*., 1998; Fitt *et al*., 2000) while others did not report any significant modifications (Venn et al., 2006, Hoegh-Guldberg and Smith, 1989a; 1989b; Jones *et al*., 1998). Nevertheless, these studies were performed with different species of the host from different regions, and such factors can lead to those different results.

Hoegh-Guldberg and Smith (1989) observed loss of pigment concentrations within *Stylophora* exposed to high light conditions for 10 days, despite no decrease in *Symbiodinium* populations. Our results reach a similar conclusion as no significant difference between 12h photoperiod and 16h photoperiod for zooxanthellae density (p-value = 0.063 > 0.05) were observed. However the result were significant when referring to concentration of pigments per 1000 cells (p-value = 0.015 < 0.05) (Table 5). Schutter *et al*. (2011) did not find significant differences in concentration of chlorophylls (Chl-*a* and -*c*) between 8h and 16h photoperiods for *Galaxea fascicularis*. But, is not possible to ignore the possible influence of the different temperature treatments in these results as the results of interaction show (Table 5). For example, at 23ºC, the amount of Chl-*a* cm^{-1} under 8h light was much higher than under 12h or 16h, where this temperature had a strong decrease compared to 26ºC. This may support the hypothesis that shorter photoperiods increase thermal stress tolerance to reductions of only a few degrees in water temperature, since at 20ºC a strong negative effect is observed. With *S. pistillata*, Titlyanov *et al*., (2001) observed, under low light conditions, an increase in photosynthetic pigments concentration in zooxanthellae and in zooxanthellae density as a strategy to maximize the light harvesting capacity. Similar results were observed in other studies (Porter *et al*., 1984; Falkowski and Dubinsky, 1981) as well as in our results.

Besides the statements of Hoegh-Guldberg and Smith (1989) and Titlyanov *et al*., (2001), Venn *et al.* (2006) documented a 50-80% loss of symbiont cells in *Montastrea cavernosa* at 32°C but observed no significant pigment loss (Chl-*a* and peridinin) concentrations in retained cells. By contrast, Dove *et al*. (2006) have reported decreased pigment concentrations without a significant loss of zooxanthellae in *Montipora monasteriata*. Keeping in mind that these responses to changes in light and temperature are very variable and unpredictable along different taxa and conditions (Strychar and Sammarco, 2012), this current study seems to suggest that zooxanthellae are more susceptible to changes in temperature, while pigments seem to be more influenced by light conditions.

It is known that *Symbidium sp*. as part of their capability to photoacclimatize can change their pigment composition (Smith *et al*., 2005; Titlyanov *et al*., 2001; Falkowski and Raven, 1997; Brown *et al.*, 1999). The main purpose of this mechanism is to create paths for dissipation of excess of energy or protection against light damage. It is known that zooxanthellae under thermal stress convert their diadinoxanthin in diatoxanthin as a method to decrease the rate of photons sent to PSII and thus helping to prevent damage to the system (Ambarsari *et al*., 1997; Falkowski and Raven, 1997; Brown et al., 1999). In our results, there was a strong reduction in diadinoxanthin absorbance in treatments exposed to 20ºC. This trend is intensified by the extension of the photoperiod (20ºC 16h). Smith *et al*. (in their references, 2005) report a correlation between the increment of carotenoids and higher light conditions. The results of the current work indicate a significant difference between the longer photoperiod and the control and the shorter photoperiod (Table 5). However, the graphical representation (Figure 11) does not seem to demonstrate such influence coming from the photoperiods, instead, the temperatures seemed to have taken a larger role in it. Nevertheless, the levels of carotenoids absorbance's at 16h treatments had lower values, a singularity that contradicts the previous statement on the function of the carotenoids. Contrarily to the expectations, the corals under extended photoperiods did not increased their amount of carotenoids due to light stress. The light damage that leads to reduced photosynthetic efficiency could have reduced the energetic surplus needed to synthesize carotenoids.

The disease outbreak, though unpredictable, provided an interesting clue on how the control of the temperatures can manage the appearance and spread of pathogens. The first signals of the disease appeared in the highest temperature tanks, the 29ºC treatments, regardless of the photoperiod regime, a fact that withdraw influence of the photoperiod over the disease and indicates that high temperatures increase growth and virulence of the pathogens (Sheridan *et al*., 2013; Vidal-Dupiol *et al*., 2011). The following days the disease spread to the 26ºC tanks, and one week later, signals of the disease were present in the 23ºC tanks. Since all the tanks were connected by the closed recirculation system, it would be expected that all the tanks were affected at the same time, but the observations suggest that lower temperatures avoid or delay the spread of pathogens. This apparent strategy of management to prevent disease outbreaks seems to be a good option to apply both for emergency and prevention protocols.

6 - Conclusions and Final Remarks

This study shows some preliminary results on the effects of changes in temperature and photoperiod on the performance of the scleractinian coral *S. pistillata*. Due to the early abortion of the experiment following the outbreak of RTN, this study can be considered as a short/medium-short term experiment to access the adaptive response of coral colonies.

How do corals respond to different photoperiods?

Corals exposed to reduced photoperiods did not have their growth rate enhanced but they kept their photosynthetic efficiency and zooxanthellar populations at normal levels. Colonies at 8h light were darker due to an increase in their pigments concentrations. In the other hand, extended photoperiod colonies did not have

an effect on growth rate, being very similar to the control photoperiod but had their hourly photosynthetic rates reduced. This reduction in photosynthesis was a consequence of a decrease in photosynthetic efficiency and/or loss of pigments. Corals at 16h light had their quantum yield of PSII reduced and they became paler, suggesting a pre-bleaching status. Furthermore, no increase in carotenoids occurred under longer exposure to light as was expected as a mechanism to protect against light stress. The results of this study demonstrated strong effects of photoperiods in coral's biology and largely these effects are similar to those found for modifications in light intensity.

How do corals respond to cold stress?

In this study corals exposed to low temperatures had slower growth rates, lower zooxanthellae density and reduced pigments' concentration. All these findings are in line with the same physiological response to heat stress. In this study, some analogous responses were seen between corals exposed to 20 and 29ºC: growth, metabolic rates, and the photosynthetic efficiency were similar among these treatments.

Do photoperiods and temperatures have any interaction on corals physiology?

Photoperiod and temperatures were factors that largely interacted on the response of the *S. pistillata*. That interaction could be seen in the photosynthetic efficiency in which the negative effects of 16h light were even larger under 20ºC and 29ºC. In addition, zooxanthellae density and pigment concentration suffered a larger reduction under the combined effects of low temperatures and longer photoperiod. These evidences also led to conclude that the combined effects of photoperiod and temperature are similar to light intensity and temperature and that shorter photoperiods can relieve some of the negative effects of thermal stress. However, with regard to growth, no interaction was found - photoperiod modifications were not able to neutralize the effects of thermal stress.

Can different combinations of photoperiods and temperatures optimize coral aquaculture?

No improvements in specific growth rates were achieved with longer or shorter photoperiods, compared with standard day length – 12L/12D. However, to produce corals with ornamental purposes a reduction on photoperiod can be an approach to enhance coloration as we got darker corals under 8h photoperiod. This approach might be used if growth rates can be sacrificed since these corals had a lower specific growth rate. 23ºC seemed to have been neutral or a had a small effect which suggests that a small reduction in production's temperatures may offer a saving of energy costs without compromising the production efficiency and in addition help to prevent disease outbreaks. Long-term effects are still missing to be evaluated since it was not possible in this work.

Some limitations of the experimental set-up created for this research can be deliberated. First, the number of replicates tanks was low, due to limitations in the room available for this experiment, but running an experiment with more tanks was not possible. This issue could have made the outcomes of the experiment more susceptible to tank effects and/or reduce the strength of the statistical analysis. Second, a closed

recirculating system connecting all the tanks allowed the propagation of the disease outbreak what led to the abortion of all experiment treatments instead of only abandoning the infected tanks. Third, the temperature regimes were hard to control. Although not recorded along 24h, for sure fluctuations of temperature along the day happened, however, this phenomenon did not create tank effects and is something that occurs in nature. The water in the individual bath was static and no thermic regulated. This proved to do not be so efficient as a common bath for all the tank at the same temperature. The lights were a large source of heat input in the system, and tanks in the 16h photoperiod were harder to chill since they were more time exposed to light. In the future, a temperature controlled common bath and a larger light distance to the water surface is highly recommended. No significant changes in salinity, alkalinity or pH were observed along the time, though we believe that the strong increase in algal growth in the tanks resulted in negative effects like turbidity of the water, competition for nutrients (McCook *et al*., 2001) and pollution of the system. It was also observed that a couple of times the organic matter created an oily film at water surface what probably reduced the exchange of gases between water and atmosphere. In future experiments, it is recommended to increase the efficiency and/or capacity of the skimmer and even add a filtration device. The feeding level was assumed to be sufficient (2 ml *Artemia*, 3x week). However, in follow up research trying to increase the *Artemia* retention in the tanks can be useful to sure a correct nutrition of the coral colonies and reduce the amount of non-consumed *Artemia* that remains in the system polluting it.

The pigment measurements can be improved recurring to a high-performance liquid chromatography (HPLC). Such was not performed due to limitations in the time since that analysis is time consuming especially if the number of samples is very large.

Regarding the colonies, in this experiment, the initial size of the colonies was not standardized since some colonies were larger and others smaller. This may represent an additional source of variation to certain parameters such as growth or metabolic rates. In following studies, colonies of similar size are recommended to be used. Additionally, corals that never had a history of disease infection are most appropriate and safe to perform a long-term experiment.

Finally, as this study represents an early step understand the effects of artificial photoperiods, follow up research on this topic is still required.

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