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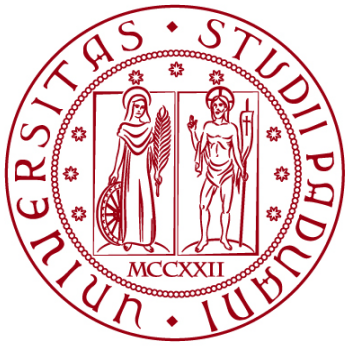

TESI DI LAUREA

Thermotolerance of early life stages of the golden kelp *Laminaria ochroleuca*

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Abstract

Kelps, classified under the order Laminariales, are brown seaweeds which can be found along all coastlines of the world – except Antarctica. These organisms play a crucial role as "ecosystem engineers" for their ability to form marine forests, three-dimensional, complex, heterogeneous and highly productive habitats which can host many different species. For this reason, their presence is fundamental to maintain ecosystem functions and to prevent biodiversity from declining. However, nowadays kelp forests are declining worldwide as they are affected by several anthropogenic stressors, some of them being overfishing, ocean warming and eutrophication. To counteract this decline, extensive efforts have been made by scientists and experts to restore these essential habitats. Numerous projects are being funded annually to either recreate lost forests or enhance the resilience of existing ones. Effective restoration strategies require comprehensive scientific knowledge regarding the tolerance, resistance and resilience of kelp species. However, extensive knowledge about the physiology and phenotypic plasticity of many species is now still lacking. This Master Thesis focuses on *Laminaria ochroleuca*, commonly known as "golden kelp". It is a warm-temperate Iberian species belonging to the order Laminariales, found in relatively warm waters ranging from southern England to Morocco. It reproduces sexually and is characterized by a heteromorphic and diplohaplontic life cycle, which features an alternance between a microscopic haploid gametophyte and a macroscopic diploid parenchymatous sporophyte. Due to variations in sea surface temperatures and habitat depths, distinct populations of *L. ochroleuca* have emerged, potentially leading to intraspecific

diversity and phenotypic plasticity in response to varying environmental conditions.

This study aims to investigate whether different populations of *L. ochroleuca* exhibit adaptive differences in gametophyte reproductive success in response to temperature variations during gametogenesis. Additionally, it explores whether the developmental temperature experienced during gametogenesis influences the thermal tolerance and the growth of microscopic sporophytes when exposed to a range of temperatures, including sub-lethal and lethal levels. If confirmed, the relationship between the temperature experienced during gametogenesis and the resulting thermal tolerance can be exploited to develop heat-resistant strains of *L. ochroleuca*. Another question addressed in this study is whether priming treatments commonly applied on terrestrial plants (specifically, chemical priming with H_2O_2 and thermal priming with sub-lethal temperatures) enhance the ability of juvenile sporophytes to tolerate both sub-lethal and lethal temperatures.

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INTRODUCTION

Thermal plasticity

Climate change is altering the environments in which species develop, driving them to redistribute and/or adapt to new environmental conditions at a global scale (King et al., 2017). More specifically, global warming is driving sea temperatures to be unpredictably variable (Suárez-Gutiérrez et al., 2018) and that is why it is becoming fundamental to gain new insight into the thermal plasticity of marine organisms. The need is to be able to predict if there will be changes in species distribution and community composition (Nicotra et al., 2010) and to understand if this will affect local ecosystems, with the aim of mitigating those impacts.

Phenotypic plasticity is defined as « the capacity for an individual genotype to produce different phenotypes in response to environmental variations » (West-Eberhard, 2003), allowing organisms to adapt and respond to environmental changes. Overall, phenotypic changes can involve fast and reversible ones, carry-over effects due to environmental exposure during early life stages (Palmer et al., 2012; Byrne et al., 2020) or parental effects (*sensu* Salinas et al., 2013), where the offspring's traits are influenced by the environmental conditions experienced by parents. The mechanisms driving phenotypic plasticity remain largely unclear, but epigenetic processes are believed to play a role in altering gene expression without modifying DNA sequences (Kelly et al., 2012). These processes include small interfering RNAs, histone modifications, chromatin remodelling and, more importantly, DNA methylation. When such changes are passed to the next generation, the plasticity is referred to as trans-generational (King et al., 2017).

Recently, phenotypic plasticity has been recognized as an important feature, a complex characteristic and a way to select the best phenotypes adapted to different environments, and it has demonstrated to be genetically controlled and inheritable. For this reason, today it has become a central topic in ecology and evolution studies (Bradshaw, 2006). Especially considering sessile species, it is easy to understand how thermal plasticity can help these organisms to adjust and buffer the non-optimal conditions in environments affected by climate change (Nicotra et al., 2010), as they are not able to migrate away from the stressors in a short time like mobile species (Bradshaw, 2006). This mechanism can result in a rapid adaptation to the new conditions, providing a fitness benefit (Chevin et al., 2010).

Intraspecific variation

When taking into account marine sessile species such as kelps, another important aspect to consider is that different populations can inhabit habitats characterized by different environmental conditions, such as temperature and depth. Throughout the species evolutionary history, this could have caused differentiations, leading to intraspecific genetic variations and phenotypic plasticity as adaptations to the diverse environmental settings (Linhart & Grant, 1996). A study performed on the brown alga *Sargassum honeri* (Turner) C.Agardh demonstrated how the adaptive divergence in benthic population is primarily driven by temperature (Li et al., 2023). Moreover, *Laminaria digitata* (Hudson) J.V.Lamouroux populations located at the species warm distribution limit exhibited higher heat tolerance in terms of growth and photoprotective performances compared to northern populations (Liesner et al., 2020a). In contrast, an Arctic population showed lower thermal adaptation in the photosynthetic performance of sporophytes

(Liesner et al., 2020a) and in reproductive traits of gametophytes (Martins et al., 2020). These findings suggest the presence of intraspecific variation in reproductive and physiological traits, likely driven by distinct abiotic conditions, especially temperature, associated with different latitudinal distributions (King et al., 2017).

In addition, in the marine environment ocean currents significantly impact the connectivity between populations (Coleman, 2013). This influence can result in low dispersal potential, leading to genetic differentiation at a fine scale (Neiva et al., 2012), or to high dispersal potential, resulting in less genetic variability (Lourenço et al., 2017). When examining the potential effects of climate change on the fitness of populations, it is crucial to understand the patterns of intraspecific differentiations. Populations with greater plasticity are more likely to persist to environmental changes, and evidence indicates that individuals exposed to high environmental variability tend to be more plastic (Sultan & Spencer, 2002). Identifying phenotypic plasticity among populations reveals essential also to predict changes in species distribution under new climate conditions (Valladares et al., 2014).

Phenotypic engineering

Phenotypic engineering involves experimentally modifying an organism's phenotypic traits to assess whether these changes enhance adaptation and increase fitness in specific environmental conditions, comparing the altered organisms with unmodified individuals (Ketterson et al., 1996). Since plants have a remarkable ability to modify their phenotypic traits in response to environmental conditions or stress without altering their genotype, revealing a great developmental plasticity (Palmer et al., 2012; De Jong & Leyser, 2012), these abilities are now being exploited to develop more productive and stress-tolerant crops.

Priming techniques have recently begun to raise interest, because they exploit the plant phenotypic plasticity to enhance its tolerance towards both biotic and abiotic stress conditions (Savvides et al., 2015; Suzuki et al., 2014). The priming process consists of a previous exposure of the organism to a stress factor, either biotic or abiotic, that can increase its resistance in a future exposure to the same or to a similar stress (Filippou et al., 2012). When exposed to the stressor, the organism enters a primed state, which enables a faster and/or stronger activation of protective mechanisms (Sani et al., 2013). Studies have shown that infecting plants with necrotizing pathogens or colonizing roots with beneficial microbes was effective to induce the primed state in the plant, activating more rapidly and efficiently the defence responses in case of attack of pathogens or insects (Conrath et al., 2006). More recently, chemical priming has also been identified as a method to induce the primed state. This approach is based on the exposure of the organism to natural or synthetic chemical compounds, such as sodium nitroprusside, hydrogen peroxide, sodium hydrosulfide, melatonin, and polyamines, that can potentially enhance its tolerance to subsequent more severe abiotic stresses. In fact, this pretreatment triggers an acclimation-like response by applying a mild stress cue (Savvides et al., 2015).

Chemical priming has been shown to reduce reactive oxygen species (ROS) accumulation under abiotic stress conditions such as heat, drought, salinity, and cold. The mitigation of oxidative stress has been linked to an enhanced activity of antioxidant enzymes, which also leads to a reduction in malondialdehyde (MDA) content and electrolyte leakage, indicating lower lipid peroxidation and less damage to cellular membrane integrity (Savvides et al., 2015). Several studies have confirmed an upregulation in the transcription of enzymatic and non-enzymatic antioxidants (Christou et al.,

2014a; Christou et al., 2014b) and components involved in the biosynthesis of ascorbate and glutathione (Christou et al., 2013).

Thermal priming has also been demonstrated to improve plant performances under more severe high temperature stresses. In a study by Wang et al. (2013), heat-primed seedlings of winter wheat *Triticum aestivum* L. showed an increased thermo-tolerance compared to non-primed individuals. This enhanced tolerance to heat was linked to reduced superoxide radical production and lower malondialdehyde levels, which resulted from the up-regulated expression of some antioxidant compounds. These changes led to higher activity of superoxide dismutase (SOD) in chloroplasts and increased glutathione reductase (GR) and peroxidase (POD) activity in mitochondria in the primed seedlings. Thermal priming has also been applied to the Mediterranean seagrass *Posidonia oceanica* (Linnaeus) Delile, resulting in a better performance of primed seedlings, characterized by stable photo-physiology due to higher expression of genes related to stress response, photosynthesis and epigenetic modifications (Pazzaglia et al., 2021).

Although priming treatments have been studied extensively in higher plants for some time, they have rarely been applied to macroalgae. However, with the expanding role of aquaculture in cultivating macroalgae for food and pharmaceuticals, along with the increasing focus on restoration actions in response to climate change, it is becoming important to investigate the potential effects of priming on seaweeds.

Kelps – habitat-forming species

Kelps are brown seaweeds belonging to the order Laminariales (Phaeophyceae), but in some ecological studies also other species of canopy-forming brown algae that play similar functions get included in this taxonomic distinction (Teagle et al., 2017). The

phylogeny is still not certain and clear (Bolton, 2010), but with the progresses which have been made in understanding evolutionary pathways and relationships, 84% of described species are included in 3 families (Alariaceae, Laminariaceae, Lessoniaceae), and 63% of them belong to only 5 genera (*Alaria, Laminaria, Saccharina, Ecklonia, Lessonia*) (Teagle et al., 2017). They are not characterized by a large taxonomic diversity, nevertheless they are diverse both structurally and functionally (Steneck et al., 2002).

Kelps can be found along all coastlines of the world – except Antarctica – (Krumhansl et al., 2016) and are present in 43% of the marine world's ecoregions (defined in Spalding MD et al., 2007). They are widespread on hard substrata – especially shallow rocky seabeds – in temperate, subpolar and deep cold tropical areas. The kelp biome, occupying 1,469,900 km^2, constitutes the second most widely distributed biome, following the seagrass one (Jayathilake & Costello, 2018; 2020).

Since kelps are photosynthetic species, their distribution is limited to the photic zone. According to a recent model developed by Jayathilake & Costello (2020), they can be found between 0 and 100m depth, but the authors did not exclude that they could be found in offshore rocky reefs (like seamounts' tops), even if available data did not report their presence (Jayathilake & Costello, 2020). However, the occurrence of *Laminaria rodriguezii* Bornet was exceptionally recorded at the maximum depth of 260 m in the Adriatic Sea (Ercegović, 1960), confirming the possible survival of kelps in deeper locations. Specifically in the NE Atlantic, dense kelp forests are commonly found in sheltered locations, from the lower shore to 20 m depth – exceptionally to more than 40 m depth (Smale et al., 2013), ranging from northern Iceland and Norway to Portugal and Morocco (Bolton, 2010). Kelps can be found in areas

with an average sea surface temperature ranging from 5°C to 25°C – they are found rarely at above 27°C (Jayathilake & Costello, 2020) – and for this reason climate change is threatening their survival and will likely alter their global distribution (Assis et al., 2016). Generally, kelp forests are rarely found at above 60° latitude and Arctic and sub-Antarctic species show a low genetic diversity, probably due to low light conditions (Henley & Dunton, 2003). Additionally, they do not often develop in waters characterized by high temperatures and low nutrient concentrations, like the tropical and subtropical ones (Bolton & Anderson, 1987), except in areas characterized by cold currents or upwelling, which carry cool and nutrients-rich waters (Steneck et al., 2002).

Kelps are considered important ecosystem engineers, since they contribute to the formation of marine forests – three-dimensional, complex, heterogeneous and highly productive habitats – which can host many different species. They provide habitat for smaller algae, invertebrates and fish that can live only thanks to the presence of the canopy and to the resources it provides (Wernberg & Filbee-Dexter, 2019). Additionally, the mature thalli (sporophyte) can provide three different microhabitats: the lamina, the stipe and the holdfast, which are structurally different and host diverse assemblages.

The growth pattern of holdfasts varies among species: *Macrocystis spp.* typically develop into a complex structure, while *Laminaria spp.* usually forms fewer but thicker haptera, with greater spacing between them. These differences affect the associated fauna, with larger holdfasts generally supporting more diverse assemblages (Teagle et al., 2017). The gaps between haptera provide an ideal habitat for colonising species due to the morphology of the structure, which offers protection from predators and shelter from

adverse environmental conditions, while also accumulating food supplies (Ojeda & Santelices, 1984). The taxa hosted by this microhabitats are mostly invertebrates, such as copepods, gastropods, bivalves, sponges, polychaetes and amphipods (Blight & Thompson, 2008; Ríos et al., 2007; Arroyo et al., 2004) and the dominant trophic level is represented by detritivores – both deposit and filter feeders – which feed on the organic particulate matter, some of which is detritus produced by the seaweeds (Schaal et al., 2011).

In contrast, the stipe forms a simpler habitat due to its long and rigid structure. Despite this, it can still support highly rich and diverse communities: sessile invertebrates can attach and grow on it (Leclerc et al., 2015), but the most common organisms are epiphytic algae, which can be either obligate or facultative epiphytes (Bartsch et al., 2008). Lastly, the blade provides a wide surface area which gets usually colonised by epibionts. Although the biodiversity on the blade is typically lower than that on the stipe and holdfast, epiphytic algae can still be found, albeit in low density due to competition with the host kelp (Teagle et al., 2017). Extensive coverage of the blade can be signal of stress in the alga; for example, an unusual abundance of encrusting coralline algae was present in *Ecklonia radiata* (C.Agardh) J.Agardh following a severe marine heatwave (Smale & Wernberg, 2012). Since many species depend on the presence of a healthy kelp canopy, deforestation can lead to severe effects on ecosystem's community structure and biodiversity. In Southern California, kelp loss, due to sea urchins grazing activity, caused a change in the ecosystem, which got dominated by ephemeral microalgae, low-lying macroalgae and phytoplankton, causing a reduction of the biodiversity of sessile invertebrates higher than 40% and the disappearance of abalone, a species which relied on the kelp presence (Graham, 2004).

Kelps – providers of ecosystem services

In addition to providing habitats for various species, kelp forests fulfil other crucial functions – fundamental also for human society – known as "ecosystem services" (Eger et al., 2023). Kelps globally support the fisheries of many commercial species, such as abalones, lobsters and many reef fishes (Steneck et al., 2002) and provide an important source of alginates, increasingly employed in the biomedical and bioengineering field, other than as a food additive (Peteiro, 2017). Although kelp forests are typically not seen as significant contributors to the blue carbon sequestration as they mostly grow on rocky substrates with low carbon burial potential (Hill et al., 2015), about 80% of their production is exported annually (Krumhansl & Scheibling, 2012). The organic matter, both particulate (POC) and dissolved (DOC), can reach new depositional habitats and become sequestered in sediments, or can be transported to the deep sea, where it gets isolated from atmospheric exchange (Krause-Jensen & Duarte, 2016). As photosynthetic organisms with large biomasses, kelps play a crucial role in oxygen production (Hatcher et al., 1977) and consequently in primary production, which generates more available food for the ecosystem and associated species (Richmond et al., 2007). Additionally, their nutrient uptake can help in bioextracting nutrients from urbanized and eutrophicated coastal waters (Kim et al., 2015).

Kelps – threatened by climate change

Unfortunately, nowadays kelps are declining worldwide due to several anthropogenic stressors, such as overfishing, ocean warming and eutrophication (Smale et al., 2013; Johnson et al., 2011). Giant kelp *Macrocystis pyrifera* (Linnaeus) C.Agardh seabeds shifted into barrens in eastern Tasmanian rocky reef systems, due to

the overgrazing by the sea urchin *Centrostephanus rodgersii* Agassiz, whose presence has increased given the absence of its predator, the rock lobster, which got overfished (Johnson et al., 2011). Eutrophication, acting together with natural disturbances, like storms, can cause a decrease in canopy-forming macroalgae by favouring their removal and their replacement with turf-forming species (Connell et al., 2008), which thrive in high nutrient and large sediment loads conditions (Gorgula & Connell, 2004).

Ocean warming has the potential to reduce kelp biomass and even extinguish some populations, altering their distribution towards latitudes where sea temperatures are lower, thus reducing environmental stress (Wernberg et al., 2010; Smale et al., 2013). As temperatures rise in the Atlantic, it is anticipated that northern kelp species such as *L. digitata* may experience declines in abundance or shift poleward (Raybaud et al., 2013). A recent study projected how kelp forest distributions may shift in response to future climatic conditions, predicting an increase in kelp biodiversity at higher latitudes, such as the Arctic and cold-temperate regions of the Northern Pacific and Atlantic Ocean. However, it also forecasted a decline in diversity at lower temperate latitudes and potential local extinctions in areas where kelp populations are already sparsely distributed (Assis et al., 2024). Changing in currents or upwelling regimes can also result in deforestation. The El Niño phenomenon, which leads to the cessation of upwelling along the West Pacific coasts and consequently to a decrease in nutrient availability and a rise in water temperatures (Dayton et al., 1999), creates stressful conditions for kelps.

Kelp restoration

As defined by the Society of Ecological Restoration (SER), restoration is “the process of assisting the recovery of an ecosystem that has

been degraded, damaged or destroyed" (SER, 2004). Given the critical ecosystem services provided by kelp forests, these habitats are increasingly being considered for restoration actions. Various methods can be employed, depending on the specific threats to the ecosystem. When seaweeds are not reproducing, the strategy of "active restoration" (Layton et al., 2020) involves introducing new reproductive individuals into the area. Layton et al. (2021) successfully transplanted adults and juvenile individuals of *Ecklonia radiata* from a healthy canopy to the restoration site, avoiding any damage to the holdfast, which proved to be the key for a successful outcome of the transplantation. These new individuals can act as a spore source, promoting the expansion of the restored forest. If excessive herbivory poses a threat to the canopy, one mitigation strategy – included in the "assisted recovery" approaches (Layton et al., 2020) – is to cull or harvest the grazers, such as herbivorous fish or more commonly, sea urchins (Otsuka et al., 2010). Additionally, installing substrata for kelp colonization, such as artificial reefs, can be an option (Terawaki et al., 2001).

A recurrent issue in kelp forest restoration is the ineffective management and policy (Carpenter et al., 2009), largely due to the inadequate quantification of the economic value of the ecosystem services they provide (Vásquez et al., 2013). Indeed, if the costs are higher than the benefits, securing funding for a restoration action becomes unlikely (Grabowski et al., 2012). Assessing the economic value of such ecosystems requires an evaluation of the benefits society perceives from the presence and the healthy functioning of the forests (Hynes et al., 2021). Kelp forests were estimated to provide a global worth of $500 billion per year focusing on just three ecosystem services, fisheries, carbon sequestration and nutrient removal (Eger et al., 2023). To facilitate the decision-making process, also the benefits of restoration must be evaluated,

recognizing the costs of implementation and maintenance when planning potential interventions (Hynes et al., 2021).

Many restoration actions have failed due to increasing natural stressors, such as rising water temperatures, stronger wave power, reduced nutrients availability, and to inefficient methods (Eger et al., 2022). However, the transplantation of both juvenile and adult individuals of *Ecklonia radiata* in a semi-exposed and sandy embayment on the east coast of Tasmania, Australia was a successful example (Layton et al., 2019). Other projects are currently active in many countries around the world, including Korea, USA (Oregon Kelp Alliance), Canada, Australia (Operation Crayweed), Europe (Italy, Spain, Portugal) and Chile (Eger et al., 2022).

Kelp aquaculture

Given their importance as commercial species, kelp aquaculture is currently experiencing global growth even in European and American regions that are not traditionally known for kelp farming, unlike China, Japan and Korea (Grebe et al., 2019). The extensive range of aquaculture products include extracts for pharmaceutical applications, biofuels, feeds for animals, biostimulants and fertilizers for agriculture, raw biomaterials and food for human consumption (Buschmann & Camus, 2019). As aquaculture expands, increased efforts are being made to minimize negative impacts and develop environmentally sustainable, ecosystem-centric strategies (Brugère et al., 2018). While the kelp farming industry often highlights the benefits provided to biodiversity, there is still limited understanding and insufficient experimental research on the ecosystem services provided by seaweed farms (Gentry et al., 2019). A review by Forbes et al. (2022) indicates that although kelp farms can alter habitats, this does not necessarily lead to a net

increase in biodiversity, as responses vary among mobile and sessile communities. There is also potential for kelp farms to drive significant ecosystem changes and facilitate the spread of invasive species and diseases (Campbell et al., 2019). Further research is needed to identify where kelp farms ecosystem services are beneficial (Grebe et al., 2019), to develop effective management strategies (Forbes et al., 2022) and to select strains and cultivars able to tolerate future environmental stressors, optimising commercial yields (Kim et al., 2017).

Research for successful kelp conservation and aquaculture

Due to the emerging interest in kelp restoration actions and aquaculture, several recent studies have focused on improving the performances of early life stages of kelps considering climate change projections. Different strategies have been employed to reach this goal, also taking inspiration from techniques used in crop production. One approach involved interspecific hybridization between *L. digitata* and *L. pallida* Grev. (Martins et al., 2019), based on the concept that hybrid vigour is often observed when different species are crossed (Fu et al., 2014). This has been shown to be effective in kelps, as demonstrated in China where it contributed to the improvement of kelp cultivation (Zhang et al., 2007). The hybrid sporophytes exhibited a 2-3°C increase in thermal tolerance compared to pure species (Martins et al., 2019), suggesting that selective breeding for heat tolerance could be valuable for both restoration actions and aquaculture industries. Additionally, outbreeding between distinct populations of a kelp species might lead to improved performances under heat stress, as suggested in *L. digitata* (Liesner et al., 2022). However, in this case it becomes crucial to carefully evaluate performances, viability and reproductive success of outbred lineages across multiple

generations before employing this approach in aquaculture or conservation efforts, as there is a potential risk of outbreeding depression. Moreover, another concern is that outbreeding and hybridization techniques do not align with current ethical standard and regulatory requirements for introducing non-native genotypes into the environment, particularly in Europe (Ramsay et al., 2022; Vissers et al., 2023).

Since phenotypic plasticity plays a key role in helping sessile organisms adapt to environmental changes, research has focused on how thermal histories influence sporophytes performances. Studies have shown that low temperatures (5°C) during gametogenesis result in higher recruitment rates and improved juvenile sporophyte growth in *L. digitata* (Liesner et al., 2020; Martins et al., 2017). Similarly, *L. digitata* sporophytes derived from gametophytes cultivated at low temperature (5°C) grew more, even at the high temperature of 20°C (Gauci et al., 2022). On the other hand, research on *L. pallida* revealed that warmer gametogenesis conditions led to offspring sporophytes with increased photosynthetic efficiency at higher temperatures (Liboureau et al., 2023). All these studies support the presence of cross-generational effects between gametophytes parents and offspring sporophytes, which could be harnessed to breed heat-tolerant kelp strains. However, the optimal gametogenesis temperature conditions that lead to better sporophyte performances can differ between species, complicating the potential adoption of this technique.

Interestingly, a recent study by Gauci et al. (2024) identified thermal priming of *Saccharina latissima* (L.) Lane, Mayes, Druehl & Saunders gametophytes as a promising technique to enhance seaweed production and to improve restoration of kelp forests affected by climate change. Under heat stress, sporophytes developed from

gametophytes primed for four weeks at 20°C exhibited tolerance over longer periods, and 1°C higher thermal tolerance over one week compared to sporophytes developed from non-primed gametophytes. Further research is needed to explore thermal plasticity, cross-generational effects, and priming techniques that could enhance sporophyte resilience under elevated temperature conditions.

Laminaria ochroleuca Bachelot Pylaie – the study species

Laminaria ochroleuca, also called "golden kelp", is a warm-temperate Iberian species belonging to the order Laminariales. It can be found in relatively warm waters ranging from southern England to Morocco, but it also occurs in the Strait of Messina (Italy), the Azores and in Gorringe Bank (Portugal; Ramos et al., 2016; Assis et al., 2009; Flores Moya, 2012). The 10°C February isotherm (van den Hoek, 1982) constrains its distribution towards the poles, and research confirms the absence of gametogenesis at this temperature (Izquierdo et al., 2001). Nevertheless, *L. ochroleuca* was observed in regions where the minimum sea surface temperature (SST) ranged from 8 to 16.3°C, and the maximum SST ranged from 15.1 to 24.6°C (Franco et al., 2017). The temperature of 24.6°C was identified as the threshold for 50% of sporophyte survival, corresponding to the mean maximum SST in southern Morocco (Franco et al., 2017), which marks the southern boundary of *L. ochroleuca*'s distribution. The environments where *L. ochroleuca* thrives encompass a variety of habitats, spanning from deep intertidal pools and subtidal zones to seabeds reaching depths of 50 meters (i.e., Messina Strait, Italy). Light availability primarily dictates its distribution (Birkett et al., 1998), enabling it to extend its presence even into deeper regions.

L. ochroleuca exhibits extensive latitudinal distribution across different environments, leading to significant intraspecific genetic diversity and phenotypic plasticity (Valladares et al., 2014). Genetic differentiations have accumulated in populations inhabiting deeper and colder regions that have acted as refugia during extreme climate events (Assis et al., 2016). It was also demonstrated that distinct genetic groups can be associated with different upwelling systems, which provide stable climates even during warmer periods (Lourenço et al., 2016). Endemic diversity for *L. ochroleuca* has been identified in upwelling regions, such as the Strait of Gibraltar, the Azores and Morocco (Assis et al., 2018). Furthermore, this species is characterized by limited long-range dispersal, preserving distinct genetic pools identifying different populations. Ocean currents have also acted as barriers to connectivity, keeping genetic hotspots of *L. ochroleuca* isolated and maintaining genetic differentiations over time (Assis et al., 2018). With climate change, upwelling refugia at lower latitudes, such as Gibraltar and Morocco, are at risk, whereas deeper sites like the Azores and the Iberian seamounts may remain safer (Assis et al., 2017). However, further research is needed to fully understand the roles of these distinct refugia (Assis et al., 2018).

The species is currently undergoing a distribution shift, with reported expansion towards the poles possibly attributed to ocean warming (Smale et al., 2013). Increase in abundance was observed along the southwest coast of the UK (Smale et al., 2014), while declines have been reported in southern Portugal over recent decades (Assis et al., 2009; Tuya et al., 2012). Besides the rising sea surface temperatures (SST), another factor contributing to the southward distribution shift is the diminishing intensity of upwelling phenomena along the Iberian coasts, as observed in the last decades (Lemos et al., 2004; Sydeman et al., 2014). Since changes

in habitat-forming species can determine large modifications in ecosystem functioning and community structure, it is becoming increasingly important to understand the consequences of these shifts. There is evidence that *L. ochroleuca* could replace a morphologically and (probably) functionally similar species, *L. hyperborea* (Gunnerus) Foslie, and possibly influence local biodiversity (Smale et al., 2014). However, little is known about its ecology and the environmental conditions which mostly affect its life cycle (Pereira et al., 2019) and further studies are needed.

L. ochroleuca is characterized by a heteromorphic and diplohaplontic life cycle, as the other species belonging to the order Laminariales, which features an alternance between a microscopic haploid gametophyte and a macroscopic diploid parenchymatous sporophyte (Lane at al., 2006). Mature diploid sporophytes generate on the blade reproductive structures called *sori*, where sporangia develop. Within the sporangium, meiotic divisions occur to form microscopic zoospores. These zoospores get released and settle on the bottom, developing into microscopic haploid gametophytes. Male gametophytes produce antheridia, while female gametophytes form oogonia, and, through mitosis, these structures produce respectively motile sperms and eggs. When a sperm cell fertilizes an egg, a zygote is formed, and this results in the formation of a new microscopic diploid sporophyte, which will grow into the next generation of large sporophytes (Dayton, 1985; Figure 1).

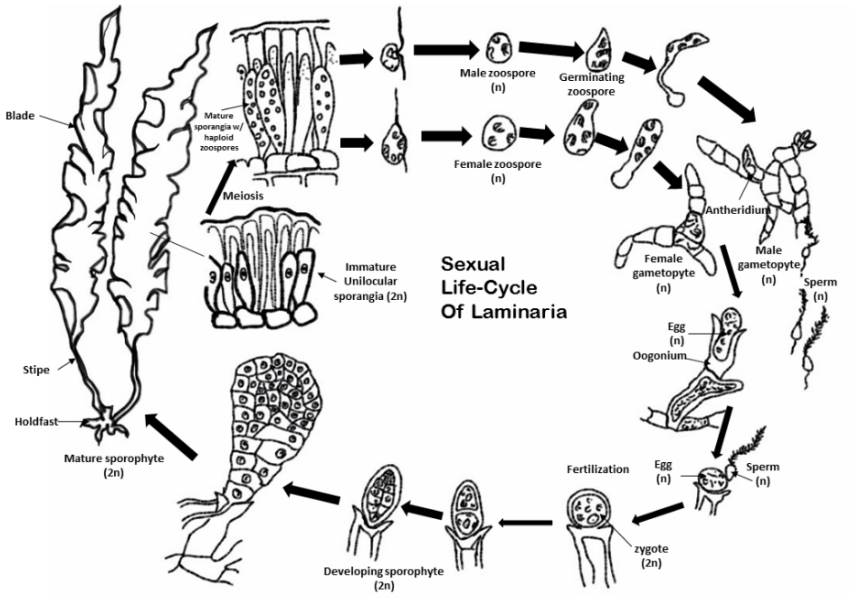

Figure 1 – **Sexual life cycle of *Laminaria spp.*** (Laminaria Life Cycle © Aivaldez94 is licensed under a CC BY-NC-SA license).

Aim of the work

Understanding how phenotypic plasticity and intraspecific adaptive variations influence the latitudinal distribution of kelps and their ability to adapt to future climate changes is crucial, yet relatively few studies have addressed these questions. To address this knowledge gap, this study investigated whether the optimal thermal reproductive range of *L. ochroleuca* varies among populations. Gametophytes from four populations (France, Morocco, Italy and Portugal) were exposed to four temperatures (11°C, 13°C, 16°C and 18°C) during gametogenesis. These temperatures represent the range allowing the gametophyte reproduction in this species. The chosen populations represent the species distribution in the North-East Atlantic and Mediterranean regions, characterized by differential environmental conditions, such as depth distributions (1-4 meters for the Morocco and France populations versus 50 meters for the Italy population) and temperature regimes (average sea surface temperature of 20°C in

Morocco versus 13°C in France). This study was conducted under common garden conditions to eliminate the influence of environmental history. The hypothesis was that different populations originating from distinct habitats, each with specific temperature regimes, could exhibit variations in reproductive success depending on the temperature they typically experience in nature.

Furthermore, this study also investigated whether the temperature during gametogenesis and sporophyte development could affect the thermal tolerance and growth of F1 microscopic sporophytes. This relationship could pave the way for developing heat-resistant strains by taking advantage of these characteristics. Sporophytes from the French population developed at the minimum and the maximum temperatures allowing successful reproduction (11°C and 16°C) were exposed to temperatures ranging from control to sub-lethal and lethal (14°C, 22°C, 23°C and 24°C) and their survival and photosynthetic efficiency were measured to assess their thermal tolerance to high temperatures.

A third experiment was also carried out to investigate whether priming treatments (chemical priming: acute exposure to hydrogen peroxide (H_2O_2) and/or thermal priming: acute exposure to sub-lethal temperature) could affect the tolerance of juvenile macroscopic sporophytes to sub-lethal and lethal temperatures (23°C, 24°C and 25°C). This research could reveal whether priming could help develop more resilient and stress-resistant seaweeds capable of withstanding future environmental challenges.

MATERIALS & METHODS

Algal material

Mature specimens of *L. ochroleuca* were collected by SCUBA diving in Italy (IT; 38°15'27.9756"N, 15°37'40.0404"E; 50 m; November 2019) and Portugal (PO; 38°45'04.1"N 9°28'33.9"W; 12 m; October 2023) or snorkelling in France (FR; 48°41’35.90”N, 3°56’28.53”N; 4 m; November 2018) and Morocco (MO; 33°14'48.46"N, 8°32'38.87"W; 1 m; August 2019; Figure 2).

The local habitats can be distinguished by latitude, but also by mean temperatures of exposure. The Morocco population is the most southern one and experiences local mean maximum temperatures of 23.58°C and mean minimum temperatures of 15.17°C. In contrast, the France population, the northernmost one, faces mean maximum temperatures of 19.34°C and mean minimum temperatures of 8.56°C, with an average of 13.63°C. The Portuguese population, situated centrally along the Atlantic area, is locally exposed to mean maximum temperatures of 20.49°C and mean minimum temperatures of 13.34°C. The Italian population, the only one in the Mediterranean, inhabits a deep habitat (50 m) in the Messina Strait. This habitat is characterized by a minimum mean temperature of 13.47°C, a mean maximum temperature of 17.81°C, and an average temperature of 15.35°C. Maximum, minimum, and average temperatures layers of each sampling location were assessed using R software environment (R Development Core Team, 2016) and Bio-Oracle (https://bio-oracle.org/index.php; Assis et al., 2017; Assis et al., 2024).

Additional information regarding temperatures at each sampling location were obtained from the E.U. Copernicus Marine Service Information (https://doi.org/10.48670/moi-00152,

https://doi.org/10.25423/CMCC/MEDSEA_MULTIYEAR_PHY_006_004_E3R1).

Figure 2 – **Map of Europe and Northern Africa**. The red dots show the sampling sites of the *L. ochroleuca* populations. Created with Google Earth.

Mature tissue was extracted from adult sporophytes, cleaned and the meiospores from each individual were released separately overnight under darkness conditions and left to germinate. The developed gametophytes were maintained in climate-controlled chambers (Fitoclima, S600, Aralab, Lisboa, Portugal) in a vegetative growth state in sterile half-strength Provasoli enriched seawater (PES; Provasoli, 1968; modifications: HEPES buffer instead of TRIS, double concentration of Na_2-glycerophosphate) at 12°C under 3-6 µmol photons m^{-2} s^{-1} of fluorescent red light to prevent gametogenesis, under a 16:8 h light-dark photoperiod (controlled via Fitolog400R software). The cultures were kept under the same conditions for 1-3 years until the start of the experiment, with a monthly medium change.

Sterile artificial seawater (Tropic Marin Sea Salt, Dr. Biener, GmbH, Wartenberg, Germany) with 30-32 ppm salinity (hand refractometer ATAGO CO., LTD) was used for culture maintenance and for all the experiments.

Experiment 1 – Influence of developmental temperature on gametophyte reproduction and subsequent sporophyte thermal tolerance and growth

1.1 Gametophyte reproductive success at different temperatures

Experimental design

For each population, an equal amount of gametophyte vegetative tissue from different individuals (FR: 6 individuals; MO, IT: 5 individuals; PO: 4 individuals) was combined and gently fragmented using a pestle and mortar (Figure 3). Each suspension was sieved using a 100 µm mesh sieve (stainless steel) and diluted in ~400 mL of half-strength medium. The four stock solutions produced were composed by gametophytes with lengths of ≤ 100 µm. The gametophyte density of the four stock solutions was determined using an inverted microscope (100× magnification, Zeiss Axio Observer D1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and the volume required to obtain ~450 gametophytes cm-2 was added to glass beakers (150 mL volume, 5.5 cm height, 5.5 cm diameter) filled with 80 mL of half-strength PES. Four replicate beakers were used per population and thermal treatment (4 populations × 4 thermal treatments × 4 replicates = 64 beakers in total).

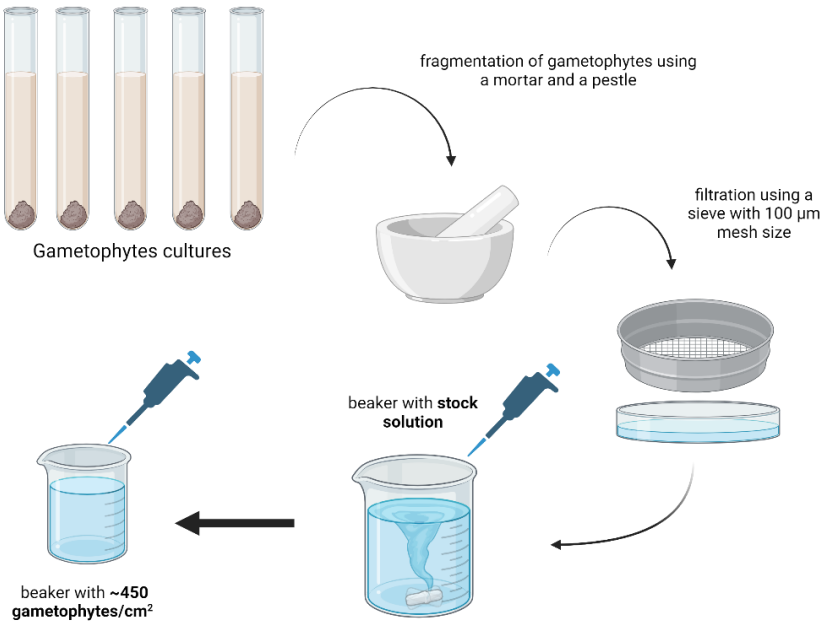

Figure 3 – **Protocol to obtain a stock solution from vegetative gametophyte tissue**. Created with BioRender.com.

The gametophytes were allowed to settle and recover from the fragmentation stress for 3 days under 6 µmol photons m^{-2} s^{-1} of red light in climate-controlled chambers.

Over the first two days of the recovery period, all the gametophytes were exposed to 14°C, while in the last day they were evenly separated and exposed to 13, 14, 14, and 16°C, corresponding later to the target temperatures of 11, 13, 16, and 18°C, respectively (Figure 4). After the recovery period, the gametophytes were transferred to four temperature-controlled water baths (Huber Variostat with Pilot ONE, Offenburg, Germany; Figure 5) with the target temperatures (11, 13, 16, and 18°C) and 20 µmol photons m^{-2} s^{-1} white LED light in a 16:8 h light:dark photoperiod for 32 days (Figure 6). The four temperatures were chosen to cover the thermal range of *L. ochroleuca* gametogenesis; 11°C is the lowest temperature at which gametophytes reproduce (tom Dieck, 1992; Izquierdo et al., 2002), while 18°C is the maximum reproductive

temperature (Izquierdo et al., 2002). The culture medium (half-strength PES) was 50% changed per beaker every 10 days. The irradiance of 20 µmol photons m^{-2} s^{-1} was chosen because it was reported as the optimal light intensity for *L. ochroleuca* gametogenesis (Izquierdo et al., 2002).

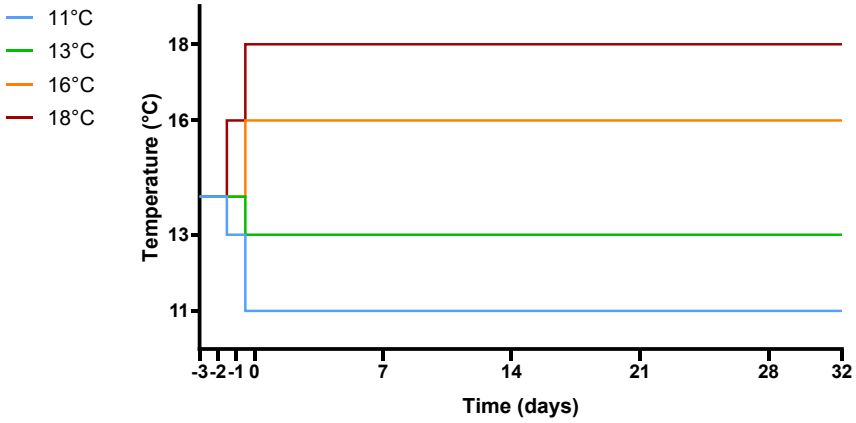

Figure 4 – **Experimental design of the thermal treatments**. The diagram shows the gradual change in temperature performed on the gametophytes coming from the 14°C culture temperature. The target temperatures of 11°C, 13°C, 16°C and 18°C were kept for 32 days. Gametophytes density was evaluated on day 0; gametogenesis was assessed on days 7, 14, 21, 18; sporophytes recruitment was quantified at the end of the experiment on day 32.

Figure 5 – **Temperature controlled water bath for one temperature treatment**.

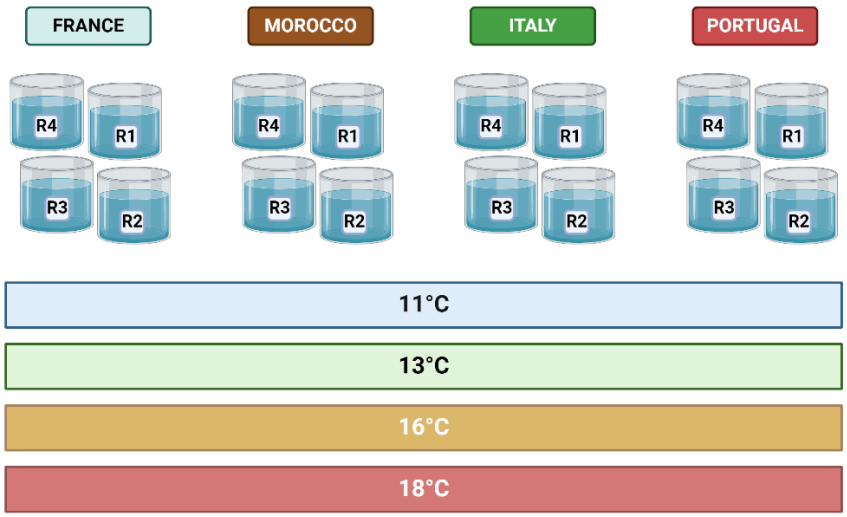

Figure 6 – **Schematic representation of the experimental setup**: 4 replicates for each population (France, Morocco, Italy and Portugal) were exposed to 4 developmental temperatures (11°C, 13°C, 16°C, 18°C). Created with BioRender.com.

Gametogenesis – quantification of ontogenetic stages

Gametogenesis was determined every 7 days over a period of 28 days by quantifying the presence of three ontogenetic stages of female gametophytes (vegetative gametophytes, gametophytes with released eggs and gametophytes with attached sporophytes; Figure 7) in a minimum of 200 female gametophytes per replicate (using an inverted microscope; 100 × magnification). When several developmental stages were present in the same gametophyte, the most advanced stage was recorded. Sporophytes were considered present as soon as the first regular cell division occurred in the zygote.

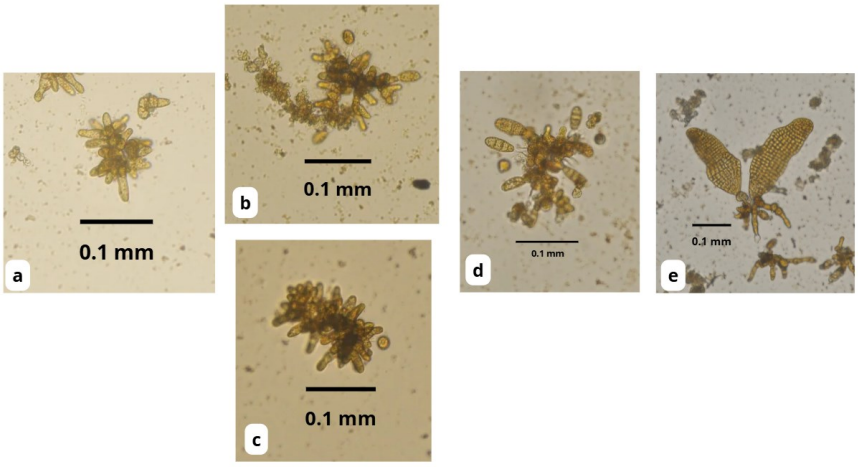

Figure 7 – **Different ontogenetic stages of a female gametophyte**: a) vegetative gametophyte; b-c) gametophyte with released egg; d) gametophyte with sporophyte attached (first cell divisions visible); e) gametophyte with juvenile sporophyte attached. Scale bar = 0.1 mm.

Sporophytes density

Reproductive success was assessed as the absolute number of sporophytes per cm^2 after 32 days. Sporophytes were quantified in a minimum of 60 fields of view per replicate using an inverted Zeiss Axio Observer D1 microscope (100 × magnification). The counting included sporophytes with normal morphology (i.e., sexually formed sporophytes; Figure 8a) and irregular morphology (i.e., possible partheno-sporophytes; Figure 8b-d).

According to Dieck & Oliveira (1993), fertilized diploid sporophytes show a normal and regular morphology (smooth blade), a clear polar differentiation in the basal rhizoid and in the proximal elongated blade and are attached to the respective female oogonium. On the other hand, asexually formed sporophytes (i. e., partheno-sporophytes because they derive via parthenogenesis) are characterized by irregular, malformed and/or twisted shapes and are not attached to female gametophytes.

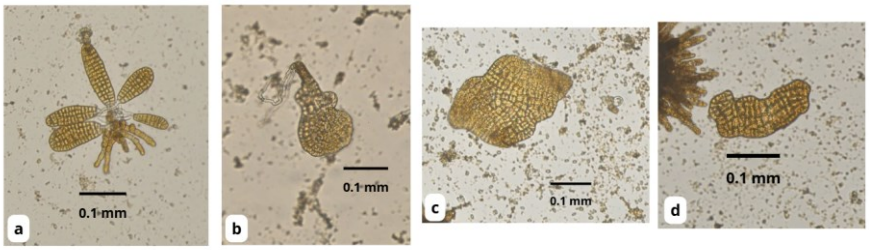

Figure 8 – **Morphological differences between normal and parthenogenetic sporophytes**: a) normal sporophytes; b-c-d) parthenogenetic sporophytes. Scale bar = 0.1 mm.

1.2 – Thermal tolerance of microscopic sporophytes

The French population of *L. ochroleuca* was chosen to analyse the thermal tolerance of sporophytes developed at a minimum and a maximum developmental temperature that allowed reproduction. The aim was to understand if the temperature at which gametogenesis occurs and the sporophytes develop influences the thermal tolerance of the obtained microscopic sporophytes.

Experimental design

Microscopic sporophytes of *L. ochroleuca* from France developed after 40 days under the minimum (11°C) and the maximum (16°C) temperature allowing reproductive success were subjected to four different thermal treatments (14°C, 22°C, 23°C and 24°C) for 14 days (Figure 9). To avoid temperature shock conditions, the sporophytes were gradually exposed to increasing or decreasing temperatures at a rate of 3-4°C day^{-1} for 3 days until reaching the target experimental temperatures (Figure 10 and 11). The thermal treatments included 14°C as the control temperature, two upper sub-lethal temperatures (22°C and 23°C) and a lethal temperature (24°C) to evaluate differences between developmental temperatures (Franco et al., 2017; Izquierdo et al., 2002; tom Dieck, 1992).

Four replicate glass beakers (150 mL volume, 5.5 cm height, 5.5 cm diameter) were used for each thermal treatment (2 developmental temperatures × 4 treatment temperatures × 4 replicates = 32 beakers in total). Each beaker was filled with 80 mL and half-strength PES and the medium was 50% changed every 10 days.

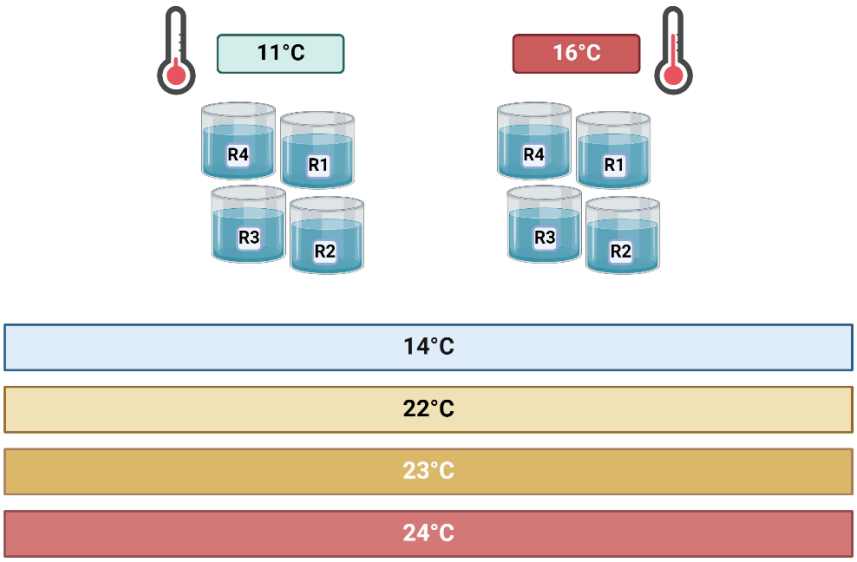

Figure 9 – **Schematic representation of the experimental setup**: The sporophytes developed under two temperatures (11°C and 16°C) were exposed to 4 treatment temperatures (14°C: control; 22°C and 23°C: sub-lethal; 24°C: lethal). Created with BioRender.com.

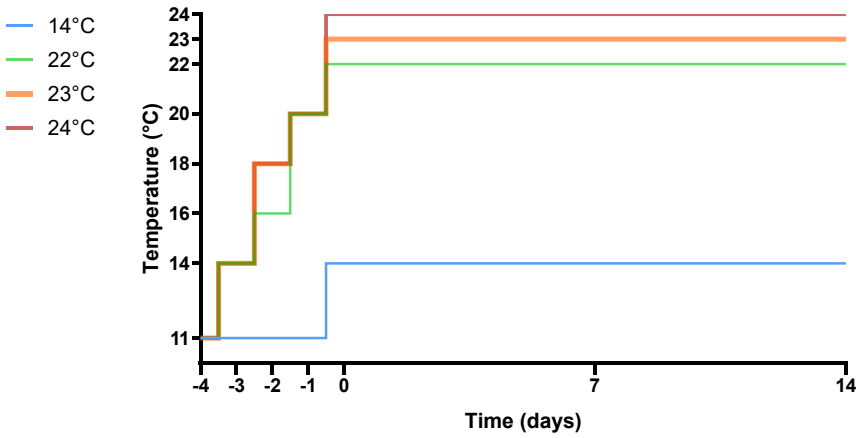

Figure 10 - **Experimental design of thermal treatments**. The diagram shows the gradual increase in temperature performed for the

sporophytes developed at 11°C. The target temperatures of 14°C, 22°C, 23°C and 24°C were reached on day 0 and were kept for 14 days.

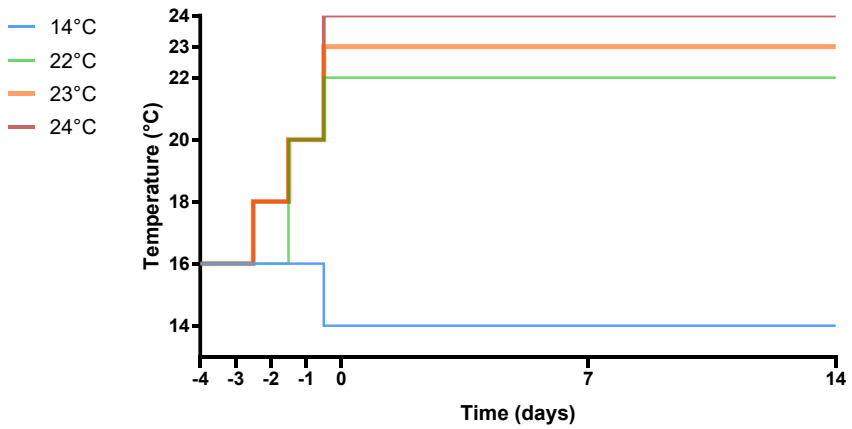

Figure 11 - **Experimental design of thermal treatments.** The diagram shows the gradual increase or decrease in temperature performed for sporophytes developed at 16°C. The target temperatures of 14°C, 22°C, 23°C and 24°C were reached on day 0 and were kept for 14 days.

Four replicate glass beakers (150 mL volume, 5.5 cm height, 5.5 cm diameter) were used for each thermal treatment (2 developmental temperatures × 4 treatment temperatures × 4 replicates = 32 beakers in total). Each beaker was filled with 80 mL and half-strength PES and the medium was 50% changed every 10 days.

The experiment was conducted in four temperature-controlled water baths (Huber Variostat with Pilot ONE, Offenburg, Germany) with 20 µmol photons m^{-2} s^{-1} white LED light in a 16:8 h light:dark photoperiod. To evaluate the thermal tolerance of microscopic sporophytes developed under different developmental temperatures, the photosynthetic efficiency, the survival and the growth were assessed.

Sporophyte photosynthetic performance

To assess the physiological performance of the microscopic sporophytes developed under the two different developmental

temperatures to the temperature treatments, the maximum quantum yield of PSII (F_v/F_m) was measured on days 0 and 14 of the experiment using an AquaPen-P AP 110P fluorometer (Photon Systems Instruments, Drásov, Czech Republic; 20% flash pulse, 70% super pulse and 20% actinic pulse). Sporophytes were acclimated to the dark for 10 minutes before the measurements. The photosynthetic measurements might also take into consideration gametophyte cells that did not become reproductive.

Sporophyte density

Sporophyte density was measured on days 1 and 15 of the experiment to quantify sporophyte survival under the different thermal treatments. Sporophytes were quantified in a minimum of 25 fields of view per replicate using a stereomicroscope (Zeiss Stemi 305, Carl Zeiss MicroImaging GmbH, Göttingen, Germany; 40 × magnification). Data were normalized with respect to initial sporophyte densities, thereby allowing comparisons between developmental temperatures.

Sporophyte relative growth rate

To assess the influence of temperature treatments on sporophyte growth, the blade length was determined on days 0 and 14.

For each replicate, 15 random fields of view were photographed using a microscope camera (Zeiss Axiocam 208 color, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) connected to a stereomicroscope (Zeiss Stemi 305, Carl Zeiss MicroImaging GmbH, Göttingen, Germany; 20 × magnification).

The 3 longest sporophyte blades were measured per picture (45 sporophytes per replicate) using ImageJ software (Schneider et al., 2012). The average sporophyte length was calculated for each

replicate and the relative growth rate (RGR) was calculated using the following formula, where T stands for time:

$$RGR\ (cm\ day^{-1}) = [\ln(length_{final}) - \ln(length_{initial})]/T$$

Experiment 2 – Effects of chemical and thermal priming on the thermal tolerance of juvenile sporophytes

Algal cultivation

A gametophyte stock solution was produced by mixing an equal amount of gametophyte vegetative tissue derived from 6 *L. ochroleuca* sporophytes from France and gently fragmenting with a mortar and a pestle. The suspension was diluted in half-strength PES, sieved using a 100 µm mesh sieve and transferred into Petri dishes (90 mm diameter, height 35 mm), containing 20-25 mL of medium (half-strength PES). The gametophytes were exposed to 13°C and 20 µmol photons m^{-2} s^{-1} of white light with a 16:8 h light:dark photoperiod to induce the gametogenesis. The medium was 50% changed every 7 days. After 2 months, the developed sporophytes were detached from the Petri dishes and transferred into aerated 2 L glass bottles and maintained under the same culture conditions. One week later, sporophytes were transferred into aerated 5 L glass bottles and kept under the same culture conditions.

After one month, the sporophytes growing in clusters were individualized and the light intensity increased to 30 µmol photons m^{-2} s^{-1} for another month until sporophytes reached a size of 5-6 cm in length. Medium was changed twice a week to enhance sporophyte growth.

Experimental design

Four priming treatments were used: 13°C (control), 23°C (thermal priming), 0.05 mM of H_2O_2 at 13°C (chemical priming) and 0.05 mM of H_2O_2 at 23°C (thermo-chemical priming). Sixty-four sporophytes were transferred into a 5 L aerated glass bottle filled with half-strength PES per treatment. All priming treatments lasted for 2 days. The temperature of 23°C was reached with a gradual increase at a rate of 3-4°C $hour^{-1}$. On the last day of priming, the temperature was slowly decreased from 23°C back to 13°C with the same rate of 3-4°C $hour^{-1}$ (Figure 12). These treatments were carried out in climate-controlled chambers (Fitoclima S600) under 30 µmol photons m^{-2} s^{-1} of white light in a 16:8 h light:dark photoperiod.

Hydrogen peroxide (H_2O_2) was selected for the priming treatment due to its beneficial effects on terrestrial plants under various stresses, such as heat, drought and cold. Pretreatment with H_2O_2 enhances the antioxidant response (Gao et al., 2010; Wang et al., 2014; Iseri et al., 2013, Bhattacharjee, 2012), induces thermotolerance (Kang et al., 2009), promotes higher growth rates (Ahmad et al., 2015) and improves photosynthetic efficiency (Zheng et al., 2018) in plants facing different types of stress.

The concentration of H_2O_2 used in the priming treatments (0.05 mM) was chosen according to priming methods involving soaking of terrestrial plants organs in H_2O_2 -enriched solutions (Uchida et al., 2002) and to a preliminary test using three different H_2O_2 concentrations (1 mM, 0.1 mM and 0.01 mM). The sporophytes grown in 1 mM H_2O_2 died after 2 days, while the ones grown in 0.1 mM and 0.01 mM were not damaged, therefore the mean concentration of 0.05 mM was selected for the experiment.

The sub-lethal temperature of 23°C (Franco et al., 2017; Izquierdo et al., 2002; tom Dieck, 1992) was chosen for the priming treatment

as it provides a heat "shock" stress to the sporophytes. This approach can help to mitigate the damages caused by prolonged exposure to constant heat stress, as demonstrated in *Laminaria japonica* Areschoug by Zhou et al. (2010), who found that the heat-pretreatment alleviated the inhibition of antioxidant enzymes, the increase in MDA content and the decrease in chlorophyll *a* content.

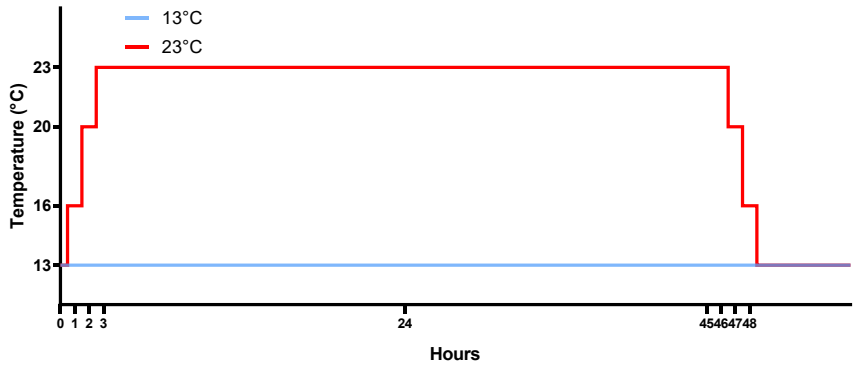

Figure 12 – **Experimental design of the priming treatment**. For the thermal priming, the temperature of 23°C was reached with a gradual increase of 3-4°C hour^{-1} and was kept for 2 days before decreasing again with the same pattern to the recovery temperature of 13°C.

After the priming treatment, sporophytes were allowed to recover at the control temperature of 13°C without H_2O_2 for 2 days. After the recovery period, the sporophytes were moved to 1 L aerated glass beakers and transferred to four temperature-controlled water baths (Huber Variostat with Pilot ONE) with the target temperatures of 13°C, 23°C, 24°C and 25°C and exposed to 30 µmol photons m^{-2} s^{-1} white LED light in a 16:8 h light:dark photoperiod (Figure 13). Four replicate beakers containing 4 sporophytes each per priming treatment were exposed to target temperatures (4 pre-treatments × 4 temperatures × 4 replicates = 64 replicates) for 14 days. Target temperatures were reached by gradually increasing from the recovery temperature of 13°C at a warming rate of 1-3°C day^{-1} (Figure 14). One set of beakers per pre-treatment was left at 13°C.

The temperature of 13°C was chosen as the control temperature since it leads to optimal sporophyte growth in *L. ochroleuca*, while 23°C, 24°C and 25°C were chosen to induce sub-lethal and lethal stress to the sporophytes (Franco et al., 2017; Izquierdo et al., 2002; tom Dieck, 1992; Figure 15). The culture medium (half strength PES) was changed twice a week.

Figure 13 - **Temperature controlled water bath for one temperature treatment**. Each 1 L aerated glass beaker contained 4 sporophytes.

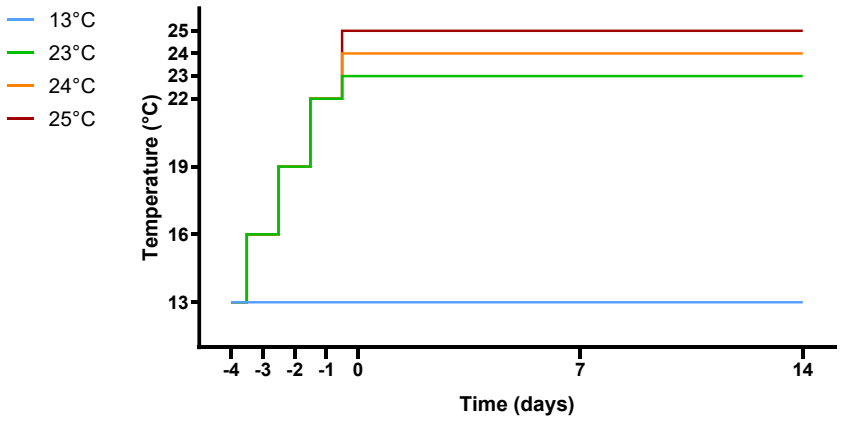

Figure 14 – **Experimental design of the thermal treatments**. The diagram shows the gradual increase in temperature performed for sporophytes coming from the 13°C recovery temperature. The target temperatures of 13°C, 23°C, 24°C and 25°C were kept for 14 days. Sporophytes growth and photosynthetic performance were evaluated on day 0 and 14.

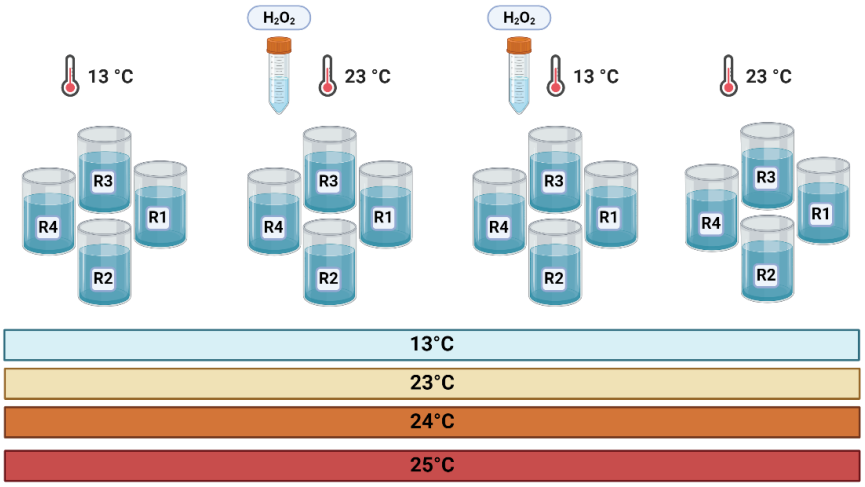

Figure 15 - **Schematic representation of the experimental setup**: sporophytes exposed to four priming treatments (control: 13°C; chemical priming: H_2O_2 at 13°C; thermo-chemical priming: H_2O_2 at 23°C; thermal priming: 23°C) were later exposed to 4 temperatures (13°C: control; 23°C and 24°C: sub-lethal; 25°C: lethal). Created with BioRender.com.

Measurements of quantum yield (F_v/F_m) and sporophyte growth rate were performed on days 0 and 14 of exposure to target temperatures, to assess the sporophytes response to the pre-treatments and their subsequent thermal tolerance.

Sporophyte relative growth rate

Sporophyte relative growth rate was calculated by measuring the sporophyte surface area, using the ImageJ software (Schneider et al., 2012). Pictures of all sporophytes were taken (using a Nikon D90 camera; Nikon, Tokyo, Japan) on days 0 and 14 of the thermal exposure experiment (13, 23, 24, 25°C).

The average sporophyte area was calculated for each priming treatment (n = 64 sporophytes) and for each replicate during the thermal exposure phase (n = 4 sporophytes per beaker).

The relative growth rate (RGR) was then calculated according to the following formula, where T indicates time:

$$RGR\ (cm^2\ day^{-1}) = [\ln(area_{final}) - \ln(area_{initial})]/T$$

Sporophytes photosynthetic performance

The photosynthetic performance of the sporophytes was measured on days 0 and 14 of the thermal exposure phase (in one random sporophyte per replicate) using a pulse amplified modulated (PAM) fluorometer (Diving PAM, Walz, Germany) through the following parameters: F_v/F_m (maximum photochemical efficiency of Photosystem II), rapid light curves (RLC) – which can provide information on the saturation status of electron transport [$rETR_{max}$] and on the overall photosynthetic performance of a plant, since it measures the effective quantum yield as a function of irradiance (Ralph & Gademann, 2005) –, maximum photosynthetic efficiency (Alpha) and light saturation parameter (Ik). Before taking the measurements, sporophytes were dark acclimated for 5 minutes.

Statistical analyses

Before each statistical analysis, the normality and the homoskedasticity (homogeneity of variances) of the residuals were tested using the Shapiro-Wilk test and the Levene's test, respectively. If the previous assumptions were not met, a permutation test on the multivariate dispersion of data points within each group was performed to ensure that sample groups exhibited similar variability in multivariate space. This step was necessary to confirm that observed significant differences identified by PERMANOVA were not confounded by heterogeneity of variance between groups.

When a significant main effect or interaction was found, post-hoc pairwise comparisons were conducted using either the BH or Bonferroni correction to detect significant differences between treatments. The choice of correction depended on the number of pairwise comparisons: the BH correction, being less stringent, was applied if more comparisons were made than required. In contrast, the Bonferroni correction, which is more stringent, was used if the exact number of requested comparisons was performed.

Differences were considered significant when *p-value* <0.05. It is worth noting that in some cases, even with the less stringent BH correction, p-values for some pairwise comparisons were equal to or slightly higher than 0.05 (up to 0.06). However, these differences were still considered significant due to the high number of unaccounted comparisons performed by the pairwise test. The correction, which was based on the total number of comparisons performed, may have artificially inflated the p-value, resulting in values slightly above the typical significance threshold. All the statistical analyses were conducted using R software (4.4.1, version RStudio 2024.04.2+764).

Gametophytes reproductive success at different temperatures (experiment 1.1)

The gametogenesis data (% of eggs and sporophytes) measured on days 14 and 28 met the assumptions of homogeneity of variances and normality of residuals, without requiring any transformation. Therefore, a Linear Mixed-Effects Model (LMM) was fitted to the data, with population and temperature as fixed factors, including their interaction. A random intercept for population was added to account for baseline differences across populations, since the samples were collected from a limited number of individuals, not fully representing the entire population. These specific days were

selected because they exhibited the greatest differences in the percentages of ontogenetic stages between treatments.

A Linear Mixed-Effects Model (LMM) was applied to assess the effects of population and temperature on the sporophyte density. The response variable was ln-transformed to meet model assumptions. Fixed factors included population, temperature and their interaction, with a random effect for population to account for variability across populations. A replicate of FR gametophytes reproduced at 13°C was identified as an outlier and excluded from the analysis, which contributed to stabilizing the model. However, it is worth noting that when the outlier was included in the analysis, no significant differences were found for the main effects or their interaction. As a result, the reliability of these findings may be less certain.

The partheno-sporophyte density data did not meet the assumptions of normality and homoscedasticity of residuals. A two-factors PERMANOVA (based on a Euclidean distance matrix and using 9999 permutations), with population and temperature as fixed factors, was applied. The analysis was stratified by population to account for the lack of independence among replicates within each population. It is important to note that the very high density of 131,9 partheno-sporophytes/cm^2 recorded in one of the four MO replicates at 13°C is likely an outlier, potentially due to a counting error. However, this potential outlier was included in the statistical analyses, since removing it would have violated the assumption of homogeneity of multivariate dispersion required to perform PERMANOVA.

Thermal tolerance of microscopic sporophytes (experiment 1.2)

F_v/F_m data met the assumptions of normality and homoskedasticity of residuals, therefore were analysed with a two-way ANOVA with

developmental temperature and experimental temperature as fixed factors. Independent t-tests performed for all the data collected on day 0 revealed significant differences in sporophytes density between replicates developed under the two developmental temperatures. For this reason, data was normalized to consider significant differences in the initial sporophyte densities between the developmental temperatures, allowing direct comparisons. Since RGR and normalized sporophytes density data did not meet the assumptions of homoskedasticity and normality of residuals, even after transformation, they were analysed using a 2-factor PERMANOVA (based on a Euclidean distance matrix and using 9999 permutations), considering as fixed factors developmental temperature and experimental temperature and including their interaction in the model. In the RGR data, an outlier from a replicate developed at 11°C and exposed to 24°C was excluded from the analysis and from the graph to improve visual clarity.

Effects of chemical and thermal priming on the thermal tolerance of juvenile sporophytes (experiment 2)

Since RGR data did not meet the assumptions of normality and homoskedasticity of residuals even after transformation, it was analysed using a two-factor PERMANOVA (based on Euclidean distances and using 9999 permutations), considering as fixed factors priming treatment and experimental temperature and including their interaction in the model. Independent t-tests performed on the photosynthetic parameters alpha, $rETR_{max}$, Ik and F_v/F_m on day 0 detected significant differences only for alpha and $rETR_{max}$ between the temperature treatments. For this reason, data was normalized to consider these initial differences and to allow direct comparisons. In the analysis of normalized alpha and $rETR_{max}$, the intercept was removed from the models (LMM and two-way

ANOVA, respectively) to prevent the introduction of unnecessary variance, as the normalized data were already centered around zero. In addition, two sporophytes pre-exposed to 13°C (control priming treatment) died when later exposed to 25°C and had photosynthetic parameter values of 0 on the last day of the experiment. These sporophytes were excluded from the analysis because their photosynthetic efficiency could not be measured.

The F_v/F_m parameter data also failed to meet the assumptions of normality and homoskedasticity of residuals even after transformation, therefore it was analysed using a two-factors PERMANOVA (based on Euclidean distances and 9999 permutations), considering as fixed factors priming treatment and experimental temperature and including their interaction in the model. The minimum saturating irradiance (Ik) data and the light-limited photosynthetic rates (α) data, which met the assumptions of normality and homoskedasticity of residuals, were analysed with a Linear Mixed-Effects Model (LMM). The fixed factors included priming treatment, experimental temperature and their interaction. A random effect was considered for the bottle to account for the variability across the four bottles in which the priming treatments were performed. The maximum electron transport rate ($rETR_{max}$) data was analysed using a two-way ANOVA, since it met the assumptions, but it was not possible to apply an LMM. The fixed factors were priming treatment and temperature, and their interaction was included in the model.

RESULTS

Experiment 1.1 – Gametophytes reproductive success at different temperatures allowing reproduction

Gametogenesis – percentage of ontogenetic stages

The development of ontogenetic stages of each population at different developmental temperatures is shown in Figure 16. By day 7, MO and PO gametophytes under all temperatures tested as well as the gametophytes from FR at 16°C exhibited a fast reproduction rate, showing high percentage of eggs and sporophyte stages (70/90%). On the other hand, gametogenesis was delayed in the IT population, being observed mostly vegetative gametophytes under all temperatures tested. After 14 days, in the MO and PO gametophytes under the temperatures between 11°C and 16°C, the percentage of eggs and sporophytes was high ranging from 72.2% to 84.2% and from 76.4% to 94.6%, respectively. The key difference between the two populations lies in the proportion of developed sporophytes. In the Morocco population, the percentage of sporophytes was higher than that of eggs (70/80% vs 5/10%), while in the PO population, the percentage of released eggs generally exceeded that of formed sporophytes (30/40% vs 45/60%).

Overall, the Atlantic populations (FR, MO and PO) displayed different temperature preferences for optimal reproduction compared to the sole Mediterranean population (IT). The Atlantic populations reproduced more efficiently at lower temperatures (up to 16°C), whereas the IT population produced more sporophytes at the highest temperature (18°C).

Interestingly, the MO population showed greater reproductive success starting at the lowest temperature (11°C), but at the highest temperature (18°C) the number of sporophytes was unexpectedly

low. This was surprising, because the sea surface temperature (SST) where the Moroccan individuals were sampled is typically higher than that characterizing the habitat of French and Portuguese individuals.

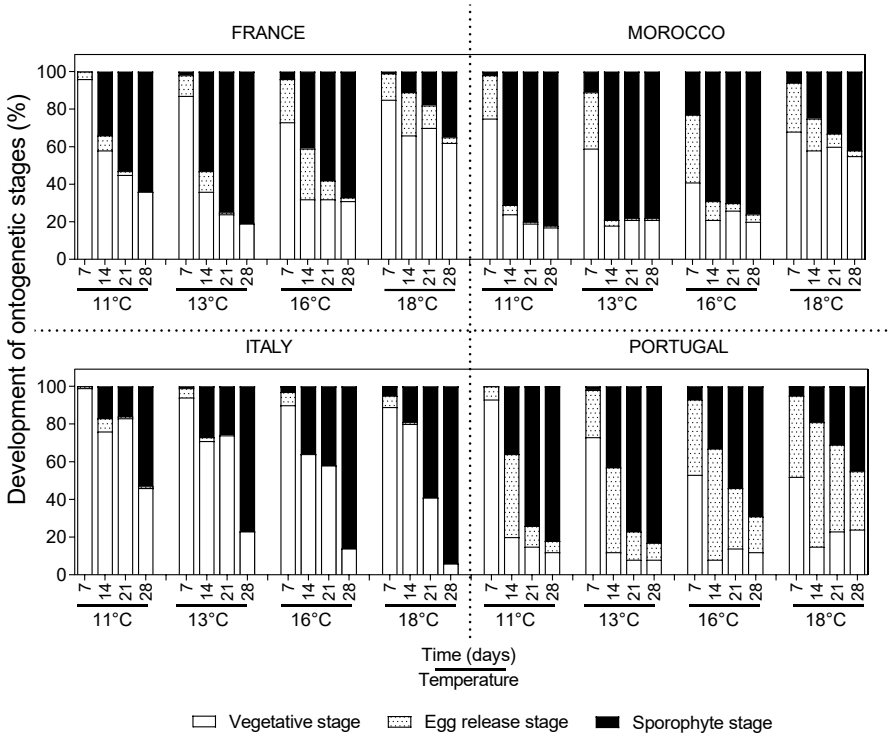

Figure 16 – **Development of ontogenetic stages (vegetative, egg release and sporophyte) over gametogenesis for all four *L. ochroleuca* populations (FR, MO, IT, PO)**. (mean values, n = 4). Counting was performed every 7 days for a period of 28 days.

The percentage of female gametophytes with eggs and/or sporophytes at day 14 revealed significant temperature × population interactions (Table 1; Figure 17). Reproduction was significantly lower in the gametophytes from FR and IT exposed to 11°C and 18°C, compared to 13°C and 16°C. The MO gametophytes showed high reproductive rates at 11°C, 13°C and 16°C, with percentages ranging from 76% to 82%, highlighting a fast reproduction. At 18°C, the reproduction significantly dropped compared to the other temperatures.

The PO population had the highest reproductive success across all temperatures with consistently high percentages of eggs + sporophytes (80%-91%). These gametophytes maintained stable reproductive performance, showing no significant differences across the tested temperatures. On the other hand, the IT gametophytes consistently exhibited the lowest reproductive success across all temperatures, with eggs + sporophytes percentages ranging from 20% to 36%.

Overall, the highest temperature (18°C) led to reduced reproductive success in all populations, except PO.

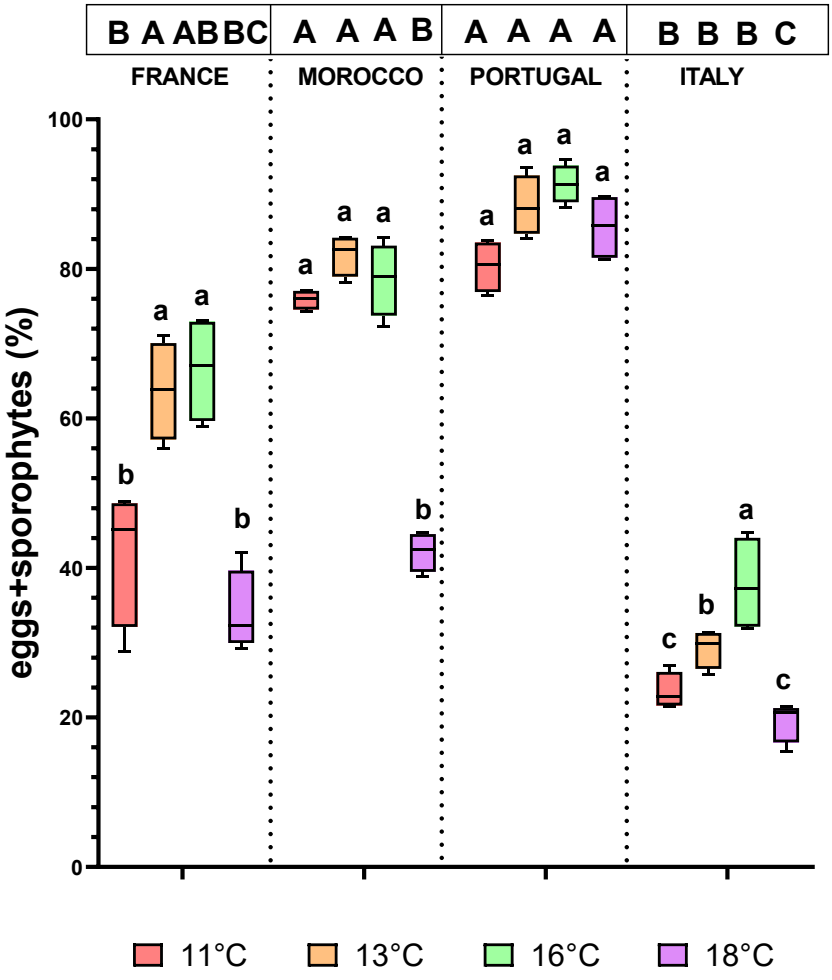

Figure 17 – **Female gametophytes reproductive potential (percentage of female gametophytes with eggs and sporophytes) of different populations of *L. ochroleuca* after 14 days over**

different developmental temperatures. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). At each temperature, different uppercase letters indicate differences between populations ($p < 0.05$). For each population, different lowercase letters above boxplots indicate differences between temperatures ($p < 0.05$). See table 1 for statistics.

Table 1 – Linear Mixed-Effects Model (LMM) for the effects of developmental temperature and population on the percentage of female gametophytes with eggs and sporophytes (ln transformed data) of *L. ochroleuca* after 14 days.

Factors	SS	Mean Sq	Df	F	Pr(>F)
population	0,25481	0,08494	3	6,9308	< **0,001**
temperature	2,49191	0,83064	3	67,7803	< **0,001**
population:temperature	1,01952	0,11328	9	9,2437	< **0,001**

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value.

After 28 days, a significant temperature × population interaction was observed for the percentage of female gametophytes with eggs and/or sporophytes (Table 2; Figure 18). The IT gametophytes exhibited a marked improvement in reproductive performance at higher temperatures (16°C and 18°C), reaching nearly 100% reproductive success at 18°C. Unlike other populations, IT had its lowest reproductive success at 11°C (around 45%-60%). The FR gametophytes showed optimal reproduction at 13°C (~83%), followed by a slight decrease at 11°C and 16°C (around 60%-75%). At 18°C, the reproductive success dropped significantly (~40%), making it the least favourable temperature for this population. The PO population once again had the most consistent reproductive performance across all temperatures, with values between 70 and 95%. The gametophytes from MO and PO showed higher reproductive success across the lower and mid-range temperatures (11°C, 13°C and 16°C) compared to 18°C.

At the highest temperature of 18°C, the highest reproductive output occurred in the gametophytes from PO and IT, highlighting their tolerance to warm temperatures, while the FR and MO gametophytes showed significantly reduced performance, with percentages of gametophytes with eggs and/or sporophytes dropping by half. In contrast, 11°C proved less favourable for the IT population, indicating different thermal optima across populations.

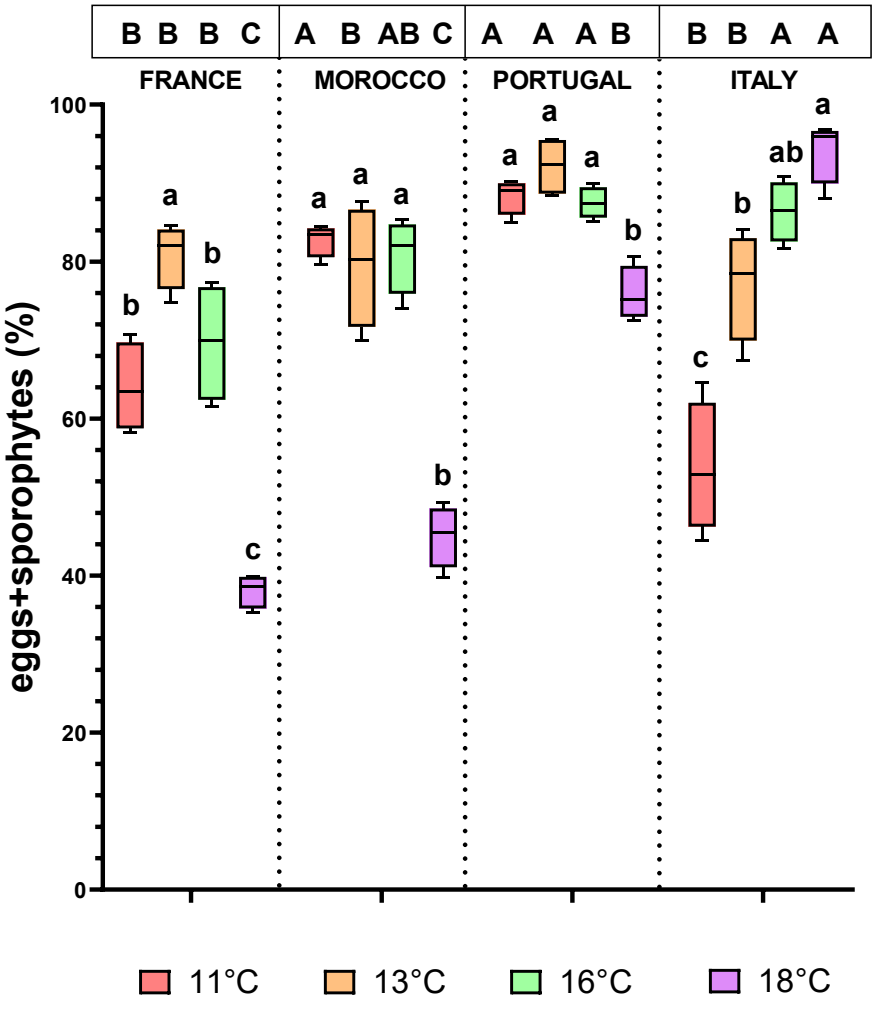

Figure 18 – **Female gametophytes reproductive potential (percentage of female gametophytes with eggs and sporophytes) of different populations of *L. ochroleuca* after 28 days over different developmental temperatures**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). At each temperature, different uppercase letters indicate differences between populations ($p < 0.05$). For each

population, different lowercase letters above boxplots indicate differences between temperatures ($p < 0.05$). See table 2 for statistics.

Table 2 – Linear Mixed-Effects Model (LMM) for the effects of developmental temperature and population on the percentage of female gametophytes with eggs and sporophytes of *L. ochroleuca* after 28 days.

Factors	SS	MeanSq	Df	F	Pr(>F)
population	702,6	234,19	3	9,2455	**< 0,001**
temperature	3795,8	1265,26	3	49,9501	**< 0,001**
population:temperature	8346,3	927,37	9	36,6106	**< 0,001**

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; MeanSq: mean square; F: F value.

Sporophytes density

The sporophytes density after 32 days showed significant temperature × population interactions (Table 3; Figure 19). The overall trends are similar to those observed for the reproductive outputs after 28 days.

The PO population showed higher sporophyte densities at 11°C and 13°C compared to 16°C and 18°C, possibly indicating a non-optimal temperature for sporophytes development. The gametophytes from FR and MO displayed higher sporophyte densities at temperatures from 11°C to 16°C (~250 to 450 individuals per cm^2) than at 18°C. The IT gametophytes followed a different thermal response pattern, with sporophyte densities increasing as temperatures rose. The highest number of sporophytes was observed at 18°C (~160 sporophytes/cm^2) compared to 11°C, a marked contrast to the other populations, but with no significant differences found with the sporophyte densities at 13°C and 16°C. At temperatures between 11°C and 16°C, lower sporophyte densities were observed in the IT gametophytes (~70-130

sporophytes/cm^2) than in the gametophytes from the other populations.

Overall, 18°C resulted in the lowest sporophyte densities for all populations except IT, which experienced its best performance at this temperature. Despite this, the IT population showed relatively low sporophyte development across all temperatures, compared to the other populations.

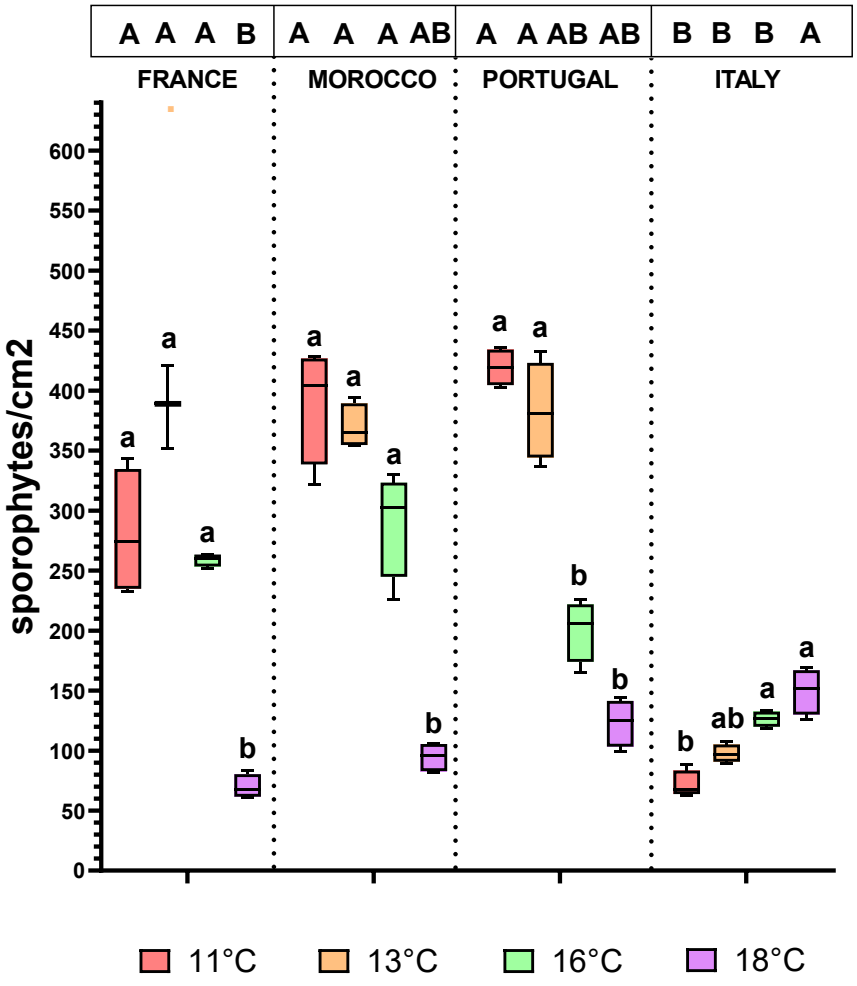

Figure 19 – **Sporophyte density of different populations of *L. ochroleuca* after 32 days over different developmental temperatures**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). At each temperature, different uppercase letters indicate differences between populations ($p < 0.05$). For each population, different lowercase letters above boxplots indicate differences between

temperatures ($p < 0.05$). See table 3 for statistics. Outliers are represented by a point.

Table 3 – Linear Mixed-Effects Model (LMM) for the effects of developmental temperature and population on the sporophyte density (ln transformed data) of *L. ochroleuca* after 32 days.

Factors	**SS**	**Mean Sq**	**Df**	**F**	**Pr(>F)**
population	1,2100	0,40332	3	27,267	**< 0,001**
temperature	1,3324	0,44413	3	30,026	**< 0,001**
population:temperature	1,3574	0,15083	9	10,197	**< 0,001**

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; Mean Sq: mean square; F: F value.

Partheno-sporophyte density

The density of partheno-sporophytes (abnormal sporophytes that develop via parthenogenesis from unfertilized eggs) on day 32 showed significant temperature × population interactions (Table 4; Figure 20). Overall, gametophytes from populations that reproduced faster, such as PO and MO, had a higher density of partheno-sporophytes. The highest densities were observed in the PO population at 11°C and 13°C and in the MO population at 13°C.

In contrast, populations that reproduced slower, such as IT and FR, exhibited lower partheno-sporophyte densities across all temperatures. Overall, the density of partheno-sporophytes across all temperatures and populations generally ranged from 5 to 50 individuals per cm^2, with the highest values reaching between 100 and 130 individuals per cm^2. The gametophytes from IT and MO populations showed no significant variations in the densities across temperatures (1 to 20 partheno-sporophytes per cm^2). The FR gametophytes showed higher partheno-sporophyte densities at 16°C (30-40 individuals per cm^2) compared to the other

temperatures. On the other hand, higher densities of abnormal sporophytes were observed at 11°C and 13°C in the PO gametophytes.

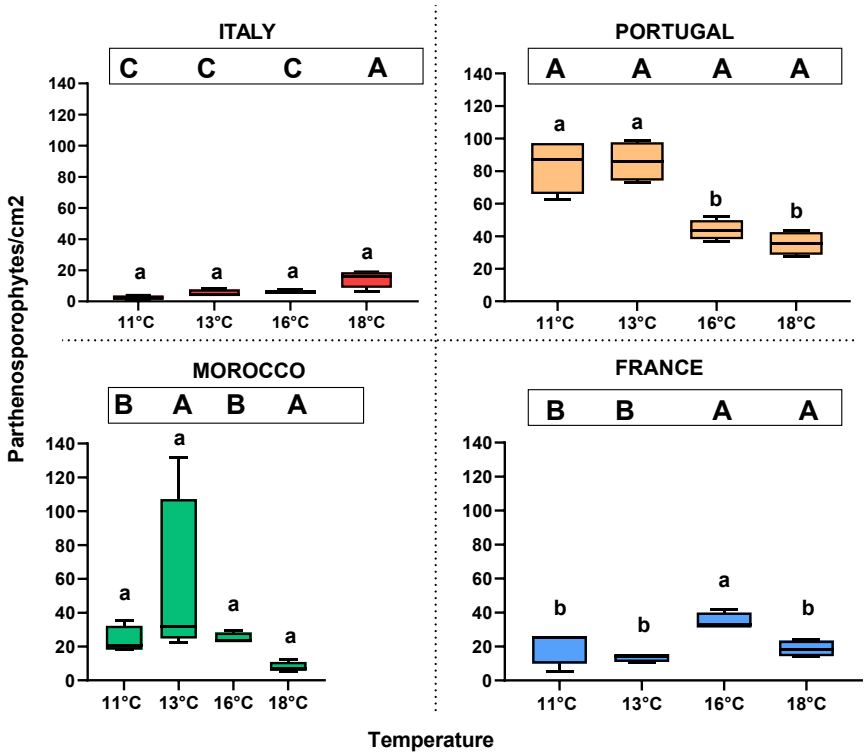

Figure 20 – **Density of partheno-sporophytes (asexually formed sporophytes) of different populations of *L. ochroleuca* after 32 days over different developmental temperatures**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). At each temperature, different uppercase letters indicate differences between populations ($p < 0.05$). For each population, different lowercase letters above boxplots indicate differences between temperatures ($p < 0.05$). See table 4 for statistics.

Table 4 – Two-factor PERMANOVA for the effects of developmental temperature and population on the partheno-sporophyte density of *L. ochroleuca* after 32 days.

Factors	**Df**	**SS**	**R2**	**F**	**Pr(>F)**
temperature	3	3624	0,07150	5,5387	**0,0016**
population	3	26082	0,51455	39,8564	< **0,001**
temperature:population	9	10512	0,20738	5,3545	< **0,001**

Residual	48	10470	0,20656		
Total	63	50688	1		

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value by permutation.

Experiment 1.2 – Thermal tolerance of microscopic sporophytes

Sporophyte relative growth rate

The sporophyte relative growth rate (RGR) was significantly affected only by the experimental temperatures (Table 5, Figure 21). Even though PERMANOVA found a significant effect of developmental temperatures, subsequent pairwise tests detected no differences. The highest RGR was observed at 14°C, showing positive growth values, indicating active growth. In contrast, RGR decreased significantly at 22°C and even more at 23°C approaching values around zero, which reflects little to no growth. The lowest RGR values were observed at 24°C, where growth rates became negative, indicating sporophytes death.

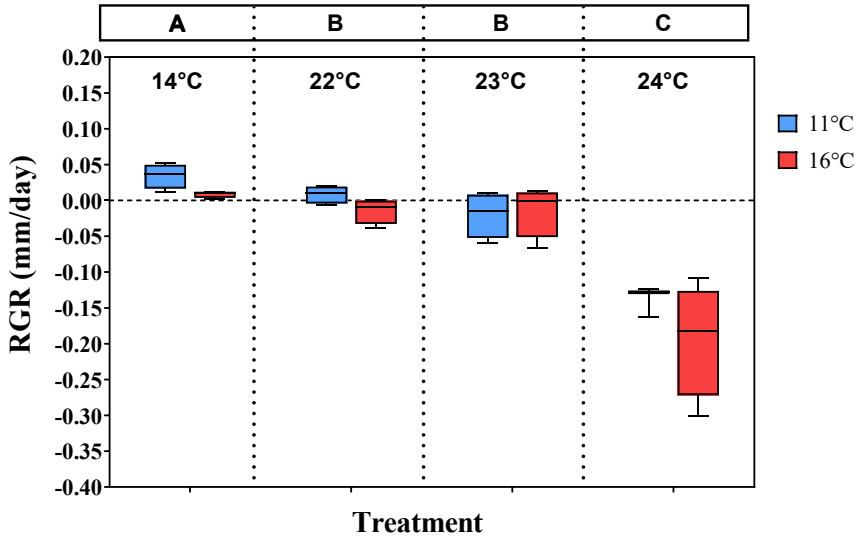

Figure 21 – **Effects of developmental temperature on the relative growth rate (length-RGR) of *L. ochroleuca* sporophytes from the France population after 14 days in different experimental temperatures (14°C, 22°C, 23°C, 24°C)**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Different letters indicate significant differences between experimental temperatures. An outlier was excluded from the graph: the RGR value is -0,942 and was measured on the replicate developed at 11°C and exposed to 24°C. See table 5 for statistics.

Table 5 – Two-factor PERMANOVA for the effects of developmental temperature (11°C and 16°C) and experimental temperature (14°C, 22°C, 23°C, 24°C) on the relative growth rate of *L. ochroleuca* sporophytes after 14 days.

Factors	**Df**	**SS**	**F**	**R2**	**Pr(>F)**
developmental temp	1	0,007784	6,0724	0,03885	**0,0191**
experimental temp	3	0,159582	41,4964	0,79649	**< 0,001**
developmental T:experimental T	3	0,003506	0,9116	0,01750	0,4525
Residuals	23	0,029484		0,14716	
Total	30	0,200355		1	

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value by permutation.

Sporophytes density

Sporophyte densities after 14 days of exposure to experimental temperatures (14°C, 22°C, 23°C and 24°C) showed significant developmental temperature × experimental temperature interactions (Table 6; Figure 22).

For sporophytes developed at 11°C, a significant reduction in densities occurred only at 24°C, while those developed at 16°C showed a significant gradual decline in the number of sporophytes (i.e., mortality) with the increase of temperatures to 22°C, 23°C and 24°C. Notably, at 23°C, the number of sporophytes differed significantly between the two developmental temperatures, with sporophytes developed at 11°C exhibiting higher survival rates. At 24°C, sporophytes developed at both developmental temperatures experienced near total mortality, as indicated by the normalized sporophyte densities near zero, identifying 24°C as the lethal temperature.

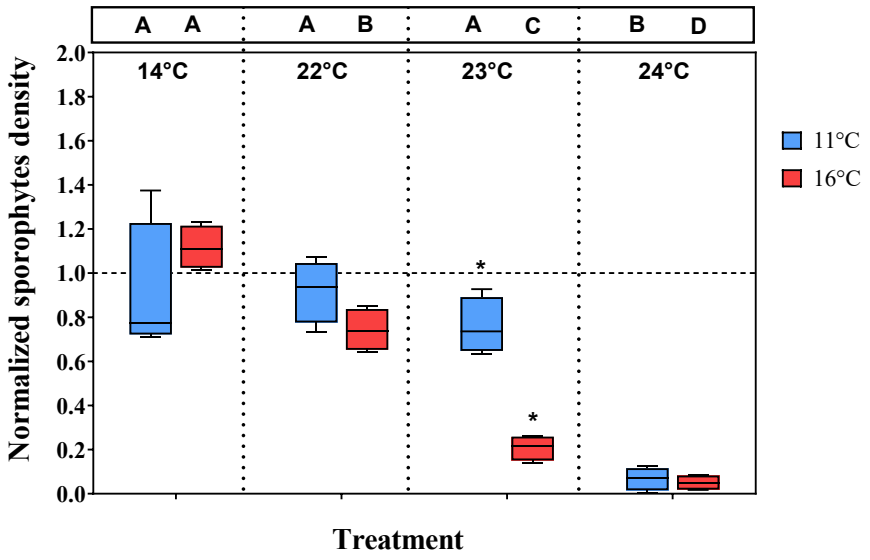

Figure 22 – **Effects of developmental temperature on sporophytes density of *L. ochroleuca* sporophytes from the France population after 14 days in different experimental temperatures (14°C, 22°C, 23°C, 24°C)**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). For each developmental temperature, uppercase letters indicate significant differences between experimental temperatures. For each experimental temperature, an asterisk indicates significant differences between developmental temperatures. See table 6 for statistics.

Table 6 - Two-factor PERMANOVA for the effects of developmental temperature (11°C and 16°C) and experimental temperature (14°C, 22°C, 23°C, 24°C) on the sporophyte density of *L. ochroleuca* after 14 days.

Factors	Df	SS	R2	F	Pr(>F)
dvelopmental temp	2	0,7303	0,13352	18,109	**< 0,001**
experimental temp	3	3,6504	0,66743	60,347	**< 0,001**
developmental temp:experimental temp	2	0,6048	0,11058	14,997	**< 0,001**
Residual	24	0,4839	0,08848		
Total	31	5,4694	1		

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value by permutation.

Maximum quantum efficiency of PSII (F_v/F_m)

The photosynthetic efficiency of PSII (F_v/F_m) of sporophytes after 14 days of exposure to experimental temperatures (14°C, 22°C, 23°C and 24°C) exhibited significant main effects of developmental temperatures and experimental temperatures, but no significant interaction between these factors (Table 7; Figure 23). The sporophytes F_v/F_m values gradually decreased with the increase of the experimental temperatures. The control temperature of 14°C resulted in the highest photosynthetic performance (~0.45-0.58). Although F_v/F_m values significantly declined at 22°C, sporophytes still maintained relatively high photosynthetic efficiency (~0.40–0.50).

At 23°C and 24°C, F_v/F_m values demonstrated a marked decline in photosynthetic performance, with the lowest efficiency recorded at 24°C. Overall, while sporophytes derived from both developmental temperatures followed a similar declining trend in photosynthetic efficiency, those developed at 16°C showed significantly higher F_v/F_m values compared to those developed at 11°C.

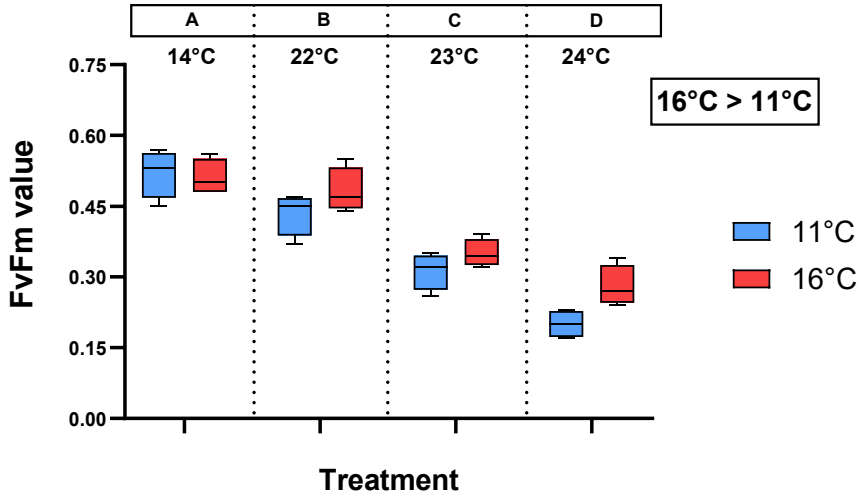

Figure 23 – **Effects of developmental temperatures on the maximum quantum efficiency of photosystem II (F_v/F_m) of *L. ochroleuca* sporophytes from the France population after 14 days in different experimental temperatures (14°C, 22°C, 23°C, 24°C)**.

Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Different letters indicate significant differences between experimental temperatures. See table 7 for statistics.

Table 7 – Two-way ANOVA for the effects of developmental temperature (11°C and 16°C) and experimental temperature (14°C, 22°C, 23°C, 24°C) on the photosynthetic efficiency of PSII (F_v/F_m) of *L. ochroleuca* sporophytes after 14 days.

Factors	**Df**	**SS**	**Mean Sq**	**Pr(>F)**
experimental temp	3	0,3699750	0,12332500	**0,01340**
developmental temp	1	0,0120125	0,012012500	**< 0,001**
experimental temp:developmental temp	3	0,0083125	0,002770833	0,20562
Residuals	24	0,0404500	0,001685417	

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; Mean Sq: mean squares.

Experiment 2 – Effects of chemical and thermal priming on the thermal tolerance of juvenile sporophytes

Sporophyte relative growth rate

The relative growth rate (RGR) of sporophytes pre-exposed to the four different priming treatments (CTR: 13°C, CTR+H_2O_2: 13°C+H_2O_2, HT: 23°C, HT+H_2O_2: 23°C+H_2O_2) and then exposed for 14 days to four experimental temperatures (13°C, 23°C, 24°C and 25°C) was significantly influenced only by the experimental temperatures (Table 8, Figure 24).

No significant differences in RGR were observed between the four priming treatments. However, sporophytes exposed to the experimental temperature of 13°C significantly showed the highest RGR, reaching positive values indicating active growth. As the temperature increased to 23°C and 24°C, RGR significantly declined, approaching values close to zero, indicating minimal or absence of growth. At 25°C, RGR became negative, indicating sporophyte death and marking 25°C as the lethal temperature.

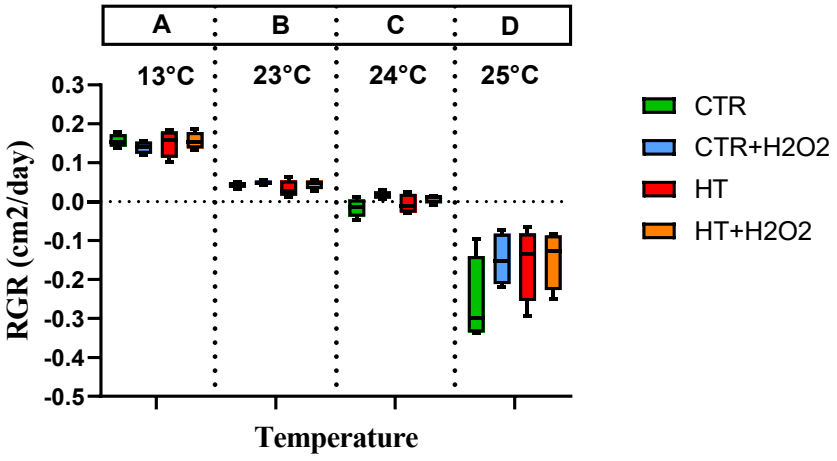

Figure 24 – **Effects of experimental temperature and priming treatment on the relative growth rate (area-RGR) of *L. ochroleuca* sporophytes after 14 days**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Uppercase letters indicate significant differences between

experimental temperatures. No differences were found between priming treatments. See table 8 for statistics.

Table 8 – Two-factor PERMANOVA for the effects of priming treatment (13°C, 13°C+H_2O_2, 23°C, 23°C+H_2O_2) and experimental temperature (13°C, 23°C, 24°C, 25°C) on the area-RGR of sporophytes of *L. ochroleuca* after 14 days.

Factors	**Df**	**SS**	**F**	**R2**	**Pr(>F)**
priming	3	0,01168	1,6889	0,01117	0,1775
temperature	3	0,89685	129,6312	0,85741	**< 0,001**
priming:temperature	9	0,02677	1,2900	0,02560	0,2677
Residuals	48	0,11070		0,10583	
Total	63	1,04601		1	

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value by permutation.

Maximum quantum efficiency of PSII (F_v/F_m)

The maximum quantum efficiency of PSII (F_v/F_m) of sporophytes was significantly affected only by the experimental temperature (Table 9; Figure 25).

F_v/F_m reached its optimal and highest values at the control temperature of 13°C (~0.70-0.75), while it significantly declined at 23°C and 24°C, with F_v/F_m values ranging from approximately 0.45 to 0.69. At 25°C, photosynthetic performance was further significantly reduced, with sporophytes showing F_v/F_m values between ~0.25 and ~0.52.

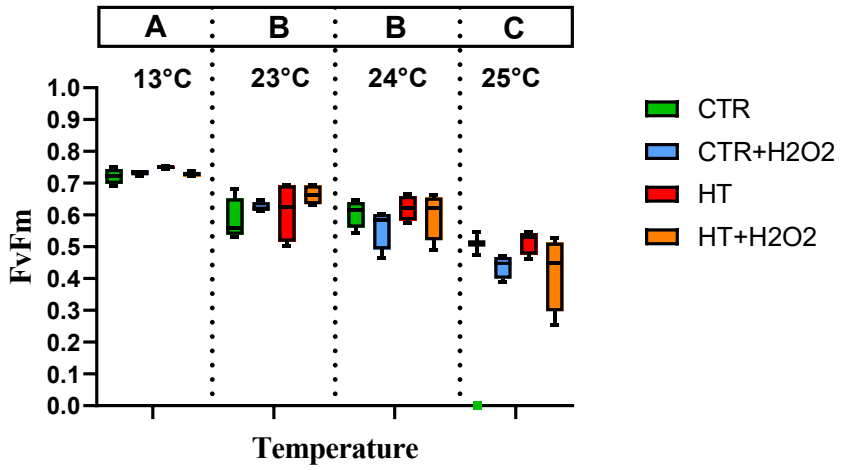

Figure 25 – **Effects of experimental temperatures and priming treatment on the maximum quantum efficiency of photosystem II (F_v/F_m) of *L. ochroleuca* sporophytes after 14 days.** Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Different letters indicate significant differences between experimental temperatures. Outliers are represented by a point. See table 9 for statistics.

Table 9 – Two-factors PERMANOVA for the effects of priming treatment (13°C, 13°C+H_2O_2, 23°C, 23°C+H_2O_2) and experimental temperature (13°C, 23°C, 24°C, 25°C) on the F_v/F_m of sporophytes of *L. ochroleuca* after 14 days.

Factors	Df	SS	F	R2	Pr(>F)
priming	3	0,01236	2,3501	0,01691	0,2642
temperature	3	0,54008	59,0037	0,73894	< **0,001**
priming:temperature	9	0,03810	1,3874	0,05213	0,2295
Residuals	46	0,14035		0,19203	
Total	61	0,73088		1	

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; R2: effect size; F: F value by permutation.

Maximum electron transport rate ($rETR_{max}$)

Significant priming treatment × experimental temperature interactions were observed on the maximum electron transport rate ($rETR_{max}$) of sporophytes (Table 10; Figure 26). At 13°C, $rETR_{max}$

values were higher in sporophytes pre-treated with 23°C+H_2O_2 compared to the sporophyte from the control (13°C) and treated with high temperature (23°C).

At higher experimental temperatures (23°C and 25°C), $rETR_{max}$ declined in a similar way across all the priming treatments. However, at 24°C, sporophytes pre-treated with 23°C showed significantly higher $rETR_{max}$ values compared to the other priming treatments.

For sporophytes pre-exposed to the control treatment (CTR at 13°C), $rETR_{max}$ values were lower in the sporophytes exposed to 25°C compared to 13°C and 23°C. Sporophytes pre-treated with 13°C+H_2O_2 and 23°C+H_2O_2 followed a similar trend, displaying the highest normalized $rETR_{max}$ values at 13°C and a gradual decline at higher temperatures, with the greatest reduction observed at 24°C and 25°C. Interestingly, sporophytes pre-treated with 23°C exhibited a different trend, showing a significant decrease in $rETR_{max}$ at 23°C and 25°C compared to 13°C and 24°C.

Overall, at the control temperature of 13°C, sporophytes from all priming treatments – except for the control at 13°C – showed $rETR_{max}$ values above 1, indicating increased maximum electron transport rates. However, sub-lethal temperatures (23°C and 24°C) caused significant reductions, except for HT (23°C) sporophytes at 24°C. The 25°C temperature was confirmed to be lethal, as indicated by the death of two sporophytes and a sharp decline in $rETR_{max}$ across all treatments.

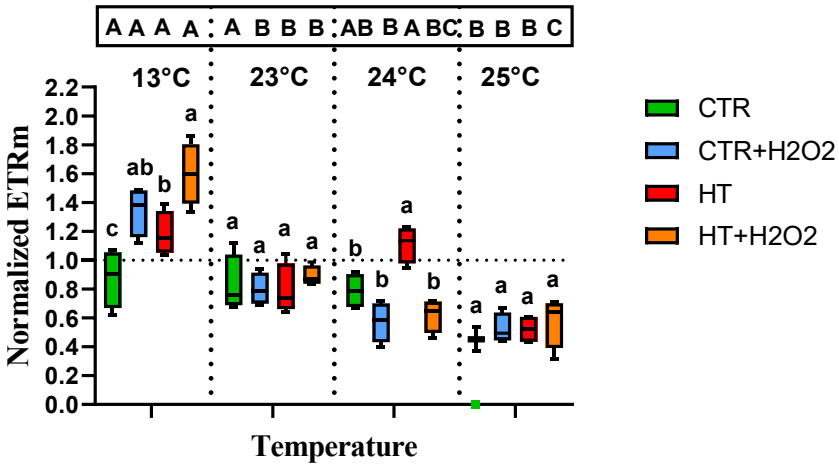

Figure 26 – **Effects of experimental temperatures and priming treatments on the $rETR_{max}$ values of *L. ochroleuca* sporophytes after 14 days**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Uppercase letters indicate significant differences between experimental temperatures. Lowercase letters indicate significant differences between priming treatments. Outliers are represented by a point. See table 10 for statistics.

Table 10 – Two-way ANOVA for the effects of priming treatments (13°C, 13°C+H_2O_2, 23°C, 23°C+H_2O_2) and experimental temperatures (13°C, 23°C, 24°C, 25°C) on the $rETR_{max}$ of sporophytes of *L. ochroleuca* after 14 days.

Factors	Df	SS	Mean Sq	F	Pr(>F)
priming	4	45,51732	11,379330	496,6879	< **0,001**
temperature	3	4,21830	1,406102	61,3739	< **0,001**
priming:temperature	9	1,50607	0,167341	7,3041	< **0,001**
Residuals	46	1,053879	0,02291042		

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; Mean Sq: mean squares; F: F value.

Minimum saturating irradiance (Ik)

The minimum saturating irradiance (Ik) was significantly affected only by the experimental temperature (Table 11; Figure 27).

Ik values were not significantly different for sporophytes exposed to 13°C, 23°C and 24°C ranging from approximately 160 to 230. However, at 25°C, a significant reduction in Ik was observed, with values ranging from approximately 140 to 190, highlighting the impact of lethal temperatures on the light saturation point of photosynthesis.

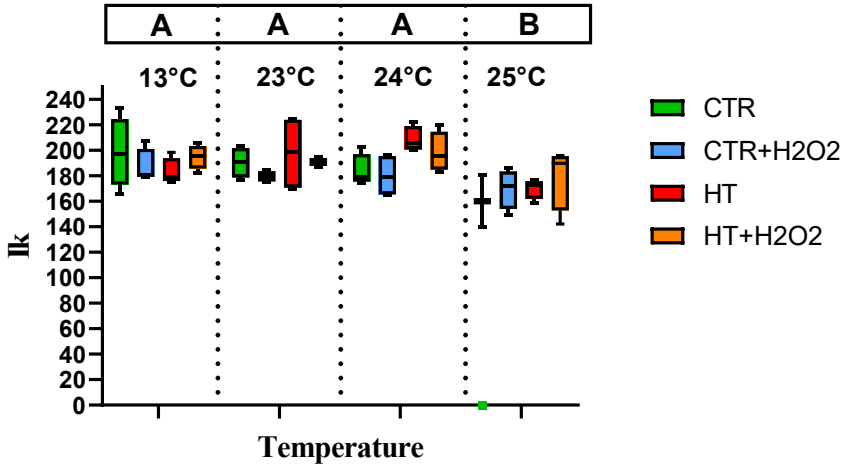

Figure 27 – **Effects of experimental temperatures and priming treatments on the Ik values of *L. ochroleuca* sporophytes after 14 days**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Different letters indicate significant differences between experimental temperatures. Outliers are represented by a point. See table 11 for statistics.

Table 11 – Linear Mixed-Effects Model (LMM) for the effects of priming treatments (13°C, 13°C+ H_2O_2, 23°C, 23°C+ H_2O_2) and experimental temperatures (13°C, 23°C, 24°C, 25°C) on the Ik of sporophytes of *L. ochroleuca* after 14 days.

Factors	SS	Mean Sq	Df	F	Pr(>F)
priming	704,4	234,81	3	0,8550	1,000
temperature	4635,0	1544,99	3	5,6258	**0,002**
priming:temperature	2457,2	273,03	9	0,9942	0,458

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; Mean Sq: mean squares, F: F value.

Light-limited photosynthetic rate (α)

Light-limited photosynthetic rates (α) exhibited significant priming treatment × experimental temperature interactions (Table 12; Figure 28), with a similar pattern to maximum electron transport rates ($rETR_{max}$).

At 13°C, the highest α values were observed in sporophytes pre-treated with 23°C+H_2O_2, while those pre-treated with 23°C and 13°C+H_2O_2 exhibited lower values. Sporophytes from the CTR (13°C) treatment exhibited an even higher significant reduction in α at this temperature. At higher experimental temperatures (23°C and 25°C), the α declined similarly across all the priming treatments. However, at 24°C, sporophytes pre-treated with 23°C showed α higher values compared to the 13°C+H_2O_2 and 23°C+H_2O_2 treatments.

Sporophytes pre-treated with CTR (13°C) showed lower α values at the highest temperature treatment of 25°C compared to 13°C. Sporophytes pre-treated with 13°C+H_2O_2 and 23°C+H_2O_2 showed the highest normalized α values at 13°C and a gradual decline at higher temperatures, with the greatest reduction observed 25°C. Interestingly, sporophytes pre-treated with 23°C (HT) exhibited higher α values at 13°C than at 23°C, while the lowest values were recorded at 25°C. Overall, at the control temperature of 13°C, sporophytes from all priming treatments, except for CTR (13°C), showed positive α values, indicating increased light-limited photosynthetic rates. However, sub-lethal temperatures (23°C and 24°C) led to significant reductions in α, except for HT (pre-treated at 23°C) sporophytes at 24°C. The 25°C temperature was confirmed to be lethal, as two sporophytes died (pre-treated with CTR) and a sharp decline in α was observed across all treatments.

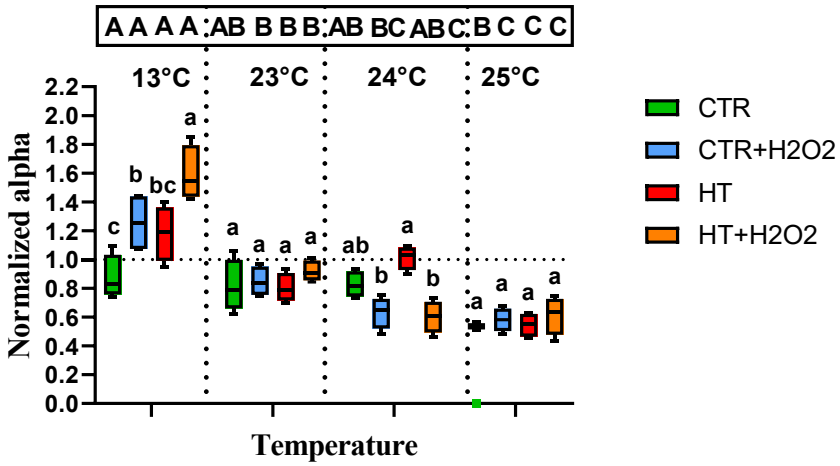

Figure 28 – **Effects of experimental temperatures and priming treatments on the α values of *L. ochroleuca* sporophytes after 14 days**. Boxplots with median, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n = 4). Uppercase letters indicate significant differences between experimental temperatures. Lowercase letters indicate significant differences between priming treatments. Outliers are represented by a point. See table 12 for statistics.

Table 12 – Linear Mixed-Effects Model (LMM) for the effects of priming treatments (13°C, 13°C+ H_2O_2, 23°C, 23°C+ H_2O_2) and experimental temperatures (13°C, 23°C, 24°C, 25°C) on the α of sporophytes of *L. ochroleuca* after 14 days.

Factors	SS	Mean Sq	Df	F	Pr(>F)
priming	13,7926	3,4482	4	194,5146	**< 0,001**
temperature	3,3537	1,1179	3	63,0614	**< 0,001**
priming:temperature	1,2647	0,1405	9	7,9269	**< 0,001**

Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; Mean Sq: mean squares; F: F value.

DISCUSSION

Experiment 1

The results demonstrated the presence of intraspecific phenotypic plasticity in *L. ochroleuca*, suggesting an evolutionary adaptation to the temperatures usually experienced across its geographical distribution. All the Atlantic populations included in this study (France, Morocco and Portugal) presented higher reproductive success at temperatures ranging from 11°C to 16°C, while the Mediterranean population (Italy) showed better reproductive performances at the highest temperature of 18°C. In addition, the F1 microscopic sporophytes developed at 11°C showed a higher survival when exposed to the sub-lethal temperature of 23°C, compared to those grown at 16°C.

Gametophytes reproductive success at different temperatures (experiment 1.1)

Differences in the reproductive behaviour of gametophytes have previously been linked to the geographical distribution of various *Laminaria* species (Bartsch et al., 2008). Northern kelp species exhibit optimal egg production at relatively low temperatures, between 5 and 10°C (tom Dieck, 1992). In contrast, southern-distributed species, such as the study species *L. ochroleuca*, show peak fertility at higher temperatures (15-18°C), aligning with the typical sea temperatures in their geographical distribution range (Izquierdo et al., 2001). Although studies have demonstrated differences in thermal tolerance among populations (King et al., 2019; Martins et al., 2020; Strasser et al., 2022), to my knowledge, no research has yet investigated the temperature range for optimal gametogenesis. This study reveals differences in reproductive outcomes within populations of *L. ochroleuca*. Specifically,

reproduction speed, fertility and sporophyte density at each temperature tested (11°C-18°C) differed across the four populations. These differences appear to reflect the sea temperatures and temperature regimes typically experienced by each population in their native environments. A notable pattern is that Atlantic populations (from France, Morocco, and Portugal) reproduce more efficiently at lower temperatures (up to 16°C) compared to the sole Mediterranean population (Italy), which showed higher recruitment at the highest temperature studied (18°C). Since spore release in *L. ochroleuca* is reported to occur from late summer to early autumn (October/November), triggered by both photoperiod and water temperature (Pereira et al., 2019; Barradas et al., 2011), and gametophytes development and subsequent gametogenesis are thought to happen directly after zoospores release, it is important to take into consideration also the summer sea surface temperature (SST) maximums.

The Italian population is locally exposed to temperatures ranging from ~ 13.5°C to ~17.8°C (Copernicus Marine Data Store, Bio-Oracle), being consistent with the obtained gradual increase in reproductive success with the increase in temperature, reaching the highest sporophyte development at 18°C. These results agree with the findings in the study of Strasser et al. (2022), where the reproductive output of Italian gametophytes reached nearly 100% and the recruitment peaked at 17°C. Moreover, there is evidence of a fast and continuous increase in the Mediterranean Sea surface temperatures (SSTs) since the late 1990s and this stands in contrast with the more stable conditions in the Atlantic Ocean (Pisano et al., 2020). In addition, due to its unique geographical and atmospheric conditions, the Mediterranean basin is more prone to long warm seasons differently from the shortest ones in the Atlantic Ocean (Benzouaï et al., 2024). For these reasons, the Italian population of

L. ochroleuca might be exposed to longer periods of warm water during the reproductive season compared to Atlantic populations, which can experience temporary high temperatures, but for a shorter period. Despite the similar temperatures experienced by the populations in this study, an important difference can be due to the prolongation of warmer seawater periods. Moreover, the oceanic waters at the geographical locations of collection of the *L. ochroleuca* individuals present a more variable pattern in terms of seawater temperature, while the Messina Strait at 40/50 meters of depth present more stable conditions (Copernicus Marine Data Store, Bio-Oracle): this can also explain why the Italian population reproduced better at 18°C, because if the water warms it tends to stay warm, while in the Atlantic ocean the maximum SST temperatures are more likely to vary.

The range of temperatures used in this study (from 11°C to 18°C) confirmed the temperatures at which gametophyte reproduction occurs in *L. ochroleuca*, as previously demonstrated in another study (Izquierdo et al., 2001) and confirmed by the northern limit of its distribution coinciding with the 10°C February isotherm (van den Hoek, 1982). However, the highest reproductive outcome was not always found at the highest temperatures used in this experiment as in Izquierdo et al. (2001), where only one population (Spain) was analysed. This was true only for the Italian population, while the others showed high reproductive outcome at temperatures between 11°C and 16°C. This can be related to the environmental conditions found in their habitat of collection during the reproductive period.

The French population is locally exposed to colder environments with minimum temperatures of 11°C and maximum of 18°C (Bio-Oracle; Gómez-Gesteira et al., 2008) during the reproductive period

of *L. ochroleuca*, from summer to late autumn with a peak in August and September (in Portugal, Pereira et al., 2018), but the reproductive outcome at 18°C was very low compared to relatively faster reproductive performance and final sporophyte densities between 11°C and 16°C. For this reason, it can be hypothesized that gametogenesis occurs late in the year after the spore release events in summer, matching in this way the reproductive optimal temperatures found in this study.

The Moroccan gametophytes also exhibited a high reproductive success (percentage of gametophytes with eggs and sporophytes and final sporophyte density) at temperatures between 11°C and 16°C. The temperatures experienced by the population at the site of collection (El Jadida) range from ~ 23.6°C to ~ 15°C, with a mean of 20°C in autumn and spring (Rezzoum et al., 2017). This stands in contrast with the optimal temperatures for reproduction success found in this study. A potential explanation might be the presence of upwelling in this region, driven by strong equatorward trade winds (Bakun, 1990). However, El Jadida is located at the border between northern and central Atlantic coasts of Morocco, which are characterized by distinct upwelling regimes. While the northern coast typically does not present or present very weak phenomena, the central coast is frequently affected by strong upwelling, especially in the summer and autumn (Benazzouz et al., 2014; Aouni et al., 2024). It is possible that the population experiences upwelling during the reproductive period, which coincides with gametogenesis. The influx of colder water masses from the depths could explain the better reproductive performance at lower temperatures. This hypothesis is further supported by a recorded minimum temperature of 10°C in early August (Copernicus Marine Data Store), suggesting an upwelling event. These findings are in accordance with a previous study performed on the same

population, which showed low reproductive output of Moroccan gametophytes at 17°C and suggested that lower temperatures might have been more suitable to induce reproduction (Strasser et al., 2022).

The Portuguese gametophytes showed high percentage of gametophytes with sporophytes and eggs at all temperatures, but high final sporophyte densities only at the coldest temperatures of 11°C and 13°C. Similarly to the Moroccan gametophytes, optimal sporophyte development under cold temperatures could be explained by the presence of upwelling along west coast of Portugal during the summer period, coinciding with the reproductive season of *L. ochroleuca* (Pereira et al., 2018; Barradas et al., 2011), bringing cold and nutrient-rich water (Fiúza, 1983). The species might be adapted to the local environmental conditions present during reproduction, since upwelling events usually occur from April to October, with an intensification of the phenomenon in July and August (Nykjær & Van Camp, 1994; Gómez-Gesteira et al., 2008). The speed in reproduction did not follow the pattern of sporophyte development. For Portuguese population, the percentage of gametophytes with eggs and sporophytes were near 100% for all temperatures after 2 weeks of gametogenesis, showing the fastest reproduction rate in all the tested populations and resilience to temperature variations. However, a high sporophyte development was only observed at the lower temperatures (11°C and 13°C). The reproduction of the Portuguese population is also linked with the highest partheno-sporophyte production, especially at the lower temperatures. The oogenesis rates were highest at 11°C and 13°C, and this has resulted in a high number of unfertilized eggs that have developed in parthenogenetic sporophytes, a phenomenon already described in culture (e.g., tom Dieck, 1992). Faster reproduction output might result in more parthenogenetic sporophytes as was

found in a study carried out on *L. digitata*, which showed a high formation of parthenogenetic individuals corresponding to a fast eggs release (Martins et al., 2017). In kelps, eggs are released shortly after nightfall, typically within the first hour of darkness (Lüning 1981; Li et al. 2013). Following their release, eggs begin to secrete the universal pheromone lamoxirene, which both triggers sperm release from male gametophyte antheridia and attracts sperm towards the eggs (Lüning & Müller 1978). Since males of many kelp species are fertile before the females (a condition known as protandry; Hsiao & Druehl, 1971; Bartsch pers. obs.), a fast gametogenesis with simultaneous egg release could theoretically lead to the release of all spermatozoids during this initial massive egg extrusion event. When additional eggs are released over the subsequent days, they might not get fertilized and may instead develop parthenogenetically, since sperm generally survive no longer than 12 hours (Li et al., 2013).

In conclusion, these results support the hypothesis of a potential intraspecific ecotype differentiation following the distribution of *L. ochroleuca*, as was found in other Laminarian species and more generally brown algae. *Ecklonia radiata* gametophyte survival and growth reflected the local environmental conditions experienced by the species, especially temperature regimes, along the Australian coasts (Mohring et al., 2014). Moreover, another example is represented by both microscopic sporophytes and gametophytes from southern and northern Chilean populations of *Lessonia nigrescens* Bory exhibiting differences in thermal tolerance (Martinez, 1999; Oppliger et al., 2012). To my knowledge, no previous study has compared the reproductive outputs and final sporophyte densities of different *L. ochroleuca* populations across the full range of temperatures in which they reproduce. This research offers preliminary insights into the potential phenotypic

plasticity that may contribute to the long-term adaptation of different ecotypes to local environmental conditions. Further research is needed to determine whether these adaptations are also linked to genetic differentiation, as has been suggested for *Undaria pinnatifida* (Harvey) Suringar (Gao et al., 2012).

These findings align with the predictions made by a study on changes in the distribution of kelp species in the North Atlantic (Assis et al., 2017). The model tested the effects of two climate scenarios (RCP2.6 and the more extreme RCP8.5), and in both cases, it projected the disappearance of the warm-temperate species *L. ochroleuca* from lower latitudes, including the Moroccan Atlantic coast, due to rising ocean temperatures, which are often associated with nutrient depletion (Kamykowski & Zentara, 1986). In this study, Atlantic populations struggled with both gametogenesis and sporophyte development at the highest temperatures of 18°C, suggesting a potential shift in their distribution to northern regions, along with the loss of southern refugia that are considered putative genetic diversity hotspots. Fortunately, deep refugia may persist (Assis et al., 2017), suggesting that the population in the Messina Strait could withstand future climate changes.

Thermal tolerance of microscopic sporophytes (experiment 1.2)

Juvenile sporophytes of *L. ochroleuca* originating from gametophytes exposed to low (11°C) and high gametogenesis temperatures (16°C) were exposed to sub-lethal and lethal temperatures to evaluate their responses to heat stress. The initial hypothesis was that sporophytes developed at the higher temperature (16°C) would exhibit greater thermal tolerance, allowing them to withstand better heat stress. However, the results, measured in terms of sporophyte survival, growth and photosynthetic efficiency, were complex. Further research is

needed to clarify whether any cross-generational effects are transferred from gametophytes (considered parental to the offspring, as defined by Byrne et al., 2019) to juvenile sporophytes.

The relative growth rate of sporophytes grown at 11°C and 16°C were similar, decreasing at sub-lethal temperatures compared to the control temperature, indicating that gametogenesis temperature did not significantly impact the subsequent sporophyte growth ability. The temperature of 24°C proved lethal, confirming earlier findings by Franco et al. (2017) that 24.6°C is fatal for macroscopic sporophytes. This suggests that microscopic sporophytes may have a heat tolerance of 1°C lower than larger individuals.

Sporophyte density revealed higher survival for sporophytes grown at 11°C when exposed to the sub-lethal temperature of 23°C, compared to those grown at 16°C. Cold-grown sporophytes exhibited stable survival rates across control and sub-lethal temperatures, whereas warm-grown sporophytes showed a gradual decline in survival as temperatures increased. In addition, sporophyte mortality (normalized sporophyte density values lower than 1) was detected for warm-grown sporophytes at sub-lethal temperatures, while the density of cold-grown individuals was stable (normalized sporophyte density near 1) at 22°C. This suggests the potential presence of cross-generational plasticity (Donelson et al., 2017; Byrne et al., 2020) in this species, which may enhance the survival of cold-acclimated individuals when exposed to heat stress. A related study on *L. digitata* found that cold conditions during gametogenesis improved F1 sporophytes growth under extreme temperatures (both low and high), with effects lasting up to three months (Gauci et al., 2022). Similarly, this experiment indicates that cold gametogenesis and sporophyte development conditions may

provide lasting benefits for offspring, highlighting potential cross-generational plasticity and carry-over effects. Another study performed on *L. digitata* also reported improved and faster growth for sporophytes originating from a 5°C gametogenesis treatment across all thermal exposures (Liesner et al., 2020).

These findings, combined with prior research on gametophytes reproductive success at varying temperatures, suggest that the positive impact of cool temperatures during gametogenesis may be tied to optimal reproductive performance in environments, where the species is naturally acclimated. In this study, *L. ochroleuca* individuals used in the experiment originated from France (Roscoff), and as previously discussed, exhibited optimal reproduction at lower temperatures (11°C and 13°C), because even though the final recruitment was similar to the one found at 16°C, the gametogenesis occurred faster and the number of parthenogenetic individuals was higher at the higher temperature. This can suggest that any beneficial effect transmitted to the juvenile sporophytes' offspring might depend on the temperature typically experienced in the *in situ* environment, leading to a better reproductive performance, rather than depending on either a gametogenesis at a low or a high temperature (Novaczek, 1984).

The potential cross-generational plasticity and carry-over effects observed here are likely not caused by irreversible changes in the DNA sequence, as the same genetic pool of individuals was used for gametophytes across all thermal treatments. Instead, it appears to involve phenotypic differentiation, potentially through epigenetic mechanisms, such as DNA methylation (Fan et al., 2019) and/or histone modifications (Pearson et al., 2019; Bourdareau, 2018). DNA methylation can lead to differential genes expression (Fan et al., 2019) in juvenile sporophytes, and some studies suggest that it

can be inherited across generations (Scheschonk et al., 2022), potentially helping species in adapting to changing environments. However, epigenetic inheritance in kelp species has yet to be demonstrated, requiring further investigation.

F_v/F_m, a measure of photosynthetic efficiency, was assessed in microscopic sporophytes, since it is commonly used as a proxy for algal physiological and cellular stress (Murchie & Lawson, 2013). Based on the sporophyte survival data, which indicated greater stress in warm-grown sporophytes when exposed to sub-lethal temperatures, we expected that these sporophytes would show lower photosynthetic efficiency. Surprisingly, photosynthetic performance followed a different pattern, as higher F_v/F_m values were found in warm-grown sporophytes compared to cold-grown ones. A peculiar relationship between respiration and acclimation temperature was previously reported in algae, such as the rhodophycean *Gracilaria vermiculophylla*, the chlorophycean *Ulva lactuca* and the closely related species, *Saccharina latissima* (Davison et al., 1991; Nejrup et al., 2013). Generally, warm acclimated algae tend to have lower respiration rates than cold-acclimated ones when exposed to high temperatures (Davison et al., 1991; Nejrup et al., 2013). This suggests that the higher photosynthetic efficiency observed in warm-grown sporophytes could be due to their lower respiration rates, whereas the higher respiration of cold-grown sporophytes may have contributed to their lower F_v/F_m values. However, this reduced photosynthetic efficiency did not seem to affect the sporophyte survival of the cold-grown juvenile sporophytes.

In conclusion, it can be suggested that exposing the early life stages of the golden kelp *L. ochroleuca* to cool environments or to temperatures that are optimal for gametophyte reproduction in

each ecotype may improve heat tolerance in grown sporophytes. These findings differ from what was observed in higher plants and seagrasses (Fan et al., 2018; Nguyen et al., 2020), where exposure to heat stress often enhances future heat tolerance. However, since this study focused on a single population, likely representing just one ecotype, further research involving other ecotypes is necessary to deepen our understanding. Identifying ways to boost kelp resilience to heat stress could be highly beneficial for both mariculture and restoration efforts (Coleman et al., 2020; Wood et al., 2019), especially considering the climate change predictions.

Experiment 2 – Effects of chemical and thermal priming on the thermal tolerance of juvenile sporophytes

Overall, only slight effects of the priming treatments were found on the performance and thermal tolerance of juvenile sporophytes. Moreover, the study confirmed that 25°C was lethal, as sporophytes died or exhibited signs of severe stress, such as negative growth rates, extensive whitening of the blade and low values of photosynthetic parameters. This aligns with previous findings (Lüning, 1984), where thermal tolerance varies across life stages, as microscopic sporophytes were found dead (bleached meristem) after two weeks at 24°C in the previously described experiment, while larger sporophytes, though stressed, survived two weeks at the same temperature. This suggests a potential weak positive correlation between thallus size and thermal tolerance, which remains to be further investigated.

Growth rates were unaffected by priming treatments and exhibited a gradual decline as temperatures increased. At 24°C, the highest sub-lethal temperature, growth rates were around zero, indicating

no growth, while a slightly positive growth was observed at 23°C. This demonstrates that juvenile sporophytes growth is severely reduced at exceptionally high temperatures, reflecting a weak thermal tolerance. In fact, kelps are already experiencing declines due to rising seawater temperatures, as seen in southern Europe (Voerman et al., 2013), and trends like the predicted reduction in upwelling intensities along the Iberian Peninsula may further contribute to the contraction of kelp forest ranges (Sydeman et al., 2014). Additionally, heat stress has already been shown to reduce kelp growth rates, as demonstrated on the brown alga *Saccharina japonica*, where high temperatures also caused blade whitening, a sign of stress (Liu & Pang, 2009).

Photosynthetic efficiency was assessed in terms of maximum quantum yield (F_v/F_m), which represents the quantum efficiency when all PSII centres are fully operational (Maxwell & Johnson, 2000). Measured values showed a trend similar to the growth rates: sub-lethal temperatures reduced photosynthetic performance compared to the control. Reductions in photosynthetic efficiency are most likely due to heat stress, as previous studies have shown that high temperatures induce photoinhibition (Bruhn & Gerard, 1996; Liu & Pang, 2009; Andersen et al., 2013). Heat stress can cause damages to the photosynthetic machinery of seaweeds, impairing the functionality of PSII, as indicated by a reduction in F_v/F_m (Maxwell & Johnson, 2000), since PSII is the most thermo-labile component of the photosynthetic apparatus (Wahid et al., 2007).

The minimum saturating irradiance (Ik) significantly decreased only at the lethal temperature of 25°C. At sub-optimal temperatures, Ik remained consistent with values measured in sporophytes under control conditions, suggesting an adaptation to light conditions that

declined only at lethal temperatures, because of photoinhibition. In fact, Ik is commonly used as an indicator of photoacclimation in microalgae (Serôdio et al., 2006), since it reflects the optimal irradiance intensity required for balancing energy capture and processing in the photosynthetic system (Salleh & McMinn, 2011).

Light-limited photosynthetic rates (α) and maximum electron transport rates ($rETR_{max}$) also exhibited a general decline with increasing experimental temperatures, with the most pronounced reductions occurring at the lethal temperature of 25°C. This highlights the severe damage to the photosynthetic systems, as also indicated by the decline in quantum yield (F_v/F_m) at the lethal temperature. Such reductions are consistent with previous findings on young sporophytes of the giant kelp *Macrocystis pyrifera*, which showed reductions in maximum electron transport rates ($rETR_{max}$) at sub-optimal temperatures for photosynthesis (Umanzor et al., 2021). Sub-optimal temperatures were associated with lower $rETR_{max}$ rates, suggesting a downregulation of key enzymes controlling the RUBISCO activity (e. g., RUBISCO activase) and other enzymes involved in the carbon fixation process (MacIntyre et al., 1997) – a pattern already observed in microalgae under similar conditions (Salleh & McMinn, 2011).

Priming refers to an artificial exposure to a given stressor in order to create a "stress memory" enhancing resilience in future encounters with the same or a similar stressor (Scheschonk et al., 2022; Jueterbock et al., 2021). In this study, thermal and chemical priming techniques were applied to assess the performance and thermal tolerance of macroscopic sporophytes. Interestingly, at the control temperature of 13°C, all primed sporophytes (chemical, thermal, and thermo-chemical) exhibited increased α and $rETR_{max}$ values compared to untreated sporophytes, suggesting a positive

effect of priming on the photosynthetic physiology. In fact, increases in α values are often associated with elevated concentrations of pigments such as chlorophyll *a* and other light-harvesting pigments (Andersen et al., 2013), while $rETR_{max}$ approximates the rate of electron flow through the photosynthetic chain (Beer et al., 2001) and it is linked to photosynthetic activity as measured by oxygen evolution or CO_2 uptake (Beer at al., 1998). This suggests that priming treatments may have enhanced photosynthetic activity of sporophytes at optimal temperature, and this could have been beneficial even though maximum quantum yields (F_v/F_m) did not show differences among treatments and highlighted similar levels of PSII activity. The chemical priming involved the addition of hydrogen peroxide (H_2O_2) to the growth medium, which was expected to activate antioxidant enzymes in the sporophytes, thereby inducing thermotolerance, as previously observed in higher plants (Kang et al., 2009; Wang et al., 2014). At higher temperatures, only the sporophytes at 24°C primed with high temperature showed an increase in both α and $rETR_{max}$. Interpreting these increases is challenging, as growth rates and photosynthetic efficiency measured through other parameters showed no effect from the priming treatments. This may have been due to an increase in the concentration of light-harvesting pigments, a potential stress response to optimize PSII activity. However, this did not lead to a higher maximum quantum yield (F_v/F_m), likely because PSII was damaged. Furthermore, the detection of a high electron flow ($rETR_{max}$) could be the result of compensatory mechanisms, such as cyclic electron flow (CEF) around PSI, aimed at preventing PSII photoinhibition (Goh et al., 2011).

The lack of significant effects from the priming treatments (chemical, thermal and thermo-chemical) could be due to the possibility that heat-stress memory lasts only a few days. For

instance, positive effects of heat priming were detected for just 5 days in the seaweed *Bangia fuscopurpurea* (Dillwyn) Lyngbye after it returned to normal growth conditions (Kishimoto et al., 2019). Other studies have shown how applying priming techniques to gametophytes contributed to an enhancement of growth and heat tolerance in the resulting sporophyte generation (Quigley et al., 2018; Jueterbock et al., 2021; Bricknell et al., 2020). This suggests that priming already grown sporophytes could not be advantageous, whereas priming early life stages could bring benefits to mature individuals.

Priming techniques in kelp sporophytes appear to work differently compared to seagrasses (Jueterbock et al., 2021) or terrestrial plants (Savvides et al., 2015), where effects tend to be more long-lasting. Epigenetic modifications, which are well-documented in higher plants, are suggested to play a role in tolerance derived from priming techniques and they may also play a role in kelps, but require further research. A deeper understanding of these modifications and their potential transgenerational transmission could improve priming techniques in kelps, potentially leading to the development of stress-tolerant strains for mariculture and restoration efforts (Jueterbock et al., 2021). Additionally, recent research suggests that priming success may be species-specific and/or influenced by the duration of the priming treatment. For instance, *Saccharina latissima* gametophytes primed at 20°C for 4 weeks produced sporophytes that showed increased thermal tolerance of 1°C over 7 days, and a tolerance at 24°C that lasted 4 days longer, compared to sporophytes developed from gametophytes primed for 2 weeks or 6 weeks and from non-primed gametophytes (Gauci et al., 2024).

In conclusion, this study did not find significant effects of priming treatments on the tolerance of juvenile sporophytes of *L. ochroleuca* to sub-lethal and lethal temperatures, indicating that these techniques may not increase the resilience or heat tolerance of this kelp species. However, further investigations should focus on how long the priming effects persist on sporophytes after the end of the treatment. In addition, it could be researched whether primed gametophytes could provide their offspring with greater heat tolerance, further exploring also the concepts of carry-over effects and trans-generational plasticity.

CONCLUSION

Intraspecific variation in gametophyte reproductive output and final sporophyte density was observed among different populations of *L. ochroleuca*, suggesting ecotype differentiation linked to the geographic distribution of the species. The Mediterranean population (Italy) reproduced better at the highest temperature of 18°C, while the Atlantic populations (France, Portugal and Morocco) performed best between 11°C and 16°C. These differences in reproductive outputs likely reflect the environmental conditions typical of each population's habitat, especially seawater temperature and presence of upwelling phenomena. Furthermore, microscopic sporophytes from the French population showed different survival rates when exposed to sub-lethal temperatures, based on the temperature at which gametogenesis occurred. Sporophytes developed in colder conditions (11°C) presented a higher survival at 23°C than those developed in warmer conditions (16°C), indicating a greater thermal tolerance. Although photosynthetic efficiency did not follow this pattern, the lower efficiency measured in cold-grown sporophytes may result from higher respiration rates when exposed to sub-lethal temperatures.

In a second experiment, chemical (H_2O_2) and thermal (23°C) priming were applied to macroscopic sporophytes of *L. ochroleuca* to assess whether these techniques could enhance their thermal tolerance to sub-lethal and lethal temperatures. No significant effects were observed in growth rates or photosynthetic performances, but further research is needed to explore the potential of these techniques. Future studies might investigate whether longer priming exposure enhances sporophyte thermal tolerance under subsequent heat stress, or if applying priming to gametophytes could improve heat tolerance in F1 sporophytes.

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Annex

Bio-Oracle temperature dataset used for France (Roscoff), Portugal (Cascais) and Morocco (El Jadida) was:

`thetao_baseline_2000_2019_depthsurf`

The selected variables to download the layers were `thetao_max`, `thetao_mean`, `thetao_min`.

For Italy (Messina Strait), since it is a deep population (40/50 meters), the dataset used was:

`thetao_baseline_2000_2019_depthmean`

The selected variables to download the layers were `thetao_max`, `thetao_mean`, `thetao_min`.

Additional information regarding temperatures at each sampling location were obtained from the E. U. Copernicus Marine Service Information.

For Atlantic locations (France, Portugal and Morocco populations), the dataset used was “European North West Shelf/Iberia Biscay Irish Seas – High Resolution ODYSSEA L4 Sea Surface Temperature Analysis”

(DOI: https://doi.org/10.48670/moi-00152).

For the Mediterranean location (Italy), the dataset used was “Mediterranean Sea Physics Reanalysis”

(DOI: https://doi.org/10.25423/CMCC/MEDSEA_MULTIYEAR_PHY_006_004_E3R1).

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